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Comm Sorts Robo to Control Axon Guidance at the *Drosophila* Midline

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Summary

Axon growth across the *Drosophila* midline requires Comm to downregulate Robo, the receptor for the midline repellent Slit. We show here that *comm* is required in neurons, not in midline cells as previously thought, and that it is expressed specifically and transiently in commissural neurons. Comm acts as a sorting receptor for Robo, diverting it from the synthetic to the late endocytic pathway. A conserved cytoplasmic LPSY motif is required for endosomal sorting of Comm in vitro and for Comm to downregulate Robo and promote midline crossing in vivo. Axon traffic at the CNS midline is thus controlled by the intracellular trafficking of the Robo guidance receptor, which in turn depends on the precisely regulated expression of the Comm sorting receptor.

Introduction

Why do some axons but not others cross the midline of the CNS? And why, having crossed once, are these axons not guided back across the midline again by the mirror-image set of cues they encounter on the opposite side? These questions reflect two more general problems in axon guidance: why do different axons behave differently at their common choice points? And how is a growth cone enticed to keep moving on once it reaches an intermediate target? In this paper we present evidence that at one such choice point and intermediate target, the midline of the *Drosophila* CNS, growth cone behavior is controlled by regulating the intracellular trafficking of a guidance receptor.

In the *Drosophila* nerve cord, as in the vertebrate spinal cord, many neurons extend axons across the midline to reach targets on the opposite (contralateral) side. These axons form the commissures that connect the two symmetric halves of the nervous system. Commissural axons cross the midline only once, even though they often continue to grow right alongside it on the opposite side. Other axons (approximately 10% in *Drosophila*) never cross the midline at all, seeking instead targets on their own (ipsilateral) side.

Axon guidance decisions at the *Drosophila* midline are made largely in response to attractants and repellents provided by midline cells. The midline attractants include the two *Drosophila* Netrins, which attract growth cones expressing the receptor Frazzled (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Attraction by Netrins is essential for at least some commissural axons to grow across the midline, but does not appear to dictate the choice of a contralateral versus ipsilateral pathway. This decision is made instead in response to the midline repellent Slit (Kidd et al., 1998a, 1999). Slit is a large secreted protein that repels growth cones expressing members of the Roundabout (Robo) family of receptors (Kidd et al., 1998a, 1999; Rajagopalan et al., 2000b; Simpson et al., 2000). Of the three Robo receptors in *Drosophila*, Robo itself (the founding member of the family) is primarily responsible for keeping ipsilateral axons from crossing the midline and commissural axons from recrossing (Seeger et al., 1993; Kidd et al., 1998a; Rajagopalan et al., 2000a; Simpson et al., 2000).

The decision to cross or not to cross (or recross) the midline can be explained by the distribution of Robo protein (Kidd et al., 1998a). Commissural growth cones express only very low levels of Robo protein as they grow across the midline, but they dramatically upregulate their Robo levels once they reach the opposite side. Ipsilateral growth cones, in contrast, express high levels of Robo from the outset. If Robo levels are made low on all growth cones (by a *robo* loss-of-function mutation), then ipsilateral axons can cross the midline and commissural axons can recross (Seeger et al., 1993). Conversely, if Robo is maintained at high levels on all growth cones (by transgenic expression of *robo* under strong neuronal promoters), then no axons cross (Kidd et al., 1999). High levels of Robo are thus both necessary and sufficient to prevent midline crossing. How, then, are Robo levels regulated?

Two important clues to this puzzle have been provided. The first is that Robo protein expressed from panneuronal transgenes is properly regulated, even if these transgenes lack the untranslated regions of the *robo* mRNA (Kidd et al., 1998a; Rajagopalan et al., 2000a). This indicates that Robo levels are regulated posttranscriptionally, probably posttranslationally. The second clue comes from the identification of the *commissureless* (*comm*) gene (Seeger et al., 1993; Tear et al., 1996). As the name indicates, commissural axons do not cross the midline in *comm* mutants. This is due to excess *robo* function, since they do cross, along with ipsilateral axons, in *robo comm* double mutant embryos (Seeger et al., 1993). Conversely, if *comm* is expressed throughout the CNS, then Robo is downregulated on all growth cones and a *robo*- or *slit*-like phenotype results (Kidd et al., 1998b; Rajagopalan et al., 2000a; Simpson et al., 2000). These data suggest that the normal function of Comm is to keep Robo levels low on commissural growth cones as they cross the midline. But how does

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Comm downregulate Robo, and why does it do so specifically in commissural growth cones before but not after they have crossed the midline?

The molecular characterization of the *comm* gene provided few hints (Tear et al., 1996), even after the subsequent characterization of *robo* (Kidd et al., 1998a, 1998b). Comm is a predicted transmembrane protein of 370 amino acids but lacks a signal sequence or any other motif that might reveal its molecular function. Homologs in other species have not been reported, although the recent completion of the *Drosophila melanogaster* (Adams et al., 2000) and *Anopheles gambiae* (Holt et al., 2002) genome sequences indicates the presence of two other *comm*-like genes in *Drosophila* and one in *Anopheles*, as we describe in more detail here.

In their initial characterization of Comm, Tear et al. (1996) proposed that it acts nonautonomously, a model further elaborated by Kidd et al. (1998b). This idea was based on the finding that *comm* mRNA is expressed in midline glia, whereas Comm protein is present not only in midline glia, but also along the midline segments of commissural axons. Some Comm protein could also be detected in neuronal cell bodies, but not *comm* mRNA. From these findings, Tear et al. (1996) inferred that Comm protein must be transferred from midline glia to commissural axons. Since both the mechanism of transfer and the reason why it would be specific to precrossing commissural axons were unknown, this curious (but not unprecedented) finding only deepened the mystery surrounding Comm function.

Recently, Georgiou and Tear (2002) have challenged this view by using transgenic RNAi experiments to demonstrate an autonomous requirement for *comm* function in neurons. Cell transplantation experiments we present here provide further evidence that *comm* is required in commissural neurons, not in midline cells, for midline crossing. Consistent with this autonomous function, but in contrast to the initial report (Tear et al., 1996), we also find that *comm* is indeed expressed in neurons, specifically in commissural but not ipsilateral neurons. Expression in commissural neurons is transient, coinciding with axon growth across the midline. We also show that Comm is a sorting receptor for Robo, diverting it from the synthetic to the late endocytic pathway even before it reaches the cell surface. These data support a model in which *comm* expression is the autonomous switch that determines if and when an axon can cross the midline, and that Comm allows axon growth across the midline by removing Robo from new membrane vesicles before they are delivered to the growth cone.

Results

Cell Transplantation Experiments Reveal an Autonomous Requirement for *comm*

We performed a series of cell transplantation experiments to resolve the question of which cells must be *comm*$^+$ for axons to cross the midline. Single neurectodermal cells were transplanted either from a *comm*$^+$ donor into a *comm*$^-$ host or vice versa. Cells were taken from and inserted into various dorsoventral positions within the thoracic or abdominal neurectoderm. Donor embryos were injected with HRP at the syncitial blastoderm stage, and cells were transplanted at the early gastrula stage (stage 7). The lineages and axon projection patterns generated by these transplanted progenitor cells were then visualized by fixing and staining the host embryos just prior to hatching (stage 16–17). In a series of 46 control transplantations from *comm*$^+$ donors into *comm*$^+$ hosts, 8 transplantations (17%) resulted in clones with only ipsilateral projections, while 38 (83%) generated both ipsilateral and contralateral projections (Table 1). This reflects the normal distribution of axon projections within the CNS (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999).

Behavior of* comm$^+$ *Neurons in a* comm$^-$ *Host

A total of 25 clones were recovered from *comm*$^+$ cells transplanted into *comm*$^-$ embryos. If *comm* function were required exclusively in midline cells, then the *comm*$^+$ neurons, like those of the *comm*$^-$ host, should all project ipsilaterally. In fact, ten clones (40%) included neurons with contralateral projections (Table 1 and Figures 1A–1D). The ability of *comm*$^+$ neurons to extend axons across the *comm*$^-$ midline, even as all other commissural axons are diverted into longitudinal pathways, is compelling evidence that wild-type *comm* function is not strictly required in midline cells for midline crossing.

Nevertheless, the percentage of *comm*$^+$ clones with contralateral projections in the *comm*$^-$ host (40%) is still only half of that observed in *comm*$^+$ hosts (83%). Equal numbers of crossing axons would be expected in these two sets of transplantations only if each commissural axon were to decide independently whether or not to cross the midline. This is unlikely to be the case, as axons often navigate at least in part by fasciculating with axons that have preceded them. Many *comm*$^+$ commissural axons may simply follow the wayward *comm*$^-$ host axons into a longitudinal pathway. While this is formally a nonautonomous function of *comm*, it is still a requirement for *comm* in commissural neurons, not in midline cells.

