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Dose-dependent functions of *Fgf8* in regulating telencephalic patterning centers

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Mouse embryos bearing hypomorphic and conditional null *Fgf8* mutations have small and abnormally patterned telencephalons. We provide evidence that the hypoplasia results from decreased *Foxg1* expression, reduced cell proliferation and increased cell death. In addition, alterations in the expression of *Bmp4*, *Wnt8b*, *Nkx2.1* and *Shh* are associated with abnormal development of dorsal and ventral structures. Furthermore, nonlinear effects of *Fgf8* gene dose on the expression of a subset of genes, including *Bmp4* and *Msx1*, correlate with a holoprosencephaly phenotype and with the nonlinear expression of transcription factors that regulate neocortical patterning. These data suggest that *Fgf8* functions to coordinate multiple patterning centers, and that modifications in the relative strength of FGF signaling can have profound effects on the relative size and nature of telencephalic subdivisions.

KEY WORDS: *Fgf8*, Forebrain, Patterning, Mouse

INTRODUCTION

Secreted molecules produced by patterning centers regulate embryonic morphogenesis. Multiple patterning centers are juxtaposed in developing tissues to provide qualitatively distinct signals that regulate regional identity and growth. In the embryonic telencephalon, at least three patterning centers extend from the midline (Crossley et al., 2001; Grove and Fukuchi-Shimogori, 2003; Garel and Rubenstein, 2004). The rostral patterning center expresses a nested set of FGF genes: *Fgf8*, *Fgf18*, *Fgf17* and *Fgf15* (Maruoka et al., 1998; Bachler and Neubuser, 2001; Gimeno et al., 2003). The dorsal patterning center expresses a nested set of BMP and WNT genes and controls the development of dorsocaudal structures (Grove et al., 1998; Galceran et al., 2000; Lee et al., 1999; Hebert et al., 2002). The ventral patterning center, the function of which has not been firmly established, expresses *Shh* (Shimamura et al., 1995; Crossley et al., 2001).

There is cross regulation between patterning centers (reviewed by Garel and Rubenstein, 2004). For instance, SHH is required to maintain *Fgf8* expression (Ohkubo et al., 2002; Aoto et al., 2002), and there is evidence that FGF8 and BMP4 reciprocally repress each other's expression (Ohkubo et al., 2002; Shimogori et al., 2004). Patterning centers are also regulated by the expression of secreted molecules such as Noggin and SFRP that restrict ligand availability (Shimamura et al., 1995; Kim et al., 2001; Anderson et al., 2002; Ohkubo et al., 2002; Shimagori et al., 2004) or molecules that function intracellularly, such as sprouty or SEF proteins, to repress signaling (Minowada et al., 1999; Lin et al., 2002; Kim and Bar-Sagi, 2004).

Previous studies support a model in which *Fgf8* expression in the mouse anterior neural ridge (the anlage of the telencephalic rostral patterning center) positively regulates the expression of *Foxg1* (Shimamura and Rubenstein, 1997; Ye et al., 1998). FOXG1 is a winged helix transcription factor that represses TGFβ signaling (Dou et al., 2000; Seoane et al., 2004) and thereby promotes proliferation and represses differentiation and dorsal telencephalic fates (Xuan et al., 1995; Dou et al., 1999; Hardcastle and Papalopulu, 2000; Hanashima et al., 2004; Mucio and Mallamaci, 2005; Martynoga et al., 2005).

Fgf8 hypomorphic mutations in both mouse and zebrafish result in a small telencephalon (Meyers et al., 1998; Shanmugalingam et al., 2000; Storm et al., 2003). Furthermore, manipulations that increase or decrease FGF signaling influence the patterning of the rostral telencephalon by modulating the expression of *Emx2*, *Otx2* and other regulatory genes (Crossley et al., 2001; Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003; Storm et al., 2003; Walshe and Mason, 2003; Sansom et al., 2005). FGF signaling in zebrafish is also implicated in regulating *Shh* expression and development of the ventral telencephalon (Shinya et al., 2001; Walshe and Mason, 2003).

An allelic series of mutations at the mouse *Fgf8* locus has facilitated the analysis of the multiple functions of the *Fgf8* gene in telencephalic development (Meyers et al., 1998; Garel et al., 2003; Storm et al., 2003; Huffman et al., 2004). These studies used four *Fgf8* alleles: *Fgf8* (wild type), $Fgf8^{flox}$ (exons 2 and 3 are present but can be deleted by Cre-mediated recombination), $Fgf8^{Neo}$ (~40% normal expression), $Fgf8^{Null}$ (exons 2 and 3 are deleted) (Meyers et al., 1998). Mice lacking *Fgf8* ($Fgf8^{Null/Null}$) die during gastrulation (Sun et al., 1999), whereas telencephalic conditional $Fgf8^{TelKO}$ nulls ($Fgf8^{flox/flox}$ recombined using *Foxg1-Cre*), $Fgf8^{Null/Neo}$, and $Fgf8^{Neo/Neo}$ mutants survive until birth and exhibit graded defects in telencephalon patterning (Storm et al., 2003; Garel et al., 2003). Previously, we reported that these mutants exhibit three general types of defects: (1) hypoplasia of rostral telencephalic structures including the frontal neocortex; (2) rostral expansion of the

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expression of transcription factors that regulate neocortical regional properties [e.g. *Emx2* and *Nr2f1* (*COUP-TF1*)]; and (3) complex interactions between the rostral and dorsal patterning centers leading to either decreases ($Fgf8^{Null/Neo}$) or increases ($Fgf8^{TelKO}$) in *Bmp4* expression and apoptosis.

