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Crossing the Midline: Roles and Regulation of Robo Receptors

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Summary

In the *Drosophila* CNS, the midline repellent Slit acts at short range through its receptor Robo to control midline crossing. Longitudinal axons express high levels of Robo and avoid the midline; commissural axons that cross the midline express only low levels of Robo. Robo levels are in turn regulated by Comm. Here, we show that the Slit receptors Robo2 and Robo3 ensure the fidelity of this crossing decision: rare crossing errors occur in both *robo2* and *robo3* single mutants. In addition, low levels of either Robo or Robo2 are required to drive commissural axons through the midline: only in *robo,robo2* double mutants do axons linger at the midline as they do in *slit* mutants. Robo2 and Robo3 levels are also tightly regulated, most likely by a mechanism similar to but distinct from the regulation of Robo by Comm.

Introduction

Navigating the midline of the central nervous system (CNS) poses several challenging problems for the growth cones of commissural axons, the axons that project across the midline to connect the two symmetric halves of the nervous system. To make a single pass across the midline, these growth cones first project toward the midline, only to then leave it again on the opposite side and never turn back. Why do commissural growth cones seek the midline, while ipsilateral growth cones avoid it? Having initially found the midline so desirable, why do commissural growth cones then immediately exit again on the opposite side? And why, if guidance cues are distributed symmetrically about the midline, do commissural growth cones respond differently to these cues before and after crossing?

An elegant series of studies on *Drosophila*, rodent, and chick embryos has provided a gratifyingly simple model for the complex behavior of commissural growth cones at the midline. The midline is a source of both attractive (Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Serafini et al., 1994, 1996; Harris et al., 1996; Mitchell et al., 1996) and repulsive (Colamarino and Tessier-Lavigne, 1995; Stoeckli et al., 1997; Brose et al., 1999; Kidd et al., 1999; Zou et al., 2000) guidance cues, and commissural growth cones switch their sensitivity to these cues as they cross the midline (Kidd et al., 1998a; Shirasaki et al., 1998; Zou et al., 2000). Prior to crossing they are sensitive only to the attractive cues, and after crossing only to the repulsive cues. Ipsilateral growth cones, which never cross the midline, are sensitive to the repulsive cues from the outset. Although not all aspects of this model have been tested in each system, and the precise molecular details may vary from species to species, this basic principle seems likely to apply to all organisms with a bilaterally symmetric nervous system.

Repulsive midline cues thus have at least three functions: to prevent ipsilateral axons from entering the midline, to drive commissural axons through the midline, and to prevent commissural axons from crossing a second time. In *Drosophila*, the midline repellent Slit fulfills each of these functions. In *slit* mutant embryos, both ipsilateral and commissural axons enter the midline and never leave it (Rothberg et al., 1990; Battye et al., 1999; Kidd et al., 1999). These projection errors demonstrate that Slit is required both to prevent ipsilateral axons from entering the midline and to prevent commissural axons from lingering at the midline. The evidence that Slit also prevents recrossing is less direct. It comes from the analysis of embryos lacking the Slit receptor Roundabout (Robo). In *robo* mutants, axons freely cross and recross the midline (Seeger et al., 1993; Kidd et al., 1998a). This suggests that Slit signals through Robo to prevent illicit crossing and recrossing. But it also raises an important question: if axons do not linger at the midline in *robo* mutants the way they do in *slit* mutants, then through what receptors does Slit act to prevent lingering?

In a companion paper (Rajagopalan et al., 2000), we report the identification of two additional Slit receptors in *Drosophila*, Robo2 and Robo3. In that paper, we show that Robo2 and Robo3 respond to a long-range Slit signal to position longitudinal axons at the appropriate distance from the midline—yet a fourth role for Slit. Here, we examine the roles of Robo2 and Robo3 in the choices growth cones make at the midline. Midline crossing errors occur at low frequency in both *robo2* and *robo3* mutants, suggesting that the decision to cross or not to cross the midline is primarily controlled by Robo, but both Robo2 and Robo3 are required to ensure the fidelity of this decision. In neither mutant, however, do axons linger at the midline. This occurs only in the *robo,robo2* double mutant. Robo and Robo2 therefore act redundantly to prevent lingering, and low levels of either receptor are sufficient to drive commissural growth cones through the midline.

How do commissural growth cones acquire sensitivity to Slit as they cross the midline? In *Drosophila*, this switch in sensitivity is mediated by the precise spatial control of Robo protein levels. Robo levels are low on commissural growth cones as they cross the midline but rise dramatically just as they emerge on the contralateral side (Kidd et al., 1998a). Robo protein levels are negatively regulated by the transmembrane protein Commissureless ([Comm]; Tear et al., 1996; Kidd et al., 1998b).

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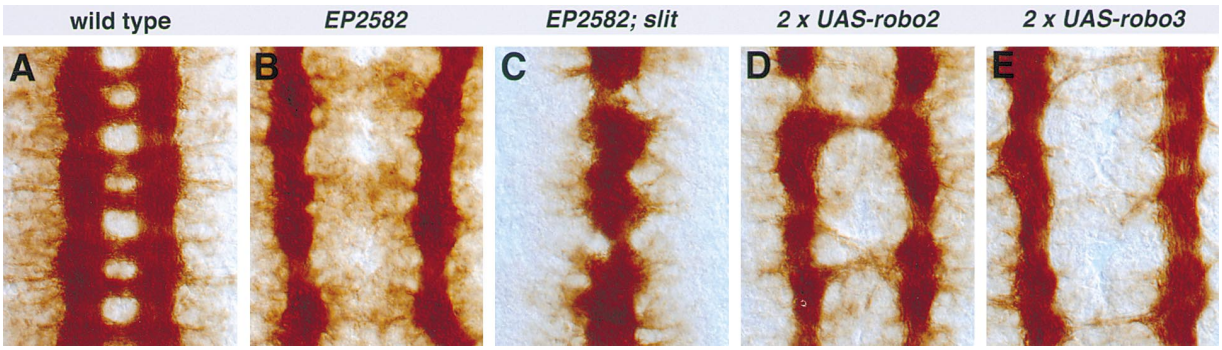

Figure 1. Overexpresson of Robo2 or Robo3 Prevents Midline Crossing

Photomicrographs of the CNS of stage 16 embryos stained with mAb BP102 to visualize all CNS axons.
(A) Wild-type embryo. The two longitudinal tracts on each side of the midline are connected in each segment by two commissures.
(B) *EP2582/+*; *elav-GAL4/+* embryo. Commissures are lost when the endogenous *robo2* gene is expressed at high levels in all neurons.
(C) *EP2582 slit^2/slit^2*; *elav-GAL4/+* embryo. In the absence of Slit, overexpression of *robo2* no longer prevents axons from entering the midline, and the entire axon scaffold collapses to the midline just as it does in *slit* mutants.
(D and E) (D) *elav-GAL4/2xUAS-robo2* and (E) *elav-GAL4/2xUAS-robo3*. High levels of either *robo2* or *robo3* expression provided by two copies of a *UAS* transgene also prevent commissure formation.

In the absence of Comm, commissural growth cones express high levels of Robo from the start and so are unable to cross the midline. Conversely, overexpression of Comm reduces Robo levels and so mimics either the *robo* mutant phenotype (at moderate levels of overexpression; Kidd et al., 1998b) or the *slit* mutant phenotype (at high levels; Kidd et al., 1999).

