Distinct Protein Domains and Expression Patterns Confer Divergent Axon Guidance Functions for *Drosophila* Robo Receptors

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SUMMARY

The orthogonal array of axon pathways in the *Drosophila* CNS is constructed in part under the control of three Robo family axon guidance receptors: Robo1, Robo2 and Robo3. Each of these receptors is responsible for a distinct set of guidance decisions. To determine the molecular basis for these functional specializations, we used homologous recombination to create a series of 9 “robo swap” alleles: expressing each of the three Robo receptors from each of the three *robo* loci. We demonstrate that the lateral positioning of longitudinal axon pathways relies primarily on differences in gene regulation, not distinct combinations of Robo proteins as previously thought. In contrast, specific features of the Robo1 and Robo2 proteins contribute to their distinct functions in commissure formation. These specializations allow Robo1 to prevent crossing and Robo2 to promote crossing. These data demonstrate how diversification of expression and structure within a single family of guidance receptors can shape complex patterns of neuronal wiring.

INTRODUCTION

Complex neuronal wiring patterns emerge during development as individual axons respond differently to a common set of extracellular guidance cues. A single guidance cue can trigger a variety of growth cone responses—attracting some axons while repelling others, or guiding some axons at a distance but others only upon direct contact with the source (Tessier-Lavigne and Goodman, 1996). How can a single guidance factor elicit such diverse responses? This question is usually answered by pointing out that most guidance molecules have multiple different receptors, that distinct receptors mediate distinct cellular responses, and that receptor expression is highly regulated at both the transcriptional and posttranscriptional levels (Dickson, 2002; Yu and Bargmann, 2001). However, this answer begs a second question: How do differences in structure and expression generate functional diversification within a family of receptors, allowing them to mediate distinct growth cone responses to their common ligand? We explore this question here, focusing on the role of the Roundabout (Robo) family receptors in patterning axonal projections in the nerve cord of the *Drosophila* embryo (Dickson and Gilestro, 2006).

Most axonal growth cones in the *Drosophila* ventral nerve cord initially extend toward the midline. Some stop or turn longitudinally before they reach the midline, but the majority continues across to the contralateral side. These crossing axons form the commissures that connect the two symmetric halves of the nervous system. Upon reaching the contralateral side many commissural axons then turn longitudinally, extending parallel to the midline but never recrossing it. Within the longitudinal pathways, axons are organized into a series of discrete fascicles, each located at a characteristic position lateral to the midline.

These various axonal trajectories are formed largely under the influence of two guidance factors produced by cells at the midline: Netrin (Harris et al., 1996; Mitchell et al., 1996) and Slit (Kidd et al., 1999). Netrin acts as a short-range attractant for commissural axons (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996), signaling through the DCC family receptor Frazzled (Fra) (Kolodziej et al., 1996). Slit, in contrast, is thought to act primarily as a repellent for CNS axons (Kidd et al., 1999), signaling through various combinations of the three Robo family receptors: Robo, Robo2, and Robo3. Here, for clarity, we refer to Robo (the founding member of the family) as Robo1, and use Robo as a generic name for the family.

The initial decision to cross or not to cross the midline is primarily controlled by Robo1 and its negative regulator Comm. Both ipsilateral and commissural axons express Robo1 (Kidd et al., 1998), but only commissural axons express Comm (Keleman et al., 2002). In these neurons, Comm is thought to function as an endosomal sorting receptor that prevents most Robo1 from reaching the axonal growth cone, thereby rendering these axons insensitive to the Slit repellent (Keleman et al., 2002; Keleman et al., 2005; Myat et al., 2002). No axons cross in embryos that lack *comm*, whereas too many axons cross in embryos that lack *robo1* (Seeger et al., 1993). Crossing errors also occur in both *robo2* and *robo3* mutants, but at much lower frequency than in *robo1* mutants (Rajagopalan et al., 2000a; Simpson et al., 2000b). Midline crossing is partly dependent on Netrin function, but many axons still cross in both *Netrin* and *fra* mutant embryos (Harris et al., 1996; Kolodziej et al., 1996;
Mitchell et al., 1996) (Brankatschk and Dickson, 2006). Hence, it has long been assumed that some other factors act alongside Netrin and Fra to promote crossing. These factors have not yet been identified.

Whether or not they initially cross the midline, many axons subsequently extend longitudinally alongside the midline. These longitudinal axons are sorted into three lateral zones, each defined by a specific combination of Robo receptors (Rajagopalan et al., 2000b; Simpson et al., 2000a). Axons in the medial zone express only Robo1, those in the intermediate zone express both Robo1 and Robo3, and those in the most lateral zone express all three Robos. Genetic loss- and gain-of-function studies have demonstrated that the Robo receptors are instructive in lateral positioning, and thus constitute a “Robo code” (Rajagopalan et al., 2000b; Simpson et al., 2000a). For example, upon loss of robo3, axons of the intermediate zone shift into the medial zone. Conversely, forced expression of Robo3 in specific medial zone neurons shifts their axons laterally into the intermediate zone. Similarly, loss of robo2 shifts some lateral axons medially, and forced expression of Robo2 also shifts medial axons laterally. Robo1 expression does not discriminate between longitudinal pathways, suggesting that it does not contribute to lateral pathway selection. Indeed, three discrete longitudinal zones still form in robo1 mutant embryos, and forced expression of Robo1 also does not shift medial axons laterally. Thus, it is primarily Robo3 and Robo2 that function in lateral positioning. They may do so in response to a gradient of Slit activity spreading laterally from the midline (Rajagopalan et al., 2000b; Simpson et al., 2000a), or through homophilic interactions (Hivert et al., 2002).

