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FGF signaling through FGFR1 is required for olfactory bulb morphogenesis

Jean M. Hébert[1,*], Mary Lin[1], Juha Partanen[2], Janet Rossant[3] and Susan K. McConnell[1,†]

[1]Department of Biological Sciences, Stanford University, Stanford, CA 94305, USA
[2]Institute of Biotechnology, University of Helsinki, FIN-00014, Finland
[3]S. Lunenfeld Research Institute, University of Toronto, Toronto M5G 1X5, ON, Canada
*Present address: Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, USA
†Author for correspondence (e-mail: suemcc@leland.stanford.edu)

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SUMMARY

During development, the embryonic telencephalon is patterned into different areas that give rise to distinct adult brain structures. Several secreted signaling molecules are expressed at putative signaling centers in the early telencephalon. In particular, *Fgf8* is expressed at the anterior end of the telencephalon and is hypothesized to pattern it along the anteroposterior (AP) axis. Using a CRE/*loxP* genetic approach to disrupt genes in the telencephalon, we address the role of FGF signaling directly in vivo by abolishing expression of the FGF receptor *Fgfr1*. In the *Fgfr1*-deficient telencephalon, AP patterning is largely normal. However, morphological defects are observed at the anterior end of the telencephalon. Most notably, the olfactory bulbs do not form normally. Examination of the proliferation state of anterior telencephalic cells supports a model for olfactory bulb formation in which an FGF-dependent decrease in proliferation is required for initial bulb evagination. Together the results demonstrate an essential role for *Fgfr1* in patterning and morphogenesis of the telencephalon.

Key words: Telencephalon, Cre/loxP, Cell fate, Neurogenesis, Forebrain, Patterning

INTRODUCTION

During development, the anterior region of the neural tube that constitutes the telencephalon becomes patterned into areas that give rise to several adult brain structures, such as the cerebral cortex, hippocampus, striatum and olfactory bulb. In addition, the cerebral cortex itself becomes partitioned into numerous functional areas, such as the somatosensory and visual cortices. Much of this patterning occurs prior to the arrival of afferent inputs, as evidenced by the restricted expression patterns of several genes (reviewed by Monuki and Walsh, 2000; Ragsdale and Grove, 2001). For example, *Emx2* and *Pax6* are expressed in counter-gradients along the anteroposterior (AP) axis of the cerebral cortex in mice. *Pax6* is expressed as early as E10.5 in the telencephalon (Walther and Gruss, 1991) and *Emx2* as early as E12.5 (Gulisano et al., 1996), and both genes are required for normal patterning (Bishop et al., 2000; Mallamaci et al., 2000, Muzio et al., 2002). In addition, *Pax6*-deficient embryos lack olfactory bulbs and olfactory epithelium (Hogan et al., 1986; Hill et al., 1991). The signals that generate these patterns of gene expression and that specify different telencephalic and cortical areas remain to be elucidated.

Several experiments suggest that *Fgf8* may play a key role in patterning the telencephalon along the AP axis. *Fgf8* is expressed just anterior to the neural plate in the anterior neural ridge as early as E8.5, and in the anterior forebrain from E9 to at least E12.5 (Crossley and Martin, 1995). Mice that are completely deficient for *Fgf8* die around the time of gastrulation (Meyers et al., 1998). However, partial loss of function of *Fgf8* in E18.5 embryos carrying an *Fgf8* null allele over a hypomorphic allele can result in a small telencephalon that lacks olfactory bulbs and a normal midline (Meyers et al., 1998), demonstrating a requirement for *Fgf8* in telencephalic development and a sensitivity of telencephalic precursors to even a partial loss of *Fgf8* expression. Results from experiments in which FGF8-coated beads were ectopically applied to forebrain tissue (Shimamura and Rubenstein, 1997; Crossley et al., 2001) and in which *Fgf8* was overexpressed in the telencephalon (Fukuchi-Shimogori and Grove, 2001) also suggest that FGF8 induces the formation of anterior telencephalic structures.

Fgf genes other than *Fgf8* may also be expressed and play a role in the developing telencephalon. For example, *Fgf3*, *Fgf15*, *Fgf17* and *Fgf18* are all expressed at the anterior end of the developing telencephalon (McWhirter et al., 1997; Maruoka et al., 1998; Shinya et al., 2001). *Fgf2* has also been implicated in telencephalic development. In particular, analysis of *Fgf2*-deficient mice demonstrates a role for this gene in regulating cortical neurogenesis and promoting neural progenitor cell proliferation (Dono et al., 1998; Ortega et al., 1998; Raballo et al., 2000), although no patterning defects were observed in *Fgf2*-deficient mice.

Although over 20 genes encode FGF ligands (reviewed by Ornitz and Itoh, 2001; Ford-Perriss et al., 2001), there are only four known genes encoding FGF receptors *Fgfr1* to *Fgfr4*. *Fgfr1*, *Fgfr2* and *Fgfr3*, but not *Fgfr4*, are expressed in the progenitor cells lining the telencephalic ventricles throughout development (Orr-Utreger et al., 1991; Peters et al., 1992;

Peters et al., 1993; Yamaguchi et al., 1992) (see Fig. 7). The genetic analysis of the Fgfr genes has thus far been uninformative as to the role of FGF signaling in telencephalic development. Embryos deficient for either *Fgfr1* or *Fgfr2* die at developmental stages that are too early for telencephalic development to be assessed (Deng et al., 1994; Yamaguchi et al., 1994; Arman et al., 1998; Xu et al., 1998), whereas *Fgfr3*-deficient mice survive and show no obvious telencephalic defects (Deng et al., 1996).

The olfactory bulb (OB), a telencephalic derivative located at the anterior most end of the rodent brain, may be particularly susceptible to a loss of FGF signaling, as observed in embryos carrying a hypomorphic allele of *Fgf8* (Meyers et al., 1998). However, it is likely that other signaling events are also involved in initial OB formation. Compared with the rest of the telencephalon, the OB undergoes unique morphological changes. In mice, OB morphogenesis is first apparent at E12.5 as an evagination at the anterior end of the telencephalon. Pre- and perinatally, olfactory axons from the olfactory epithelium in the nose enter the anterior telencephalon and, together with the dendrites from mitral cells, the projection neurons in the OB, form structures called glomeruli. It has been proposed that initial OB morphogenesis is induced by the arrival of axons from the olfactory epithelium, because these axons reach the anterior telencephalon prior to any sign of OB formation and contact ventricular zone cells just before changes in the proliferation rate of these cells is observed (Gong and Shipley, 1995). Another proposed mode of OB induction involves retinoic acid signaling from the frontonasal mesenchyme to the telencephalon and induction of *Pax6* expression (LaMantia et al., 1993; Anchan et al., 1997). The OB is also a site that continues to acquire new neurons throughout life as neuroblasts born in the anterior subventricular zone migrate anteriorly along the rostral migratory stream to reach the OB and regenerate the interneuron population (Luskin, 1993; Lois and Alvarez-Buylla, 1994).

We have previously described a CRE/*loxP* conditional genetic approach for knocking out or overexpressing genes in the telencephalon using the Foxg1-Cre mouse line (Hébert and McConnell, 2000). In mice that carry the Foxg1-Cre allele and a gene flanked by *loxP* sites ('floxed'), recombination of the floxed allele occurs efficiently in the telencephalon from its earliest stages of development. To investigate the role of FGF signaling further in telencephalic development and patterning, we have specifically disrupted *Fgfr1* in the telencephalon using Foxg1-Cre mice. We find that *Fgfr1* is essential for the formation of the OB, the most anterior telencephalic structure, whereas AP patterning in the rest of the telencephalon is largely normal in the *Fgfr1* mutants.