The identification of two additional Robo receptors expressed on CNS axons raises the question of whether they too are subject to such tight spatial regulation, and if so, whether this control is exerted by Comm. We show here that, as for Robo, so too Robo2 and Robo3 levels are actively kept low on commissures. We also show that, when overexpressed at high levels, Comm downregulates not only Robo but also Robo2 and Robo3. This accounts for the *slit*-like phenotype that results. Surprisingly, however, there are strong indications that the endogenous Comm protein may not be required to keep Robo2 and Robo3 levels low on commissures. We therefore propose that, just as the three Robos have both distinct and overlapping functions in controlling pathway choices within the CNS, their protein levels might also be regulated by distinct (but possibly also overlapping) mechanisms.

Results

A Gain-of-Function Genetic Screen for Midline Crossing Defects

Axonal growth cones navigate by constantly assessing the relative balance of multiple attractive and repulsive guidance cues (Tessier-Lavigne and Goodman, 1996; Winberg et al., 1998). This is borne out by the many reported instances in which the loss of a single cue has only a minor effect on pathway choices, while ectopic or increased levels of the same signal dramatically alter axonal trajectories. We therefore decided that a gain-of-function approach might reveal important components of the midline crossing decision that had escaped detection in the extensive loss-of-function screens that had previously been performed (Seeger et al., 1993; Hummel et al., 1999a). To facilitate such gain-of-function genetic screens, Rørth and colleagues have generated a set of 2300 random insertions of a P element transposon, the *EP* element, that places any 3′ flanking gene under the control of the GAL4 transcriptional activator (Rørth et al., 1998). We screened these *EP* lines for midline guidance defects using the *slit-GAL4* driver to force strong expression of any flanking gene in midline cells and the *1407-GAL4* driver to force expression in neurons. We identified one insertion, *EP2582*, that, when combined with either *1407-GAL4* or another neuronal driver, *elav-GAL4*, results in a severe reduction or the complete absence of commissures in most segments (Figure 1B).

That fewer axons cross the midline in these embryos might be due either to a loss of commissural neurons or to a misrouting of commissural axons. Using a variety of neuronal cell fate markers, we were unable to detect any cell fate changes in the ventral nerve cord of *elav-GAL4*, *EP2582* embryos. In particular, the commissural neurons stained with Even-skipped and Engrailed antibodies are present in their normal positions. We therefore conclude that, in *elav-GAL4*, *EP2582* embryos, commissural neurons are formed but are unable to extend axons toward or across the midline. This misrouting of commissural axons requires the midline repellent Slit: in a *slit* mutant background, forced expression of the gene flanking *EP2582* is no longer able to prevent axons from entering the midline, and the entire axon scaffold collapses onto the midline just as it normally does in *slit* mutants (Figure 1C).

Overexpression of Robo2 or Robo3 Prevents Midline Crossing

To determine the identity of the gene placed under GAL4 control in the *EP2582* line, we isolated genomic DNA flanking the insertion site. Sequence analysis revealed that the *EP2582* element was inserted in the 5′ untranslated region of the *robo2* gene. The further molecular characterization of *robo2* and the closely linked *robo3* gene, as well as the isolation of loss of function muta-

tions in both genes, is reported in the companion paper (Rajagopalan et al., 2000).

We were at first surprised that overexpression of *robo2* from the *EP2582* insertion prevents axons from crossing the midline, as Kidd et al. (1998a) had previously reported that overexpression of *robo* from a *UAS* transgene does not. However, it is important to note that the *EP* element drives expression of the endogenous gene from a promoter containing 14 GAL4 binding sites, while the *UAS* transgene drives expression of a cDNA from a promoter containing only five GAL4 binding sites. The experiments therefore cannot be directly compared. When we generated similar *UAS* transgenes for *robo2* and *robo3*, we found that, just like *UAS-robo*, they too fail to prevent commissure formation when expressed as single copy transgenes together with the *elav-GAL4* driver. However, two copies of either the *UAS-robo2* or *UAS-robo3* transgenes are sufficient to prevent axons from crossing the midline (Figures 1D and 1E), as Kidd et al. (1999) have since shown to be the case also for *UAS-robo*. Thus, all three Robos, when overexpressed under identical conditions, are similarly potent in preventing midline crossing.

Robo2 Is Expressed on Pioneer Growth Cones

These gain-of-function experiments demonstrate that both Robo2 and Robo3 can respond to the short-range repulsive signal provided by Slit at the midline. Furthermore, both Robo2 and Robo3 are expressed on CNS growth cones and bind to Slit (Rajagopalan et al., 2000). Both Robo2 and Robo3 are therefore both strong candidates to mediate some of the growth cone responses to Slit at the midline. In particular, we wondered whether Robo2 or Robo3 might account for some of the differences in growth cone behavior between *slit* and *robo* mutants. The clearest examples of this are the pCC and aCC growth cones. In wild-type embryos, the pCC growth cone pioneers an ipsilateral pathway close to the midline and the aCC motorneuron projects laterally away from the midline to pioneer the intersegmental nerve. In *robo* mutants, the pCC growth cone is redirected across the midline to the contralateral side (Seeger et al., 1993). The aCC growth cone usually follows its normal path away from the midline in *robo* mutants, but at a very low frequency it crosses the midline to exit the CNS on the opposite side (Seeger et al., 1993; Wolf and Chiba, 2000). pCC also projects toward the midline in *slit* mutants, but rather than continuing across as it does in *robo* mutants, the pCC growth cone instead remains at the midline (Kidd et al., 1999). In addition, aCC projects across rather than directly away from the midline with much higher frequency in *slit* mutants than it does in *robo* mutants (Kidd et al., 1999). These different behaviors of the pCC and aCC growth cones in *slit* versus *robo* mutants indicate that they can respond to Slit using some receptor other than Robo. We were therefore interested in determining whether Robo2 and/or Robo3 is expressed on the pCC and aCC growth cones.

Late stage 12 and early stage 13 embryos were stained for Robo2 or Robo3 and counterstained with anti-Fasciclin II (Fas II) mAb 1D4 (van Vactor et al., 1993) to visualize the pCC and aCC axons. At this stage, the first commissural axons have already crossed the midline and the pCC and aCC growth cones are pioneering their respective longitudinal and motor pathways. Robo2 expression at this stage is largely coincident with Robo, being absent on the commissural pioneers but expressed at high levels on growth cones pioneering the ipsilateral pathways (Figure 2A). Notably, Robo2 can be detected at high levels on the aCC growth cone and at somewhat lower levels on the pCC growth cone (Figures