In summary, then, Robo1, Robo2, and Robo3 each have distinct functions in axon guidance in the ventral nerve cord: Robo1 prevents inappropriate crossing, whereas Robo2 and Robo3 primarily act to specify the lateral positions of longitudinal axons. To what extent do differences in gene expression or receptor structure account for these functional specializations? For midline crossing, the expression pattern is thought to be critical. For example, Robo3 is not expressed early enough to influence the initial crossing decisions of pioneer commissural and longitudinal axons. Conversely, lateral pathway selection is thought to rely on critical biochemical differences between the three Robo proteins: “Robo3 and Robo2 must differ from one another either in their ectodomains (and thus their abilities to read the Slit gradient), or in their cytoplasmic domains (and thus have different abilities to signal), or both (Simpson et al., 2000a)”.

Whether such biochemical differences indeed exist between the Robos, and to what extent they contribute to their various guidance functions, has not been resolved. To address this question, we have used homologous recombination to construct a set of 9 “robo swap” alleles, expressing each of the three Robo receptors in each of the three distinct spatial and temporal patterns of the three robo genes. Surprisingly, and in contrast to previous models (Rajagopalan et al., 2000b; Simpson et al., 2000a), we found that lateral positioning does not rely on structural differences between the Robo receptors—neither in their ectodomain nor cytoplasmic domains. Expression differences alone can account for lateral pathway selection. In contrast, structural differences are critical in the midline crossing decisions, both for the role of Robo1 in preventing crossing and for an unexpected positive role of Robo2 in promoting crossing. Using these Robo swap alleles, we have thus been able to demonstrate how differences in both receptor structure and expression account for diverse guidance functions among members of a single receptor family.

RESULTS

The robo Swaps

The three Robo receptors differ in both their expression patterns and structure (Kidd et al., 1998; Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b). Robo1 and Robo2 are expressed in most, possibly all, neurons as the initial axon pathways are pioneered during embryonic stage 12 (Figure 1A, left). Robo3 is not expressed until stage 13, and remains limited to a subset of neurons. Robo1 expression persists throughout embryogenesis, but Robo2 is extinguished in many neurons as development proceeds. Thus, from stage 14 onward, most neurons express one of three specific combinations of Robo receptors, according to which longitudinal axons are sorted into one of three lateral zones (Figure 1A, right).

All three Robo receptors are single-pass transmembrane proteins. Their ectodomains are similar, each comprising a series of 5 immunoglobulin domains and 3 fibronectin type III domains, and all three Robos bind Slit (Brose et al., 1999; Kidd et al., 1999) with similar affinity (Howitt et al., 2004). The Robos are more divergent in their cytoplasmic domains. Robo1 contains each of the four short conserved consensus sequences (CC0-CC3) common to most Robo proteins in other species, but Robo2 and Robo3 lack the CC2 and CC3 motifs.

The primary goal of the present study was to determine which of these differences—expression or structure—account for the distinct functions of each of the three Robos in axon guidance. Our general strategy was to create a set of 9 robo swap alleles, each driving the expression of one Robo receptor in the pattern of another (or itself as a control). We modified each of the robo loci by gene targeting (Rong and Golic, 2000), replacing the exons that encode the mature protein, as well the small intervening introns, with a single exon encoding a full length Robo protein (Figure 1B). The replacement exon also introduced 3 tandem HA epitope tags at the amino terminus, allowing us to use the same anti-HA antibody to assess the distribution of each Robo swap protein. We refer to a specific robo swap allele as roboXroboY, where X indicates the targeted locus and Y indicates the substituted coding region. For example, robo1robo2 is the knock-in of the robo2 coding region into the robo1 locus. We verified the molecular structure of each of these 9 swap alleles by genomic PCR and DNA sequencing, and by staining ventral nerve cords of these embryos with anti-HA to visualize the knock-in proteins. We confirmed that the expression pattern of each HA-Robo protein perfectly matched the pattern of the endogenous Robo protein it replaced, both in stage 13 (Figure S1) and in stage 16 (Figures 1C and 1D) embryos.

A potential pitfall of our strategy is that the deletion of most introns and/or the inclusion of the epitope tags could disrupt the function of one or other robo gene. To test this, we used
anti-FasII mAb 1D4 to examine axonal pathways in the ventral nerve cord of each of the three “iso-robo” alleles (in which a given robo was replaced with itself). In wild-type stage 16 embryos, anti-FasII labels several longitudinal fascicles on each side of the midline, appearing in dorsal views as 3 discrete pathways, one in each “Robo zone.” Each of the robo null mutants has a characteristic phenotype with this marker (Figure 1E). In robo1 mutants, the medial pathways from each side of the midline are generally fused into a single pathway that meanders back and forth across the midline (Kidd et al., 1998, Figure 1E and Table 1). In robo2 mutants, axons of the medial pathways cross in ~25% of segments, while the lateral fascicle is disrupted in ~35% of hemisegments (Rajagopalan et al., 2000b; Simpson et al., 2000a; Figure 1E and Table 1). In robo3 mutants, the intermediate fascicle is shifted medially in every hemisegment, fusing with the medial fascicle (Rajagopalan et al., 2000b; Simpson et al., 2000a; Figure 1E and Table 1).
et al., 2000a; Figure 1E and Table 1). We did not observe these characteristic phenotypes in any of the 3 iso-robo swap alleles (Figure 1F and Table 1). Additionally, whereas robo1 and robo2 null mutants are homozygous lethal, all three iso-robo swap alleles were viable and fertile as homozygotes (Table 1). We thus conclude that the general modifications common to all robo swap alleles do not interfere with robo function.

### Lateral Pathway Selection Relies on Differences in Robo Expression

Using the “hetero-robo” swaps, we first asked whether lateral pathway selection depends on structural differences between the Robo proteins, as generally assumed (Rajagopalan et al., 2000b; Simpson et al., 2000a), or on differences in their expression profiles. If structural differences are indeed critical, the longitudinal pathways should be highly disorganized in each of the hetero-robo swap alleles. If it is only differences in expression that matter, the longitudinal pathways should be normal. We focused on the role of robo3, since robo3 loss-of-function embryos have a clear and highly penetrant phenotype in which axons of the intermediate zone are displaced medially (Rajagopalan et al., 2000b; Simpson et al., 2000a). In these embryos, the intermediate FasII pathway merges with the medial pathway in every hemisegment (Figure 2, Table 1). Axons of the Sema2b neurons also project in the intermediate zone, just medial to the FasII fascicle, and they too shift into the medial zone in robo3 mutants (Figure 2).

