Roundabout signalling, cell contact and trophic support confine longitudinal glia and axons in the *Drosophila* CNS

Edward F. V. Kinrade¹, Tamar Brates¹, Guy Tear² and Alicia Hidalgo¹,*

¹NeuroDevelopment Group, Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK
²Molecular Neurobiology Group, New Hunt’s House, Guy’s Hospital Campus, King’s College, London SE1 1UL, UK

*Author for correspondence (e-mail: a.hidalgo@gen.cam.ac.uk)

Accepted 3 November; published on WWW 21 December 2000

SUMMARY

Contrary to our knowledge of the genetic control of midline crossing, the mechanisms that generate and maintain the longitudinal axon pathways of the *Drosophila* CNS are largely unknown. The longitudinal pathways are formed by ipsilateral pioneer axons and the longitudinal glia. The longitudinal glia dictate these axonal trajectories and provide trophic support to later projecting follower neurons. Follower interneuron axons cross the midline once and join these pathways to form the longitudinal connectives. Once on the contralateral side, longitudinal axons are repelled from recrossing the midline by the midline repulsive signal Slit and its axonal receptor Roundabout. We show that longitudinal glia also transiently express roundabout, which halts their ventral migration short of the midline. Once in contact with axons, glia cease to express roundabout and become dependent on neurons for their survival. Trophic support and cell-cell contact restrict glial movement and axonal trajectories. The significance of this relationship is revealed when neuron-glial interactions are disrupted by neuronal ablation or mutation in the *glial cells missing* gene, which eliminates glia, when axons and glia cross the midline despite continued midline repellent signalling.

Key words: robo, Connectives, Glia, Cell survival, *Drosophila melanogaster*, CNS

INTRODUCTION

Flies, like vertebrates, are bilateral symmetrical organisms, in which the two sides are separated by the midline. The fly ventral nerve cord (VNC) of the central nervous system (CNS) extends along the main body axis, like the spinal cord of vertebrates, linking the brain with the sensory and motor systems. Interneurons project axons that cross the midline once and then extend along the longitudinal pathways (Goodman and Doe, 1992). The insect ventral midline and its vertebrate equivalent, the floorplate, are sources of antagonistic repulsive (Slit) and attractive (Netrins) signals that regulate the crossing of axons (Tear, 1999; Tessier-Lavigne and Goodman, 1996; Thomas, 1998). The combination of these molecules instructs axons to cross the midline, to leave the midline once they have reached it and never to cross it again. However, despite our understanding of the control of midline crossing, not enough is known about how longitudinal axons are maintained laterally.

Repulsion from the midline is a key mechanism to keep axons along longitudinal pathways (Tear, 1999; Tessier-Lavigne and Goodman, 1996; Thomas, 1998). The repulsive signal Slit (Sli) is produced by the midline glia (Kidd et al., 1999). Interneuron axons express the Sli receptor Roundabout (Robo) and thus remain parallel to the midline from a certain distance (Kidd et al., 1998a). To allow *robo*-expressing axons to reach the midline prior to extending along the longitudinal pathways, the midline glia also express the Commissureless protein, which is responsible for downregulating *robo* expression as axons approach the midline (Kidd et al., 1998b; Tear et al., 1996). At the end of axonogenesis, all longitudinal axons express *robo* and remain contralateral, away from the midline.

The longitudinal pathways are pioneered by four neurons per hemisegment, pCC, MP1, dMP2 and vMP2, whose axons never cross the midline (Bastiani et al., 1986; Bate and Grunewald, 1981; Hidalgo and Brand, 1997; Jacobs and Goodman, 1989; Lin et al., 1994). These ipsilateral pioneer axons form a scaffold for the later selective fasciculation of follower axons. During the formation of the first longitudinal fascicle, pioneer growth cones also express the Robo receptor, which prevents them from crossing the midline (Kidd et al., 1998a). During their pathfinding, the pioneer axons interact with a class of glial cells, the interface glia (Ito et al., 1995), which at the end of embryogenesis overlie the longitudinal axons (Hidalgo and Booth, 2000). The longitudinal glia are the interface glia derived from the segmentally repeated lateral glioblasts, located at the edge of the neuroectoderm. Longitudinal glia, like the midline glia, are reminiscent of vertebrate oligodendrocytes since they originate from highly migratory and proliferative precursors and enwrap CNS axons (Halter et al., 1995; Jacobs et al., 1989; Schmidt et al., 1997). The longitudinal glioblasts divide and migrate ventrally until they contact the cell bodies of the pioneer neurons, where they halt at a certain distance from the midline. The first longitudinal fascicle is formed as the descending axons of
dMP2 and MP1 meet the ascending axons of pCC and vMP2 (Bate and Grunewald, 1981; Hidalgo and Brand, 1997). Longitudinal glia migrate anteroposteriorly slightly ahead, but in close contact with, the extending pioneer growth cones and stall at choice points relevant for axon guidance and fasciculation (Hidalgo and Booth, 2000). Following the formation of the first longitudinal fascicle, glia continue migrating, occupying choice points to instruct axonal defasciculation and refasciculation. The final pattern of pioneer axon trajectories is dictated by glia (Hidalgo and Booth, 2000).