Behavior of* comm$^-$ *Neurons in a* comm$^+$ *Host

In the reciprocal set of transplantations, 65 clones were recovered from *comm*$^-$ cells transferred into *comm*$^+$ hosts. Contralateral projections were lacking in 45 (69%) of these *comm*$^-$ clones, compared to just 17% of the control clones (Table 1 and Figures 1E–1H). The hypothesis that *comm* function is required in midline cells predicts these ratios to be equal and is rejected with high probability ($p < 0.0001$, χ^2 test).

In some cases, it was also possible to identify the transplanted precursor based on the positions and projections of the neurons it generated. Among those clones that could be identified, we detected one NB3-1 clone and one NB5-6 clone. Normally, both NB3-1 and NB5-6 generate contralateral projections (Figures 1I and 1J; Bossing et al., 1996; Schmidt et al., 1997), as we also observed for these two neuroblasts in the control transplantations. In contrast, the *comm*$^-$ NB3-1 and NB5-6 clones recovered in *comm*$^+$ hosts consisted exclusively of ipsilateral projections (Figures 1E and 1F).

These data clearly demonstrate that wild-type *comm* function is required in the commissural neurons themselves. However, a small number of *comm*$^-$ neurons did still cross in these experiments. These *comm*$^-$ commissural axons may have followed the *comm*$^+$ host commissural axons across the midline, rather than responding

Table 1. Transplantation Results

Series	Total Clones		No Contralateral Projections		Contralateral Projections	
			n	%	n	%
comm$^+$ → *comm*$^+$	46	observed	8	17.4	38	82.6
comm$^+$ → *comm*$^-$	25	observed	15	60.0	10	40.0
		expected[a]	25	100.0	0	0.0
comm$^-$ → *comm*$^+$	65	observed	45	69.2	20	30.8
		expected[a]	11	17.4	54	82.6

Clones were scored for contralateral projections in stage 16–17 embryos, following transplantation of single progenitor cells at stage 7.
[a]The expected distribution if *comm* were required exclusively in midline cells.

independently to the midline guidance cues. It is possible that some axons also cross the midline without requiring *comm* in wild-type embryos. The fully penetrant phenotype of *comm* null mutations implies only that the commissural pioneers need *comm*, not that all commissural neurons need it.

Dynamic Expression of *comm* in Neurons

Our finding of an autonomous requirement for *comm* in neurons is difficult to reconcile with the report of Tear et al. (1996) who observed strong midline *comm* expression but did not detect any expression in CNS neurons beyond mid-stage 12. We therefore reexamined the distribution of *comm* mRNA in the developing CNS, from the beginning of axonogenesis at early stage 12 until shortly before hatching.

We confirm the prominent expression of *comm* at the CNS midline throughout this period. However, we also observe a strong and dynamic pattern of *comm* expression in CNS neurons (Figure 2). At mid-stage 12, as the first commissural axons extend across the midline, the neurons expressing *comm* include RP1 and RP3, as noted by Tear et al. (1996), as well as a single neuron located more laterally that was not described in that study (Figure 2A). As development proceeds, many more neurons express *comm*, with the number of *comm*-positive neurons peaking at about stage 14 and gradually subsiding toward the end of embryogenesis (Figures 2B–2G). Although not as persistent, expression of *comm* in neurons is generally as strong as it is at the midline.

comm Is Expressed Specifically in Commissural Neurons and Is Extinguished after Crossing

We have shown that *comm* is required in commissural neurons for crossing. Conversely, forced expression of *comm* in ipsilateral neurons is sufficient to reroute them across the midline (Kidd et al., 1998b; Bonkowsky et al., 1999). We therefore anticipated that *comm* would be expressed in commissural neurons but not in ipsilateral neurons. Furthermore, since *comm* is expressed in such a dynamic pattern, we wondered whether commissural neurons might only express *comm* as their axons grow

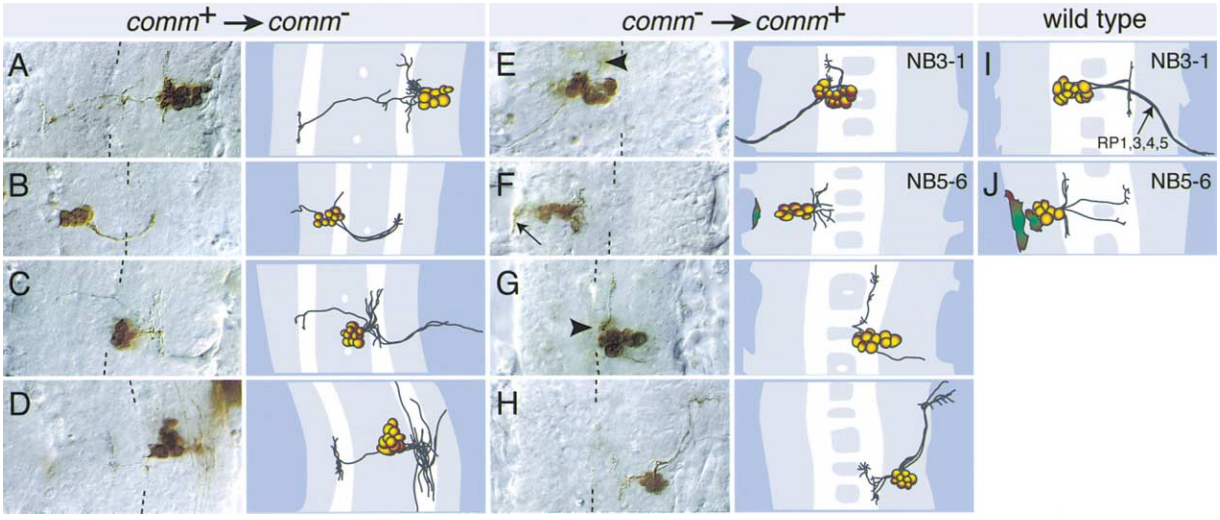

Figure 1. Cell Transplantations Reveal an Autonomous Requirement for *comm*

Neurectodermal cells were transplanted from wild-type (*comm*$^+$) donors into *comm*$^-$ null mutant hosts (A–D) or vice versa (E–H), and the resulting clones visualized in stage 16–17 embryos. Left panels show a dorsal view of the ventral nerve cord, with anterior up. The midline is indicated by dashed lines. Right panels are drawings of the same preparations. Arrowheads in (E) and (G) indicate axons which grew a short distance toward the midline, but then turned away. Clones identified as NB3-1 and NB5-6 are shown in (E) and (F), respectively, with corresponding drawings of wild-type clones shown in (I) and (J) (redrawn from Bossing et al., 1996, and Schmidt et al., 1997). NB3-1 clones include the normally contralateral RP1, 3, 4, and 5 motor neurons. The NB5-6 clones include subperineural glia (arrow on photomicrograph, green in drawing).

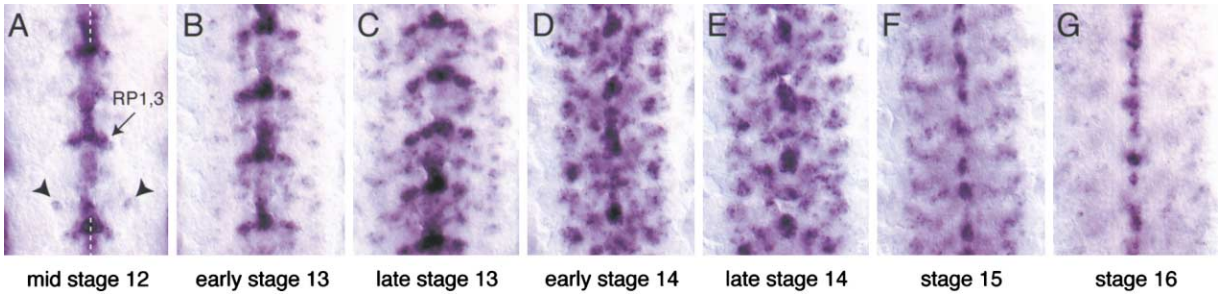

Figure 2. Expression of *comm* in CNS Neurons during Axon Pathfinding

Dynamic pattern of *comm* mRNA accumulation in the ventral nerve cord from stages 12 to 16. Dorsal views of the ventral nerve cord are shown, with anterior up. The midline is indicated by the dashed line in (A). The arrow in (A) indicates *comm* expression in the RP1 and RP3 motor neurons. Arrowheads indicate expression in a lateral pair of cells, which are not in focus in every segment.

across the midline. To explore these ideas, we surveyed *comm* expression in a set of identifiable neurons for which specific axonal markers are available, thus allowing us to correlate *comm* expression with growth cone behavior. *comm* expression was detected by fluorescent in situ hybridization, followed by immunofluorescent staining to reveal the axonal marker (Figure 3).

