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FGF signalling generates ventral telencephalic cells independently of SHH

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Sonic hedgehog (SHH) is required to generate ventral cell types throughout the central nervous system. Its role in directly specifying ventral cells, however, has recently been questioned because loss of the *Shh* gene has little effect on ventral development if the *Gli3* gene is also mutant. Consequently, another ventral determinant must exist. Here, genetic evidence establishes that FGFs are required for ventral telencephalon development. First, simultaneous deletion of *Fgfr1* and *Fgfr3* specifically in the telencephalon results in the loss of differentiated ventromedial cells; and second, in the *Fgfr1*;*Fgfr2* double mutant, ventral precursor cells are lost, mimicking the phenotype obtained previously with a loss of SHH signalling. Yet, in the *Fgfr1*;*Fgfr2* mutant, *Shh* remains expressed, as does *Gli1*, the transcription of which depends on SHH activity, suggesting that FGF signalling acts independently of SHH to generate ventral precursors. Moreover, the *Fgfr1*;*Fgfr2* phenotype, unlike the *Shh* phenotype, is not rescued by loss of *Gli3*, further indicating that FGFs act downstream of *Shh* and *Gli3* to generate ventral telencephalic cell types.

KEY WORDS: Basal ganglia, Ganglionic eminence, FGF, SHH, GLI3, Telencephalon, Patterning, Mouse

INTRODUCTION

The vertebrate brain comprises a multitude of neural cell types organized into distinct functional areas. How are these areas generated during development? The adult cerebral hemispheres, derived from the embryonic telencephalon, are composed largely of two areas: the dorsal cerebral cortex, which is the seat of our highest cognitive and perceptual functions; and the ventral basal ganglia, which have multiple functions related to motor coordination, cognition and emotions. Homeobox transcription factors are required to specify precursor cells as dorsal or ventral (Wilson and Rubenstein, 2000; Campbell, 2003; Zaki et al., 2003; Hébert, 2005). For example, in embryos mutant for *Pax6* and *Emx2*, two genes expressed in the dorsal telencephalon, precursor cells fail to adopt or maintain a cortical fate and instead adopt, at least in part, the fate of basal ganglia cells (Muzio et al., 2002). Conversely, homeobox genes expressed in the ventral telencephalon, such as *Gsh2*, *Nkx2.1* and *Dlx2*, and the bHLH gene *Mash1* (*Ascl1* – Mouse Genome Informatics) are essential for proper specification of ventral fates (Anderson et al., 1997; Szucsik et al., 1997; Casarosa et al., 1999; Horton et al., 1999; Sussel et al., 1999; Corbin et al., 2000; Toresson and Campbell, 2001; Yun et al., 2003). Although progress has been made in understanding how morphogens might restrict expression of these genes to ventral or dorsal domains, many points remain unclear (Hébert, 2005).

Sonic hedgehog (SHH) is a secreted factor that is required for the induction of ventral cell types throughout the neural tube, including the forebrain. In the telencephalon, loss of SHH signalling leads to a loss of ventral cell types that normally express *Dlx2*, *Gsh2* and *Nkx2.1*, at the expense of dorsal cell types that express *Pax6* and *Emx2* (Ericson et al., 1995; Chiang et al., 1996; Ohkubo et al., 2002; Fuccillo et al., 2004). Likewise, ectopic expression of the *Shh* gene can induce expression of *Dlx2* and *Nkx2.1* in the dorsal telencephalon of zebrafish and mice (Ericson et al., 1995; Barth and Wilson, 1995; Hauptmann and Gerster, 1996; Shimamura and Rubenstein, 1997; Kohtz et al., 1998). In the spinal cord, SHH is thought to act directly on neural precursor cells to induce ventral features (Jessell, 2000; Briscoe and Ericson, 2001).

Although SHH signalling is essential for telencephalic precursor cells to adopt a ventral fate, it is not strictly necessary for this process. The zinc-finger transcription factor gene *Gli3* antagonizes *Shh* and dorsalizes the telencephalon (Grove et al., 1998; Theil et al., 1999; Aoto et al., 2002; Kuschel et al., 2003; Rallu et al., 2002). Remarkably, embryos mutant for both *Shh* and *Gli3* exhibit grossly normal dorsoventral patterning, implying that other factors can pattern this axis of the telencephalon (Aoto et al., 2002; Rallu et al., 2002). Candidates for the ventralizing signal are the fibroblast growth factors (FGFs).

The anterior neural ridge at the anterior end of the neural plate forms the rostral midline once the neural tube has closed. In mice, both the ridge and rostral midline express several FGF genes, *Fgf8*, *Fgf14*, *Fgf15*, *Fgf17* and *Fgf18* (McWhirter et al., 1997; Maruoka et al., 1998; Xu et al., 1999; Crossley et al., 2001; Wang et al., 2000). Three FGF receptor genes are widely expressed in neural precursor cells, *Fgfr1*, *Fgfr2* and *Fgfr3* (Orr-Urtreger et al., 1991; Peters et al., 1992; Peters et al., 1993; Hébert et al., 2003). FGFs have been postulated to specify both dorsal and ventral cell types in the telencephalon. In chick embryos, FGF signalling, following activation of the Wnt pathway is required for inducing expression of dorsal features, such as expression of the neocortical marker *Emx1* (Gunhaga et al., 2003). In zebrafish, FGF signalling is required for the ventral telencephalon to form. The expression of markers for specific populations of ventral neurons is diminished or absent in *fgf8* mutants (Shanmugaligam et al., 2000). When FGF signalling is further decreased by inhibition of the intracellular signalling pathway or by knocking down *fgf8* and *fgf3* expression using morpholinos, ventral precursor cells expressing *dlx2* or *nk2.1b* are reduced or absent (Shinya et al., 2001; Walshe and Mason, 2003). In

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chick embryos, application of soluble FGFR4 leads to a loss of *Nkx2.1*-expressing telencephalic cells (Marklund et al., 2004). Moreover, in mice, FGF8-soaked beads can induce expression of ventral markers in dorsal telencephalic explants in culture (Kuschel et al., 2003). Whether FGF signalling is required in the embryonic mammalian telencephalon for specifying either dorsal or ventral telencephalic cells remains unknown. Moreover, how FGFs interact with other signalling molecules, such as SHH, is unclear.

In this study, the role of FGF signalling in patterning the dorsoventral axis of the telencephalon is examined. Using a conditional genetic approach in the mouse, FGF signalling is disrupted specifically in the telencephalon at the earliest stages of its development. All combinations of two receptors are deleted and examined for patterning defects. Although *Fgfr2*;*Fgfr3* double mutants that retain only one functional copy of *Fgfr1* appear grossly normal, *Fgfr1*;*Fgfr3* and *Fgfr1*;*Fgfr2* mutants exhibit severe ventral phenotypes. In the *Fgfr1*;*Fgfr3* mutant, ventromedial precursor cells are specified but fail to differentiate, whereas in the *Fgfr1*;*Fgfr2* mutant, precursor cells with ventral characteristics fail to develop and cells all along the dorsoventral axis adopt a dorsal fate. This occurs despite evidence of active SHH signalling. Furthermore, although the *Shh* phenotype can be rescued by loss of *Gli3* expression (Rallu et al., 2002), the *Fgfr1*;*Fgfr2* phenotype cannot, placing FGF signalling downstream of these genes in generating ventral cells.

