Commissureless is required both in commissural neurones and midline cells for axon guidance across the midline

Marios Georgiou and Guy Tear*

Molecular Neurobiology Group, MRC Centre for Developmental Neurobiology, Guy’s Hospital Campus, King’s College, London SE1 1UL, UK

*Author for correspondence (e-mail: guy.tear@kcl.ac.uk)

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SUMMARY

In the absence of Commissureless (Comm) function, axons are unable to extend across the central nervous system midline. Comm downregulates levels of Roundabout (Robo), a receptor for the midline repellent Slit, in order to allow axons to cross the midline. comm transcript is expressed at high levels in the midline glia and Comm protein accumulates on axons at the midline. This has led to the hypothesis that Comm moves from the midline glia to the axons, where it can reduce Robo levels. We have found that expression of Comm in the midline cells is unable to rescue the comm phenotype and that tagged versions of Comm are not transferred to axons. A re-examination of Comm protein expression and the use of targeted RNA interference reveal that correct midline crossing requires that Comm is expressed in the commissural axons and midline glia. We suggest that accumulation of Comm protein at the midline spatially limits Comm activity and prevents it from being active on the contralateral side of the central nervous system.

Key words: Commissureless, Drosophila, Midline, Axon guidance

INTRODUCTION

During development of the nervous system the constituent neurones must navigate to and connect with their appropriate targets. This feat of navigation often requires that axons migrate over a long distance. To enable this to occur accurately, axons can make use of intermediate targets along their route toward their final target (Tessier-Lavigne and Goodman, 1996). One intermediate target used by axons in both vertebrates and invertebrates is the midline of the central nervous system (CNS) (Stoeckli and Landmesser, 1998; Tear, 1999). The commissural axons within the CNS project towards the midline cells as they extend towards their targets on the opposite side of the body. Other populations of axons in the CNS do not extend towards the midline and remain on their own side (Colamarino and Tessier-Lavigne, 1995; Klambt et al., 1991). Whether axons are attracted towards or away from the midline is dependent on how they respond to the different signals produced by the midline cells. The midline acts as a source for both attractive and repulsive axon guidance molecules (Brose et al., 1999; Harris et al., 1996; Kennedy et al., 1994; Kidd et al., 1999; Mitchell et al., 1996; Serafini et al., 1994; Stoeckli et al., 1997; Zou et al., 2000). An initial differential sensitivity to these cues allows CNS axons to choose a path that takes them towards the midline or one that keeps them away. For example axons that express the DCC/Frazzled group of receptors alone on their growth cones are receptive to the attractive activity of the netrin molecules secreted from the midline (KeinoMasu et al., 1996; Kołodziej et al., 1996), whereas the expression of Roundabout (Robo) on growth cones makes them sensitive to the midline repellent Slit and routes the axons away from the midline (Kidd et al., 1998a).

The differential expression of receptors defines how axons make their initial growth decision but fails to explain why the commissural growth cones then leave the intermediate target that they formerly found so attractive, in order to progress along their pathway. This change in behaviour is possible because the commissural growth cones can adapt their sensitivity to midline signals (Kidd et al., 1999; Kidd et al., 1998a). Subsequent to reaching the midline commissural growth cones lose their sensitivity to attractive cues and become responsive to the midline repellent cues. This switch also serves to explain why commissural axons do not re-cross the midline as they now behave as ipsilaterally projecting neurones and remain away from the midline.

In Drosophila, the switch in sensitivity displayed by commissural axons is explained by the precise spatial regulation of the Robo receptor molecule (Kidd et al., 1998a; Kidd et al., 1998b). Before crossing the midline, the commissural neurones express the robo mRNA but do not express high levels of Robo protein on their surface in contrast to the ipsilaterally projecting neurones, which do express the Robo protein. Upon crossing the midline, the Robo protein levels on the commissural neurones increases and they become responsive to Slit. Similarly vertebrate axons become sensitive to the repellents Slit and Semaphorin.
during crossing (Zou et al., 2000). In addition, an increase in functional Robo levels silences the attractive response by commissural neurones to the midline cue netrin (Shirasaki et al., 1998; Stein and Tessier-Lavigne, 2001) through a direct interaction between Robo and DCC. Thus, regulation of Robo protein levels is crucial for correct axonal migration at the midline. The only molecule known to regulate Robo protein levels is the Drosophila transmembrane protein Commissureless (Comm) (Kidd et al., 1998b; Tear et al., 1996).

