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Development of midline cell types and commissural axon tracts requires *Fgfr1* in the cerebrum

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Abstract

The adult cerebral hemispheres are connected to each other by specialized midline cell types and by three axonal tracts: the corpus callosum, the hippocampal commissure, and the anterior commissure. Many steps are required for these tracts to form, including early patterning and later axon pathfinding steps. Here, the requirement for FGF signaling in forming midline cell types and commissural axon tracts of the cerebral hemispheres is examined. *Fgfr1*, but not *Fgfr3*, is found to be essential for establishing all three commissural tracts. In an *Fgfr1* mutant, commissural neurons are present and initially project their axons, but these fail to cross the midline that separates the hemispheres. Moreover, midline patterning defects are observed in the mutant. These defects include the loss of the septum and three specialized glial cell types, the indusium griseum glia, midline zipper glia, and glial wedge. Our findings demonstrate that FGF signaling is required for generating telencephalic midline structures, in particular septal and glial cell types and all three cerebral commissures. In addition, analysis of the *Fgfr1* heterozygous mutant, in which midline patterning is normal but commissural defects still occur, suggests that at least two distinct FGF-dependent mechanisms underlie the formation of the cerebral commissures.
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Introduction

To function in its environment, the left and right sides of an animal's nervous system need to exchange information. In the vertebrate forebrain, the cerebral hemispheres are connected directly by three major axon tracts: the corpus callosum (CC), which connects neocortical neurons in layers 5 and 2/3 from each hemisphere; the hippocampal commissure (HC), which connects the hippocampus; and the anterior commissure (AC), with posterior and anterior branches, which connects the piriform cortex and olfactory bulbs, respectively. During development, several steps are required for axons in each of these tracts to extend across the midline and reach their targets. For instance, callosal axons must first extend from the cortical plate to the intermediate zone, turn medially and project ventrally within this zone, and upon approaching the midline turn sharply toward it and cross the boundary between the two hemispheres. On the contralateral side, the axons project along a mirror image trajectory (reviewed by Richards et al., 2004). It is unclear which cues in the environment of commissural axons guide them along each step of their trajectories and how the cellular structures that produce these cues are formed.

In the embryonic cerebrum, or telencephalon, several populations of midline glia are likely to play important roles in guiding commissural axons from one hemisphere to the other. First, midline "zipper" glia are thought to be responsible for fusing the hemispheres, which facilitates the passage of axons across the midline (Silver et al., 1993; Richards et al.,

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2004). Second, two other midline glial structures are likely to be critical for promoting the crossing of at least callosal axons, the "glial wedge" and the indusium griseum glia (Silver et al., 1982; Shu and Richards, 2001). These two populations of glia express *Slit2*, which encodes a chemorepellent, and are avoided by callosal axons in vivo. Moreover, the glial wedge in cultured explants can repel or inhibit the growth of cortical axons. In slice cultures in which the glial wedge is removed, or in the *Nfia* mutant in which it does not form, callosal axons continue their ventral trajectory along the midline instead of turning toward it and crossing (Shu and Richards, 2001; Shu et al., 2003b). Despite the importance of midline glial structures in guiding commissural axons, little is known about what factors induce their formation.

Commissural defects in the mammalian telencephalon can result from mutations in over 50 genes (Richards et al., 2004). Some of these genes encode axon guidance molecules. For example, *netrin-1*, *Npn2*, *EphB2*, and *Slit2* mutant mice all lack one or more telencephalic commissures (Serafini et al., 1996; Henkemeyer et al., 1996; Chen et al., 2000; Bagri et al., 2002). In addition to these familiar pathfinding molecules (Chisholm and Tessier-Lavigne, 1999; Kaprielian et al., 2001), molecules traditionally thought of as morphogens, such as Sonic Hedgehog, Bone Morphogenetic Proteins, and Wnts, also directly guide commissural axons, at least in the spinal cord (Butler and Dodd, 2003; Charron et al., 2003; Lyuksyutova et al., 2003). However, many of the genes implicated in commissure formation are unlikely to encode axon guidance molecules and instead may be required to generate and pattern the cellular environments through which commissural axons travel (Richards et al., 2004).

Fibroblast Growth Factors (FGFs) act as morphogens to pattern tissues throughout development, including the neocortex (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003). A role for FGFs in forming the telencephalic midline and its commissures, however, is less clear. In humans, reduced *FGFR1* expression leads to Kallmann syndrome, which consists of several developmental defects and in some cases can include agenesis of the CC (Dode et al., 2003). In zebrafish, loss of FGFs results in telencephalic midline defects and loss of commissures (Shanmugalingam et al., 2000; Walshe and Mason, 2003). In these studies, however, the missing midline cell types and the cause for the loss of commissures was not elucidated. A mouse model would facilitate the distinction between several possible mechanisms by which FGFs contribute to forming the CC, and potentially the HC and AC. For instance, FGFs have been implicated in patterning the environment through which certain axons must navigate; *Fgf8* is required in mice for organizing intra-neocortical projections, presumably by patterning neocortical areas (Huffman et al., 2004), and in *C. elegans*, FGF receptor gene expression is required in epidermal cells for the growth of adjacent axons (Bulow et al., 2004). Alternatively, FGF signaling may be required in neurons themselves; in *Xenopus*, FGF signaling is required in retinal ganglion neurons for targeting their axons to the correct destination (McFarlane et al., 1996), and FGF8 acts as a chemoattractant on trochlear axons in the hindbrain (Irving et al., 2002).

Here, the requirement for FGF signaling in generating midline cell types and in forming the three telencephalic commissural tracts is explored in mice using embryos that are mutant for *Fgfr1*, *Fgfr3*, or both. Loss of *Fgfr3* has no phenotype on its own and does not worsen the phenotype obtained with loss of *Fgfr1*. On the other hand, mice that are homozygous mutant for *Fgfr1* specifically in the telencephalon lack all three commissural tracts, demonstrating an essential role for this gene in forming not only the CC, but also the HC and AC. In this mutant, neurons that project to their contralateral targets are present and extend axons, suggesting that commissural neurons are generated normally. However, there is a dramatic perturbation in the development of specific midline structures that are implicated in the crossing over of commissural axons. These include the septum, a major ventral forebrain structure, and specific midline glial populations. Therefore, one potential mechanism underlying the absence of forebrain commissures in the *Fgfr1* mutant appears to be an early patterning defect at the midline. Surprisingly, however, in the *Fgfr1* heterozygous mutant, midline patterning is normal and the AC crosses the midline, but the CC and HC still fail to cross. This suggests that in addition to a requirement for FGF signaling in generating key midline structures a second FGF-dependent mechanism underlies commissure formation.

