FGF signaling is strictly required to maintain early telencephalic precursor cell survival

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The FGF family of extracellular signaling factors has been proposed to play multiple roles in patterning the telencephalon, the precursor to the cerebrum. In this study, unlike previous ones, we effectively abolish FGF signaling in the anterior neural plate via deletion of three FGF receptor (FGFR) genes. Triple FGFR mutant mice exhibit a complete loss of the telencephalon, except the dorsal midline. Disruption of FGF signaling prior to and coincident with telencephalic induction reveals that FGFs promote telencephalic character and are strictly required to keep telencephalic cells alive. Moreover, progressively more severe truncations of the telencephalon are observed in FGFR single, double and triple mutants. Together with previous gain-of-function studies showing induction of Foxg1 expression and mirror-image duplications of the cortex by exogenous FGF8, our loss-of-function results suggest that, rather than independently patterning different areas, FGF ligands and receptors act in concert to mediate organizer activity for the whole telencephalon.

KEY WORDS: Forebrain, FGF receptors, Neural plate, Neural patterning, Mouse

INTRODUCTION

How a morphologically uniform sheet of cells becomes patterned into molecularly, functionally and structurally distinct areas remains one of the principal puzzles facing developmental biologists. In particular, the mechanisms involved in patterning the central nervous system, which begins as a sheet of neuroepithelial cells during embryogenesis, remain poorly understood. The anterior region of the neuroepithelium develops into the telencephalon, the precursor to the cerebral hemispheres in which our highest cognitive processes reside. This study addresses how the anterior neuroepithelium acquires and maintains a telencephalic fate.

The earliest and most specific marker for telencephalic fate is the expression of Foxg1, a gene encoding a winged helix transcription factor (Shimamura and Rubenstein, 1997; Tao and Lai, 1992). Foxg1 expression initiates shortly after the anterior neuroepithelium (or neural plate) bends to form the head folds. Other genes (Pax6, Nkx2.1, Otx1, Six3, Dlx2 and Emx genes) are also expressed in the telencephalic anlage at early stages, although their expression either extends beyond telencephalic precursors to diencephalic ones or turns on later than Foxg1 (Puelles and Rubenstein, 2003; Shimamura et al., 1995; Wilson and Houart, 2004).

The anterior neural ridge (ANR, or anterior neural border, ANB, in zebrafish), which is the junction between the anterior neural and non-neural ectoderm, is essential for induction of the telencephalon. In mice and zebrafish, removal of the ANR/ANB prior to the specification of telencephalic character causes a failure to induce Foxg1 expression (Houart et al., 1998; Shimamura and Rubenstein, 1997). Moreover, in zebrafish, transplanting the ANB to the diencephalon or midbrain at the mid-gastrula stage induces ectopic expression of markers for the dorsal and ventral telencephalon (Houart et al., 1998). This indicates that the ANR acts to induce the telencephalon, perhaps acting as a classic organizer. However, the molecular mediators of this activity remain unknown.

There are two leading candidates for ANR-expressed telencephalic inducers. The first are secreted frizzled-related WNT antagonists (sFRPs). Tlc, an sFRP in zebrafish, is expressed in the ANB and is necessary and sufficient to induce telencephalic gene expression (Houart et al., 2002). In mice, however, evidence supporting the role of sFRPs in telencephalon induction is lacking (Satoh et al., 2006). The second candidate for ANR-derived telencephalic inducers are fibroblast growth factors (FGFs). FGFs are developmentally important intercellular signaling molecules that can activate the Ras/MAPK, PLCγ, PI3 kinase and STAT1 pathways upon binding to FGF receptors (Mason, 2007). During early telencephalic development in mice, at least 5 of the 22 genes encoding FGF ligands, Fgf3, Fgf8, Fgf15, Fgf17 and Fgf18, are expressed in the ANR or its early derivatives, and three of the four receptor genes, Fgfr1, Fgfr2 and Fgfr3, are expressed broadly in the anterior neuroectoderm (Maruoka et al., 1998; McWhirter et al., 1997; Orr-Urtreger et al., 1991; Peters et al., 1993; Peters et al., 1992; Rash and Grove, 2007; Shinya et al., 2001; Yamaguchi et al., 1992).

FGF signaling acts at the end of gastrulation to posteriorize the neural plate and later to pattern the telencephalon (Mason, 2007; Wilson and Houart, 2004; Wilson and Rubenstein, 2000). However, as no FGF or FGFR mutant in either mouse or zebrafish has yet to result in a failure to form a telencephalon, FGF signaling has not been considered a telencephalic inducer or a mediator of organizing activity for this tissue. Nevertheless, implanting FGF8-soaked beads in anterior neural explants lacking an ANR induces Foxg1 expression (Shimamura and Rubenstein, 1997), consistent with a role in inducing the telencephalon. One reason why a telencephalon is still observed in the mutants examined to date is that functional compensation may occur between family members with overlapping expression domains. Alternatively, studies suggest distinct roles for FGFs in the developing telencephalon (Borello et al., 2008; Cholfin et al., 2003; Cholfin and Rubenstein, 2007; Cholfin and Rubenstein, 2008).

