Hedgehog regulated Slit expression determines commissure and glial cell position in the zebrafish forebrain

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Summary

Three major axon pathways cross the midline of the vertebrate forebrain early in embryonic development: the postoptic commissure (POC), the anterior commissure (AC) and the optic nerve. We show that a small population of Gfap+ astroglia spans the midline of the zebrafish forebrain in the position of, and prior to, commissural and retinal axon crossing. These glial 'bridges' form in regions devoid of the guidance molecules *slit2* and *slit3*, although a subset of these glial cells express *slit1a*. We show that Hh signaling is required for commissure formation, glial bridge formation, and the restricted expression of the guidance molecules *slit1a*, *slit2*, *slit3* and *sema3d*, but that Hh does not appear to play a direct role in commissural and retinal axon guidance. Reducing Slit2 and/or Slit3 function expanded the glial bridges and caused defasciculation of the POC, consistent with a 'channeling' role for these repellent molecules. By contrast, reducing Slit1a function led to reduced midline axon crossing, suggesting a distinct role for Slit1a in midline axon guidance. Blocking Slit2 and Slit3, but not Slit1a, function in the Hh pathway mutant *yot* (*gli2DR*) dramatically rescued POC axon crossing and glial bridge formation at the midline, indicating that expanded Slit2 and Slit3 repellent function is largely responsible for the lack of midline crossing in these mutants. This analysis shows that Hh signaling helps to pattern the expression of Slit guidance molecules that then help to regulate glial cell position and axon guidance across the midline of the forebrain.

Key words: Zebrafish, Cyclopamine, Morpholino, Axon guidance, POC, Chiasm, Gfap, Glia, *hedgehog*, *slit*, *you-too*

Introduction

In bilateral organisms, some axons cross the midline of the CNS to form commissures that functionally connect the two sides of the nervous system. Axonal growth cones navigate towards, across, and then away from the midline in response to precise signals present along the growth substrate, including intermediate cellular and molecular guidance cues (reviewed by Goodman, 1996; Grunwald and Klein, 2002; Kaprielian et al., 2001; Steward, 2002). A variety of secreted molecules, including Netrins and Sonic Hedgehog, function to attract axonal growth cones towards the midline of the spinal cord, whereas other guidance cues, including Slits, Semaphorins and Ephrins, typically function to repel growth cones. In the *Drosophila* nerve cord, the midline repellent Slit plays a major role in establishing which commissural axons cross the midline and how far from the midline non-crossing axons are positioned (Battye et al., 1999; Harris and Holt, 1999; Kidd et al., 1999; Simpson et al., 2000a; Simpson et al., 2000b). Slit repulsion is mediated by Roundabout (Robo) receptors expressed in growth cones (Keleman et al., 2002).

In the vertebrate forebrain, Slit-Robo-mediated repulsion is crucial for the appropriate dorsoventral position of the optic chiasm (Rasband et al., 2003). In mice lacking both Slit1 and Slit2 function, retinal axons cross the midline in multiple locations around the true chiasm (Plump et al., 2002). Similarly, zebrafish lacking *astray* (*robo2*) function show aberrant retinal axon growth across the midline (Hutson and Chien, 2002; Karlstrom et al., 1996). Analyses of these two phenotypes led to the proposal that gradients of Slit expression descending toward the optic chiasm channel Robo-expressing axons within the appropriate pathway (Hutson and Chien, 2002; Plump et al., 2002; Rasband et al., 2003; Richards, 2002b). Slit1 and Slit2 have also been shown to channel callosal axons across the cortical midline in the mouse forebrain (Bagri et al., 2002; Shu et al., 2003d). It is not known whether the Robo/Slit system also influences formation of the first forebrain commissures in vertebrates, the postoptic commissure (POC) and the anterior commissure (AC). However, the fact that commissures form in *astray* (*robo2*) mutants indicates that Robo2 alone is not necessary for POC and AC midline crossing (Karlstrom et al., 1996).

The cellular growth substrate for retinal axons as they cross the midline consists of neurons, neuroepithelial cells and glia (Marcus and Easter, 1995; Mason and Sretavan, 1997). CD44/SSEA1-positive neurons border the optic nerve in and across the mammalian midline, and may provide a barrier that directs RGC axons to cross the midline in the correct position (Jeffery, 2001; Marcus et al., 1995; Marcus and Mason, 1995; Mason and Sretavan, 1997). Other identifiable midline cells

include a radial glial 'palisade' (Misson et al., 1988) that helps to guide ipsilaterally projecting retinal axons (Mason and Sretavan, 1997). More dorsally, midline glial populations that make up the 'glial sling', 'glial wedge', and indusium griseum have all been implicated in midline crossing in the corpus callosum (Richards, 2002a; Shu et al., 2003a; Shu et al., 2003b; Shu et al., 2003c; Shu and Richards, 2001; Silver et al., 1982; Silver and Ogawa, 1983). Little is known about how midline cells influence the formation of the forebrain commissures.

The zebrafish forebrain provides an easily accessible system for the study of chiasm and commissure formation. Prior to chiasm formation, the POC forms in the diencephalon, while the AC forms more anterodorsally in the telencephalon (Bak and Fraser, 2003; Wilson et al., 1990). RGC axons grow across the ventral midline in close proximity to the POC (Wilson et al., 1990). Pioneering growth cones of the POC and optic nerve grow superficially (Wilson and Easter, 1991; Wilson et al., 1990), and their growth substrate primarily consists of poorly-characterized neuroepithelial cells and basal lamina (Burrill and Easter, 1995; Wilson and Easter, 1991; Wilson et al., 1990). It is currently unknown whether these neuroepithelial cells are neural and/or glial precursor cells, neurons or glia (Burrill and Easter, 1995), although some express Glial Fibrillary Acidic Protein (Gfap) and have a radial glial morphology, and so appear to be astroglia (Marcus and Easter, 1995). These Gfap+ cells come in direct contact with POC and optic axons at the midline at 48 hours post-fertilization (Marcus and Easter, 1995).

Several achiasmatic zebrafish mutants have been identified in which forebrain axons fail to cross the midline and most RGC axons project ipsilaterally (Karlstrom et al., 1997; Karlstrom et al., 1996). Many of these ipsilateral/midline mutants affect Hedgehog (Hh)-mediated midline patterning, with *detour* (*dtr*) and *you-too* (*yot*) encoding the Hh-responsive transcription factors Gli1 and Gli2, respectively (Karlstrom et al., 1999; Karlstrom et al., 2003). Complex regulation and processing of the vertebrate *gli* genes results in either repression or activation of Hh target gene expression (Koebernick and Pieler, 2002), and both *dtr(gli1)* and *yot(gli2)* mutations block the ability of cells to activate Hh signaling (Karlstrom et al., 2003). The cellular and molecular basis for the axon guidance defects in the Hh/midline mutants is currently unknown. Besides playing an important role in directing cell differentiation in the forebrain (Nybakken and Perrimon, 2002), Hh proteins directly attract commissural axons to the midline in the mouse spinal cord (Charron et al., 2003; Ogden et al., 2004) and may repel RGC axons in the chick forebrain (Trousse et al., 2001). It is thus possible that the midline guidance errors in Hh pathway mutants are due to a loss of direct Hh-mediated axon guidance. Alternatively, they may be indirectly caused by cell specification defects in the forebrain (Karlstrom et al., 2003; Sbrogna et al., 2003).

Although much has been learned about midline guidance in the forebrain, many fundamental questions remain. First, what is the molecular and cellular architecture of the midline growth substrate that leads to proper commissure and then chiasm formation? Second, how do Slits influence the first midline-crossing axons in the forebrain, those of the POC and AC? Finally, given the loss of midline crossing in Hh pathway mutants, does Hh directly or indirectly affect axon guidance in the forebrain? We show that Gfap+ cells express *slit1a* and form a 'bridge' across the midline prior to commissure and chiasm formation, and that Slit molecules help to establish the POC. We show that Hh is not needed directly to guide commissural and retinal axons near the midline, but instead is necessary to establish the proper patterning of midline glia and the patterned expression of *slit* guidance cues. We propose a model in which *slit1a* expressed by a glial bridge provides a substrate for axon crossing at the midline, whereas *slit2* and *slit3* repulsion helps to channel growth cones into commissures.

Materials and methods

Wild-type and mutant fish lines

Fish lines were maintained in the University of Massachusetts-Amherst Zebrafish Facility. Wild-type embryos were from the TL line; the *you-too* (yot^{ty119}) and *detour* (dtr^{ts269}) lines were maintained in TL and Tü backgrounds. The *you-too* (yot^{ty119}) allele encodes a truncated Gli2 protein that acts as a dominant repressor (Gli2DR) to block Gli-mediated activation, while dtr^{ts269} is a *gli1* loss-of-function mutation (Karlstrom et al., 2003). Embryos were grown at 28.5°C and staged in hours post-fertilization (hpf) (Kimmel et al., 1995), dechorionated with 0.2 mg/ml pronase (Sigma). 0.003% 1-phenyl-2-thiourea (PTU) was added starting at 23 hpf to reduce pigmentation (Westerfield, 1993). No differences were seen between PTU-treated and untreated embryos in overall growth or assayed phenotypes.

Antibodies

Anti-acetylated tubulin (IgG2b monoclonal, Sigma) was used at a dilution of 1:800 to label axons (Wilson et al., 1990). A polyclonal anti-goldfish-Gfap antibody (generous gift of Sam Nona, University of Manchester, UK) (Nona et al., 1989) was used at 1:400 to label astroglial cells (Marcus and Easter, 1995). F59 (IgG1 monoclonal, Developmental Studies Hybridoma Bank) is specific for slow muscle myosins in chicken (Crow and Stockdale, 1986), and diluted at 1:10 preferentially labels slow muscle fibers in zebrafish (Devoto et al., 1996). Secondary antibodies from Jackson ImmunoResearch Laboratories were diluted as follows: FITC goat anti-mouse IgG, 1:200; Cy5 goat anti-mouse IgG, 1:200; TRITC goat anti-Rabbit, 1:500; FITC goat anti-Rabbit, 1:200.

