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Roundabout Controls Axon Crossing of the CNS Midline and Defines a Novel Subfamily of Evolutionarily Conserved Guidance Receptors

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Summary

The *robo* gene in *Drosophila* was identified in a large-scale mutant screen for genes that control the decision by axons to cross the CNS midline. In *robo* mutants, too many axons cross and recross the midline. Here we show that *robo* encodes an axon guidance receptor that defines a novel subfamily of immunoglobulin superfamily proteins that is highly conserved from fruit flies to mammals. For those axons that never cross the midline, Robo is expressed on their growth cones from the outset; for the majority of axons that do cross the midline, Robo is expressed at high levels on their growth cones only after they cross the midline. Transgenic rescue experiments reveal that Robo can function in a cell-autonomous fashion. Robo appears to function as the gatekeeper controlling midline crossing.

Introduction

Bilaterally symmetric nervous systems, such as those found in insects and vertebrates, have special midline structures that establish a partition between the two mirror image halves. Axons that link the two sides of the nervous system project toward and across the midline, forming axon commissures. These commissural axons project toward the midline, at least in part, by responding to long-range chemoattractants emanating from the midline. One important class of midline chemoattractants are the netrins (Kennedy et al., 1994; Serafini et al., 1994), guidance signals whose structure, function, and midline expression are evolutionarily conserved from nematodes and fruit flies to vertebrates (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996; Wadsworth et al., 1996).

The midline also provides important short-range guidance signals. Although some growth cones in the nerve cord of insects extend away from the midline, most extend toward or along the midline during some segment of their trajectory. Certain classes of growth cones either extend toward the midline or longitudinally along it and yet never cross it. Most growth cones (~90% in the *Drosophila* CNS), however, do cross the midline. After crossing, the majority of these growth cones turn to project longitudinally, growing along or near the midline. Interestingly, these axons never cross the midline again.

What midline signals and growth cone receptors control whether growth cones do or do not cross the midline? After crossing once, what mechanism prevents these growth cones from crossing again? Studies in the chick (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997) and grasshopper (Myers and Bastiani, 1993) embryos have led to the suggestion that the midline contains a contact-mediated repellent and that commissural growth cones must overcome this repellent to cross the midline.

What is the function of this putative midline repellent? One likely role would be to prevent commissural axons from recrossing the midline after their initial crossing. Another role would be to prevent growth cones from crossing that normally project along the midline but do not cross it.

One approach to find the genes encoding the components of such a midline guidance system is to screen for mutations in which either too many or too few axons cross the midline. Such a large-scale mutant screen was previously conducted in *Drosophila* and led to the identification of two key genes: *commissureless* (*comm*) and *roundabout* (*robo*) (Seeger et al., 1993; reviewed by Tear et al., 1993). In *comm* mutant embryos, commissural growth cones initially orient toward the midline but then fail to cross it and instead recoil and extend on their own side. *comm* encodes a novel surface protein expressed on midline cells. As commissural growth cones contact and traverse the CNS midline, Comm protein is apparently transferred from midline cells to commissural axons (Tear et al., 1996).

In *robo* mutant embryos, many growth cones that normally extend only on their own side instead now project across the midline, and axons that normally cross the midline only once instead appear to cross and recross multiple times (Seeger et al., 1993; Kidd et al., 1998). Double mutants of *comm* and *robo* display a *robo*-like phenotype. Thus, although Comm is normally essential for axons to cross the midline, in the absence of Robo it is not required at all for crossing.

In this paper we report on the cloning and characterization of *robo* in *Drosophila*, and on the existence and expression of Robo-like sequences in mammals. *robo* encodes a novel guidance receptor that defines a novel subfamily of immunoglobulin (Ig) superfamily proteins that is highly conserved from fruit flies to mammals. The results of protein expression and transgenic rescue experiments lead us to propose that in *Drosophila* Robo functions as the gatekeeper controlling midline crossing. Another paper (Kidd et al., 1998) presents evidence showing that both the *robo* and *comm* genes function

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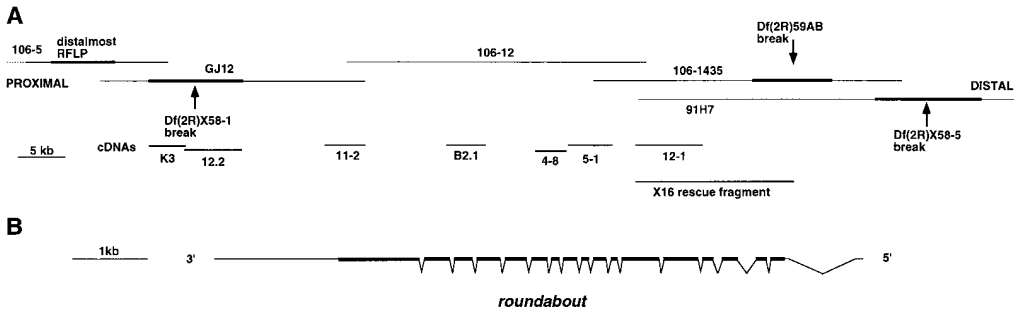

Figure 1. Organization of the *robo* Genomic Locus

(A) Cosmid chromosome walk through the 58F/59A region of the second chromosome. The position of deficiency breakpoints within the cosmids used are shown in the top two rows. Identified transcripts from the walk are shown below the cosmids. The 12–1 transcript corresponds to the *robo* gene; the direction of transcription is distal to proximal. The location of the 16 kb XbaI genomic rescue fragment is indicated below.

(B) Position and size of introns within the *robo* transcript. Coding sequence is indicated by the thicker part of the line. Introns are represented by gaps. The transcript is shown 3′-5′ to reflect its orientation in (A).

in a complementary and dosage-sensitive fashion in controlling midline crossing. A further paper (Zallen et al., 1998 [this issue of *Cell*]) describes the multiple axon guidance phenotypes of a *robo* homolog, *sax-3*, in the nematode.

Results

Cloning of the *roundabout* Gene

Standard recombination and deficiency mapping placed *robo* in the 58F/59A region. We initiated an ~150 kb chromosomal walk through the region and used RFLP and deficiency analysis to define the limits of the walk (Figure 1). Reverse Northern analysis identified regions containing transcripts. The cDNA 12–1 appeared by its size (given the relatively large number of independent EMS-induced *robo* alleles recovered from our mutant screen) and expression to be the most likely candidate for *robo*. A 16 kb XbaI fragment including the 12–1 transcript and a region 5′ to it is capable of rescuing the *robo* mutant. Further evidence that the 12–1 transcript represents the *robo* gene is presented below.

roundabout Encodes a Member of the Immunoglobulin Superfamily

We recovered and sequenced overlapping cDNA clones corresponding to the 12–1 transcription unit. A single long open reading frame (ORF) that encodes 1395 amino acids was identified (D1 in Figure 2). Conceptual translation of the ORF reveals the Robo protein to be a member of the Ig superfamily; Robo's ectodomain contains five Ig-like repeats followed by three fibronectin (FN) type-III repeats. The predicted ORF also begins with a putative signal sequence, in the middle contains a transmembrane domain, and ends with a large, 457 amino acid cytoplasmic domain.