In this study, the in vivo requirement for FGF signaling in initiating telencephalon formation is investigated. In order to effectively abolish FGF signaling in the anterior neural plate, floxed alleles of Fgfr1 and Fgfr2 are deleted using two different Cre driver lines in an Fgfr3−/− background. Our results indicate that except for the dorsal midline, formation of the telencephalon is entirely...
dependent on FGF signaling. Moreover, the phenotype observed in the FGFR triple mutant together with those observed in less severe single and double FGFR mutants suggest that FGF ligands and receptors, rather than having distinct functions, act in concert to pattern the early telencephalon.

MATERIALS AND METHODS
Generation of mutant embryos
To study the role of FGF signaling in initiating telencephalon formation, two crosses were used to generate mice mutant for all three FGFR genes expressed in the neural plate (Fig. 1A, Fig. 6A). A loss of Fgfr1 or Fgfr2 in the whole embryo leads to lethality at gastrulation (Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994), therefore floxed alleles of these genes (Trokovic et al., 2003; Yu et al., 2003) were used to generate knockouts when crossed to Foxg1lox or CAGG-CreER mice (Hayashi and McMahon, 2002; Hébert et al., 2003a) in an Fgfr3–/– background (Deng et al., 1996). Control mice used were heterozygous for Foxg1Cre and Fgfr2 and carried the Cre allele, and these controls did not exhibit any of the phenotypes described in this study. Triple FGFR mutant embryos were obtained at the ages indicated in the expected ratios with no signs of necrosis (1:32 using the Foxg1lox driver and 1:8 using CAGG-CreER). A total of 25 triple homozygous mutant embryos from the Foxg1lox cross and 28 from the CAGG-CreER cross were used.

RNA in situ hybridization
In situ hybridizations were performed according to two protocols; one using radioactive RNA probes on sections and the other using digoxigenin (DIG)-labeled probes in whole mount. For the radioactive in situ, frozen sections were prepared and hybridized to 35S-labeled probes as previously described (Frantz et al., 1994). For in situ hybridizations on whole-mount mouse embryos using DIG-labeled probe, 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.

Cell proliferation and TUNEL assays
Embryos were collected at E9.0 (14- to 18-somite stages) and frozen in O.C.T. (Tissue-Tek). Fresh frozen sections were used for either phospho-histone H3 (P-HH3) staining, as previously described (Storm et al., 2006), or for TUNEL analysis, according to the manufacturer’s specifications (Roche, 2156792). Horizontal sections throughout the anterior neural tissue of control and mutant embryos were compared. Sections were counterstained with Hoechst 33342. The fraction of P-HH3+ or TUNEL + cells was determined by counting the number of these cells in a segment of Foxg1-expressing neuroepithelium divided by the total number of cells (Hoechst3+) in that segment. An example of a segment of Foxg1+ cells used for counting is illustrated by the neuroepithelial area between the arrowheads in Fig. 5 and contains ~200 cells. At least three segments were counted in each case.

Tamoxifen treatment
For the experiments using the CAGGA-CreER mice, 5, 9 or 10 mg of tamoxifen per 40 g body weight was administered via intraperitoneal injection to pregnant females at E6.75 and, in cases where a second injection was performed, again at E7.75. Tamoxifen-treated embryos were collected, fixed and subjected to whole-mount RNA in situ hybridization as described above.

Explant cultures
Telencephalic neuroepithelium was dissected from E8.5 (5- to 8-somite stage) embryos and when indicated the ANR was removed with a ‘Bonn’ Microprobe (Fine Science Tools, 10030-13) as described previously (Shimamura and Rubenstein, 1997). Isolated tissue was electroporated with 20 μg of a plasmid vector expressing a human Foxg1 cDNA (OriGene, SC116871). The pEGFP-N1 vector (Clontech, 6085-1) was used to monitor the efficiency of electroporation. For electroporation, five pulses of 100 V, each for 50 msconds, were administered. Electroporated telencephalic tissue was cultured on a Micropore floating filter (Falcon, 353090) on Dulbecco’s Modified Eagle Medium (DMEM; GIBCO, 11960) supplemented with 10% fetal bovine serum and 1× streptomycin/penicillin (GBCO) in a 5% CO2 incubator at 37°C for 24 hours.