Two mechanisms are likely to promote the cell interactions that generate the longitudinal pathways, although the molecules involved are largely unknown. The first is cell contact, which is manifested in axon–axon and axon–glia adhesion. Axon–axon contact is responsible for selective fasciculation, such as fasciculation of follower with pioneer axons (Bastiani et al., 1984; Hidalgo and Brand, 1997; Raper et al., 1983; Raper et al., 1984). Axon–glia contact enables the glia to guide pioneer growth cones and provoke their fasciculation or defasciculation at choice points (Hidalgo and Booth, 2000). The second mechanism is trophic support. Survival of follower neurons depends on glia, thus contributing to the maintenance of contralateral follower axons (Booth et al., 2000). However, it is not known whether these interactions can prevent axons and glia from re-crossing the midline.

Here we address the question of what are the mechanisms that confine longitudinal axons and glia away from the midline. We show that robo is transiently required to stop longitudinal glia migration short of the midline. We also show, however, that from the time that the glia contact axons, robo is no longer responsible for restricting glial movement. When glia are in contact with axons, trophic support and cell–cell contact maintain glia and axons in lateral positions. These later mechanisms operate on axonal patterns contemporarily with, but independently of, midline-derived repulsion, since upon interference with neuron–glia interactions glia and axons can cross the midline despite expressing the repulsion receptor Robo.

**MATERIALS AND METHODS**

**Fly stocks**

Wild-type: Canton-S. Mutants: gcm<sup>AP1</sup>/CyO[acman] (Jones et al., 1995) (null), gcm<sup>AP1</sup>/CyO[acman]; Df (3L) H99/TM6B. Both robo alleles are nulls that do not produce any protein: robo<sup>214</sup>/CyO[acman]; robo<sup>21772</sup>/CyO[acman] (Kidd et al., 1998a); Df (3L) H99/TM6B (White et al., 1994). Ablations: w; ftz<sup>NGAL4</sup> (Lin et al., 1995), w; ftz<sup>NGAL4</sup> Df (3L) H99/TM6B, w; UAS ricin (UFR1.1)/CyO[acman] (Hidalgo et al., 1995), w; UAS rpr/TM3 LacZ, w; UFR1.1; Df (3L) H99; Sm6a-TM6B and w; UAS rpr Df (3L) H99/TM6B (Booth et al., 2000). Other: w; 158 (Booth et al., 2000), w; UAS sli (Kidd et al., 1999). Robo rescue: sgcmGAL151; robo<sup>214</sup>/CyO, w; rob<sup>214</sup>/CyO; UAS robo, sgcmGAL151; robo<sup>21772</sup>/CyO, w; rob<sup>21772</sup>/CyO; UAS robo.

**Cell ablations**

Ablation of neurons was carried out with the GAL4 system driving either the toxin, ricin (Hidalgo et al., 1995) or reaper (rpr) expression (Booth et al., 2000). The line ftz<sup>NGAL4</sup> drives expression in all four pioneer neurons, pCC, MP1, dMP2 and vMP2 from their initial specification and in many other neurons (Hidalgo and Brand, 1997; Lin et al., 1995). It also drives expression transiently in some of the glioblast progeny. Expression does not affect most glia derived from the glioblast, since ricin or rpr expression with this line leaves many longitudinal glia intact at stage 13 and since longitudinal glia survival can be rescued in ablated embryos in a rpr mutant background (Fig. 7D). This line is not expressed in midline glia. Only embryos in which ablation had taken place were analysed. Furthermore, non-ablated embryos were also identified by the expression of lacZ from the reporter balancer chromosomes, which was visualised with anti-β-gal antibodies.

**Rescue of robo mutant phenotype**

Homozygous robo<sup>214</sup> mutants do not express any Robo protein. robo expression was provided to glia in a mosaic fashion with sgcmGAL151 (Booth et al., 2000). Only embryos with mosaic robo expression were analysed.