We identify the 12–1 transcript as encoding *robo* based on several criteria. First, the embryonic *robo* phenotype can be rescued by the 16 kb XbaI genomic fragment containing this cDNA; no other transcripts are contained in this 16 kb XbaI fragment. Second, we identified a CfoI RFLP associated with the allele $robo^6$. This polymorphism is due to a change of nucleotide 332 of the ORF from G to A, which results in a change of Gly111 to Asp. Gly111 is in the first Ig domain (Figure 3) and is conserved in all Robo homologs thus far identified (Figure 2). The change is specific to the allele $robo^6$ and is not seen in the parental chromosome or in any of the other seven alleles, all of which were generated from the same parental genotype. Third, the production of antibodies (below) which recognize the Robo protein reveals that the alleles $robo^1$, $robo^2$, $robo^3$, $robo^4$, and $robo^5$ do not produce Robo protein (Table 1). Finally, transgenic neural expression of *robo* rescues the midline crossing phenotype of *robo* mutants (see below).

Developmental Northern blot analysis using both cDNA and genomic probes suggests that *robo* is encoded by a single transcript of ~7500 bp (our longest cDNA clone was 6.4 kb). We sequenced genomic DNA and identified 17 introns within the sequence, of which 14 are only 50–75 bp in length, plus three introns of 843, 236, and 110 bp (Figure 1B). The precise start point of the transcript has not been determined.

A Family of Evolutionarily Conserved Robo-like Proteins

The presence of five Ig and three FN domains, a transmembrane domain, and a long (457 amino acid) cytoplasmic region suggests that Robo may be a receptor and signaling molecule. The netrin receptor DCC/Frazzled/UNC-40 has a related domain structure, with 6 Ig and 4 FN domains and a similarly long cytoplasmic region (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). The only currently known protein with a "5 + 3" organization is CDO (Kang et al., 1997). However, CDO is only distantly related to Robo (15%–33% amino acid identity between corresponding Ig and FN domains).

We identified several more highly related 5 + 3 proteins in vertebrates whose amino acid identity exceeds that of CDO and thus are likely to represent true Robo homologs. A human expressed sequence tag (EST; yu23d11, GenBank #H77734) shows high homology to the second Ig domain of *robo* and was used to probe a human fetal brain cDNA library (Stratagene). The clones recovered correspond to a human gene with five Ig and

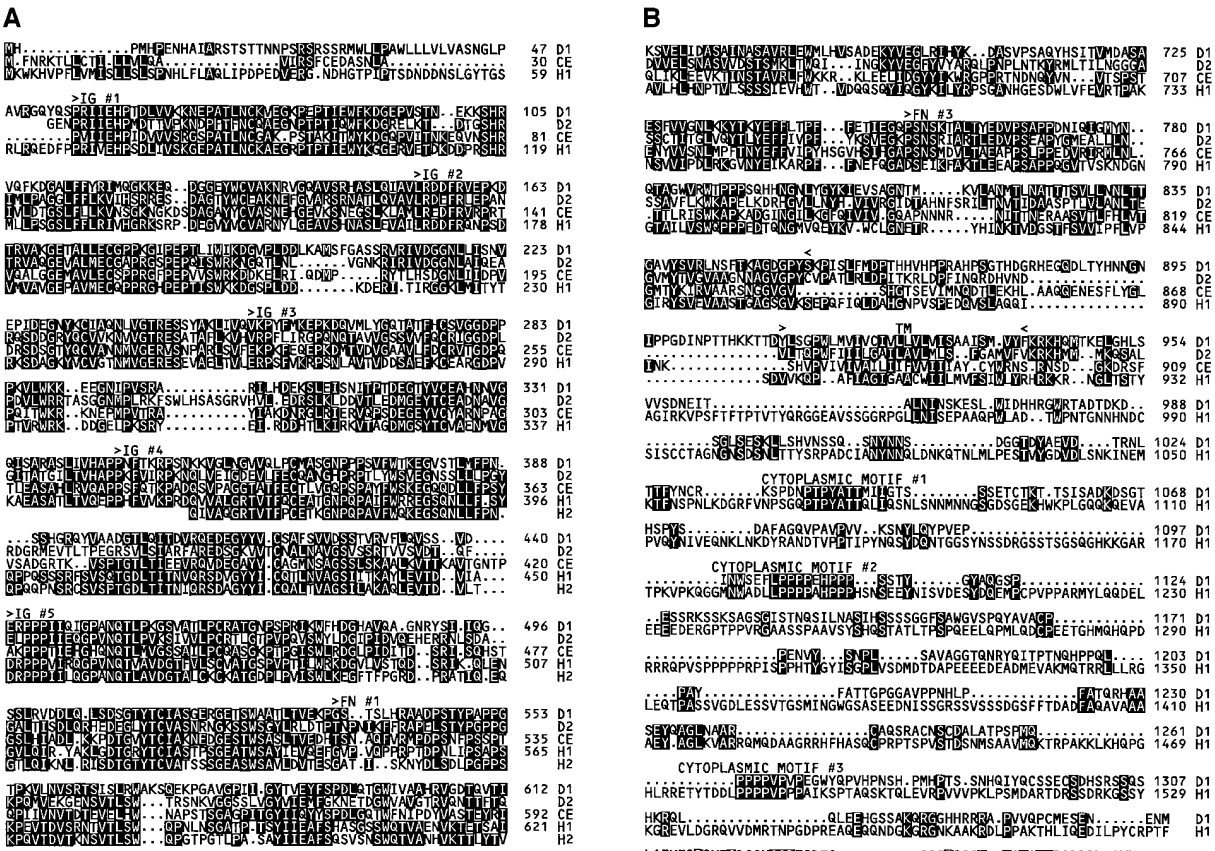

Figure 2. Sequence Alignment of Robo Family Members

The complete amino acid alignment of the predicted Robo proteins encoded by *D-robo1* (D1) and *H-robo1* (H1) are shown. The extracellular domain of *C. elegans robo* (CE; SAX-3; Zallen et al., 1997), the predicted extracellular domain of *D-robo2* (D2), and partial sequence of *H-robo2* (H2) are also aligned. The D2 sequence was predicted by the gene-finder program Grail. The position of immunoglobulin domains (Ig), fibronectin domains (FN), the transmembrane domain (TM), and conserved cytoplasmic motifs are indicated. Amino acid number is only indicated for proteins for which the complete sequence is known. The extracellular domain of rat *robo1* is nearly identical to H1 and is not shown for reasons of space.

three FN domains (Figure 2). The homology is particularly high in the first two Ig domains (58% and 48% amino acid identity, respectively, compared to 26% and 30% for the same two Ig domains between D-Robo1 and CDO) and together with the overall identity throughout the extracellular region and the presence of three conserved cytoplasmic motifs has led us to designate this as the human *roundabout 1* gene (*H-robo1*). Database searching reveals a nucleotide sequence corresponding to *H-robo1* in the database, *DUTT1*, which differs in the signal sequence, suggesting alternative splicing. There is also a 9 bp insertion and seven single base pair changes, presumably polymorphisms. Five ESTs (see Experimental Procedures) show high sequence similarity to the cytoplasmic domain of *H-robo1*. Partial sequencing of cDNAs isolated using one of these ESTs as a probe confirm that there is a second human *roundabout* gene (*H-robo2*).