The previous studies concentrated on the phenotype of the $Fgf8^{TelKO}$ and $Fgf8^{Null/Neo}$ mutant telencephalon beginning at E10.5 and did not examine primary phenotypes in the neural plate or just following neural tube closure. Because prosencephalic expression of *Fgf8* begins at neural plate stages (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997; Crossley et al., 2001), it is essential to investigate the mutant phenotypes shortly after this stage to elucidate the mechanisms underlying $Fgf8^{TelKO}$ and $Fgf8^{Null/Neo}$ phenotypes. Therefore, here we report studies of *Fgf8* dose-dependent effects on neural plate and early post-neurulation stage embryos. Furthermore, Storm et al. (Storm et al., 2003) focused on the effects of reducing *Fgf8* dose on telencephalic midline development; here we concentrate on the effect of reducing *Fgf8* dose on telencephalic patterning centers, regionalization and growth.

We report our finding that specification of the prosencephalon is intact in *Fgf8* mutants; however, a major reduction in *Foxg1* expression, a reduced mitotic index, and increased apoptosis contribute to telencephalic hypoplasia. We also demonstrate that *Fgf8* regulates the expression of *Bmp4*, *Wnt8b* and *Shh*, which in turn affect patterning of both dorsal and ventral structures. Nonlinear effects of *Fgf8* dose on *Bmp4* expression correlate with a holoprosencephaly phenotype and alterations in the expression of transcription factors that regulate neocortical patterning. The nexus of regulatory interactions between patterning centers that control gradients of transcription factor expression demonstrates that modifications in the relative strength of FGF/BMP/WNT/SHH signaling have profound effects on the relative size and nature of telencephalic subdivisions that are likely to contribute to their phylogenetic and intra-individual diversity.

MATERIALS AND METHODS

Mice and genotyping

All *Fgf8* mutant alleles were maintained on a mixed 129/CD1 Swiss genetic background. $Fgf8^{Null/+}$ and $Fgf8^{Neo/+}$ mice were crossed to produce $Fgf8^{Null/Neo}$ embryos. $Fgf8^{flox/flox}$ and *Foxg1-Cre*;$Fgf8^{Null/+}$ mice were crossed to produce $Fgf8^{flox/Null}$;*Foxg1-Cre* ($Fgf8^{TelKO}$) embryos. PCR genotyping was performed as described previously (Hebert and McConnell, 1999; Storm et al., 2003). For staging of embryos, noon on the day of the vaginal plug was considered as embryonic day 0.5 (E0.5). Heterozygous $Fgf8^{Null/+}$ embryos did not show any discernable phenotype and were used with wild-type embryos as controls

Immunohistochemistry, TUNEL and in situ hybridization

For immunohistochemistry, TUNEL assays and in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C. Immunohistochemistry was performed on 10-16 μm cryostat sections as described previously (Yun et al., 2001). Rabbit anti-phosphohistone-3 (PH3) 1/400 (Upstate) were used as primary antibodies. Hoechst counterstaining and fluorescent immunohistochemical staining were analyzed under a Leica microscope and images were acquired using a Spot CCD camera. TUNEL analysis was performed on 10-16 μm cryostat sections using the Apoptag Kit following the manufacturer's recommendations (Intergen). In situ hybridization was performed on whole-mount embryos as described previously (Depew et al., 2002).

Cell proliferation and apoptosis analyses:

E9.0 embryos (~14-17 somites) were sectioned in the horizontal plane. Every other section was stained using either PH3 or TUNEL histochemistry (counter-stained with DAPI). The number of $PH3^+$ cell nuclei and $TUNEL^+$ cells in the neuroepithelium was counted in two regions of the forebrain: the rostral midline and the rostroventral telencephalon (box 1 and box 2, respectively in Figs 3 and 5). Box 1 was ~45 μm wide and spanned the rostral midline. Box 2 was ~170 μm wide and approximated the rostroventral telencephalon, extending laterally from the edge of box 1 towards the optic stalk region (probably encompassing the anlage of the basal ganglia, septum and rostral cortex). We counted labeled cells in sections from ventral telencephalic regions (rows A-D in Figs 4 and 6); sections that were dorsal to the optic stalk were not analyzed (i.e. row E in Figs 4 and 6). In a given section, box 2 was drawn both to the left and to the right of the midline, and cells were counted on both sides. Precise quantification of positive cell numbers was complicated by: (1) different levels of PH3 staining; (2) the small size of dots generated by TUNEL staining; (3) uncertainty about whether a single cell can have more than one TUNEL reaction product. However, these complications appeared independent of genotype, and should not have led to systematic biases between genotypes, but could affect the absolute numbers.

RESULTS

Rostral and ventral telencephalic morphological defects in $Fgf8^{Null/Neo}$ hypomorphic and $Fgf8^{TelKO}$ mutants

Comparative analysis of Cresyl Violet-stained horizontal sections prepared from E14.5 wild-type, $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ embryos revealed the dysmorphologies in the mutant forebrains (Fig. 1). Reducing *Fgf8* dose caused progressive prosencephalic hypoplasia. There was some variation in the severity of the phenotypes of $Fgf8^{Null/Neo}$ mutants, whereas no clear phenotypic variation was observed in $Fgf8^{TelKO}$ mutants. In all mutants, rostral and rostroventral structures showed the most profound alterations. Both $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants had a single ganglionic eminence and lacked identifiable septal and preoptic nuclei, an optic chiasm and olfactory bulbs (Fig. 1, and not shown); these phenotypes were more severe in $Fgf8^{TelKO}$ mutants. The rostral midline of the mutants differed: the $Fgf8^{Null/Neo}$ midline was thicker than that in $Fgf8^{TelKO}$ mutants. Caudal and dorsal telencephalic structures appeared to be relatively more preserved, as choroidal, hippocampal and amygdaloid structures were present (Fig. 1 and not shown), although they appeared smaller and thinner than normal. To elucidate the mechanisms underlying these morphological defects, we studied the earlier development of these mutants.