Materials and methods

Generation of mutant embryos

To study the role of FGF signaling in telencephalic commissure formation, a breeding paradigm was designed to generate mice mutant for *Fgfr1*, *Fgfr3*, or *Fgfr1* and *Fgfr3* (Fig. 1A). A loss of *Fgfr1* in the whole embryo leads to lethality at gastrulation (Deng et al., 1994; Yamaguchi et al., 1994), therefore a floxed allele of *Fgfr1* was used to generate a knockout of this gene in the telencephalon when crossed to *Foxg1*^{Cre} mice, as previously described (Pirvola et al., 2002; Hébert et al., 2003). *Fgfr1* heterozygous brains were found to have an intermediate phenotype (see Figs. 6, 7), hence the controls that are used throughout this study carry two functional alleles of *Fgfr1* (i.e. they do not carry a *Foxg1*^{cre} allele, or if they do, they do not carry a *Fgfr1*^{lox} allele). The *Fgfr3* allele used is a null allele (Deng et al., 1996). Since *Fgfr3* homozygous null mice are viable but breed poorly, heterozygotes were used in the cross. Mutant embryos were obtained in the expected ratios with no signs of necrosis.

Immunohistochemistry

Tissue, freshly frozen in OCT embedding medium, was sectioned at 20–40 μm, fixed, blocked in 10% goat serum/PBS, stained with primary antibody for 2 h at room temperature or 4°C overnight, and stained with 1:100 dilution of secondary antibody coupled to FITC (for visualizing neurofilament and Tag1) or Texas Red (for visualizing GFAP), 2 h at room temperature. The 2H3 anti-neurofilament antibody, developed by Tom Jessell and Jane Dodd, and 4D7 anti-Tag1 antibody, developed by M. Yamamoto, were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences. These antibodies were used as supernatants without dilution. The anti-Tag1 antibody was found to preferentially stain the CC and AC, whereas the anti-neurofilament antibody, preferentially the HC. The anti-GFAP antibody (Dako, # Z 0334) was used at a dilution of 1:500.

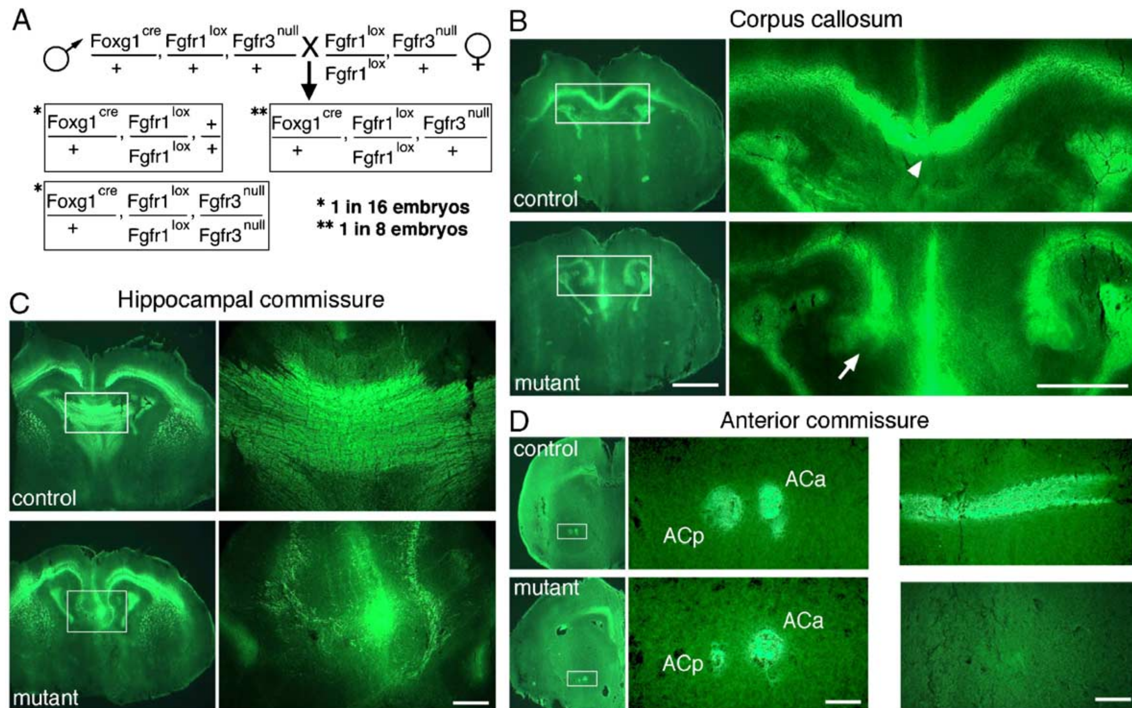

Fig. 1. Telencephalic loss of *Fgfr1* results in a loss of all axonal midline crossing. (A) Cross used to generate mutant embryos deficient for both copies of *Fgfr1* and zero, one, or two copies of *Fgfr3*. These three mutant genotypes resulted in indistinguishable phenotypes. (B–D) Coronal sections of control and mutant telencephalons. Areas outlined in white in the left panels are magnified in the right panels. Scale bar for the left panels (shown in panel B only), 1 mm. (B) P0 callosal axons from an *Fgfr1* homozygous mutant telencephalon are stained with anti-Tag1 antibody. In the mutant, although the callosal axons initially project normally toward the midline, they fail to cross as in controls (arrowhead) and instead form Probst-like bundles (arrow). Scale bar in right panel, 0.5 mm. (C) P0 axons that form the hippocampal commissure are stained with anti-Neurofilament antibody and do not cross the midline in the *Fgfr1* homozygous mutant. Scale bar: 200 μm. (D) Axons that form the anterior commissure are stained with an anti-Tag1 antibody in the control and mutant (*Fgfr1* homozygous, *Fgfr3* heterozygous) E18.5 telencephalon. Initially, these axons project normally along their posterior (ACp) and anterior (ACa) branches (left panels), but in the mutant, unlike the control, they fail to ultimately form a tract that crosses the midline (right panels). Scale bars: left, 100 μm; right, 200 μm.

RNA in situ hybridization

In situ hybridizations were done according to two protocols, one using radioactive RNA probes and the other DIG-labeled probes. For the radioactive in situs, frozen sections were prepared and hybridized to ^{35}S-labeled probes as previously described (Frantz et al., 1994a). A minimum of three mutant and three control embryos were analyzed for each probe. For the DIG in situs, riboprobes were made using reagents from Roche Applied Science. Embryonic brains were fixed in 4% paraformaldehyde, equilibrated in a solution of 30% sucrose made in 4% paraformaldehyde, and 30–35 μm frozen sections were obtained using a microtome and processed for in situ hybridization as described previously (Tole et al., 1997). Hybridization was carried out overnight at 70°C, in a buffer containing 50% formamide, 4XSSC, and 1% SDS. Posthybridization washes were at 70°C in 2× SSC and 50% formamide.

DiI labeling

Crystals of DiI were placed in fixed embryonic brains in the frontal cortex. Placements were unilateral to permit better visualization of midline crossing defects. After incubation at 37°C for 21 days, the brains were embedded in 3% agarose and sectioned at 100 μm thickness on a vibratome. Sections were imaged using a Zeiss Axioscope2.