Comm and Robo activities are closely intertwined. In the absence of Comm commissural growth cones are unable to cross the midline, suggesting that they are particularly sensitive to the repellent molecules expressed at the midline (Seeger et al., 1993; Tear et al., 1996). Overexpression of Comm in all neurones results in the downregulation of Robo protein levels which mimics the robo phenotype where axons are no longer repelled from the midline (Kidd et al., 1998b). Comm is expressed at high levels at the midline where Robo protein levels are low, consistent with the ability of Comm to regulate Robo levels negatively. Once away from the midline, Comm is unable to affect Robo and its levels on the axon can increase so preventing the axon from returning to the midline. Previous work describing this process suggested that Comm transcript was only found in the midline glia (Tear et al., 1996). Comm protein was proposed to move from these midline cells to accumulate on the commissural region of the commissural neurones, as the cell bodies of the commissural neurones did not appear to express high levels of comm transcript. This provided a mechanism whereby Comm could locally downregulate Robo levels to allow midline crossing. In the absence of Comm, there is no downregulation of Robo and the commissural axons are unable to cross the midline.

We describe further characterisation of Comm distribution and its mode of action. Re-expression of Comm in all midline cells in comm mutant animals cannot rescue the comm phenotype and we are unable to see the transfer of a tagged version of Comm from the midline cells to commissural axons. Using a targeted RNA interference strategy in combination with a re-analysis of Comm protein distribution, we have found that Comm is expressed and required in commissural neurones but not ipsilateral neurones. We suggest that Comm function in commissural neurones aids in the initial selection of commissural versus ipsilateral pathway choice. Comm protein is found to accumulate on the surface of commissural axons within the commissure where it could interact with Comm on the surface of the midline cells. This interaction may prevent Comm protein reaching the contralateral portions of the commissural axons so allowing Robo protein levels to increase and a switch in the responsiveness of the commissural axons to midline cues.

For rescue experiments, we used a transformant line carrying wild-type comm-coding sequence on the X chromosome (Kidd et al., 1998b) and also a line carrying a Comm-GFP fusion transgene on the second chromosome (constructed here), both under the control of the UAS promoter. P-element transformation was performed using standard procedures. For each UAS transgene, multiple lines were generated and checked for expression.

Re-expression of Comm at the midline in comm mutants was achieved by crossing flies heterozygous for comm (E39 or A490) and UAS-Comm to flies heterozygous for comm and either sim-GAL4 or slit-GAL4. The progeny from this cross will contain 25% comm mutant embryos. Twenty percent of these comm mutants will possess both UAS-Comm and a GAL4 driver. When re-expressing two copies of Comm at the midline, flies heterozygous for comm, UAS-Comm and UAS-Comm-GFP were crossed to flies heterozygous for comm and slit-GAL4. In this case, 37.5% of comm mutant embryos will possess one or two copies of UAS-Comm and a slit-GAL4 driver.

DNA constructs

All manipulations of the comm cDNA are based on the published full-length cDNA clone (Tear et al., 1996). Comm-GFP was constructed by using the PCR on both comm and mGFP6 (Schuldt et al., 1998) cDNAs to introduce compatible cloning sites ensuring that the entire coding sequence was in frame. The PCR products were then ligated into the pUAST vector (Brand and Perrimon, 1993) and the construct sequenced on both strands. The entire open reading frame was excised using EcoRI and KpnI cloned into the pRmHa-3 vector for use in S2 cell assays.

The comm hairpin consists of an inverted repeat of comm sequence, separated by a linker region. A fragment of 375 bp of comm-coding sequence (positions 818 to 1192) and the linker region were synthesised by the PCR. The linker region consisted of AAGG (which provokes the loop) flanked by SplI and EcoRI restriction sites (linker region size, 16 bp). Two PCR products were created with either XbaI or XhoI at the 5’ ends. comm 5′-3′ plus linker was subcloned into the XbaI and EcoRI sites of Bacterial expression vector pBluescript (Stratagene). comm 3′-5′ plus linker was then inserted into the resultant construct using the XhoI and SplI restriction sites. This construct was amplified in Epicurian Sure Cells (Stratagene). The entire hairpin sequence was then cloned into pUAST using XbaI and Xhol sites.

**S2 cell assay**

Transfections were carried out as described (Di Nocera and Dawid, 1983). Twenty-four hours after transfection, protein expression was induced with 0.7 mM CuSO4. Sixteen hours post-induction, cells were processed for immunohistochemistry using mAb 13C9 (Kidd et al., 1998a) and rabbit polyclonal αGFP (Molecular Probes). pRmHa-3-robo construct was provided by V. McCabe. pUAS-comm-HP was driven in S2 cells using the pMT-GAL4 construct, which was a kind gift from Kai Zinn (Caltech).

**Comm antibodies and immunohistochemistry**

cDNA corresponding to the extracellular region of Comm (amino acids 1 to 130) was amplified using the PCR and cloned into pQE30 (Qiagen) to generate QEEC9. The His-tagged protein was induced and purified using standard techniques and the protein injected into rabbits by a commercial company (Eurogentec). Rabbit serum was affinity purified using a column bearing the immunogen. Antibody was eluted from the column using 3M sodium thiocyanate and the eluate dialysed against PBS. The antibody was pre-absorbed against Drosophila embryos and used at a final concentration of 1:10. All immunostaining was performed using standard techniques (Patel, 1994). Anti-myc (9E10) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Rabbit anti-Eagle was provided by J. Urban.