MATERIALS AND METHODS

Maintenance of mouse lines

Mice were maintained in a mixed strain background (>25% 129Sv) and genotyped as described (Hébert and McConnell, 2000) (R. Trokovic, N. Trokovic, S. Hernesniemi, U. Pirvola, J. R., A. P. McMahon, W. Wurst and J. Partanen, unpublished). Because Foxg1-Cre mice were generated by targeting *Cre* to the *Foxg1*-locus, creating a *Foxg1* null allele, only heterozygous Foxg1-Cre mice are used in the experiments described here. Although mice that are heterozygous for *Foxg1* show no phenotype on their own (Xuan et al., 1995; Hébert et al., 2002) (this report), it should be noted that the severity of the phenotypes observed with loss of *Fgfr1* could in theory be affected by loss of one functional copy of *Foxg1*.

RNA in situ hybridization analysis

Frozen sections were prepared and hybridized to ^{35}S-labelled probes as previously described (Frantz et al., 1994). A minimum of three mutant and three control embryos were analyzed for each probe at each age. Plasmids used to make probes were kindly provided by Heike Popperl (*Pbx3*) and Brian Condie (*Gad67*).

DiI tracing

Whole E12.5 embryos, E18.5 heads or E18.5 brains were immersion fixed in 4% paraformaldehyde for 1 week. A single DiI crystal on the point of a pin was inserted in the olfactory epithelium (anterograde labeling in whole E12.5 embryos or E18.5 heads) or olfactory cortex (retrograde labeling in E18.5 brains); extraneous crystals were washed away carefully. Diffusion occurred over 1-3 weeks in the dark at room temperature in 4% formalin/PBS. Samples were then embedded in 3% low melting temperature agarose in PBS and vibratome sectioned (125 μm) into phosphate buffer. Sections were mounted onto glass slides and examined wet using rhodamine optics on an inverted fluorescence microscope.

BrdU and TUNEL analysis

Females pregnant with E12.5 embryos received an intraperitoneal injection with BrdU and were euthanized 1 hour later. Embryos were collected, frozen in OCT and sectioned on a cryostat. Fresh frozen sections were used for either BrdU staining, as previously described (O'Rourke et al., 1997), or for TUNEL analysis according to the manufacturer's specifications (Roche, Catalog # 2 156 792). Sections were counterstained with Syto11 (Molecular Probes) to reveal cell nuclei. The fraction of BrdU- or TUNEL-positive cells was determined by counting the number of labeled cells in a radial segment spanning from the ventricular surface to the pial surface and dividing by the total number of Syto11-positive cells in the segment. Segments were taken either at the anterior telencephalic end, which corresponds to the location of the OB primordium, or 500 μm caudally in the precursor to the cerebral cortex. At least two segments from each of three separate embryos were counted for each area. Expression of *Cre* in mouse cells has previously been reported to increase the frequency of chromosomal abnormalities both in mice (Schmidt et al., 2000) and in cultured cells (Loonstra et al., 2001). In our studies, a higher rate of apoptosis was observed in the lateral and ventral telencephalon of embryos carrying the *Foxg1-Cre* allele (*Foxg1-Cre;Fgfr1^{flox}* embryos), than of controls not carrying the Foxg1-Cre allele (*Fgfr1^{flox}*, data not shown). However, the increased rate of apoptosis due to CRE was not in itself sufficient to cause a phenotype. Embryos that carried the *Foxg1-Cre* allele were used as controls in the TUNEL and BrdU incorporation assays presented here, and all other experiments presented in this report included *Foxg1-Cre;Fgfr1^{flox}* embryos among the controls.

Fos induction assay

Lateral cortical tissue was dissected from the brains of control and *Fgfr1*-deficient embryos at E13.5. Cells from each embryo were dissociated separately for 10 minutes with trypsin EDTA, split in duplicate 15 mm wells, and allowed to recover 5 hours in DMEM supplemented with N2. FGF2 was added at 50-100 ng/ml to one of each of the duplicate wells 1 hour before cells were collected for northern blot analysis. The intensity of northern blot bands from five separate control and mutant samples was quantified by measuring the integrated density using NIH Image 1.62. The intensity of bands obtained with *Fos* was normalized to the intensity of bands obtained with *Gapdh*. Fold induction in levels of *Fos* expression was calculated by dividing the normalized intensity for *Fos* bands in FGF2-treated samples by those in untreated samples.

RESULTS

Failure of olfactory bulb development in the *Fgfr1*-deficient telencephalon

To investigate the role of FGF signaling in telencephalic development, we generated mice that lacked *Fgfr1* in this tissue using a CRE/*loxP* approach. Previously, a conditional allele of *Fgfr1* was generated in which essential exons 8-15 are flanked by *loxP* sites ($Fgfr1^{flox}$) (R. Trokovic, N. Trokovic, S. Hernesniemi, U. Pirvola, J. R., A. P. McMahon, W. Wurst and J. Partanen, unpublished). This allele confers wild-type function prior to recombination, whereas the excision of exons 8-15 generates a null allele. To disrupt the *Fgfr1* gene in the telencephalon, mice carrying both a *Foxg1-Cre* allele, which confers recombination in the telencephalon (Hébert and McConnell, 2000), and a $Fgfr1^{flox}$ allele were crossed to homozygous $Fgfr1^{flox/flox}$ mice to generate *Foxg1-Cre;*$Fgfr1^{flox/flox}$ mutants. Mutants with *Fgfr1*-deficient telencephalons are recovered in Mendelian ratios until birth. The mutants then die within 24 hours of birth. To determine the efficiency of recombination of the $Fgfr1^{flox}$ allele, DNA was isolated from E12.5 *Foxg1-Cre;*$Fgfr1^{flox/flox}$ telencephalons and examined by southern blot analysis. No unrecombined $Fgfr1^{flox}$ allele could be detected in telencephalic DNA from all three embryos studied, using a probe whose sequence is contained between the *loxP* sites (Fig. 1A). In addition, RNA in situ hybridization analysis using an *Fgfr1* probe to sequences contained within the *loxP* sites indicates a loss of transcript in the telencephalon of mutant embryos (Fig. 1B,B′). As expected, *Fgfr1* transcript is also lost in other tissues that express the *Foxg1-Cre* allele, such as the olfactory epithelium (Fig. 1C,C′).

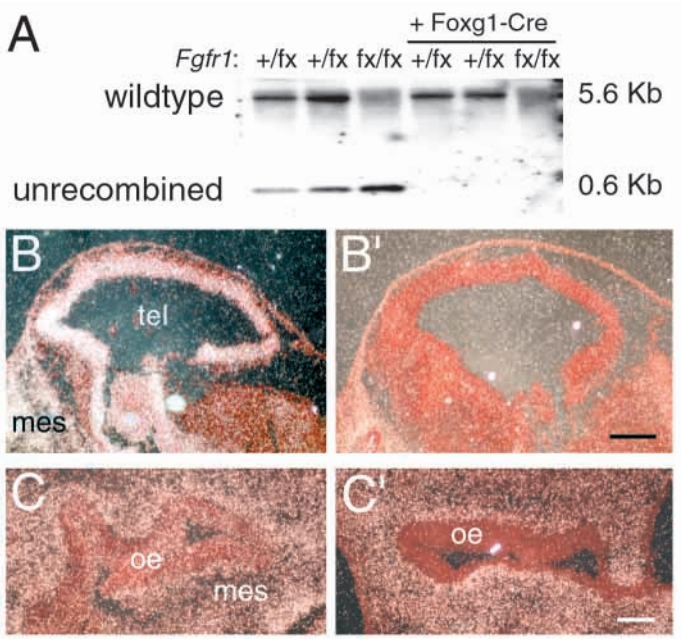

Fig. 1. *Fgfr1* expression is efficiently abolished in the telencephalon of *Foxg1-Cre;*$Fgfr1^{flox/flox}$ mutants. (A) Southern blot analysis showing that the floxed allele of *Fgfr1* undergoes complete recombination in the telencephalon of *Foxg1-Cre/+;Fgfr1fx* mice. DNA was isolated from the dorsolateral telencephalon of E12.5 mice, digested with *Eco*RI, and probed with a ~500 bp *Eco*RI-*Bam*HI genomic DNA fragment containing exon 15. (B) RNA in situ hybridization analysis for *Fgfr1* expression in a sagittal section through the telencephalon of an E12.5 control embryo. *Fgfr1* is expressed throughout the neuroepithelium lining the telencephalic ventricle (tel) as well as in the facial mesenchyme (mes). Anterior is leftwards, dorsal is upwards. (B′) *Fgfr1* expression is lost from the telencephalon in mutant embryos. Scale bar: 0.25 mm. (C) Sagittal section through the nose of an E12.5 control embryo. *Fgfr1* expression is widespread in the mesenchyme (mes) and, compared with the mesenchyme, is at a lower level in the olfactory epithelium (oe). (C′) In the E12.5 mutant embryo, *Fgfr1* expression is also lost from the olfactory epithelium, but not the surrounding mesenchyme, as expected given the recombination pattern observed previously with *Foxg1-Cre* mice (Hébert and McConnell, 2000). Scale bar: 0.1 mm.