Degenerate PCR primers based on conserved sequences between *H-robo1* and *D-robo1* were used to isolate a PCR fragment from a rat embryonic E13 spinal cord cDNA library. The fragment was used to probe an E13 spinal cord cDNA library, resulting in the isolation of a full-length rat *robo* gene (*robo1*). The predicted protein shows high sequence identity (>95%) with *H-robo1* over the entire length. The 5′ sequences of different rat *robo1* cDNA clones suggests that this gene is alternatively spliced in a similar fashion to *H-robo1*/*DUTTI*. We used a similar approach to isolate rat *robo2* cDNA clones.

The mouse EST vi92e02 is highly homologous to the cytoplasmic portion of *H-robo1*. The *C. elegans sax-3* gene is also a *robo* homolog (Figure 2; Zallen et al., 1998). A second *Drosophila robo* gene (*D-robo2*) is also predicted from analysis of genomic sequence in the public database. Taken together, these data indicates that Robo is the founding member of a novel Ig subfamily with at least one member in nematode, two in *Drosophila*, two in rat, and two in human (Figure 2).

The alignment of the Robo family proteins reveals that

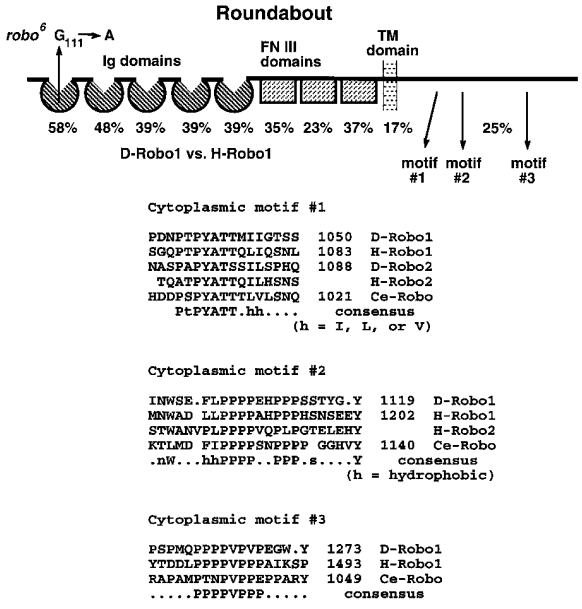

Figure 3. Structure of Robo Protein

Schematic of the structure of D-Robo protein. The position of the Immunoglobulin (Ig), fibronectin (FN), and transmembrane (TM) domains and the amino acid substitution in *robo*^{6} are shown. Percent amino acid identity between D-Robo1 and H-Robo1 is indicated for each domain. Amino acid alignments of the three conserved cytoplasmic motifs are shown below the structure; in *C. elegans robo*, motifs #2 and #3 have been switched to provide a better alignment.

the first and second Ig domains are the most highly conserved portion of the extracellular domain. The cytoplasmic domains are highly divergent except for the presence of three highly conserved motifs (Figure 3). The consensus for the first motif is PtPYATTxhh, where x is any amino acid and h is I, L, or V. The presence of a tyrosine in the center of the motif suggests that it could be a site for phosphorylation. The other two motifs consist of runs of prolines separated by one or two amino acids. All three of these conserved sites could function as the binding sites for cytoplasmic adapter proteins (see Discussion) which would transmit signals generated by ligand binding.

Robo is Regionally Expressed on Longitudinal Axons in the *Drosophila* Embryo

To determine the role that *robo* might play in regulating axon crossing behavior, we examined the *robo* expression pattern in the embryonic CNS. The in situ hybridization pattern of *robo* mRNA in *Drosophila* shows it to have elevated and widespread expression in the CNS. We raised multiple monoclonal and serum antibodies against portions of Robo protein and observed the same staining pattern with all of them. The data shown here uses a monoclonal antibody (MAb 13C9) against part of the extracellular portion (amino acids 404–725) of Robo protein. Robo is first seen in the embryo weakly expressed in lateral stripes during germband extension. At the onset of germband retraction, Robo expression is observed in the neuroectoderm. By the end of stage 12, as the growth cones first extend, Robo is seen on growth cones which project ipsilaterally, including pCC (Figure 4E–4G), aCC, MP1, dMP2, and vMP2. Strikingly, little or no Robo expression is observed on commissural growth cones as they extend toward and across the midline (Figure 4A). However, as these growth cones turn to project longitudinally, their level of Robo expression dramatically increases. Robo is expressed at high levels on all longitudinally projecting growth cones and axons (Figures 4B and 4C). In contrast, Robo is expressed at nearly undetectable levels on commissural axons. This is striking since ~90% of axons in the longitudinal tracts also have axon segments crossing in one of the commissures. Thus, Robo expression is regionally restricted. Robo expression is also seen at a low level throughout the epidermis and at a higher level at muscle attachment sites. In stage 16–17 embryos, faint Robo staining can be seen in the commissures (Figure 4C) but at levels much lower than observed in the longitudinal tracts.

Table 1. *robo* Mutant Alleles

Allele	Synonym	Class
robo^1	GA285	Protein null
robo^2	GA1112	Protein null
robo^2	Z14	Protein null
robo^2	Z570	Protein null
robo^2	Z1772	Protein null
robo^2	Z1757	Protein positive; Gly_{111} to Asp
robo^2	Z2130	Reduced protein levels
robo^2	Z3127	Protein positive

All alleles were generated by EMS mutagenesis of *FasIII* null chromosomes. Each of these alleles appear to represent a complete, or near complete, loss-of-function phenotype for *robo*, since the mutant phenotype observed when these alleles are placed over a chromosome deficient for the *robo* locus [Df(2R) X58-5] is indistinguishable from the homozygous allele.

Immunoelectron Microscopy of Robo

We used immunoelectron microscopy to examine Robo localization at higher resolution. In stage 13 embryos, Robo is expressed at higher levels on growth cones and filopodia in the longitudinal tracts than on the longitudinal axons themselves (Figures 5A and 5D). This localization is consistent with the model that Robo functions as a guidance receptor. The increased sensitivity of immunoelectron microscopy reveals the presence of very low levels of Robo protein on the surface of commissural axons (Figures 5A and 5B). In addition, Robo-positive vesicles can be seen inside the commissural axons (Figure 5A), possibly representing transport of Robo to the growth cone or internalization of Robo from the commissural axons. Finally, by reconstructing the path of single axons by use of serial sections, we confirm that Robo expression is greatly up-regulated after individual axons turn from the commissure (Figure 5B) into a longitudinal tract (Figure 5C). The expression of Robo on noncrossing and postcrossing axons and its higher level of expression on growth cones and its filopodia suggest a model where Robo functions as an axon guidance receptor for a repulsive midline cue.