RESULTS
Deletion of FGFR genes in the anterior neural plate leads to a loss of FGF signaling
The role of FGF signaling in the induction and initial formation of the telencephalon remains obscure. All FGF and FGFR mutants examined to date in either zebrafish or mice have a telencephalon, albeit in some cases mispatterned. Therefore, either expression of FGF and FGFR genes is not required for initial telencephalon formation or compensation by related genes has masked the full role of FGF signaling in the mutants examined. To distinguish between these two possibilities, FGF signaling was abolished in this study by conditionally deleting floxed alleles of Fgfr1 and Fgfr2 in an Fgfr3-null background (Fig. 1A). Cre-mediated recombination was first

Fig. 1. Knocking out FGF signaling leads to a loss of telencephalic structures. (A) Genetic cross required to generate embryos that lack Fgfr expression in the anterior neural plate. The Foxg1lox allele is used to recombine floxed alleles of Fgfr1 and Fgfr2 in an Fgfr3-null background. (B) RNA in situ hybridization on horizontal sections (anterior up, posterior down) using a radioactive probe that recognizes the sequences between the lox sites in the floxed Fgfr1 allele. By the 10- to 12-somite stage (E9.75), Fgfr1 expression is not detectable above background levels in a Fgfr1;Fgfr2 double mutant (right). Bottom panels are enlargements of the boxed regions in the top panels. (C) Whole-mount RNA in situ hybridization for Spry1, a reporter for FGF signaling. Expression is absent in the 13-somite stage triple FGFR mutant (right) in the anterior neuroepithelium that normally gives rise to the telencephalon (arrowheads). (D) By E10.5, the bilaterally symmetrical telencephalic hemispheres are apparent in the control (left), but not the mutant (right; arrowheads).
carried out using the Foxg1Cre mice in which recombination can start as early as embryonic day (E) 7.5 in the anterior neuroectoderm (Fuccillo et al., 2004), but is consistent and uniform in telencephalic precursors by E8.75-9 (Fuccillo et al., 2004; Hébert and McConnell, 2000; Storm et al., 2003).

In order to examine whether Foxg1Cre-mediated recombination is efficient in the anterior neural plate, RNA in situ hybridization analysis was performed on sections of E8.75 controls and Foxg1Cre;Fgfr1fx/fx;Fgfr2fx/fx;Fgfr3–/– double mutants using an antisense probe complementary to the transcribed Fgfr1 sequences found between the loxP sites. In E8.75 controls (10- to 12-somite stage), Fgfr1 was expressed ubiquitously throughout the embryo, whereas in mutants of the same age, the expression of Fgfr1 was greatly reduced or absent specifically in the anterior neural plate (Fig. 1B), indicating that recombination began earlier and was essentially complete by E8.75. Similarly, Fgfr2 is presumably also deleted from the anterior neural plate in the Foxg1Cre;Fgfr1fx/fx;Fgfr2fx/fx;Fgfr3–/– mutants, as Fgfr1;Fgfr3 double mutant littermates did not display any of the phenotypes described here.

To confirm loss of FGF signaling in the Foxg1Cre;Fgfr1fx/fx;Fgfr2fx/fx;Fgfr3–/– triple mutants (from now on referred to as Foxg1Cre;FGFR mutants), we examined the expression of the downstream gene sprouty 1 (Spry1), which, as in other species is induced by FGF signaling (Kim and Bar-Sagi, 2004; Liu et al., 2003; Minowada et al., 1999). At E8.75 (12-13 somites), Spry1 expression normally occurs in the anterior neural plate and mid-hindbrain junction, whereas in the Foxg1Cre;FGFR triple mutant, it was specifically absent from the anterior neural plate (Fig. 1C, arrowheads), consistent with an absence of FGF signaling in this region by E8.75.

**FGF signaling is required for telencephalic tissue to develop**

Examination of whole Foxg1Cre;FGFR triple mutant embryos at E10.5 revealed an apparent truncation of anterior structures, including the telencephalon and facial tissues (Fig. 1D, arrowheads). At E12.5, a stage at which the major structures of the telencephalon are morphologically distinguishable, mutants lacked all anterior head structures, with no visible telencephalon, nasal process, upper and lower jaw (Fig. 1D, Fig. 7D). The facial phenotype is not analyzed in this study and is not altogether unexpected, as FGF signaling in the branchial arch ectoderm is important for facial development, and Foxg1Cre is also expressed in these cells (Hébert and McConnell, 2000; Trumpp et al., 1999). The morphology of the diencephalon, midbrain, hindbrain and rest of the body remained unaffected. No variability in this morphological phenotype could be detected and it was 100% penetrant (5/5 mutants at E10.5, 2/2 at E11.5 and 9/9 at E12.5).

To ascertain whether telencephalic tissue is completely missing in the E12.5 mutant, we examined the expression of several telencephalic markers, starting with Foxg1, which is expressed in all undifferentiated and differentiated telencephalic cells, except dorsal midline cells after E9.5, as well as in part of the optic stalk (Hatini et al., 1994; Pratt et al., 2004; Tao and Lai, 1992). In the mutant embryos, expression of Foxg1 was completely lacking except for a small region underlying the diencephalon, in the region of the anterior hypothalamus (Fig. 2A, arrowhead). To test whether the remaining small region of Foxg1 expression corresponds to the optic stalk, expression of Foxg1 [a marker for the ventral optic stalk and hypothalamus (Hatini et al., 1994)] and Tcf4b [Tcf7l2 – Mouse Genome Informatics; a marker for the dorsal diencephalon (Cho and Dressler, 1998)] was examined. In the triple mutant, the Foxg1 expression domain was immediately adjacent to the Foxd1 and Tcf4b domains underlying the diencephalon (Fig. 2B and data not shown), suggesting that the remaining Foxg1 expression in the mutant is primarily in the optic stalk rather than in the telencephalon, and that the mutant completely lacks telencephalic areas that express Foxg1 at E12.5. Consistent with this interpretation, expression of the dorsal telencephalic marker Emx1 and ventral markers Dlx2, Nx2.1 and Gli1 was also missing at E12.5 (Fig. 2C, D and data not shown), and at least Emx1 and Dlx2 were already missing by E10-10.5 (see Fig. S1 in the supplementary material). The only area expressing the ventral markers was diencephalic, and little or no neuroectodermal tissue was present in a position anterior to these expression domains. Together, loss of expression of both dorsal and ventral markers demonstrates a requirement for FGF signaling in forming not only the ventral telencephalon, as previously reported (Gutin et al., 2006; Kusche1 et al., 2003; Shainmugalingam et al., 2000; Shinya et al., 2001; Storm et al., 2006; Walshe and Mason, 2003), but also the dorsal telencephalon.