**Immunohistochemistry**

Antibody stainings were carried out following standard procedures. Rabbit anti-Repo (reversed polarity) was used at 1:300; rabbit anti-Heatless at 1:1000; mouse Fasciclin 2 (FasII/Fas2/mAb1D4) at 1:5; mouse anti-Robo at 1:5; mouse anti-Sli at 1:10; rabbit anti-β-gal at 1:500. Antibody concentrations were doubled in fluorescent stainings and fluorescence signal was amplified with Streptavidin-Alexa 488 (Molecular Probes). Fluorescent stainings were analysed in an MRC 600 confocal microscope.

**RESULTS**

**Longitudinal glia migrate over the midline in robo mutants**

Interface glia normally overlie the longitudinal connectives of the CNS and are not found over the midline (Ito et al., 1995). Mutations in robo cause interface glia to migrate over the midline (n=12/12 embryos, Fig. 1C,D). However, not all glia migrate over the midline: some remain along the longitudinal tracts (Fig. 1D), whose position is nevertheless closer to the midline than in normal embryos (compare Fig. 1C,D with A,B). This differential effect of robo mutations on glia correlates with similar differences in the axonal phenotype. For instance, only the pCC/MP2 (central) fasII fascicle, but not the outer two fascicles, is affected in robo mutants (Fig. 1C; Kidd et al., 1998a). The longitudinal glia are responsible for the formation of the three fasII fascicles (Hidalgo and Booth, 2000). Hence, these data suggest that either there is a differential requirement for robo amongst the longitudinal glia, or that other members of the robo family may also play a role in these glia or that further factors, other than robo function, may determine glial positions along the longitudinal fascicles.

**robo is transiently expressed in longitudinal glia**

Since in robo mutants longitudinal glia migrate over the midline, we wondered if robo may be expressed in glia as well as in axons. If so, robo would keep glia away from the midline in normal embryos and would render them insensitive to midline repulsion in robo mutants. Prior to axonogenesis, robo is expressed in two broad longitudinal bands at either side of the sli-expressing midline (Fig. 2A, stage 12.3). Sli protein is also found within these bands at stages 12.2 and 12.1 (Fig. 2B). By stage 13 robo expression resolves into segmental clusters (Fig. 2C), preceding the overt expression in axons.

robo is also expressed in one transverse stripe per hemisegment at stage 11 (Fig. 2D). These lateral stripes include the tracheal pits. The longitudinal glioblasts originate just ventrally of the tracheal pits, and they divide as they migrate medially within these lateral bands of robo expression.
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(Fig. 2D). Glia stop migrating medially as they enter the longitudinal bands of robo expression (Fig. 2E,F). Within these robo expression domains glia express robo themselves. This was confirmed by double labelling experiments. Firstly, glial nuclei were visualised with the glial marker anti-Repo (Halter et al., 1995) and found to be totally surrounded by robo-expressing cytoplasm and membranes (stages 12.4-12.1, Fig. 3A,C-E). Secondly, glia were found to coexpress the longitudinal glia cell membrane marker Heartless (Shishido et al., 1997) and Robo (stages 12.4-12.2, Fig. 3B,F-H). Thirdly, longitudinal glia, expressing lacZ under GAL4 control by the glial line 158 (Booth et al., 2000), were found to coexpress β-gal and Robo (stage 12.3, Fig. 3I-K, note view of glial projections in a 0.5 μm section).

In glial cells missing (gcm) mutants, in which glial fate is transformed to neuronal fate (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), there is a reduction of robo expression between stages 12.2 and 13 (Fig. 4C,D). Furthermore, the remaining signal is often concentrated on what appears to be individual cells, which could correspond to pCC (stage 13, Fig. 4D). robo signal increases to normal levels in gcm mutants from stage 14. If robo expression were limited to neurons, in gcm mutants an increase (not a reduction) in robo expression at stages 12.2-13 would have been expected.

These data show that longitudinal glia express robo and respond to midline-derived repulsion within a narrow time window.

Expression of robo in normal embryos disappears from glia from stage 13, as glia occupy more dorsal positions over the longitudinal tracts. By stage 14, Robo is clearly present only in axons (see Kidd et al., 1998a). Since glia maintain lateral positions in the longitudinal pathways throughout embryogenesis despite ceasing to express robo, further mechanisms must restrict glial movement.

robo is required cell-autonomously in the longitudinal glia

To test whether robo function is required cell-autonomously in the longitudinal glia, we analysed whether ectopic expression of the repulsive signal Sli affects glial migration.