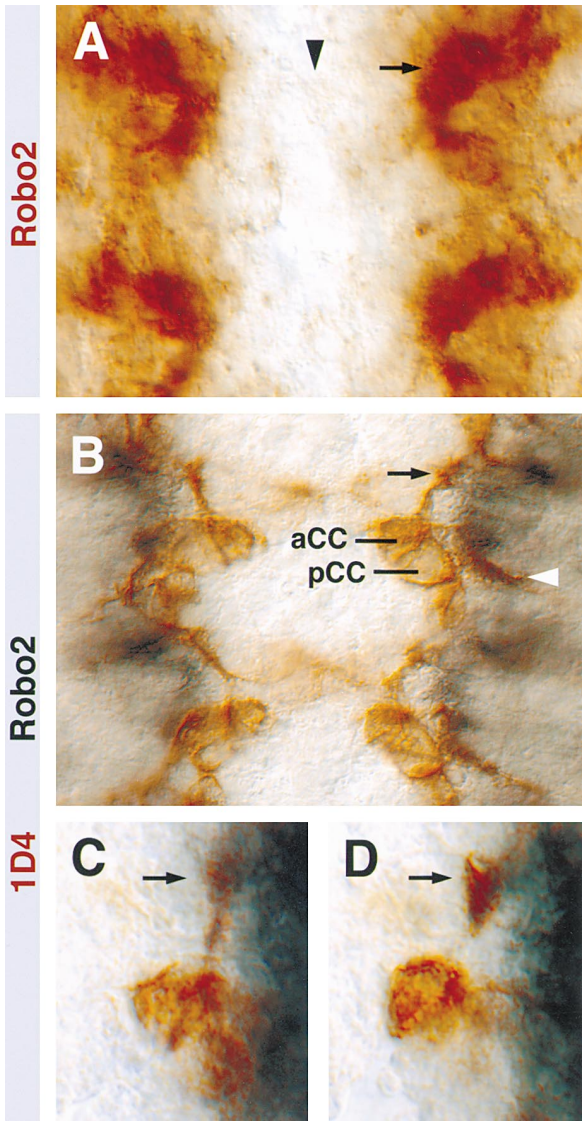

Figure 2. Expression of Robo2 on Pioneer Growth Cones

CNS of late stage 12 embryos stained with anti-Robo2 (brown in [A] and black in [B–D]) and mAb 1D4 (anti-Fas II, brown in [B–D]).

(A) As the first axon pathways are pioneered, Robo2 is expressed at high levels on longitudinal growth cones (arrow) but is undetectable on commissural growth cones (arrowhead).

(B) Double staining with 1D4 reveals the pCC and aCC axons. Low levels of Robo2 are seen on the pCC growth cone (arrow). Higher levels can be detected on other growth cones, including that of the aCC motorneuron (arrowhead).

(C and D) Higher magnification view of embryos double stained with 1D4 and anti-Robo2, as in (B), but this time overstained for Robo2. Robo2 protein is now clearly visible on the pCC growth cone (arrows). The pCC cell body is below the focal plane in (D).

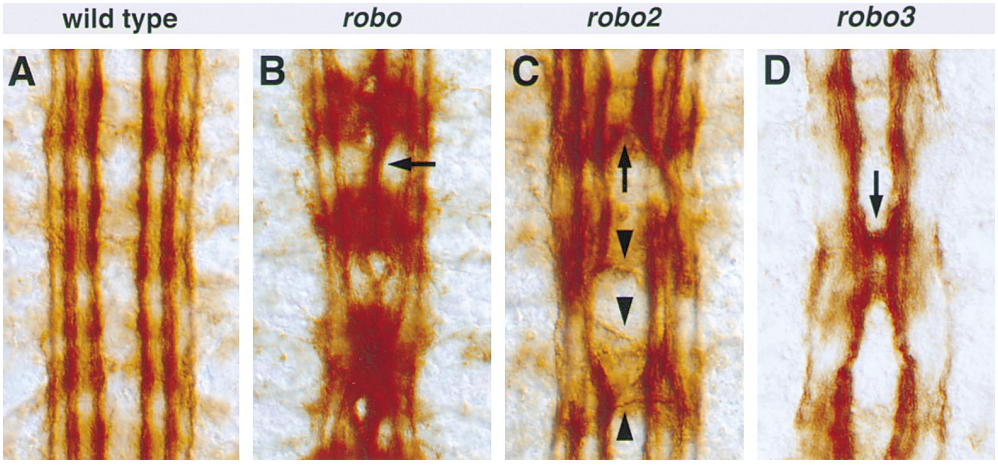

Figure 3. Midline Crossing Defects in *robo*, *robo2*, and *robo3* Single Mutant Embryos

CNS of stage 16 embryos stained with the anti-Fas II mAb 1D4. (A), wild-type; (B), *robo*[1]; (C), *robo2*[1]/*robo2*[4]; and (D), *robo3*[1].

(A) In wild-type embryos, staining with 1D4 reveals three longitudinal fascicles on each side of the midline.

(B) In *robo* mutant embryos, the innermost fascicles from each side of the midline generally combine to form a single bundle that meanders back and forth across and along the midline (arrow).

(C) In *robo2* mutants, such midline crossing errors are much less frequent. This embryo is a relatively rare case in which Fas II–positive axons are misrouted across in the midline in each of three adjacent segments. These axons generally take a more direct route across the midline and form thinner bundles than those observed in *robo* mutants. The arrowheads indicate bundles scored as "thin" for the quantification in Table 1; the bundle indicated with the arrow (slightly above the focal plane) was scored as "normal."

(D) A rare instance of Fas II–positive axons crossing the midline in the *robo3* mutant (arrow).

2B–2D). Robo3 could not be detected on any growth cones at this stage; not until late stage 13 are there detectable levels of Robo3 on longitudinal growth cones (Rajagopalan et al., 2000).

The Projections of pCC and aCC Are Normal in *robo2* and *robo3* Mutants

These expression patterns suggested that Robo2 rather than Robo3 might mediate some of the Robo-independent responses to Slit in the pCC and aCC growth cones. In addition, it seemed likely that both Robo2 and Robo3 might function in other longitudinal growth cones to keep them away from the midline.

To test these possibilities, we first examined the projections of the pCC and aCC axons in *robo2* and *robo3* mutants in late stage 12 and early stage 13 embryos using the 1D4 marker. At this stage in *slit* mutants, staining with 1D4 captures the pCC and aCC growth cones as they set off on their aberrant projections toward the midline (Kidd et al., 1999). In over 100 hemisegments of stage 12 and 13 *robo2* and *robo3* embryos stained with 1D4, we did not observe a single pCC or aCC growth cone straying from its normal pathway. These results provided the first indication that there may be some functional redundancy among the Robo receptors, an issue to which we will return shortly.

Midline Crossing Errors in *robo2* and *robo3* Mutants

Although we could not detect any midline crossing defects by these pioneer axons at early stages, staining with 1D4 did reveal a low frequency of crossing errors at later stages in both *robo2* and *robo3* mutant embryos. In stage 16–17 embryos, 1D4 labels three longitudinal fascicles on each side of the midline (Figure 3A). The first of these Fas II–positive fascicles, the pathway pioneered by the pCC growth cone, extends tightly alongside the midline. In *robo* mutant embryos, this first Fas II fascicle in particular wanders back and forth across and along the midline in every segment (Figure 3B; Table 1; Seeger et al., 1993). The second and third fascicles lie closer to the midline, and occasionally some of these axons also venture across it. At this stage, with many more ipsilaterally projecting axons to examine, we could now detect a low level of aberrant midline crossing in both *robo2* and *robo3* mutants: Fas II–positive bundles extended across the midline in ~25% of segments examined in *robo2* mutants and in 7% of segments in *robo3* mutants (Figures 3C and 3D; Table 1). These misrouted axon bundles are not only less frequent but also generally much thinner than those observed in *robo* mutants (Table 1). Their passage across the midline is also far more fleeting: axons labeled by 1D4 casually meander across and along the midline in *robo* mutants, but in *robo2* and *robo3* mutants the few misrouted axons take a direct route straight across the midline.