Molecular patterning defects in late neural plate stage $Fgf8^{Null/Neo}$ mutants: expansion of *Otx2* and reduction of *Foxg1* expression

Fgf8 expression begins in the anterior neural plate at approximately the 4-somite stage (Crossley and Martin, 1995; Crossley et al., 2001). Thus, we analyzed the expression of three transcription factors that are important for prosencephalic development, *Otx2, Six3* and *Foxg1*, in the $Fgf8^{Null/Neo}$ mutant embryos at the 9- to 10-somite stage (Fig. 2) and other neural plate stages (data not shown) (Xuan et al., 1995; Simeone et al., 2002; Lagutin et al., 2003; Gestri et al., 2005). We did not examine $Fgf8^{TelKO}$ mutants at the 9- to 10-somite stage, because Cre-mediated recombination may not yet be complete at this stage. In each case, we observed subtle molecular and morphological defects in the anterior neural plate, which appeared wider and flatter (Fig. 2). *Otx2* expression appeared to have expanded both rostrally in the prosencephalon (Fig. 2B,B′) and caudally into the rhombencephalon (Fig. 2A,A′), consistent with evidence FGF8 can repress *Otx2* expression (Martinez et al., 1999; Crossley et al., 2001; Chi et al., 2003). *Six3* expression remained strong rostrally, and may have expanded caudally (Fig. 2C,C′,D,D′). In contrast, expression of *Foxg1* (*Bf1*) was reduced in the neural

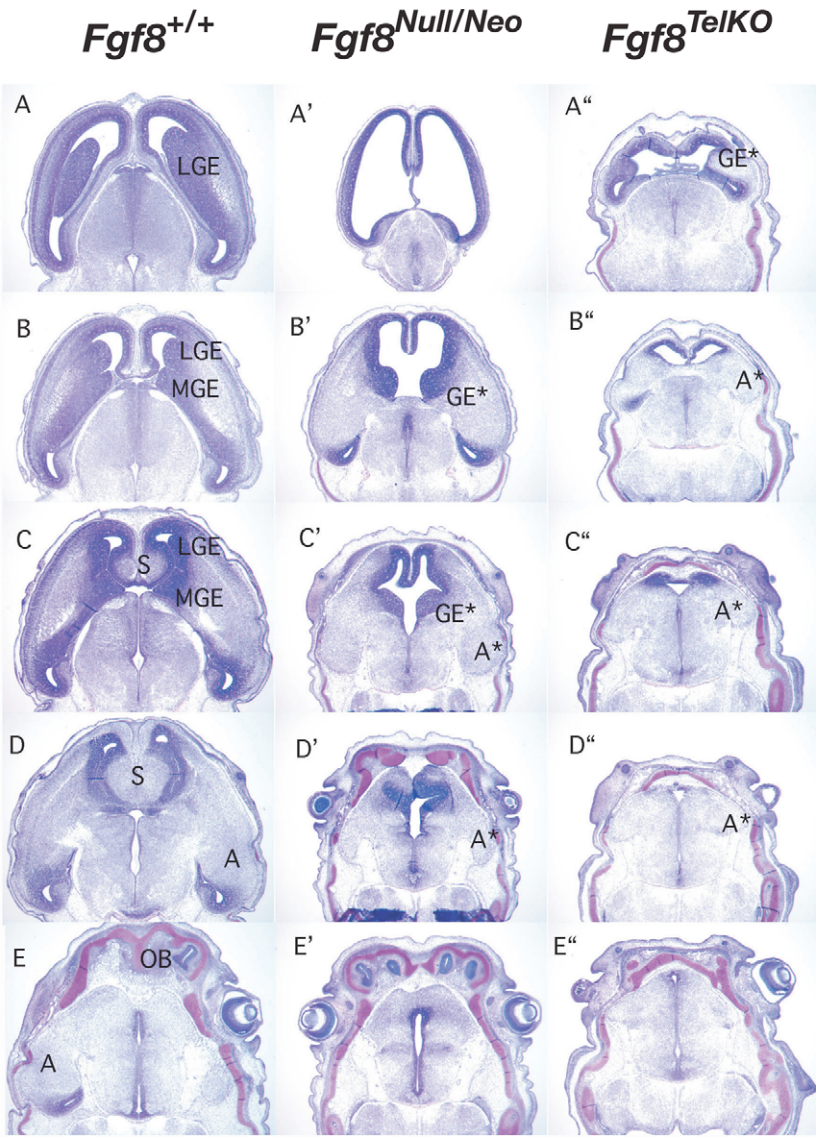

Fig. 1. *Fgf8* hypomorphic (*Null/Neo*) and telencephalon null (*TelKO*) mutants are hypoplastic and have morphological defects in rostral and midline regions of the telencephalon. (**A-E″**) Horizontal sections of E14.5 wild-type (A-E), $Fgf8^{Null/Neo}$ (A′-E′) and $Fgf8^{TelKO}$ (A″-E″) brains were stained with Cresyl Violet. Structures were defined by their location and morphology. A,A′,A″ are most dorsal and E,E′,E″ are most ventral. A, amygdala region; A*, mutant amygdala region; GE*, mutant ganglionic eminence region; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; OB, olfactory bulb; S, septum.

plate; *Foxg1* expression was even more reduced in the branchial arches (Fig. 2E,E′,F,F′). Thus, based on the expression of *Six3* and *Foxg1*, some aspects of telencephalic molecular specification appear intact in $Fgf8^{Null/Neo}$ mutants.