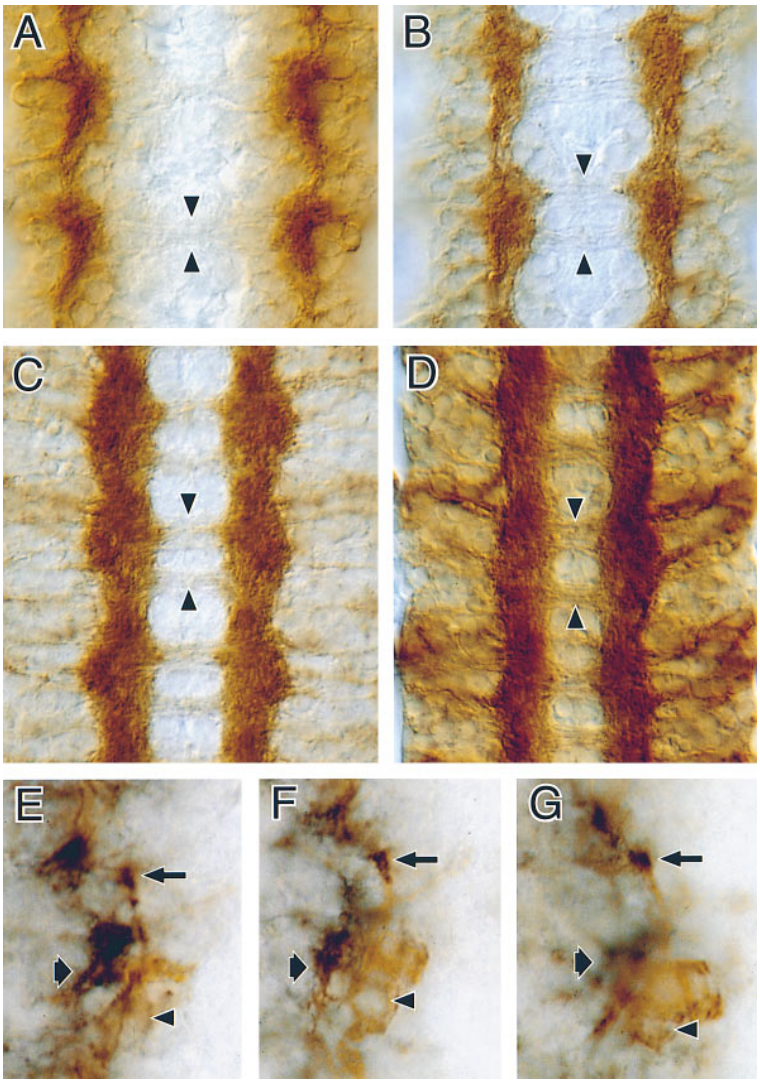

Figure 4. Expression of Robo Protein

The central nervous systems of wild type (A–C, E–G) embryos and a transgenic embryo overexpressing Robo (D; *elav-GAL4::UAS-robo*) are stained with anti-Robo MAb 13C9.

(A) During commissure formation and prior to commissure separation (arrowheads), Robo protein is expressed at high levels on axons in the longitudinal tracts, but is low or absent from commissural axons.

(B) After commissure separation, Robo protein is still low or absent from the commissural axons (arrowheads).

(C) By stage 16, a very low level of Robo protein is detectable in the commissural axons (arrowheads). A higher level of Robo protein is evident on the longitudinal axons.

(D) In a stage 16 embryo overexpressing Robo protein, increased Robo protein is visible in the neuronal cell bodies and the longitudinal axon tracts. Robo protein is still largely absent from the commissural axons (arrowheads).

(E–G) Embryos double stained for Robo protein (black) and Fas II (1D4 MAb, brown) reveal the presence of Robo protein on the pCC neuron growth cone (long arrows). Robo is largely absent from the pCC cell body (stained with anti-Fas II, arrowheads). The other black blobs show the presence of Robo on other growth cones, such as that of the aCC neuron, as indicated by the thick arrows.

Transgenic Expression of Robo

We hypothesized that if Robo is indeed a growth cone receptor for a midline repellent, then panneural expression of Robo protein during the early stages of axon outgrowth might lead to a *robo* gain-of-function phenotype similar to the *comm* loss-of-function and opposite of the *robo* loss-of-function. To test this hypothesis, we cloned a *robo* cDNA containing the complete ORF but lacking most of its untranslated regions (UTRs) downstream of the UAS promoter in the pUAST vector and generated transgenic flies for use in the GAL4 system (Brand and Perrimon, 1993). Expression of *robo* in all neurons was achieved by crossing the *UAS-robo* flies to either the *elav-GAL4* or *scabrous-GAL4* lines.

Surprisingly, panneural expression of *robo* mRNA did not produce a strong axon scaffold phenotype as assayed with MAb BP102. Staining with anti-Fas II (MAb 1D4) revealed subtle fasciculation defects, but overall the axon scaffold looked quite normal. Interestingly, the Robo protein, although expressed at higher levels than in wild type, remains restricted as in wild type, i.e., high levels of expression on the longitudinal portions of axons and very low levels on the commissures (Figure 4D). This result indicates that there must be strong regulation of Robo expression, probably posttranslational, that assures its localization to longitudinal axon segments. Such a mechanism could operate by the regulation of protein translation, transport, insertion, internalization, and/or stability.

We used these transgenic flies to rescue *robo* mutants. Expression of *robo* by the *elav-GAL4* line in both $robo^3$ and $robo^5$ homozygotes rescued the midline crossing of Fas II positive axons, including pCC and other identified neurons, in all segments in all embryos.

Robo Appears to Function in a Cell-Autonomous Fashion

To test whether Robo can function in a cell-autonomous fashion, we used the *UAS-robo* transgene with the ftz_{ng}-*GAL4* line (Lin et al., 1994). The ftz_{ng}-*GAL4* line expresses in a subset of CNS neurons, including many of the earliest neurons to be affected by the *robo* mutation such as pCC, vMP2, dMP2, and MP1. Expression of *robo* by the ftz_{ng}-*GAL4* line is sufficient to rescue these identified neurons in the *robo* mutant; pCC, which in robo mutants heads toward (Figure 6H) and crosses the midline, in