MATERIALS AND METHODS

Generation of mutant embryos

To study the role of FGF signalling in patterning the ventral telencephalon, crosses were designed to generate mice mutant for all three combinations of two FGFR genes expressed in the telencephalon (Fig. 1A). A loss of *Fgfr1* or *Fgfr2* in the whole embryo leads to lethality at gastrulation (Deng et al., 1994; Yamagushi et al., 1994; Arman et al., 1998), therefore floxed alleles of these genes were used to generate telencephalic specific knockouts when crossed to *Foxg1*^{cre} mice, as previously described (Hébert et al., 2003; Yu et al., 2003). *Fgfr1*$^{lox/+}$, *Fgfr1*$^{lox/+}$;*Fgfr3*$^{null/+}$ and *Fgfr1*$^{lox/+}$;*Fgfr2*$^{lox/+}$ carrying the *Foxg1*^{cre} allele do not exhibit the phenotypes described in this study and were used as controls. The *Fgfr3* allele used is a null allele (Deng et al., 1996). As *Fgfr3* homozygous null mice are viable but breed poorly, heterozygotes were used in the cross. Mutant embryos were obtained at the ages indicated in the expected ratios with no signs of necrosis. A total of 19 *Foxg1*$^{cre/+}$;*Fgfr1*$^{lox/lox}$;*Fgfr3*$^{null/null}$, three *Foxg1*$^{cre/+}$;*Fgfr2*$^{lox/lox}$;*Fgfr3*$^{null/null}$ and 31 *Foxg1*$^{cre/+}$;*Fgfr1*$^{lox/lox}$;*Fgfr2*$^{lox/lox}$ embryos were used and at least three embryos of each genotype were used for each experiment. Mice carrying the *Gli3*-null (*extra toes*) allele were obtained from Jackson Laboratories.

RNA is situ hybridization and immunohistochemistry

In situ hybridization was carried out according to two protocols: one using radioactive RNA probes on sections and the other DIG labelled probes in whole mount. For the radioactive in situ, frozen sections were prepared and hybridized to ^{35}S-labelled probes, as previously described (Frantz et al., 1994). For the in situ hybridization of whole-mount mouse embryos using digoxigenin-labelled probes, the procedure was performed as described (Henrique et al., 1995) with the exception that BM purple (Roche) was used instead of NBT/BCIP to reveal expression patterns. A minimum of three mutant and three control embryos were analyzed for each probe (except for the E16.5 *Fgfr1;Fgfr2* double mutant in Fig. 4 for which only one viable embryo was obtained and analysed). Immunohistochemistry for NPY was performed as previously described (Marin et al., 2000) with a 1:2500 dilution of polyclonal rabbit serum (generous gift from S. A. Anderson).

BrdU incorporation and TUNEL assays

Female mice pregnant with E9.5 and E10.5 embryos receive an intraperitoneal injection with BrdU and were euthanized 1 hour later. At these embryonic ages, Cre-mediated deletion of *Fgfr1* and *Fgfr2* is complete (Hébert et al., 2003) and precursor cells acquire ventral fates. Embryos were collected and frozen in OCT. Fresh frozen sections were used for either BrdU staining, as previously described (Hébert et al., 2003), or for TUNEL analysis according to the manufacturer's specifications (Roche, Catalogue # 2 156 792). Coronal sections for control and mutants were matched so that they correspond to the same position along the anteroposterior axis of the telencephalon. Sections were counterstained with Syto11 (Molecular Probes). The fraction of BrdU or 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). Segments used to count dorsal cells are at 45° ventral to the dorsal midline and ventral segments are 45° dorsal to the ventral midline. Midline segments are defined as an area encompassing 200 cells and obtained by drawing a line through the points were the telencephalic hemispheres meet dorsally and ventrally. A 100 Syto11-positive cells are counted on either side of these points to establish the 200-cell segment. TUNEL- or BrdU-positive cells within this segment are then counted. At least two segments from each of three separate embryos are counted in each case.

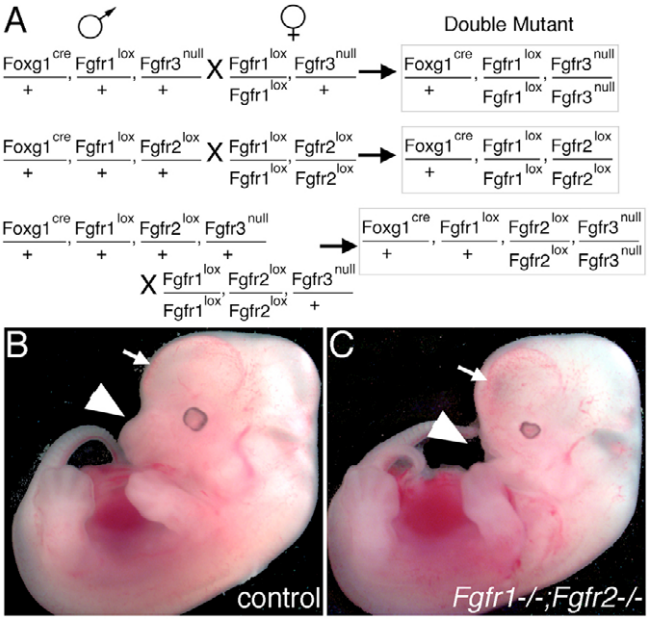

Fig. 1. All three FGFR double mutants are generated. (**A**) Crosses designed to obtain the three possible combinations of FGFR double mutants. The *Fgfr1* and *Fgfr2* mutant alleles are floxed, whereas the *Fgfr3* allele is a null. The embryos homozygous mutant for *Fgfr2* and *Fgfr3* are also heterozygous mutant for *Fgfr1*. Whole E12.5 control (**B**) and *Fgfr1;Fgfr2* double mutant (**C**) embryos. The mutant, as well as having a smaller telencephalon (arrow), loses the frontonasal process (arrowhead).

Cell cycle exit assay

Female mice carrying E10.5 embryos were injected with BrdU. Embryos were collected at E11.5 and embedded in OCT. Cryosections (10 μm) were fixed (4% paraformaldehyde), boiled in 10 mM sodium citrate for Ki67 antigen retrieval, blocked (5% goat serum, 0.1% Triton X-100), incubated overnight at 4°C with Ki67 antibody (1:100, Novocastra monoclonal), incubated 1 hour with FITC-coupled secondary, post-fixed, treated with 2 N HCl, neutralized with 100 mM Na Borate, blocked, incubated with rat anti-BrdU (1:1,000, Oxford Biotechnology), incubated with Texas Red-coupled anti-rat secondary and counterstained with Hoechst 33342. Cells in sectors corresponding to the MGE area were counted as described above for BrdU and TUNEL cell counts.

RESULTS

Mutants that lack any two FGFR genes in the telencephalon are viable to at least E14.5

To test the role of FGFs in patterning the ventral telencephalon, FGF signalling was disrupted genetically. Genes encoding FGF receptors rather than ligands were targeted because there are only three receptors expressed in the telencephalon, *Fgfr1*, *Fgfr2* and *Fgfr3*, compared with at least five ligands (Orr-Urtreger et al., 1991; Peters et al., 1992; Peters et al., 1993; McWhirter et al., 1997; Maruoka et al., 1998; Xu et al., 1999; Wang et al., 2000; Crossley et al., 2001; Hébert et al., 2003). In addition, the disruption of ligand gene expression can have indirect effects on telencephalic precursors by affecting the development of neighbouring ectodermal or mesodermal tissues. *Fgfr1* and *Fgfr2* were deleted in the telencephalon using a Cre/*loxP* approach to bypass the early embryonic lethality associated with whole-embryo knockouts of these genes (Deng et al., 1994; Yamaguchi et al., 1994; Arman et al., 1998). Loss of *Fgfr3* or *Fgfr2* on their own does not lead to significant patterning defects (data not shown), whereas loss of *Fgfr1* in the telencephalon leads to an olfactory bulb defect (Hébert et al., 2003). In this study, all combinations of FGFR double mutants are generated using the crosses depicted in Fig. 1A. All three double mutant embryos were viable to at least E14.5. Except for the lack of a nasal process and a smaller telencephalon in the *Fgfr1*;*Fgfr2* double mutant (Fig. 1C), no gross morphological defect could be identified upon observing whole E12.5 embryos.