Reduced expression of *Foxg1* and *Six3* and expanded expression of *Emx2* in the early telencephalon

Defects in both $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants were obvious by E9.0. At this age, the evagination of the optic vesicles (as marked by *Six3* expression) was roughly normal in the mutants but the size of the telencephalic vesicles was reduced (Fig. 3A,A′,A″,B,B′,B″). *Six3* expression in the rostral midline was reduced in both mutants (arrowhead; Fig. 3B,B′,B″). Expression of *Foxg1* was greatly reduced in $Fgf8^{Null/Neo}$ mutants and was even more difficult to discern in $Fgf8^{TelKO}$ embryos, whereas *Foxg1* expression in the olfactory placode appeared unaffected (Fig. 3C,C′,C″,D,D′,D″). By contrast, *Emx2* expression showed a subtle rostral shift at E9.0 (not shown) and a more profound rostral expansion at E9.5 (arrowheads in Fig. 3F,G), which was maintained at later stages (see Fig. 7) (Garel et al., 2003; Storm et al., 2003).

Reduced cell proliferation in the early rostroventral telencephalon

Because the inactivation of *Foxg1* reduces cell proliferation in the telencephalon (Hanashima et al., 2002), we assessed the expression of the M-phase cell cycle marker phosphohistone-3 (PH3) in *Fgf8* mutants at E9.0. In the rostral midline (box 1, Fig. 3E,E′,E″, Fig. 4) no differences were detected between the genotypes. In the rostroventral telencephalon (box 2; Fig. 3E,E′,E″, Fig. 4), wild-type embryos had roughly a twofold higher mitotic index than either mutant (Fig. 3E,E′,E″ and Fig. 4; Table 1).

$Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants exhibit distinct changes in the expression of regulators of dorsal midline development.

Previously, we provided evidence that the mutations in $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ embryos have distinct effects on the expression of *Bmp4* and the level of apoptosis in the dorsal midline of E10.5 embryos (Storm et al., 2003). To confirm and extend these findings, we examined the expression at E9.5 of signaling molecule and transcription factor genes implicated in regulating dorsal midline differentiation (Fig. 5). *Bmp4* expression, which marks the

rostrodorsal midline at this age, was not detected in this region of either mutant (Fig. 5A,A′,A″). Expression of *Msx1*, a homeobox transcription factor positively regulated in the forebrain by BMP signaling (Furuta et al., 1997; Shimamura and Rubenstein, 1997; Feledy et al., 1999), was also not detected in the rostrodorsal midline of $Fgf8^{Null/Neo}$ mutants but was maintained in $Fgf8^{TelKO}$ brains (Fig. 5B,B′B″), similar to previous observations of *Bmp4* expression at E10.5 (Storm et al., 2003).

Consistent with the results of in utero electroporation experiments (Shimogori et al., 2004), we found that reductions in *Fgf8* gene dose led to a rostral expansion of the WNT expression domain in the dorsal telencephalon. Thus we observed that telencephalic *Wnt8b* expression (which is in paramedian dorsal longitudinal domains) extended rostrally and encompassed much of the hypoplastic telencephalon in both *Fgf8* mutants (Fig. 5C,C′,C″). Its expression pattern resembled that of *Emx2* (Fig. 3F,F′,F″,G,G′,G″ and not shown).

We then examined the expression of *Sp8*, a buttonhead-like zinc-finger transcription factor gene, which in the limb bud is regulated by FGF10 and WNT/β-catenin signaling, and is implicated in regulating *Fgf8* expression (Bell et al., 2003; Kawakami et al., 2004). In control embryos, *Sp8* expression was present throughout much of the telencephalic vesicle, but was reduced or absent from the rostrodorsal midline (Fig. 5D). *Sp8* expression was almost eliminated in $Fgf8^{Null/Neo}$ mutants (Fig. 5D′). In contrast, in $Fgf8^{TelKO}$ embryos, *Sp8* expression not only persisted but also encompassed the dorsal midline (Fig. 5D″). Thus reduction in the dose of *Fgf8* resulted in the rostral expansion of *Wnt8b* in both mutants, whereas $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants exhibited distinct patterns of *Msx1* and *Sp8* expression. These changes reflect the complexity of regulatory interactions in the dorsal midline.