In normal embryos, longitudinal glia migrate towards the pioneer neurons. We expressed Sli ectopically in all postmitotic neurons with elavGAL4. This has been shown to cause axonal misrouting across the midline late in embryogenesis, as visualised with BP102 antibodies (Kidd et al., 1999). Upon ectopic Sli expression, in old embryos we observed defasciculation defects and rather subtle misroutings across the midline with fasII (not shown). For the analysis of glial patterns we focused on early embryogenesis, at the time when the interface glia migrate and establish contact with the pioneer neurons. We observed a range of phenotypes between stages 12.5 and 13 (altogether with 60% penetrance, n=46 embryos), comprising delayed glial migration (Fig. 5D), defective migration of glia along axons (Fig. 5E), increased glial numbers (Fig. 5F) and increased distance from the midline between the connectives (not shown). After stage 13, we observed more glia clustered at the exit of the nerves from the
CNS, and missing interface glia along the connectives (not shown). However, older embryos look to have recovered somewhat compared to the earlier defects and can look normal. These data show that glial migration is affected upon ectopic expression of Sli. The increase in glial numbers suggests that whereas some glia do not migrate, the glia that reach the pioneer neurons divide further, thus increasing the overall numbers of glia. This increase in glial numbers would explain the remarkable recovery of glial presence along the connectives in late embryogenesis. The presence of glia in the connectives despite the ectopic expression of sli in axons is consistent with the fact that glia do not express robo after stage 13 (see above).

**Robo is insufficient to restrict all glial movement**

Interface glia express robo only transiently, and they can overlie longitudinal axons despite their ectopic expression of Sli. We therefore determined whether robo expression is sufficient to restrict glia to lateral positions in the longitudinal pathways.

We targeted robo expression to the glioblast in robo mutant embryos. Glia (marked with anti-Repo antibodies) expressing robo are most often found in lateral positions, indicating that robo expression can keep glia away from the midline compared to their usual position in robo mutants (n=16/18 clones) (Fig.

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**Fig. 3.** The repulsion receptor Robo is expressed in the longitudinal glia. Confocal images, representing projections of 0.5 μm thick sections. (A,C-E) Robo protein (green) surrounds glial nuclei visualised with anti-Repo antibodies (red) at stage 12.2. (A) Robo protein is distributed in broad longitudinal bands at this stage. Total thickness: 1.5 μm. (C) Robo, green; (D) merged images of C and E; (E) Repo, red. Robo protein is located in the cytoplasm and membranes of individual glia. Higher magnification details. Total thickness: 1.5 μm. (B,F-H) Robo (red) protein is colocalised with Htl (green) in longitudinal glia (colocalisation in yellow) at stage 12.4. (B) Total thickness: 4.5 μm. Note the black nuclei, which do not express Robo or Htl, surrounded by both signals. (F) Htl, green; (G) merged images of F and H (colocalisation in yellow); (H) Robo, red. Higher magnification detail of one cell. Total thickness: 2 μm. Note the black nucleus in the centre of the cell surrounded by both signals. (I) Robo, green; (J) merged images of I and K (colocalisation in yellow); (K) β-gal, red. Glia visualised with anti-β-gal antibodies (red) in embryos expressing lacZ in glia (genotype: 158 GAL4/UAS tau lacZ and Robo (green), at stage 12.2. Total thickness: 0.5 μm. Note colocalisation in cytoplasmic projections (arrows). Note also black nuclei (arrowheads), surrounded by both signals. White bar represents position of midline. Anterior is up, except for C-E, in which anterior is to the left.

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**Fig. 4.** Decrease in Robo protein in gcm mutant embryos. (A,B) Wild-type embryos stained with anti-Robo (brown) and anti-Sli (blue) at stages 12.2 (A) and 13 (B). Note the clusters of Robo protein (arrowheads), more pronounced at stage 13. (C,D) gcm mutant embryos stained with anti-Robo antibodies (brown). Note the reduced extent of the Robo clusters, and the concentration of Robo signal in individual round spots, which appear to be individual cells. Anterior is up.
6B). However, these robo-expressing glia remain in contact with axons and thus are still closer to the midline than in normal embryos (see also Fig. 6E,F). In some cases glia were found over the midline despite expressing robo (n=2/18 clones, Fig. 6C). These data suggest that robo is not sufficient to restrict glial migration and that glial positions also depend on contact with axons.

Since glia are required for formation of normal axonal trajectories, we wondered if targeting robo expression to glia alone in robo mutants would restore the mutant axonal phenotypes. Targeting robo expression to the longitudinal glioblast, in mutants lacking robo expression, does not rescue the robo axonal phenotype. The pCC fascicle misroutes across the midline despite the expression of robo in adjacent glia (n=40 clones, Fig. 6E,F). These data confirm the notions that the pCC fascicle requires robo autonomously to remain ipsilateral (Kidd et al., 1998a) and that pathfinding by the pCC growth cone does not require glia (Hidalgo and Booth, 2000; Hosoya et al., 1995; Jones et al., 1995).