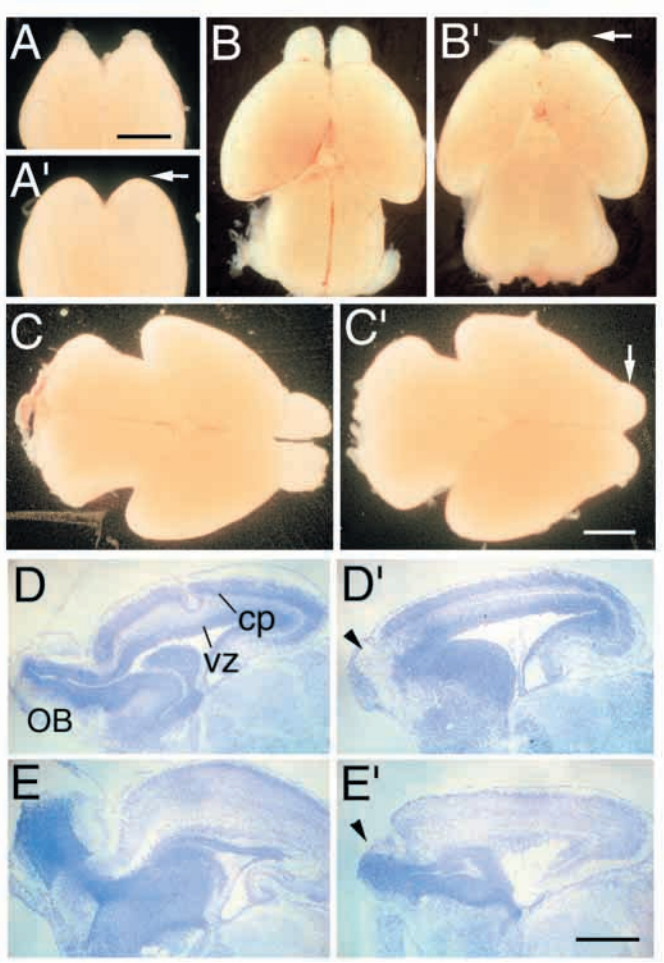

Fig. 2. Telencephalons that lack *Fgfr1* do not develop normal olfactory bulbs. Ventral views of E13.5 control (A) and mutant (A′) brains showing a lack of OB development in the mutant (arrow; anterior is upwards). Scale bar: 0.75 mm. Dorsal views of E16.5 control (B) and mutant (B′) brains; OBs are absent from the mutant (arrow). At P0, control brains (C) have well developed OBs, whereas mutants (C′) have a small bulb-like protrusion (arrow). Scale bar for B-C′: 1 mm. Cresyl Violet stained sagittal sections through the brains of control (D) and mutant (D′) mice at E16.5 showing a lack of cellular organization in the anterior mutant telencephalon (arrowhead); the anterior telencephalon of mutants is no longer arranged in layers as it is in control embryos. Cresyl Violet stained sections through control (E) and mutant (E′) brains at P0 showing a small bulb-like protrusion (arrowhead). cp, cortical plate; OB, olfactory bulb; vz, ventricular zone. Scale bar for D-E′: 0.75 mm.

A striking defect caused by loss of *Fgfr1* in the telencephalon is a failure of normal olfactory bulb (OB) formation. The OB appears absent in *Foxg1-Cre;Fgfr1*$^{flox/flox}$ embryos from E12.5, the age at which it is first morphologically distinguishable, to E16.5 (Fig. 2A-B′,D,D′). The phenotype is completely penetrant: 27/27 E12.5, 22/22 E13.5, 3/3 E15.5 and 8/8 E16.5 brains from mutant embryos lacked any morphologically apparent OB compared with embryos of control genotypes (*Fgfr1*^{flox}, *Fgfr1*$^{flox/flox}$ or *Foxg1-Cre;Fgfr1*^{flox}) in which the OBs are evident at these ages. From E18.5 to birth, a protrusion resembling a small bulb becomes progressively apparent at the anterior end of the mutant telencephalon (Fig. 2C′,E′). Sagittal sections through the telencephalon at E16.5 reveals that the cellular organization of the anterior telencephalon of mutants is disrupted and is no longer arranged in layers as it is in control embryos (Fig. 2D,D′). Therefore, *Fgfr1* is required for OB formation. All the other major areas of the telencephalon, the cerebral cortex, the hippocampus, and the medial and lateral ganglionic eminences are present in mutants and appear histologically normal.

Anterior-posterior patterning is largely normal in the *Fgfr1*-deficient telencephalon

There are several possible mechanisms by which *Fgfr1* may be necessary for OB formation. One possibility is that the cells that form the OB are never specified in the absence of *Fgfr1* because of an AP patterning defect. An anterior shift of posterior cell fates leading to a loss of the most anterior types might be expected given that the source of FGF signaling is likely to be at the anterior end of the telencephalon, where *Fgf8*, *Fgf15*, *Fgf17* and *Fgf18* are most strongly expressed (McWhirter et al., 1997; Maruoka et al., 1998). To address the possibility that an AP patterning defect in mutants results in a failure of OB cell specification, we examined the expression of genes that are regionally expressed along the AP axis in the telencephalon.

Two genes that are thought to play roles in telencephalic patterning are the homeodomain transcription factors *Emx2* and *Pax6* (Bishop et al., 2000; Mallamaci et al., 2000; Muzio et al., 2002). *Emx2* is expressed at low levels anteriorly and higher levels posteriorly in the E12.5 cerebral cortex (Gulisano et al., 1996) (Fig. 3A). *Pax6* is expressed in a counter-gradient to *Emx2*, with higher levels anteriorly and lower levels posteriorly (Walther and Gruss, 1991) (Fig. 3B). To examine whether these patterns are disrupted in the *Fgfr1*-deficient telencephalon, expression of *Emx2* and *Pax6* was assessed using RNA in situ hybridization. In *Foxg1-Cre;Fgfr1*$^{flox/flox}$ E12.5 embryos, *Emx2* and *Pax6* expression remain in gradients in the developing cerebral cortex (Fig. 3A′,B′), indicating that AP patterning has occurred despite the lack of *Fgfr1*. It should be noted, however, that it remains possible that the levels of expression of these genes have been shifted quantitatively along the AP axis in response to loss of *Fgfr1*.

At later stages of cortical development, genes other than *Emx2* and *Pax6* are also expressed in regionally restricted patterns in wild-type embryos. For example at E16.5 and P0, *Cdh8* (cadherin 8) is expressed strongly in the anterior cortex and weaker posteriorly (Fig. 3C). In mutants, the higher anterior expression of *Cdh8* in the cortex is lost (Fig. 3C′), suggesting a slight perturbation in anterior cortical cell fates. However, other genes with regionally restricted expression patterns, such as ephrin A5 and *Fgfr3*, show no apparent change in their expression patterns in mutants (Fig. 3D,D′; see Fig. 7B,B′).