The loss of one copy of *Foxg1* resulting from insertion of *cre* at this locus could in theory contribute to the defects observed with loss of FGFR genes described below. However, (1) as *Foxg1* heterozygosity on its own does not lead to any patterning defect between E9.5 and E12.5, (2) as no genetic interaction is revealed between *Foxg1* and FGFR genes even in the $Foxg1^{cre/+};Fgfr1^{+/-};Fgfr2^{-/-};Fgfr3^{-/-}$ mutant (Fig. 2B,F,J), and (3) as phenotypes analogous to the ones described here implicating FGF signalling in ventral telencephalic development have been obtained in other systems in which *Foxg1* is wild type (see Discussion), it is unlikely that loss of one copy of *Foxg1* contributes to the observed phenotypes.

One copy of *Fgfr1* is largely sufficient for normal dorsoventral patterning

The telencephalon of E12.5 double mutant embryos was examined by RNA in situ hybridization analysis using probes for *Pax6* and *Emx2* (dorsal markers), *Dlx2* and *Mash1* (ventral markers), and *Nkx2.1* (ventromedial marker). The *Fgfr2*;*Fgfr3* mutant displayed grossly normal morphology at E12.5 (Fig. 2B,F,J). This mutant, in addition to being deficient in *Fgfr2* and *Fgfr3*, lacks one copy of *Fgfr1* (Fig. 1A). Despite this, normal patterns of *Pax6*, *Dlx2* and *Nkx2.1* expression are observed (Fig. 2B,F,J), indicating that one copy of *Fgfr1* is largely sufficient to pattern the dorsoventral axis of the telencephalon. Owing to the lack of an obvious patterning defect in this mutant, further analyses focused on the *Fgfr1*;*Fgfr3* and *Fgfr1*;*Fgfr2* mutants.

Generation of ventral telencephalic precursor cells requires FGF signalling

In the *Fgfr1*;*Fgfr3* mutant, the dorsoventral borders of *Pax6*, *Dlx2*, *Nkx2.1* and *Mash1* expression match those found in control embryos (Fig. 2C,G,K arrowheads; Fig. 4K), suggesting that the specification of dorsal and ventral precursors has occurred normally. However, the morphology of the ventral telencephalon in this mutant is abnormal. No sulcus forms between areas where the medial and lateral ganglionic eminences normally differentiate. In addition, the septum is missing. This phenotype is also observed in the *Fgfr1* single mutant (Tole et al., 2006). Loss of the normal ventral morphology, without loss of ventral precursor cells, suggests that a process other than specification of ventral precursors, such as cell differentiation or survival, is disrupted in the *Fgfr1*;*Fgfr3* mutant (addressed below).

By contrast, in the *Fgfr1*;*Fgfr2* mutant, not only is ventral morphology disrupted, but normal dorsoventral patterning is lost. In this mutant, a high level of *Pax6* and *Emx2* expression extends to the ventral midline instead of ending approximately midway down the dorsoventral axis (Fig. 2D; Fig. 7I), whereas the domains of *Dlx2, Nkx2.1* and *Mash1* expression are greatly reduced or completely lost in the telencephalon (Fig. 2H,L; Fig. 4L; Fig. 7L). The remaining *Dlx2* and *Nkx2.1* expression near the ventral midline is likely to be hypothalamic as, in adjacent sections, expression of the

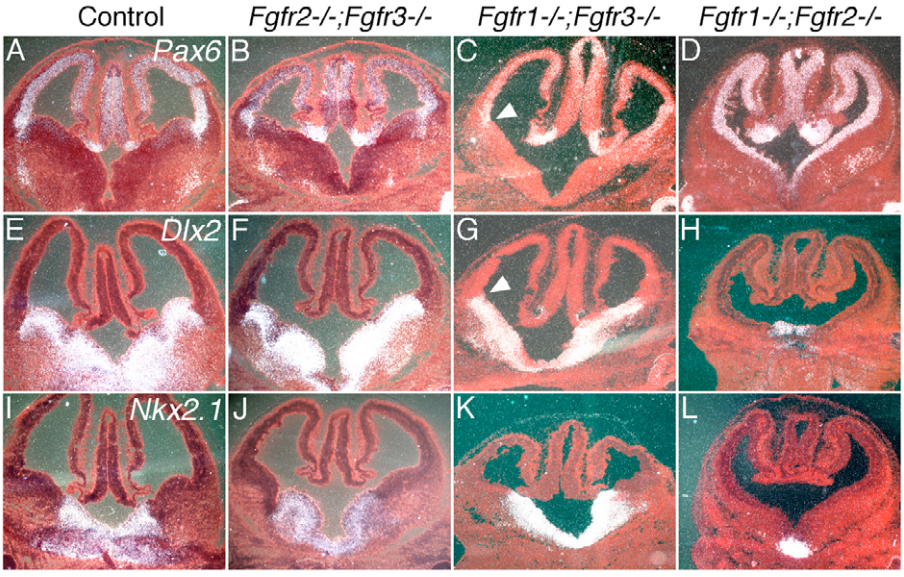

Fig. 2. Ventral cell types are lost in FGFR double mutants. RNA in situ hybridization analysis of E12.5 coronal brain sections using radioactive probes. (**A**,**E**,**I**) In the control embryo, expression of *Pax6* (A), *Dlx2* (E) and *Nkx2.1* (I) are used to identify the dorsal, ventral and ventromedial areas of the telencephalon. (**B**,**F**,**J**) In the $Fgfr2^{-/-};Fgfr3^{-/-}$ mutant, dorsoventral patterning and morphology of the telencephalon are grossly normal. (**C**,**G**,**K**) In the $Fgfr1^{-/-};Fgfr3^{-/-}$ mutant, although the borders of expression of Pax6 (C), Dlx2 (G) and Nkx2.1 (K) are normal (arrowheads), indicating proper dorsoventral specification of precursor cells, the morphology of the ventral telencephalon is abnormal and flattened in appearance. (**D**,**H**,**L**) In the $Fgfr1^{-/-};Fgfr2^{-/-}$ mutant, ventral precursor cells are lost, indicated by expression of *Pax6* that extends to the ventral midline (D) and by the loss of *Dlx2* (H) and *Nkx2.1* (L) expression.

telencephalic marker *Foxg1* is reduced or absent in this area, whereas expression of the hypothalamic marker *Foxd1* is increased (see Fig. S1 in the supplementary material). This phenotype demonstrates that *Fgfr1* and *Fgfr2* are required together to generate ventral telencephalic precursor cells.