The set of neurons examined included both commissural and ipsilateral neurons, and in each class both motor neurons and interneurons. The commissural neurons examined were (1) the RP1, 3, 4, and 5 motor neurons, which express the *lim3A-τmyc* reporter (Thor et al., 1999); (2) the cluster of 10–15 lateral EG interneurons labeled by *eg-GAL4* (Higashijima et al., 1996; Dittrich et al., 1997); (3) the three EW interneurons, also labeled with *eg-GAL4*; (4) the drlU intersegmental interneuron, identified with *drlU-τmyc* (Bonkowsky and Thomas, 1999); and (5) the Sema2b intersegmental interneuron in each of the A4-A8 hemisegments, identified with *Sema2b-τmyc* (Rajagopalan et al., 2000b). The ipsilateral neurons examined were (1) the aCC motor neuron and (2) the pCC intersegmental interneuron, both of which are labeled by anti-FasciclinII MAb 1D4; and (3) the dorsal Ap intersegmental interneuron, labeled with *ap-GAL4* (O'Keefe et al., 1998).

Our analysis of *comm* expression in these neurons revealed a striking correlation between *comm* expression and a contralateral projection (Figure 3M): all of the commissural neurons and none of the ipsilateral neurons express *comm*. At least for this set of neurons, the correlation is perfect, with just two minor caveats. First, for the EG neurons, and to a lesser extent the RP and EW neurons, we cannot be entirely confident that every single neuron in these clusters expresses *comm*, though we gained impression that this is likely to be the case. Second, the Sema2b neuron can only be identified after its axon has crossed the midline, at which time *comm* expression appears to be stochastic. Stochastic expression of *comm* is also seen in the Ap ipsilateral neuron at later stages, although it is consistently negative for *comm* as its axon first contacts the midline and turns to avoid it.

We also found a striking temporal correlaton between *comm* expression and midline crossing (Figure 3N). The RP, drlU, EG, and EW neurons all extinguish their *comm* expression shortly after their axons have crossed the midline. Interestingly, the EG and EW neurons, which are the only commmissural neurons we can identify before their axons reach the midline, are also clearly negative for *comm* prior to crossing (Figures 3A and 3B). In particular, the EW axons grow anteriorly for a short distance before turning medially to cross the midline. These neurons do not appear to express *comm* until they make this medial turn (Figures 3B and 3C).

Comm Associates with Robo and Recruits It to Endosomes

Ipsilateral neurons and postcrossing commissural neurons express *robo* but not *comm*, and Robo levels are high in the growth cone, whereas crossing commissural neurons express both *robo* and *comm*, and Robo levels are low. How does coexpression of Comm prevent Robo from accumulating in the growth cone? To address this question, we sought to mimic these two situations by expressing *comm* and *robo* alone or together in cultured cells.

In COS cells that express *robo* alone, Robo protein is present mainly at the plasma membrane, as well as in the Golgi and endoplasmic reticulum (Figures 4A and 4H). Only a small amount of Robo can be detected in endosomes (Figures 4K and 4N). In contrast, in cells that express both *robo* and *comm*, most Robo protein is in late endosomes and lysosomes, where it colocalizes with Comm (Figures 4C, 4M, and 4P). This endosomal staining of Comm is also seen in the absence of Robo (Figures 4B, 4L, and 4O) and is reminiscent of the punctate distribution of endogenous Comm in neurons (Tear et al., 1996; Georgiou and Tear, 2002). Comm can also usually be detected in the Golgi (Figure 4I) but not at the plasma membrane. Plasma membrane staining is normally only seen in cells expressing particularly high levels of Comm, suggesting that the machinery that sorts Comm to the endosomal compartment can be saturated.

We conclude that Comm is normally sorted to the late endosomal-lysosomal system and can also recruit Robo to this compartment. To test whether this effect might be specific for Robo, we asked whether Comm could also recruit the Netrin receptor Frazzled (Fra) to endosomes. It could not (Figure 4D). Using Fra-Robo and Robo-Fra chimeric receptors, in which the cytoplasmic domains of the two receptors had been swapped, we

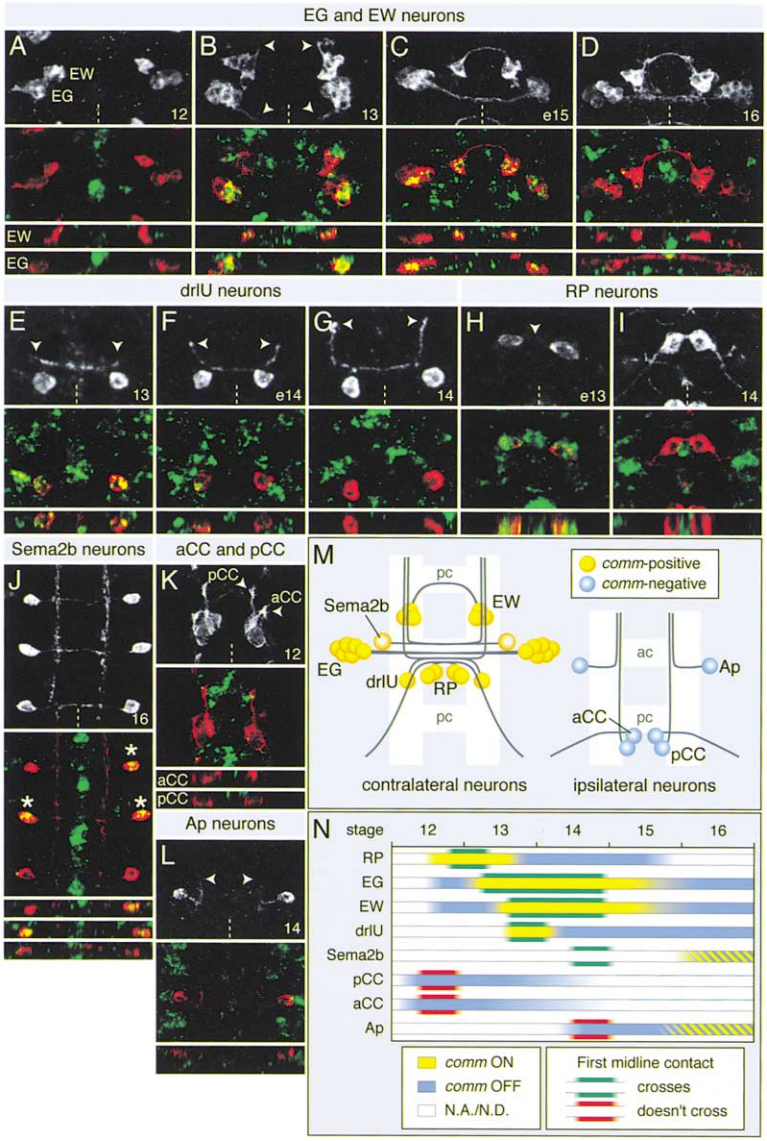

Figure 3. *comm* Expression Correlates Spatially and Temporally with Midline Crossing

(A–L) Confocal micrographs of the CNS of embryos stained for *comm* mRNA (green) and various axonal markers (red). In each set of panels, the upper panel is a maximum intensity projection of the red channel for the entire confocal stack, to show both axons and cell bodies. The second panel is a single xy confocal section at the level of the relevant neuronal cell bodies, and the lower panel(s) are xz sections. The axonal marker does not always evenly fill the cell bodies, and so the *comm* signal in the labeled neurons does not always appear yellow. For (A)–(D), the xy images are montages of a ventral section at the level of the EG neurons and a dorsal section through the EW neurons. In (J), the three xz sections correspond to each of the three segments shown. Numbers in the upper panels indicate the stage (e, early). Arrowheads indicate growth cones. Asterisks in (J) indicate Sema2b neurons positive for *comm*. Genotypes and antibodies were as follows: (A–D) *eg-GAL4/UAS-τlacZ*, stained with anti-β-galactosidase; (E–G) *drlU-τmyc*, anti-myc; (H and I) *lim3A-τmyc*, anti-myc; (J) *Sema2b-τmyc*, anti-myc; (K) wild-type, MAb 1D4; (L) *ap-GAL4/UAS-τlacZ*, anti-β-galactosidase.

(M) Positions and projections of the surveyed neurons at the end of embryogenesis, indicating the status of *comm* expression as their axonal growth cones initially confront the midline. For the Sema2b neurons, this could not precisely be determined, and these neurons have been colored to reflect the stochastic expression observed after crossing. Abbreviations: pc, posterior commissure; ac, anterior commissure.

(N) Time course of *comm* expression and midline guidance decisions of identified neurons. N.A./N.D. indicates that the relevant marker is not expressed or does not uniquely identify the selected neurons. Diagonal stripes indicate stochastic expression.

could further show that the ability of Comm to recruit Robo to endosomes requires only the extracellular and/or transmembrane domains of Robo (Figures 4E and 4F).