Immunocytochemistry and in situ hybridization

Embryos were fixed in 4% formaldehyde (Ted Pella) for 2 hours at room temperature and antibody-labeled, as previously described (Karlstrom et al., 1999) with several modifications. Briefly, embryos were dehydrated, incubated in 100% acetone for 7 minutes at –20°C, rehydrated in PBS+0.2% Triton X-100 (PBS-Tx), digested with 10 μg/ml Proteinase K for 3-5 minutes depending on age, washed for 3×5 minutes in PBS-Tx, and incubated in blocking solution (PBS-Tx-2%BSA-5%NGS-1%DMSO) for 1 hour at room temperature. All primary and secondary antibodies were diluted in blocking solution and applied for 2 hours at room temperature.

Digoxigenin-labeled anti-sense mRNA probes were synthesized using SP6/T7/T3 Dig RNA Labeling Kits (Roche). The probes used were *pax2a* (Krauss et al., 1991), *shh* (Krauss et al., 1993), *ptc1* (Concordet et al., 1996), *sema3d* (Halloran et al., 1998), *slit1a* (Hutson et al., 2003), *slit2* and *slit3* (Yeo et al., 2001), *robo1*, *robo2* and *robo3* (Lee et al., 2001), and *robo4* (Park et al., 2003). A standard in situ hybridization protocol (Jowett, 1997) was modified to enable multi-antibody labeling following mRNA probe hybridization. Briefly, embryos were fixed with 4% formaldehyde (Ted Pella) for 2 hours at room temperature. Anti-acetylated tubulin and anti-Gfap primary antibodies were added with anti-DIG antibodies. Following a Fast Red (Roche) color reaction, embryos were washed for 2×15 minutes with PBS-0.1% Tween20, and then for 3×15 minutes with

PBS-Tx, blocked, and incubated in secondary antibodies. Embryos were never post-fixed.

Embryos were cleared in 75% glycerol and examined using a Zeiss LSM510 laser-scanning confocal microscope at a magnification of 40× or 63×. For lateral views of the forebrain, eyes were removed. Three-dimensional stacks were acquired using multitracking for double- and triple-labeled embryos to ensure sequential acquisition and preclude signal crossover. Stacks ranged from 20 μm to 50 μm in Z-distance with constant pinhole settings of 105.7 μm (~1.0 Airy unit) for all channels and an (optimal) interval of 0.4 μm. Images are maximum intensity projections unless otherwise noted. For consistency, throughout this paper we use 'anterior' to mean closer to the telencephalon, and 'posterior' for closer to the diencephalon, despite changes in orientation due to flexure of the nervous system.

Cyclopamine treatment

At several different time points, wild-type embryos were treated with 100 μM or 200 μM Cyclopamine (Toronto Chemical) in embryo medium + 0.5% EtOH (Incardona et al., 1998) at 28.5°C. No effect on the assayed phenotypes was seen in control embryos incubated in embryo medium + 0.5% EtOH. Thirty embryos were treated in 2 ml volumes in 12-well plates.

Morpholino and mRNA microinjection

Translation-blocking (Nasevicius and Ekker, 2000) zebrafish *slit1a* (GACAA CATCC TCCTC TCGCA GGCAT), *slit2* (CATCA CCGCT GTTTC CTCAA GTTCT) and *slit3* (TATAT CCTCT GAGGC TGATA GCAGC) morpholinos (MOs, GeneTools) were kept as 10 mg/ml stocks in Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM Hepes, Phenol Red) and diluted in Danieau's solution. Injections of 400-500 pl were made into the yolk of one- to four-cell stage embryos obtained from wild-type crosses or *yot*^{ty119} *(gli2DR)* heterozygous intercrosses. *slit1a* MOs were injected at 1 ng, *slit2* or *slit3* at 2-4 ng, with no phenotypic differences seen between these doses of *slit2* or *slit3* MOs. Co-injections of *slit2* and *slit3* MOs used 2.5 ng of each. Injected embryos were fixed at 28 hpf for triple antibody labeling of axons, glial cells and slow muscle fibers. Overexpression of Slit genes can cause convergent extension defects (Yeo et al., 2001), as can high doses of *slit1a*, *slit2* or *slit3* MOs (L.D.H. and C.-B.C., unpublished). To rule out the possibility that defects in axon guidance were due to indirect morphogenetic effects, each injected embryo was stained with F59, and only those that displayed normal body shape, somite formation and patterning of the superficial slow-muscle layer were used to score axon and glial defects. Homozygous *yot (gli2DR)* mutant embryos were identified by the loss of embryonic slow muscle fibers at 28 hpf (Stickney et al., 2000). Zebrafish *shh* was transcribed from the T7TS*shh* plasmid (Ekker et al., 1995) and microinjected as previously described (Barresi et al., 2000).

Results

Forebrain commissural and retinal axons grow along a midline-spanning glial bridge

To better characterize the cellular growth substrate for commissural axons, we examined the relationship between Gfap+ cells, gene expression borders, and commissural and retinal axons in the forebrain (Figs 1 and 2). In the regions of the AC and POC, Gfap+ cells span the midline superficial to neuroepithelial cells and send radial fibers into the forebrain (Fig. 1B, see also Movies 1, 2 and 3 in the supplementary material) with AC and POC axons arranged perpendicular to these fibers. To orient these cells in relation to the optic stalk and diencephalic midline, we examined Gfap+ cells in relation to *pax2a* (Macdonald et al., 1997) and *shh* (Ekker et al., 1995; Krauss et al., 1993) expression. The anteriormost Gfap+ cells in the diencephalon are adjacent to, but do not overlap with, *pax2a*-expressing cells of the optic stalk (Fig. 1C,D). These Gfap+ cells lie superficial to neuroepithelial cells that express *sonic hedgehog* (*shh*; Fig. 1E,F). The astroglial fibers that stretch into the brain appear to overlap with *shh*-expressing cells at the midline from 15 hpf to 27 hpf of development; however, by 36 hpf the Gfap+ fibers no longer overlap with the zone of *shh* expression (Fig. 1E,F, data not shown).

Previous studies reported that Gfap+ cells appear in the

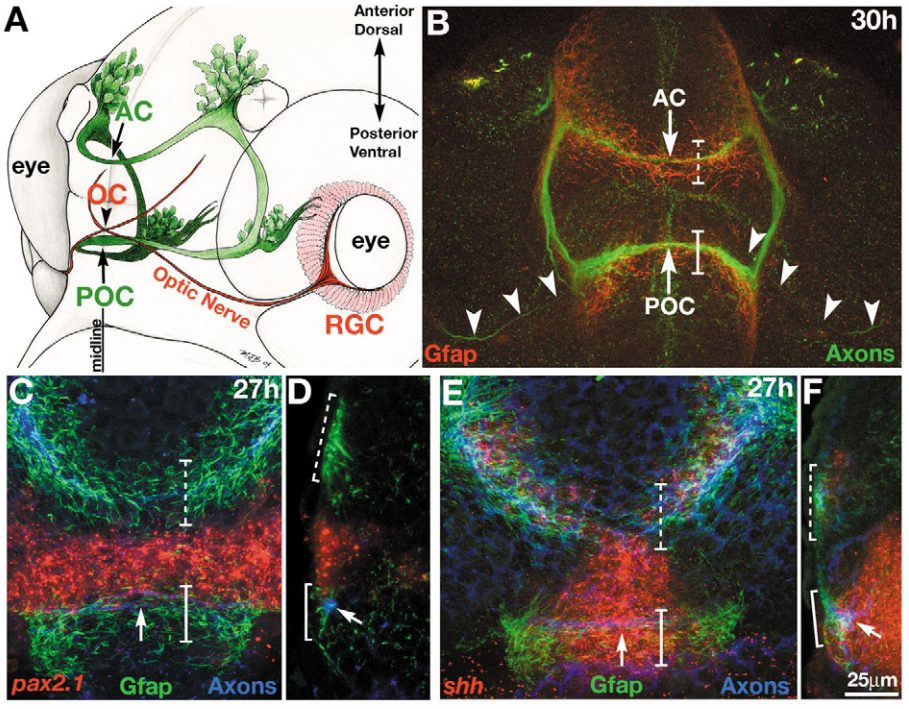

Fig. 1. Gfap-expressing cells in the commissure regions of the zebrafish forebrain. (A) Diagram of forebrain tracts and commissures. Commissural axons (green) cross the midline to form the anterior commissure (AC) and the postoptic commissure (POC). Retinal ganglion cells (RGCs) from the eye form the optic nerve and optic chiasm (OC). (B) Localization of RGC (arrowheads), AC and POC axons [immunolabeled with anti-acetylated tubulin (AT); green] in relation to Gfap+ cells (anti-Gfap; red) at 30 hpf. Gfap+ cells span the midline in the region of the AC (dashed bracket) and POC (solid brackets). (C,D) *pax2a* in situ labeling (red) of the optic stalk region in relation to axons (blue, arrows) and Gfap+ cells (green, brackets) at 27 hpf. (E,F) *shh* in situ labeling (red) in relation to axons (blue, arrows) and Gfap+ cells (green, brackets). (B,C,E) Frontal views of the forebrain, anterior/dorsal up; (D,F) lateral views, anterior left, anterior/dorsal up. Because brain flexure brings dorsal structures into an anterior position, 'anterior' and 'posterior' will be used in subsequent figures, to reflect the eventual positions of these two commissures.

midline region only after forebrain commissures have formed, suggesting that astroglia probably do not guide axons across the midline (Marcus and Easter, 1995). However, our confocal analysis indicates that Gfap+ cells span the midline of the forebrain at 15 hpf, well before the appearance of any axons in the brain (Fig. 2) (Wilson et al., 1990). Gfap+ cells span the diencephalic midline in the future POC region (Fig. 2A), and by 18 hpf Gfap+ cells span the midline where both the POC and AC will form (Fig. 2B). As development proceeds, Gfap+ cells become increasingly restricted to the locations of the AC and POC (Fig. 2C, bracket; Fig. 2D-F). The first POC axons begin crossing the midline at approximately 23-24 hpf (Bak and Fraser, 2003) in association with Gfap+ cells (Fig. 2C, arrow). RGC axons (Fig. 2E,F, arrowheads) then grow towards the midline close to, but not in direct contact with, POC axons (Fig. 2E,F, bottom arrow) (Burrill and Easter, 1995). A small cluster of Gfap+ cells extends anteriorly from the glial bridge directly in the position where RGC axons cross the midline (Fig. 2F, arrowhead).