**Axon-glial contact contribute to keep glia laterally**

Longitudinal glia require contact with axons to maintain their survival. In normal embryos some glia die from the time when they reach the longitudinal axons (A. H., E. K. and M. Georgiou, unpublished data). Consistently, ablation of neurons leads to loss of glia (genotype: ftzNG4/UAS ricin; Fig. 7C; also A. H. and A. Brand, unpublished data). In rpr mutant embryos in which apoptosis is blocked, the distribution of interface glia along the connectives is normal (Fig. 7B). If neuronal ablation takes place in rpr embryos (genotype: rpr ftzNG4 / UAS ricin rpr; n=10/10 embryos), interface glia are rescued, as monitored with anti-Repo (Fig. 7D). These results show that interface glia apoptosis is induced in the absence of neurons.

Although the rescued glia in the experiment above no longer die, they do not migrate normally (Fig. 7D). Glia do not acquire their normal lateral positions and are found over the remaining axons, or clustered around their original location, or scattered throughout, or along the edges of the ventral nerve cord, or clustered over the midline (Fig. 7D). These observations imply that interface glia require contact with longitudinal axons to survive. Furthermore, interface glia require contact with axons to acquire their normal positions along the longitudinal pathways.

Interface glia are also in contact across the midline. We have observed that cytoplasmic projections of the longitudinal glia reach the midline and contact glia from the contralateral side (Fig. 8). Glial projections were visualised with reporter lacZ expression driven by the glial-specific GAL4 line 158 (Booth et al., 2000; Fig. 8A) and with anti-Heartless (Htl) antibodies (Fig. 8B). These midline-reaching longitudinal glia projections were observed between stages 13-14 (in all segments of all embryos stained with anti-Htl), after robo expression has disappeared from the longitudinal glia. Since glia dictate axonal patterns (Hidalgo and Booth, 2000), these glial projections may provide a pathway across the midline when axons are deprived of their normal lateral fasciculation cues.

**Interference with axon-glial interactions causes axons to cross the midline despite expressing robo**

To test whether longitudinal axon-glial interactions play a role in maintaining longitudinal axons away from the midline, we disrupted the normal axon-glial interactions to answer several questions: (1) does elimination of glia provoke midline crossing by axons?; (2) does elimination of pioneer neurons provoke midline crossing by axons or glia?; (3) are the potential effects dependent or independent of Robo-mediated repulsion?

To analyse the consequences of absence of glia in longitudinal pathways, we analysed gcm mutant embryos lacking functional glia, since they are transformed to neurons.

![](image) Fig. 5. robo is required cell-autonomously in longitudinal glia. Effects in glia of ectopic panneural expression of sli (genotype: elavGAL4/UAS sli) in embryos stained with fasII (brown, pioneer neurons and axons) and anti-Repo (black, glia). (A-C) Wild-type embryos at stage 12.3 (A) and 12.1 (B,C) focusing on a dorsal (B) or ventral (C) plane; (D-F) elavGAL4/UAS sli embryos at stage 12.3 (D) and 12.1 (E,F) focusing on a dorsal (E) or ventral (F) plane. Note in A that glia are in contact with pioneer neurons at this stage (arrowheads), but if neurons express sli, glia do not migrate as far (D, arrowheads; severely affected specimen). Note in B that at stage 12.1 in normal embryos the longitudinal glia migrate along the pioneer axons. Upon neuronal sli expression (E) glia are repelled from establishing contact with axons (arrowheads; arrows indicate glia that migrate normally in the same specimen). Interface glia remain clustered around the neuronal cell bodies. Note in C the ventral glia also labelled with anti-Repo (arrowheads). Upon neuronal sli expression there are higher numbers of ventral glia, which are also disorganised (F, arrowheads). These glia could correspond to interface glia which failed to migrate. Anterior is up.
We find that in gcm mutant embryos, axonal fascicles, which normally extend along the longitudinal pathways, may cross the midline (in at least one segment – and most frequently in several – in 17 out of 18 embryos examined; Figs 8D and 9B).

To analyse the consequences of absence of pioneer neurons on the positions of axons and glia, we ablated all pioneer neurons and other neurons with ftzNGAL4 (Lin et al., 1995) driving ricin expression (Hidalgo et al., 1995). Such ablation causes fasII positive fascicles, which would normally project along the longitudinal pathways, to cross the midline within the commissures (most segments in 16/16 embryos, Fig. 9C; see also Hidalgo and Brand, 1997). Longitudinal glia also migrate over the midline in these embryos (n=4/7 embryos; Fig. 7C).