The low frequency of these crossing errors makes them difficult to detect at the level of single identified neurons. We therefore cannot determine whether the axons that stray across the midline in *robo2* and *robo3* mutants are ipsilateral axons that cross or commissural axons that recross. Most likely both types of error occur in these mutants, just as they do in *robo* mutants.

We also used a panel of specific neuronal markers to examine cell fate specification in the ventral nerve cords of both *robo2* and *robo3* mutant embryos (see Experimental Procedures). All markers examined showed the wild-type staining pattern. Importantly, Slit and both of the remaining Robo receptors are expressed at normal levels in *robo2* and *robo3* embryos.

Table 1. Midline Crossing Defects in *robo*, *robo2*, and *robo3* Mutants

Genotype	Segments Scored	Segments with Fas II–Positive Axon Bundles Crossing Midline (%)			
		Thin	Normal	Thick	Total
Wild-type	133	0	0	0	0
$robo^1/robo^1$	144	0	0	100.0	100.0
$robo2^1/robo2^4$	202	8.9	6.4	7.9	23.3
$robo2^8/robo2^8$	148	6.1	10.9	8.1	25.0
$robo3^1/robo3^1$	235	1.7	2.6	3.0	7.2

Stage 16–17 embryos were stained with anti-Fas II mAb 1D4, dissected, and examined for segments in which any labeled axons inappropriately crossed the midline. Fas II–positive axon bundles at the midline were scored as thin, normal, or thick by comparison to the normal longitudinal Fas II fascicles.

Axons Linger at the Midline in *robo,robo2* Double Mutants

In *slit* mutants, axons linger at the midline, whereas in each of the *robo*, *robo2,* and *robo3* single mutants those axons that enter the midline— whether appropriately or not—continue across to the contralateral side. As there is no other known Slit receptor encoded in the *Drosophila* genome, we suspected that two or perhaps all three Robo receptors may share the responsibility of forcing commissural axons through the midline. To test this, we generated both *robo,robo2* and *robo,robo3* double mutants. The close proximity of the *robo2* and *robo3* genes has thus far hindered our efforts to recover a *robo2,robo3* recombinant.

We first examined the projections of the pCC and aCC neurons in these double mutant embryos. In early stage 13 *slit* mutant embryos stained with 1D4, the pCC axon has already reached the midline, where it meets and fasciculates with its contralateral homolog and then extends anteriorly on a pathway right along the midline. In many segments, the aCC axons also extend toward the midline (Figure 4A; Kidd et al., 1999). The pCC and aCC growth cones behave in exactly the same way in *robo,robo2* double mutants (Figure 4E). In contrast, *robo,robo3* double mutants appear identical to *robo* single mutants at this early stage; aCC axons project normally, but sluggishly, away from the midline, while the two misrouted pCC axons meet at the midline but then continue on their opposing paths across it (Figure 4I).

By stage 16 in *slit* mutant embryos, all CNS axons have converged to form a single axon bundle at the midline (Figures 4B and 4C; Rothberg et al., 1990; Kidd et al., 1999). This is also the case in *robo,robo2* double mutant embryos (Figures 4F and 4G). In *robo,robo3* double mutants stained with 1D4, axons of both the first and second Fas II fascicles project across and along the midline, while the third fascicle remains for the most part the same distance from the midline as in the *robo* single mutant (Figure 4J).

These data reveal an important difference between the two double mutants. The *robo,robo3* double mutant phenotype represents the mere addition of the two single mutant phenotypes. In contrast, the *robo,robo2* double mutant shows defects that are not observed in either single mutant but do occur in *slit* mutants. Robo and Robo2 therefore share some functions in midline guidance; Robo and Robo3 do not.

slit-like Defects in the Mesoderm of *robo,robo2* Double Mutants

Not only axonal growth cones but also muscle precursors rely on Slit to repel them away from the midline. Following gastrulation, myoblasts migrate away from the ventral midline over the dorsal surface of the CNS. Some of the ventral muscles later extend back toward the midline to attach themselves to the ventral body wall beneath the developing CNS, still some distance from the midline. In *slit* mutant embryos, many muscles stretch abnormally across the dorsal surface of the CNS (Figure 4D; Kidd et al., 1999). This same muscle phenotype is also seen in *robo,robo2* double mutants (Figure 4H), but not in *robo,robo3* double mutants (Figure 4L) or any of the three single mutants (Kidd et al.,1999; E. N. and V. V., unpublished data).

Robo2 and Robo3 Are Downregulated on Commissures

Like Robo, both Robo2 and Robo3 accumulate specifically on longitudinal axon segments but remain at barely detectable levels on commissures (Figure 2; see also Figure 2 of Rajagopalan et al., 2000). For Robo, it has been demonstrated that panneural expression does not alter this pattern (Kidd et al., 1998a). To determine whether this is also the case for Robo2 and Robo3, we prepared *UAS* transgenes in which coding sequences for either *robo*, *robo2*, or *robo3* were fused to heterologous 5′ and 3′ untranslated sequences. The 5′ coding sequences for each gene were additionally modified in order to tag each protein with an amino-terminal HA epitope, allowing us to directly compare the expression patterns for each protein. Embryos carrying a single copy of one of these *UAS* transgenes, together with an *elav-GAL4* driver, were stained with anti-HA antibodies. Not only Robo, but also Robo2 and Robo3, remained confined to longitudinal axon segments (Figure 5). The tight spatial regulation of Robo2 and Robo3 is dramatically revealed by the fact that their expression domains now expand medially across the entire width of the longitudinal tract but end abruptly at the commissures.

Overexpressed Comm Downregulates Robo2 and Robo3

Robo protein levels are negatively regulated by Comm (Tear et al., 1996; Kidd et al., 1998b). Does Comm also regulate Robo2 and Robo3 protein levels? That this