Hedgehog signaling does not directly affect axon guidance, but does pattern midline glia

The expression of *shh* adjacent to the POC and optic chiasm (Fig. 1D), combined with the loss of midline axon crossing in Hh mutants (reviewed by Russell, 2003) and the recent findings that Shh can directly guide midline crossing axons in mouse (Charron et al., 2003) and chick (Trousse et al., 2001), suggest that Shh may directly guide POC and/or RGC axons near the midline of the forebrain. To test this, we used cyclopamine (CyA) to pharmacologically block Smoothened-mediated Hedgehog signaling starting just prior to the times when POC (22 hpf) or RGC (27 hpf) axons grow toward the midline (Fig. 3A). In embryos treated with 100 μM CyA from 27 hpf to 36 hpf, RGC axons crossed the midline normally, forming a wild-type chiasm (Fig. 3E,F). Similarly, the POC and optic chiasm were completely formed in embryos treated with CyA from 22 hpf to 36 hpf (Fig. 3D,F). To verify that CyA was completely blocking Hh signaling at these ages, we examined late-onset expression of the Hh receptor and the Hh target gene *patched1* (*ptc1*) in the fin bud, which normally begins at 32 hours of development (Concordet et al., 1996; Lewis et al., 1999). Treatment with 100 μM CyA from 27 hpf to 36 hpf led to a complete loss or major reduction of *ptc1* expression in the fin bud (data not shown), whereas 200 μM CyA treatment eliminated all *ptc1* expression in the fin bud, indicating a complete block of Hh signaling (Fig. 3B-F; upper insets)

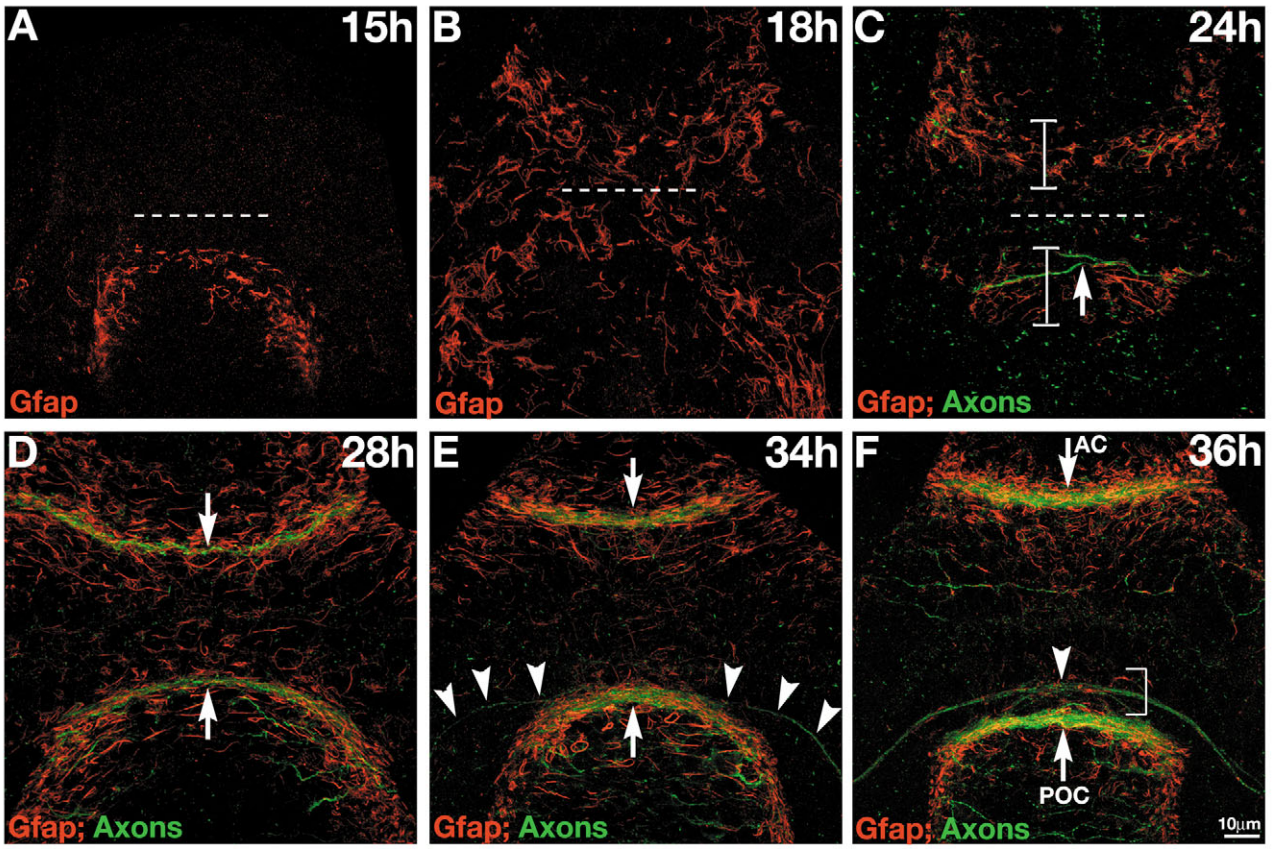

Fig. 2. Gfap+ cells span the midline prior to formation of forebrain commissures. (A-F) Double labeling for axons (anti-AT; green) and Gfap+ cells (red). (A) Gfap+ cells span the midline posterior to the optic recess (dashed line) at the presumptive POC at 15 hpf. (B) By 18 hpf, Gfap+ cells span the midline, anterior and posterior to the optic recess (dashed line), at the presumptive AC and POC. (C) By 24 hpf, more distinct glial bridges (brackets) have formed at the presumptive AC and the forming POC (arrow). (D) At 28 hpf both commissures (arrows) have formed in association with the glial bridges. (E) The first RGC axons (arrowheads) grow towards the midline along Gfap+ cells at 34 hpf. (F) The optic chiasm (arrowhead) forms in association with a distinct cluster of Gfap+ fibers just anterior to the POC (bracket). At 15 hpf and 18 hpf, no axons are present in the forebrain (these data) (Wilson et al., 1990), so the green channel was omitted to reduce background. All panels show frontal views of the forebrain. Scale bar: 10 μm.