Given that the midline is the source of the repulsive signal Sli and longitudinal axons express the Sli receptor Robo (Kidd et al., 1999; Kidd et al., 1998a), midline crossing by axons was unexpected. Misrouting across the midline could occur if the midline glia were damaged, leading to loss of the Sli repulsive signalling molecule. However, neither the gcm gene nor the ftzNGAL4 line are expressed in midline glia, (Hidalgo and Brand, 1997; Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) and sli expression is normal in the midline of both gcm mutants (Fig. 9G) and ablated embryos (Fig. 9H). In both gcm mutants (n=13/17 embryos) and ablated embryos (n=8/8 embryos) the misrouted axons cross the midline despite expressing normal levels of the Sli receptor Robo (Fig. 9G,H). These data show that, unexpectedly, interference with axon-glia interactions results in midline crossing despite midline derived repulsion.

These data suggest that longitudinal axon-glia interactions are involved in keeping both axons and glia away from the midline independently of midline-derived repulsion.

**Trophic support confines axonal and glial lateral positions**

Trophic support between neurons and glia is a means of restricting glial movement and axonal trajectories. There are reciprocal (although asymmetric) trophic interactions between neurons and glia during pathfinding. Pioneer neurons maintain the survival of longitudinal glia (see above and Fig. 7C), and glia maintain the survival of follower neurons (Booth et al., 2000). When axon-glia interactions are intact, axonal CNS patterning is virtually normal in the absence of cell death (rpr mutants, Fig. 9F), although subtle axonal defects are found, such as longitudinal growth cones projecting towards the midline (Fig. 8C). However, when neuron-glia interactions are perturbed in rpr mutant embryos, that are unable to undergo programmed cell death, the misrouting of axons across the midline is dramatically enhanced, compared either with rpr mutants alone or with perturbing neuron-glia interactions in embryos in which apoptosis can occur normally. In embryos double mutant for gcm and rpr...
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(gcm rpr, n=9/9 embryos, Fig. 9D) or upon neuronal ablation in a rpr mutant background (ftzNGAL4 rpr/UAS ricin rpr, n=10/10 embryos, Fig. 9E), axonal extension along the longitudinal pathways is severely affected, as visualized with fasII. Longitudinal connectives are virtually missing and fasII-positive axons cross the midline in every segment. In the case of ablated rpr mutant embryos the phenotype often resembles (albeit more severely) the robo mutant phenotype (Fig. 9E).

These midline crossing axons express robo. In embryos double mutant for gcm and rpr (n=6/11 embryos, Fig. 9I) or upon neuronal ablation in a rpr mutant background (n=9/9 embryos Fig. 9J) there is no robo expression along the longitudinal connectives, but instead all axons expressing robo cross the midline. These observations imply that attraction towards the midline is the default pathway taken by axons and glia despite midline repulsion in the absence of normal axon-glia interactions in the longitudinal pathways. They also mean that the pressure for survival normally keeps cells in contact with their normal neighbours, in this case in lateral positions.

Taken together, these data show that in normal embryos control of cell survival and cell-cell contact are means of confining glia and axons laterally.

DISCUSSION

Pioneer axons, interface glia and interactions between them are necessary for the formation and maintenance of longitudinal
pathways of the VNC. Here, we show that the longitudinal glia also respond transiently to the Sli/Robo repulsive mechanism, which enables them to stop migrating ventrally, short of the midline. In fact, we have shown that longitudinal glia migrate within broad dorsoventral and subsequently longitudinal bands of robo expression. Within these domains, glial nuclei are surrounded by robo-expressing cytoplasm and membranes. This was confirmed by demonstrating that glial cell cytoplasm and membranes coexpress robo and the longitudinal glia membrane marker htl as well as cytoplasmic lacZ driven by GAL4 control in glia. Moreover, in gcm mutants, in which glial fate is transformed to a neuronal one (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996), there is a reduction in robo expression during the time window in which robo is normally expressed in glia. Furthermore, ectopic expression of sli can affect glial migration. And finally, expression of robo in glia in robo mutants favours the lateral distribution of glia.

Our data have also shown that the sensitivity of glia to repulsion is transient, consistent with the fact that the expression of robo in glia disappears as it is switched on in axons. From this time on, glia associate with longitudinal axons to maintain their lateral positions. From the moment when glia contact axons, trophic support and cell-cell contact prevail to restrict the position of glia. In fact, interface glia can overlie longitudinal connectives that express sli ectopically, particularly at stages when glia do not normally express robo. Furthermore, some glia with targeted robo expression in robo mutants can still overlie the midline despite expressing robo. Our data show that these additional cellular mechanisms also participate in confining axons to the longitudinal pathways. The involvement of trophic support and cell contact in maintaining the lateral positions of axons and glia is revealed upon interference with neuron-glia interactions. Firstly, in the absence of pioneer neurons, glia die, but if their survival is not compromised, they do not acquire their normal positions. Instead, they become dispersed within the VNC migrating over the midline and to other positions. This means that glia need contact with axons to acquire their normal positions. Secondly, in the absence of either glia or pioneer neurons, longitudinal axons that would not normally cross the midline, now cross the midline despite expressing the repulsion receptor Robo. This midline crossing by follower axons is likely to be due to a combined loss of axonal fasciculation cues, glial contact and trophic support by glia. Thirdly, since midline crossing is enhanced in embryos also lacking programmed cell death, the dependence on neuron-glia contact for survival forces these two cell types to remain associated along the lateral pathways.