To assess whether the observed phenotypes might be due to changes in cell survival or proliferation, *Fgfr1*;*Fgfr3* and *Fgfr1*;*Fgfr2* mutants were analyzed using TUNEL and BrdU incorporation assays at E10.5, a stage when recombination of the floxed alleles in the mutant is complete and prior to the start of neurogenesis. Sections of telencephalons were partitioned into defined sectors that include dorsal, ventral and dorsomedial components (see Materials and methods). No difference in the percentage of cells incorporating BrdU or staining with TUNEL is observed in any sector between control and *Fgfr1*;*Fgfr3* mutant embryos. However, statistically significant differences are observed between control and *Fgfr1*;*Fgfr2* mutant embryos (Fig. 3A,B). Namely, slightly less proliferation is observed in the ventral telencephalon of this mutant compared with control (42.9±1.2% versus 48.8±2.1%, P=0.02) and an increase in apoptosis is observed in the dorsal midline of the mutant (21.3±2.6% versus 13.5±1.5%, P<0.0001). The reduced levels of ventral proliferation in the *Fgfr1*;*Fgfr2* double mutant could in part explain the loss of ventral precursor cells.

Another possibility is that ventral precursor cells fail to be specified in the *Fgfr1*;*Fgfr2* mutant. To address this point, we have analyzed the expression of the ventral telencephalic marker *Nkx2.1* in the E9.25-9.75 mutant. Unlike other ventral markers, *Nkx2.1* is readily detectable in control embryos at this age. In addition, at this age, expression of *Nkx2.1* can be associated on a morphological basis with the telencephalon as opposed to the hypothalamus (or ventral diencephalon) where it is also expressed. Consistent with a role for FGF signalling in specifying ventral precursor cells, telencephalic, but not diencephalic, expression of *Nkx2.1* is lost in the E9.25-E9.75 *Fgfr1*;*Fgfr2* double mutant (Fig. 3C-F).

Differentiation of ventral telencephalic cells requires FGF signalling

In the *Fgfr1*;*Fgfr3* mutant, the loss of the septum and of the sulcus between the ganglionic eminences without a loss of ventral precursor cells suggests that differentiation of ventral cell types in the telencephalon is impaired. Therefore the expression of genes that mark differentiating ventral cells was examined. Expression of *Lhx6*, *Lhx7* and *Shh*, which is normally found in the differentiating field of the medial ganglionic eminence and septal areas, is almost absent in the mutant (Fig. 4B,E and not shown), whereas expression of *Ebf1*, which marks differentiating cells of the lateral ganglionic eminence, is unaffected (Fig. 4H). Together with the TUNEL and BrdU results described above, this suggests that FGF signalling is required to promote the differentiation, rather than the survival, of ventromedial cell types. In the *Fgfr1*;*Fgfr2* mutant, not only is *Lhx6*, *Lhx7* and *Shh* expression lost, but so is *Ebf1* (Fig. 4C,F,I), as would be expected, given the loss of ventral precursor cells (Fig. 2D,H,L).

In order to determine whether differentiation of ventral cell types was affected in older FGFR mutant embryos, we examined the presence of ventral and dorsal neurons at E16.5. Consistent with the early loss of all differentiated ventral cell types in the *Fgfr1*;*Fgfr2* mutant (Fig. 4C,F,I), expression of a ventral marker for GABAergic interneurons, *Gad67*, is lost in the ventral telencephalon at the expense of the dorsal neuronal marker, *Tag1* (Fig. 4O,R,S). However, in the *Fgfr1*;*Fgfr3* mutant, the domains of *Gad67* and *Tag1* expression are similar to those in controls (Fig. 4M,N,P,Q), as might be expected, as neurons derived from the LGE are still produced at earlier ages (Fig. 4H). However, expression of NPY, a marker for a subclass of migratory interneurons derived from *Nkx2.1*-positive precursors in the MGE (Marin et al., 2000; Anderson et al., 2001), are missing in the *Fgfr1;Fgfr3* mutant at E18.5 (see Fig. S2 in the supplementary material), consistent with the early loss of MGE-derived neurons (Fig. 4B,E).

To address the nature of disrupted neurogenesis in the ventral medial area of the *Fgfr1;Fgfr3* mutant, the expression of genes that normally mark the germinative layers of the MGE, the ventricular zone (VZ) and subventricular zone (SVZ), was examined. At E12.5, expression of *Hes5* and *Lhx2* normally marks the VZ, whereas *Prox1* marks the SVZ. In the mutant, however, elevated levels of *Prox1* expression are found in the VZ, coincident with apparent reductions in the levels of *Hes5* and *Lhx2* expression (Fig. 5A-C). This suggests that there is a loss of VZ precursor cells at the expense of SVZ precursors, which does not readily explain the loss of neurons.

To further address the cause underlying the disrupted neurogenesis, the rates of cell cycle exit and cell death were examined at E11.5, a stage when neurons have just begun to be generated. Although mutants exhibited a significantly higher percentage of TUNEL-labelled cells in the ventral medial area compared with controls (0.8% versus 0.2%, P<0.03; Fig. 5F), it is

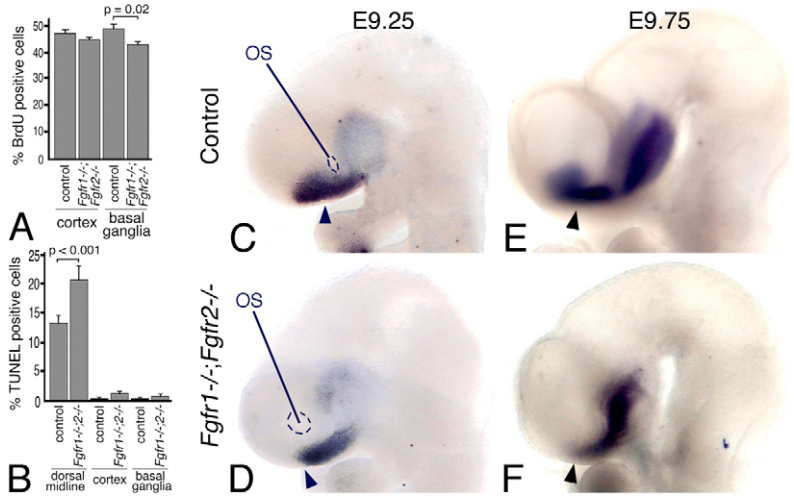

Fig. 3. Cell fate, proliferation, and death are perturbed in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant. (**A**,**B**) BrdU incorporation and TUNEL analyses of E10.5 control and mutant telencephalons. Slightly lower rates of BrdU incorporation in the ventral telencephalon (A) and higher rates of cell death in the dorsal midline (B) are observed in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant compared with controls. (**C-F**) Whole-mount in situ hybridization. The more anterior domain of *Nkx2.1* expression in the forebrain is absent as early as E9.25 in the mutant. Arrowheads indicate the putative telencephalic-diencephalic ventral border. OS, optic stalk.