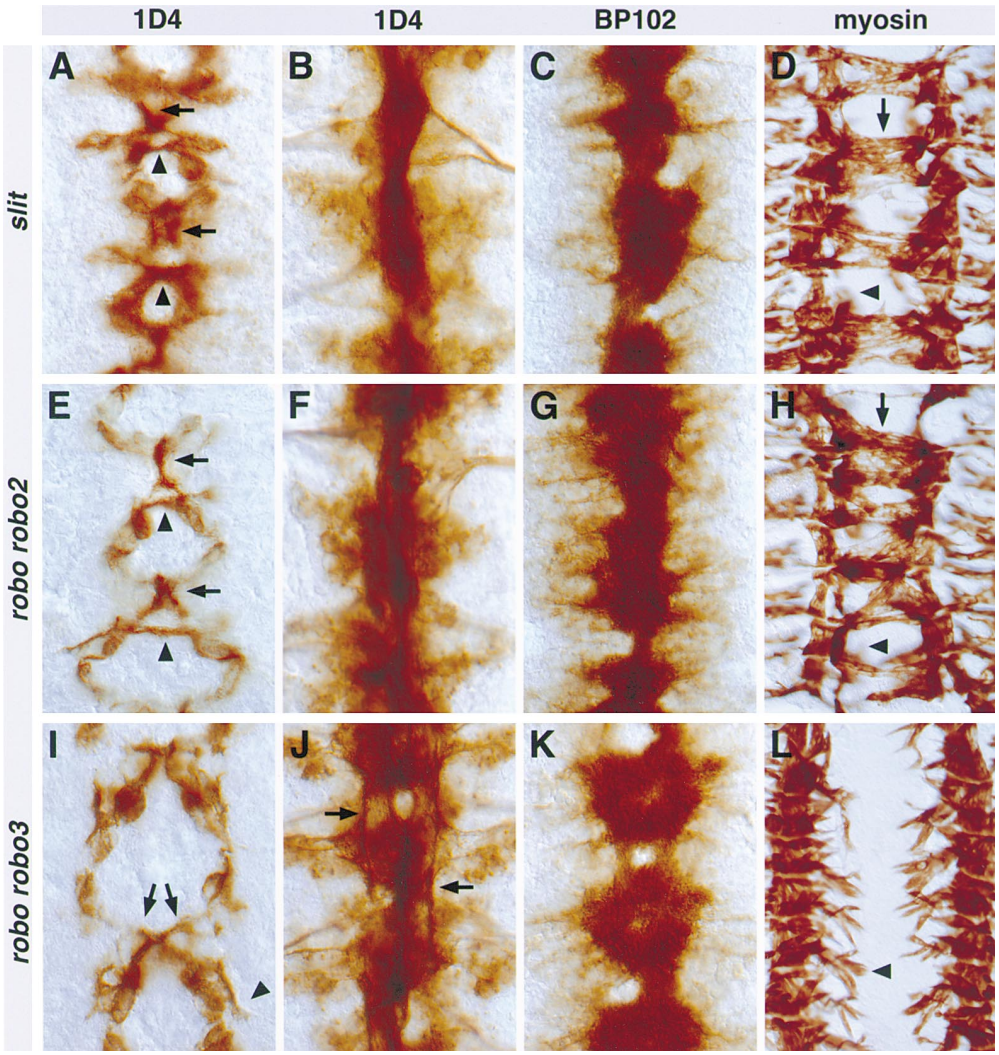

Figure 4. Midline Repulsion Defects in *slit* Single Mutant Embryos and *robo,robo2* and *robo,robo3* Double Mutants

(A–D) *slit*^{2}, (E–H) *robo*^{1},*robo2*^{5}, and (I–L) *robo*^{1},*robo3*^{1} embryos stained with mAb 1D4 (A, B, E, F, I, and J), mAb BP102 (C, G, and K), or mAb FMM5 against muscle myosin (D, H, and L). (A, E, and I) show early stage 13 embryos; the remaining panels show embryos at stage 16. Embryos stained for myosin are shown at low magnification.

(A, E, and I) The pCC growth cone, which normally extends anteriorly and slightly lateral (see Figure 2), extends abnormally toward the midline in *slit* single mutants and both *robo,robo2* and *robo,robo3* double mutants. In *slit* and *robo,robo2* embryos, the pCC axon fasciculates with its contralateral homolog to extend anteriorly right along the midline (arrows in [A] and [E]; the entire pCC axon does not lie in the same focal plane in the lower segment shown in [A]). In *robo,robo3* embryos, the pCC growth cones continue across the midline (arrows in the lower segment of [I]; in the upper segment the two pCC growth cones are only just beginning to interact). The aCC axons also frequently cross the midline in *slit* and *robo,robo2* embryos (arrowheads in [A] and [E]), while in *robo,robo3* aCC projects away from the midline, although often not as robustly as in wild-type embryos (arrowhead in [I], compare to Figure 2B).

(B, F, and J) By stage 16, all axons have joined the single midline bundle in *slit* and *robo,robo2* embryos. In *robo,robo3* embryos, the fused first and second FasII fascicles wander back and forth across the midline as the first fascicle does in *robo* single mutants, while the third FasII fascicle still avoids the midline (arrows in J).

(C, G, and K) The midline collapse phenotype in *slit* and *robo,robo2* mutants, as revealed by the BP102 marker. Stained with BP102, the *robo,robo3* double mutant more closely resembles the *robo* single mutant.

(D, H, and L) Ventral muscles normally attach to the epidermis beneath the lateral CNS. In both *slit* and *robo,robo2* mutants, these muscles frequently extend across the dorsal surface of the CNS (arrows in [D] and [H]). Some muscles do still attach beneath the CNS, though much closer to the midline than usual (arrowheads indicate such attachments below the focal plane in [D] and [H]). In *robo,robo3* mutants, as in *robo* single mutants, ventral muscles only very rarely stretch across the dorsal CNS, attaching themselves instead correctly beneath the CNS though often somewhat closer to the midline than usual (arrowhead in [L]). Note that (L) is focused on the ventral body wall while (D) and (H) are focused on the dorsal CNS.

would be the case was largely anticipated by the fact that overexpression of Comm at high levels throughout the CNS results in a phenotype identical to that of *slit* mutants (Kidd et al., 1999), which we have found to be mimicked in turn by loss of both Robo and Robo2. To test this prediction, we examined Robo2 and Robo3 levels in such Comm overexpression embryos. As expected, not only Robo staining but also Robo2 and

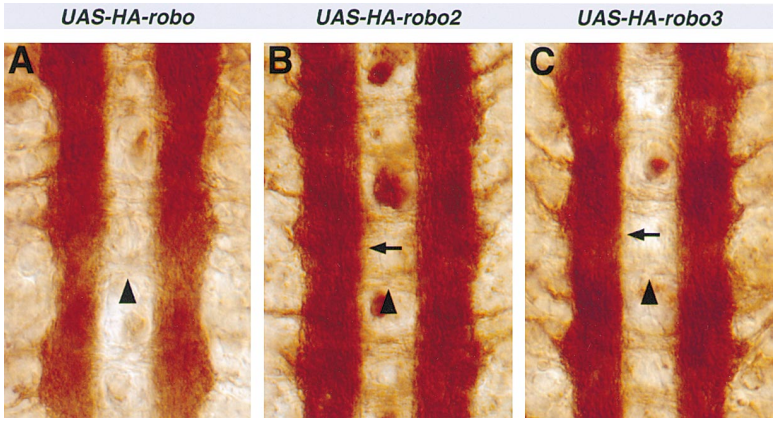

Figure 5. Robo2 and Robo3 Are Downregulated on Commissures

Stage 16 embryos carrying an *elav-GAL4* driver and either a (A) *UAS-HA-robo*, (B) *UAS-HA-robo2*, or (C) *UAS-HA-robo3* transgene, stained with anti-HA. At the relatively low levels of panneural expression provided by the *UAS* transgenes, none of the three *robos* prevents normal commissure formation. Each of the HA-tagged Robo proteins accumulates at high levels on longitudinal axon tracts but still remains at low levels on commissural axon segments (arrowheads). Note that the Robo2 and Robo3 expression domains have expanded medially to span the entire width of the longitudinal tract (arrows).

Robo3 staining is greatly reduced within the CNS (Figure 6). In the periphery, where Comm is not expressed, Robo and Robo2 levels remain unaltered.

Spatial Regulation of Robo2 and Robo3 May Not Require Comm

These Comm misexpression experiments demonstrate that, when expressed at high levels, Comm can downregulate not only Robo but also Robo2 and Robo3. But is this also an essential function of the endogenous Comm protein? This is a difficult issue to address. Ideally, one would like to know whether, in a *comm* mutant, Robo2 and Robo3 are still excluded from commissures. But as there are no commissures in a *comm* mutant, this cannot be directly tested. Commissures do however form in the *comm* mutant provided *robo* function is also removed. Indeed, too many axons cross and recross the midline in *robo;comm* double mutants just as they do in *robo* single mutants (Figure 7D; Seeger et al., 1993). We therefore examined Robo2 and Robo3 expression in *robo;comm* double mutant embryos. Despite the excessive midline crossing in these embryos, both Robo2 and Robo3 are still confined to longitudinal axon segments (Figures 7A–7C).