Misrouting of axons across the midline despite the expression of robo has also been observed in calmodulin and Son of sevenless mutants (Fritz and VanBerkum, 2000). In this case, calmodulin and Son of Sevenless are required to transduce the Sli signal by Robo. It is conceivable that interfering with neuron-glia interactions similarly alters the response of cells to Robo signalling. However, this is unlikely to explain our cases of midline misrouting, since we eliminated cells, and thus interfered with cell-cell communication, but molecular functions were not directly altered.

**Temporal sequence of robo expression**

robo is initially expressed in the growth cones of pCC and in other pioneer axons, and subsequently in all contralateral longitudinal axons (Kidd et al., 1998a). We found that prior to its expression in axons, robo expression is dynamic. robo is initially expressed in broad transverse and longitudinal domains (these longitudinal domains demarcate the future positions of the longitudinal pathways); subsequently the transverse domains vanish and the longitudinal domains become more restricted and include glia. robo is then further restricted to one cell cluster per hemisegment, and finally robo expression disappears from these clusters and becomes apparent in axons. Thus, robo expression is switched on in a strict temporal and spatial manner prior to its expression in axons.

Presumably robo expressing glia receive the repulsive signal Sli emanating from the midline, thus halting their migration at
a certain distance from the midline. We can also detect Sli expression in the longitudinal domains of robo expression from the time when glia reach these positions and pioneer axons project longitudinally. Presumably this detection of Sli is a protein diffused from the midline and bound by robo-expressing cells within the lateral domains (Kidd et al., 1999).

**Axons and glia seek to establish normal or alternative contacts**

Several pieces of evidence suggest that cell-cell contact – presumably in the form of adhesion – plays a major role in the formation of longitudinal pathways. There is evidence for contact between axons, in the form of fasciculation; contact between axons and glia, and glia-glia contact.

Axons fasciculate selectively with different pioneer fascicles (Bastiani et al., 1984; Hidalgo and Brand, 1997; Raper et al., 1983; Raper et al., 1984). Therefore, the need to maintain fasciculation is likely to contribute to abnormal midline crossing under our experimental conditions. In fact, since follower axons normally fasciculate with pioneer axons, when the pioneer neurons are eliminated, follower axons are likely to cross the midline in search of alternative axonal as well as glial contact.

Interface glia at either side of the midline are in physical contact through their cytoplasmic projections, which could facilitate midline crossing of both axons and longitudinal glia. For instance, in the absence of pioneer axons, follower axons may follow these glial projections across the midline. When lateral neuron-glial interactions are disturbed both axons and glia migrate to locations where they can re-establish axon-glia contact. For instance, glial ablation causes both axons and remaining glia to associate over the commissures (Hidalgo et al., 1995). In the case of gcm mutants, it is conceivable that some of the misrouting we have found in fact correspond to the normal trajectories of the transformed neurons. In fact, in gcm mutants, the transformed neurons have unique and stereotypic projections, which cross the midline (Jones et al., 1995). However, since in gcm mutants there are no functional glia, we do not know if the projections of the transformed neurons would have still crossed the midline in the presence of interface glia. We have provided two pieces of evidence indicating that in gcm mutants there is ectopic misrouting across the midline. Firstly, midline misrouting, visualised with fasII and anti-Robo, were found primarily in later embryogenesis (from stage 15), indicating problems relating to the midline in the maintenance rather than the establishment of axonal trajectories. Secondly, in gcm rpr double mutant embryos there are virtually no longitudinal axons expressing either fasII or Robo, and axons project mostly across the midline.

Embryos lacking programmed cell death have an almost normally patterned VNC, but when axon-glial interactions are disturbed in these embryos axons project by default towards the midline. This implies that axon-glial interactions are necessary to maintain axons longitudinally, and they are sufficient if cell survival is not compromised. Several molecules are known to be involved in cell adhesion and are expressed in the longitudinal pathways of the CNS, for instance Neuroglian (Bieber et al., 1989), Neurotactin (Speicher et al., 1998), FasII (Lin et al., 1994) and Connectin (Gould and White, 1992). Mutations in these molecules cause fasciculation defects. However, it is not known whether these molecules are involved only in axonal fasciculation or also in axon-glia interactions.