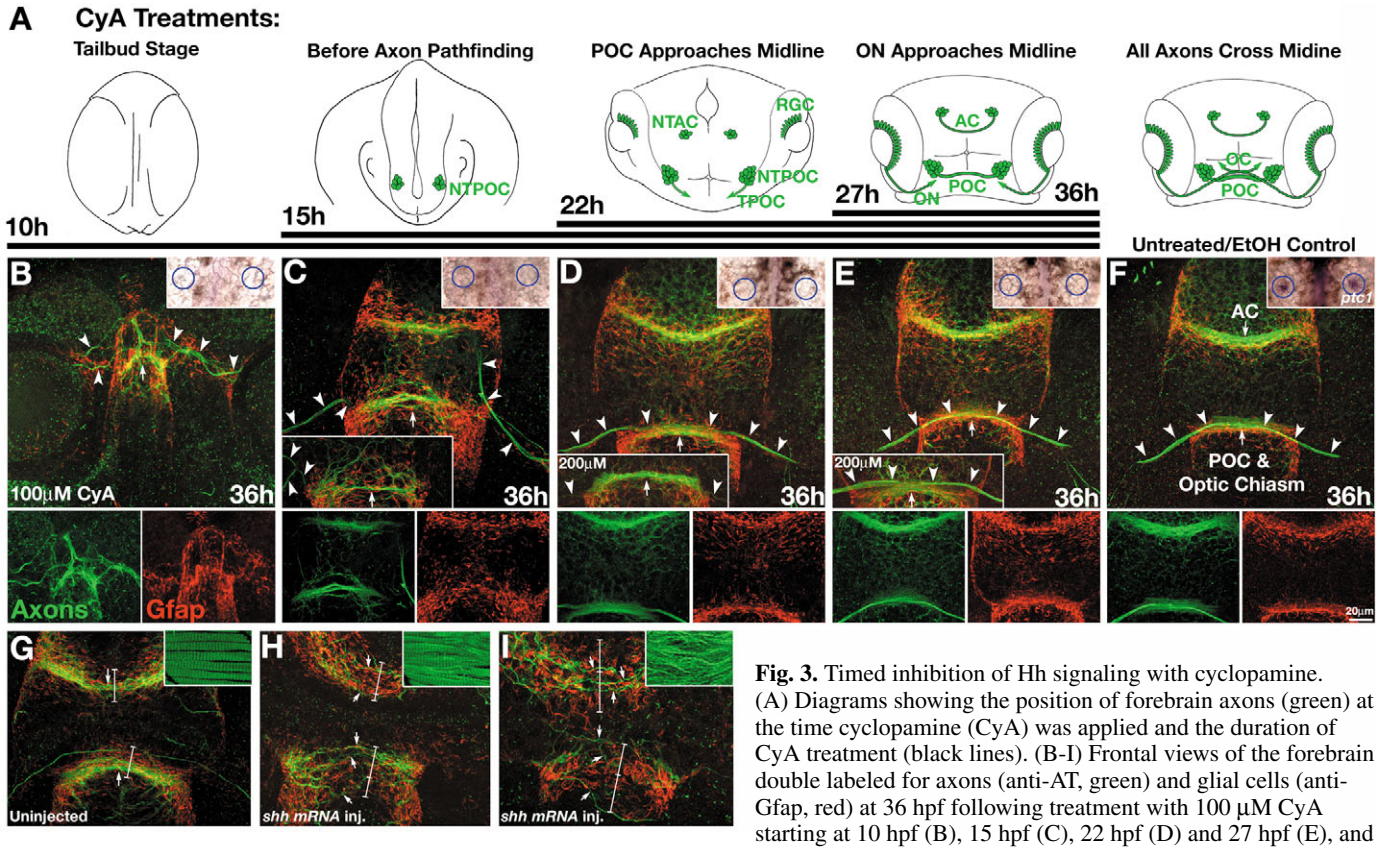

Fig. 3. Timed inhibition of Hh signaling with cyclopamine. (A) Diagrams showing the position of forebrain axons (green) at the time cyclopamine (CyA) was applied and the duration of CyA treatment (black lines). (B-I) Frontal views of the forebrain double labeled for axons (anti-AT, green) and glial cells (anti-Gfap, red) at 36 hpf following treatment with 100 µM CyA starting at 10 hpf (B), 15 hpf (C), 22 hpf (D) and 27 hpf (E), and at 34 hpf in uninjected (G) and *shh* mRNA-injected (H-I) embryos. (F) EtOH (CyA carrier)-treated control embryo. (B-F) Bottom panels show separated red and green channels; upper insets show *ptc1* gene expression in the fin buds (circled). (B) Treatment with 100 µM CyA from 10 hpf to 36 hpf resulted in major disruption of forebrain midline development, complete disorganization of forebrain axons (arrowheads) and glial cells, and complete loss of *ptc1* expression in fin buds (circles, upper right inset). (C) Treatment with 100 µM CyA from 15 hpf to 36 hpf resulted in ipsilateral RGC projections (arrowheads), a reduced (n=13; C, lower inset) or defasciculated (n=16) POC (arrows), disorganized glial cells around the POC, and complete loss of *ptc1* in fin buds (circles, upper right inset). (D,E) AC, POC (arrows) and RGC (arrowheads) axons cross the midline normally in embryos treated with CyA starting at 22 hpf and 27 hpf. CyA treatment (200 µM) gave the same results (lower insets). Again, the limb buds lacked *ptc1* expression (circles, upper right insets). (F) Normal commissure, chiasm and glial bridge formation in EtOH-treated control embryos. Inset shows normal limb bud *ptc1* expression (circles, upper right inset). (G-I) Analysis of axons and astroglial cells following *shh* mRNA injection. (G) Uninjected embryo. (H) Following low levels of Hh activation (approximate doubling of superficial slow muscle fibers; anti-F59, green, inset), the AC was dramatically reduced, the POC was highly defasciculated, RGC axons were absent (green, arrows), and glial bridges were expanded (red, brackets). (I) Following high levels of Hh activation (complete transfating of the entire somite into slow muscle fibers; inset), the AC was similarly reduced, the POC was severely reduced and exhibited wandering axons, RGC axons were absent (green, arrows), and expansion of the glial bridges was more pronounced (red, brackets). NTPOC, Nucleus of the tract of the POC; NTAC, nucleus of the tract of the AC; ON, optic nerve; OC, optic chiasm.

(Neumann et al., 1999). Higher doses of CyA (200 µM) applied between 22-36 hpf also led to a reduction in the number of RGCs, consistent with a role for Hh in RGC specification (Neumann and Nuesslein-Volhard, 2000). However, both POC and RGC axons correctly crossed the midline in these treated embryos (Fig. 3D,E; lower inset).

Because axons fail to cross the midline of the forebrain in Hh signaling pathway mutants (Culverwell and Karlstrom, 2002), we next treated embryos with 100 µM CyA at earlier time points to determine when Hh plays a role in commissure formation. CyA treatment from 10 to 36 hpf resulted in major axon pathfinding errors of all commissural and the retinal axons (Fig. 3B, green) phenocopying the more severe Hedgehog pathway mutants (Culverwell and Karlstrom, 2002; Russell, 2003; Varga et al., 2001). In addition, Gfap+ cells were reduced and highly disorganized (Fig. 3B, red) following these early CyA treatments. Similar to the Hh mutants, these embryos exhibited a loss of midline forebrain tissues and partial cyclopia (Fig. 3B), suggesting that axon and glial cell disruptions could be due to the loss of midline cells. However, embryos treated with CyA from 15 h to 36 h of development showed defects in axon guidance despite the presence of midline tissue (Fig. 3C). In these embryos, POC axons were disorganized and often formed several separate bundles (Fig. 3C, arrow). In some embryos, the POC was extremely reduced and many POC axons wandered into the preoptic area (Fig. 3C; lower left inset). In addition, RGC axons completely failed to cross the midline and grew ipsilaterally (Fig. 3C, green, arrowheads). Importantly, CyA treatment at these same stages disrupted the glial bridge that spans the midline in the region of the POC (Fig. 3C, red).

To better understand the relationship between Hh signaling and formation of the midline glial bridge, we examined *yot (gli2DR)* mutants that have a repressed transcriptional response to Hh without a major loss of midline tissue (Karlstrom et al., 1999). In *yot(gli2)* mutants, Gfap+ cells were reduced at the midline and many cells were aberrantly positioned in the region between the two commissures (Fig. 4). This disorganization of Gfap+ cells was apparent before axons are present (Fig. 4A,D). As seen in the 15-36 hpf CyA-treated embryos, misguided POC axons in *yot* mutants were predominantly associated with mis-positioned Gfap+ cells (Fig. 3C, Fig. 4F). To assess whether ectopic expression of Hh would disrupt axon and glial guidance, we injected Shh-encoding mRNA at the two-cell stage. The POC was reduced and defasciculated following global Shh overexpression (Fig. 3G-I, arrows) and Gfap+ cells were similarly expanded along the anteroposterior axis (Fig. 3G-I, brackets). Together, these results suggest that Hh signaling is required for the proper patterning of glial bridges in the forebrain, and that axon errors seen in Hh pathway mutants may be the indirect effect of disruptions in this axon substrate.

Hh signaling patterns the forebrain expression of *slit1a, slit2, slit3* and *sema3d*

To better understand how Hh signaling is involved in establishing the commissural axon growth substrate, we next examined axon guidance molecule expression in *yot (gli2DR)* mutants. Given the role for Robo/Slit-mediated growth cone repulsion in RGC axon guidance (Hutson and Chien, 2002; Hutson et al., 2003), we first examined the expression of *slit1a* and *slit2/3* during commissure formation (Fig. 5). Consistent with the surround repulsion guidance mechanism seen for retinal axons (Rasband et al., 2003), *slit2* and *slit3* were expressed adjacent to the commissures, with expression being absent from regions where commissural axons and glial bridges cross the midline (Fig. 5A,C). In *yot(gli2)* mutants, *slit2* and *slit3* were expanded, with expression filling the commissural regions normally devoid of *slit2*/*slit3* expression (Fig. 5B,D). In contrast to *slit2*/*slit3*, *slit1a* was expressed in the preoptic area and AC region, with expression overlapping both POC and AC axons (Fig. 5E). A subset of Gfap+ cells in the glial bridge and the majority of the Gfap+ cells in the AC region expressed *slit1a* (Fig. 5G,H, data not shown), although none of these cells expressed either *slit2* or *slit3*. *slit1a* expression was reduced in *yot (gli2)* mutants in the preoptic area (Fig. 5F), unlike the expansion of expression seen for *slit2*/*slit3*.

The secreted guidance molecule *sema3d* (Halloran et al., 1998) is expressed at the midline directly underneath the POC and is thus in a position to guide axons at the midline (Fig. 5G). Similar to *slit1a*, *sema3d* was severely reduced in the diencephalon of *yot (gli2DR)* mutants (Fig. 5H). We also examined expression of *ephrinB2a* and *ephrinB3*, secreted molecules that are expressed near the chiasm and POC, and that are known to play a role in RGC axon guidance (Chan et al., 2001). Surprisingly, despite other midline patterning

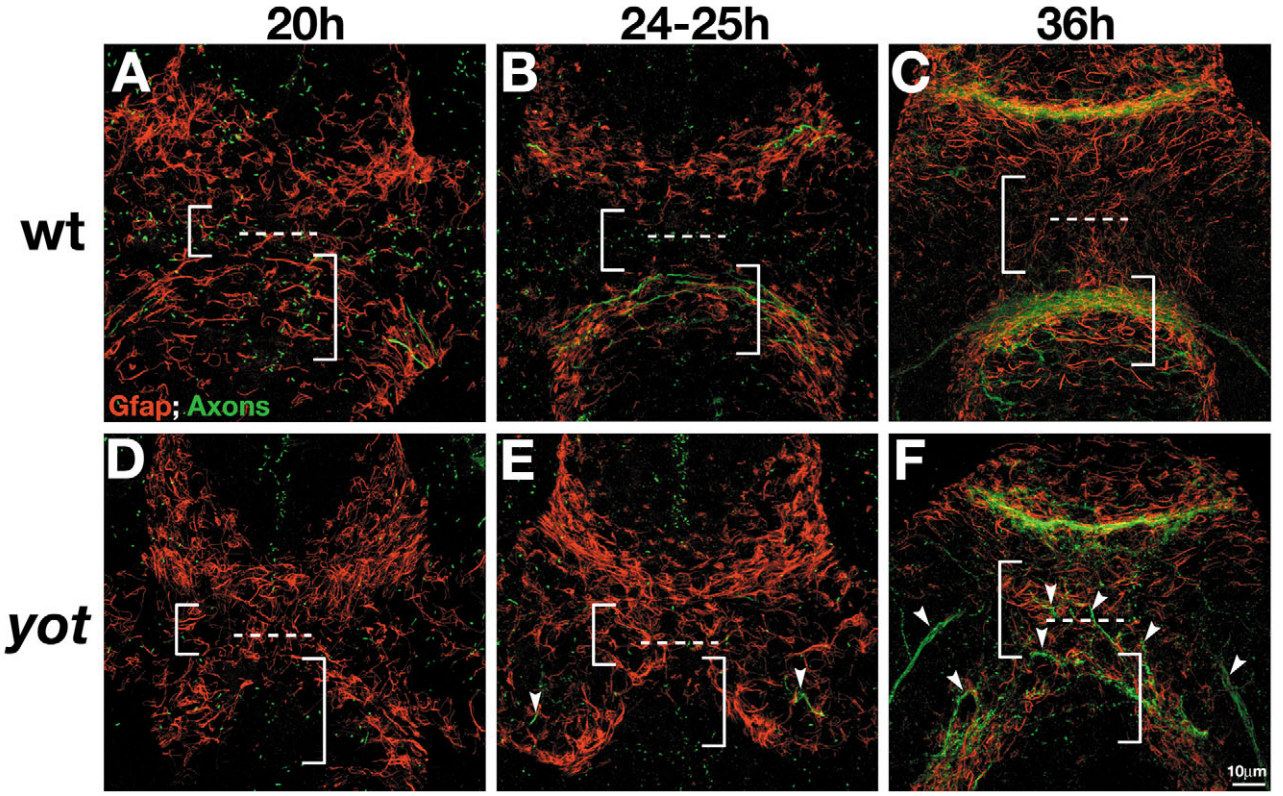

Fig. 4. The post-optic glial bridge is disrupted in *yot (gli2DR)* mutants. Axons (anti-AT; green) and Gfap+ cells (red) at 20 hpf (A,D), 24-25 hpf (B,E), and 36 hpf (C,F) in wild type (WT; A-C) and *yot (gli2DR)* mutants (D-F). (D-F) POC and RGC axons do not cross the midline in *yot (gli2DR)* mutants (E,F, arrowheads) (Karlstrom et al., 1999). Gfap+ cells are reduced at the midline in the region where the POC would normally form (right brackets), and spread into the pre-optic area (left brackets). Frontal views, anterior up, dotted line marks the optic recess.

defects in the region (Karlstrom et al., 1997), expression of *ephrinB2a* and *ephrinB3* was largely unaffected in *yot (gli2DR)* mutant embryos (data not shown).