The anterior telencephalon is specified in *Fgfr1*-deficient animals

Although patterning appears for the most part normal in the *Fgfr1*-deficient telencephalon, the results described above fail to ascertain clearly whether the anterior telencephalon, from which the OB forms, has been specified or not in mutants. To address this question, we examined the expression of anterior telencephalic markers. Ephrin A5 and *Pou3f1* (*Scip*/*Oct6*) are two genes expressed at E12.5 in the anterior tip of the telencephalon, where the OB is just beginning to form in control embryos (Fig. 4A,C). Ephrin A5 is also expressed in the OB itself at E16.5 (Fig. 4B). Surprisingly, in mutant E12.5

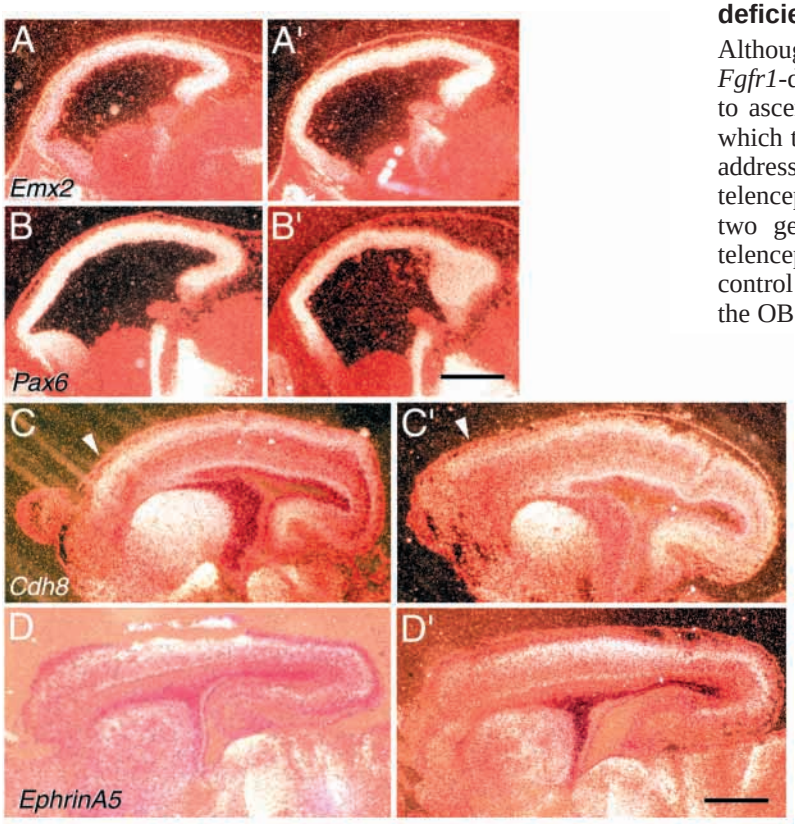

Fig. 3. Patterns of gene expression that distinguish anterior and posterior regions of the telencephalon are for the most part maintained the in *Fgfr1*-deficient mice. RNA in situ hybridization on sagittal sections of E12.5 telencephalons using *Emx2* (A,A′) and *Pax6* (B,B′) probes. In control embryos (A,B), *Emx2* and *Pax6* form opposing gradients in the cerebral cortex. *Emx2* and *Pax6* gradients are also established in mutants (A′,B′). Scale bar: 0.4 mm. (C,C′) *Cdh8* expression at E16.5; the anterior domain of *Cdh8* expression is reduced in mutants (C′) compared with control (C) (arrowheads). (D,D′) Ephrin A5 expression at P0; in control animals (D), ephrin A5 is expressed most strongly in the presumptive sensorimotor cortex (Mackarehtschian et al., 1999) and this location of strongest expression is unchanged in *Foxg1-Cre;Fgfr1*$^{flox/flox}$ mutants (D′). Scale bar for C-D′: 0.6 mm.

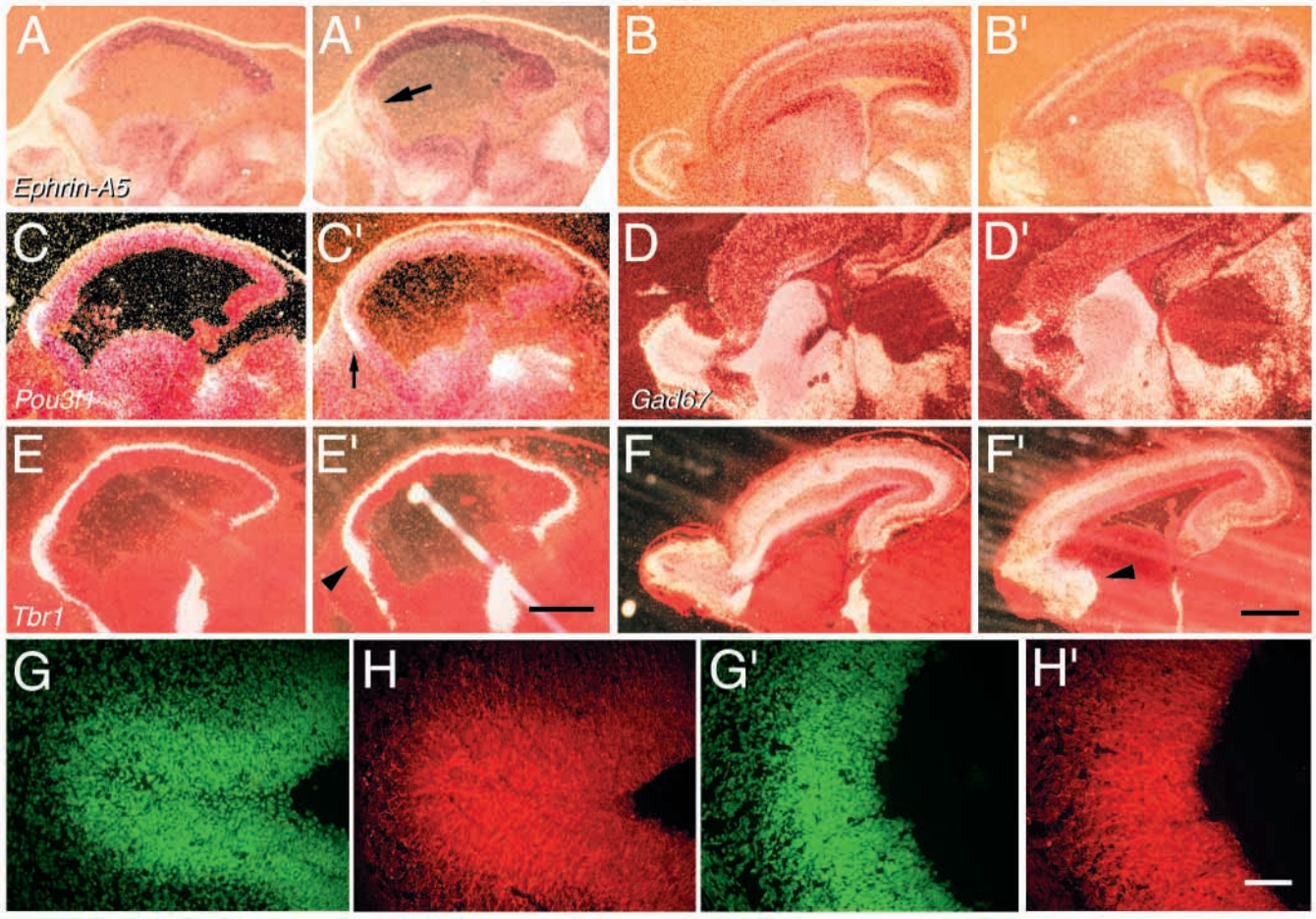

Fig. 4. The specification of anterior cell types appears normal in the *Fgfr1*-deficient telencephalon. RNA in situ hybridization of sagittal sections of control (A,B) and mutant (A′,B′) brains with a probe against ephrin A5; anterior expression is maintained in mutants (arrow) at E12.5 (A,A′) and E16.5 (B,B′; note that expression at this age is no longer organized in layers). Sections of E12.5 control (C) and mutant (C′) brains probed for *Pou3f1* shows expression in both control and mutant anterior telencephalon (arrow). (D) *Gad67* marks interneurons migrating anteriorly into the olfactory bulb of P0 control (D) and mutant (D′) brains. Sections of control (E,F) and mutant (E′,F′) brains at E12.5 (E,E′) and E16.5 (F,F′) hybridized to a *Tbr1* probe; the accumulation of *Tbr1*$^{+}$ neurons at the anterior tip of the brain occurs normally in mutants, although the cells are displaced ventrally (arrowheads). Scale bar in E′, 0.4 mm; in F′, 0.6 mm. (G,G′) Nuclei of cells in the olfactory bulb region of E13.5 embryos stained with Syto11. (H,H′) The same sections stained with antibodies against RC-2 to visualize radial glial cells. Scale bar: 0.05 mm.

embryos, cells in the anterior tip of the telencephalon still express *Pou3f1* and ephrin A5, even though no morphological changes indicative of OB formation are occurring (Fig. 4A′,C′). At E16.5, ephrin A5 remains expressed in the anterior end of both control and mutant telencephalons (Fig. 4B,B′). These results suggest that the anterior-most end of the telencephalon is specified in the absence of *Fgfr1*.