Much to our surprise, in both robo3/robo1 and robo3/robo2 homozygous embryos, all three FasII fascicles formed normally and the Sema2b axons remained in the intermediate zone (Figure 2).

#### Table 1. Midline Crossing and Longitudinal Pathway Errors in robo Swap Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viable</th>
<th>n (Hemisegments)</th>
<th>Segments with FasII-Positive Axons at Midline (%)</th>
<th>Hemisegments with Fused or Broken FasII Fascicles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intermediate</td>
<td>Lateral</td>
</tr>
<tr>
<td>Wild-type</td>
<td>yes</td>
<td>610</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>robo1^+/robo1^b</td>
<td>no</td>
<td>288</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>robo1^b/robo2^b</td>
<td>yes</td>
<td>272</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>robo1^b/robo2</td>
<td>yes</td>
<td>330</td>
<td>97.6</td>
<td>0.9</td>
</tr>
<tr>
<td>robo1^b/robo3</td>
<td>yes</td>
<td>264</td>
<td>99.2</td>
<td>0.8</td>
</tr>
<tr>
<td>robo2^b/robo2^b</td>
<td>no</td>
<td>186</td>
<td>23.7</td>
<td>10.2</td>
</tr>
<tr>
<td>robo2^b/robo1^b</td>
<td>yes</td>
<td>300</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>robo2^b/robo2</td>
<td>yes</td>
<td>308</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>robo2^b/robo3</td>
<td>yes</td>
<td>284</td>
<td>14.8</td>
<td>2.2</td>
</tr>
<tr>
<td>robo3^1</td>
<td>yes</td>
<td>340</td>
<td>4.1</td>
<td>100.0</td>
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<tr>
<td>robo3^b/robo1^b</td>
<td>yes</td>
<td>266</td>
<td>0.6</td>
<td>0.4</td>
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<tr>
<td>robo3^b/robo2</td>
<td>yes</td>
<td>384</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>robo3^b/robo3</td>
<td>yes</td>
<td>304</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>robo2^b/robo1^b-2</td>
<td>yes</td>
<td>280</td>
<td>0.0</td>
<td>3.6</td>
</tr>
<tr>
<td>robo2^b/robo2-1</td>
<td>yes</td>
<td>350</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td>robo2^b/robo3</td>
<td>yes</td>
<td>304</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Stage 16–17 embryos stained with anti-FasII were scored for the presence of FasII-positive axons extending across or along the midline, and for breaks in the intermediate and lateral FasII fascicles, often due to fusion with the more medial fascicle.

Figure 2. Lateral Positioning Is Normal in All Three robo3 Swap Mutants

Stage 16 embryos of the indicated robo swap alleles, stained with either anti-FasII to visualize the three FasII fascicles (green) or anti-GFP to reveal the Sema2b axons carrying a Sema2b-GAL4 UAS-mCD8-GFP reporter (red, bottom row). The neuropil is counterstained with anti-HRP (magenta, top; blue, bottom). Note that the intermediate zone axons are shifted medially in the robo3 mutant, but positioned normally in all three robo3 swap alleles (arrowheads; for quantification of anti-FasII phenotypes, see Table 1). Schematics indicate the Robo proteins expressed from each robo locus, according to the color scheme of Figure 1A. Lateral position in robo2 swap alleles is shown in Figure S2.
Quantiﬁcationally, there were no signiﬁcant differences in FasII pathway formation between either of these robo3 swap embryos and the wild-type and robo3robe3 controls (Table 1). These results were particularly unexpected, as previous gain-of-function experiments had suggested that Robo1 could not direct axons into more lateral pathways (experiments had suggested that Robo1 could not direct axons were particularly unexpected, as previous gain-of-function rescue in that the defects in the lateral FasII fascicles were partially by the additional roles of Robo2 in midline crossing (robo2 is less clear. This lateral FasII fascicle is only partly disrupted in embryos, but cross the midline in robo1 and each of the robo1 swap alleles (A).

**Figure 3. Unique Features of Robo1 Protein and Expression Are Needed to Prevent Crossing**

Embryos of the indicated genotypes and stages, stained with anti-FasII (green). In stage 16 embryos, ectopic crossing of FasII-positive axons is observed in robo1 mutants and all swap alleles with the exception of the robo1robe1 control (A and B; for quantiﬁcation, see Table 1). In stage 13 embryos, the ipsilateral pCC axons (arrowheads) project normally in the robo1robe1 control, but cross the midline in robo1 and each of the robo1 swap alleles (A).

**Unique Features of Robo1 Protein Prevent Midline Crossing**

Robo1 has a critical role in preventing longitudinal axons from crossing the midline (Kidd et al., 1998; Seeger et al., 1993). To test whether Robo2 and Robo3 could functionally substitute for Robo1, we examined midline crossing phenotypes in robo1robo2 and robo1robo3 homozygous embryos. A priori, we could envision three possible outcomes for this experiment. If the Robo2 and Robo3 proteins expressed from the robo1 locus cannot be adequately downregulated by Comm, then commissural axons should fail to cross the midline—as they do, for example, when Robo2 or Robo3 is expressed from a strong pan-neuronal promoter (Rajagopalan et al., 2000a; Simpson et al., 2000b). A, if, however, Robo2 and Robo3 are correctly regulated by Comm, we would then predict either a wild-type phenotype or a robo1-like phenotype, depending on whether or not Robo2 and Robo3 can substitute for Robo1 in preventing longitudinal axons from crossing.