Slit1a functions distinctly from Slit2 and Slit3 in POC formation

To test whether and how Slit proteins regulate glial bridge and commissure formation, we next used morpholino antisense oligonucleotide (MO) injections to reduce Slit function in wild-type and mutant embryos. Inhibition of *slit2*, *slit3*, and *slit2*/*slit3* together in wild-type embryos resulted in defasciculation of the POC, with many distinct axon bundles crossing throughout the pre- and post-optic areas (Fig. 6A-D,K). To semi-quantify these POC defects, we generated a crossing index of POC formation based on a scale of 1 to 7 (with 7 being an ideal wild-type commissure and 1 being no commissure) for individual embryos injected with different MOs (Fig. 6L). Inhibition of *slit2*, *slit3*, and *slit2*/*slit3* together in wild type embryos significantly disrupted POC formation (Fig. 6L, purple bars). Besides leading to defasciculation, *slit2* or *slit2*/*slit3* MO injections caused some POC axons to wander into inappropriate regions of the forebrain in most embryos (Table 1). *slit3* MO and *slit1a* MO injections did not significantly increase axon wandering (Fig. 6A-D,K; Table 1). In some *slit* MO-injected embryos the AC

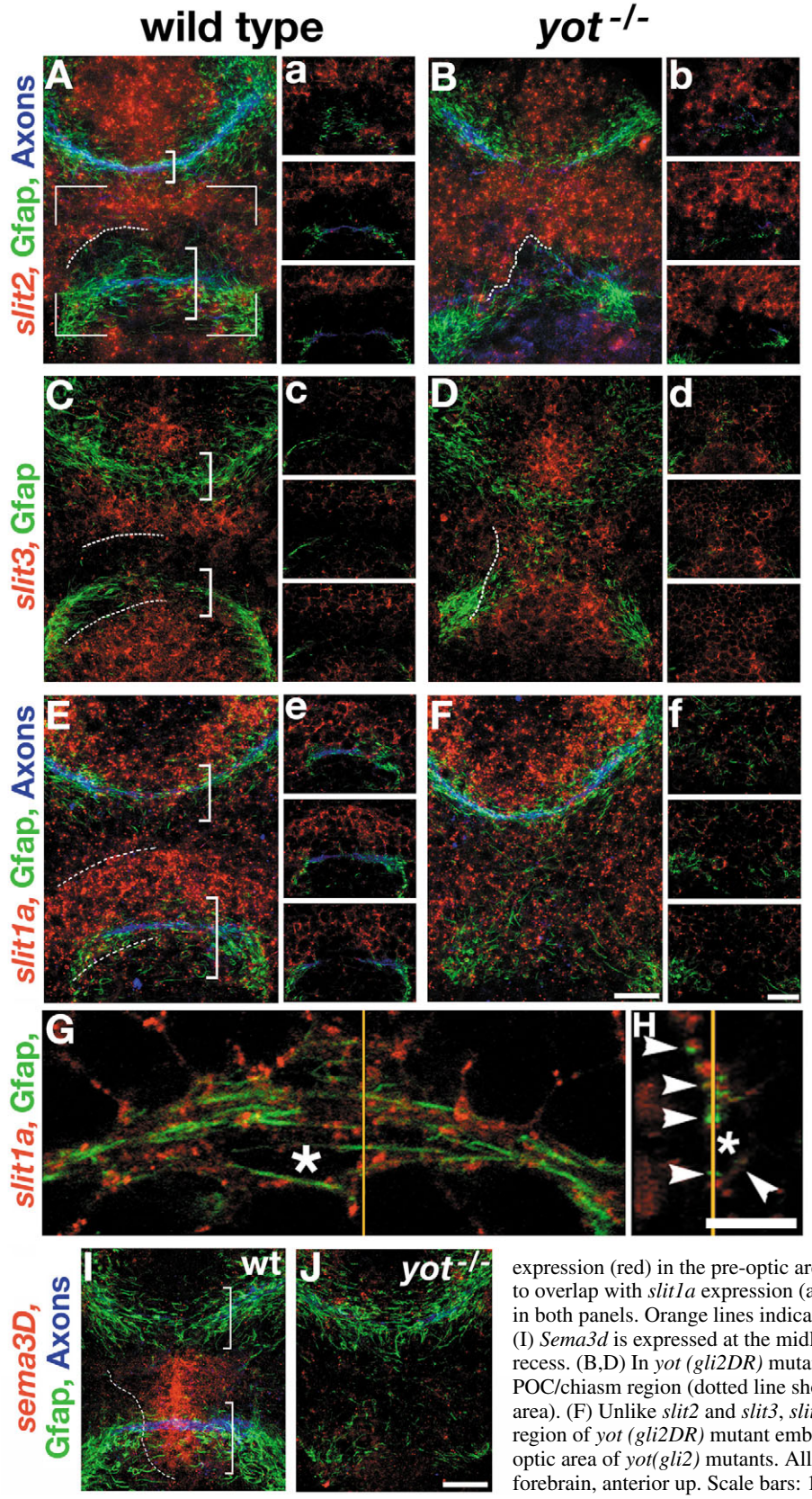

Fig. 5. *slit* and *sema3d* expression in wild type and *yot(gli2)* mutants. (A-J) Triple (A-F,I,J) and double (G,H) labeling shows mRNA expression (red) in relation to axons (anti-AT, blue, not shown in C and D) and glial cells (anti-Gfap, green) in the commissural region of the forebrain at 27 hpf in wild type (A,C,E,G-I) and mutants (B,D,F,J). (a-f) Single confocal slices through the preoptic and POC/chiasm region (boxed in A) from superficial (top) to deep (bottom). (A,C) *slit2* and *slit3* expression is mostly absent from the pre-optic area, the POC/chiasm region (lower bracket), and the AC region (upper bracket) in wild-type embryos. Dotted lines show borders of expression. (E) *Slit1a* is expressed in complementary domains to *slit2*/*slit3* and is present in the pre-optic area (between dotted lines). (G,H) Higher power single 0.56 μm confocal slices of wild-type embryo showing frontal (G) and lateral (H) views of *slit1a* expression (red) in the pre-optic area and the glial bridge cells. Gfap+ fibers appear to overlap with *slit1a* expression (arrowheads). The asterisk marks the same nucleus in both panels. Orange lines indicate the position of cross section in other panels. (I) *Sema3d* is expressed at the midline from the POC (lower bracket) to the optic recess. (B,D) In *yot (gli2DR)* mutants, *slit2* and *slit3* expression is expanded into the POC/chiasm region (dotted line shows the border of expression in the pre-optic area). (F) Unlike *slit2* and *slit3*, *slit1a* expression is reduced in the POC/chiasm region of *yot (gli2DR)* mutant embryos. (J) *sema3d* expression is absent in the pre-optic area of *yot(gli2)* mutants. All panels except H show frontal views of the forebrain, anterior up. Scale bars: 10 μm.