To test for a physical association between Robo and Comm, lysates from cells expressing both proteins were immunoprecipitated with antibodies against either the HA tag on Robo or the myc tag on Comm, and then probed on Western blots with anti-myc (Figure 4G). Comm protein precipitated with anti-myc appears to exist in three major forms, one that migrates at around 40 kDa, the predicted size of the unmodified protein, and two slower-migrating forms of about 52 kDa and 55 kDa that presumably carry some posttranslational modification. Comm could also be detected in the anti-HA precipitates, indicating that Robo indeed associates with Comm. Interestingly, Robo associates preferentially with the modified forms of Comm. Using Fra and the chimeric receptors, we could show that this association is specific and that it also requires the extracellular and/or transmembrane domains of Robo. The association between Robo and Comm does not require their colocalization in endosomes, as Robo and the Robo-Fra chimera also associate with a mutant form of Comm (L229A,P230A) that is not sorted to endosomes but instead delivered to the plasma membrane (Figure 4G, see below).

Comm Prevents Robo from Reaching the Cell Surface

Comm could recruit Robo to endosomes either by stimulating its endocytosis from the plasma membrane or by sorting it directly from the trans-Golgi network. We next performed a series of experiments to distinguish between these two possibilities. We reasoned that if an appreciable fraction of Robo were trafficked via the plasma membrane in cells that coexpress Comm, then it should be possible to label Robo at the cell surface using antibodies against its extracellular HA tag and to observe the subsequent internalization of these anti-HA antibodies. Furthermore, in cells treated with nocoda-

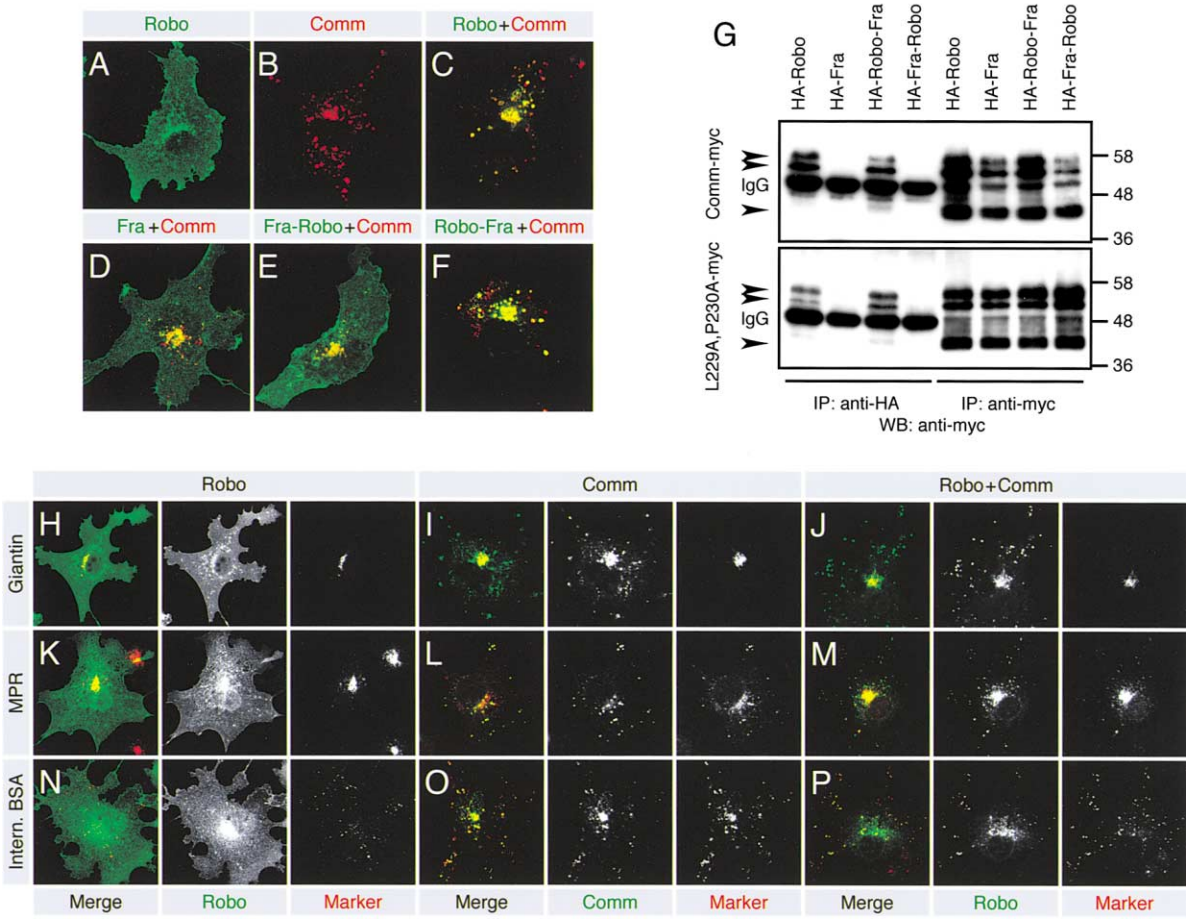

Figure 4. Comm Recruits Robo to Late Endosomes

(A–F) Confocal micrographs of COS-7 cells expressing Robo, Fra, or chimeric Fra-Robo and Robo-Fra proteins (green), in the presence (C–F) or absence (A) of Comm (red). (B) shows a cell expressing Comm alone.

(G) Coimmunoprecipitation of wild-type Comm (top) and the mislocalized L229A,P230A mutant (bottom) with Robo and Robo-Fra, but not Fra or Fra-Robo. Molecular weight markers are indicated on the right, in kDa.

(H–P) Costaining of cells expressing Robo (H, K, and N), Comm (I, L, and O), or both (J, M, and P) with the Golgi marker Giantin (H–J), mannose-6-phosphate receptor (MPR), a Golgi and late endosome marker (K–M), or fluorescently labeled BSA internalized for 3.5 hr to label the entire endocytic pathway (N–P). Comm, and Robo if coexpressed, did not colocalize with the early endosomal marker EEA1 (not shown).

zole to block trafficking from early to late endosomes, Robo, Comm, and the internalized anti-HA antibodies should all accumulate in early endosomes.

To test this, cells were chilled for 60 min to block endocytosis, and during this period they were incubated with anti-HA antibodies. After washing off excess antibody, the cells were returned to 37°C for a 45 min chase, then fixed, permeabilized, and stained for Comm, if present (using anti-myc), and for the total pool of Robo (using antibodies against a C-terminal V5 epitope tag). In cells expressing Robo alone, anti-HA antibodies were internalized into large vesicles, presumably late endosomes and lysosomes (Figure 5A). In contrast, in cells expressing both Robo and Comm, little or no anti-HA was internalized (Figure 5B). When we treated the cells expressing Robo alone with 20 μM nocodazole during the entire chase period, internalized anti-HA antibodies were blocked in smaller peripheral vesicles, presumably early endosomes (Figure 5C). In cells expressing both Robo and Comm, and similarly treated with nocodazole, neither anti-HA, Robo, nor Comm appeared to accumulate in early endosomes (Figure 5D).

As a further test for trafficking of Robo via the cell surface, we incubated the cells in the presence of anti-HA antibodies for 90 min at 37°C. This longer incubation at 37°C should allow for multiple rounds of endocytosis and recycling at the plasma membrane, increasing the opportunity for recognition of any surface Robo by the exogenous anti-HA antibodies. An incubation period of 90 min was found to give only barely detectable levels of anti-HA internalized by fluid-phase endocytosis, as observed in untransfected cells or cells expressing Comm alone (not shown), but yielded high levels of receptor-mediated endocytosis of anti-HA in cells expressing Robo alone (Figure 5E). In cells expressing both Robo and Comm, the amount of anti-HA internalized could be explained entirely by fluid-phase endocytosis (Figure 5F). Indeed, when observed in the anti-HA channel, cells expressing both Robo and Comm could not be distinguished from untransfected cells. As a con-