Sonic hedgehog (Shh) expression in the prechordal plate maintains the expression of FGF ligands in the ANR and depends on FGF signaling to form the ventral telencephalon (Hébert and Fishell, 2008). This does not exclude the possibility that FGF signaling also promotes the expression of Shh in a positive-feedback
FGF signaling is dispensable for dorsal midline formation

Although Foxg1 is not expressed at detectable levels in the dorsal midline area from E9.5 onwards, the use of Foxg1Cre mice results in efficient recombination of floxed alleles in most or all dorsal midline cells of the telencephalon (Hébert and McConnell, 2000; Hébert et al., 2002; Hébert et al., 2003b; Fernandes et al., 2007). A particularly good example of this was observed with the Bmp4lox-lacZ allele, in which the coding region for Bmp4 is replaced by lacZ upon recombination. In the Foxg1Cre;Bmp4lox-lacZ embryos, all cells of the dorsal midline, including those of the cortical hem and choroid plexus, are recombined and express lacZ (Hébert et al., 2003b). Moreover, at a functional level, using Foxg1Cre mice to recombine the BMP receptor gene BMPR1dΔ leads to a loss of midline characteristics in most or all dorsal midline cells (Fernandes et al., 2007).

In the E12.5 FGFR triple mutant, small bilateral ‘outpocketings’ are observed where the telencephalic hemispheres would normally be found (asterisks in Fig. 2A,D). Since these outpocketings do not express Foxg1 (Fig. 2A), this raises the possibility that they are remnants of the telencephalic dorsal midline. The presence of dorsal midline cell types was assessed in the E12.5 control and Foxg1Cre;FGFR triple mutant telencephalon using dorsal midline markers Wnt3a (a marker for the cortical hem), Tr (choroid plexus), Msx1 (choroid plexus and cortical hem) and Lhx5 (eminence thalami and septum). In the most anterior region of the mutant, the remaining tissue expressed only Tr and Msx1 (Fig. 3A) and data not shown), indicating that the remaining cells are choroid plexus in nature. In more posterior regions, Tr, Wnt3a, Mx1 and Lhx5 were all expressed (Fig. 3), indicating that the dorsal telencephalic dorsal midline remains in the Foxg1Cre;FGFR triple mutant. Consistent with this finding, this tissue does not express the ventral or dorsal telencephalic markers Dlx2 and Emx1, or a marker for the diencephalon, Tcl4b (Fig. 2).

Two possibilities can explain why the dorsal midline remains in the FGFR triple mutant. The first is that FGF signaling is not required for dorsal midline formation. The second is that Fgfr1 and Fgfr2 are not recombined early or efficiently enough in dorsal midline precursor cells so that the remaining signaling is sufficient to promote dorsal midline formation. Several points argue against this latter possibility. First, no residual Fgfr1 and Fgfr2 expression was detectable in presumptive dorsomedial precursors from E8.75 onwards (Fig. 1B and data not shown). Second, the loss of expression observed at E8.75 is likely to be early and efficient enough, as recombination using Foxg1Cre mice can cause the loss of the dorsal midline in another mutant, Bmp1r1Δ (Fernandes et al., 2007). Although it remains possible that the cell fate of dorsal midline precursors is already induced by FGF signaling before Cre recombination effectively abolishes it, or that some cells still express Fgfr1 and Fgfr2 in the FGFR triple mutant, the results presented here suggest that FGF signaling is not required for forming the dorsal midline.

FGF signaling is essential to keep telencephalic cells alive

At least two possible mechanisms, which are not mutually exclusive, can underlie the loss of Foxg1-expressing tissue observed at E12.5 (Figs 2 and 3). First, FGF signaling might be required to induce anterior neural plate cells to adopt a telencephalic fate and express telencephalic markers. Second, anterior neural plate cells might adopt a telencephalic fate, but then fail to proliferate or survive. To distinguish between these possibilities, expression of Foxg1 was examined shortly after anterior neural tissue first exhibits telencephalic character (E8.75-9.25), times at which no FGFR or Spry1 transcripts are detected in the mutant (Fig. 1B,C). Surprisingly, at E8.75 (11-somite stage), Foxg1 expression was clearly detected in the telencephalic anlage of mutants (3/3 mutant embryos), whereas at E9.25 (20-somite stage) it was undetectable (4/4 mutant embryos) (Fig. 4). This suggests that FGF signaling is required to maintain the survival of telencephalic cells. Alternative explanations that involve a loss of Foxg1 expression without a loss of cells are not likely because they would not account for the truncation of anterior neural tissue that is apparent from E9.75 onwards (Fig. 1D, Fig. 7D), as well as the loss of expression of other telencephalic markers (Fig. 2).