**MATERIALS AND METHODS**

**Drosophila lines and genetics**

Commissureless null (commE39) and loss-of-function (commA490) mutants are as described (Tear et al., 1996). Eg-GAL4 flies were provided by A. Prokop, Sema2b-Gal4 flies were provided by B. Dickson, UAS-tauGFP were provided by J. Thomas. sim-GAL4 and slit-GAL4 were provided by C. Klämbt.
RESULTS

Comm is required for axons to cross the CNS midline

Comm was identified in a large-scale genetic screen for mutations that affect the development of CNS axon tracts in the *Drosophila* embryo (Seeger et al., 1993). In the normal embryo, axons form an orthogonal structure with longitudinal axon tracts extending anteroposteriorly, positioned at either side of the midline, and a pair of commissural tracts that join the longitudinal pathways in each segment of the embryo (Fig. 1A). The majority of the axons that extend within the longitudinal tracts have their cell bodies located on the opposite or contralateral side of the midline and must extend across the midline in one of the two commissural tracts. In comm mutant animals, the commissural tracts fail to form giving rise to a unique and distinctive phenotype (Fig. 1B).

Comm has been cloned and shown to encode a novel transmembrane protein (Tear et al., 1996). Studies at that time revealed that the comm transcript is expressed at high levels at the midline at the stage when axons are first extending across the midline. The Comm protein was revealed as being expressed at the midline and appearing to accumulate on the surface of the commissural portion of the crossing axons (Fig. 1C). Little Comm protein was identified within the cell bodies of these neurones and no Comm protein was seen on the longitudinal portions of the commissural axons (Tear et al., 1996). This led to the suggestion that Comm is locally transferred from the midline cells to the commissural axons to regulate Robo.

Comm at the midline is not sufficient for axon crossing

To identify whether the Comm protein expressed by the midline glial cells is sufficient to regulate Robo protein on the commissural axons we attempted to rescue the comm phenotype by driving Comm expression at the midline. Re-expression of Comm at the midline was tested for its ability to rescue two comm alleles: commE39 and commA490. These alleles represent null and hypomorph conditions respectively. In the commE39 allele the comm transcription unit has been deleted and no Comm protein is produced, whereas in the commA490 allele a frameshift mutation at amino acid 217 results in the production of a Comm protein which is truncated within the cytoplasmic domain. In commE39 embryos, no commissures form, whereas commissures do form on average in approximately 20% of the commA490 mutant embryos (Table 1). However commissure formation in the commA490 embryos is usually restricted to one or two thoracic segments. The rescue experiments were performed by driving Comm expression in the midline cells using UAS-comm (Kidd et al., 1998b) and two GAL4 drivers: slit-GAL4 and sim-GAL4.

The combination of either midline GAL-4 driver with a single copy of UAS-Comm were able to drive levels of Comm protein at the midline corresponding to that observed in the wild-type embryo (data not shown). However, there was no significant rescue of the comm phenotype in either of the comm alleles (Fig. 1). We observed a mild rescue when comm was

<table>
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<th>Genotype</th>
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<th>Number of commissures per embryo</th>
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<td>24 (60.0%)</td>
<td>4.79</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>commE39/UAS-comm/Sim GAL4</td>
<td>62</td>
<td>0</td>
<td>0</td>
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<td>1 (2.2%)</td>
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Fig. 1. The commissureless mutant phenotype is not significantly rescued by expression of Commissureless at the midline. Axon tracts within the ventral nerve cord of (A) wild-type and (B) comm mutant embryos labelled with mAb BP102 at stage 15. In the wild-type embryo, the axons are organised into two longitudinal tracts, which are connected via two commissural tracts per segment. In comm mutants, the commissures do not form. (C) A wild-type embryo stained with a polyclonal Comm antibody. Comm protein accumulates at the commissure and is absent from the longitudinals. (D-F) Comm expression is driven at the midline using either the sim- (D) or slit- (E,F) GAL4 drivers and the resultant axon scaffold visualised with mAb BP102. (D,E) Driving wild-type Comm expression at the midline leads to a partial rescue of commA490 loss-of-function mutants. However there is no rescue of the null allele, commE39 (F). All panels are a view of the dorsal surface of the CNS and are shown anterior upwards.
expressed at the midline in the comm\textsuperscript{A490} background, but no rescue whatsoever in a comm\textsuperscript{E39} null background (Fig. 1D-F). In the rescued comm\textsuperscript{A490} embryos, partial or abnormal commissures formed in up to six segments (Fig. 1D,E). However, the overall rescue is weak and commissure formation occurs most often within the thoracic region, where relatively normal commissures can also be produced in the mutant. In the rescue experiment, a greater proportion of the mutant embryos are now able to form commissures (an increase from 21% to 35%) (Table 1).