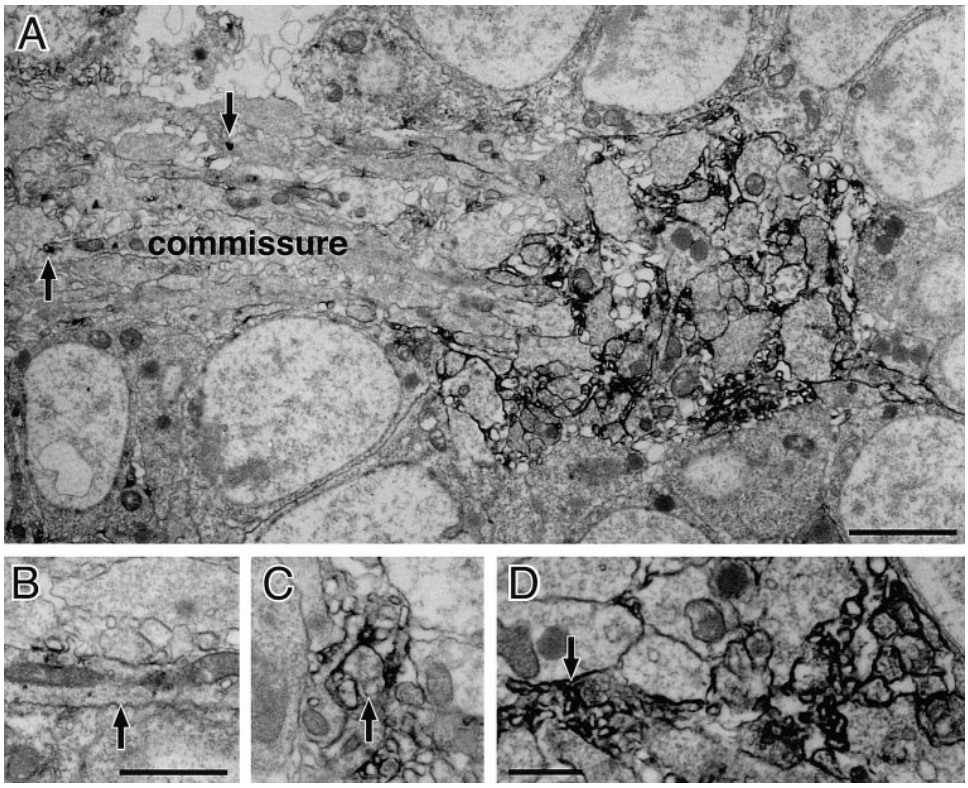

Figure 5. Immuno–Electron Microscopy of Robo Protein Expression

Cross section of wild-type stage 13 nerve cords stained for immuno-EM with anti-Robo MAb 13C9.

(A) Cross section at the level of the anterior commissure. Robo protein is largely absent from the surface of the commissural axons; Robo-positive vesicles are visible within the commissural axons (arrows). In the longitudinal tract, Robo staining is seen on axons but is concentrated on growth cones and in particular on small filopodial processes.

(B) Higher magnification of a commissural axon. Very light Robo staining is visible on the axon surface (arrow).

(C) Higher magnification of the same axon as in (B) (followed in serial sections) but after crossing the commissure and turning into the longitudinal tract. Increased Robo expression is evident on the longitudinal axon surface (arrow) as compared to the commissural axon segment shown in (B).

(D) Higher magnification of part of a longitudinal tract showing Robo protein concentrated on filopodial processes (arrow).

Scale bars = (A) 2 μm; (B and C) 1 μm; (D) 0.5 μm.

these rescued embryos now projects ipsilaterally and does not cross the midline (Figure 6I). When the same embryos were stained with the anti-Robo MAb 13C9, we observed that all Robo-positive axons did not cross the midline (Figure 6C). The *ftz$_{ng}$-GAL4* line drives expression in many of the axons in the pCC pathway (Lin et al., 1994), a medial longitudinal fascicle (arrow in Figure 6D). In *robo* mutants, this axon fascicle freely crosses and circles the midline, joining with its contralateral pathway (arrows, Figure 6E). When rescued by the *ftz$_{ng}$-GAL4* line driving *UAS-robo*, this pathway now largely remains on its own side of the midline (arrow, Figure 6F), even though occasionally a few axons cross the midline (arrowhead, Figure 6F). These experiments support the notion that Robo can function in a cell-autonomous fashion.

Expression of Mammalian *robo1* in the Rat Spinal Cord

The isolation of several vertebrate Robo homologs suggests that Robo may play a similar role in orchestrating midline crossing in the vertebrate nervous system as it does in *Drosophila*. In the vertebrate spinal cord, the ventral midline is comprised of a unique group of cells called the floor plate. As in the *Drosophila* nervous system, the vertebrate spinal cord contains both crossing and noncrossing axons. Spinal commissural neurons are born in the dorsal half of the spinal cord; commissural axons project to and cross the floor plate before turning longitudinally in a rostral direction. In contrast, the axons of two other classes of neurons, dorsal association neurons and ventral motor neurons, do not cross the floor plate (Figure 7A; Altman and Bayer, 1984).

To address the possibility that Robo may play a role in organizing the projections of these spinal neurons, we examined the expression of rat *robo1* by RNA in situ hybridization. At E13, when many commissural axons will have already extended across the floor plate (Figure 7D; Altman and Bayer, 1984), rat *robo1* is expressed at high levels in the dorsal spinal cord in a pattern corresponding to the cell bodies of commissural neurons. Rat *robo1* is also expressed at lower levels in a subpopulation of ventral cells in the region of the developing motor column (Figure 7B). Interestingly, this expression

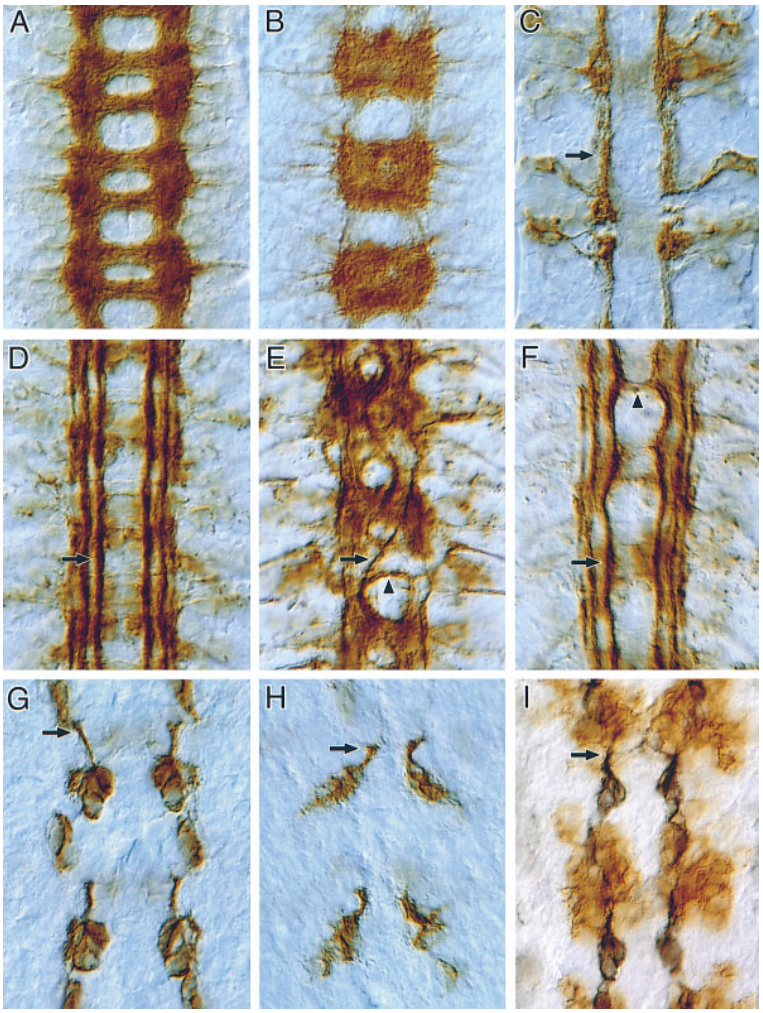

Figure 6. Transgenic Rescue of the *robo* Phenotype

Wild type (A, D, and G), *robo* (B, E, and H), and *ftz*$_{ng}$*-GAL4::UAS-robo* rescued *robo* embryos (C, F, and I) stained with BP102 (A and B), anti-Fas II (D–I), and anti-Robo (C and I) antibodies. The *robo* alleles used were protein nulls.