To test the hypothesis that telencephalic cells die between E8.75 and E9.25 in the triple mutant, TUNEL staining was performed on E9.0 embryos (16-somite stage) to determine the amount of cell death in the anterior neuroectoderm. High levels of cell death were detected specifically in the anterior Foxg1-expressing domain of Foxg1Cre;FGFR mutants but not in controls that also carry Foxg1Cre and are heterozygous for the FGFR receptor genes (Fig. 5A,B,D).
recombination was found to be variable. Nevertheless, levels of two injections of tamoxifen at the highest concentrations, administered once at E6.75 or twice, with a second administration regimens of tamoxifen administration were assessed: tamoxifen was the number of cells that lack functional FGF receptor signaling, several Foxg1Cre;FGFR mutants. Surprisingly, despite the high levels of cell cycle marker phospho-histone H3 (P-HH3) in controls and analyzed the expression at E9.0 (16 somites) of the M-phase cell expression, and loss of any Foxg1 expression is essential for the expression of at least one FGF ligand gene, Fgf8 because they reciprocally promote each others expression and FGFs at this stage. In fact, removal of the ANR recapitulates the FGF triple mutant phenotype. Whereas most explants displayed from E8.5 (5- to 8-somite stage) embryos were cultured with and promoting cell survival. Wild-type explants of anterior neural plate exhibit similar loss-of-function phenotypes. An explant culture expressing telencephalic cells, demonstrates a strict requirement for FGF signaling in keeping telencephalic precursor cells alive.

To test whether a decrease in cell proliferation also contributes to the truncation of anterior neural tissue observed in the mutants, we analyzed the expression at E9.0 (16 somites) of the M-phase cell cycle marker phospho-histone H3 (P-HH3) in controls and Foxg1Cre;FGFR mutants. Surprisingly, despite the high levels of cell death in this region, no significant difference in the percentage of M-phase cells over the total number of neuroepithelial cells could be detected in controls versus mutants (Fig. 5C,E), suggesting that proliferation is not a key factor in the loss of telencephalic precursors observed in the mutants from E8.75 to E9.25.

FGF signaling promotes expression of Foxg1 in the anterior neuroectoderm

The requirement for FGF signaling in maintaining telencephalic precursor cell survival between E8.75 and E9.25 does not preclude an earlier role for FGFs at E8.5 (7-9 somites) in inducing anterior neuroectoderm to adopt telencephalic character. Such a role could have been missed, as Foxg1 cis regulatory elements drive Cre expression, and Foxg1 expression itself is used as the earliest telencephalic marker (first detectable at E8.5 in the neuroectoderm) (Shimamura and Rubenstein, 1997). Therefore a lag, albeit a short one, is expected between initial Foxg1 expression and loss of any FGF-mediated induction of telencephalic character.

To assess the role of FGF signaling in inducing the earliest signs of telencephalic character to the anterior neural plate, FGFR expression would need to be disrupted as early as E7.5-8.0. For this reason, a CAGG-CreER driver was used, in which recombination is reported to occur ubiquitously in embryos upon tamoxifen administration (Hayashi and McMahon, 2002). To maximize recombination of the Fgfr1 and Fgfr2 alleles and increase the number of cells that lack functional FGF receptor signaling, several regimens of tamoxifen administration were assessed: tamoxifen was administered once at E6.75 or twice, with a second administration at E7.75, with doses of 5, 9 or 10 mg/40 g of body weight. Even with two injections of tamoxifen at the highest concentrations, recombination was found to be variable. Nevertheless, levels of FGFR expression and signaling in mutants were reduced compared with controls (Fig. 6B,C) and this resulted in a decrease in the level of Foxg1 expression in the anterior neuroepithelium from the earliest stages at which it is detected (Fig. 6D,E arrowhead).

One possible cause for decreased Foxg1 expression could be cell death. TUNEL labeling revealed an increase in the percentage of dying cells in these mutants compared with controls at E8.5, from 1.2±0.3% (mean±standard error) to 4.1±1.1%; P=0.013. However, this remains a small percentage of cells that are dying and is unlikely to be the sole contributor to the reduction in Foxg1 expression. Together, these results suggest that FGF signaling is a positive regulator of Foxg1 expression. Moreover, these loss-of-function studies are consistent with previous gain-of-function studies suggesting that FGFs induce telencephalic character (Shimamura and Rubenstein, 1997).