Analysis of cell death

Embryos were collected and frozen in OCT. Fresh frozen sections were used for TUNEL analysis according to the manufacturer's specifications (Roche, Cat. # 2 156 792). Coronal sections for control and mutants were matched so that they correspond to the same position along the anterior–posterior axis of the telencephalon. Sections were counterstained with Syto11 (Molecular Probes). The fraction of TUNEL positive cells is determined by counting the number of these cells in a radial segment spanning from the ventricular surface to the outer edge of the neural tissue and dividing by the total number of cells in the segment (segments contain ∼200 total cells).

Results

Mice deficient in telencephalic FGF signaling lack commissural tracts

To determine whether *Fgfr1* or *Fgfr3* plays a role in forming the commissures of the telencephalon, mutant embryos derived from the cross illustrated in Fig. 1A were examined. Throughout this study, no difference in phenotype could be observed between any of the three classes of mutants that lack both copies of *Fgfr1* (Fig. 1A); that is, loss of one or both copies of *Fgfr3* did not worsen the phenotype obtained with loss of both copies of *Fgfr1* alone. Therefore, the *Fgfr1* homozygous mutant is used, unless otherwise indicated, to illustrate the phenotypes described below. Similarly, loss of one or both copies of *Fgfr3* does not reproducibly worsen the phenotype observed in the heterozygous *Fgfr1* mutant (Fig. 6 and not shown) nor does loss of *Fgfr3* exhibit a discernable commissural phenotype on its own (Figs. 2D–F). Therefore, *Fgfr3* does not appear to play any significant role in commissural formation in the telencephalon.

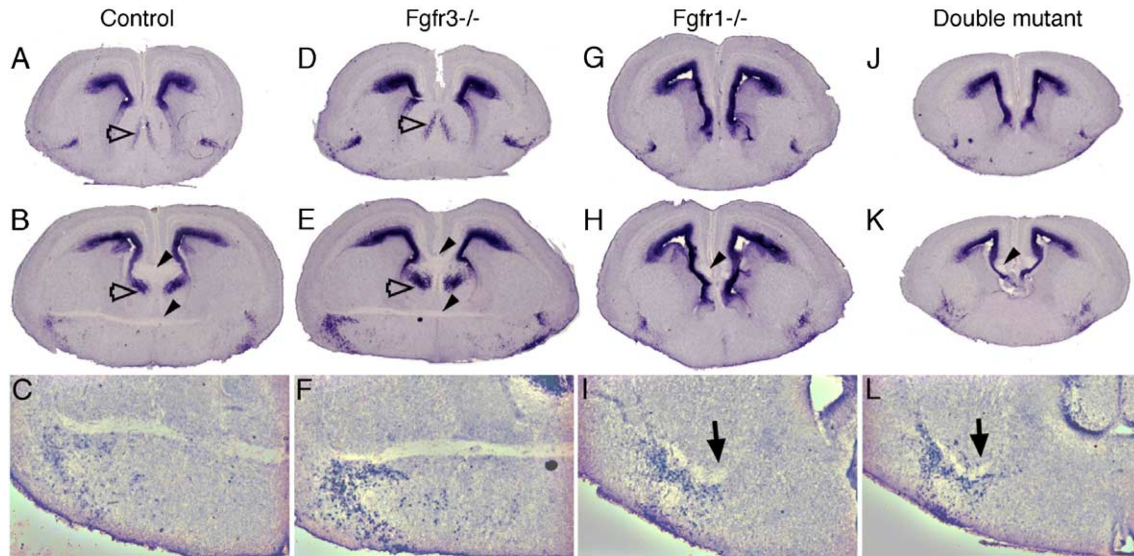

Fig. 2. RNA in situ hybridization analysis using a *Pax6* DIG probe on coronal sections of E16.5 control (A–C), *Fgfr3−/−* (D–F), *Fgfr1−/−* (G–I), and double mutant (J–L) brains reveal similar defects in the absence of *Fgfr1* or the combined absence of *Fgfr1* and *Fgfr3*. In contrast, the absence of *Fgfr3* alone does not appear to cause detectable defects. Panels A, D, G and J are more rostral than panels B, E, H, and K. The septum, which expresses a characteristic pattern of *Pax6* mRNA (open arrowheads), appears to be missing in *Fgfr1* mutant and in the double mutants. The CC, HC, and AC, seen in control and *Fgfr3−/−* brains are not detectable in the absence of *Fgfr1*, but Probst bundles are seen (filled arrowheads). Bottom panels are magnifications of sections adjacent to panels B, E, H, K showing the AC axons extending toward the midline in control and *Fgfr3−/−* (C, F) or arresting in the lateral region in mutants (I, L, arrows). Note that the double mutant is smaller in this figure, but this finding is not reproducible (e.g. Figs. 3, 5).

Cortical neurons destined to project their axons through the corpus callosum begin to extend these axons at E15.5 in the mouse (Ozaki and Wahlsten, 1992). The first callosal axons, the pioneering axons, cross the midline and reach their contralateral targets between E15.5 and E16 and originate in the presumptive cingulate cortex (Koester and O'Leary, 1994; Ozaki and Wahlsten, 1998; Rash and Richards, 2001). The first callosal axons originating from the neocortex cross the midline a day later at E16.5 (Ozaki and Wahlsten, 1992, 1998). Midline crossing of hippocampal axons is strain-dependent and begins between E14.5 and E17.5 (Livy and Wahlsten, 1997). The timing of the formation of the anterior commissure has not been described in detail, but the axons that form this tract can be observed to cross the midline as early as E14.5 (e.g. Serafini et al., 1996; data not shown).

In the *Fgfr1*-deficient mutant, all three commissural tracts fail to cross the midline (Figs. 1B–D, 2G–L). This phenotype was found to be 100% penetrant (n = 31). Brains from embryonic day 18.5 (E18.5) and postnatal day zero (PO) mutant and control littermates were collected, sectioned, and stained with antibodies against neurofilament and Tag1 to visualize the commissural tracts. In the *Fgfr1* mutant, callosal axons project along the intermediate zone of the cortical plate and begin their ventral descent along the midline, but then fail to turn and cross it, and instead form Probst-like bundles (Fig. 1B arrow, Figs. 2H, K filled-arrowheads, Fig. 6J). Likewise, hippocampal axons fail to reach and cross the midline (Figs. 1C, 2G, H, J, K). The formation of Probst-like bundles at the end of the callosal axons suggests that the loss of midline crossing is not simply due to a delay in axonogenesis. Consistent with this, mutant brains as late as P0 are still missing all three commissures (Fig. 1 and data not shown).