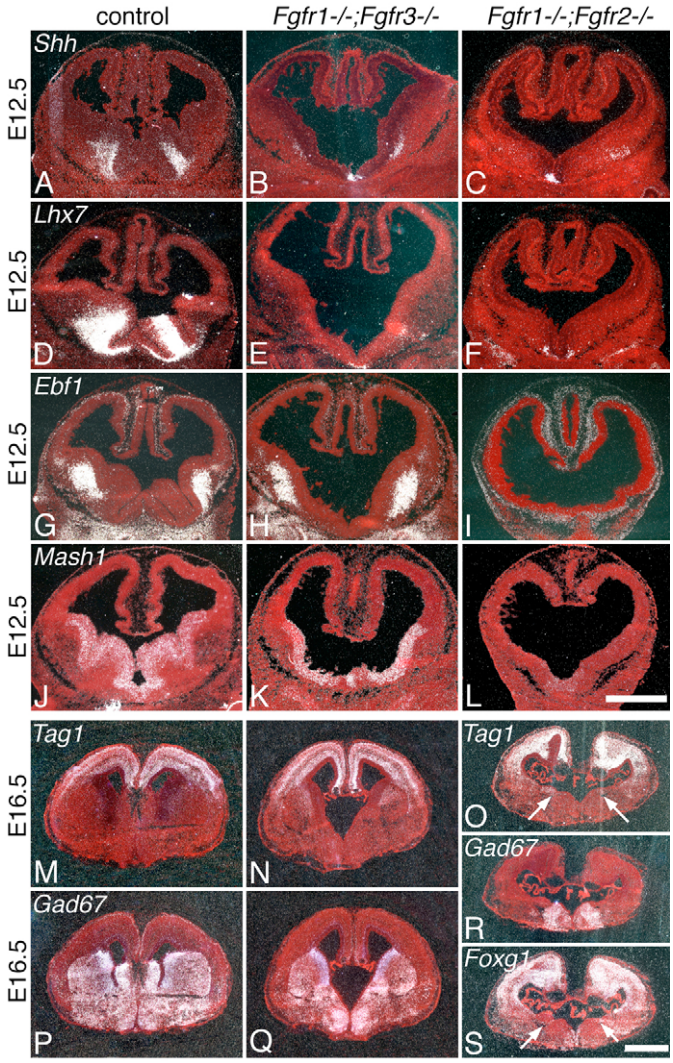

Fig. 4. Neurogenesis is perturbed in the *Fgfr1*$^{-/-}$;*Fgfr3*$^{-/-}$ and *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutants. (**A-L**) RNA in situ hybridization analysis of E12.5 coronal brain sections using radioactive probes. *Shh* and *Lhx7* are normally expressed in differentiating cells of the medial ganglionic eminence (A,D); however, this expression is lost in the mutants (B,C,E,F). *Ebf1*, on the other hand, which is normally expressed in differentiating cells of the lateral ganglionic eminence (G), remains expressed in the *Fgfr1*$^{-/-}$;*Fgfr3*$^{-/-}$ mutant (H), but not the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant (I). Mash1, which is normally expressed in ventral precursors (J), remains expressed in the *Fgfr1*$^{-/-}$;*Fgfr3*$^{-/-}$ (K) but not the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ (L) mutant. (**M-S**) RNA in situ hybridization of E16.5 coronal brain sections. *Gad67* expression is lost in *Foxg1*-expressing areas (arrows) of the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant at the expense of *Tag1* expression (O,R,S), whereas in the *Fgfr1*$^{-/-}$;*Fgfr3*$^{-/-}$ mutant, the boundaries of *Gad67* and *Tag1* expression are normal (M,N,P,Q). Scale bars: 0.5 mm in A-L; 1 mm in M-S.

questionable whether this increased level of cell death, which is still low, can fully account for the dramatic loss of neurons. To assess the rate of cell cycle exit, pregnant females were injected with BrdU 24 hours prior to collecting E11.5 brains. These were then double labelled in sections for Ki67, which marks all cycling cells (Scholzen and Gerdes, 2000), and BrdU, which marks cells that had been cycling a day before. The fraction of double-labelled (BrdU+Ki67) cells over total BrdU-labelled cells is 66% in mutants compared with 25% in controls (P<0.0001; Fig. 5D,E). This change is likely to be in large part responsible for the lack of neurons observed in the *Fgfr1;Fgfr3* mutant.

Shh and *Gli1* remain expressed in the *Fgfr1;Fgfr2* mutant

The loss of ventral precursor cells in the *Fgfr1*;*Fgfr2* mutant mimics the phenotype observed in mice in which SHH signalling is abolished in the telencephalon using a tissue-specific knockout of the smoothened (*Smo*) gene (Fuccillo et al., 2004). In the *Smo* mutant, *Pax6* expression expands to the ventral midline, while telencephalic expression of *Nkx2.1* and *Gsh2* are lost. Given these matching phenotypes, the question arises as to whether FGF signalling acts either through or independently of SHH to generate ventral cell types.

To address this question, the expression of *Shh* was examined in the *Fgfr1*;*Fgfr2* mutant at E10.5, a time at which expression of this gene in the mesendoderm underlying the telencephalon is still likely to be required for inducing ventral cell types. *Shh* expression shows little or no difference between control and *Fgfr1*;*Fgfr2* mutant embryos (Fig. 6A), indicating that FGF signalling through *Fgfr1* and *Fgfr2* is not required to maintain *Shh* expression in ventral mesendoderm. To address whether SHH is actively signalling in the mutant, the expression of *Gli1*, a gene whose transcription is dependent on SHH activity (Bai et al., 2002), was examined. *Gli1* is expressed in *Foxg1*-positive telencephalic cells in both the control and mutant at E10.5 (Fig. 6B,C), suggesting that not only is SHH expressed, but that it is also active in the *Fgfr1*;*Fgfr2* mutant and that it is not sufficient to generate ventral cell types. This indicates that SHH requires *Fgfr1* and *Fgfr2* to generate ventral telencephalic cells and, consequently, that FGF signalling acts independently or downstream of SHH. Consistent with these results, *Shh* is required to maintain *Fgf8* expression (Ohkubo et al., 2002; Aoto et al., 2002) and FGF8-soaked beads can induce ventral gene expression in dorsal telencephalic explants in culture, even when SHH signalling is inhibited (Kuschel et al., 2003).

Loss of Gli3 does not rescue the Fgfr1;Fgfr2 phenotype

Shh and *Gli3* act antagonistically to pattern the dorsoventral axis of the telencephalon (Rallu et al., 2002). In the *Shh* mutant, ventral cell types are lost and dorsal ones expand, and the opposite is observed in the *Gli3* mutant – ventral cell types expand and dorsal ones are lost. However, grossly normal dorsoventral patterning is restored in mutants that lack both copies of *Shh* and one or both copies of *Gli3* (Aoto et al., 2002; Rallu et al., 2002). Can *Gli3* also rescue the loss of ventral cell types in the *Fgfr1*;*Fgfr2* mutant?

This question was addressed by analyzing *Fgfr1*;*Fgfr2* mutants that carry one or two mutant alleles of *Gli3*. In these mutants, loss of *Gli3* does not rescue the loss of ventral cell types. In the *Fgfr1*;*Fgfr2* mutant that carries just one mutant copy of *Gli3*, *Pax6* and *Emx2* expression extends to the ventral-most area of the telencephalon and *Dlx2* expression is largely or completely lost as it is in the *Fgfr1*;*Fgfr2* double mutant (Fig. 7E-M). The ventromedial area that is devoid of *Pax6* and *Emx2* expression but positive for *Dlx2* expression in the mutants is likely to coincide with the anteroventral hypothalamus, as revealed in serial sections by reduced or absent *Foxg1* expression (not shown). The failure of one mutant copy of *Gli3* to rescue the *Fgfr1*;*Fgfr2* phenotype, in contrast to its ability to rescue the *Shh* phenotype (Rallu et al., 2002), indicates that FGF signalling acts downstream of *Gli3* and *Shh*.