In addition, expression of another gene, *Tbr1*, which marks all early-born telencephalic neurons including the projection neurons of the OB (Bulfone et al., 1995), suggests that neurogenesis occurs normally at the anterior end of the *Fgfr1*-deficient telencephalon. At E12.5, *Tbr1* expression reveals a relatively large number of neurons at the anterior end of the telencephalon compared with most other areas (Fig. 4E). This pattern of *Tbr1* expression at the anterior tip is consistent with a previous report showing an increase in postmitotic cells in this region, an early step in the initial formation of the OB (Gong and Shipley, 1995). The *Fgfr1*-deficient telencephalon also shows an increase in neuronal differentiation at the anterior end, as evidenced by *Tbr1* expression (Fig. 4E′). This result is confirmed by staining E12.5 with a TUJ1 antibody, which marks an accumulation of newborn neurons in both the control and mutant anterior telencephalon (data not shown). However, in mutant animals the accumulation of neurons is displaced ventrally and inwards towards the ventricle when compared with control embryos, in which the differentiating cells begin to protrude anteriorly (Fig. 4E,E′; arrowhead). This displacement of anterior neurons in mutants is even more evident at E16.5 (Fig. 4F,F′; arrowhead). These results suggest that the anterior telencephalon in mutants has been specified to produce the normal, relatively large number of neurons compared with the rest of the telencephalon, despite a lack of normal OB morphogenesis. *Tbr1* expression outside of the OB also suggests that *Fgfr1* is not required for normal neurogenesis throughout the rest of the telencephalon (Fig. 4E-F′).

Another cell class present in the anterior telencephalon at the time of initial OB evagination is the radial glial cell. It is likely that radial glia are required to guide the migration of

early-born projection neurons into the OB, as is the case in other CNS areas, and are thus required for OB morphogenesis. To address the possibility that defects in radial glial cells are the root of OB malformations in the *Fgfr1*-deficient telencephalon, we used antibodies against the radial glial marker RC-2 (Misson et al., 1988) in E13.5 and E16.5 control and *Fgfr1*-deficient telencephalon. Radial glia are present in similar numbers and with normal morphologies at the anterior end of both the control and mutant telencephalon at E13.5 and E16.5 (Fig. 4G-H′), suggesting that radial glia specification and differentiation are unaffected by loss of *Fgfr1*.

Anterior telencephalic neurons in mutants share characteristics with OB neurons

Although neurons still accumulate at the anterior end of the telencephalon in mutant embryos, the results above do not establish whether these neurons are in fact normal OB neurons. The primary projection neuron found within the OB is the mitral cell. Mitral cells receive inputs through their dendrites directly from olfactory receptor neurons in the olfactory epithelium of the nose. The mitral cells then project axons to the olfactory cortex. To ascertain whether the neurons that accumulate at the anterior end of the *Fgfr1*-deficient telencephalon develop connections typical of mitral cells, we used the tracer DiI to track the afferent and efferent projections of these cells.

To ascertain whether cells in the anterior telencephalon of mutants receive their normal inputs from olfactory sensory neurons, a DiI crystal was placed in the olfactory epithelium in the posterior part of the nasal cavity in each of three control and mutant embryos for each age. Sections (125 μm) were collected using a vibratome and examined by fluorescence microscopy. Olfactory sensory axons contacted the anterior telencephalon by E12.5 in both control and mutant embryos (Fig. 5A,A′). At E18.5 in control embryos, large numbers of olfactory neurons have penetrated the bulb where they presumably make connections with OB neurons (Fig. 5B). In mutant embryos, these projections are indistinguishable from those in controls (Fig. 5B′). Thus, the pattern of sensory projections to the anterior telencephalon from the olfactory epithelium is indistinguishable in control and mutant brains.

We next ascertained whether cells in the anterior-most region of the *Fgfr1*-deficient telencephalon project normally to olfactory cortex. DiI crystals were placed in the olfactory (or piriform) cortex of three control and three mutant E18.5 embryos to retrogradely label neurons projecting to the olfactory cortex. Neuronal cell bodies in both the OB of control embryos and the bulb-like region of mutants are labeled with DiI (Fig. 5C,C′). In both cases, the retrogradely labeled cells were small and multipolar with short dendrites, as is typical of young mitral neurons before their dendritic innervation of glomeruli which have yet to form (Ramón y Cajal, 1995). These results indicate that not only do neurons accumulate at the anterior end of the *Fgfr1*-deficient telencephalon in the absence of a normal OB, but that these neurons also establish olfactory pathway projections characteristic of mitral cells.

Consistent with the finding that mitral cells are likely to be present in mutants, but displaced so that they now reside within the wall surrounding the lateral ventricle as opposed to in a morphologically distinguishable bulb, the size of the telencephalon in mutants appears enlarged compared with the telencephalon in controls (not including the OB) (Figs 2, 3). Although no significant difference was found in the dorsoventral thickness of the telencephalon, the length of the telencephalon from the most anterior point to the most posterior point were measured in whole-mount mutant and control brains (excluding the OB in controls) at E16.5 and P0. The mutant telencephalon was found to be 11% longer ($P<0.01$).

The rostral migratory stream in *Fgfr1* mutant embryos

Neuroblasts from the ventral telencephalon migrate to the OB before birth and continue to do so after birth from the anterior

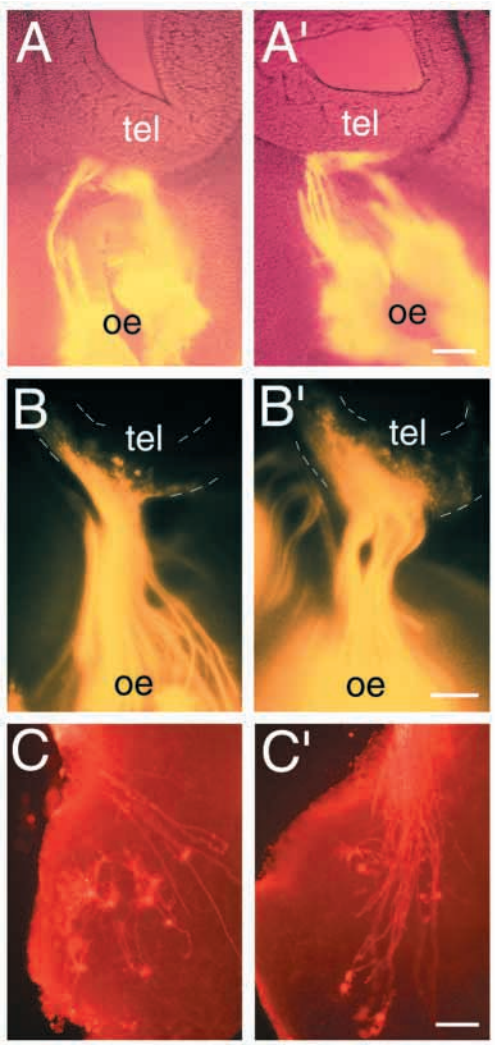

Fig. 5. Neurons at the anterior end of the *Fgfr1*-deficient telencephalon make connections characteristic of mitral cells. DiI labeling of sensory axons from the olfactory epithelium (oe) at E12.5 shows that axons have reached the anterior telencephalon (tel) in both control (A) and *Fgfr1*-deficient (A′) mice. Scale bar: 0.1 mm. (B,B′) DiI labeling of the olfactory epithelium (oe) at E18.5 reveals that sensory axons have penetrated the anterior telencephalon in both control (B) and mutant (B′) mice; ventricular and basal borders of the telencephalic neuroepithelium are indicated (broken lines). Scale bar: 0.15 mm. (C) Retrograde labeling of mitral cells in the olfactory bulb of control mice following DiI injection into lateral (piriform) cortex (in which DiI penetrated the tract). Similar cells are labeled in *Fgfr1* mutants (C′). Scale bar: 0.1 mm.