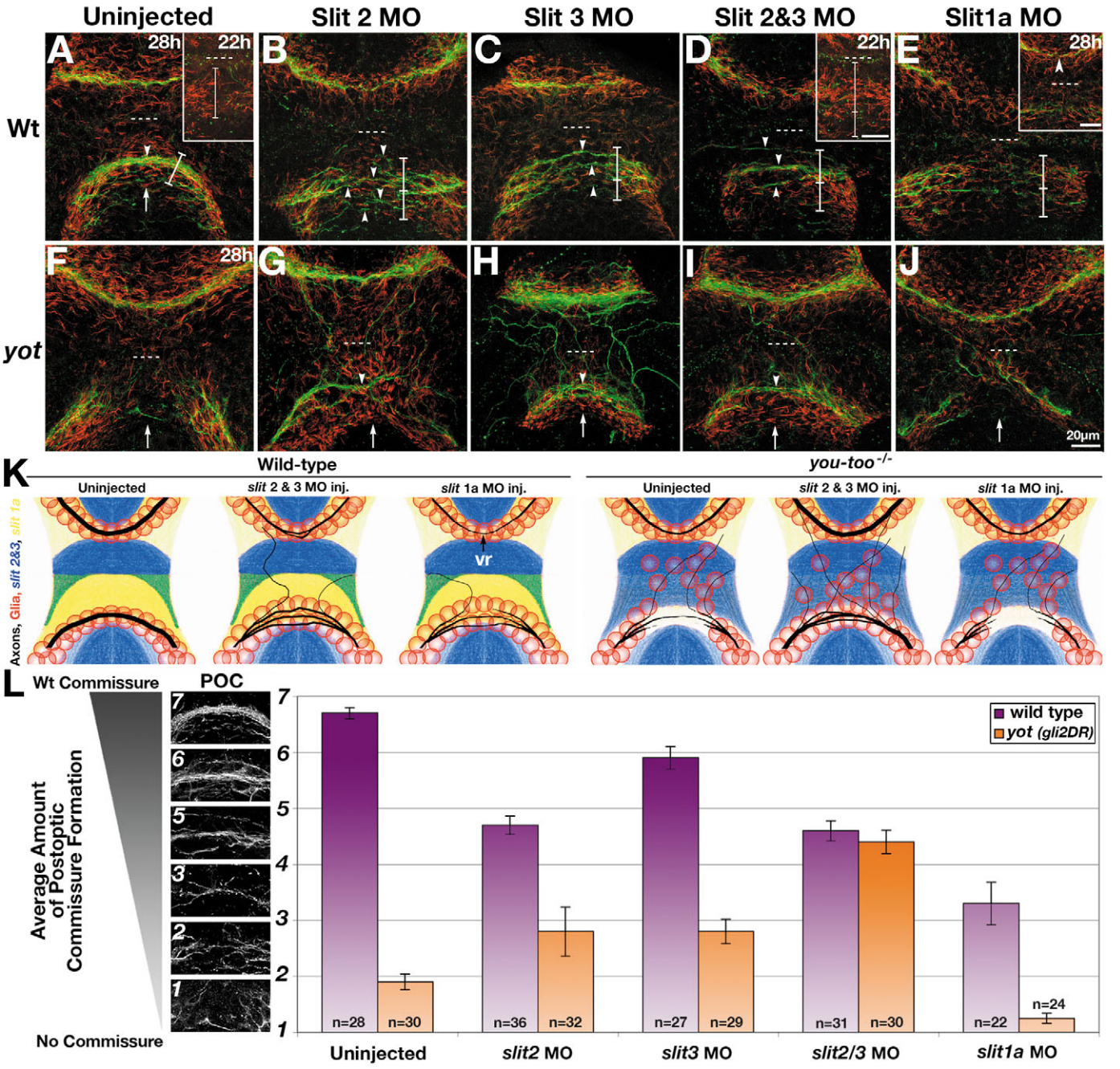

Fig. 6. Morpholino knockdown of *slit2*, *slit3* and *slit1a* in wild type and *yot (gli2DR)* mutants. (A-J) Double labeling showing axons (anti-AT, green) and glial cells (anti-Gfap, red) in the forebrain at 28 hpf. (A) wt uninjected controls at 28 hpf and 22 hpf (inset). (B-D) Injection of MOs to *slit2* (B), *slit3* (C), or *slit2* and *slit3* together (D), into wild type resulted in defasciculation of the POC (arrowheads) and spreading of the glial bridge (brackets, lower division of bracket shows expansion beyond the bridge width seen in uninjected controls). This spreading of the glial bridge was also present prior to the appearance of POC axons (A,D, insets of POC region at 22 hpf, brackets). (E) *slit1a* MO-injected embryo with reduced AC and POC. Inset shows *slit1a* morphant with normal AC (arrowhead) and reduced POC. (F-J) *yot (gli2DR)* mutant embryos. (G-I) Injection of *slit2* MOs (G), *slit3* MOs (H), or *slit2* and *slit3* MOs together (I), into *yot (gli2DR)* mutant embryos resulted in a dramatic rescue of commissure (arrowheads) and glial bridge (arrow) formation. (J) By contrast, MO knockdown of *slit1a* in *yot (gli2DR)* mutants did not rescue POC or glial bridge formation. Dotted lines mark the optic recess. (K) Summary of results of *slit* MO knockdown on axons (black) and glia (red) relative to *slit2*/*slit3* (blue) and *slit1a* (yellow) expression in wild type and *yot (gli2DR)* mutant embryos. vr, variably reduced. (L) Semi-quantitative analysis of POC formation following *slit* MO injections into wild type (purple bars) and *yot (gli2DR)* mutants (orange bars). Axon phenotypes were binned into seven categories ranging from no axons crossing (1) to a qualitatively wild-type POC (7); illustrated on left. Values represent the mean±s.e.m. All differences between injected embryos and corresponding uninjected controls are significant (Mann-Whitney U-Test, P=0.003-0.0001). No significant difference in commissure formation was found between *slit2* + *slit3* MO injection into wild type versus *yot (gli2 DR)* mutant embryos.

was variably reduced, whereas in others it appeared to be unaffected, despite clear POC defects (Fig. 6A-E,K).

Inhibition of Slit1a function in wild-type embryos disrupted POC formation more severely than inhibition of Slit2 and/or Slit3, and produced distinct axon guidance errors with a marked reduction in crossing fibers (Fig. 6A,D,E,K). *slit1a* MO injection reduced POC formation in wild-type embryos by approximately 50% (Fig. 6E,K,L), with only a small increase in the number of wandering POC axons (Table 1). Reduction in POC formation by *slit1a* MOs suggests that Slit1a promotes midline crossing, whereas defasciculation and increased wandering following Slit2/Slit3 inhibition suggests that Slit2/Slit3 restrict where POC axons can cross the midline.

Inhibition of Slit2/Slit3, but not Slit1a, rescues POC formation in *yot (gli2DR)* mutants

Because injection of *slit2*/*slit3* MOs led to axon defects consistent with the known roles for Slit proteins as axon repellents, we wondered whether the expansion of *slit2*/*slit3* expression in *yot (gli2DR)* mutants could be responsible for the inability of POC and RGC axons to grow across the midline. To answer this question, we assayed POC formation in *yot (gli2DR)* mutants injected with *slit* MOs (Fig. 6F-I,K). *slit2* and *slit3* single MO injections partially rescued POC formation in *yot (gli2DR)* mutants (Fig. 6L), whereas injection of both *slit2* and *slit3* MOs together rescued POC formation to a level that was not significantly different from that seen in *slit2*/*slit3* MO-injected wild-type embryos. *yot (gli2DR)* mutations led to a significant increase in POC axon wandering, which was further increased after injection of *slit2* and/or *slit2*/*slit3* MOs (Fig. 6; Table 1). Together, these results support the idea that Slit2 and Slit3 act as repellent guidance cues for POC axons, and that their misexpression in *yot (gli2DR)* mutants is a major cause of POC defects seen in this mutant. By contrast, *slit1a* MO injection did not rescue POC formation in *yot* mutants, and instead caused a further reduction in axon crossing (Fig. 6J-L). These knockdown results support the idea that Slit1a functions distinctly from Slit2/Slit3, with Slit1a being necessary for POC axons to grow across the midline and Slit2/Slit3 acting as repellent guidance cues.

Inhibition of Slits leads to disorganization and spreading of the glial bridge

Given the *slit2*/*slit3* expression on either side of the forebrain glial bridges (Fig. 5), and the correlation between Gfap+ cell position and axonal defects at the midline in *yot (gli2DR)* mutants (Figs 1, 2, 4), we next wondered whether Slit molecules might play a role in positioning glial cells in the forebrain. Indeed, loss of Slit1a, Slit2, Slit3, or both Slit2 and Slit3 function in wild-type embryos led to an anteroposterior spreading of Gfap+ cells in the pre- and post optic areas (Fig. 6A-E). This spreading was apparent at 22 hpf, prior to midline axon growth in the forebrain (Fig. 6A,D, insets).

Glial cells responded more drastically to Slit knockdown when we injected *slit* MOs into *yot (gli2DR)* mutants. In this case, injection of *slit2*, *slit3* and *slit2*/*slit3* MOs often led to a complete restoration of the glial bridge across the midline in the POC region (Fig. 6A,F-I). The extent of glial bridge rescue correlated with rescued POC formation. In contrast to Slit2 and Slit3, knocking down Slit1a function did not rescue glial bridge formation in *yot (gli2DR)* mutants (Fig. 6F,J). Again, in all *slit* MO-injected *yot (gli2DR)* embryos, aberrantly positioned axons appeared to be associated with Gfap+ cells.

Overlapping and distinct expression of Robo receptors in commissural neurons and midline glia

To determine which cell populations are potentially capable of responding directly to Slit cues, we determined whether commissural neurons and midline glial cells express one or more of the Roundabout (Robo) family of Slit receptors. Double labeling using different *robo* in situ probes in combination with the anti-tyrosine hydroxylase (TH) antibody that labels commissural neurons of the nucleus of the tract of the POC (ntPOC) (Holzschuh et al., 2001) showed that *robo1*, *robo2* and *robo3* are expressed in the majority of ntPOC cells, but that *robo4* is not expressed in cell bodies in the ntPOC region (data not shown).

To determine whether Robo receptors are expressed in cells of the diencephalic glial bridge, we triple labeled 28 hpf embryos with the anti-AT antibodies, anti-Gfap antibodies, and *robo1*, *robo2*, *robo3* or *robo4* in situ probes (Fig. 7). This triple labeling revealed that Gfap+ cells of the glial bridge appear to express *robo1* and *robo3*, but not *robo2* (Fig. 7A-L). Interestingly, *robo4* expression overlaps with only a subset of Gfap+ cells in the anterior portion of the glial bridge; those Gfap+ cells that come in direct contact with POC axons and RGC axons (Fig. 7M-P). *robo4* expression was not seen in Gfap+ cells ventral to the commissure (Fig. 7M). Thus, both glial cells and commissural neurons express different combinations of Robo receptors, and may therefore be capable of responding directly to Slit guidance signals.

Discussion

A model for commissure formation in the forebrain: a glial bridge and Slit guidance cues

We have characterized a population of midline-spanning glial cells (a glial bridge) that precedes the formation of the major commissures in the zebrafish forebrain, and have shown that Slit molecules play a central role in positioning both these glial cells and the commissures themselves. Our data suggest that Gfap and *slit1a* co-expressing cells form a midline bridge that

Table 1. POC axon wandering in *yot* and *slit* MO-injected embryos

	Uninjected	*slit2* MO	*slit3* MO	*slit2*/*slit3* MO	*slit1a* MO
Wild type	0.4±0.2, *n*=17	2.8±0.5, *n*=19	0.8±0.3, *n*=16	2.4±0.4, *n*=30	1.0±0.3, *n*=12
yot	2.4±0.4, *n*=19	7.7±0.8, *n*=26	4.8±0.7, *n*=18	4.5±0.4, *n*=27	3.0±0.5, *n*=14

Values represent the average number of wandering POC axons ± the standard error of the mean.