We performed two additional experiments to further explore the question of whether Comm might regulate Robo2 levels. First, if Comm were required to downregulate Robo2 and Robo3, then we might expect fewer axons to cross the midline in *robo;comm* double mutants than in *robo* single mutants. To address this, we examined these two genotypes with the 1D4 antibody, a sensitive marker for midline crossing defects. We could not detect any differences between the two genotypes. In particular, midline crossing is just as rampant in the *robo;comm* double mutant as it is in the *robo* single mutant.

In a final experiment, we asked if the lack of commissures in the *comm* mutant might be due not only to elevated Robo levels but also to elevated Robo2 levels. If so, one might expect to find a significant number of axons crossing the midline in *robo2;comm* double mutant embryos. This is not the case. When stained

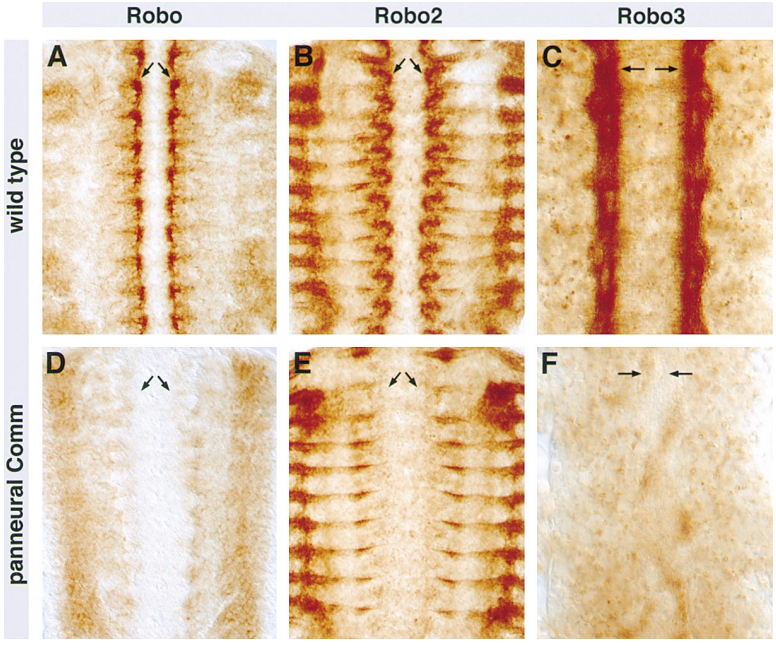

Figure 6. Comm Overexpression Reduces Levels of All Three Robos

Wild-type (A–C) and *elav-GAL4/UAS-comm* (D–F) embryos were stained in parallel for Robo, Robo2, and Robo3. The *UAS-comm* transgene used provides high levels of Comm expression, resulting in a *slit*-like phenotype. Robo (A and D) and Robo2 (B and E) expression are shown at stage 13. At this stage, the peripheral expression provides an internal control. Panneural Comm does not alter Robo and Robo2 protein levels in the periphery but dramatically reduces the levels of both Robos within the CNS (arrows). For Robo3 there is no such internal control, and so these embryos are shown at stage 16 when Robo3 levels are highest in wild-type embryos (C). In Comm overexpression embryos (F), only very low levels of Robo3 protein can be detected on CNS axons, which have now converged at the midline (arrows).

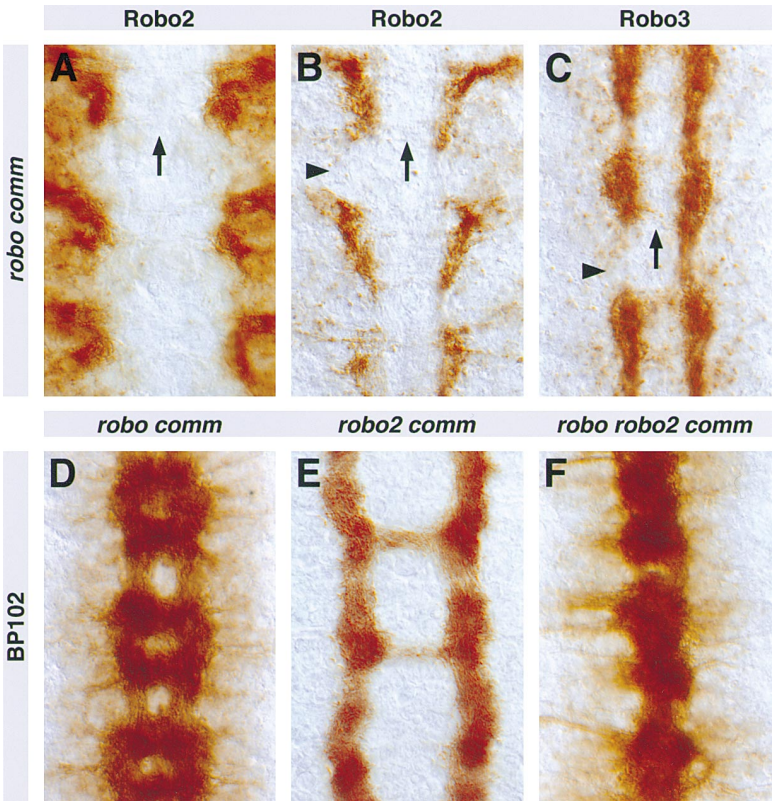

Figure 7. Evidence that Robo2 and Robo3 Can Still Be Regulated in the Absence of Comm

(A–D) *robo*^{1};*comm*^{5}, (E) *robo2*^{5};*comm*^{1}, and (F) *robo*^{1},*robo2*^{5};*comm*^{1} embryos stained with (A and B) anti-Robo2, (C) anti-Robo3, or (D–F) mAb BP102. (A) shows a stage 13 embryo; (B–F) are stage 16 embryos. Only very low levels of Robo2 and Robo3 can be detected on commissures in *robo;comm* embryos (arrows in A–C). For Robo2, this is particularly striking at early stages, when both *robo* and *robo2* mRNA expression is ubiquitous and the distribution patterns of the two proteins are almost identical. In some segments at later stages, Nomarski optics reveals a complete break in the longitudinal tract (arrowheads in [B] and [C]), suggesting that in these segments in particular many more axons cross the midline. Even in these segments, Robo2 and Robo3 levels still remain low on commissures. Stained with BP102 to reveal all axons, *robo;comm* (D) appears identical to *robo*, but *robo2;comm* (E) very closely resembles *comm*. The *robo,robo2;comm* mutant (F) is indistinguishable from *slit* and *robo,robo2*.

with mAb BP102, the *robo2;comm* double mutant very closely resembles the *comm* single mutant (Figure 7E). In other words, whereas *robo* is epistatic to *comm*, *comm* is epistatic to *robo2*. The failure of axons to cross the midline in *comm* mutants is therefore due almost entirely to the action of Robo, not of Robo2, lending further support to the hypothesis that Comm is not required for Robo2 regulation. Nevertheless, in some segments we did detect a small number of axons crossing the midline in *robo2;comm* embryos. It is therefore possible that a few axons do indeed fail to cross the midline in *comm* mutants in part due to the action, though not necessarily the elevated levels, of Robo2.