The ability of one copy of comm at the midline to rescue commissure formation in comm\textsuperscript{A490} embryos is very mild. Increasing the number of copies of comm expressed to two does not increase the average number of commissures formed per embryo (Table 1). The complete lack of any rescue of the comm\textsuperscript{E39} null allele, even when two copies of Comm are re-supplied at the midline, suggests that comm expression is required in cells other than the midline cells.

We also attempted to rescue the comm phenotype by re-expressing Comm in all neurones in both comm\textsuperscript{E39} and comm\textsuperscript{A490}. This resulted in the production of a robo-like phenotype in which axons misroute towards the midline (data not shown). The phenotype matches that seen when Comm is overexpressed in the wild-type embryo (Kidd et al., 1998b), suggesting that endogenous Comm at the midline is not necessary for this phenotype.

**Comm-GFP driven at the midline does not transfer onto commissural axons**

In addition to testing whether Comm at the midline is able to rescue the comm phenotype we investigated whether Comm in the commissural axons originates from the midline cells. To do this we expressed a C-terminal GFP tagged version of Comm (Comm-GFP) at the midline using sim-GAL4 and slit-GAL4 simultaneously (Fig. 2A). The fusion protein is fully functional, able to downregulate Robo and has the same subcellular localisation pattern as the wild-type protein, both in vivo and in vitro (M. G. and G. T., unpublished). We observed that Comm-GFP does not transfer from the midline cells to commissural axons but remains within the cells where the gene is expressed. Rather than transfer between cells we found that Comm-GFP can move to fill the cell where it is expressed. When large amounts of Comm-GFP are driven the protein is observed within the axons of neurones produced at the midline, such as the MP1s and the VUMs (Fig. 2B). However, no Comm protein is detected at the commissures. This suggests that the midline cells do not supply the Comm protein found on commissural axons.

**Dissecting where Comm expression is required: Targeted RNAi**

Our investigations suggest that Comm expression in the midline cells is not sufficient to allow commissural axons to cross the midline. Furthermore, the Comm protein observed on Comm axons does not originate from the midline. These results suggest that Comm function is necessary in cells other than or in addition to the midline cells. To address directly the question of where comm expression is required, we sought to disrupt gene expression in specific cell types using targeted RNA interference (RNAi). Targeted RNAi uses the same rationale as conventional RNAi in which double stranded RNA from a gene is used to disrupt the function of that gene (Fire et al., 1998), but in this modification, gene disruption is targeted to specific cells using the UAS/GAL4 system (Brand and Perrimon, 1993; Hidalgo et al., 2001). To direct the generation of double stranded comm RNA in targeted cells a construct was generated (UAS-comm-HP) that consists of an inverted repeat of a 375 bp region of comm sequence, with the repeats separated by a linker region, downstream from a GAL4 UAS. GAL4 will drive transcription of this construct resulting in the production of a RNA hairpin loop, thus producing double stranded RNA specific to comm.

**Comm-hairpin specifically disrupts comm expression in vitro and has no effect on neuronal survival or general axon guidance in vivo**

To verify that the comm-hairpin transcript (comm-HP) inhibits comm expression and that this inhibition is specific to comm, the hairpin was driven in S2 cells. When Comm-GFP is co-expressed with comm-HP, very few cells show GFP fluorescence in contrast to transfections where comm-HP is not co-expressed (Fig. 3A,B).

In the S2 cell assay comm-HP does not have any nonspecific effects on cell growth or on the expression of an unrelated protein such as Robo when co-expressed in the same cells (Fig. 3C). We also tested whether expression of comm-HP affected the development of neurones in the peripheral nervous system (PNS) in vivo. These neurones do not express Comm and are unaffected in comm mutants. comm-HP was activated in the PNS using the pan-neural driver elav-GAL4. PNS axon...
guidance was unaffected suggesting that comm-HP does not have a nonspecific effect on axon outgrowth or guidance (Fig. 3D).

To confirm that comm-HP is able to disrupt Comm expression in the embryo, we expressed comm-HP in a subset of neurones using the eagle-GAL4 driver (MZ360) (Dittrich et al., 1997). The eagle (eg)-positive neurones were identified using an anti-Eg antibody and examined for co-expression of Comm. In wild-type embryos the eg-positive neurones express Comm protein (Fig. 3E); however, when comm-HP is expressed in these cells, the levels of Comm protein is reduced (Fig. 3F). This reduction in Comm expression occurs in most eg-positive neurones but not all suggesting that the comm-HP is not completely effective in vivo or that it is not correctly expressed in all cells where it is driven.