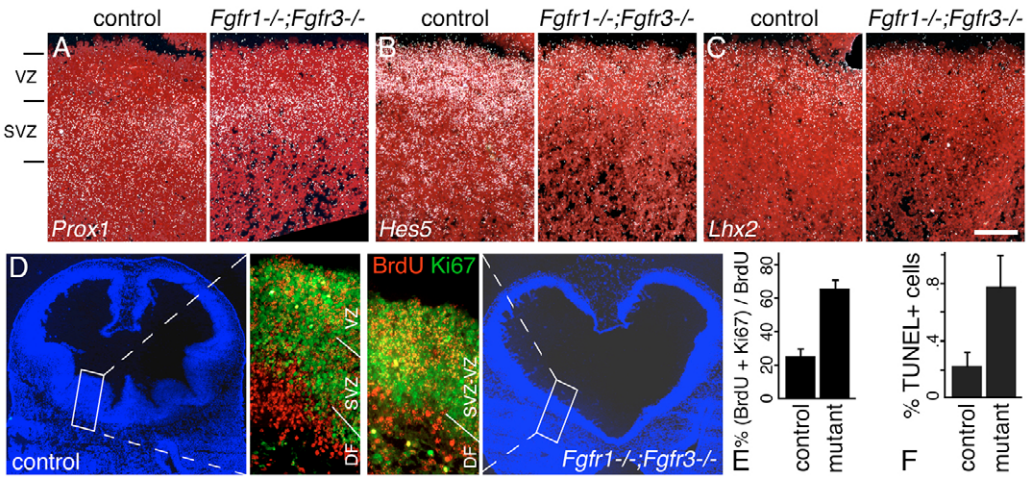

Fig. 5. The *Fgfr1*$^{-/-}$;*Fgfr3*$^{-/-}$ SVZ is expanded and undergoes more cell death and less cell cycle exit. (**A-C**) RNA in situ hybridization of E12.5 coronal sections through the ventromedial area. Unlike the control, *Prox1* expression in the mutant expands from the SVZ to the ventricular surface (A). This expansion coincides with an apparent decrease in the expression of the VZ markers *Hes5* and *Lhx2* (B,C). Scale bar: 0.02 mm. (**D**) Control and mutant E11.5 brains doubled-labelled for Ki67 and BrdU (injected 24 hrs prior to sacrifice). (**E**) A larger percentage of Ki67+BrdU labelled cells over total BrdU-labelled cells is present in the mutant compared with the control, indicating an abnormally low rate of cell cycle exit. TUNEL analysis (**F**) indicates a higher rate of cell death in the mutant at E11.5.

In the *Fgfr1*;*Fgfr2* mutant that carries two mutant copies of *Gli3*, the loss of ventral precursor cells is also not rescued. However, in this case development of the telencephalon is greatly compromised (Fig. 7A-D). Unlike the *Shh*;*Gli3* double homozygous mutant (Rallu et al., 2002), the *Fgfr1*;*Fgfr2*;*Gli3* triple homozygous mutant does not exhibit a high frequency of exencephaly (less than 20%), but rather a flattening and significant reduction in the size of the telencephalon (Fig. 7D). Nevertheless, in *Foxg1*-positive areas, *Pax6* and *Emx2*, but not *Dlx2*, are expressed (Fig. 7N-P; see Fig. S3 in the supplementary material), suggesting that complete loss of *Gli3* does not rescue the loss of ventral cells observed in the *Fgfr1*;*Fgfr2* mutant and that FGF signalling in fact acts downstream of *Gli3*.

Consistent with previous reports indicating that *Gli3* is required for the cortical hem to form (Grove et al., 1998), expression of *Wnt3a* and *Wnt2b*, markers for the hem, are absent in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$;*Gli3*$^{-/-}$ mutant (Fig. 7T-U and not shown), but present in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ and *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$;*Gli3*$^{+/-}$ mutants (Fig. 7Q-S and not shown).

DISCUSSION

The results of this study demonstrate that FGF signalling is required in the telencephalon for both the generation of ventral precursors and their differentiation. Using a conditional genetic approach in the mouse, simultaneous loss of *Fgfr1* and *Fgfr2* leads to a loss of ventral precursor cells, whereas loss of *Fgfr1* and *Fgfr3* leads to a loss of differentiated ventromedial cells (Figs 2-4). Hence, FGF signalling is required both for generating ventral precursors and for promoting their differentiation. The loss of ventral precursors in the *Fgfr1*;*Fgfr2* mutant mimics the phenotype obtained in embryos in which *Smo* is conditionally deleted in the telencephalon starting no earlier than E8.75 using the *Foxg1*^{Cre} allele (Fuccillo et al., 2004). The same phenotype is also obtained in embryos in which *Fgf8* is deleted (Storm et al., 2006). Therefore, both SHH and FGF signalling are similarly required for generating ventral precursors. In this process, FGFs could be acting upstream, downstream or in parallel to SHH signalling.

FGFs act downstream of SHH

Previous evidence supports a model in which FGFs act downstream of *Shh* to generate ventral cell types (Fig. 8). First, although SHH is required to induce ventral telencephalic cell types (Ericson et al., 1995; Chiang et al., 1996; Ohkubo et al., 2002; Fuccillo et al., 2004), ventral cells can still develop if both SHH and GLI3 are lost, indicating that another factor can generate ventral cells independent of SHH (Aoto et al., 2002; Rallu et al., 2002). Second, the maintenance of *Fgf8* expression requires *Shh* (Aoto et al., 2002; Ohkubo et al., 2002). Third, FGF8-soaked beads can induce expression of ventral markers and repress expression of dorsal ones when placed on cultured explants of dorsal telencephalon, even when SHH signalling is inhibited with cyclopamine (Kuschel et al., 2003). And finally, zebrafish embryos that lack *Fgf8* or are treated with morpholinos against *Fgf8* and *Fgf3* show loss of ventral cell types (Shanmugalingam et al., 2000; Shinya et al., 2001; Walshe and Mason, 2003) and chick embryos treated with soluble FGFR4 show a loss of *Nkx2.1* expression (Marklund et al., 2004). However, direct genetic evidence in mammals demonstrating a role for FGFs in generating ventral telencephalic cell types has been lacking.

Three results described in this study establish that FGFs in fact act downstream of *Shh* and *Gli3*. First, *Shh* expression is not affected in the *Fgfr1*;*Fgfr2* mutant (Fig. 6). This is also the case with loss of *Fgf8*, whereby *Shh* expression is maintained (Kawauchi et al., 2005). Second, *Gli1* expression, which is dependent on SHH activity (Bai et al., 2002), is maintained in the *Fgfr1*;*Fgfr2* mutant (Fig. 6), indicating that SHH activity is not sufficient to generate ventral cells and depends on FGF signalling. And finally, although loss of *Gli3* rescues the lack of ventral precursors in the *Shh* mutant (Rallu et al., 2002), it does not do so in the *Fgfr1*;*Fgfr2* mutant (Fig. 7; see Fig. S3 in the supplementary material), placing FGF signalling downstream of *Shh* and *Gli3*.

The question remains as to whether FGF signalling, acting downstream of SHH to generate ventral telencephalic cells, directly specifies these cells or whether it is required to promote their proliferation or survival once they have already been specified.