Taken together, these data strongly suggest that Comm is not strictly required for the downregulation of Robo2 and Robo3 on commissures. This conclusion must however be considered tentative pending the availability of specific axonal markers that will allow us to confirm that the axons of individual *robo2*- or *robo3*-expressing neurons do indeed cross the midline in *robo;comm* embryos.

We also examined *robo,robo2;comm* triple mutants (Figure 7F) and *slit;comm* double mutants with BP102. As expected, but in contrast to a previous report for the *slit;comm* double (Hummel et al., 1999b), both combinations are phenotypically indistinguishable from *slit* single mutants.

Discussion

Distinct and Overlapping Roles for Robo Receptors

In *robo* mutant embryos, axons freely cross and recross the midline (Seeger et al., 1993). In *slit* mutants, axons enter the midline and stay there (Rothberg et al., 1990; Battye et al., 1999; Kidd et al., 1999). At first, these two phenotypes seemed so distinct that the link between the two genes was not apparent, even once their products became known. An astute genetic insight led Kidd et al. (1999) to the discovery that Slit is the ligand for the Robo receptor, raising the question of why mutations in the genes encoding a ligand and its receptor would result in such different mutant phenotypes. This paradox is now resolved with the identification of two additional Slit receptors, Robo2 and Robo3, and the demonstration that one of them, Robo2, shares some of Robo's tasks in controlling axon traffic at the midline.

Genetic studies have defined four functions for the midline repellent Slit in shaping axonal pathways in the CNS: as a short-range signal, Slit prevents ipsilateral axons from crossing the midline, prevents commissural axons from lingering at the midline, and prevents axons that have crossed the midline from turning back to cross again (Rothberg et al., 1990; Seeger et al., 1993; Kidd et al., 1998a; Battye et al., 1999; Kidd et al., 1999). Slit also acts at a distance to control lateral pathway choices (Rajagopalan et al., 2000; Simpson et al., 2000). These four functions of Slit are mediated by different combinations of Robo receptors. In the companion paper (Rajagopalan et al., 2000), we show that long-range patterning of the longitudinal tracts is mediated by the distinct actions of Robo2 and Robo3. In this paper, we have investigated the roles of Robo2 and Robo3 in the short-range guidance decisions at the midline. Our analysis of midline guidance decisions in *robo2* and *robo3* single mutants, as well as *robo,robo2* and *robo,robo3* double mutants, has revealed an interesting pattern of distinct

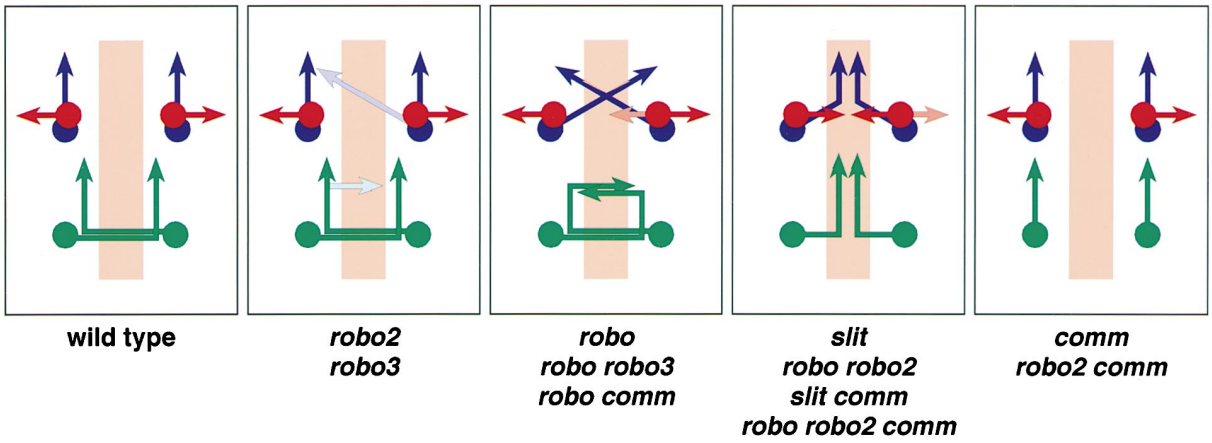

Figure 8. Short-Range Responses to Slit at the Midline

Schematic diagram showing the behavior of axons at the midline in single, double, and triple mutant combinations for *slit*, *robo*, *robo2*, *robo3*, and *comm*. The midline is represented by the thick orange bar. Typical pathway choices are shown for an ipsilateral interneuron (blue), a commissural interneuron (green), and an ipsilateral motorneuron (red). These neurons are exemplified by pCC, SP1, and aCC, respectively, although these specific neurons have not been examined in each case. In wild-type embryos, the axons of ipsilateral interneurons never cross the midline, while the axons of commissural interneurons cross only once. Ipsilateral motorneurons project their axons away from the midline and out of the CNS. In *robo2* and *robo3* mutants, some axons that should avoid the midline instead project across it. These defects are rare in *robo2* mutants and rarer still in *robo3* mutants, making them difficult to detect for single neurons. In *robo* mutants, these midline crossing errors are far more frequent. At a very low frequency, ipsilateral motoraxons also project across the midline. In a *robo* mutant background, additional loss of *robo3* is without phenotypic consequence (since it only acts to ensure the fidelity of the response to Robo), nor does loss of *comm* function (since it is only required to downregulate Robo). In both *slit* and *robo,robo2* mutants, both ipsilateral and commissural interneurons grow toward and then linger at the midline. Ipsilateral motoraxon axons also often project toward the midline, usually continuing across it to exit the CNS on the opposite side. Additional loss of *comm* function does not alter the phenotype of these mutants. In *comm* mutants, no axons cross the midline. This is due to the action of Robo, not Robo2, and so the *comm* phenotype is unaltered when *robo2* function is also removed.

and overlapping functions for the three receptors (Figure 8).

Midline crossing errors occur in all three single mutants and so all three Robo receptors have important functions in keeping longitudinal axons out of the midline. However, these crossing defects are far less frequent in *robo2* than in *robo* mutants and rarer still in *robo3* mutants. The decision to cross or not to cross the midline is therefore controlled primarily by Robo, but Robo2 and Robo3 are also required to ensure the fidelity of this decision. In both the *robo* and *robo2* single mutants, axons that enter the midline continue across it. Only in the *robo,robo2* double mutant do axons linger at the midline as they do in *slit* mutants. Robo and Robo2 therefore act redundantly in mediating this antilinger response to Slit. The *robo,robo2* double mutant also reveals other redundant functions of the two receptors, including their roles in directing the migration of both myoblasts and some motorneuron growth cones away from the midline. Robo3, in contrast, does not share any redundant functions with Robo, as the *robo* and *robo3* single mutant phenotypes are combined additively in the *robo,robo3* double mutant.

Why Can Robo2 Prevent Lingering but Not Crossing?

One puzzling conclusion that we draw from these studies is that, in the absence of Robo, low levels of Robo2 are sufficient to prevent commissural axons from lingering at the midline but not to prevent either ipsilateral axons from crossing nor commissural axons from recrossing. For example, the pCC growth cone expresses both Robo and Robo2. In a *robo* mutant, Robo2 alone is not able to keep the pCC growth cone from entering the midline but is sufficient to drive it through the midline. How might this be explained?