Disruption of comm function in either neurones or midline cells results in guidance defects at the midline

comm-HP was expressed in midline cells and/or neurones to identify if interference with Comm function in these cells affect axon outgrowth in the CNS. In order to look at specific neurones that cross the midline, we used the Sema2b-tmyc marker (Rajagopalan et al., 2000). This marker labels the cell bodies and axons of two to three neurones per hemisegment within five posterior abdominal segments, A4-8. These laterally positioned neurones normally send their axons immediately across the midline at the anterior margin of the anterior commissure, then turn to project anteriorly within the contralateral longitudinal tract (Fig. 4A). Thus, in each segment, three Sema2b tracts can be scored, two longitudinal tracts and a commissural tract. Absence of a commissural tract will occur when Sema2b axons from both sides of the CNS fail to cross the midline, whereas a defect in a longitudinal tract will occur when some axons fail to extend across the midline to the contralateral longitudinal pathway. In the wild-type embryo, the Sema2b commissural and longitudinal axon tracts are rarely defective (five tracts affected in 34 embryos; i.e. 0.15 defective tracts per embryo). When comm-HP is expressed, either pan-neurally or at the midline, we observe an increased incidence of failures to form Sema2b tracts (Fig. 4B,C). These failures occur as the Sema2b axons now stall and fail to extend across the midline or do not fasciculate with one another appropriately. Counting the number of Sema2b tracts severely affected reveals an increase from an average of 0.15 defective tracts per embryo (n=34) in wild type to 0.78 tracts affected per embryo when comm-HP is expressed at the midline (n=40) or 1.96 tracts affected per embryo when comm-HP is expressed in neurones (n=27). This phenotype suggests a requirement for Comm in both midline cells and CNS neurones. If the observed defects were due to a limited inhibition of Comm function, we would expect the phenotype to be enhanced if Comm levels in the embryo are reduced by removing one copy of the gene. Indeed we find that the phenotype is enhanced when comm-HP is driven in a comm heterozygous background (Fig. 4D,E). In these embryos, an average of 2.9 Sema2b tracts are severely affected or absent per embryo (n=21), in addition many or all of the Sema2b tracts are reduced in thickness in the embryos. In all cases the same phenotypes are observed using several different UAS-Comm-HP inserts at various sites in the genome. The observed failure of axons to cross the midline and the genetic interaction with comm shows that comm-HP is indeed disrupting comm expression in vivo, as was observed in vitro.

The phenotypes observed with the Sema2b marker suggest that comm-HP affects comm levels but that the expression or effect of the comm-HP transgene when activated by GAL4 is mosaic and that Comm is not completely eliminated in all cells. As a result, the visualisation of the axon scaffold with BP102 reveals only minor defects. Expression of comm-HP throughout the CNS using elav-Gal4 results in thinner longitudinals, stalling of axons around the commissures and occasional thinner commissures (Fig. 4G,I). An identical, but

Fig. 3. comm-HP specifically disrupts comm expression in vitro and in vivo but has no effect on sensory axon guidance. (A) Drosophila S2 cells expressing Comm-GFP in which Comm localises to intracellular vesicles (inset). (B) When comm-HP is co-expressed with Comm-GFP, the number of fluorescing cells is very low. Most cells that do fluoresce show either low levels of fluorescence or a disrupted localisation (inset). (C) robo and comm-HP co-transfected cells. comm-HP does not affect the expression level or numbers of Robo-transfected cells. (D) When comm-HP is driven throughout the nervous system using elav-GAL4, sensory axon extension and guidance is unaffected. The elav-GAL4 driver was used to drive both UAS-comm-HP and UAS-tGFP and axons were visualised with αGFP. (E) Eagle-expressing neurones revealed with anti-Eagle antibody (green); these neurones also express Comm (F, yellow). (G) When comm-HP is driven in the Eg-positive neurones, Comm levels are reduced in these cells (arrows). However not all Eg-positive neurones are equally affected, as Comm protein remains within a subset of the Eg-positive neurones (arrowhead in G). The embryo in D shows a view of the ventral region of the PNS at stage 16, the embryo is oriented with anterior to the left and dorsal upwards. (E-G) show views of ventral regions of the CNS in stage 13 embryos oriented anterior upwards.
generally milder phenotype is observed when driving the hairpin at the midline (Fig. 4J). This phenotype suggests a requirement for Comm in both midline cells and CNS neurones; however, *comm-HP* is unable to phenocopy the comm phenotype. Expression of *comm-HP* in neurones in a comm heterozygous background results in an enhanced phenotype where crossing errors are frequent and stalling occurs (arrow). Commissures and longitudinal sections are often absent or significantly reduced. (F-J) BP102 staining of the ventral nerve cord at (F-H) stage 13 or (I,J) stage 16. Arrows indicate thinner commissures, arrowheads indicate axon stalling. (F) Wild-type embryo. (G) When *comm-HP* is expressed in neurones, some axon stalling around the commissures is evident (arrowhead) together with thinner longitudinals and occasional thinner commissures (arrow). (H) This phenotype is enhanced when *comm-HP* is expressed in neurones in a comm heterozygous background. At later stages in development, some axon stalling and thinner longitudinals are observed when *comm-HP* is expressed in (I) neurones or (J) midline cells. All panels show a dorsal view of the CNS and are oriented with anterior upwards.