(A) Wild-type embryo showing the normal scaffold of longitudinal and commissural axon tracts in three adjacent segments. Each neuromere has two commissures.

(B) A *robo* embryo showing thickening of commissures and thinning of longitudinal tracts in three adjacent segments.

(C) *robo* mutant embryo rescued with *ftz*$_{ng}$*-GAL4::UAS-robo* transgenes showing Robo-positive axons not crossing the midline (arrow).

(D) Wild-type embryo showing the characteristic pattern of three Fas II-positive longitudinal fascicles on either side of the midline. The innermost Fas II fascicle, the pCC fascicle, is indicated by an arrow.

(E) *robo* embryo showing the different Fas II-positive fascicles and in particular the pCC pathway inappropriately crossing and circling the midline (arrowhead) and forming fused pathways (arrow).

(F) Rescued mutant embryo showing normal ipsilateral projection of the pCC pathway (arrow). In this example, the rescue is not complete as a few axons still cross the midline (arrowhead).

(G) Wild-type embryo showing the normal trajectory of the pCC growth cone extending anterior and then a bit away from the midline (arrow).

(H) *robo* embryo showing the pCC growth cone extending anterior and toward the midline (arrow).

(I) Rescued mutant embryo double stained for Fas II (black) and Robo (brown). The pCC growth cone is extending parallel to the midline (arrow).

pattern is similar to and overlaps partly with the mRNA encoding DCC (Figure 7C), another Ig superfamily member that is also expressed on commissural and motor neurons and encodes a receptor for Netrin-1 (Keino-Masu et al., 1996). Rat *robo1* is not, however, expressed in either the floor plate or the roof plate of the spinal cord or in the dorsal root ganglia. This is in contrast to rat *cdo*, which is strongly expressed in the roof plate (K. B., M. T.-L., and R. Krauss, data not shown). Therefore, like its *Drosophila* homolog, rat *robo1* RNA appears to be expressed both by neurons with crossing axons and neurons with noncrossing axons, suggesting that it may encode the functional equivalent of D-Robo1.

Discussion

In *robo* mutants, too many axons cross the midline (Seeger et al., 1993) (Figure 6B); axons that normally extend along the midline without crossing it now do cross (Figure 6E), and axons that normally cross the midline once and then turn to project longitudinally now recross multiple times (Kidd et al., 1998). Midline cells appear to develop normally, but growth cones no longer avoid them. The cloning of *robo* and the characterization of the sequence and expression of its encoded protein lead us to propose that Robo is the growth cone receptor for a putative midline repellent and that Robo functions as the gatekeeper controlling midline axon crossing. Our focus in this paper has been on the function of Robo in controlling axon crossing of the midline based on the major phenotype we observe in *robo* mutants. However, given the expression of Robo both within the nervous system and in other tissues during development, our analysis does not preclude other functions for Robo.

Robo Defines a Novel Subfamily of the Ig Superfamily

Robo appears to define a novel family of guidance receptors with five Ig domains followed by three FN-type III domains (herein called the 5 + 3 family of Robo-like receptors; Figure 3). We have identified one Robo-like protein sequence in the nematode database, two in *Drosophila*, two in rat, and two in human. Only one other protein (in rat and human) is known to have a 5 + 3

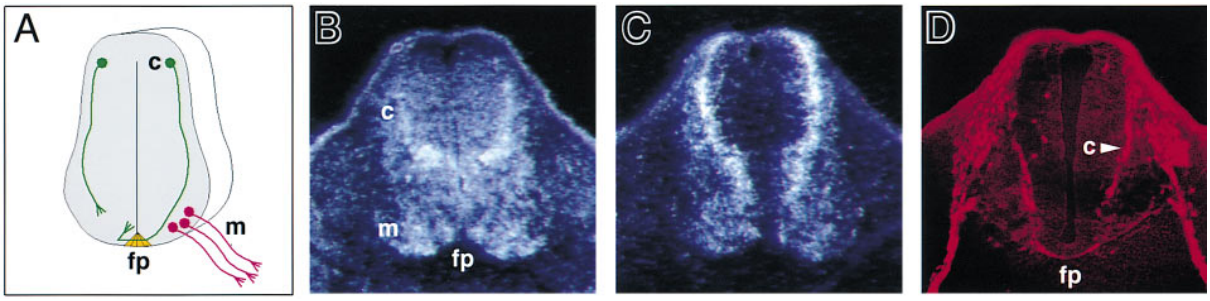

Figure 7. Expression of Rat *robo1* in the Developing Rat Spinal Cord

Expression of rat *robo1* and *DCC* mRNA in semiadjacent transverse sections of E13 rat spinal cord visualized by in situ hybridization (B and C) and TAG-1 protein visualized by immunohistochemistry (D).

(A and D) As shown in schematic and by TAG-1 immunohistochemistry, by E13 a majority of commissural axons (c) have crossed the floor plate (fp). Motoneurons (m) project away from the midline.

(B) At this stage, *robo1* mRNA is detected over the cell bodies of commissural (c) and motor (m) neurons. Lower levels of expression are detected in the intermediate zone of the spinal cord, but expression is largely absent from the floor plate, the roof plate, and the dorsal root ganglia.

(C) The pattern of *robo* mRNA expression overlaps with that of *DCC* mRNA, a marker for commissural neurons.

(D) TAG-1 protein expression at E13 shows commissural axons already crossing the floor plate.

structure, and that is CDO (Kang et al., 1997). However, based on sequence analysis, CDO is much more distantly related to Robo than are the Robo family members described here. All of these Robo family proteins share their greatest sequence similarity in the first two Ig domains (48% and 56% identity, respectively, between D-Robo1 and H-Robo1, compared to 26% and 30% between D-Robo1 and H-CDO).

Drosophila Robo Appears to Encode an Axon Guidance Receptor for a Midline Repellent

Like the DCC family of axon guidance receptors (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996), Robo family members have long cytoplasmic domains. The cytoplasmic domain varies considerably in length across species and has very little sequence similarity with the exception of three proline-rich motifs of about ten or more amino acids in length. These three short regions are highly conserved and might potentially function as binding sites for SH3 domains or other domains in linker proteins functioning in Robo-mediated signal transduction. The first conserved cytoplasmic motif contains a tyrosine and is a potential site for phosphorylation. The second conserved motif contains the sequence LPPPP and is a potential site for *Drosophila* Enabled or its mammalian homolog Mena (Niebuhr et al., 1997). Given the role of *Drosophila* Abl tyrosine kinase in midline guidance (Elkins et al., 1990) and the function of *enabled* as a suppressor of mutations in *Abl* (Gertler et al., 1995), it will be of interest to determine whether Enabled binds the Robo cytoplasmic domain.

Although many guidance molecules with multiple Ig and FN domains function as cell adhesion molecules (CAMs), we think it unlikely that Robo regulates midline crossing by functioning as a CAM. The midline crossing *robo* mutant phenotype shows no evidence of an adhesive function for Robo protein. Moreover, when Robo is expressed in S2 cells, we see no evidence that Robo can mediate homophilic adhesion, in contrast to many *Drosophila* IgCAMs (K. Bland et al., unpublished data). These results do not exclude the possibility that Robo might function as a CAM in a heterophilic fashion.