One possibility would be that Robo2 is a specialized antilinger receptor that issues a qualitatively different signal from Robo: a "get out" rather than a "keep out" signal. In controlling lateral positioning, Robo2 does indeed transduce a qualitatively different signal from Robo (Rajagopalan et al., 2000; Simpson et al., 2000). We do not think, however, that this is also the case for their short-range responses to Slit at the midline. First, midline crossing errors do occur in *robo2* mutants, so Robo2 cannot just be a dedicated antilinger receptor. Second, when overexpressed, Robo2 is as potent as Robo in issuing a "keep out" instruction.

Assuming then that Robo and Robo2 do provide qualitatively similar repulsive signals at the midline, we must conclude that only low levels of repulsion are needed to prevent growth cones from lingering at the midline, while high levels are necessary to keep them out. There are two possible explanations for this. Experiments in grasshopper embryos suggest that the passage out of the midline might be facilitated by fasciculation with the axon of the contralateral homolog (Myers and Bastiani, 1993), though as the behavior of the pCC axons in *slit* and *robo,robo2* mutants demonstrates, fasciculation with the contralateral homolog is not in itself sufficient. Another and perhaps more likely possibility is suggested by experiments in rodents, demonstrating a loss of sen-

sitivity to attractive cues once axons reach the midline (Shirasaki et al., 1998). This remains to be demonstrated in *Drosophila*, but our data lend strong support to the idea that growth cones that have not yet reached the midline are indeed more strongly attracted to it than those that have.

Spatial Regulation of Robo Receptors

Tight spatial regulation is exerted on the levels of all three Robos. Robo levels are kept low on commissural growth cones that cross the midline but then rise dramatically as these growth cones emerge on the opposite side (Kidd et al., 1998a). Strong panneural expression does not alter this pattern: Robo is still excluded from commissural axon segments (Kidd et al., 1998a). Here and in the companion paper (Rajagopalan et al., 2000), we show that Robo2 and Robo3 levels are also spatially regulated. Both are low on commissures and high on a laterally restricted subset of longitudinal axons. In this paper, we have also shown that, as for Robo, panneural expression of *robo2* or *robo3* does not raise their protein levels on commissures, even though it results in high expression levels on axons across the entire longitudinal tract. This demonstrates that, as for Robo, there must also be some mechanism, most likely posttranslational, that prevents the accumulation of Robo2 and Robo3 on commissural axon segments.

The nature of this regulatory mechanism is presently unknown. It may involve regulation of protein synthesis, stability, transport, insertion, or internalization. The only thing that is clear at present is that Robo levels are negatively regulated by the transmembrane protein Comm. The evidence for this is compelling. In wild-type embryos, Comm levels are high on commissures where Robo is low and low on longitudinal axons where Robo is high (Tear et al., 1996; Kidd et al., 1998a, 1998b). If Comm levels are experimentally raised, Robo levels are reduced and more axons cross the midline (Kidd et al., 1998b; Bonkowsky et al., 1999). If Comm is reduced (in a *comm* hypomorph), fewer axons cross the midline, and those that do so express higher levels of Robo (Kidd et al., 1998b).

Naturally, we wondered whether Robo2 and Robo3 might also be regulated by Comm. They too are low where Comm is high, and vice versa. We therefore first asked whether high levels of Comm would also downregulate Robo2 and Robo3. This proved to be the case. However, this does not necessarily mean that the endogenous Comm protein is responsible for keeping Robo2 and Robo3 levels low on commissures. Indeed, and to our surprise, it seems that Comm may not be required to regulate Robo2 and Robo3. The evidence for this is as follows: first, in *robo;comm* double mutant embryos, in which many axons cross and recross the midline despite the loss of *comm* function, both Robo2 and Robo3 are still restricted to longitudinal axon segments. Second, in a *robo* mutant background, removing *comm* does not reduce the number of axons that inappropriately cross the midline, as one would expect if the loss of Comm resulted in increased Robo2 or Robo3 levels. And third, while *robo* is epistatic to *comm*, *comm* is epistatic to *robo2*. In other words, in a *comm* mutant, axons are prevented from crossing the midline due to the function of *robo*, not *robo2*. Together, these observations strongly suggest that regulation of Robo2 does not require Comm.

Nevertheless, if, as these data suggest, Robo2 and Robo3 are indeed regulated by a mechanism distinct from Comm, it must still be very similar in both its localization and mode of action. One intriguing possibility is that there is not only a family of *robo* genes in *Drosophila* but also a family of *comm* genes to regulate their protein levels. Indeed, we and our collaborators have identified two other *comm*-like genes in *Drosophila* (S. R., J. H. Simpson, T. Kidd, C. S. Goodman, and B. J. D., unpublished data) and found that at least one of these new Comms can also downregulate Robo2 when overexpressed (S. R., unpublished data). We therefore speculate that, just as the three Robos have distinct and overlapping functions, they may also be regulated by a set of Comm proteins that themselves have both distinct and overlapping functions. The specificity and mechanism of these regulatory interactions will be the focus of future studies performed in collaboration with the Goodman laboratory.

Evolutionary Diversification of Robo Receptors

The decision to cross or not to cross the midline is an ancient one. Even the most primitive bilaterally symmetric nervous systems are organized into an orthogon of commissural and longitudinal fibers (Samat and Netsky, 1981; Raikova et al., 1998). The system of Slit at the midline and Robo on CNS growth cones is also ancient, dating back at least to the separation of the vertebrate and invertebrate lineages. Of the three *Drosophila* Robos, it is clearly Robo itself that retains most of the features and functions of the ancestral Robo. Robo2 and Robo3 appear to be more recent additions to the family, quite possibly even being unique to the insect lineage. They have diverged both structurally and functionally, acquiring in the process a new role in controlling the lateral position of axons within the longitudinal pathways (Rajagopalan et al., 2000; Simpson et al., 2000). Robo3 is now largely dedicated to this role, while Robo2 still retains some of its original functions in controlling guidance decisions at the midline. A short-range repulsive mechanism that originally evolved to control guidance at the midline has thus more recently been coopted as a long-range mechanism to regulate lateral positioning. It would be interesting to examine the expression of Slit and Robo in the most primitive nervous systems and to try to reconstruct the steps that led to the recent diversification of the Robo family in *Drosophila*. Such studies might reveal some of the key events during nervous system evolution.

Experimental Procedures

The initial screen of the *EP* collection was performed using a *slit-GAL4* driver provided by Christian Klämbt and the *1407-GAL4* driver provided by Liqun Luo. Lines were initially screened for lethality in combination with either driver and then rescreened for CNS defects using mAb BP102. The *elav-GAL4* driver we used was generated by Aaron DiAntonio and provided by Corey Goodman. For the Comm overexpression experiments, a *UAS-comm* transgene was prepared by cloning the entire *comm* open reading frame, stripped of its 5′ and 3′ untranslated region, into the pUAST vector (Brand and Perrimon, 1993). A transgenic line providing particularly strong ex-

pression levels was selected, and *UAS-comm* and wild-type embryos fixed and stained in parallel using the same set of reagents. For all single, double, and triple mutant analysis, CyO,P[*wg-lacZ*] and TM3,P[*Ubx-lacZ*] balancers were used in order to genotype embryos. Cell fate specification in the ventral nerve cord was examined using anti-Engrailed mAb 4D9 (at a dilution of 1:2), anti-Even-Skipped (1:30), anti-Elav mAb F8A9 (1:30), and anti-Repo (1:500). mAb FMM5 against muscle myosin was used at 1:8 and mAb BP102 at 1:10. All other methods were as described in Rajagopalan et al. (2000).

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