**Fig. 4.** Expression of *comm-HP* in either neurones or midline cells leads to guidance defects at the midline. (A) Sema2b neurones in the wild-type stage 16 embryo. (B) When *comm-HP* is driven in midline cells, the Sema2b neurones make crossing errors. The commissures are thinner and occasionally fail to form, as do sections of the longitudinal tract. Growth cone stalling at the midline is also seen (arrow). (C) When *comm-HP* is driven in neurones, the commissures are more severely affected and longitudinal sections can be completely absent. (D,E) Expression of *comm-HP* in neurones in a comm heterozygous background results in an enhanced phenotype where crossing errors are frequent and stalling occurs (arrow). Commissures and longitudinal sections are often absent or significantly reduced. (F-J) BP102 staining of the ventral nerve cord at (F-H) stage 13 or (I,J) stage 16. Arrows indicate thinner commissures, arrowheads indicate axon stalling. (F) Wild-type embryo. (G) When *comm-HP* is expressed in neurones, some axon stalling around the commissures is evident (arrowhead) together with thinner longitudinals and occasional thinner commissures (arrow). (H) This phenotype is enhanced when *comm-HP* is expressed in neurones in a comm heterozygous background. At later stages in development, some axon stalling and thinner longitudinals are observed when *comm-HP* is expressed in (I) neurones or (J) midline cells. All panels show a dorsal view of the CNS and are oriented with anterior upwards.

Driving Comm-HP in specific populations of neurones

The widespread expression of *comm-HP* within the CNS can cause axonal outgrowth defects; however, *comm-HP* is mosaic in its action and axon outgrowth defects are best-observed using markers for subsets of neurones. Therefore we tested whether expression of *comm-HP* could cause axon outgrowth defects when it is expressed in small populations of neurones within the CNS. For these experiments, we used three GAL4 drivers: eg-GAL4, 1512 and MZ465 (Dittrich et al., 1997; Hidalgo and Brand, 1997). eg-GAL4 is expressed in four neuroblasts and their progeny. The neuronal progeny includes a group of lateral EL neurones that project within the anterior commissure and a smaller group of more medial neurones that extend via the posterior commissure (Fig. 5A), the commissural tracts they form are complete by stage 13 (Dittrich et al., 1997; Dormand and Brand, 1998; Higashijima et al., 1996). *eg-GAL4* was used to drive *comm-HP* and a tau-GFP reporter in these commissural neurones. The expression of *comm-HP* within the neurones causes the axon bundles in certain segments to fail to cross and in some cases appear to turn away from the midline (Fig. 5B). When driving one copy of the hairpin construct, 13% of hemisegments show midline crossing errors (n=368) (Fig. 5B). With two copies of *comm-HP*, the number of midline crossing failures is increased to 61% of hemisegments (n=160) (Fig. 5C). When driving one copy of *comm-HP* the disruption is limited to the more medial neurones; when driving two copies, the more lateral neurones now fail to cross in certain hemisegments (Fig. 5B,C). Interestingly, by late stage 15, the *eg*-positive neurone crossing errors are overcome and the staining pattern appears normal. If one copy of *comm-HP* is expressed in the *eg*-positive neurones and one copy at the midline the number of crossing...
failures remains similar to the level seen when driving one copy of comm-HP in the eg-positive neurones alone at 13% of hemisegments (Fig. 5D).

Thus, expression of comm-HP causes strong midline crossing defects when expressed in commissural neurones. We also tested the ability of comm-HP to affect the development of ipsilaterally projecting neurones. The GAL-4 line15J2 is expressed in the vMP2 and dMP2 interneurones, and occasionally in pCC. While line MZ465 drives expression in pCC, aCC and RP2 (Hidalgo and Brand, 1997), driving expression of comm-HP using either of these lines resulted in no guidance errors for any of these ipsilateral neurones (Fig. 5E,F). Therefore, in contrast to the contralateral projecting neurones, no guidance errors were observed when driving Comm-HP in the ipsilaterally projecting neurones.

**Comm protein is detectable in neuronal cell-bodies**

Our rescue and targeted RNAi experiments both suggest a requirement for Comm function not only in the midline cells but also in the commissural neurones. This has led us to re-examine the Comm protein distribution in the embryo and to do this we developed a rabbit polyclonal antibody against Comm. This antibody is specific for Comm as no staining is observed in the comm-HP embryos and has the same distribution as was reported previously (Tear et al., 1996). Using confocal microscopy, it is apparent that Comm is expressed on the commissural tracts and throughout the nerve cord (Fig. 6A,B). The staining within the neuronal cell bodies is qualitatively different from that seen at the commissure in that it appears punctate (commissural axons have what appears to be cell surface staining) (Fig. 1C). This punctate staining was previously suggested to result from transfer of Comm to the axons from the midline cells. We now believe that this transfer does not take place and that the vesicular Comm is produced by the neurones themselves. Our data from expression of comm-HP suggests that Comm is required in commissural but not ipsilateral neurones. To identify whether all neurones express Comm, we labelled the Sema2b, eg-positive and 15J2 neurones and looked for colocalisation with Comm. Punctate Comm staining clearly co-localised with both the Sema2b and eg-positive cell bodies (Fig. 3F, Fig. 6A-D). However no obvious colocalisation was apparent when using the 15J2 driver to drive GFP in the vMP2 and dMP2 neurones (Fig. 6E,F). It therefore appears, from both the hairpin and colocalisation experiments, that these ipsilateral interneurones possess no Comm protein or RNA.