The pattern of expression of Robo is also consistent with Robo functioning as a guidance receptor. EM analysis shows that Robo protein in the *Drosophila* embryo is expressed at its highest levels on growth cones and filopodia and at lower levels on axons (Figure 5). Robo protein is expressed from the outset on those growth cones that do not cross the midline, while it is dramatically up-regulated from very low levels to high levels on commissural growth cones once they cross the midline and turn to project longitudinally. Consistent with the model of Robo as a guidance receptor, transgenic rescue experiments suggest that Robo can function in a cell-autonomous fashion (Figure 6).

These results are all consistent with the model that Robo functions as a guidance receptor on growth cones and that Robo responds to an unknown midline repellent. While the identity of this midline repellent is still unknown, it appears to be neither Netrin A nor Netrin B (Harris et al., 1996; Mitchell et al., 1996), since *robo* mutants display no genetic interactions with *Netrin* mutants, and Robo-positive axons do not cross the midline in *Netrin* mutants (T. K. et al., unpublished data).

An alternative model is that Robo is a guidance receptor not for a midline repellent, but rather for an unknown longitudinal attractant. Since most guidance decisions appear to require growth cones to assess the relative balance of positive and negative forces in multiple directions (Tessier-Lavigne and Goodman, 1996), it is possible that growth cones might abnormally cross the midline because the alternative choice, extending longitudinally, is no longer as attractive. While formally possible, we think this hypothesis unlikely for several reasons. First, in *robo* mutants growth cones that abnormally cross the midline still tend to extend in the correct direction and project longitudinally the normal distance, and they display their normal patterns of selective fasciculation with the appropriate axons on both sides of the

midline. Second, other mutants that perturb longitudinal extension do not automatically lead growth cones to cross the midline (e.g., *lola* and *logo*; Seeger et al., 1993). Crossing the midline is not the default pathway for abnormal longitudinal guidance cues. Rather, the midline appears to represent a repulsive barrier that is not easily penetrated, even when growth cones do not find their normal longitudinal pathways.

These arguments lead us to favor the model in which Robo functions as a guidance receptor for a midline repellent. Regardless of which model is correct, Robo clearly functions as a guidance receptor, and this receptor's major role is as the gatekeeper controlling crossing and recrossing of the midline. Growth cones expressing high levels of Robo are prevented from crossing the midline, whereas growth cones expressing very low levels of Robo are allowed to cross the midline.

Robo Expression Is Regionally Restricted

Robo protein is regionally restricted to longitudinal axons but not to commissural axons (Figure 4). This regional expression of Robo is similar to what was previously observed for Fasciclin I and Fasciclin II in grasshopper (Bastiani et al., 1987) and TAG-1 and L1 in rat (Dodd et al., 1988). The Robo regional expression is tightly regulated in ways we do not yet fully understand, as indicated by the highly regional protein expression that is observed after panneural expression of the *robo* mRNA. Such a pattern of regulated expression is consistent with a role for Robo in preventing growth cones from crossing the midline; growth cones expressing high levels do not cross, while growth cones expressing very low levels do cross.

Function of Robo Family Members in Other Species

The nematode genome appears to have only a single Robo family member, and this protein is encoded by the *sax-3* gene (Zallen et al., 1998). Mutations in *sax-3* lead to a variety of axon guidance defects, including abnormal crossing of the ventral nerve cord and other directional and fasciculation phenotypes. The crossover phenotype is highly analogous to the midline-crossing phenotype in *Drosophila* and suggests a similar receptor function for *C. elegans* Robo. However, some of the other axon guidance defects suggest that Robo in the nematode may have additional roles in guidance.

We have identified two human Robo family genes (*H-robo1* = *DUTTI*, and *H-robo2*), two rat Robo family members, and a second Robo-like gene in *Drosophila* (*D-robo2*). At the moment, we know the most about *robo1* in the rat. Rat *robo1* mRNA is expressed by commissural neurons in the developing spinal cord in a manner consistent with this mammalian receptor playing a role in midline guidance similar to its *Drosophila* counterpart. Determining whether or not rat Robo1 protein, like its homolog in *Drosophila*, is differentially expressed by commissural axons before or after crossing of the midline awaits the generation of antibodies against rat Robo1. Likewise, genetic loss-of-function perturbation experiments will be required to determine if rat Robo1 truly does function as the gatekeeper controlling axon crossing of the midline in mammals as it does in *Drosophila*. However, given the remarkable conservation of the structure and function of axon guidance ligands and receptors such netrins, DCC, semaphorins, and IgCAMs (reviewed by Tessier-Lavigne and Goodman, 1996), we anticipate that Robo functions in a similar fashion to control midline axon crossing in all bilaterally symmetric nervous systems.

Relationship Between Roundabout and Commissureless

In *comm* mutant embryos, too few axons cross the midline, while the opposite phenotype is observed in *robo* mutant embryos (Seeger et al., 1993). Previous studies showed that the double mutant (*comm*; *robo*) displays a phenotype that is indistinguishable from *robo* alone, thus leading to the model that the Comm protein is required for certain growth cones to cross the midline in the presence of Robo. Comm protein is expressed on the surface of midline cells (Tear et al., 1996). These results suggest a model where Robo functions as a guidance receptor for a midline repellent and Comm is required to down-regulate Robo expression or function on commissural axons, thus allowing them to cross the midline. In another paper (Kidd et al., 1998), we examine the dosage sensitivity and complementary functions of Comm and Robo. We show that Comm regulates Robo protein expression and that both proteins function in a dosage-sensitive mechanism that regulates the ability of axons to cross the midline. In light of the fact that the *C. elegans* ventral nerve cord lacks commissures, it is interesting that, as yet, no *comm* homologs have been discovered in the ~80% sequenced regions of the *C. elegans* genome (C. Bargmann and J. Zallen, personal communication). It may be that Robo had an early phylogenetic function in axon guidance, in part preventing midline crossing. The evolution of complex bilaterally symmetric central nervous systems containing longitudinal axon tracts connected by commissures in the arthropod and chordate lineages led to the concomitant guidance decision of whether to cross or not to cross the midline. With this choice, Comm may have evolved to help regulate the expression and thus the function of Robo.

Experimental Procedures

Genetic Stocks

All eight independent *robo* alleles were isolated on chromosomes deficient for *Fasciclin III* as described in Seeger et al., 1993. Subsequent use of a duplication that includes *FasIII*, and recombination of the *robo* chromosomes, indicates that the *robo* phenotype is independent of the absence of *FasIII*. Deficiencies were obtained from the *Drosophila* stock center at Bloomington, Indiana, and from the laboratories of C. Lehner and T. Orr-Weaver. A homozygous viable insert of *elav-GAL4* on the third chromosome was obtained from A. DiAntonio.