In both robo1robo2 and robo1robo3 homozygous stage 16 embryos stained with anti-FasII, we observed midline crossing errors that were qualitatively (Figure 3A) and quantitatively (Table 1) similar to those observed in robo1 null mutants. We also examined stage 13 embryos in order to follow the projections of the ipsilateral pioneer neuron pCC. In robo1robo2 and robo1robo3 embryos, as in robo1 null mutants, the pCC axons projected aberrantly across the midline (Figure 3A). The misrouting of pCC is particularly telling, as its growth cone expresses both Robo1 and Robo2, yet it requires only Robo1 for its ipsilateral projection (Kidd et al., 1998; Rajagopalan et al., 2000a). Thus, regardless of whether it is expressed from its endogenous locus or the robo1 locus, Robo2 cannot prevent pCC from crossing. We conclude that midline repulsion of longitudinal axons requires features of the Robo1 protein that are not shared with either Robo2 or Robo3. We note however that both robo1robo2 and robo1robo3 homozygotes are viable and fertile, whereas robo1 null mutants are lethal (Table 1). This conﬁrms that these alleles do express functional Robo proteins that can substitute for Robo1 in other contexts.

Is Robo1’s expression pattern also relevant to its speciﬁc function in regulating midline crossing? To test this, we asked whether Robo1 could prevent inappropriate crossing even if it
were provided exclusively from either the robo2 or robo3 locus. Specifically, we examined robo1 mutant embryos additionally heterozygous for either the robo2<sup>robo1</sup> or robo3<sup>robo1</sup> allele. In these embryos, Robo2 and Robo3 are expressed normally, whereas Robo1 is expressed aberrantly in the manner of either Robo2 or Robo3. In both cases we observed the characteristic robo1 phenotype (Figure 3B), indicating that Robo1 must be expressed in its endogenous pattern to prevent ipsilateral axons from crossing. For the robo3 locus this result was expected, as there is little robo3 expression at the stage in which axon pathways are pioneered (Rajagopalan et al., 2000b; Simpson et al., 2000b). That expression from the robo2 locus is not sufficient for Robo1 to prevent crossing was however somewhat surprising, as robo1 and robo2 have similar expression patterns at early stages (Rajagopalan et al., 2000b; Simpson et al., 2000b). Thus, the specific role of Robo1 in preventing midline crossing can be attributed to both its unique pattern of expression as well as unique features of the Robo1 protein.

Midline crossing errors also occur in both robo2 and robo3 mutant embryos, albeit at a much lower frequency (Rajagopalan et al., 2000a; Simpson et al., 2000b; Table 1). For example, in robo2 null mutant embryos, we observed FasII-positive axons crossing the midline in 23.7% of segments. This phenotype was observed in only 2.0% of segments in the robo2<sup>robo1</sup> swap and 14.8% in the robo3<sup>robo1</sup> swap (Table 1). Similarly, crossing errors were observed with anti-FasII in 4.1% of segments in robo3 null mutants, but only 0.6% and 0.5% in the robo2<sup>robo1</sup> and robo3<sup>robo1</sup> swaps (Table 1). Thus, in contrast to Robo1, neither the Robo2 nor Robo3 protein has unique features that are required to prevent longitudinal axons from crossing. These results also confirm that both robo2<sup>robo1</sup> and robo3<sup>robo1</sup> express a functional Robo1 protein, capable of rescuing the robo2 and robo3 phenotypes, respectively, but not the robo1 phenotype.

**Cytoplasmic Regions Confer Robo1’s Specific Function in Midline Crossing**

We next sought to map the unique structural features of the Robo1 protein that are critical for its role in preventing midline crossing. To do this, we generated a series of chimeric receptors between Robo1 (which can prevent crossing) and Robo3 (which cannot, even when expressed from the robo1 locus). We took advantage in this case of the fact that robo1 mutants can be fully rescued by a transgene that includes ~4.5 kb of flanking regions from the genomic robo1 locus (Kidd et al., 1998). This allowed us to generate the chimeric receptors in the context of a rescuing transgene (Figure 4A). Site-specific integration (Groth et al., 2004) was used to insert these transgenes into a defined genomic locus, thereby eliminating any additional complications that might arise through variable expression of the different transgenes (Figure 4A).

We first tested this general strategy by preparing intronless robo1::robo2 and robo3 transgenes in the context of robo1 flanking sequences. We refer to these transgenes as robo1::robo<sub>X</sub>, where X indicates the coding region inserted into the robo1 genomic rescue transgene. Consistent with the results obtained with the robo swap knock-in alleles, the midline crossing errors observed in the robo1 mutant background were almost fully rescued by robo1::robo1, but not by either robo1::robo2 or robo1::robo3 (Figure 4B and Table 2). Having thus validated this transgenic rescue approach, we next generated a series of transgenes that encode reciprocal pairs of chimeric receptors, recombining distinct regions of Robo1 and Robo3. We designate these chimeric rescue transgenes as robo1::robo<sub>XY</sub>-robo<sub>YX</sub>, whereby the N-terminal region is from RoboX, the C-terminal region from RoboY, and the fusion point located in a predicted unstructured region between domains DX and DY.

To test whether the critical features lie in the ectodomain or cytoplasmic domain, we exchanged either the entire ectodomains of Robo1 and Robo3, or just the Slit binding site in the first two immunoglobulin domains. Regardless of which Robo protein contributed the extracellular domain, we obtained almost full rescue if the cytoplasmic domain derived from Robo1 (robo1::robo3<sup>robo1</sup>), but only 0.6% and 0.5% in the robo1::robo3<sup>robo1</sup> and robo1::robo3<sup>robo1</sup> transgenes (Figure 4B and Table 2). Conversely, no rescue was observed if the cytoplasmic domain derived from Robo3 (robo1::robo1<sup>robo3</sup> and robo1::robo1<sup>robo3</sup>), but was completely abolished once the CC2 motif was also exchanged (robo1::robo1<sup>CC1</sup>-robo3<sup>CC3</sup> and robo1::robo1<sup>CC1</sup>-robo3<sup>CC2</sup>; Figure 4B and Table 2). These results map the unique requirements of Robo1 in midline crossing to its cytoplasmic domain.