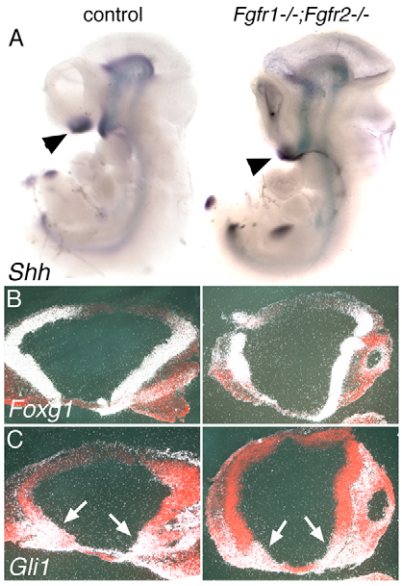

Fig. 6. SHH signalling is insufficient to generate ventral precursors in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant. (**A**) Whole-mount RNA in situ hybridization analysis of E10.5 control and *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant embryos indicates that *Shh* remains expressed in the mutant in the ventral mesendoderm underlying the telencephalon (arrowheads). (**B**,**C**) RNA in situ hybridization analysis of serial E10 coronal sections through the anterior prosencephalon. In telencephalic areas expressing *Foxg1* (B), expression of *Gli1*, which requires SHH activity, is localised to the ventral areas of the mutant (C, arrows).

Several lines of evidence suggest that FGF signalling does in fact induce or specify ventral cells. First, FGF8-soaked beads can ectopically induce ventral telencephalic cells (Kuschel et al., 2003). Second, even at early stages of telencephalon development, ventral telencephalic cells that normally express *Nkx2.1* are not present in the *Fgfr1*;*Fgfr2* or *Fgf8* mutants (Fig. 3) (Storm et al., 2006), consistent with a role for FGF signalling in specifying ventral cells. This does not exclude the possibility that FGF signalling also promotes the proliferation and survival of ventral precursor cells. In fact, reduced proliferation in the ventral telencephalon is observed in the *Fgfr1*;*Fgfr2* mutant at E10.5 (Fig. 3A) and both reduced proliferation and increased apoptosis are observed in the *Fgf8* mutant as early as E9 (Storm et al., 2006).

SHH antagonizes GLI3 to relieve repression of FGF gene expression

In *Shh*;*Gli3* double mutants, ventral cells are generated (Aoto et al., 2002; Rallu et al., 2002), presumably owing to FGF signalling. But how can FGFs generate ventral cells if *Shh* is required to maintain expression of FGF genes, in particular *Fgf8* (Ohkubo et al., 2002)? The likely answer is that FGF gene expression is normally repressed by *Gli3* (Fig. 8). In the *Shh* mutant, *Fgf8* expression is lost because of unchecked repression by *Gli3*, but in the *Shh*;*Gli3* double mutant, *Fgf8* is no longer repressed by *Gli3*. Consistent with *Gli3* repressing FGF gene expression, *Fgf8* expression expands in the *Gli3* mutant (Theil et al., 1999; Aoto et al., 2002; Kuschel et al., 2003). Furthermore, in the *Shh*;*Gli3* double mutant, *Fgf8* expression also expands (Aoto et al., 2002), suggesting that *Shh* promotes *Fgf8* expression only indirectly by repressing *Gli3* (Fig. 8).

Whether similar interactions between *Gli3*, *Shh*, and FGF signalling occur in other parts of the developing CNS remains unclear. Interestingly, loss of *Gli3* can also rescue loss of SHH signalling in the spinal cord. In *Shh*;*Gli3* or *Smo*;*Gli3* double mutants, ventral neuron generation is rescued as is dorsoventral patterning, indicating that other yet to be identified factors pattern the spinal cord (Litingtung and Chiang, 2000; Persson et al., 2002; Wijgerde et al., 2002). Given the results presented here and that FGF genes are expressed within or immediately adjacent to the spinal cord (Crossley and Martin, 1995; Borja et al., 1996; Maruoka et al., 1998; Xu et al., 1999), FGFs become excellent candidates for factors that generate ventral cell types in the spinal cord. It should be noted that at least in the spinal cord, but perhaps also in the telencephalon, loss of *Gli3* does not completely rescue the loss of *Shh* phenotype (Litingtung and Chiang, 2000; Persson et al., 2002; Wijgerde et al., 2002), suggesting that SHH has a role in patterning neural tissue that goes beyond inactivating GLI3.

The nature of the signals that dorsalize the telencephalic cells in the *Fgfr1;Fgfr2;Gli3* triple mutant remain unclear. Previous evidence has suggested that FGF signalling in combination with Wnt signalling dorsalizes telencephalic precursor cells (Gunhaga et al., 2003). It is possible that in the *Fgfr1;Fgfr2;Gli3* mutant, the remaining FGF signalling through FGFR3 along with a Wnt that is expressed beyond the cortical hem area (such as Wnt7a) (Grove et al., 1998) accounts for the dorsalization of the remaining telencephalon.

FGFR1 is the likely receptor for FGF8 in the early telencephalon

Of the three FGF receptor genes expressed in telencephalic precursor cells, *Fgfr1* plays a dominant role. Telencephalons that are deficient for both copies of *Fgfr2* and *Fgfr3* but that retain only one copy of *Fgfr1* exhibit grossly normal patterning (Fig. 2), indicating that FGFR1 produced from one allele is sufficient to transmit most of the FGF signalling load. Conversely, although loss of *Fgfr2* or *Fgfr3* on their own have little or no patterning defect in the telencephalon, loss of *Fgfr1* alone results in an olfactory bulb defect (Hébert et al., 2003), a loss of midline glia, cerebral commissures and differentiated ventromedial cell types (Tole et al., 2006), indicating that *Fgfr1* on its own is necessary for telencephalic development. The *Fgfr1* and *Fgfr1*;*Fgfr3* phenotypes are similar, as are the *Fgfr2* and *Fgfr2*;*Fgfr3* phenotypes (Fig. 2; G.G., S.K.M. and J.M.H., unpublished), suggesting that *Fgfr3* does not play a significant role in the patterning processes described in this study. The key roles played by *Fgfr1* are likely to be conserved across species. In humans, reduction or loss of expression of this gene leads to Kallmann syndrome, which includes olfactory bulb and commissural defects (Dode et al., 2003).

Reduction or loss of *Fgf8* expression leads to comparable phenotypes to those obtained with loss of *Fgfr1*. In zebrafish, ventral, midline and commissural defects are observed when *fgf8* expression is lost or reduced (Shanmugalingam et al., 2000; Shinya et al., 2001; Walshe and Mason, 2003). Mouse embryos in which *Fgf8* is hypomorphic can lack olfactory bulbs (Meyers et al., 1998) and those in which *Fgf8* is null lack ventral precursor cells (Storm et al., 2006). However, in the latter case the phenotype is more similar to the *Fgfr1*;*Fgfr2* phenotype described here, suggesting that FGF8 acts not only through FGFR1, but also through FGFR2. Given the similarities in the phenotypes obtained to date with reductions or loss of *Fgf8* and with the different FGFR mutants, it is likely that in the early telencephalon FGF8 acts primarily through FGFR1 and secondarily through FGFR2.