**DISCUSSION**

Neurones navigating towards their target cells must make a series of decisions as they extend along their trajectory. Within the CNS, extending axons decide whether to grow towards or away from the midline (Colamarino and Tessier-Lavigne, 1995; Seeger et al., 1993). Those axons that do grow towards the midline also switch their behaviour so that they do not remain at the midline but extend across to the contralateral side. It is clear that there is tight spatial and temporal regulation of the surface expression of guidance receptors, as well as of the activation of novel molecular interactions that change the nature of the signals received by the axons as they cross the midline (Dodd et al., 1988; Stein and Tessier-Lavigne, 2001; Stoeckli et al., 1997; Zou et al., 2000). Comm is one of the few molecules to have been identified as having an important role to regulate the levels of a receptor protein necessary for axon guidance at the midline. It is able to downregulate levels of the Robo protein, which acts as a receptor for the midline-derived axon outgrowth inhibitor molecule Slit (Kidd et al., 1998b). Downregulation of Robo is necessary for axons to cross the midline; however, very little is known about how or in which cells Comm functions. We have shown that Comm is expressed and required in both commissural neurones and midline cells for correct midline crossing.
Comm protein does not transfer from the midline glia onto commissural axons

To allow commissural axons to cross the midline, Comm specifically downregulates Robo on these axons. Once across the midline, Robo levels increase, suggesting Comm acts only at the midline. It has been suggested previously that Comm acts specifically at the midline via a mechanism whereby Comm is supplied to axons by the midline cells (Tear et al., 1996). In this model, Comm transfers from the surface of midline cells to the commissural axons during cell/cell contact. If Comm were then unable to extend far along the axon it could act locally to downregulate Robo in the commissures and allow extension across the midline. This model predicts that Comm can transfer from midline cells to the commissural axons and that Comm expression is required only at the midline. We have shown that the expression of Comm protein at the midline alone is not sufficient for commissure formation. We were unable to generate any rescue of midline crossing when re-expressing wild-type Comm at the midline in comm-null mutant embryos. However, a mild rescue was observed when Comm was re-expressed at the midline in commA490 mutants. As no rescue is seen in null embryos and only partial rescue is seen in commA490 embryos, Comm is necessary in other CNS cells and we show Comm is also necessary within the commissural axons themselves. Thus, in the commA490 embryos, the truncated protein is expressed in commissural axons in addition to the midline cells and it retains some weak function. The re-expression of Comm protein adds function to the midline cells, thereby slightly increasing the number of axons able to cross the midline. Unfortunately we cannot rescue the comm phenotype by overexpression of Comm in all neurones as this results in the efficient downregulation of Robo and promiscuous midline crossing. However we were able to reveal a requirement for Comm in neurones using a targeted RNAi approach.

We also examined Comm transfer from the midline cells using a C terminal GFP-tagged Comm protein, Comm-GFP. The addition of GFP to the Comm protein allowed us to differentiate the re-expressed Comm from that expressed endogenously and to identify whether Comm could move from one cell to another in the embryo. Comm-GFP retains full function, as revealed by its ability to phenocopy the robo phenotype when overexpressed in all neurones (data not shown). Yet when Comm-GFP was expressed at the midline, we failed to observe transfer into commissural axons. The Comm-GFP did not appear to be tightly associated with any cellular component, as the protein was clearly able to move within the cells. Comm-GFP was visible within the axons of the neurones derived from the midline cells, e.g. MP1 and the VUMs. Despite clear labelling of these axons, no staining of commissural axons was visible. Thus, Comm is unable to move from the midline cells to the commissural neurones, confirming that Comm must function in cells other than or in addition to the midline cells to regulate commissural axon extension at the midline. Interestingly when Comm is driven in MP1 it does not affect its guidance, even though Robo is normally required in this neurone to keep it away from the midline (Seeger et al., 1993).

Our investigations examining rescue of the comm phenotype by re-expression of Comm at the midline and analysis of the ability of Comm-GFPs to move from midline cells leads to the conclusion that the Comm protein observed on the commissural tracts in wild-type embryos is not provided by the midline cells. It is possible that low levels of comm transcript are present in neurones and that Robo downregulation at the midline is due to neuronal Comm protein accumulating at the midline.

Comm is expressed and required in both commissural neurones and midline glia

To identify which cells in the CNS require Comm function, we
made use of the technique of targeted RNAi. This method inhibits the production of Comm by expressing double-stranded RNA, in the form of a hairpin-loop (comm-HP).