subventricular zone as part of the rostral migratory stream. Upon reaching the OB, the cells differentiate into GABAergic interneurons. It is possible that the failure of the OB to form in mutants might arise if these neurons no longer migrated to the anterior telencephalon in the *Fgfr1*-deficient telencephalon. To address this possibility, we examined the expression of several genes that mark these migrating cells by in situ RNA hybridization analysis. In E16.5 and P0 control animals, *Dlx2*, *Gad67* and *Pbx3* are all expressed in the ganglionic eminence and in cells migrating anteriorly and dorsally (Fig. 4D and data not shown). In mutant embryos, this pattern of expression is maintained for all three genes, although the labeled cells appear to accumulate within the anterior telencephalon at E16.5 and in the bulb-like protrusion at P0 (Fig. 4D′ and data not shown). This result is consistent with previous findings demonstrating that cells from the ventral telencephalon still migrate towards the anterior tip of the telencephalon even when the OB itself has been surgically removed (Jankovski et al., 1998; Kirschenbaum et al., 1999).

Proliferation and morphogenesis are abnormal in mutant OB primordia

The experiments described above suggest that OB neurons are born at the right time and accumulate normally at the anterior end of the telencephalon in *Fgfr1* mutants and that many of these neurons display connections characteristic of mitral cells. Despite the normal fate specification of anterior telencephalic cells, OB development is highly abnormal in the mutant mice. This raises the possibility that the OB phenotype is due to a defect in the process of morphogenesis itself, whereby anterior telencephalic cells normally evaginate to form a bulb. It has been suggested that projections from the olfactory epithelium, which contact the anterior telencephalon, induce bulb morphogenesis by reducing the number of proliferating telencephalic cells in this region (Gong and Shipley, 1995). The reduction in proliferation and the accompanying increase in neuronal differentiation at the anterior end of the telencephalon are thought to trigger the evagination of the OB relative to the rest of the telencephalon, where proliferation continues at a high rate. In this model, if cells in the anterior telencephalon were proliferating at the same rate as those in the rest of the telencephalon, expansion would occur uniformly in the lateral plane of the telencephalon and the OB would thus be morphologically indistinguishable from neighboring areas. Decreased proliferation at the anterior tip of the telencephalon thus enables the formation of a morphologically apparent OB.

As illustrated in Fig. 5, projections from the olfactory epithelium to the anterior telencephalon appear normal in the *Fgfr1*-deficient telencephalon, both at an early age when these projections have just reached their target, and at a later age when functional connections are presumably being established. Therefore, contact between olfactory epithelial projections and the anterior telencephalon is not in itself sufficient to induce OB formation.

To address the second part of the model, that a decrease in the proliferation of anterior telencephalic cells is required for evagination, we asked whether proliferation in the mutant telencephalon at the time of initial bulb formation is affected by loss of *Fgfr1*. E12.5 embryos were exposed to BrdU in utero for 1 hour and were then analyzed in situ for BrdU incorporation. The percentage of BrdU-positive cells was counted in two sections from each of three separate experiments for both control and mutant embryos. In control embryos, markedly fewer cells incorporated BrdU in the OB region (23.0%) compared with neighboring cerebral cortex (45.0%, Fig. 6A-C, $P<0.0001$), consistent with previous findings in rat at a comparable developmental age (25.4% versus 50.2%) (Gong and Shipley, 1995). In mutant embryos, proliferation in the OB region remains nearly as high as in neighboring cortex (41.6% compared with 44.0%, Fig. 6A′,B′,C). These results indicate that *Fgfr1* is required to lower the number of proliferating cells at the anterior end of the telencephalon and suggest that OB morphogenesis depends on a reduced rate of proliferation in this area. In addition, our data show that the cerebral cortex and other telencephalic areas do not require *Fgfr1* to maintain normal rates of proliferation (Fig. 6B′ and data not shown).

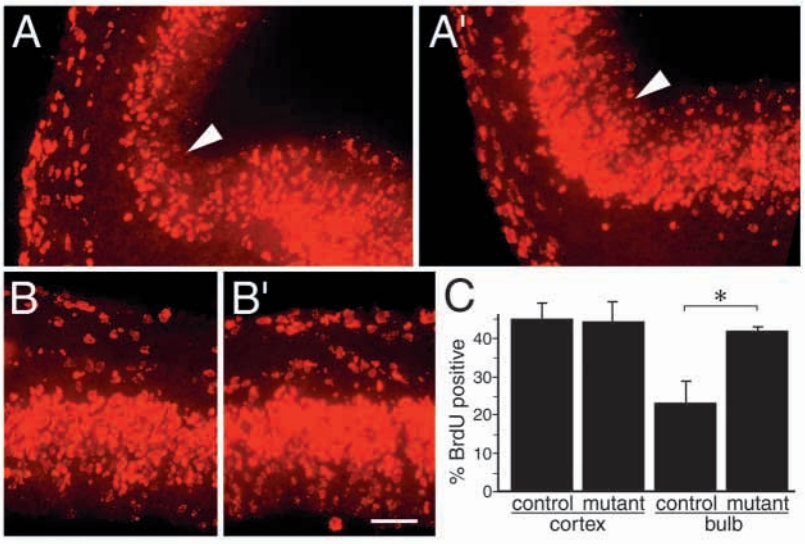

Fig. 6. The failure of olfactory bulb development in *Foxg1-Cre/+;Fgfr1fx/fx* mice involves a failure of cells in the anterior telencephalon to show decreased levels of proliferation. BrdU incorporation in E12.5 control (A,B) and mutant (A′,B′) telencephalons at the site of normal olfactory bulb development (A,A′) and in the cerebral cortex (B,B′). Levels of BrdU incorporation are reduced at the anterior tip of the telencephalon in control whereas in the mutant levels remain high (arrowheads). Scale bar: 0.08 mm. (C) Quantitation of BrdU incorporation. Unlike cells in the anterior telencephalon of controls, cells in the anterior telencephalon of mutants continue to proliferate at rates comparable with those in neocortex (asterisk, $P<0.0001$).

To address the possibility that FGF signaling might affect the rate of apoptosis in the telencephalon, cell death was also examined in control and mutant embryos at E12.5 using TUNEL staining. The percentage of TUNEL-positive cells was counted in two sections from each of three separate experiments for both control and mutant embryos. No significant differences between controls and mutants were observed within the OB region (2.6±2.1% in controls versus 2.0±1.4% in mutants), the cerebral cortex