In a previous study, it was shown that neurons in the anterior part of the *Fgfr1*-deficient telencephalon still project to their normal target in the olfactory cortex despite the lack of normal olfactory bulb morphogenesis (Hébert et al., 2003). Consistent with this finding, the anterior branch of the anterior commissure still forms in the mutant, as does the posterior branch (Fig. 1D). However, once these branches have joined at the anterior olfactory nucleus, a major relay station for the olfactory system, they fail to project to the midline as they normally do and instead curl slightly in the dorsal direction before arresting in the striatum (Figs. 1D, 2I, L arrows). Therefore, FGF signaling is specifically required for all commissural axons of the telencephalon to project toward and cross the midline.

Midline glia and the septum are missing in the Fgfr1-deficient telencephalon

Examination of the *Fgfr1* mutant brain in sections revealed morphological anomalies along the midline. This suggests that a possible mechanism underlying the failure of commissural tracts to cross from one hemisphere to the other is that the midline is not properly formed. In particular, three midline glial structures have been described, the midline zipper glia, the indusium griseum, and the glial wedge. These glia are thought to play critical roles in fusing the midline and guiding callosal axons across it (Silver et al., 1982, 1993; Shu and Richards, 2001; Richards et al., 2004). Little is known about what factors are required to induce these midline glial structures to form. In this study, the use of the *Foxg1*^{Cre} allele leads to a loss of *Fgfr1* expression at the earliest stages of telencephalon development (Hébert and McConnell, 2000; Hébert et al., 2003). This raises the possibility that early patterning defects, such as the loss of

midline cells including the specialized glial structures, underlie the late commissural phenotype.

To explore this possibility, the presence of midline glia was examined using an anti-GFAP antibody in E14.5 to 17.5 control and mutant embryos. GFAP staining was undetectable at E14.5 and only faintly discernable at E15.5 in control brains (data not shown). By E16.5, when GFAP staining clearly marks all three glial structures in control brains, it becomes apparent that three of these structures are absent in the *Fgfr1*-deficient telencephalon, the indusium griseum glia, the midline zipper glia, and the glial wedge ($n = 3$) (Fig. 3). Hippocampal glia are present in both the control and the mutant in more posterior regions at E16.5 and E17.5 (Fig. 3; data not shown). This suggests that FGF signaling through FGFR1 is essential for the development of the indusium griseum glia, the midline zipper glia, and the glial wedge.

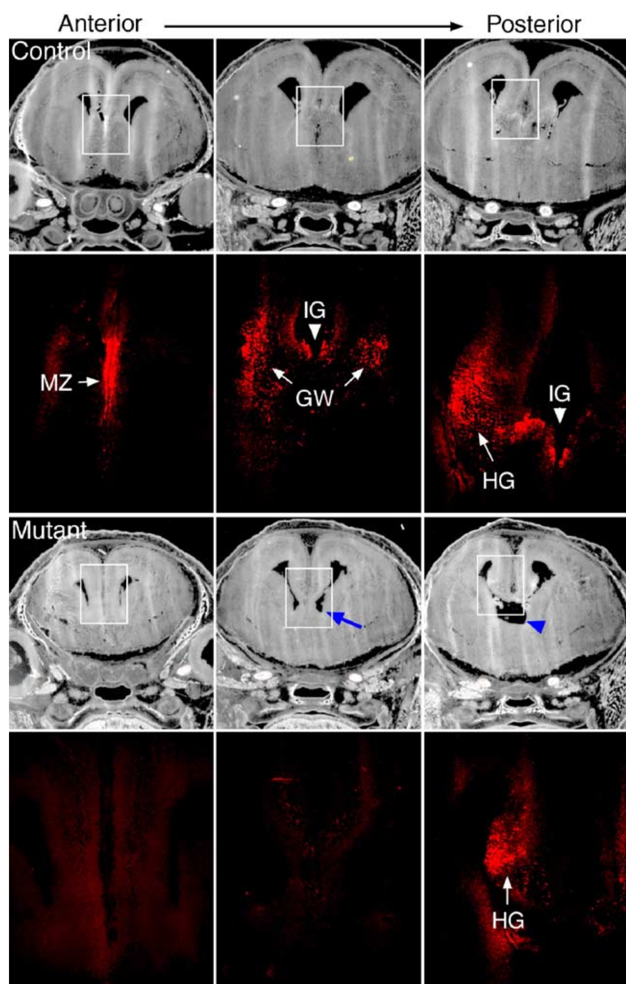

Fig. 3. Three midline glial structures do not develop normally in the *Fgfr* mutants. Coronal sections of E16.5 control and *Fgfr1*;*Fgfr3* double mutant brains stained with anti-GFAP antibody. Areas outlined in white in the top panels are magnified in the lower panels. From left to right, sections go from anterior to posterior. In the control (top), the midline zipper glia (MZ), indusium griseum glia (IG), and glial wedge (GW) are present whereas in the mutant (bottom) they are missing. In more posterior regions, the hippocampal glia (HG) are present in both the control and mutant. Note the gap in the mutant where the septum should be (blue arrowhead) and the ventrally extended ventricles (blue arrow).

In addition, the mutant lacks a septum (Figs. 2, 3, 5). *Pax6* expression marks different regions of the septum along the rostro-caudal axis in control embryos, but this expression is absent at all levels in the mutant (Fig. 2, open arrowheads). Morphologically, loss of the septum in the mutant is also apparent as a gap in the ventral midline area (Figs. 3, 5). The absence of the septum is due to a failure of ventro-medial precursors to generate differentiated cells earlier in development, rather than to an increase in apoptosis or a decrease in proliferation, and will be extensively described in an accompanying study. Together, these results are consistent with the view that defects in midline structures underlie the failure of commissural axons to cross the midline.

FGF8 and FGFR1 are specifically expressed in midline cells

As presented above, loss of *Fgfr3* has little or no effect on the development of telencephalic commissures, and does not worsen the phenotype observed with loss of *Fgfr1*. The explanation for this is likely to lie in the expression pattern of these genes. Of the three Fgfr genes expressed in telencephalic precursor cells (*Fgfr4* is not expressed in the telencephalon; data not shown), only *Fgfr1* is expressed in midline precursors at E12.5, prior to the appearance of midline glial structures (Figs. 4B–D, arrowheads; Bansal et al., 2003). At E15.5, *Fgfr1* remains expressed in midline precursor cells, including the region that gives rise to the glial wedge (Fig. 4E, arrowhead). In addition, *Fgf8*, which encodes a putative ligand for telencephalic FGFR1 (Hébert et al., 2003), is also expressed in the midline (Fig. 4A).

It is worth noting that *Fgfr1* is also expressed in cortical neurons (Bansal et al., 2003), with the highest levels of expression in the midline cingulate cortex at E15.5 (Fig. 4E, arrow), a stage at which pioneering callosal axons from this region are projecting toward the midline. This leaves open the possibility that *Fgfr1* is essential not only in forming midline structures that guide commissural axons, but may also be required in commissural neurons themselves for their normal differentiation.