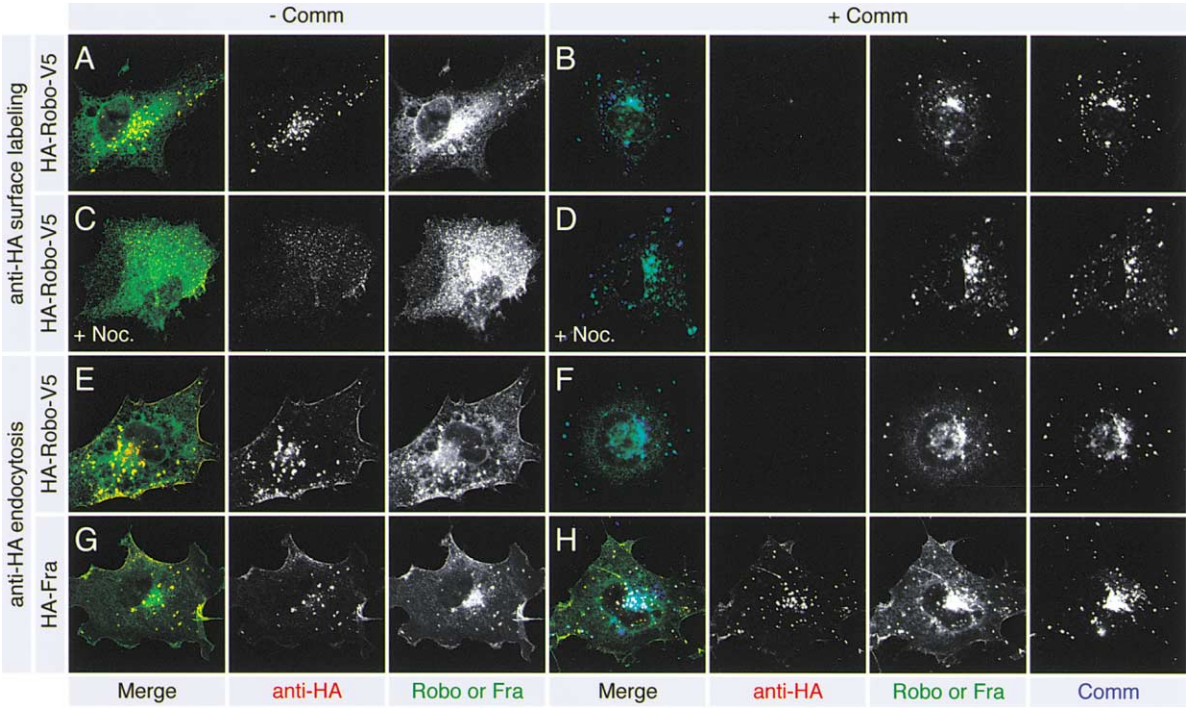

Figure 5. Comm Does Not Recruit Robo to Endosomes via the Cell Surface

Confocal micrographs of COS-7 cells expressing HA-Robo-V5 (A–F) or HA-Fra (G–H). Cells shown in (B), (D), (F), and (H) also expressed Comm-myc. Each set of panels shows a representative cell from the indicated experiment, stained with anti-V5 or anti-HA to reveal the total pool of Robo or Fra, respectively (green in merge) and, if necessary, anti-myc to visualize Comm (B, D, F, and H, blue in merge). Anti-HA antibodies (red in merge) were internalized prior to fixation and permeabilization. All cells were fixed and stained under identical conditions, and images were acquired and processed with identical settings.

(A and B) Surface labeling of cells expressing HA-Robo-V5 with anti-HA, followed by 45 min chase.

(C and D) As for (A) and (B), except that 20 μM nocodazole was added during the chase.

(E–H) Continuous uptake of anti-HA during a 90 min incubation at 37°C.

trol, we monitored the uptake of anti-HA by cells expressing the analogous HA-tagged Fra protein, both in the presence and absence of Comm. Without Comm, anti-HA endocytosis mediated by Fra is similar to that mediated by Robo (Figure 5G). However, in striking contrast to Robo, Fra also mediates the uptake of exogenous anti-HA in the presence of Comm, even though much less Fra is localized to endosomes (Figure 5H).

Together, these data provide strong evidence that, in the presence of Comm, very little if any Robo is trafficked to endosomes via the cell surface. We therefore conclude that Comm does not collect Robo at the plasma membrane, but rather sorts it directly from the trans-Golgi network to late endosomes.

The Comm Endosomal Sorting Signal Is a Conserved LPSY Motif

We next sought to identify the endosomal sorting signal in the Comm cytoplasmic domain. As Wolf et al. (1998) have noted, Comm contains a predicted binding site for heterotetrameric adaptor (AP) proteins, which could potentially mediate endosomal sorting. Otherwise, there is no obvious candidate sorting signal, nor any region of significant homology to other known proteins. We therefore began by looking for additional *comm*-like genes in *Drosophila* and in the mosquito *Anopheles gambiae*. *Drosophila* has two other genes with some similarity to *comm*, which we refer to as *comm2* and *comm3*, and for both of these we recovered full-length cDNAs. In the *Anopheles* genome we identified a single predicted gene related to *comm*. Our functional characterization of these genes is still in progress, but preliminary data indicate that *Drosophila comm2*, at least, is also able to downregulate Robo proteins in vivo (S.R. and B.J.D., unpublished). These four predicted insect Comm proteins are of a similar size and structure but are poorly conserved, with only 15%–20% identity between any pair (Figure 6A). Their cytoplasmic domains do, however, contain a highly conserved region of 22 amino acids (residues 215–236 in Comm; Figure 6B). The putative AP binding site in Comm (YPSL, residues 251–254) is conserved in Comm2 (YPSV) but not in Comm3 or *Anopheles* Comm.

To map Comm's endosomal sorting signal, we generated a series of deletion and alanine-scanning mutations within the cytoplasmic domain (Figures 6C and 6D). We tested the localization of these mutant Comm proteins in COS cells, both in the absence and presence of Robo. In all cases where Comm was correctly targeted to endosomes, Robo went with it, consistent with our view that the interaction between Comm and Robo does not require their respective cytoplasmic domains. These studies defined a region of 25 amino acids that, together with the 15 amino acids we left untouched in the juxta-

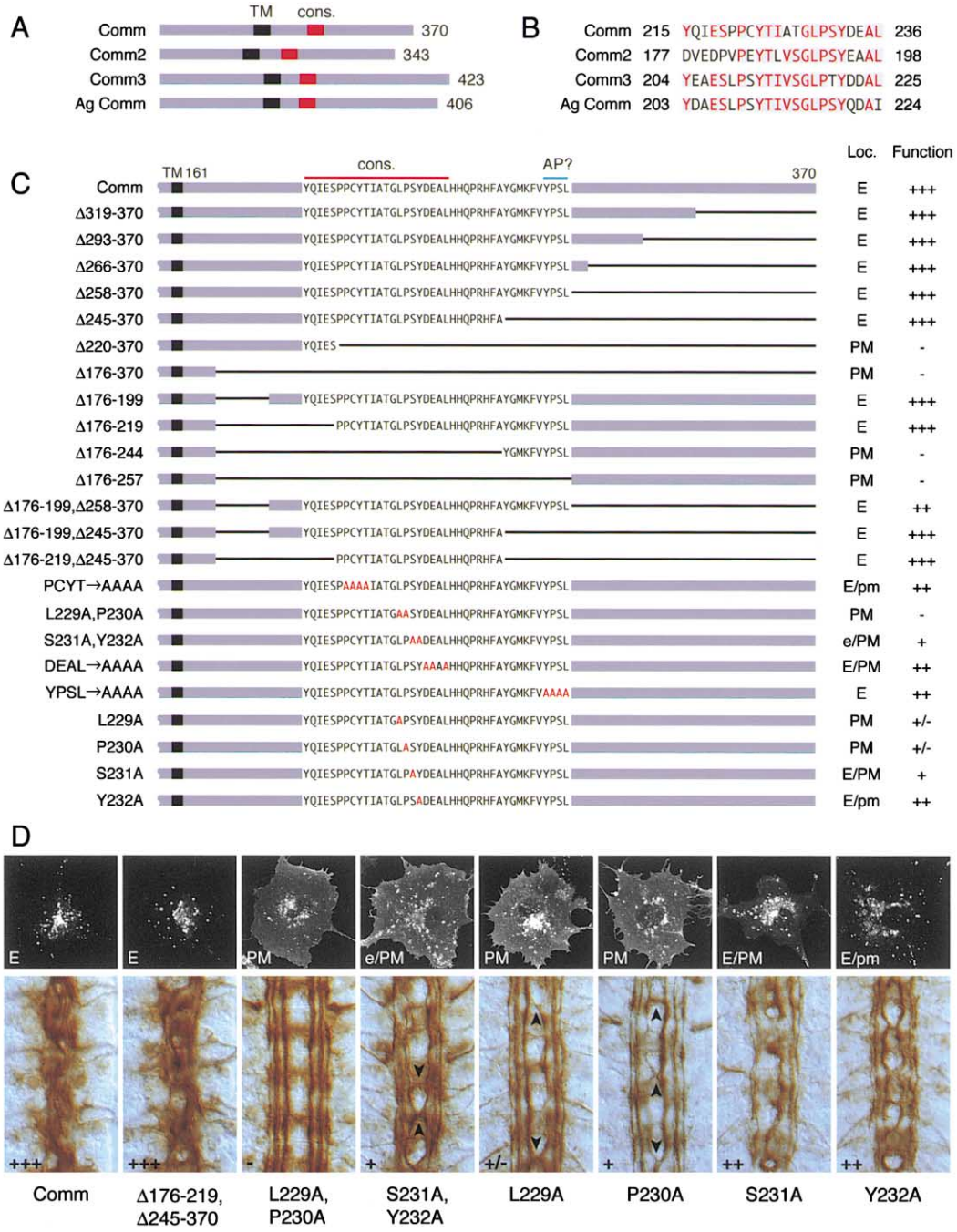

Figure 6. A Conserved LPSY Sorting Signal Is Required for Comm Function in Midline Crossing

(A) Structure of *Drosophila* and *Anopheles* Comm proteins, drawn to scale. "TM" indicates the position of the predicted transmembrane domain, and "cons." indicates the position of the highly conserved 22 amino acid region.