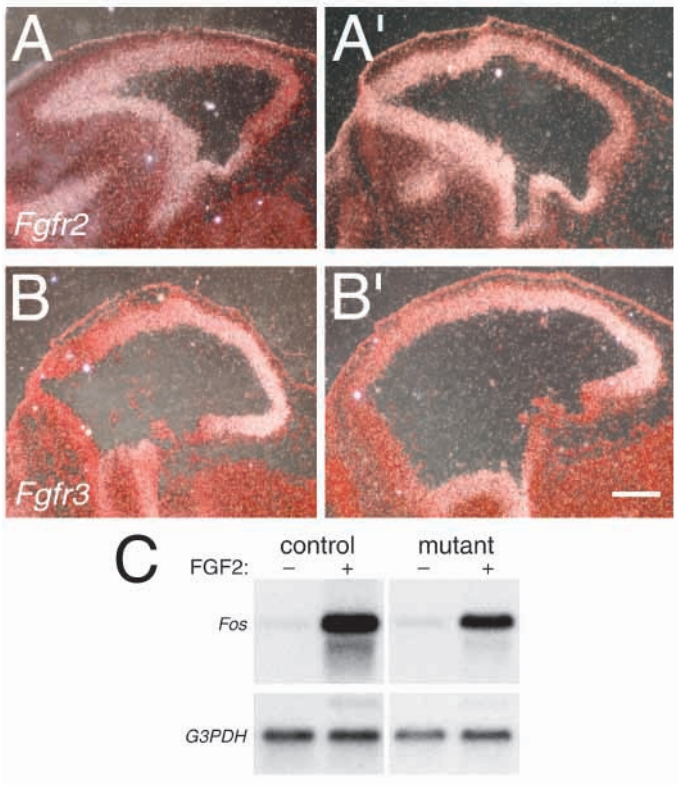

Fig. 7. Expression of FGF receptor mRNAs in E12.5 wild-type and *Fgfr1*-deficient mice. Sagittal sections showing in situ hybridization in control (A,B) and mutant (A′,B′) brains for *Fgfr2*, (A,A′) and *Fgfr3* (B,B′). (C) FGF2 induces *Fos* expression in control cells from the dorsolateral telencephalon. FGF2-induced *Fos* expression is reduced but not eliminated in *Fgfr1*-deficient cells. *Gapdh* is used as a loading control (*G3PDH* in figure). Scale bar: 0.25mm.

(1.8±1.6% in controls versus 1.2±1.1% in mutants) or in any other telencephalic areas (data not shown).

Fgfr2 and *Fgfr3* may partially compensate for loss of *Fgfr1* in the telencephalon

Although *Fgfr1* has been effectively deleted in the telencephalon of *Foxg1-Cre;Fgfr1*$^{flox/flox}$ mice (Fig. 1), most areas outside of the OB region remain largely normal. One reason for this might be that *Fgfr2* and *Fgfr3* are still being expressed in telencephalic progenitor cells (Orr-Utreger et al., 1991; Peters et al., 1992; Peters et al., 1993; Yamaguchi et al., 1992). In fact, expression of *Fgfr2* and *3* is unaffected by loss of *Fgfr1* (Fig. 7A-B′); consequently, these receptors may partially compensate for the absence of *Fgfr1*. To test the possibility that *Fgfr1*-deficient telencephalic cells are still responsive to FGF signaling, cells from the dorsal telencephalon of E13.5 control and mutant embryos were dissociated and cultured in the presence or absence of FGF2. Responsiveness to FGF2 was assessed by examining induction of *Fos*, a gene whose expression is induced by FGFs in neural cells (Ghosh and Greenberg, 1995). Telencephalic cells from both control and mutant animals expressed *Fos* upon FGF2 treatment (Fig. 7C), confirming that *Fgfr1*-deficient dorsal telencephalic cells are still responsive to FGF. However, cells from mutant brains showed a diminished response to FGF2 compared with control cells, as control cells showed a 41-fold induction of expression (±15), whereas mutant cells showed a 24-fold induction of expression (±4, P=0.03). It is unlikely that this responsiveness in mutants is due to residual *Fgfr1*, because recombination of the *Fgfr1*^{flox} allele is complete by E12.5 (Fig. 1). Based on the findings that dorsal telencephalic cells express *Fgfr2* and *3* and are still responsive to FGF2, it is likely that FGF signaling takes place in vivo in the absence of *Fgfr1*, but that signaling levels are reduced relative to levels encountered in the wild-type brain.

DISCUSSION

In this report, we demonstrate an essential role for *Fgfr1* in forebrain development. By crossing *Foxg1-Cre* mice to mice that carry an *Fgfr1* allele in which the essential exons 8-15 are flanked by *loxP* sites, we generated embryos in which the telencephalon lacks functional *Fgfr1*. In these mutants, the OB does not form normally. This failure occurs despite specification of anterior telencephalic cells, a normal early accumulation of anterior neurons that go on to form connections characteristic of mitral cells, and the presence of radial glia. What differs between the control and *Fgfr1*-deficient telencephalon is that the normal decrease in cell proliferation at the anterior tip at E12.5 fails to occur during the initial stages of OB morphogenesis, indicating that *Fgfr1* is directly or indirectly required to inhibit anterior cell proliferation and promote OB evagination. Although FGF signaling has been implicated in cortical area formation (Fukuchi-Shimogori and Grove, 2001), the *Fgfr1*-deficient cerebral cortex remains patterned along the AP axis. However, minor changes in the expression levels of the genes examined here, such as *Emx2* and *Pax6*, may nevertheless occur in mutants and could underlie subtle patterning defects. Overall, these results demonstrate an essential role for *Fgfr1* in telencephalic development, particularly in OB morphogenesis.

FGF signaling and anterior-posterior patterning of the telencephalon

FGF signaling has been hypothesized to pattern the AP axis of the telencephalon (Shimamura and Rubenstein, 1997; Meyers et al., 1998; Crossley et al., 2001; Fukuchi-Shimogori and Grove, 2001). More specifically, FGF8 expressed from its source at the anterior end of the telencephalon might instruct cells along the AP axis to express different levels of regulatory genes that in turn could result in the adoption of different fates. Telencephalic patterning is grossly normal in *Fgfr1*-deficient mutants. The expression of *Emx2* and *Pax6* in counter gradients indicates that AP patterning has occurred and, except for the OB, all the major structures – cerebral cortex, hippocampus, choroid plexus, and medial and lateral ganglionic eminences – are present and morphologically normal (Fig. 2 and data not shown).

The role of different FGF receptors in brain patterning remains unclear. One possibility for the lack of a major patterning defect in the *Fgfr1*-deficient telencephalon is that *Fgfr1*, *Fgfr2* and *Fgfr3*, all of which are expressed in the ventricular zone of the telencephalon throughout development, have overlapping functions. Alternatively, each receptor may

support distinct developmental outcomes. The results presented here suggest that levels of FGF signaling are dampened by deleting *Fgfr1*, rather than completely ablated, and that this does not result in a global disruption or shift in patterning. This is most consistent with the possibility that the different FGF receptors are working in concert to pattern the telencephalon.

FGF receptor-ligand specificities in the early telencephalon

Both *Fgf8* and *Fgfr1* are essential for normal telencephalic development, as both *Fgfr1*-deficient and *Fgf8*-hypomorphic telencephalons lack normal OBs (Meyers et al., 1998) (this report). This suggests that FGF8 acts through FGFR1 in the early anterior telencephalon, an unexpected finding given previous reports in which FGF8 had little or no significant affinity for FGFR1 in cell mitogenicity and binding assays (Ornitz et al., 1996; Chellaiah et al., 1999). The basis for this discrepancy remains unexplored.

Although our data are consistent with the view that FGF8 acts through FGFR1, FGF8 must also act through at least one other FGF receptor because the telencephalic phenotype of *Fgf8* hypomorphic embryos can be more severe than that seen in *Fgfr1*-deficient brains. In addition to loss of the OBs, some *Fgf8* hypomorphs also have smaller telencephalons with midline defects (Meyers et al., 1998). FGF8 is reported to have little affinity for FGFR2 and significant affinity for FGFR3 (Ornitz et al., 1996; Chellaiah et al., 1999), but it is clear that FGFR3 on its own can not be a predominant receptor for transmitting FGF8 signals in the telencephalon because mice deficient for *Fgfr3* have normal OBs and the rest of the telencephalon appears phenotypically normal (Deng et al., 1996). Elucidation of the pertinent interactions between FGF receptors and ligands in the developing telencephalon will benefit from further analysis of animals bearing mutations in single Fgf and Fgfr genes, as well as mutants deficient in several genes at once.