Conversely, Foxg1 expression is essential for the expression of at least one FGF ligand gene, Fgf8 (Martynoga et al., 2005). It has been difficult to determine the epistatic relationship between Foxg1 and FGFs because they reciprocally promote each others expression and exhibit similar loss-of-function phenotypes. An explant culture paradigm was used to address this question, at least with regards to promoting cell survival. Wild-type explants of anterior neural plate from E8.5 (5- to 8-somite stage) embryos were cultured with and without an ANR, which is likely to be the main or only source of FGFs at this stage. In fact, removal of the ANR recapitulates the FGFR triple mutant phenotype. Whereas most explants displayed scattered cell death within ~25 μm from their periphery regardless of the presence or absence of an ANR, only explants devoid of an ANR displayed extensive cell death throughout the tissue (n=5/5; see Fig. S3 in the supplementary material), suggesting that the source of FGFs that conveys cell survival in vivo is the ANR. We then tested whether electroporation of a Foxg1 expression construct could rescue the cell death observed in the explants lacking an ANR. A GFP expression construct was coelectroporated to monitor the

**Fig. 4.** Foxg1-expressing tissue disappears between the 11- and 20-somite stages. (A,B) Whole-mount RNA in situ hybridization of E8.75 (11-somite stage) embryos indicates apparently normal levels of Foxg1 expression (arrowheads) in the FGFR triple mutant (B). (C,D) At E9.25 (20-somite stage), Foxg1 expression (arrowheads) is undetectable in the FGFR triple mutant (D).

This high level of cell death, along with the complete loss of Foxg1-expressing telencephalic cells, demonstrates a strict requirement for FGF signaling in keeping telencephalic precursor cells alive.

**Fig. 5.** Without Fgfr expression, Foxg1-expressing cells die. (A-C) Serial horizontal sections from 16-somite stage control (left) and mutant (right) embryos (anterior up). The areas between the arrowheads are the Foxg1-expressing telencephalic precursors, as determined by in situ hybridization (B). (D) Few TUNEL+ cells (A, purple) are detected in the Foxg1 domain of the control, whereas in mutants ~30% of cells are labeled. (E) The number of P-HH3+ (C, purple) mitotic cells, however, is similar in both controls and mutants. Hoechst-labeled DNA is blue.
GFP efficiency of electroporation (all controls were electroporated with GFP alone). Expression of *Foxg1* was found to significantly reduce the amount of cell death observed in the explants lacking ANRs (n=3/3; see Fig. S3 in the supplementary material), suggesting that *Foxg1* can act downstream of FGFs in mediating survival. Importantly, exogenous *Foxg1* does not induce Fgfl expression in the explants (data not shown), consistent with *Foxg1* acting downstream of FGFs.

**FGF signaling patterns and expands the telencephalon**

FGF8 mediates organizer activity for the midbrain and cerebellum. In conditional mutants deficient for *Fgfl* at the mid-hindbrain junction, or isthmus, early cell death of precursors leads to the loss of the midbrain and cerebellum (Chi et al., 2003), whereas ectopic application of FGF8-soaked beads in the diencephalon leads to a repatterning of this tissue into an ectopic mirror-imaged midbrain (Crossley et al., 1996; Martinez et al., 1999). The results of the present study suggest that FGF signaling might act in a similar manner to pattern and expand the telencephalon. The extent of telencephalic truncation was compared in littermates in which an increasing number of FGF receptor genes are deleted in the anterior neural plate (Fig. 7). In the mutant in which only *Fgfl* was lost, there was only a slight reduction in telencephalon size, with loss of only the anterior olfactory bulbs and differentiated ventromedial neurons (Fig. 7B) (Gutin et al., 2006; Hébert et al., 2003a). In a mutant in which both *Fgfl* and *Fgfr2* were lost, the telencephalon was more severely truncated along the anterior-posterior axis and there was a complete loss of all ventral cell types (Fig. 7C) (Gutin et al., 2006). Finally, when *Fgfl*, *Fgfr2* and *Fgfr3* were deleted, the telencephalon was essentially gone (Fig. 7D).

When levels of cell death were compared between controls, *Fgfl;Fgfr2* double mutants and FGFRII triple mutants at the 15-somite stage (~E8.75), a progressive increase in cell death was observed. In controls, an average of 2.3% of cells (±0.8%) were TUNEL-positive. In *Fgfl;Fgfr2* double mutants, no significant difference in the level of cell death was observed in the dorsal neuroepithelium at this age compared with controls, but an average of 8.8% of cells (±3.5%) were TUNEL positive in the ventral neuroepithelium, consistent with the loss of this part of the telencephalon. Finally, in the FGFRII triple mutant, 32.5% of cells (±5.2%) were TUNEL-positive throughout the telencephalic neuroepithelium (Fig. 5A,D). Coincident with the incidences of cell death was the loss of expression of *Dlx2* in the *Fgfl;Fgfr2* double mutant (see Fig. S4 in the supplementary material) and of both *Emx1* and *Dlx2* in the triple mutant (see Fig. S1 in the supplementary material).