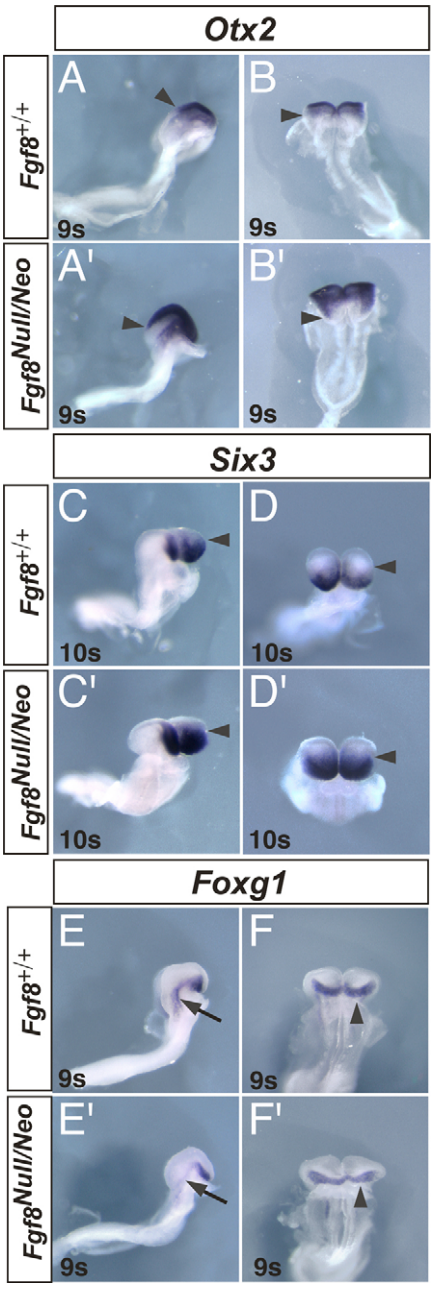

Fig. 2. Analysis of neural plate patterning in $Fgf8^{Null/Neo}$ embryos. Whole-mount in situ hybridization showing *Otx2* expression (**A-B′**), *Six3* expression (**C-D′**) and *Foxg1* expression (**E-F′**) in 9- to 10-somite embryos. The developmental stage of embryos is indicated in the lower left corner by the number of somites (s). On lateral views of embryos, anterior is located to the right; on frontal views, anterior is located towards the top. In the neural plate of the mutants, *Otx2* expression is slightly expanded rostrally (arrowheads in B,B′) and caudally at the level of the midbrain/hindbrain boundary (arrowheads in A,A′); *Six3* expression is intensified in caudal regions (arrowheads in C-D′); and *Foxg1* expression is slightly reduced (arrowheads in F,F′). *Foxg1* expression is not detected in the mutant pharyngeal region (arrows in E,E′).

$Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants exhibit distinct patterns of apoptosis in the rostral midline

Alterations in the expression of *Fgf8* are associated with cell death (Trumpp et al., 1999; Crossley et al., 2001; Abu-Issa et al., 2002; Storm et al., 2003; Chi et al., 2003), therefore we examined apoptosis in *Fgf8* mutants at E9.0 using the TUNEL assay. $TUNEL^+$ cells are normally abundant in the rostral midline of the telencephalon at this age (box 1, Fig. 5E, Fig. 6A-E). However, approx. threefold fewer $TUNEL^+$ cells were visible in the rostral midline of the $Fgf8^{Null/Neo}$ mutants (Fig. 5E′, Fig. 6A′-E′), whereas $Fgf8^{TelKO}$ mutants appeared similar to wild-type controls (Fig. 5E″, Fig. 6A″-E″; Table 1). These distinct effects on apoptosis are similar to those observed in mutants at E10.5 (Storm et al., 2003) and were

Table 1. PH3 and TUNEL analysis in the rostral midline and rostroventral telencephalon

Marker assayed	Genotype	Number of embryos	Box 1	Box 2
PH3	Wild type	3	1.4±1.2	26±5.7
PH3	Null/Neo	2	1.5±0.7 [0.37]	12±4.6 [0.016]
PH3	TelKO	2	1.7±0.6 [0.39]	13±3.7 [0.003]
TUNEL	Wild type	3	5±1.2	2.5±1.3
TUNEL	Null/Neo	2	1.6±0.9 [0.005]	38±18 [0.005]
TUNEL	TelKO	2	5.5±1.2 [0.37]	38±20 [0.001]

The data show the average number±s.d. of cellular nuclei labeled with the M-phase marker (phosphohistone 3; PH3) and the average number of $TUNEL^+$ profiles (apoptosis marker) in the rostral midline (Box 1) and in the rostroventral telencephalon (Box 2) (see PH3 and TUNEL data in Figs 3-6). We do not know whether or not an individual cell can have more than one $TUNEL^+$ signal. See Materials and methods for a description of how the boxes were drawn. Averages were determined by counting the total number of labeled cells in Box1 or Box2 (which was drawn to both the left and the right of the midline) in three or four sections of two or three embryos and dividing by the number of boxes counted. The standard deviation (in parentheses) reflects the variance in the average number of positive cells/embryo. Results of *T*-test analyses are shown in square parentheses for each mutant in comparison with wild type. Values below 0.05 are statistically significant.