(B) Alignment of the conserved cytoplasmic regions.

(C) Mutant *Drosophila* Comm proteins assayed for localization in COS-7 cells and for function in vivo. All Comm proteins additionally carried two C-terminal c-*myc* epitopes. The sequence including the conserved region ("cons.") and the putative AP binding site ("AP?") is shown; the rest of the cytoplasmic domain is indicated by the gray box. Columns on the right indicate the localization of the protein expressed in COS-7 cells ("loc.") and its function in promoting ectopic midline crossing in vivo ("function"). For localization, E indicates predominantly endosomal, PM indicates predominantly plasma membrane, and E/PM indicates mixed endosomal and plasma membrane. Lower-case indicates a relatively minor amount of the protein in that compartment. At least two separate transfection experiments were performed for all constructs, and in each case they yielded the same distribution. For ectopic midline crossing, three plus signs indicates a strong *robo*- or *slit*-like phenotype, two plus signs indicates a moderate phenotype, similar to *robo* null mutants, one plus sign indictes a weak *robo*-like phenotype, and a minus sign indicates no or only rare crossing defects. The extent of Robo downregulation in these embryos was consisent with the severity of the *robo*-like phenotype. At least two different insertions of each transgene were tested, and in each case they were phenotypically indistinguishable.

(D) Localization and misexpression phenotypes for selected Comm proteins. Upper images are confocal micrographs of representative cells expressing the indicated Comm protein, stained with anti-myc. The localization for these selected cells is classified as for these cells generally

membrane domain, is sufficient for targeting Comm (and Robo, if coexpressed) to endosomes. This region excludes the putative AP binding site, but contains most of the highly conserved residues. Within this region, an LPSY motif is critical for endosomal sorting. If it is mutated, then Comm is found mainly at the plasma membrane. Only a minor fraction of the mutant Comm protein is found in endosomes, possibly reaching this compartment by endocytosis from the plasma membrane rather than direct trafficking from the Golgi. This LPSY motif is also present in each of the other Comms (as LPTY in Comm3). Comm3 and *Anopheles* Comm even have a second LPSY motif within the conserved region, where Comm has PPCY.

The LPSY Motif Is Required for Comm to Downregulate Robo and Promote Midline Crossing In Vivo

Finally, we tested each of these mutant Comm proteins for its ability to downregulate Robo and promote midline crossing in vivo. We reasoned that if Comm also sorts Robo to late endosomes in vivo and this is its only function in midline crossing, then the LPSY sorting motif should be the only part of Comm's cytoplasmic domain needed for its function in vivo. The mutant Comm proteins that were mislocalized to the plasma membrane should all be nonfunctional in vivo, while those that are correctly sorted should still be functional, even though they lack most of the cytoplasmic domain.

To test these predictions, we generated flies carrying *UAS* transgenes encoding each of the mutant Comm proteins that we had tested in COS cells. These transgenes were then expressed in all CNS neurons using the *elav-GAL4* driver. We examined these embryos with anti-myc antibodies to determine the expression and localization of the transgenic Comm protein, with anti-Robo MAb 13C9 to test for the ability of the mutant Comm protein to downregulate Robo, and with MAb 1D4 to detect any misrouting of longitudinal axons across or along the midline, the hallmarks of the *robo* and *slit* loss-of-function phenotypes (Kidd et al., 1998a, 1999).

Consistent with the hypothesis that Comm sorts Robo to endosomes in vivo, we found a striking correlation between the sorting of a mutant Comm protein to endosomes in COS cells and its function in vivo (Figures 6C and 6D). In particular, the 25 amino acid region of Comm's cytoplasmic domain that is sufficient for endosomal sorting in vitro is also sufficient for Comm to downregulate Robo and promote midline crossing in vivo. Conversely, point mutations in the LPSY motif completely abolish Comm function in vivo, just as they prevent endosomal sorting in vitro. Comm mutants that were sorted to endosomes in COS cells also showed a punctate intracellular localization in vivo, but in general were difficult to detect, even though they were fully functional. Indeed, it was only the nonfunctional Comm proteins that could readily be detected in vivo. This seemingly paradoxical result is, however, in complete agreement with our view that the essential function of Comm in vivo is to be degraded in lysosomes, taking Robo with it. This function is critically dependent on the same LPSY motif that targets Comm (and Robo) directly from the Golgi to late endosomes and lysosomes in vitro.

Discussion

In the *Drosophila* CNS, some axons but not others cross the midline. Those that do cross, cross only once. The decision to cross or not to cross the midline is controlled by Comm. The data presented here suggest a model in which Comm controls this decision by regulating the intracellular trafficking of Robo, the receptor for the midline repellent Slit (Figure 7).

Comm Acts Autonomously

Previous models for Comm function in midline crossing have proposed that it acts nonautonomously (Tear et al., 1996; Kidd et al., 1998b). Comm was thought to be produced in midline glia and transferred specifically to commissural axons as they cross the midline. Our cell transplantation experiments provide strong evidence that *comm* in fact acts autonomously. Wild-type neurons in an embryo that otherwise lacks all *comm* function are still able to cross the midline. Indeed, they are the only axons that cross in these embryos. Conversely, *comm* mutant neurons in an otherwise wild-type embryo only rarely cross the midline.

An autonomous requirement for *comm* in commissural neurons has also recently been demonstrated by the transgenic RNAi studies of Georgiou and Tear (2002). These authors also suggest that *comm* function is additionally required in midline cells. However, our transplantation studies show that, at least for some neurons, *comm* function at the midline is dispensible for crossing. Nevertheless, we do see a partial nonautonomous requirement for *comm*: axons of wild-type neurons are less likely to cross the midline in a *comm* mutant host than in a wild-type embryo, and, conversely, axons of *comm* mutant neurons do occasionally cross in wild-type hosts. These results are consistent with a partial requirement for *comm* function in midline cells but could also be explained by a "community effect" in midline guidance. For example, if the commissural pioneers are

in (C). Lower panels show dissected stage 16 embryos carrying the *elav-GAL4* driver and the relevant *UAS-comm* transgene, stained with MAb 1D4. Dorsal views are shown, with anterior up. In wild-type embryos, 1D4 labels five longitudinal axon fascicles on either side of the midline, three of which are visible in dorsal views such as these. These fascicles cross or converge at the midline in *robo* or *slit* mutants, or if a functional *UAS-comm* transgene is expressed using *elav-GAL4*. The extent of ectopic crossing observed for the mutant *comm* transgenes provides a measure of the relative activity of the Comm protein and is indicated for these selected embryos as in (C). Arrowheads indicate the relatively rare cases of ectopic midline crossing in the S231A,Y232A double mutant and the L229A and P230A single mutants. In weak and moderate *robo*-like phenotypes (single and double plus signs), the two medial fascicles often fuse and meander back and forth across the midline (arrows), but the outer two fascicles are only slightly disrupted. This distinguishes these embryos from those showing a stronger *slit*-like phenotype, as exemplified by the wild-type Comm and Δ176-219,Δ245-370 embryos.