Significance values (Mann-Whitney U-test) (Sokal and Rohlf, 1995): wild type versus *yot* for all conditions, $P \leq 0.001$; *slit2* MO or *slit2*/*slit3* MO versus uninjected wild type or *yot*, $P < 0.001$.

slit3 MO and *slit1a* MO injections did not produce significant wandering in wild type or *yot* mutants.

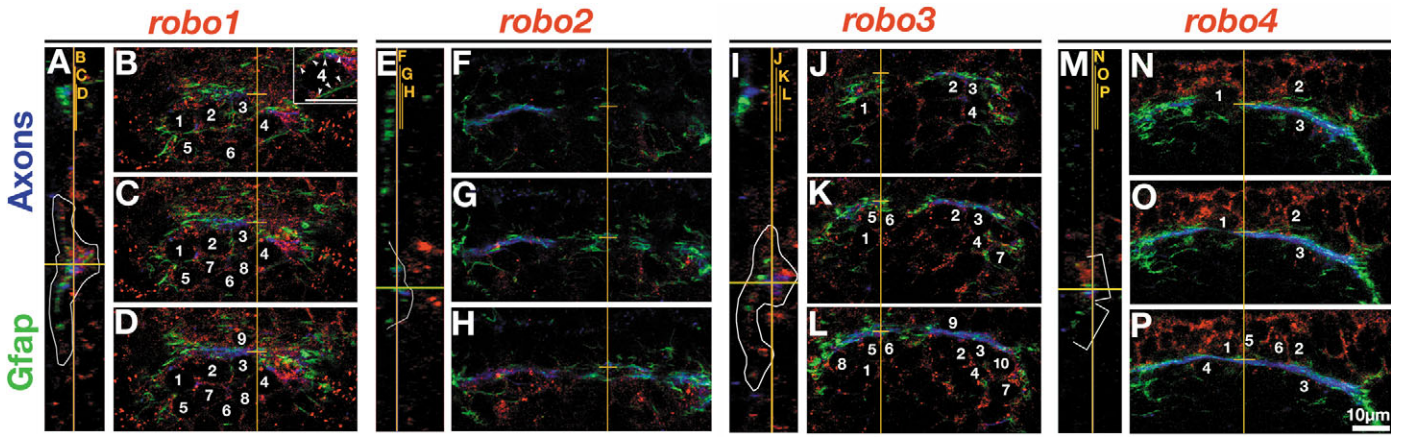

Fig. 7. Single slice confocal analysis of Robo expression (red) in relation to axons (anti-AT, blue) and glial cells (anti-Gfap, green) in the POC/chiasm region. (A,E,I,M) Sagittal slice of anterior forebrain, anterior to the left with the POC/chiasm region outlined in white. Lettered orange lines show the positions of accompanying sections. (B-D) Frontal views of the POC/chiasm region displayed in consecutive 0.4 μm slices from superficial (B) to deep (D). Vertical and horizontal orange lines denote the location of the sagittal section in A. *robo1* expression directly overlaps with Gfap+ fibers (A, outlined area; B-D, numbered nuclei). Inset in B shows a higher magnification of cell #4, arrowheads point to overlapping expression of *robo1* and *gfap*. (F-H) Single slices separated by 0.8 μm. *robo2* is expressed exclusively below Gfap+ cells (E, white line denotes separation between Gfap+ fibers and *robo2* expression). (J-L) Single slices separated by 1.2 μm. *robo3* expression directly overlaps with Gfap+ fibers in the POC/chiasm region (I, outlined area; J-L, numbered nuclei). (M-P) Single slices separated by 0.8 μm. Only the anterior subset of Gfap+ cells overlaps with *robo4* expression (M, top bracket; N-P, numbered nuclei). Scale bar: 10 μm. Nuclei of Robo/Gfap co-expressing cells are numbered in all frontal views.

provides a favorable substrate for POC and RGC axon growth (Fig. 8A). Based on Slit and Robo expression analysis and knockdown experiments, we propose that Slit2- and Slit3-mediated repulsion excludes Robo-expressing glia from Slit2/Slit3-expressing regions, restricting these cells to bands that span the midline and prefigure the commissures (Fig. 8A). Similarly, Slit2- and Slit3-mediated repulsion constrains growth of Robo-expressing commissural and RGC axons to the same Slit2/Slit3-negative region of the forebrain where this glial bridge is positioned. In contrast to Slit2 and Slit3, our data show that Slit1a is required for commissural axons to cross the midline, but does not play a major role in patterning the glial bridge. Sema3d does not appear to be necessary for commissure formation, as the POC forms in *slit2*/*slit3* MO-injected *yot (gli2DR)* mutants that lack *sema3d* expression. We show that Hh signaling is needed to establish the proper expression of Slit genes (Fig. 8D,E), and for formation of the glial bridge (Fig. 8B). Finally, we show that cell-type-specific Robo receptor 'codes' could differentially mediate the response of RGC axons, POC axons and glial cells to Slit signaling in the POC/chiasm region (Fig. 8C).

Slit guidance cues and forebrain commissure formation

slit2 and *slit3* expression surrounds the POC and AC (see also Hutson and Chien, 2002), and loss of Slit2 and/or Slit3 function results in the abnormal spreading of POC axons into anterior and posterior regions of the diencephalon (Figs 5, 6). Thus both Slit2 and Slit3 appear to constrain POC axon growth. The finding that Slit2 but not Slit3 knockdown also led to increased axon wandering (Table 1) suggests that Slit2 plays a more significant role in preventing growth outside the POC region. These Slit2/Slit3 loss-of-function data are consistent with the surround-repulsion model proposed for Robo/Slit retinal axon guidance (Hutson and Chien, 2002; Plump et al., 2002; Rasband et al., 2003; Richards, 2002b). Interestingly, although these POC 'morphotypes' are relatively subtle in wild-type embryos, *slit* MO knockdowns in the context of Hh pathway mutants (which have altered Slit expression; Fig. 5, Fig. 8B) uncovered both overlapping and distinct functions for different Slits in the formation of the POC and the diencephalic glial bridge. The dramatic rescue of *yot (gli2DR)* commissure and glial bridge defects following *slit2*/*slit3* knockdown was surprising given the general forebrain patterning defects associated with the loss of Hh signaling, including the reduction of the guidance molecule Sema3d at the midline (Fig. 5). The fact that *slit2*/*slit3* MO injection can rescue commissure formation in *yot (gli2DR)* mutants to the same level seen in *slit2*/*slit3* MO-injected wild-type embryos strongly suggests that the expansion of *slit2*/*slit3* expression in *yot (gli2DR)* mutants is the major cause of POC (and most likely RGC) midline crossing errors (Fig. 8), and that Hh signaling and Sema3d are not directly required for axons to cross the midline.

A distinct function for Slit1a in commissure formation

Several lines of evidence show that Slit1a (Hutson et al., 2003) may function distinctly from Slit2/Slit3 during commissure formation in the zebrafish forebrain. First, *slit1a* expression is complementary to *slit2*/*slit3* expression and unlike *slit2*/*slit3*, commissural axons grow in *slit1a*-expressing regions (Fig. 5). Second, expression of *slit1a* is distinctly affected by the loss of Hh signaling, with *slit1a* expression being reduced rather than expanded in *yot (gli2DR)* mutants (Fig. 5F). Third, blocking Slit1a function in wild-type embryos led to a marked reduction in POC crossing rather than the defasciculation seen in *slit2*/*slit3* morphants (Fig. 6). Fourth, reducing Slit1a