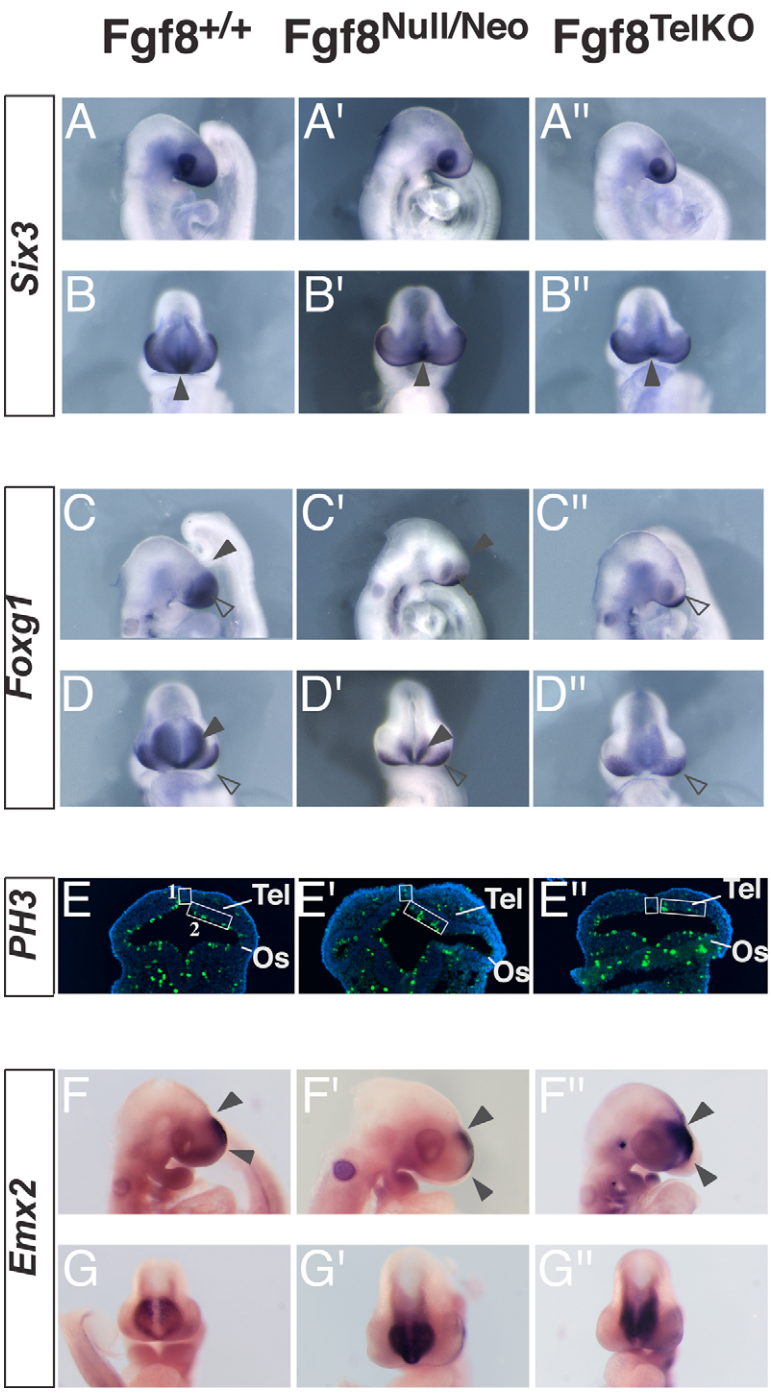

Fig. 3. *Foxg1* expression and proliferation are reduced in the forebrain of *Fgf8*$^{Null/Neo}$ and *Fgf8*^{TelKO} mutant embryos. Whole-mount in situ hybridization on E9 embryos showing *Six3* (**A-B″**), *Foxg1* (**C-D″**) and *Emx2* (E9.5 embryos; **F-G″**) expression. For each probe, the top panel shows lateral views of the embryos, and the lower one frontal views. The *Six3* expression domain in the rostroventral telencephalon appears smaller (arrowheads in B-B″) and *Emx2* expression in the caudodorsal telencephalon expands rostrally in *Fgf8*$^{Null/Neo}$ and *Fgf8*^{TelKO} mutants (arrowheads in F-F″). By contrast, the *Foxg1* expression domain in the telencephalon is severely reduced in *Fgf8*$^{Null/Neo}$embryos and almost absent in *Fgf8*^{TelKO} embryos (solid arrowheads in C-D″). By contrast, *Foxg1* expression in the olfactory placodes is still detected in both mutants (open arrowheads). Anti-phosphohistone3 (PH3) immunofluorescence on horizontal sections through the forebrain labels the nuclei of mitotic cells in wild-type (**E**), *Fgf8*$^{Null/Neo}$ (**E′**) and *Fgf8*^{TelKO} (**E″**) embryos (additional sections are in Fig. 4). The reduction in PH3$^+$-labeled cells correlates with the reduction in *Foxg1* expression (D,D′,D″). *Foxg1* is required for proliferation in the telencephalon (Xuan et al., 1995). Note, the panels showing PH3 labeling are turned 180° with respect to the panels showing frontal views of in situ hybridizations, such that the rostral regions face each other; this was done to facilitate comparison of *Foxg1* expression and the number of PH3$^+$ cells. The boxes indicate the regions in which the numbers of PH3$^+$ cells were counted in the rostral midline (box 1) and in the rostroventral telencephalon (box 2) (see Table 1). Os, optic stalk; Tel, telencephalon.

positively correlated with the expression of *Msx1* (Fig. 5B,B′,B″), a transcription factor that is a positive regulator of apoptosis (Liu et al., 2004; Ramos et al., 2004; Park et al., 2005). In the rostroventral telencephalon (box 2, Fig. 5E,E′E″, Fig. 6), both the *Fgf8*$^{Null/Neo}$ and *Fgf8*^{TelKO} embryos had approx. tenfold more TUNEL$^+$ cells than wild-type embryos (Table 1).

Changes in the numbers of TUNEL$^+$ cells were also observed in other parts of *Fgf8* mutant brains. In wild-type embryos, apoptotic cells were present in caudal regions of the optic vesicles and in the hypothalamus (Fig. 6A,B,C). Large numbers of TUNEL$^+$ cells were observed in the optic stalk, hypothalamus and mesencephalon of *Fgf8*$^{Null/Neo}$ mutant embryos (Fig. 6A′-E′ and not shown) [see Chi et al. (Chi et al., 2003) for apoptosis in the mesencephalon of *Fgf8* mutants]. The high levels of cell death in the optic stalk and hypothalamus correlate with early expression of *Fgf8* in these regions (Crossley et al., 2001; Treier et al., 2001). Scattered TUNEL$^+$ cells were visible in the optic stalks of *Fgf8*^{TelKO} mutants (Fig. 6A″-E′), but these brains showed less apoptosis than the *Fgf8*$^{Null/Neo}$ mutants in the hypothalamus and mesencephalon, consistent with the lower levels of Cre recombinase expression in these regions.