We used a Drosophila S2 cell assay to test the ability of comm-HP to inhibit comm expression. Here, comm-HP caused a marked reduction in Comm expression, but did not affect the expression of Robo. Expression of comm-HP does not lead to general, nonspecific axon guidance defects on PNS axons. Furthermore, when comm-HP was driven in specific ipsilateral neurones, guidance of these neurones was unaffected. However, expression of comm-HP in commissural neurones does lead to midline crossing defects and these defects are enhanced when one copy of comm is removed. This suggests that comm-HP specifically inhibits Comm expression in vivo.

Several conclusions can be made from the targeted RNAi experiments. First, midline crossing errors were observed when Comm expression was inhibited specifically in neurones. Thus, comm transcript and Comm activity are present in neurones. Second, an identical yet milder phenotype was observed when driving the hairpin specifically at the midline. Comm function is therefore required in both neurones and the midline glia. The identical nature of the neuronal and glial phenotypes suggests that comm within both of these cell types is required for the same process, i.e. allowing a growth cone to cross the midline. Finally, the lack of any guidance errors in ipsilateral neurones, when driving the hairpin either throughout the nervous system or in specific neurones, suggests that Comm activity is not required in these ipsilateral neurones.

The CNS defects produced by the expression of Comm-HP in the CNS are fairly mild when observed with BP102, a reduction in the size of the commissural tracts and some longitudinal breaks can be observed. These phenotypes are more obvious when the outgrowth of a smaller population of neurones, the Sema-2b neurones, are examined. Here, there are clear defects in extension across the midline and stalling of some commissural axons. These axons also appear to fail to extend in the longitudinalis. This affect is likely to be specific to Comm, as removal of one copy of comm enhances this phenotype. However, it is apparent that comm-HP is unable to fully inhibit comm in all cells. This might explain why the phenotype differs from that seen in comm loss-of-function embryos. In comm mutants, when all cells lose Comm function and all axons do not cross the midline, the default is to extend in the longitudinalis. In the comm-HP mutants, a subset of commissural axons fail to extend and stall. This may affect the extension of neighbouring axons producing the longitudinal defects seen with BP102. Expression of comm-HP in neurones or midline cells gives rise to the same phenotype, suggesting that a slight reduction in Comm levels at the midline also leads to crossing failures. By contrast, driving large amounts of fully functional Comm-GFP at the midline has no effect on guidance. This would suggest that a certain threshold level of Comm is required at the midline.

Our results suggest that the Comm protein identifiable in neuronal cell bodies is not the result of transfer from midline cells. This punctate vesicular neuronal staining was seen to be restricted to commissural neurones, with no protein observed within ipsilateral neurones. Thus, the targeted RNA interference and protein localisation results suggest that Comm is not present in ipsilateral neurones. It is therefore possible that the presence or absence of neuronal Comm determines whether or not an axon crosses the midline. Indeed expression of Comm in the normally ipsilateral projecting Ap neurones (O'Keefe et al., 1998) using an Ap-GAL4 driver causes all the Ap neurones to cross the midline (Bonkowsky et al., 1999). Thus, individual ipsilateral neurones can be converted to midline crossing neurones by the introduction of comm activity, suggesting that the presence of neuronal Comm dictates axon pathway choice.

Comm protein accumulates on the commissural portion of the contralaterally projecting axons. The appearance of this staining is very different to the vesicular localisation seen in the cell body. Rather it appears that Comm protein can accumulate at the cell surface within the commissure. Perhaps Comm protein is transported from the cell bodies within the vesicles to be presented at the cell surface during commissure formation. Furthermore, Comm protein does not seem to extend beyond the midline region onto the contralateral segments of the axon. Comm thus appears to be targeted to the commissural tract or somehow sequestered in this region. This may either concentrate its activity in this region and/or serve as a device for preventing Comm activity spreading further along the axon. In this way Comm could act to prevent Robo-mediated sensitivity to the midline Slit signal in commissural axons prior to crossing, but be unable to act on Robo once the axon has extended beyond the midline. It is possible that an interaction between Comm at the midline and Comm in the neurone provides the means to trap or concentrate Comm at the commissure. An accompanying paper provides evidence of the ability of Comm to bind itself through its extracellular domain (M. G. and G. T., unpublished).

We reveal that comm activity is necessary in commissural neurones and midline glial cells. A similar requirement for Comm in neurones has been observed using cell transplantation techniques (Diana Cleppien, Gerd Technau and Barry Dickson, personal communication). We suggest the presence of Comm in the commissural neurones may encourage midline crossing. This tendency is promoted by Comm activity in the midline cells. The combined action is predicted to allow inhibition of Robo activity specifically in the commissural neurones, allowing growth across the midline. Comm protein accumulates at the axon surface within the commissural region, using a mechanism that is likely to involve Comm in the midline glia. However, Comm activity does not extend beyond the midline, allowing Robo levels to increase at the growth cone surface and initiate a sensitivity to the midline inhibitor Slit that encourages extension away from the midline and prevents re-crossing.

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