The midline expresses guidance cues in the Fgfr1−/− mutant

In the *Fgfr1*-deficient telencephalon, commissural axons not only fail to cross the midline, but they also fail to turn toward it from a distance (Figs. 1, 2), suggesting that long-range guidance cues may no longer be emanating from midline cells. However, the midline of the *Fgfr1* mutant still expresses several putative guidance cues including *Slit2*, *Netrin1*, *Shh*, *Fgf8*, and *Bmp4* (Fig. 5 and data not shown), although expression of genes that normally extends into septal areas is partially lost due to the septum phenotype. For example, *Slit2* is normally expressed in midline precursor cells that line the ventricle from the cortex dorsally to the septum ventrally, whereas in mutants, dorsal expression is maintained while ventral expression is reduced or lost (Fig. 5B). In contrast, although *Wnt8b* expression, which is normally restricted to dorsal midline precursors, is maintained in more caudal

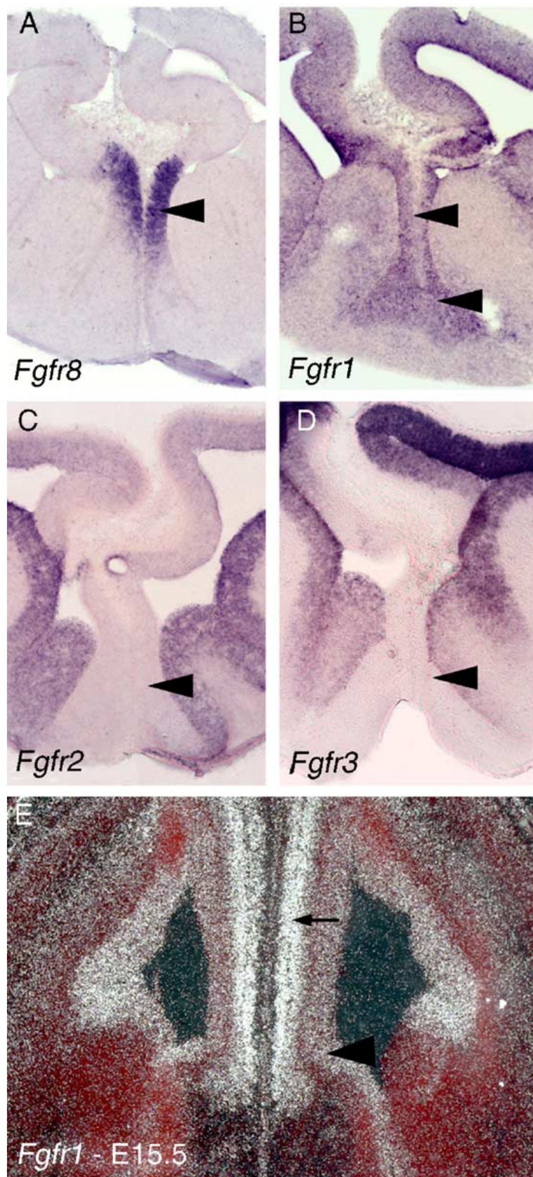

Fig. 4. *Fgf8* and *Fgfr1* are expressed in midline precursor cells. (A–D) Coronal serial sections of an E12.5 wild-type telencephalon showing RNA hybridization for *Fgf8* (A), *Fgfr1* (B), *Fgfr2* (C), and *Fgfr3* (D) using DIG probes. *Fgf8* along with *Fgfr1*, but not *Fgfr2* or *Fgfr3* is expressed in the midline of the telencephalon. (E) Coronal section of an E15.5 wild-type telencephalon showing RNA hybridization for *Fgfr1* using an ^{35}S-labeled probe; expression is observed in the area that will form the glial wedge (arrowhead) and in midline neurons (arrow).

hippocampal areas of the mutant (not shown), it is lost in the area that gives rise to the glial wedge (Fig. 5C, arrow), consistent with the loss of these cells in the mutant (Fig. 3).

It remains possible that other guidance cues, or substrates on which commissural axons extend, are missing, even at a distance from the midline. Certainly within the vicinity of the midline itself, a loss of substrates is supported by the finding that midline structures are lost, including the septum, indusium griseum, and midline zipper glia, which are thought to be required for fusing the hemispheres and providing a passageway for crossing axons (Silver et al., 1993; Richards et al., 2004).

The midline crossing defect is not due to a loss of contralaterally projecting neurons

An additional potential mechanism by which commissures might fail to develop normally in the *Fgfr1*-deficient telencephalon is that neurons that normally project their axons to the contralateral hemisphere may not have been generated. However, anti-neurofilament and Tag1 staining reveal that axons that normally form the CC and AC initially project normally in the mutant (Figs. 1, 2), indicating that not only are commissural neurons generated, but that they can also project axons.

To assess whether loss of Fgfr expression might affect the positional identities of the neurons in the cortical plate, we examined the expression of two transcription factor genes, *Pax6* and *Emx2*, which are required for specifying the normal positional identities of cortical precursor cells (Bishop et al., 2000; Mallamaci et al., 2000; Muzio et al., 2002). As previously reported for the E12.5 *Fgfr1* mutant telencephalon (Hébert et al., 2003), these genes are expressed in their respective gradients in E13.5 *Fgfr1* heterozygous as well as homozygous mutant telencephalons (Supplementary Fig. 1). However, we cannot exclude the possibility that the levels of expression of these genes have been shifted quantitatively along the A–P or D–V axes in response to loss of *Fgfr1*. Note that the expression of other genes, including *EphrinA5* and *Fgfr3*, shows no shifts in expression levels at later ages in the *Fgfr1* mutant (Hébert et al., 2003). Together, these results suggest that the areal identity of cortical neurons has not been significantly perturbed by loss of *Fgfr1*.

To further characterize the state of the cortical neurons, we examined the expression of genes that they normally express, such as the transcription factor genes *Otx1*, *Cux2*, *Pou3f1*, and *Tbr1*, the axon pathfinding receptor genes *Robo1*, *Robo2*, *Dcc*, *EphB2*, *Npn2*, and two genes important in axon extension *Tag1* and *Gap43*. In the *Fgfr1* mutant, cortical layer 5 neurons are present and still express all these genes, four of which are shown in Figs. 5E–H and four in Supplementary Fig. 2. The patterns of expression are consistent with previously described patterns, e.g. for *Otx1*, *Pou3f1*, *Cux2*, *Robo1*, *Robo2*, and *Dcc* (Frantz et al., 1994a,b; Gad et al., 1997; Shu et al., 2000; Zimmer et al., 2004).