One deficiency [*Df(2R)P*, which deletes 58E3/F1 through 60D14/E2] fails to complement *robo* mutations, two other deficiencies [*Df(2R)59AB* and *Df(2R)59AD*, which delete 59A1/3 through 59B1/2 and 59A1/3 through 59D1/4, respectively] complement *robo*, and a duplication [*Dp(2;Y)bw+Y*, which duplicates 58F1/59A2 through 60E3/F1] rescues *robo* mutations. This mapping places *robo* in the 58F/59A region. *Df(2R)X58–5* and *Df(2R)X58–12* remove *robo*, while

Df(2R)X58–1 does not. By mapping the breakpoints of these deficiencies, we were able to define the limits of the region that contained *robo*.

Cloning and Molecular Analysis of the *robo* Genes

Start points for a molecular walk to *robo* were the P1 clones, DS02204 and DS05609, that were obtained from the Berkeley and Crete *Drosophila* Genome Projects. Chromosomal walking was performed using standard techniques to isolate cosmids from the Tamkun library (Tamkun et al., 1992). cDNAs were isolated from the Zinn 9–12 hr *Drosophila* embryo λgt11 library (Zinn et al., 1988) and from a human fetal brain library (Stratagene). Northern blot of poly-A^+ RNA and reverse Northern blots were hybridized using sensitive Church conditions. Sequencing of the cDNAs and genomic subclones was performed by standard methods.

A full-length *D-robo1* cDNA was generated by ligating two partial cDNAs at an internal HpaI site and subcloning into the EcoRI site of pBluescript.SK^+. A full-length *H-robo1* cDNA was synthesized by ligating an XbaI-SalI fragment from a cDNA and a PCR product coding for the carboxy-terminal 222 amino acids at an SalI site. The PCR product has an EcoRI site introduced at the stop codon. The ligation product was cloned into pBluescript.SK^+ digested with XbaI and EcoRI.

To clone the rat *robo1* cDNA, degenerate oligonucleotide primers designed against sequences conserved between the 5′ ends of D-Robo1 and H-Robo1 were used to amplify a 500 bp fragment from an E13 rat brain cDNA by PCR. This fragment was used to screen an E13 spinal cord library at high stringency, resulting in the isolation of a 4.2 kb cDNA clone comprising all but the last 700 nucleotides. Subsequent screenings of the library with nonoverlapping probes from this cDNA led to the isolation of four partial and seven full-length clones. To clone the rat *robo2* cDNA, we screened the same library with a fragment of the *H-robo2* cDNA.

Expressed Sequence Tag and Genomic Sequences

The ESTs yu23d11 (GenBank #H77734), zr54g12 (GenBank #AA236414) and yq76e12 (Genbank #H52936 and #H52937) code for portions of H-RoboI. The EST yq7e12 is aberrantly spliced to part of the human glycophorinB gene. Five ESTs, yn50a07, yg02b06, yg17b06, yn13a04, and ym17g11, code for part of *H-robo2*. The *Drosophila* P1 clone DS00329 encodes the genomic sequence of *D-robo2*. Sequences 1825710 and 1825711 (both GenBank #U88183; locus ZK377) code for the predicted sequence of *C. elegans robo*. The EST vi62e02 (GenBank #AA499193) codes for mouse *robo1*.

Identification of Molecular Defects in *robo* Alleles

Southern blots of *robo* alleles and their parental chromosomes were hybridized with fragments from cosmid 106–1435 or partial cDNA clones to identify RFLPs affecting the *robo* transcription unit. DNA was obtained from homozygous mutant embryos. 35 cycles of the PCR was performed on the DNA obtained from half of an embryo. Primers specific for the region flanking the CfoI polymorphism used were ROBO6 (5′-GCATTGGGTCATCTGTAGAG-3′) and ROBO23 (5′-AGCTATCTGGAGGGAGGCAT-3′). The PCR products were sequenced directly.

Transformation of *Drosophila*, *robo* Rescue, and Overexpression

The 16 kb XbaI fragment from cosmid 106–1435 was cloned into the *Drosophila* transformation vector pCaSpeR3. Transformant lines were generated and mapped by standard procedures. Four independent lines were shown to rescue $robo^{1,3,5}$ alleles as judged by MAb 1D4 staining.

PCR amplification of the *D-robo* ORF using the primers 5′-GAGTG GTGAATTCAACAGCACCAAAACCACAAAATGCATCCC-3′ and 5′-CGGGGAGTCTAGAACACTTCATCCTTAGGTG-3′ produced a PCR product with an altered ribosome binding site that more closely matches the *Drosophila* consensus (Cavener, 1987) and has only 21 bp of 5′ UTR and no 3′ UTR sequences. The PCR product was cloned into the EcoRI and XbaI sites of pUAST (Brand and Perrimon, 1993). Transformant lines were crossed to *elav-GAL4* and *sca-GAL4* lines, which express GAL4 in all neurons, or ftz_{ng}*-GAL4*, which expresses in a subset of CNS neurons (Lin et al., 1994). Embryos were assayed by staining with MAbs BP102, 1D4, and 13C9. For ectopic expression in the *robo* mutant background, the stocks $robo^3$ and $robo^5$ (both protein nulls) were used.

Generation of Fusion Proteins and Antibodies

A six-histidine-tagged fusion protein was constructed by cloning amino acids 404–725 of the D-Robo protein into the PstI site of the pQE31 vector (Qiagen). Fusion proteins were purified under denaturing conditions and subsequently dialyzed against PBS. Immunization of mice and MAb production followed standard protocols (Patel, 1994).

RNA Localization and Protein Immunocytochemistry

In situ tissue hybridization was performed as described in Tear et al., 1996. Immunocytochemistry was performed as described by Patel, 1994. MAb 1D4 was used at a dilution of 1:5 and BP102 at 1:10. For anti-Robo staining, MAb 13C9 was diluted 1:10 in PBS with 0.1% Tween-20, and the embryos were fixed and cracked so as to minimize exposure to methanol. The presence of triton and storage of embryos in methanol were both found to destroy the activity of MAb 13C9.

In situ hybridization of rat spinal cords was carried out essentially as described in Fan and Tessier-Lavigne, 1994. E13 embryos were fixed in 4% paraformaldehyde, processed, embedded in OCT, and sectioned to 10 μm. A 1.0 kb ^{35}S antisense rRobo riboprobe spanning the first three immunoglobulin domains was used for hybridization. An additional nonoverlapping probe was also used with identical results. DCC transcripts were detected as described in Keino-Masu et al., 1996. Immunohistochemistry against TAG-1 was carried out on 10 μm transverse spinal cord sections using MAb 4D7 (Dodd et al., 1988).

Electron Microscopy

Canton S embryos were hand devitellinized, opened dorsally to remove the gut, and prepared for immuno–electron microscopy according to the procedures described previously (Lin et al., 1994) with the following modifications. The fixed embryos were incubated sequentially with MAb 13C9 (1:1) for 1–2 hr, biotinylated goat anti-mouse secondary antibody (1:250) for 1.5 hr, and then streptavidin-conjugated HRP (1:200) for 1.5 hr. Hydrogen peroxide (0.01%) was used instead of glucose oxidase for the HRP–DAB reaction.

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GenBank Accession Numbers

GenBank accession numbers for the *robo* genes described in this paper are as follows: *H-robo1*, AF040990; *H-robo2*, AF040991; rat *robo1*, AF041082; and *D-robo1*, AF040989.