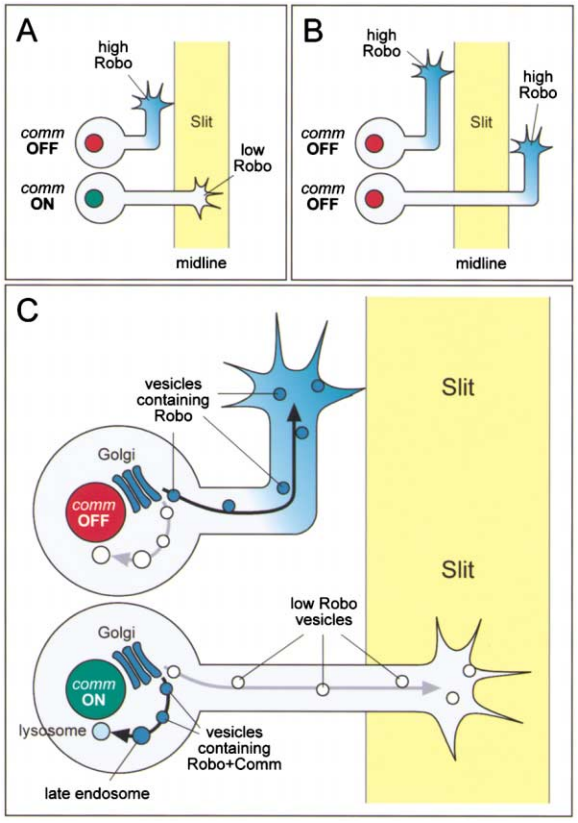

Figure 7. Model for Comm Function in Midline Crossing

(A and B) *comm* expression is the switch that controls midline crossing. In an ipsilateral neuron, *comm* is OFF. As a consequence, the growth cone carries high levels of Robo protein and is repelled by Slit at the midline. In a commissural neuron, *comm* is initially ON (A), keeping Robo levels low to allow crossing. Once the commissural growth cone reaches the other side, *comm* is turned OFF in order to increase Robo levels and prevent recrossing (B).

(C) Comm regulates Robo trafficking. If *comm* is OFF, Robo is packaged into vesicles delivered to the growth cone. If *comm* is ON, most (but not all) Robo is instead sorted by Comm into vesicles bound for late endosomes and lysosomes, where both Robo and Comm are degraded. Vesicles travelling to the growth cone thus contain very little Robo protein, and insertion of these vesicles at the axon tip allows it to extend across the midline.

mutant and their axons thus diverted into an ipsilateral pathway, then wild-type axons might follow. Conversely, axons of some *comm* mutant neurons might simply follow wild-type axons across the midline.

Comm Expression Specifies Midline Crossing

What is the basis for the specificity of Comm's action? Why are only commissural axons allowed across the midline, and why only once? Previous models have proposed that Robo levels may initially be lower in commissural neurons than in ipsilateral neurons, or that only commissural neurons might express a cell surface receptor needed for the uptake of Comm from midline glia (Kidd et al., 1998b). Our analysis of *comm* expression in the CNS offers a much simpler explanation for its specificity: in general, only commissural neurons express *comm*, and only as they cross.

The tight correlation we have observed between *comm* expression and midline crossing is complemented by genetic data showing that neuronal *comm* expression is both necessary and sufficient for midline crossing. Necessity is evident from the *comm* loss-of-function phenotype (Seeger et al., 1993) and from our transplantation experiments. Sufficiency is demonstrated by studies showing that the forced expression of *comm* reroutes ipsilateral neurons across the midline (Kidd et al., 1998b; Bonkowsky et al., 1999) and entices commissural neurons to recross or linger at the midline (Kidd et al., 1998b, 1999). Whereas previously these gain-of-function phenotypes were attributed to shifting the initial bias of Robo-Comm antagonism or bypassing the need to transfer Comm from the midline glia, they can now more readily be understood as the result of misexpressing *comm* in neurons that should not express it and persistently expressing *comm* in neurons that should extinguish it.

These data thus suggest a simple model in which *comm* expression is the intrinsic switch that specifies an ipsilateral versus a contralateral projection—OFF for ipsilateral, ON for contralateral (Figure 7A). This switch appears to be regulated not only spatially but also temporally, as *comm* generally goes OFF in a commissural neuron after crossing (Figure 7B). We lack the early markers needed to determine whether *comm* is usually ON or OFF before crossing, but we note that for the few commissural neurons we can identify early (the EW and EG neurons), *comm* is initially OFF. What turns *comm* ON and then OFF again to allow just a single passage across the midline? One possibility is that each commissural neuron is intrinsically programmed for a brief pulse of *comm* expression. Alternatively, *comm* expression might be controlled by retrograde signals sent from the growth cone to inform the nucleus of its arrival at the midline and its successful passage across. This is an appealing idea, since such a mechanism would uncouple the ability of the growth cone to cross the midline from the precise time of arrival and duration of transit.

Comm Is a Sorting Receptor for Robo

Understanding Comm's function in midline crossing has also been hindered by the fact that its molecular function was unknown and its amino acid sequence provided no obvious clues. Our data suggest that Comm is a sorting receptor, recognizing Robo via its lumenal and/or transmembrane domain and consigning it for delivery from the trans-Golgi network to late endosomes. Robo may not be the only cargo for Comm. From gain-of-function genetic experiments (Rajagopalan et al., 2000a; Simpson et al., 2000), we infer that Comm also selects Robo2 and Robo3 for delivery to endosomes. Analogous gain-of-function studies suggest that Comm2 also sorts Robo receptors, with a preference for Robo2, while Comm3 may not sort any of the three Robos (S.R. and B.J.D., unpublished). The Comm proteins thus define a new family of sorting receptors, the cargo of which include, but may not be limited to, the Robo family of guidance receptors.

We used a COS cell assay to map the endosomal sorting signal in the cytoplasmic domain of Comm, and in parallel we determined which parts of Comm's cyto-

plasmic domain are required for its function in vivo. Both studies converged on an LPSY motif. Point mutations in this motif result in relocalization of Comm to the plasma membrane, both in vitro and in vivo, and abolish Comm's ability to downregulate Robo and promote midline crossing in vivo. In contrast, large cytoplasmic deletions do not destroy Comm function, provided they leave the endosomal sorting signal intact. This LPSY motif is also conserved in *Drosophila* Comm2 and Comm3, as well as *Anopheles* Comm, proteins that otherwise have very little sequence similarity. Together, these data provide strong evidence that the only critical function of the Comm cytoplasmic domain in vivo is to mediate endosomal sorting.

Robo Trafficking Controls Midline Crossing

Our view that Comm is an endosomal sorting receptor and that *comm* expression is a cell-autonomous switch for midline crossing leads us to a model in which axon traffic at the midline is controlled by regulating the intracellular trafficking of Robo, the receptor for the midline repellent Slit (Figure 7C). If *comm* is OFF, Robo is packaged into vesicles for delivery to the growth cone. The insertion of these vesicles at the growth cone confers sensitivity to Slit, thereby preventing growth across the midline. Conversely, if *comm* is ON, Comm sorts Robo into vesicles destined for late endosomes and lysosomes. Membrane vesicles delivered to the growth cone contain only very low levels of Robo, and so the axon can grow unimpeded across the midline by inserting these vesicles at its tip.

One requirement for this model is that, in order to prevent a commissural axon from recrossing, Comm protein, like *comm* mRNA, should rapidly disappear after crossing, or at least lose its ability to sort Robo. We believe that Comm is indeed rapidly degraded in vivo. In contrast to COS cells, very little Comm protein can be detected in vivo, even when we use the *GAL4-UAS* system to express high levels of *comm* mRNA throughout the CNS. Only if the LPSY endosomal sorting motif is mutated can Comm accumulate to appreciable levels in vivo, in this case at the plasma membrane. This suggests that, unlike other sorting receptors, Comm may not be recycled back to the Golgi for repeated rounds of sorting but instead be degraded along with its cargo in lysosomes. Other mechanisms may also exist to inactivate Comm after crossing, for example by altering the posttranslational modifications that appear to be necessary for it to recognize Robo.

An appealing feature of this model for Comm function is that it offers an explanation for the striking spatial distribution of Robo: high on the longitudinal segments of commissural axons but low on their midline segments (Kidd et al., 1998a). Such a distribution could arise simply as a consequence of the precise temporal regulation of Robo trafficking. For this, we need only to posit that new membrane and newly synthesized Robo are both added predominantly at the growth cone and that membrane flow and the diffusion of Robo along the axon shaft are limited (possibly even by a barrier established through contact with midline cells). In this context it is interesting to note that, although little if any Robo can be detected on the midline segments of commissural axons as they first cross the midline, appreciable levels of Robo protein do start to accumulate on commissures toward the end of embryogenesis (Kidd et al., 1998a). This may be due to the slow diffusion of Robo along the axon shaft.

Although not required by our model, we also assume that the sorting of Robo by Comm occurs primarily at the cell body. Endosomes and lysosomes have, however, also been observed along axon shafts, and studies using a pH indicator to monitor the acidification of endosomes in growing axons suggest that sorting also occurs along the axon, in particular in its most proximal and distal regions (Overly and Hollenbeck, 1996). This might explain the initial observation that Comm is not only localized in vesicles at the cell bodies, but can also be detected along the proximal segments of commissural axons (Tear et al., 1996).