Hippocampal and olfactory bulb neurons also appear to be generated normally. Both the morphology and the expression of the hippocampal marker, *Lhx9*, show no difference between control and mutant embryos at E16.5 (Fig. 5D). The hippocampus does appear slightly thinner in the mutant. Therefore, to assess whether this could be due to cell death, a detailed TUNEL analysis of the hippocampal anlagen at E10.5, E13.5, and E15.5 was performed. No significant differences in the number of dying neurons or precursor cells between the control and the mutant were detected (Supplementary Fig. 3 and data not shown). Hence, it is unlikely that cell death or a slight change in the size of the hippocampus accounts for the complete loss of the HC.

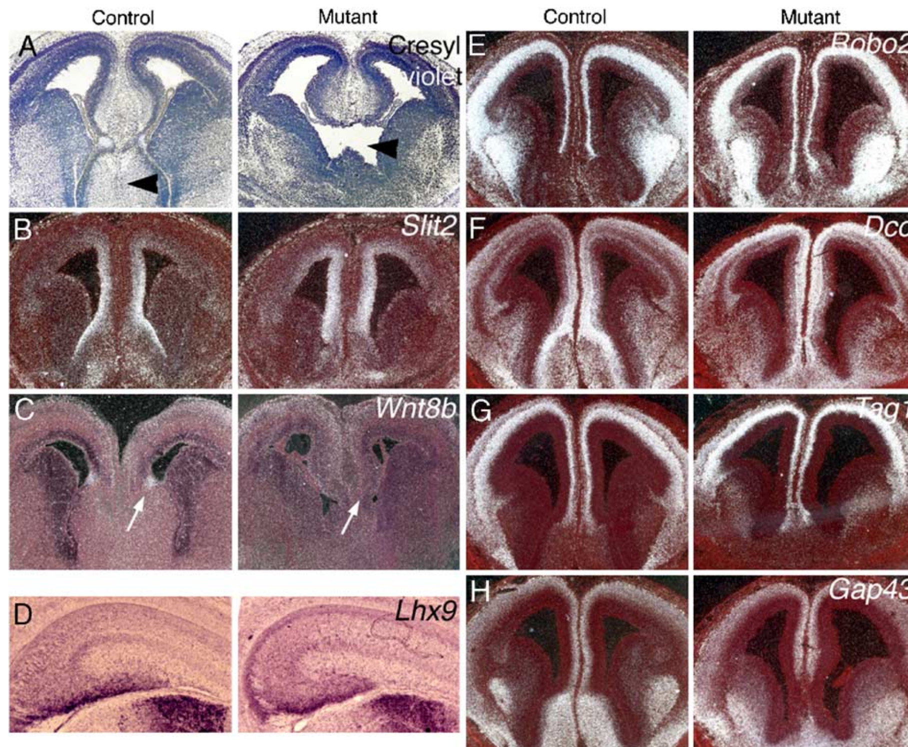

Fig. 5. In the Fgfr-deficient telencephalon, the midline expresses putative axon guidance cues and cortical neurons express axon guidance receptors. Coronal sections of E16.5 controls and *Fgfr1*;*Fgfr3* double mutants stained with Cresyl Violet (A), or hybridized to *Slit2* (B), or *Wnt8b* (C), *Robo2* (E), *Dcc* (F), *Tag1* (G), and *Gap43* (H) (^{35}S-labeled probes, white denotes expression). Note the lack of septal formation (arrowheads in panels A, B, E, G, H). The morphology and expression pattern of *Lhx9* in the hippocampus at E16.5 is normal in the mutant (D) (DIG-labeled probe, purple denotes expression).

A previous report has shown that olfactory bulb neurons, although not organized in a typical bulb like structure, are present in the *Fgfr1*-deficient telencephalon and extend axons (Hébert et al., 2003). Together, these results suggest that most or all neurons that project from one hemisphere to the other are generated normally in mutant embryos.

Commissure defects persist in the heterozygote despite normal midline patterning

Loss of both copies of *Fgfr1* in the telencephalon results in a complete loss of axonal midline crossing (Figs. 1, 2, 6G, J). To investigate whether commissural defects are sensitive to the level of FGF signaling, embryos heterozygous, instead of homozygous, for the *Fgfr1* allele were analyzed at E17.5 with DiI tracing and E18.5 with anti-Tag1 staining. In heterozygous embryos, an intermediate phenotype is observed: axons that form the CC and HC fail to cross the midline (n = 5; Fig. 6 arrow in panels C, I, arrowhead in panel D), whereas those that form the AC project normally (not shown). Interestingly, unlike in the homozygote, CC and HC axons in the heterozygote turn toward the midline before failing to cross it. This suggests that the axons are more capable of responding to a midline cue in the heterozygous mutant compared with the homozygous mutant. This difference in the behavior of the axons could reflect a sensitivity to *Fgfr1* gene dose either directly in the commissural neurons themselves or indirectly in cells that make up their environment.

If, in the *Fgfr1* heterozygous and homozygous mutant, callosal and hippocampal axons fail to cross the midline only because of the loss of midline glia, then a loss of these cells is also expected in the *Fgfr1* heterozygote, as observed in the homozygote (Fig. 3). Surprisingly, however, deletion of one copy of *Fgfr1* does not lead to a loss of midline glia; the midline zipper glia and the indusium griseum are present and appear normal (n = 3/3; Figs. 7B, D), as does the glial wedge (not shown). The septum is also present in the *Fgfr1* heterozygote (n = 11/11; not shown), consistent with the presence of the AC. This suggest that an FGF-dependent mechanism, other than the generation of specialized midline glia, is required for callosal and hippocampal axons to cross the midline.

Note that all the mutants analyzed, in addition to carrying mutations in Fgfr genes, also carry a mutant allele of *Foxg1*, due to insertion of the Cre recombinase at this locus. However, *Foxg1* heterozygosity is unlikely to contribute to the observed phenotypes. First, reduced FGF signaling without *Foxg1* heterozygosity in humans and zebrafish is sufficient to cause phenotypes comparable to the ones observed here (see Discussion). Second, loss of one copy of *Foxg1* does not result in a loss of midline cell types or in a failure of CC, HC, or AC axons to cross the midline (Figs. 6A, B, 7, and data not shown). This is consistent with what has previously been observed in heterozygous mutant *Foxg1* animals in which the corpus callosum develops regardless of strain background (Stewart Anderson, personal communication).