Thus, at E9.0-E10.0, as the telencephalic vesicles are forming, a reduction of *Fgf8* dose leads to an alteration in the expression of rostrodorsal patterning signals, and in the cellular responses to these signals (proliferation and apoptosis). These modifications undoubtedly contribute to the telencephalic hypoplasia observed in

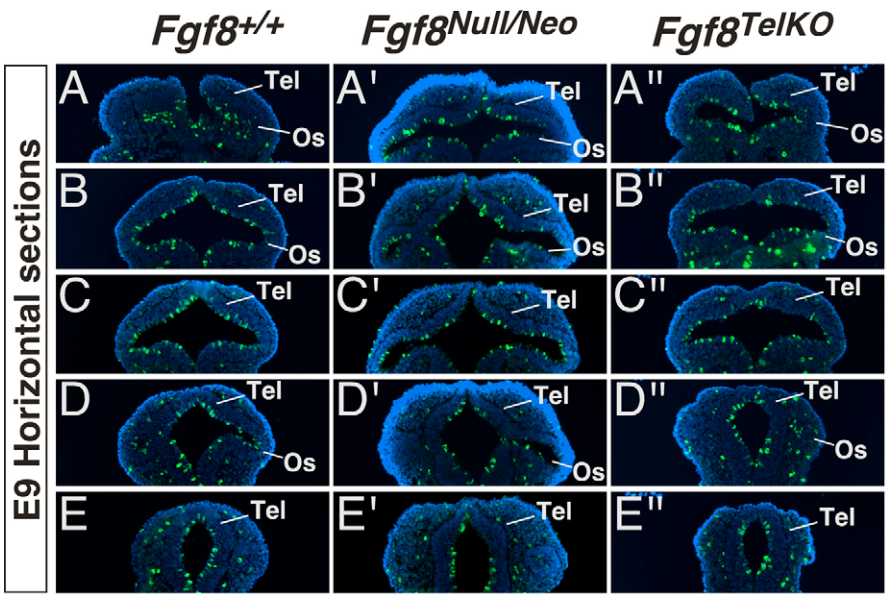

Fig. 4. Analysis of proliferation in the *Fgf8* mutants at E9.0. Anti-phosphohistone3 (PH3) immunofluorescence on horizontal sections through the forebrain labels the nuclei of mitotic cells in wild-type (**A-E**), *Fgf8*$^{Null/Neo}$ (**A′-E′**) and *Fgf8*^{TelKO} (**A″-E″**) embryos. A-A″ are the most ventral; E-E″ are the most dorsal. Note, sections B,B′,B″ are the same as in Fig. 3. Os, optic stalk; Tel, telencephalon.