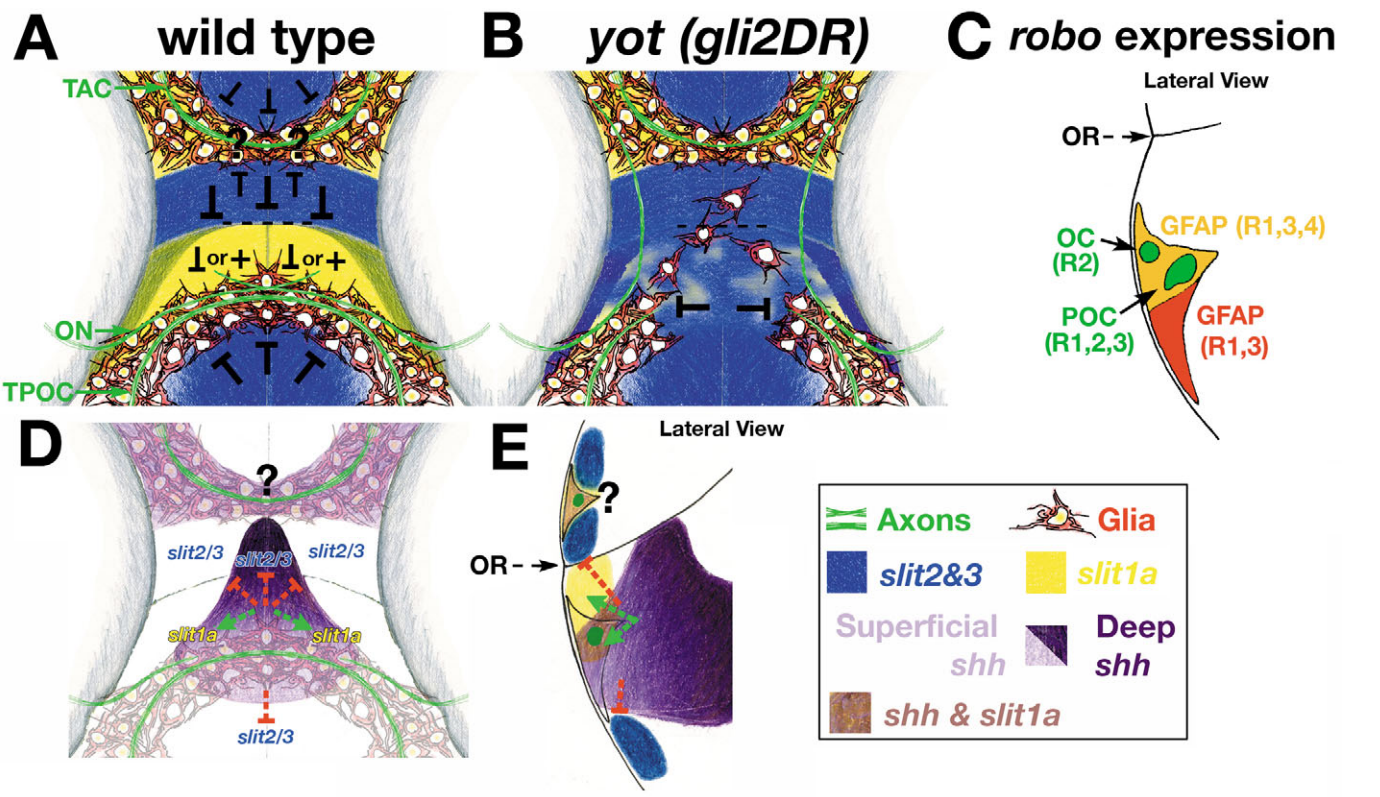

Fig. 8. Model for Slit-mediated glial bridge and commissure formation in wild-type and Hh mutant zebrafish embryos. (A) *slit2* and *slit3* (blue) are normally expressed posterior to the POC and in bands adjacent to the AC. We propose that Slit2 and Slit3 repulsion creates corridors that prevent Robo-expressing glial and axonal cell migration into *slit2*/*slit3*-expressing domains. By contrast, *slit1a* expression (yellow) in the preoptic area and dorsal telencephalon overlaps with the forebrain glial bridges and forebrain commissures, suggesting either that (1) Slit1a provides a permissive or attractant cue for these cells, or (2) that Slit1a is less repellent for glia and commissural axons than Slit2/Slit3, causing glial cells and commissural axons to position themselves on *slit1a*-expressing cells just anterior to the *slit2*/*slit3* expression domain. Slit expression in the telencephalon also suggests a role in AC formation. Gfap+ cells (red) may provide a positive or permissive substrate for AC, POC and RGC axon (green) growth across the midline. (B) In *yot (gli2DR)* mutants, RGC axons and POC axons fail to cross the midline. In addition, Gfap+ cells are reduced in the POC/chiasm region and spread into the pre-optic area. *slit2*/*slit3* expression is expanded across the commissure regions, whereas *slit1a* expression is reduced. *slit* MO injections show that expansion of *slit2*/*slit3* expression is the major cause of midline crossing errors in *yot (gli2DR)* mutants (black bars). (C) Schematic lateral view of Robo expression in the POC/chiasm region. RGC axons express *robo2* (Lee et al., 2001), POC neurons express *robo1*, *robo2* and *robo3* (green), all Gfap+ cells express *robo1* and *robo3* (red and orange), but anterior Gfap+ cells also express *robo4* (orange). (D,E) Illustration of *shh* expression (purple) in relation to axons and glial cells in the forebrain at 30 hpf from frontal (D) and lateral (E) views. Shh leads to the repression of *slit2*/*slit3* expression but positively regulates *slit1a* expression (dashed arrows). Currently, it is unknown whether Shh regulates Slit expression in the AC region (?).

function in *yot (gli2DR)* mutants failed to rescue axon crossing in *yot (gli2DR)* mutants and instead led to an even further decrease in POC axon crossing (Fig. 6). Interestingly, retinal axons in the optic tract also navigate across domains of *slit1a* expression, and knocking down Slit1a function causes retinal axon guidance errors that are not easily explained by removal of a repulsive signal (L.D.H. and C.-B.C., unpublished).

How might Slit1a influence commissural and retinal axon guidance? Based on our loss of function experiments, Slit1a does not appear to be a major repellent for commissural axons. One possibility is that *slit1a* expression on a subset of Gfap+ cells in the glial bridge contributes to the permissive/attractive growth environment provided by these cells (Fig. 8A). If true, our *slit1a* MO experiments indicate that Slit1a is not the only guidance molecule needed for commissural axons to recognize these glial cells, as axons continue to follow aberrantly placed glial cells in *slit1a* MO-injected embryos (Fig. 6). An alternative model is that Slit1a acts as a repellent molecule for commissural axons, but is less repellent than Slit2/Slit3 (Fig. 8A). Thus, when confronted with a choice between Slit1a-, Slit2- and Slit3-expressing domains, commissural axons choose to travel on the 'path of least repulsion', growing on *slit1a*-expressing cells that border *slit2*/*slit3*-expressing cells (Fig. 8).

Slit molecules help to position a glial bridge

Our studies have uncovered a novel role for Slit molecules in the positioning of astroglia in the vertebrate forebrain (Fig. 8). We show that Gfap+ cells are positioned in *slit1a*-expressing regions that do not express *slit2* or *slit3* (Fig. 5), and that loss of Slit2/Slit3 (but not Slit1a) function results in an expansion of the glial bridge in the POC region prior to axon growth (Fig. 6). In addition, glial cell defects in *yot (gli2DR)* mutants were largely rescued following *slit2*/*slit3* MO knockdown, suggesting that the mis-positioning of glial cells in *yot (gli2DR)* mutants is due to the expanded zones of *slit2*/*slit3*

expression in these embryos (Figs 6, 8). Such a role for Slits in cell positioning has been shown for a variety of cell types, including glial cells and mesodermal cells (for reviews, see de Castro, 2003; Piper and Little, 2003). In the *Drosophila* CNS, Slit/Robo signaling is required to position glial cells that later serves to guide longitudinal and commissural axons (reviewed by Hidalgo, 2003). A role of Slits in cell migration has also been shown in mouse, where Slit1 is required cell autonomously for subventricular neuroblasts to migrate in vitro (Nguyen-Ba-Charvet et al., 2004). The fact that a subset of Robo receptor genes are expressed in zebrafish forebrain glial cells (Fig. 7, Fig. 8C) indicates that Robo-mediated Slit repulsion might directly prevent glial cells from occupying *slit2*- and *slit3*-expressing domains, perhaps by influencing cell migration in a way that is analogous to growth cone repulsion.

Our confocal analysis of Gfap expression during forebrain development revealed that two glial bridges span the midline prior to AC, POC and RGC formation, and that these Gfap-expressing cells are present at the right position to provide cues for midline axon growth. These astroglial cells send short fibers perpendicular to the pial surface into the forebrain, and the developing commissure forms as a ribbon along these fibers (see Movies 1, 2, 3 in the supplementary material) (Marcus and Easter, 1995). A previous immuno-EM study used the same Anti-Gfap antibodies to show that retinal and commissural axons directly contact Gfap+ radial glia-like fibers (Marcus and Easter, 1995). These anatomical data strongly suggest that the glial bridge provides the growth substrate for the first commissural axons to cross the midline (Fig. 8A). Disruption of the glial bridge correlates with midline axon crossing errors, and glial cell defects precede axon defects both in *yot (gli2DR)* mutants (Figs 3, 4) and in *slit2*/*slit3* morphants (Fig. 6D). These functional data suggest that these glial cells provide a positive or at least permissive cue for midline axon crossing (Fig. 8A,B). A positive role for glial cells is also supported by the observation that aberrantly growing axons appear to preferentially grow along mis-positioned Gfap+ cells rather than following their normal pathway or an alternative non-glial route. It will now be important to directly test whether glial cells are both necessary and sufficient for midline guidance of commissural and retinal axons.

An indirect role for Hh in commissure and glial bridge formation

shh is expressed at the right place and time to influence both cell fate and axon guidance at the midline in the diencephalon (Fig. 1E,F, Fig. 8D,E). Secreted Shh was recently shown to play a direct role in attracting axons to the ventral midline of the mouse neural tube (Charron et al., 2003). In addition, in vitro and in vivo experiments in chick suggest that Shh may inhibit RGC axon growth (Trousse et al., 2001). However, by using CyA to block Smoothened-mediated Hh signaling at different times, we show that Hh signaling does not appear to directly guide commissural or retinal axons in the zebrafish forebrain (Fig. 3). The rescue of POC formation in *yot (gli2DR)* mutants by injection of *slit2*/*slit3* MOs (Fig. 6) also indicates that Hh signaling does not play a major role in the midline guidance of POC axons. Instead, Hh signaling appears to affect axon guidance indirectly through its role in patterning of the midline (Dale et al., 1997; Karlstrom et al., 2003; Mason and Sretavan, 1997), including the formation of the glial bridge and the regulation of axon guidance-molecule expression (Figs 3, 4, 5).

How can we reconcile our CyA results with the direct inhibitory affect seen for Shh in chick retinal explants? One possibility is that this particular guidance function for Shh has not been evolutionarily conserved between fish and chick. However, this seems unlikely given the high degree of conservation in other Hh-mediated developmental processes (Ingham and McMahon, 2001), and the similar expression of Hh in the chiasm region in chick and fish (Trousse et al., 2001) (Fig. 1E,F). Alternatively, loss-of-function approaches may be more informative than ectopic expression studies regarding the direct requirement for Hh in vivo. Thus, although retinal axons may be able to directly respond to ectopic Hh signals in vitro, direct Hh-mediated growth-cone guidance may not play a significant role in the context of other guidance cues present in the chiasm region. The finding that overexpression of *shh* in zebrafish not only disrupted AC, POC and RGC axon crossing but also disrupted glial bridge formation (Fig. 3G-I) is also consistent with a primary role for Hh signaling in patterning the axon growth substrate.

Rescue of POC formation in *yot (gli2DR)* mutants following *slit2*/*slit3* MO injections, combined with the expansion of *slit2*/*slit3* expression in Hh pathway mutants, suggests that the indirect effect of Hh on glial and axon guidance in the diencephalon is largely mediated by Slit proteins. Hh may regulate Slit gene expression directly, or through its regulation of cell fates. By contrast, loss of Hh or Slit function had little effect on AC formation, indicating that other patterning and guidance molecules are sufficient to pattern the ventral telencephalon and establish the AC. These data are consistent with the known role for Hh in ventral forebrain patterning, and begin to shed light on the downstream molecular targets that result in proper glial position and neural connectivity in this region.

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Supplementary material

Supplementary material for this article is available at
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