To further subdivide the critical regions of Robo1, we tested reciprocal pairs of chimeric receptors with fusions between each of the conserved cytoplasmic motifs of Robo1: CC0, CC1, CC2, and CC3. We found that Robo1’s ability to rescue was preserved if CC3 was exchanged with the corresponding region of Robo3 (robo1::robo1<sup>CC1</sup>-robo3<sup>CC3</sup>, Figure 4B and Table 2), but was completely abolished once the CC2 motif was also exchanged (robo1::robo1<sup>CC1</sup>-robo3<sup>CC3</sup> and robo1::robo1<sup>CC1</sup>-robo3<sup>CC2</sup>; Figure 4B and Table 2). Conversely, the Robo3 chimera did not rescue robo1 at all if it contained only the CC3 motif of Robo1 (robo1::robo3<sup>CC2</sup>-robo1<sup>CC3</sup>; Figure 4B and Table 2), but rescued partially if it also included CC2 (robo1::robo3<sup>CC1</sup>-robo1<sup>CC2</sup>; Figure 4B and Table 2) and almost fully if it included CC1 (robo1::robo3<sup>CC0</sup>-robo1<sup>CC1</sup>; Figure 4B and Table 2). We conclude that the critical features for preventing midline crossing are located within the cytoplasmic region of Robo1 surrounding the CC2 motif, with an additional minor contribution from the CC1 region.

**A Positive Role for Robo2 in Midline Crossing**

Our robo swap alleles provided an excellent opportunity to explore other possible functions of Robo receptors in midline axon guidance. In particular, we wondered whether one or more of the Robos might additionally act as a positive factor in midline crossing. Although Netrins have a key role in promoting midline crossing, many commissures do still form in embryos that lack the two Netrin genes, NetA and NetB (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al., 1996). Thus, some other system must operate alongside Netrin-Fra to promote crossing. We hypothesized that this system might involve one of the Robos, and that the prominent repulsive role of Robo receptors may have hitherto obscured any additional positive role in midline crossing. To test this prediction, we examined the consequence of removing one or more Robo receptors in embryos devoid of Netrin function (NetAB<sup>−/−</sup>; Brankatschk and Dickson, 2006)). A priori, the loss of a receptor for a midline repellent should, if anything, increase the number of commissures in the NetAB background. If instead fewer
commissures were observed, this would be a strong indicator of a positive role in commissure formation.

The phenotypes we observed in both NetAB robo1 and NetAB robo3 embryos appeared to be simple combinations of the respective NetAB, robo1 and robo3 mutants (Figure 5A and Table 2). In contrast, NetAB robo2 embryos had a surprising phenotype that could not be predicted from either single mutant. Whereas commissures are only mildly disrupted in NetAB mutants, and normal or even excessive crossing occurs in robo2 single mutants, commissures were almost completely eliminated in the NetAB robo2 embryos (Figure 5A and Table 3).

Removing pairs of robo genes in the NetAB background did not reveal any further synergistic interactions, other than the expected midline collapse phenotype in NetAB robo1 robo2 embryos (Figure 5A) that is also seen in robo1 robo2 embryos (Rajagopalan et al., 2000a; Simpson et al., 2000b). We conclude that Robo2, and Robo2 alone, has an additional positive role in commissure formation. This positive role is also independent of Fra, because NetAB fra embryos resemble NetAB (and fra), and fra robo2 resembles NetAB robo2 (Figure S3 and Table 3). Thus, Netrin-Fra and Robo2 act independently, and the positive role of Robo2 cannot be explained by cross-talk between Robo2

Figure 4. Mapping the Robo1 Domain that Prevents Midline Crossing
(A) Strategy used for site-specific transgenic rescue of the robo1 mutation. Transgenes were inserted into the VIE-274b landing site on the tip of chromosome arm 2L (see Experimental Procedures).
(B) Stage 16 embryos mutant for robo1 and carrying the indicated transgene, stained with anti-FasII (green). Schematics illustrate the structure of the chimeric receptor, using the color scheme of Figure 1A (blue for Robo1, green for Robo3). The region comprising CC1 and CC2 of Robo1 is critical to prevent inappropriate crossing of FasII-positive axons (for quantification of midline crossing phenotypes, see Table 2).
and Fra (as proposed for their vertebrate counterparts; Stein and Tessier-Lavigne, 2001).

We further used our robo2 swap alleles to assess whether this positive role of Robo2 relies on its unique structural properties. We crossed both robo2\textsuperscript{1} \textendash \textsuperscript{robo1} and robo2\textsuperscript{2} \textendash \textsuperscript{robo3} into the NetAB background, and found that neither Robo1 nor Robo3 can substitute for Robo2 in promoting midline crossing (Figure 5B and Table 3). For example, the anterior commissure appeared normal in just 6.2% of segments in NetAB robo2\textsuperscript{1} \textendash \textsuperscript{robo1} embryos, but in 27.2% of segments in NetAB robo2\textsuperscript{2} \textendash \textsuperscript{robo3} embryos (Figure 5C and Table 3), suggesting that Robo2’s positive role in midline crossing is more critically dependent on unique features of its ectodomain.