Hence with diminishing levels of FGF signaling there is a gradual truncation of telencephalic areas, starting with ventrorostral areas and progressing to include dorsocaudal areas. Furthermore, the fact that all combinations of double mutants show a similar and relatively intact dorsal telencephalon strongly suggests that there is a remarkable differential in the need for FGF signaling along the dorsoventral axis. The data presented here, together with previous reports demonstrating roles for FGF ligands in patterning both the ventral and dorsal telencephalon (Hébert and Fishell, 2008), suggest that FGF signaling acts as an organizer to induce, pattern and expand the telencephalon, as it does in the mid-hindbrain region.

**DISCUSSION**

**FGFs as candidate mediators of organizer activity for the telencephalon**

Previous studies in which FGF signaling was partially disrupted demonstrated essential roles for this pathway in specifying, patterning and expanding anterior and ventral areas of the telencephalon (Gutin et al., 2006; Kuschel et al., 2003; Marklund et al., 2004; Shanmugalingam et al., 2000; Shinya et al., 2001; Storm et al., 2006; Walshe and Mason, 2003). From these studies, however, it remained unknown whether FGF signaling is required as a telencephalic inducer and a mediator of organizing activity. The results presented here are consistent with these functions. First, reducing FGFRI gene expression prior to telencephalon induction leads to a corresponding decrease in *Foxg1* expression. And second, deletion of the FGFRII genes coincident with induction leads to death of all *Foxg1*-expressing telencephalic precursors, resulting in a lack of not only all basal ganglia but also all cortical areas. This role for FGFs in the anterior neural plate is reminiscent of FGF functions in mediating organizer activity in other tissues such as the midbrain and limb, where FGFs are required to keep cells alive and where progressive reduction of FGF signaling leads to progressive loss of tissue (Basson et al., 2008; Mariani et al., 2008; Chi et al., 2003; Crossley et al., 1996; Martinez et al., 1999).

In addition to being necessary to form tissues such as the limbs, mid-hindbrain and telencephalon, FGFs are also sufficient to induce the formation of normally organized ectopic midbrains and limbs. Although this has not been demonstrated for the telencephalon,
FGFs mediate telencephalic cell survival

FGFs are also likely to be sufficient to induce ectopic telencephalic tissue. An FGF8-soaked bead can rescue induction of Foxg1 expression in cultured explants of anterior neural plate from which the ANR is removed (Shimamura and Rubenstein, 1997). Moreover, ectopically expressing Fgf8 in the early posterior telencephalon can result in a mirror-image duplication of parts of the dorsal telencephalon (Fukuchi-Shimogori and Grove, 2001; Shimogori and Grove, 2005). Earlier ectopic expression prior to telencephalon induction, if technically feasible, would be likely to result in a complete duplication.

It is unknown how FGFs exert both a survival and a patterning function. In several tissues in which FGFs have a role in patterning or in mediating organizer activity, including the mid-hindbrain and the limb, loss of FGFs leads to massive cell death (Chi et al., 2003; Crossley et al., 1996; Martinez et al., 1999). In particular in the mid-hindbrain, FGF signaling, at least in part mediated by FGF8, is required from the 14- to the 20-somite stages for both survival and developmental and that it is difficult to separate them. The simplest explanation is that continuous FGF signaling as a single event conveys both survival and patterning functions. FGFs are also likely to be sufficient to induce ectopic telencephalic tissue. An FGF8-soaked bead can rescue induction of Foxg1 expression in cultured explants of anterior neural plate from which the ANR is removed (Shimamura and Rubenstein, 1997). Moreover, ectopically expressing Fgf8 in the early posterior telencephalon can result in a mirror-image duplication of parts of the dorsal telencephalon (Fukuchi-Shimogori and Grove, 2001; Shimogori and Grove, 2005). Earlier ectopic expression prior to telencephalon induction, if technically feasible, would be likely to result in a complete duplication.

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**Interaction between FGF signaling and Foxg1**

The function of FGFs and Foxg1 appear tightly linked through a positive-feedback loop in early telencephalon development. Both Foxg1 and Fgf8 expression are turned on at apparently the same time in the ANR and anterior neural plate (Shimamura and Rubenstein, 1997). FGF signaling is necessary and sufficient for Foxg1 expression and Foxg1 is necessary for the expression of at least one FGF ligand gene, Fgf8 (Figs 2, 4 and 6) (Martynoga et al., 2005; Shimamura and Rubenstein, 1997). In addition, both Foxg1 mutants and Fgfr1:Fgfr2 double mutants exhibit similar phenotypes in that the ventral areas fail to develop and the overall size of the telencephalon is reduced (Gutin et al., 2006; Hanashima et al., 2002; Martynoga et al., 2005; Xuan et al., 1995). From the data presented here, it appears that at least for maintaining cell survival, Foxg1 can act independently of FGFs (see Fig. S3 in the supplementary material).

Despite these overlapping and cooperative functions, the finding that the FGFR triple mutant phenotype is more severe than the Foxg1 mutant phenotype indicates that genes other than Foxg1 must regulate FGF gene expression, and conversely FGFs must function through genes other than Foxg1 in forming the telencephalon, the cortical regions in particular. The identity of these other genes remains unclear. It also remains untested whether FGFs act directly in a dose-dependent manner to assign cell fates as a morphogen along the anterior-ventral to posterior-dorsal axis.