Our model for the regulated sorting of Robo receptors in *Drosophila* neurons has an interesting parallel in the sorting of Gap1p, the general amino acid permease in yeast. If cells are grown on a poor nitrogen source, Gap1p is inserted into the plasma membrane to stimulate amino acid uptake. In contrast, if cells are grown on a rich nitrogen source, Gap1p is sorted from the trans-Golgi network to the vacuole (the yeast lysosome), where it is degraded (Roberg et al., 1997). As in the case of Robo, here too a newly synthesized membrane protein is selected for apparently futile destruction as it passes through the trans-Golgi network. Rather than wasting energy making these proteins only to destroy them, why not simply synthesize them on demand? For the yeast cell, it has been suggested that this mechanism may provide a means of very rapidly adjusting plasma membrane Gap1p levels in response to changing nutrient conditions (Helliwell et al., 2001). We hypothesize that the regulated sorting of Robo receptors may similarly ensure a rapid response in a changing environment. If, as we propose, Comm is degraded or otherwise inactivated as soon as a commissural axon has crossed the midline, then a preexisting pool of Robo receptors from the Golgi and endoplasmic reticulum could be delivered to the growth cone within minutes—in ample time to prevent recrossing.

Growth cones navigating their way through the developing embryo often need to adjust their sensitivity to extracellular guidance cues. Regulating the intracellular trafficking of guidance receptors is an efficient way to achieve this and may prove to be a common mechanism in axon pathfinding.

Experimental Procedures

Cell Transplantation

Cell transplantations were performed as described in Prokop and Technau (1993). *comm* mutant embryos were collected from a *comm*^{5}/TM3,*Kr-GFP* stock and identified by the lack of the "green balancer." Clones obtained from *comm*^{5}/TM3,*Kr-GFP* and TM3,*Kr-GFP* homozygote donor cells showed no obvious abnormalities compared to wild-type cells (Bossing et al., 1996; Schmidt et al., 1997) and were taken as controls.

Analysis of *comm* Expression

Embryos were fixed with 4% paraformaldehyde (PFA), permeabilized by acetone treatment (Nagaso et al., 2001), refixed, preincubated in 50% formamide in 5× SSC, and prehybridized for 1–2 hr at

56°C in 1 ml hybridization buffer. Hybridization with *comm* antisense probes was performed in 500 μl hybridization buffer at 56°C (Tear et al., 1996). Embryos were then washed for 2 hr at 56°C with 50% formamide in 5× SSC, followed by 1 hr each in 2× SSC and 0.2× SSC. All solutions additionally contained 0.1% Tween-20 and 0.1% Triton-X100. Embryos were then rinsed in TTX (0.1 M Tris [pH 7.5], 137 mM NaCl, 3 mM KCl, 0.1% Tween-20, 0.1% Triton-X100) and blocked for 1 hr in TTX containing 5% normal goat serum and 0.1% BSA, prior to overnight incubation with either alkaline phophatase (AP)-conjugated anti-DIG Fab fragments (1:2000, Roche) or with mouse anti-DIG MAb (1:1000, Jackson Immunochemicals). AP detection was performed as described (Tear et al., 1996). For fluorescent detection, embryos were incubated for 2 hr with biotinylated anti-mouse secondary antibodies (1:500, Jackson Immunochemicals) and for 1 hr with streptavidin-HRP (1:100, Perkin Elmer). Fluorophore was incorporated around the hybridized probe using biotinyl-tyramide and streptavidin-Alexa488 (1:500, Molecular Probes) with the TSA Indirect kit (Perkin Elmer).

For counterstaining to detect specific axonal markers, rabbit anti-myc (1:1000) or rabbit anti-β-galactosidase (1:2000, Cappel) was added together with the anti-DIG, and anti-rabbit-Alexa568 together with the biotinylated anti-mouse antibody. For counterstaining with MAb 1D4, the in situ hybridization was first completed and any remaining mouse epitopes were blocked with anti-mouse FAb fragments (1:10, Jackson) prior to incubation with 1D4 and anti-mouse-Alexa568 (1:500, Molecular Probes). Dissected embryos were mounted in Vectashield mounting medium (Vector Labs), and confocal images were acquired on a Zeiss LSM confocal microscope.

Plasmids

HA-robo-V5 was prepared in the pUB6/V5-HisA vector (Invitrogen), and *HA-fra*, *HA-fra-robo*, and *HA-robo-fra* in pcDNA3.1$^+$ (Invitrogen) for COS cell transfections. Standard PCR-based cloning procedures were used, and the integrity of each plasmid was confirmed by sequence analysis. Each of these predicted proteins contains the Wingless signal sequence followed by three HA epitope tags, and then the following residues: *HA-robo-V5*, Robo residues 56–1395 (followed by the V5 epitope and H_6 tags); *HA-fra*, Fra residues 35–1526; *HA-robo-fra*, Robo 56–945 + SF (encoded by a HindIII site) + Fra 1256–1526; *HA-fra-robo*, Fra 35–1253 + SF + Robo 948–1395.

comm-myc was prepared by cloning the entire *comm* open reading frame into a pcDNA3.1$^+$-derivative that includes two C-terminal c-*myc* epitope tags. Mutations within the *comm* open reading frame were generated using the overlap extension PCR method and confirmed by sequencing. These wild-type and mutant *comm-myc* inserts were then subcloned into pUAST to generate *UAS-comm* transgenes.

COS Cell Transfections and Immunofluorescence Staining

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics and transfected at 80% confluency using Lipofectamine Plus Reagent (Invitrogen). Cells were split 24 hr after transfection to glass coverslips and fixed at 48 hr after transfection with 4% PFA in CB (10 mM PIPES [pH 6.8], 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$) for 10 min at room temperature. After permeabilization for 2 min in CB containing 0.2% Triton-X100, cells were blocked in 2% gelatine, 50 mM NH_4Cl in CB for 30 min, and incubated for 1 hr at room temperature with the appropriate combination of anti-V5 MAb (1:500, Invitrogen), anti-HA MAb 16B12 (1:500, BabCo), rabbit anti-myc (1:1000, Gramsch Laboratories), anti-Giantin MAb (1:1000; Linstedt and Hauri, 1993), rabbit anti-EEA1 (1:100; Rubino et al., 2000), or rabbit anti-MPR (1:300 after acetone-methanol fixation; Griffiths et al., 1988). After extensive washes, cells were then incubated with the appropriate combination of Alexa488- or Alexa568-conjugated goat anti-mouse or anti-rabbit secondary antibodies (all 1:1000, Molecular Probes). Cells were mounted in mowiol solution and confocal images were acquired on a Zeiss LSM confocal microscope. For BSA internalization experiments, cells were incubated with 30 μg/ml BSA-AlexaFluor594 (Molecular Probes) in serum-free DMEM for 3.5 hr at 37°C prior to fixation.

Internalization of Anti-HA Antibodies

For surface labeling, cells were rinsed 48 hr after transfection in ice-cold PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and then incubated for 60 min on ice in binding medium (DMEM, 7.5% heat-inactivated fetal calf serum, 20 mM HEPES [pH 7.4]) containing Cy3-conjugated anti-HA (1:200, prepared by conjugating Cy3 to MAb 16B12 using the Cy3 Antibody Labeling Kit, Amersham). Cells were then extensively rinsed on ice and incubated in binding medium at 37°C for 45 min. If required, nocodazole was added at 20 μM. Cells were then rinsed extensively on ice, fixed in ice-cold 4% PFA in CB for 7 min and then in 4% PFA in CB at room temperature for 8 min, and permeabilized with 0.2% Triton-X100 in CB for 2 min. To monitor the continuous internalization of anti-HA antibodies, cells were incubated at 37°C for 90 min in binding medium containing Cy3-conjugated anti-HA (1:200), followed by washing, fixation in 4% PFA in CB at room temperature for 10 min, and permeabilization in 0.2% Triton-X100 in CB for 2 min. Cells were stained for Robo, Fra, and Comm as described above, except that Comm was detected using goat anti-rabbit-Cy5 secondary antibodies (1:500, Amersham).

Immunoprecipitation

Cells were lysed 48 hr after transfection in 1 ml RIPA buffer (50 mM Tris-HCl [pH 7.5], 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate) containing Complete Protease Inhibitors (Roche), then briefly sonicated and cleared by centrifugation. 250 μg total protein was immunoprecipitated with either anti-HA MAb 16B12 (BabCo) or anti-myc MAb 9E10 (Santa Cruz Biotechnology) using Protein G Plus-Agarose beads (Oncogene). Precipitates were washed with RIPA buffer followed by PBS, denatured by heating in sample buffer, resolved by SDS-PAGE, and transferred to Hybond-P membrane (Amersham) for Western blotting with either anti-HA or anti-myc, followed by goat anti-mouse-HRP.

Analysis of Mutant Comm Proteins In Vivo

The various *UAS-comm* transgenic lines were crossed to a third chromosome *elav-GAL4* insertion, and embryos were fixed and stained with MAb 9E10, MAb 13C9, or MAb 1D4 as described (Rajagopalan et al., 2000b).

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