**DISCUSSION**

**The robo Swaps: Dissecting the Distinct Functions of Each Robo**

The midline guidance cue Slit is thought to act through each of three different Robo family receptors to help form the orthogonal axonal pathways of the *Drosophila* ventral nerve cord. Each of the three Robos has a distinct role in forming these projections. Robo1 is primarily required to prevent longitudinal axons from crossing the midline (Kidd et al., 1998; Seeger et al., 1993). Robo2 has a minor role in preventing longitudinal axons from crossing (Rajagopalan et al., 2000a; Simpson et al., 2000b), and, as we have shown here, also facilitates the crossing of commissural axons. Finally, Robo3 may also help prevent some longitudinal axons from crossing, but its major function is to direct the formation of the intermediate longitudinal pathways (Rajagopalan et al., 2000b; Simpson et al., 2000a).

The goal of this study was to assess whether these functional specializations reflect structural differences in the Robo proteins themselves or differences in *robo* gene regulation. To this end, we used gene targeting to replace the coding region of each robo gene with that of each other robo, creating a series of robo swap alleles. We found that commissure formation relies on the unique structural features of both Robo1 (to prevent crossing) and Robo2 (to promote crossing). In contrast, lateral positioning of longitudinal axons does not rely on structural differences between the Robo proteins, but rather differences in *robo* gene expression.

**A Robo Expression Code for Lateral Pathway Selection**

In the longitudinal pathways, axons are organized into discrete and stereotyped fascicles. In part, this requires selective fasciculation mediated by contact-dependent attractive or repulsive surface proteins that “label” specific axon fascicles (Goodman and Bastiani, 1984). This includes the Fasll protein we have explored here as a marker (Lin et al., 1994). In addition to these pathway labels, the lateral pathways are also segregated into three broad zones according to the distinct combination of Robo receptors they express (Rajagopalan et al., 2000b; Simpson et al., 2000a). Loss- and gain-of-function genetic experiments have shown that these Robo proteins are instructive in lateral pathway selection and, hence, define a “Robo code” (Rajagopalan et al., 2000b; Simpson et al., 2000a).

A popular model for lateral pathway selection posits that the three Robo proteins have distinct signaling properties, and that they position axons on a lateral gradient of their common ligand Slit (Rajagopalan et al., 2000b; Simpson et al., 2000a). In this model, the Robo proteins are assumed to differ in either their affinity for Slit, the strength of their “repulsive output,” or both. However, direct evidence for a role of Slit in lateral pathway is still

### Table 2. Midline Crossing Errors in robo Mutants Carrying Chimeric robo Transgenesi

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Segments with Fasll-Positive Axons at Midline (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.4</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, VIE-274b</td>
<td>99.1</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1</td>
<td>2.4</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo2</td>
<td>97.4</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo3</td>
<td>99.1</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>98.8</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>95.4</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>94.4</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>96.5</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>2.5</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>2.0</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>15.0</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>22.0</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>80.1</td>
</tr>
<tr>
<td>robo1\textsuperscript{1}, robo1::robo1~\textsuperscript{robo3}~\textsuperscript{robo2}</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Stage 16–17 embryos stained with anti-Fasll were scored for the presence of Fasll-positive axons extending across or along the midline. The null allele robo1\textsuperscript{1} was recombined with the VIE-274b landing site prior to integration of the indicated transgene at this site.
lacking, and alternative models have to be considered. One such possibility is that the Robo proteins might act instead as homophilic adhesion molecules (Hivert et al., 2002). In such a model, the Robo proteins might operate in a manner similar to other pathway labels such as FasII, but over broader zones. Regardless of whether they invoke a role for Slit, homophilic adhesion, or some other unidentified ligand, all models presented to date have assumed that there must be critical structural differences in the Robo proteins. These structural differences would form the basis of a combinatorial Robo code for lateral pathway selection.

Our data demonstrate that this cannot be the case. Lateral positioning does not rely on structural differences between the Robo proteins. This is particularly clear for the distinction between the medial and the intermediate zones, which relies entirely on the selective expression of Robo3 on intermediate axons. We find, however, that lateral positioning of these axons works surprisingly well even when Robo3 protein is replaced by either Robo1 or Robo2. Although we cannot exclude some minor disruption in specific pathways, the overall structure of the longitudinal pathways appears normal in these embryos. Notably, this includes the formation of the intermediate FasII pathway and the projections of the Sema2b axons, both of which were diagnostic for Robo3’s role in lateral positioning (Rajagopalan et al., 2000b; Simpson et al., 2000a). Thus, at least for the medial and intermediate axons, the only relevant differences between the Robos are in their patterns of gene expression. The “Robo code” is not a protein code; it is a gene-expression code.

At first glance, this result is difficult to reconcile with the previously published gain-of-function experiments (Rajagopalan et al., 2000b; Simpson et al., 2000a). In these experiments, the various Robo proteins were expressed from GAL4/UAS transgenes in specific neurons (the Ap neurons). These Ap neurons normally express only Robo1 and hence project ipsilaterally in the medial zone. In both reports, expression of Robo3 shifted these axons into the intermediate zone, as expected, but expression of Robo1 did not. Why might Robo1 be able to replace the endogenous Robo3 in our swap experiments, but not the transgenic Robo3 in these gain-of-function studies? A trivial but unsatisfying explanation is that this was merely an artifact of the GAL4/UAS system. Prior to the advent of site-specific transgenesis (Groth et al., 2004), it was notoriously difficult to control for the varying expression levels from different transgene insertions, which rarely match endogenous levels. More interesting possibilities are that the discrepancy may reflect differences resulting from assaying the behavior of neurons that normally express Robo3 versus those that don’t, or perhaps a “community effect”
that results from manipulating an entire cohort of neurons, not just a single neuron. In this regard it is also important to note that the Ap axons are likely to be follower axons for their specific pathway, not pioneers. Whatever the reason for this discrepancy, the substitution of the robo1 coding region into the robo3 locus is presumably the more physiologically relevant assay.