**FGFs and dorsal midline development**

The only area that appears unaffected by the loss of FGF signaling in the Foxg1Cre;FGFR mutants described here is the dorsal midline, which suggests that FGFs are not required for the formation of this part of the telencephalon. However, hypomorphic levels of Fgf8 lead to a lack of dorsal midline characteristics, suggesting that FGFs may indeed play a role (Storm et al., 2003; Storm et al., 2006). An explanation that might reconcile these opposing findings is that, in the Fgf8 hypomorph, FGF signaling is reduced throughout development, whereas in the conditional FGFR triple mutant it is not abolished until E8.75. Therefore, if FGF signaling is required earlier, a dorsal midline phenotype might occur in the Fgf8 hypomorph, but not the FGFR triple mutant. The likelihood of this possibility is lessened, however, as if the Fgf8 hypomorphic allele is converted to a null allele using the same Foxg1Cre mice used in this report, dorsal midline features develop, indicating that it is not the timing of FGF8 loss that matters, but the level of FGF8 (Storm et al., 2003). Hence there may be a window within the level of FGF signaling that disrupts dorsal midline formation, perhaps by repressing dorsal midline determinants such as Zic2 and/or BMPs (Brown et al., 1998; Fernandes et al., 2007; Nagai et al., 2000; Storm et al., 2003).

**FGF ligands and receptors act in part redundantly to set the level of signaling**

The finding that simultaneous deletion of Fgfr1, Fgfr2 and Fgfr3 leads to a dramatically more severe truncation of the telencephalon than loss of any one or two FGF genes alone indicates that, rather than having distinct functions, these receptors can to a large extent compensate for each other. This compensation is not complete, however, as knockouts of individual receptor genes and pairs of genes do result in phenotypes, albeit mild compared with the triple mutant phenotype described here (Hébert et al., 2003a; Gutin et al., 2006). This partial non-overlap in function could be due to the receptors either having somewhat divergent mechanisms of intracellular signaling or simply functioning with different efficiencies to more or less affect the overall level of signaling.

This latter possibility appears likely when one compares the phenotypes of all FGFR and FGFR mutants. As progressively more FGF genes are deleted, the phenotypes observed increase in severity, with truncations expanding from the anterior regions of the ventral-medial telencephalon to the posterior regions of the dorsal telencephalon. Disruption of Fgfr2 and Fgfr3 on their own or together does not lead to gross patterning defects (Gutin et al., 2006). In fact, in mutants in which Fgfr2 and Fgfr3 are deleted, a single functional allele of Fgfr1 is sufficient to confer grossly normal
patterning of the telencephalon. Disruption of both copies of Fgfr1 alone, on the other hand, leads to a lack of olfactory bulbs, differentiated ventral medial neurons and specialized midline glia (Gutin et al., 2006; Hébert et al., 2003a; Tole et al., 2006). Similar phenotypes are also observed in the Fgfr1/Fgfr3 double mutants. In the Fgfr1/Fgfr2 double mutants, the anterior ventral truncations are more severe, with a lack of most or all ventral precursors and of all anterior telencephalic regions (Fig. 7) (Gutin et al., 2006). These phenotypes are recapitulated by progressive loss of Fgf8 expression (Storm et al., 2006). Finally, if FGF signaling is abolished by eliminating expression of Fgfr1, Fgfr2 and Fgfr3, as shown in this study, then all telencephalic regions are lacking except for a small dorsomedial region from which the choroid plexus and cortical hem are derived. Since loss of the dorsal telencephalon is not observed in any of the double mutants and is only observed when all three receptors are eliminated, dorsal precursor cells are very likely to require less FGF signaling than ventral ones. Moreover, the FGFR mutant phenotypes suggest that FGFR1 carries the bulk of the signaling load, followed by FGF2, then FGF3, and that together they set the overall level of signaling.

Given that the disruption of any single FGF ligand gene does not result in as severe a phenotype as that observed in the FGFR triple mutant, FGF ligands must also act in a large part redundantly. In fact, of the five FGF genes expressed in the most anterior part of the early mouse CNS, Fgf3, Fgf8, Fgf15, Fgf17 and Fgf18, only mutations in Fgf8 and Fgf17 on their own lead to patterning defects (Cholfín and Rubenstein, 2007; Garel et al., 2003; Storm et al., 2006). Comparing the phenotypes of severely hypomorphic and null alleles of Fgf8 reveals a progressive loss of ventral structures along with a gradual reduction in the size of the neocortex, with preferential loss of anterior precursors (Storm et al., 2006). In moderately hypomorphic mutants or in embryos in which FGF signaling is reduced after neural specification, the size of the dorsal part of the frontal cortex (Cholfín and Rubenstein, 2003) is coordinately regulated by Fgf8, Fgf17, and Emx2. J. Comp. Neurol. 509, 144-153.


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