FGF signalling generates ventral telencephalic cells independently of SHH

Grigoriy Gutin1,*†, Marie Fernandes1,†, Laura Palazzolo1, HunKi Paek1, Kai Yu2, David M. Ornitz2, Susan K. McConnell3 and Jean M. Hébert1,†

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; Susel 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, Fgfl4, 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

* These authors contributed equally to this work
† Author for correspondence (e-mail: jhebert@aecom.yu.edu)

Accepted 30 May 2006
chick embryos, application of soluble FGFR4 leads to a loss of Nkx2.1-expressing telencephalic cells (Marklund et al., 2004). Moreover, in mice, FGFR8-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 FoxI1lox/lox mice, as previously described (Hébert et al., 2003; Yu et al., 2003). Fgfr1flox/flx, Fgfr1flox/flx,Fgfr3null/null and Fgfr1flox/flx,Fgfr2fllox/fllox carrying the FoxI1lox 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 FoxI1lox/lox,Fgfr3fllox/fllox,Fgfr2fllox/fllox,homo/homo (Hébert et al., 2003) and 31 FoxI1lox/lox,Fgfr3fllox/fllox,Fgfr2fllox/fllox 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 35S-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 or four embryos were hybridized to each probe: the numbers of embryos hybridized are indicated in the figure legends. 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 where 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.

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 monocolonal), 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.

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).
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, GFG signalling was disrupted genetically. Genes encoding GFG 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 neighboring 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<sup>cre+</sup>;Fgfr1<sup>+/+</sup>;Fgfr2<sup>–/–</sup>;Fgfr3<sup>–/–</sup> mutant (Fig. 2B,F,J), and (3) as phenotypes analogous to the ones described here implicating FGFR 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 FGFR 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
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 defined sectors that include dorsal, ventral and dorsomedial neurogenesis. Sections of telencephalons were partitioned into floxed alleles in the mutant is complete and prior to the start of incorporation assays at E10.5, a stage when recombination of the (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 E9.25-9.75 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 Fgfr1 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), is 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 Proxl marks the SVZ. In the mutant, however, elevated levels of Proxl 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
differentiating cells of the lateral ganglionic eminence (G), remains, on the other hand, which is normally expressed in Ebf1 (B,C,E,F).

eminence (A,D); however, this expression is lost in the mutants normally expressed in differentiating cells of the medial ganglionic

not the of expression (O,R,S), whereas in the Fgfr1–/– mutant (I). Mash1, which is normally expressed in expressing areas (arrows) of the Fgfr1–/–;Fgfr2–/– mutant at the expense of 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.

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).
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 Foxg1Cre 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 Fgfg8 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.
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 dorsalventral 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 FGF3 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 (Shamugalingam 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 FGF1, but also through FGF2. Given the similarities in the phenotypes obtained to date with reductions or loss of Fgf8 and with the different FGF receptors, it is likely that in the early telencephalon FGF8 acts primarily through FGF1 and secondarily through FGF2.

**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).
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 Fgr3 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 forebrain development (Miyake et al., 2005), but its expression or role in the mouse is unknown. Although mutations in 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–/–;Fgfr2–/– 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–/–;Gli3–/– mutant (D) compared with 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.

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–/–;Fgfr3–/– 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 rostrocaudal 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
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.

We thank Gerd Fishell and members of his laboratory for insightful discussions; Juha Partanen and Janet Rossant (Fgfr1), and Chu-Xia Deng and Philip Leder (Fgfr3) for mice; and Sonia Garel, Sonia Garel, Alex Joyner, John Rubenstein, Stewart Anderson and Celine Zimmer for plasmids. This work was supported in part by NIMH MH65261 (S.K.M.), by a Howard Hughes Medical Institute Junior Faculty Start-Up Award, by the Alexandrine and Alexander L. Sinsheimer Foundation, NIMH 1R21MH075779, 1R01MH70596 (J.M.H.).

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

References


Marklund, M., Sjodal, M., Beeler, B. C., Jessell, T. M., Edlund, T. and