How might differences in robo gene expression explain lateral positioning? One possibility is that it is only the total Robo levels that are important, with higher levels sending axons further laterally on the presumptive Slit gradient. This model fits with the results of “supershifting” experiments, in which additional copies of the Robo3 transgene displaced the Ap axons even further from the midline (Rajagopalan et al., 2000b). It is also supported by mathematical modeling of the Robo code (Goodhill, 2003). This model still invokes a role for the Slit gradient, for which there is admittedly no direct evidence. Alternatively, lateral pathway selection might rely on critical differences in the precise spatial and temporal pattern of expression, rather than differences in total Robo levels.

### Table 3. Commissure Formation in NetAB, fra, and robo Swap Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (Segments)</th>
<th>Anterior Commissure</th>
<th>Posterior Commissure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Thin</td>
</tr>
<tr>
<td>Wild-type</td>
<td>437</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>NetAB</td>
<td>276</td>
<td>80.4</td>
<td>13.8</td>
</tr>
<tr>
<td>NetAB; robo1/robo1</td>
<td>154</td>
<td>79.5</td>
<td>12.8</td>
</tr>
<tr>
<td>NetAB; robo2</td>
<td>247</td>
<td>1.6</td>
<td>27.6</td>
</tr>
<tr>
<td>NetAB; robo3</td>
<td>184</td>
<td>88.0</td>
<td>6.5</td>
</tr>
<tr>
<td>NetAB; robo2, robo3</td>
<td>150</td>
<td>4.0</td>
<td>34.7</td>
</tr>
<tr>
<td>fra; fra</td>
<td>222</td>
<td>93.7</td>
<td>5.4</td>
</tr>
<tr>
<td>fra; fra; robo2/robo2</td>
<td>208</td>
<td>12.5</td>
<td>61.5</td>
</tr>
<tr>
<td>NetAB; robo2/robo1</td>
<td>286</td>
<td>7.0</td>
<td>39.2</td>
</tr>
<tr>
<td>NetAB; robo2/robo2</td>
<td>287</td>
<td>82.5</td>
<td>14.0</td>
</tr>
<tr>
<td>NetAB; robo2/robo3</td>
<td>278</td>
<td>6.2</td>
<td>67.7</td>
</tr>
<tr>
<td>NetAB; robo2/robo1-2</td>
<td>436</td>
<td>4.5</td>
<td>57.0</td>
</tr>
<tr>
<td>NetAB; robo2/robo2-1</td>
<td>162</td>
<td>27.2</td>
<td>30.9</td>
</tr>
</tbody>
</table>

Stage 16–17 embryos stained with BP102 were scored for defects in the anterior and posterior commissures. Data in italics indicate a robo1-like phenotype, for which “normal” also includes thicker commissures. The robo1+, robo1−, robo2−, and robo2− alleles are null, and robo3− is a strong hypomorph.

By examining a series of chimeric receptors consisting of distinct parts of Robo1 and Robo3, we have mapped this critical and unique function of Robo1 in midline repulsion to a region of the cytoplasmic domain containing the CC1 and CC2 motifs. This conclusion is broadly consistent with previous studies that have examined Robo1 deletion mutants lacking specific CC motifs, in this case in a pan-neuronal transgenic rescue assay (Bashaw et al., 2000). Although there are subtle differences that may reflect the use of chimeric receptors versus single domain deletions, and the consequences of expressing them under the control of endogenous versus heterologous gene regulatory elements, the two studies together strongly suggest that the proline-rich CC2 motif is the critical structural determinant of Robo1’s unique capability of preventing midline crossing. This domain is thought to serve as a docking site for a number of factors that contribute to Slit-dependent repulsion through Robo1, including Enabled (Bashaw et al., 2000), the Rac GTPase activating protein Vlise/CrGAP (Hu et al., 2005; Lundstrom et al., 2004), and the SH2-SH3 adaptor Dock (Fan et al., 2003), the latter recruiting in turn the Rac guanine nucleotide exchange factor Sos (Yang and Bashaw, 2006) and p21 activated kinase (Fan et al., 2003). CC2 is also the most broadly conserved of the cytoplasmic domains in Robo1, with the insect Robo2 and Robo3 proteins being the only known Robo receptors that lack CC2. The lack of CC2 in Robo2 and Robo3 cautions against the inference that the distinct guidance functions of these two receptors are necessarily mediated by repulsive signaling in response to activation by Slit.

Indeed, we have presented here evidence that Robo2 can even act in opposition to Robo1 to promote crossing. We assume that Robo2 normally exerts this positive function autonomously in commissural neurons, acting in parallel to Netrin-Frazzled signaling to allow midline crossing. We can envision two models to account for the positive role of Robo2 in midline crossing. In one scenario, Robo2 transduces an attractive signal that...
promotes crossing, possibly in response to its midline ligand Slit. Such a model has previously been proposed for Robo2 in the guidance of ganglionic tracheal branches (Englund et al., 2002). Alternatively, Robo2 might promote crossing by antagonizing the repulsive function of Robo1, thus mediating an "anti-repulsion" rather than an "attraction" signal. Formally, this model is analogous to the role of Comm in Drosophila (Seeger et al., 1993), and of Robo3/Rig-1 in vertebrates (Sabatier et al., 2004).

Our preliminary data are more consistent with this latter scenario.

We now know of three factors that promote midline crossing: Comm, Netrin-Frazzled, and Robo2. Of these, only Comm appears to be instructive. Comm is expressed in commissural neurons, and are required but not sufficient for crossing (Kolodziej et al., 1996; Rajagopalan et al., 2000a; Simpson et al., 2000b). They are also partially redundant and independent, as crossing is severely disrupted only when both are eliminated. A conceptual model for midline crossing (Dickson and Gilestro, 2006) proposes a bistable switch created by the mutual inhibition between high Robo1 levels and midline crossing: high Robo1 levels prevent crossing due to repulsive signaling, whereas crossing the midline leads to clearance of Robo1 protein from the midline axon segment. In such a model, the permissive factors (Frazzled and Robo2) may act to ensure the appropriate balance between midline attraction and midline repulsion, bringing this feedback loop into the dynamic range at which the instructive factor (Comm) can operate. In principle, any one of the three factors—Comm, Robo2, or Frazzled—could have taken on the instructive role. Comm has evidently done so in Drosophila. To the extent that a similar feedback loop operates in mice, the instructive role may have fallen in this species to the Robo2 analog, Robo3 (Sabatier et al., 2004).