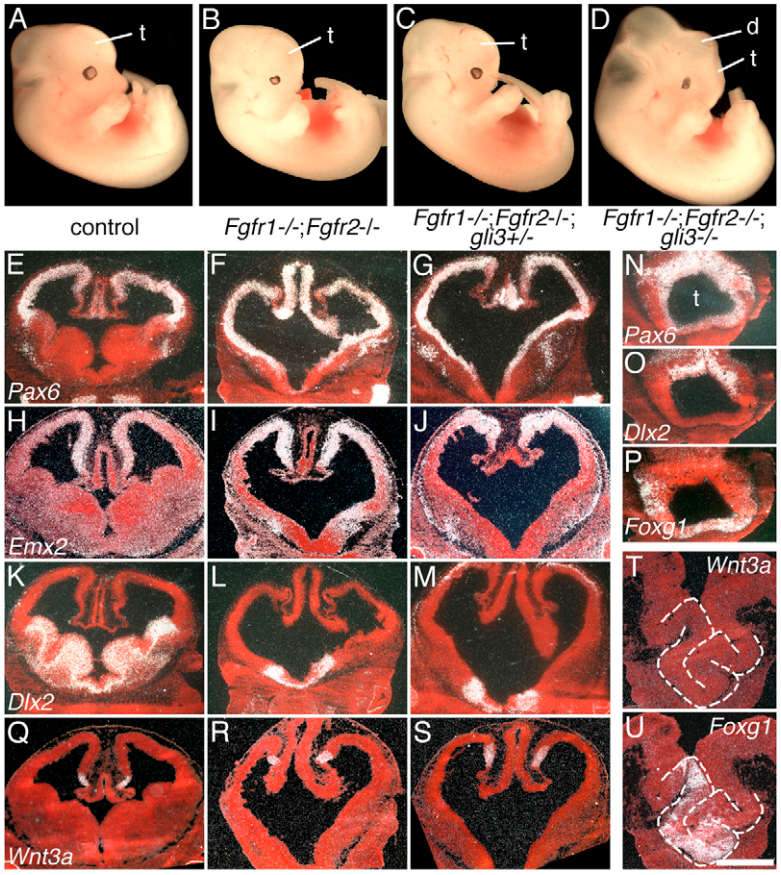

Fig. 7. Loss of *Gli3* does not rescue the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ phenotype. (**A-D**) E12.5 whole embryos. Development of the telencephalon is severely compromised in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$;*Gli3*$^{-/-}$ mutant (D) compared with the other genotypes (A-C). t, telencephalon; d, diencephalon. (**E-U**) RNA in situ hybridization analysis of E12.5 coronal brain sections. In the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$;*Gli3*+/– mutant, as in the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$ mutant, *Pax6* and *Emx2* expression extends to the ventromedial area of the telencephalon at the expense of ventral markers such as *Dlx2* (E-M). The remaining expression of *Dlx2* in both mutants (L,M) is likely to be hypothalamic rather than telencephalic as it coincides with areas with decreased or absent *Foxg1* expression in neighbouring sections (not shown). In the *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$;*Gli3*$^{-/-}$ mutant, *Foxg1*-positive areas express *Pax6* but not *Dlx2* (N-P). *Wnt3a* expression in the cortical hem appears normal for all genotypes except *Fgfr1*$^{-/-}$;*Fgfr2*$^{-/-}$;*Gli3*$^{-/-}$ (Q-U). Broken lines indicate the *Foxg1*-positive telencephalic area in an exencephalic mutant (T,U). Scale bar: 0.5 mm in E-U.

This is surprising, given previous studies suggesting that FGF8 has little or no affinity for FGFR1 in cell mitogenicity and binding assays, and that FGFR3 is the highest affinity receptor for this ligand (Ornitz et al., 1996; Chellaiah et al., 1999). This discrepancy could potentially be explained by the presence of alternatively spliced variants of the *Fgfr3* transcript in the different cell types or the differential expression of unidentified co-factors. Nevertheless, whether *Fgfr3* in any way compensates for loss of *Fgfr1* and/or *Fgfr2* in the early telencephalon awaits analysis of the triple FGFR mutant.

Fgf8 is not the only Fgf gene expressed in the early telencephalon. *Fgf14*, *Fgf15*, *Fgf17* and *Fgf18* are expressed in an overlapping or similar pattern to *Fgf8* (McWhirter et al., 1997; Maruoka et al., 1998; Xu et al., 1999; Wang et al., 2000), *Fgf7* is expressed in the lateral telencephalon (Mason et al., 1994), *Fgf1* and *Fgf2* are believed to be widely expressed throughout the telencephalic neuroepithelium (e.g. Dono et al., 1998), and other FGF genes for which the telencephalic expression pattern in mice has not yet been characterized may also be expressed. Of note, *Fgf19* plays a role in zebrafish in promoting ventral forebrain development (Miyake et al., 2005), but its expression or role in the mouse is unknown. Although in this study no role was uncovered for *Fgfr3* in generating ventral cell types, this gene is required for regulating the onset of oligodendrocyte terminal differentiation that occurs postnatally (Oh et al., 2003). Given that the expression of FGF ligands is poorly characterized postnatally, and that mutations in FGF ligand genes have not yet revealed a similar oligodendrocyte differentiation phenotype, it remains unclear which ligands are likely to interact with FGFR3.

A model for the formation of the telencephalic midline

Holoprosencephaly, the incomplete separation of the telencephalic hemispheres, is the most common developmental forebrain defect in humans (Muenke and Beachy, 2001). Although mutations in the SHH pathway are known to cause holoprosencephaly in both humans and mice, it remains a mystery how the absence of *Shh* expression, which is normally present only ventrally, can result in not only the loss of ventral cells but also loss of the rostral and dorsal midline (Hayhurst and McConnell, 2003). FGF signalling in the telencephalon is also likely to be required for midline formation. The *Fgfr1*;*Fgfr3* mutant lacks rostral midline glial cell types, the indusium griseum and midline zipper glia, and all three cerebral commissures (Tole et al., 2006); and the *Fgfr1*;*Fgfr2* and *Fgf8* mutants have a higher rate of apoptosis (Fig. 3B) (Storm et al., 2006) and a wider domain of *Bmp4* expression in the dorsal midline (data not shown) (Storm et al., 2003), indicating that FGFs regulate formation of the rostrodorsal midline.

Consistent with a role for FGF signalling in midline formation, in zebrafish, loss of FGF activity also leads to disruption of the midline and severe commissural axon pathway defects (Shanmugalingam et al., 2000; Walshe and Mason, 2003). Furthermore, the level of *Fgf8* expression appears to regulate dorsal midline formation by regulating *Bmp4* expression (Storm et al., 2003); and ectopic FGF8 can induce structures resembling a dorsal midline (Crossley et al., 2001). *Gli3* is also required for promoting the expression of BMP and WNT genes and for forming the dorsal midline (Grove et al., 1998; Theil et al., 1999; Kuschel et al., 2003). Therefore *Gli3* and FGF signalling are likely to interact in regulating formation of the

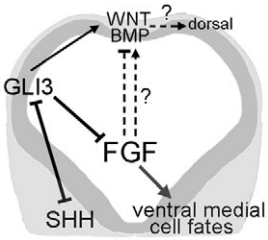

Fig. 8. Model for the generation of ventral and midline cell types in the telencephalon. SHH, which is expressed ventral to the developing telencephalon, is required to generate not only ventral cell types, but also rostral and dorsal midline ones. SHH is likely to be required for these processes only indirectly by antagonizing GLI3 and thus relieving repression of Fgf gene expression (see Discussion). FGFs are then necessary to generate ventral and at least some rostral midline cell types. Previous evidence indicates that GLI3 is required for expression of WNTs and BMPs and for formation of the dorsal midline, and that FGF8 regulates *Bmp4* expression in a dose-dependent manner. Hence, SHH is likely to promote ventral and midline development indirectly by regulating GLI3 and FGF signalling.

dorsal midline (Fig. 8). These previous reports, along with the results presented here, point to a model whereby SHH promotes ventral and midline development indirectly by regulating GLI3 and FGF signalling.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/15/2937/DC1

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