The role of FGF signaling in OB morphogenesis

The most striking phenotype of *Fgfr1*-deficient brains is the absence of normal OBs. Indeed, it was initially tempting for several reasons to speculate that the lack of OBs in *Fgfr1* mutants arises from a failure in the induction or specification of the most anterior telencephalic cells. First, as summarized above, FGF signaling is hypothesized to specify cell fates along the AP axis (Meyers et al., 1998; Fukuchi-Shimogori and Grove, 2001), and mice hypomorphic for *Fgf8*, which is expressed at the anterior end of the telencephalon (Crossley and Martin, 1995; McWhirter et al., 1997), also lack OBs (Meyers et al., 1998), although the mechanism leading to this defect remained unexplored. Second, like the *Fgfr1* mutants described here, mice deficient for *Pax6*, a gene that is expressed at high levels in the anterior telencephalon and is known to be required for normal telencephalic patterning, lack OBs (Hogan et al., 1986). These correlations are consistent with a role for FGF signaling in elevating *Pax6* expression to promote OB formation. However, there are at least two significant differences between the *Pax6* mutants and those described here. While both lack OBs, *Pax6* mutants also lack nasal cavities and olfactory epithelia, which may play a role in OB induction. In addition, *Pax6* mutants completely lack *Pax6* expression, whereas *Pax6* expression is at best reduced in the *Fgfr1*-deficient telencephalon.

Our data argue that the absence of the OB in *Fgfr1*-deficient mice is not due to defective anterior cell fate specification. First, markers for the early anterior telencephalon, ephrin A5 and *Pou3f1*, are induced and expressed normally in mutants. Second, *Tbr1* expression in the anterior telencephalon indicates that the normal increase in neurogenesis at the anterior end occurs in mutants. And third, these neurons make connections that are characteristic of mitral cells, receiving afferents from the olfactory epithelium and projecting axons to the olfactory cortex. Moreover, it has been shown previously that in *Tbr1*$^{-/-}$ mice, mitral cells are lacking, but morphogenesis of the bulb still occurs (Bulfone et al., 1998), indicating that mitral cell differentiation and bulb morphogenesis can occur independently of one another. Therefore, it seems likely that in the *Fgfr1*-deficient telencephalon, the deficiency in OB formation is not likely to be due to the absence of mitral cells, which appear to be specified and to differentiate, but instead to abnormal morphogenesis that results in the misplacement of these cells. In addition, a global delay in OB development cannot account for the phenotype because anterior telencephalic markers are expressed normally and anterior neurogenesis occurs on a normal schedule in the mutants.

The failure of bulb morphogenesis in the *Fgfr1*-deficient telencephalon is also not likely to result from defects in radial glia differentiation, which appears normal, or neuronal migration from the ventral telencephalon (Fig. 4D,D′,G-H′). In E16.5 to P0 mutants, neuroblasts from the ventral telencephalon migrate as they would normally towards the anterior telencephalon. Once these cells reach the anterior telencephalon, however, they accumulate abnormally in this location (perhaps because of the misplacement of OB neurons such as mitral cells), instead of continuing anteriorly as they normally would into a protruding OB. Nevertheless, because cells still migrate from the ventral telencephalon, it is unlikely that defects in this process are responsible for the initial lack of bulb evagination in the *Fgfr1*-deficient telencephalon. Moreover, previous reports indicate that cell migration to the OB from the ventral telencephalon is not required for initial OB morphogenesis (Anderson et al., 1997; Bulfone et al., 1998).

A model for initial OB evagination has been proposed previously (Gong and Shipley, 1995). According to this model, just prior to the earliest signs of OB evagination, sensory axons from the olfactory epithelium arrive at the anterior telencephalic end, where they initiate OB morphogenesis (Fig. 8, step 1). Subsequent to the arrival of axons from the olfactory epithelium and their contact with anterior telencephalic progenitor cells, a decrease in the proliferation of these cells is observed (Fig. 8, step 2), suggesting that olfactory epithelial axons inhibit progenitor cell proliferation to trigger bulb evagination. In this model, a decrease in the number of proliferating cells along with an increase in the number of differentiating cells leads to an initial radial (or outwards) expansion at the anterior telencephalic tip (Fig. 8, step 3). If cells at the anterior telencephalon were proliferating at the same rate as the rest of the telencephalon, uniform expansion would occur in the lateral plane of the telencephalon and the OB would be morphologically indistinguishable from neighboring areas (Gong and Shipley, 1995).

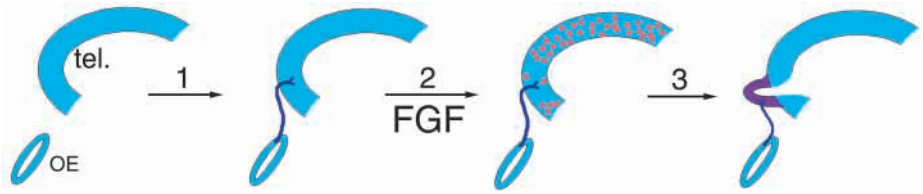

Fig. 8. Model of the steps involved in early OB morphogenesis. In the first step, axons from the olfactory epithelium (OE) project to and make contact with the anterior telencephalon (tel.). In a second step that is dependent on FGF signaling, a reduction in the number of proliferating cells (dots) at the anterior end of the telencephalon occurs (around the axons from the OE), concurrent with an increase in the number of neurons (not shown). In the third step, the decrease in cell proliferation causes initial bulb evagination (purple).

As shown in Fig. 1, the olfactory epithelium normally expresses *Fgfr1* and this expression is lost in mutants, raising the possibility that the olfactory epithelium itself is defective and thus cannot induce OB formation. However, the olfactory epithelium is histologically normal in the *Fgfr1*-deficient telencephalon, and olfactory axons reach the telencephalon on time at E12.5 (Fig. 5A). The normal behavior of olfactory epithelial axons suggests that projections from the olfactory epithelium to the telencephalon are not sufficient to induce OB evagination. However, it remains possible that the arrival of olfactory axons still plays an important role in OB formation, as the olfactory axons could either exert their effect on the anterior telencephalon directly or indirectly through an FGF-dependent mechanism (e.g. if the axons secreted an FGF to which the anterior telencephalon normally responds), or through an FGF-independent mechanism that would be required in addition to FGF signaling to initiate OB evagination. In this latter case, disrupting either the FGF-dependent or -independent pathways would cause a failure of OB evagination.

It is interesting to note that between E16.5 and birth, a bulb-like structure begins to appear in the *Fgfr1* mutants. Previous studies in mice have shown that olfactory epithelial axons can induce OB-like neurons in the anterior telencephalon of bulbectomized neonates (Graziadei and Monti-Graziadei, 1986). It is possible that during later development, the inductive effect of sensory axons, along with the arrival of migrating neurons from the ventral telencephalon, account for the late forming bulb-like evagination in the *Fgfr1*-deficient telencephalon.

What is clear from the results presented here is that in the *Fgfr1*-deficient telencephalon, a decrease in proliferation in the region of normal bulb evagination fails to occur, indicating that FGF signaling, either directly or indirectly, is required to inhibit proliferation at the anterior tip of the forebrain (Fig. 8, step 2) and that the initial evagination of the OB requires a decrease in the number of proliferating cells at the anterior end of the telencephalon (Fig. 8, step 3). The mechanism by which *Fgf8* hypomorphic embryos fail to form the OB remains to be elucidated, but the results presented here suggest that it may also be due to a failure to decrease anterior telencephalic cell proliferation. Consistent with this possibility, FGF8-soaked beads placed ectopically in the chick telencephalon generate extra sulci, which are thought to be the result of reduced proliferation (Crossley et al., 2001).

FGF signaling has previously been implicated in promoting proliferation of neuroepithelial progenitor cells in vitro (Gensburger et al., 1987; Ghosh and Greenberg, 1995) and in vivo (Dono et al., 1998; Ortega et al., 1998; Raballo et al., 2000). Hence, the result that *Fgfr1* is required to inhibit proliferation of anterior telencephalic cells was unexpected. However, FGF signaling has previously been implicated in inhibiting the proliferation of cell types other than those described here. For example, *Fgfr3*-deficient mice show an increase in chondrocyte proliferation (Deng et al., 1996), whereas activation of FGFR3 inhibits their proliferation (Sahni et al., 1999), demonstrating a role for FGF signaling in inhibiting proliferation. FGF5 either directly of indirectly inhibits proliferation of hair cells (Hébert et al., 1994). Hence, the roles that FGF signaling plays throughout telencephalic development may yet be through unexpected mechanisms.

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