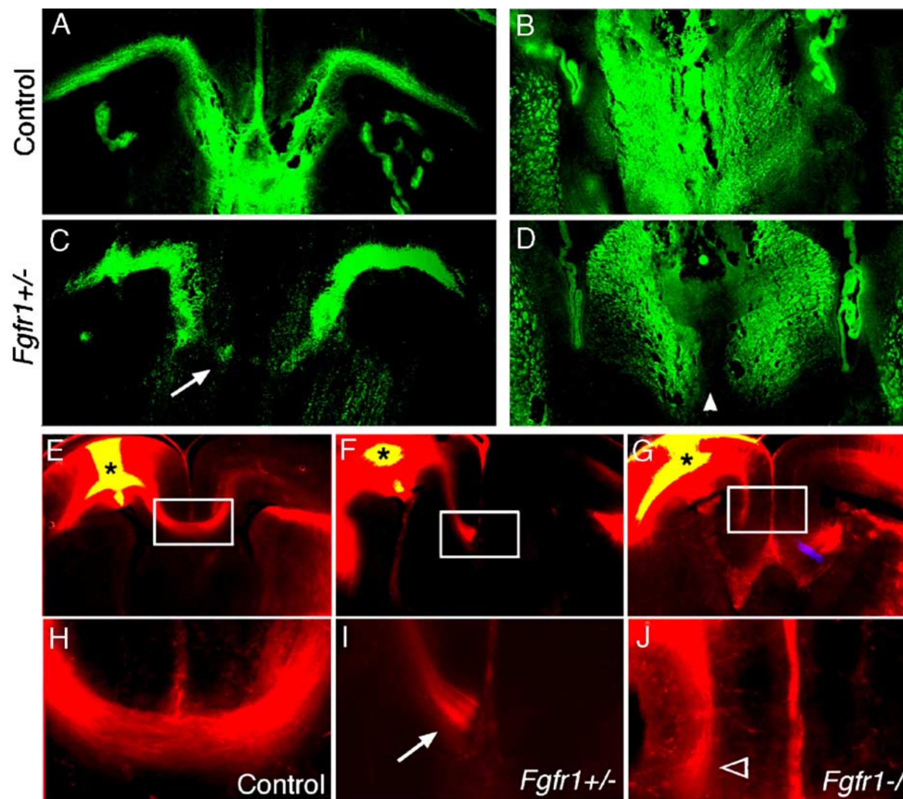

Fig. 6. Development of the telencephalic commissures is sensitive to gene dosage. E18.5 coronal sections of control (*Foxg1*$^{Cre/+}$) (A, B) and *Fgfr1* heterozygous mutant (C, D) brains stained with anti-Tag1 antibody and E17.5 coronal sections of DiI injected control (E, H), *Fgfr1* heterozygous (F, I), and *Fgfr1* homozygous mutant (G, J) brains. Sites of DiI crystals in the neocortex are indicated (asterisks in panels E, F, G) and the callosal axon tract is magnified in the bottom panel (H, I, J). In the *Fgfr1* heterozygote, callosal axons turn toward the midline (C, I; arrows) whereas in the homozygote they fail to turn toward the midline (J; open arrowhead). Likewise, in the heterozygote, HC axons accumulate near the midline, but do not cross (arrowhead in panel D). Note that the CC and HC develop normally in control mice that lack one copy of *Foxg1* due to the insertion of Cre (A, B).

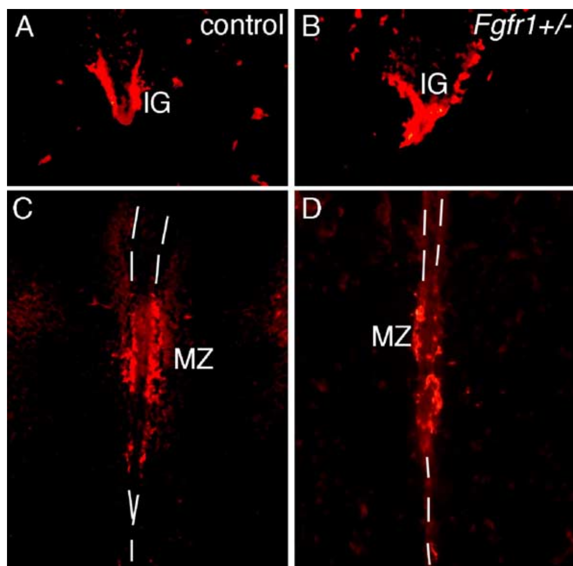

Fig. 7. The midline zipper glia and indusium griseum are normal in the *Fgfr1* heterozygote. Coronal sections of E16.5 control (left) and *Fgfr1*+/− mutant (right) brains stained with anti-GFAP antibody (dorsal is up). (A, B) Sections showing the indusium griseum (IG) in the control and mutant. (C, D) Sections anterior to those shown in panels A and B, showing the midline zipper glia (MZ); dotted white lines outline the medial border of the right and left hemispheres.

Discussion

Several steps are required for axons in each of the commissural tracts of the telencephalon to extend across the midline and reach their targets. Fig. 8 depicts these trajectories and how they are disrupted in the *Fgfr1*-deficient telencephalon. FGF signaling through FGFR1 is not required for early pathfinding steps. In the *Fgfr1* mutant, both CC and AC axons extend normally until they reach the area where they would normally turn toward the midline and instead stop or form Probst-like bundles. For instance, mutant callosal axons extend from the cortical plate to the intermediate zone, turn medially and project ventrally within this zone, but upon approaching the midline, they fail to turn sharply to cross it. Hence, FGF signaling is required for axons to cross the telencephalic midline.

Dual functions for Fgfr1 in commissure formation in the cerebrum

It is likely that there are two *Fgfr1*-dependent mechanisms required for the midline crossing of commissural axons in the telencephalon. The first is the FGF-dependent generation of midline cell types. In the *Fgfr1* mutant, several midline structures along which the axons normally project fail to

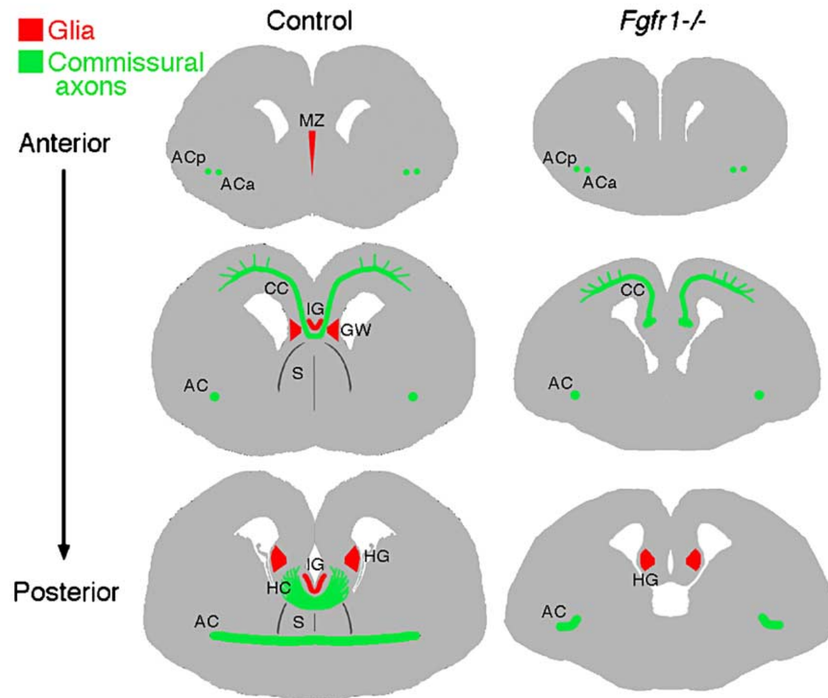

Fig. 8. FGF-dependent midline structures are essential for commissural axon crossing. In anterior regions of the telencephalon in E16.5 animals, commissural axons (green) cross the midline, whereas in control embryos, they fail to do so. In the mutant, axons from the corpus callosum (CC) and both branches of the anterior commissure (AC), the anterior (ACa) and posterior (ACp) branches, initially project normally compared to the control, but then fail to turn and cross the midline. Hippocampal commissural axons (HC) also fail to reach the midline in the mutant. The septum (S) and three midline glial structures, the midline zipper (MZ), the indusium griseum (IG), and the glial wedge (GW), which fail to develop in the mutant, are likely to play key roles in guiding commissural axons across the midline. The hippocampal glia (HG) are still present in posterior regions of the mutant.