**EXPERIMENTAL PROCEDURES**

**Generation of robo Swap Alleles**

Each of the 9 robo swap and 2 chimeric robo alleles was generated by ends-in homologous recombination (Rong and Golic, 2000). Donor constructs were prepared in P-element vectors, each containing a single exon encoding the desired HA-tagged Robo protein in the correct reading frame for fusion with the signal sequence encoded in the first exon of each robo gene. This replacement exon was flanked on the 5′ side by 7.5–7.7 kb of genomic DNA from the locus to be targeted, and 1.4–1.9 kb on the 3′ side. An I-SceI site was included roughly in the middle of the 5′ homology region. Two I-CreI sites at the distal end of the 3′ homology region, separated by a mini-white marker. The entire targeting cassette was flanked by FR Target sites. These constructs were prepared using standard PCR-based cloning procedures, using genomic DNA from the W1118 strain and plasmids containing the robo1, robo2, or robo3 cDNAs as templates. All coding regions and cloning junctions were confirmed by DNA sequencing. Transgene insertions on the X or third chromosome were used for targeting, as all three robo genes are located on the second chromosome.

The targeting fragment was then liberated and linearized in the female germ line using FLP and I-SceI, respectively, and progeny were screened for movement of the mini-white marker to the 2nd chromosome, as well as its resistance to eYFP (indicating that it is no longer flanked by FR Target sites, as in the donor; (Newsome et al., 2000)). The successful generation of 1–6 homologous recombinants per allele was initially confirmed using a set of specific PCRs to detect the insertion of the replacement robo sequence and the disruption of the endogenous locus. The initial recombinants contained a duplication at the intended locus, which was subsequently resolved by using I-CreI to induce a double-stranded break and selecting in the progeny for the loss of the intervening white+ marker. These recombinants were then screened by PCR to identify those that retained the replacement allele and had lost the endogenous allele, prior to the more extensive histological characterization as described in the Results.

**Transgenic Rescue of robo1**

robo1 genomic rescue transgenes were prepared in an attB vector for integration into the VIE-274b attP landing site, located on the tip of chromosome arm 2L (K. Keleman and B.J.D., unpublished data). This landing site was created by P-element-mediated insertion of a vector containing the attP site, a 3′ fragment of the white+ marker and an attB site, such that cDNA-mediated integration restores the white+ marker. We converted this vector into a transgene for robo rescue experiments by adding 2386 bp of the robo1 5′ flanking region (nucleotides 2R:18588619…18587234 of the Drosophila genome release 5.20) and 2177 bp of the 3′ flanking region (nucleotides 2R:18582049…18579863). This transgene includes the genomic regions encoding the Robo1 signal peptide up to residue G190 (GenBank NM_057551), and is followed by 4 tandem HA epitope tags. The robo1::robo1, robo1::robo2, and robo1::robo3 transgenes were prepared by cloning the corresponding open reading frames into this vector, spanning the regions from residues Y59, G190 (NM_080531), and H72, (NM_134748), respectively, to the corresponding stop codons. Chimeric receptors were constructed to encode precise fusions at the following residues: robo1(y95)::robo3(y95), I520F-V521; robo1(y95)::robo3(y95), F187S-G188; robo1(y95)::robo3(y95), R292C-G293; robo1(y95)::robo3(y95), Y1028F-G1029; robo1(y95)::robo3(y95), Y1028F-G1029; robo3(y95); S1028E-G1029. robo1(y95)::robo3(y95); G1185E-G1186; robo3(y95)::robo1(y95), K1256F-S1257, robo3(y95)::robo1(y95), R1185F-S1186; robo3(y95)::robo1(y95), G1185E-G1186; robo1(y95)::robo3(y95), G1185E-G1186.

**Immunohistochemistry**

Immunofluorescence stainings of staged and fixed embryos were performed as described (Patel, 1994). Robo (mouse), Robo2 (rabbit), and Robo3 (mouse) antisera were used at a dilution of 1:1000, 1:200, and 1:500, respectively (Rajagopalan et al., 2000b). Other primary antibodies used were anti-FasII mAb 1D4 (1:1000, (van Vactor et al., 1993)), anti-HA mAb 3F10 (1:750, Roche Diagnostics), anti-HA mAb 16B12 (1:1000, BabsCO, Berkeley Antibody Company), anti-β-galactosidase (1:1000, Promega), anti-GFP (1:1000, Molecular Probes), and Cy3-conjugated sheep anti-HRP (1:500, Jackson Immunoresearch). Secondary antibodies used were anti-mouse Alexa Fluor-488, anti-rat and anti-rabbit Alexa Fluor-568-conjugated (1:1000, Molecular Probes). Homozygous embryos were identified by selecting against anti-β-galactosidase staining indicating the presence of Cy3, P[wg-lacZ] the balancer chromosome. Selected embryos were dissected, mounted in Vectashield mounting medium (Vector Labs), and images were acquired with a Zeiss LSM 510 confocal microscope.

**Quantification of Midline Crossing and Lateral Positioning Defects**

For the quantification of defects in the commissural or longitudinal axon pathways, stage 16 or 17 embryos were stained with mAb BP102 or anti-FasII, and mounted in 70% glycerol. Phenotypes were scored blind to the genotype included to allow identification of the mutant embryos, which were dissected ways, stage 16 or 17 embryos were stained with mAb BP102 or anti-FasII, and mounted in 70% glycerol. Phenotypes were scored blind to the genotype included to allow identification of the mutant embryos, which were dissected

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.cell.2010.01.002.

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REFERENCES