**Trophic support is a means of restricting cell movement**

Cells in animals are programmed to die unless they receive input from their neighbours (Raff et al., 1993). In the nervous system, target cells provide trophic factors to extending axons, thus ensuring correct innervation. In the *Drosophila* CNS, trophic support between neurons and glia plays an instructive role during the formation of longitudinal pathways. We have provided further evidence by showing that glia numbers are depleted upon neuronal ablation, and that they can be rescued by blocking programmed cell death. Longitudinal glia normally undergo apoptosis at the time when they first come into axonal contact (A. H., E. K. and M. Georgiou, unpublished data). Pioneer neurons do not require longitudinal glia for survival, but they require glia for pathfinding (Booth et al., 2000; Hidalgo and Booth, 2000). Thus by regulating glia survival, the pioneer neurons anchor longitudinal glia to their axons to enable their pathfinding. Subsequently, longitudinal glia maintain the survival of follower neurons, thus aiding the maintenance of the axonal fascicles in lateral positions (Booth et al., 2000). Altogether these data show that survival pressure is instructive in determining the positions of glia and axons during pathfinding.

We have provided further evidence here in support of this notion. Firstly, in embryos lacking programmed cell death, some axons project across the midline. Secondly, when neuron-glial interactions are disturbed in embryos lacking programmed cell death, axons and glia dramatically cross over the midline. These are more severe misroutings than if only neuron-glial interactions are disturbed. This reveals the roles of axon-glial interactions in keeping both axons and glia laterally, and it also shows that combining lack of programmed cell death with other genotypes does not lead to additive but synergistic phenotypes. This means that cells respond differently if their survival needs are removed. Interestingly, blocking programmed cell death has been used as a means of unravelling functions of neurotrophins other than in survival (Patel et al., 2000). Our observations, however, imply that preventing cell death does not recreate a normal although death-free environment, but instead generates a novel one in which cells are subject to different kinds of pressures. In the normal *Drosophila* embryo, the pressure for cell contact to survive keeps axons and glia away from the default midline pathway, and along lateral positions.

**Sequential model of lateral confinement of axons and glia**

We present a model for longitudinal pathway formation that integrates the response to repulsive signalling and interactions between axons and glia at lateral positions (Fig. 10). There are two key features. (1) Temporal sequence: robo is expressed in glia transiently to confine their migration, and it disappears from glia when they become dependent on axons for survival. (2) Balance of forces: subsequently robo is expressed in axons confining them to extend parallel to the midline (Kidd et al., 1998a; Kidd et al., 1998b), whereas axon-glia interactions drive the need to establish cell contact and maintain survival of both cell types within lateral positions (Booth et al., 2000) (A. H., E. K. and M. Georgiou, unpublished data). If pioneer neurons or glia are eliminated, follower axons and glia will cross the midline despite midline repellent signalling.

Initially, robo is expressed in longitudinal glia (this work)
and subsequently in pioneer axons (Kidd et al., 1998a), confining both glia and pioneer axons to a fixed distance from the midline. This may depend solely on midline-derived SlI (Kidd et al., 1999). Expression of robo in the pCC growth cone (Kidd et al., 1998a) alone, independent of interactions with glia, confines its extension parallel to the midline. In fact, whereas other pioneer axons are affected by the absence of glia, pCC can extend normally in the absence of glia (Hidalgo and Booth, 2000) but it cannot in the absence of robo (Kidd et al., 1998a). Hence, pCC defines the initial trajectory of longitudinal fascicles. As robo is switched on in the pioneer axons, it is switched off in glia. From this time on, glia depend on pioneer axons for their survival (see also A. H., E. K. and M. Georgiou, unpublished data). Hence, trophic dependence on neurons anchors the longitudinal glia to the pioneer axons during growth cone guidance. Subsequently, axon–glia contact and the mutual dependence of follower neurons and glia for trophic support (Booth et al., 2000) keeps both axons and glia along the lateral pathways. If the normal interactions between axons and glia are disrupted, the need for cell-cell contact forces axons and glia to establish alternative contacts. The requirement to establish contacts that maintain survival predominates and axons can cross the midline despite normal repellent signalling. Thus, trophic support is instructive to keep both axons and glia in their lateral positions.

We thank G. Booth and M. Landgraf for discussions and comments on the manuscript; P. Badenhurst, A. Brand, J. Castelli-Gair, C. Goodman, K. Hosono, M. Landgraf, C. Mirth, J. Roote, N. Sanchez-Soriano and A. Travers for antibodies and flies. This work was supported by a Wellcome Trust Fellowship to A. H. and a MRC Fellowship to G. T. T. B. held a BBSRC studentship.

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