develop. The CC normally extends across the midline between the indusium griseum and glial wedge; the HC crosses between the dorsal part of the septum and indusium griseum; and the AC crosses along the ventral part of the septum and the preoptic area of the ventral diencephalon. In the *Fgfr1* mutant, the septum, indusium griseum glia, and glial wedge are missing, suggesting that the axons may no longer be able to reach the midline because the cell types and structures that normally provide outgrowth and guidance cues are absent. In addition, the midline zipper glia, which are likely to be required for bridging the telencephalic hemispheres (Silver et al., 1993; Shu et al., 2003a; Richards et al., 2004), are missing in the mutant. Without this bridge, commissural axons do not have a substrate on which to extend axons through the midline (Fig. 3).

The glial wedge has previously been shown to guide callosal axons (Shu and Richards, 2001). In organotypic slice cultures in which the glial wedge is removed or in *Nfia* mutants in which it does not form, callosal axons continue projecting ventrally into the septum instead of turning toward the midline and crossing (Shu and Richards, 2001; Shu et al., 2003b). Consistent with this, in the *Fgfr1* mutant, in which the glial wedge is also missing, callosal axons also fail to turn toward and cross the midline. However, callosal axons were not observed to project as far ventrally, perhaps because of the lack of a septum in the mutant.

The second mechanism by which *Fgfr1* is required for at least the callosal and hippocampal axons to cross the midline is independent of the generation of the indusium griseum, midline zipper glia, glial wedge, and septum. In the heterozygous *Fgfr1* mutant, unlike the homozygous mutant, these midline cell types are present and appear normal, but the CC and HC still fail to cross the midline. One possibility is that FGF signaling, in addition to forming essential midline structures, could play a direct role in promoting the outgrowth or guidance of commissural axons toward and across the midline. *Fgfr1* is expressed at high levels in telencephalic precursor cells throughout development, including midline precursors, at low levels in the lateral neocortical plate, and at high levels in midline cortical neurons (Fig. 4E). Receptor expression in cortical and midline neurons could potentially play a role in guiding their commissural axons. Consistent with this possibility, CC and HC axons in the *Fgfr1* heterozygous mutant progress further toward the midline than those in the homozygous mutant, suggesting that these axons may be especially sensitive to levels of FGF signaling (Fig. 6). FGFs have previously been shown to act directly on non-commissural neurons to guide the trajectories of their axons (McFarlane et al., 1996; Irving et al., 2002), lending further support to their potential direct role in guiding commissural axons.

Alternatively, in the *Fgfr1* mutant (Figs. 1, 2, 6G, J), commissural axons may fail to turn toward the midline and cross it due to the absence of guidance pathways other than FGF signaling. However, an analysis of potential midline-derived pathfinding molecules, *Slit2*, *netrin1*, and *Bmp4*, failed to show a loss of expression of these molecules. It remains

possible that the FGF-dependent expression of other, yet to be identified, midline guidance cues may be critical.

Fgfr1 and its interaction with other genes in forming midline structures

Interestingly, mouse telencephalons that are null for *Ext1*, a gene that encodes an enzyme that polymerizes heparan sulfate, have a very similar phenotype as *Fgfr1*-deficient telencephalons. Similarities include loss of olfactory bulbs, of all three commissural tracts, and of midline structures (Inatani et al., 2003; Hébert et al., 2003). FGFs have previously been shown to bind heparan sulfate (Selleck, 2000). Although the *Ext1* mutant mice were not stained for GFAP, the similarity between the *Ext1* and *Fgfr1* mutants suggests that heparan sulfate is required specifically for signaling through FGFR1 in forming critical midline glial and septal structures. In addition, growing evidence suggests that heparan sulfate proteoglycans are directly involved in axon guidance (reviewed in Lee and Chien, 2004), opening up the possibility that guidance in these cases occurs in part via FGFs.

In humans, partial or complete loss of the corpus callosum has been associated with over 50 congenital syndromes (Richards et al., 2004). Although many genes have been identified that lead to loss of the CC in both humans and mice, fewer genes have been found to be required for the HC or AC to form, and fewer still are required for all three commissural tracts to form. Most of the genes implicated are not suspected of playing direct roles in guiding growth cones, but the mechanism by which they lead to commissural defects is unknown. In humans, reduced levels of signaling through *FGFR1* lead to Kallmann syndrome, which can include a loss of the CC and olfactory bulbs (Dode et al., 2003), phenotypes mimicked by loss of *Fgfr1* in mice (Hébert et al., 2003; this study), indicating that the function of this receptor in patterning the telencephalon is conserved in mammals and that the *Fgfr1*-deficient mouse used here provides a useful model for studying Kallmann syndrome.

The conservation of FGF function in patterning the midline of the telencephalon is likely to extend to all vertebrates. In zebrafish, loss of *Fgf8* leads to disruption of the midline, resulting in severe commissural axon pathway defects (Shanmugalingam et al., 2000; Walshe and Mason, 2003). The midline structures affected, however, were not identified. In mammals, two midline glial structures, the indusium griseum and midline zipper, share morphological and molecular characteristics that are distinct from other glial cell types (including the glial wedge) and are thus likely to both be derived from a common group of precursor cells (Shu et al., 2003a). FGFs are the first factors specifically implicated in the induction of this cell type. The study presented here also demonstrates the novel requirement for FGFR1 in the normal development of the septum. Given the expression pattern of *Fgf8* and the different Fgfr genes in the telencephalon (Fig. 4) and that loss of *Fgf8* also results in commissural defects in zebrafish, it is likely that FGF8 acts through FGFR1 to specify midline glial and septal cell types.

Loss of SHH signaling also causes loss of midline cell types, leading to holoprosencephaly in humans and mice (Hayhurst and McConnell, 2003). It now becomes of interest to examine the interactions between SHH and FGF signaling in inducing midline cell types. In sum, FGF signaling plays a critical role in bridging the hemispheres by forming midline cell types and promoting the midline crossing of commissural axons.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.10.020.

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