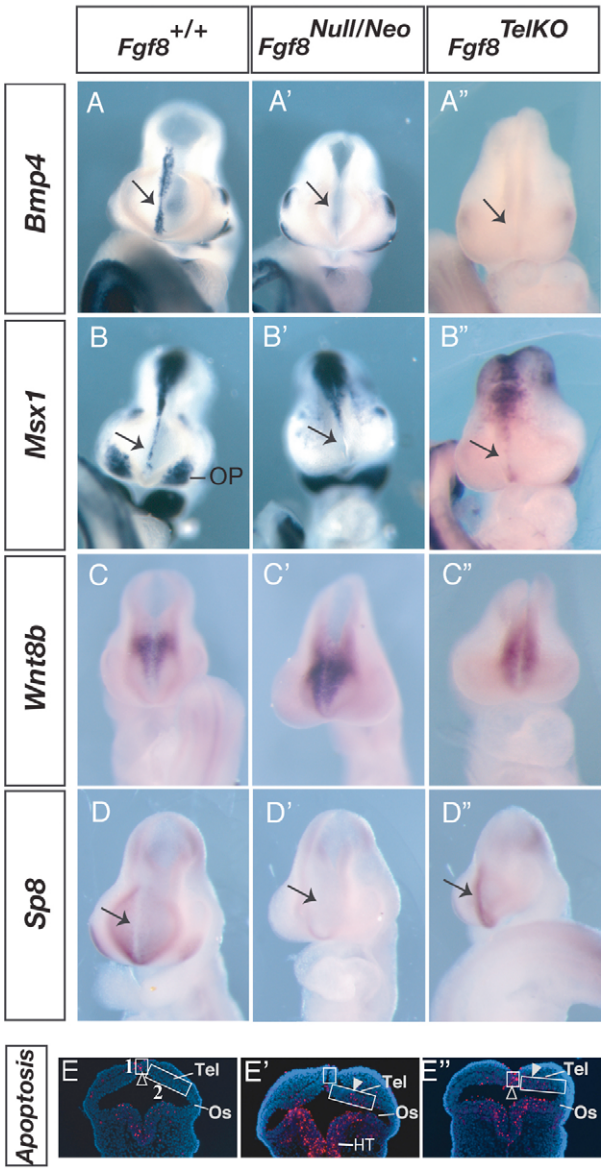

Fig. 5. Expression of patterning signals and transcription factors implicated in dorsal midline/paramedial development in E9-9.5 *Fgf8*$^{Null/Neo}$ and *Fgf8*^{TelKO} embryos. Frontal views of embryos hybridized in whole mount with probes for *Bmp4* (**A-A″**), *Msx1* (**B-B″**), *Wnt8b* (**C-C″**) and *Sp8* (**D-D″**). *Bmp4* expression is absent in the prosencephalic dorsal midline in both mutants (arrows A-A″). *Msx1* expression in prosencephalic dorsal midline is indicated by an arrow (B-B″); this expression is not detectable in the *Fgf8*$^{Null/Neo}$ mutant. *Msx1* expression is also not detectable in the olfactory placodes. *Wnt8b* expression is expanded rostrally in the *Fgf8*$^{Null/Neo}$ mutant and even more so in the *Fgf8*^{TelKO} mutant (C-C″) (note that the embryo in C″ is tilted backward, so the rostral expansion can be more readily observed). *Sp8* expression is greatly reduced in the *Fgf8*$^{Null/Neo}$ mutant, but is present in the midline of the *Fgf8*^{TelKO} mutant (arrow points to midline in D-D″). TUNEL analysis on horizontal sections through the E9.0 forebrain labels apoptotic cells in wild-type, *Fgf8*$^{Null/Neo}$ and *Fgf8*^{TelKO} embryos (**E-E″**) (see additional cell death analysis in Fig. 6). In wild-type embryos, apoptotic cells are detected in the telencephalic midline (open arrowhead in E), the optic stalks and hypothalamus. In *Fgf8*$^{Null/Neo}$ embryos, the rostral midline has fewer TUNEL$^+$ cells, E′, although it does exhibit evidence for cell death in the telencephalon (white arrowhead), optic stalk, and particularly in the hypothalamus. *Fgf8*^{TelKO} embryos have a higher concentration of TUNEL$^+$ cells in the rostral midline (open arrowhead, E′), and have scattered TUNEL$^+$ cells in the telencephalon (white arrowhead), optic stalk, and hypothalamus. The number of TUNEL$^+$ cells in the rostral midline correlates with the expression of *Msx1* at E9.0 (B-B″). Note that the panels showing TUNEL labeling are turned 180° with respect to the panels showing frontal views of in situ hybridization, such that the rostral regions face each other; this was done to facilitate the comparison of *Msx1* expression with the number of TUNEL$^+$ midline cells. The boxes demarcate the regions in which TUNEL$^+$ cells were counted in the rostral midline (box 1) and rostroventral telencephalon (box 2) of embryos (see Table 1). Abbreviations: HT: hypothalamus, OP, olfactory placode; Os, optic stalk; Tel, telencephalon.

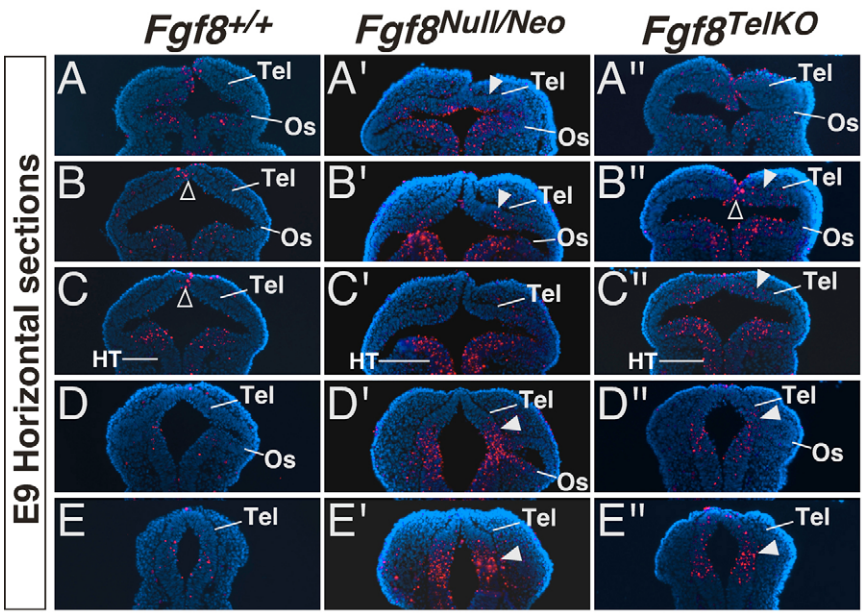

Fig. 6. Analysis of cell death in the *Fgf8* mutants at E9.0. TUNEL analysis on horizontal sections through the E9.0 forebrain labels apoptotic cells in wild-type (**A-E**), *Fgf8*$^{Null/Neo}$ (**A′-E′**) and *Fgf8*^{TelKO} embryos (**A″-E″**). A-A″ are the most ventral; E-E″ are the most dorsal. Note, sections B,B′,B″ are the same as in Fig. 5. Black arrowheads indicate the rostral midline; white arrowheads indicate the apoptotic cells in the telencephalic primordium (A′,B′,B″,C″) and at the junction of the dorsal optic stalk with the telencephalon (D′,D″,E′,E″). HT, hypothalamus; Os, optic stalk; Tel, telencephalon.

these mutants. We next turned our attention to the effect of reducing the *Fgf8* dose on the expression of transcription factors that control telencephalic regionalization.

Fgf8 mutants show shifts in the graded expression of the *Emx2* and *COUP-TF1* transcription factors in the telencephalon

Previous reports suggested that FGF8 can repress the expression of *Emx2* and *COUP-TFI* (*Nr2f1* – Mouse Genome Informatics) (Crossley et al., 2001; Garel et al., 2003; Storm et al., 2003; Fukuchi-Shimogori and Grove, 2003), transcription factor genes that are expressed in caudal-to-rostral gradients in the telencephalon (Liu et al., 2000; Zhou et al., 2001; Bishop et al., 2002; O'Leary and Nakamgawa, 2002; Muzio and Mallamaci, 2003; Grove and Fukuchi-Shimogori, 2003; Hamasaki et al., 2004; Garel and Rubenstein, 2004). We found that the levels of *Emx2* expression were increased in the E11.5 telencephalic vesicle as the dose of *Fgf8* was reduced (Fig. 7A,A′,A″). These data are consistent with observations made in mildly hypomorphic *Fgf8* mutants (*Fgf8*$^{Neo/Neo}$) (Garel et al., 2003).

In contrast, *COUP-TF1* expression was affected differently in the two mutants. The expression of *COUP-TF1* spread rostrally in *Fgf8*$^{Null/Neo}$ mutant embryos, but its rostral and dorsal expression did not expand in *Fgf8*^{TelKO} mutants (Fig. 7B,B′,B″). Although *Emx2* and *COUP-TF1* expression share similar caudorostral gradients, their ventrodorsal expression differs. *Emx2* is expressed in a dorsal-to-ventral gradient, whereas *COUP-TF1* has a ventral-to-dorsal

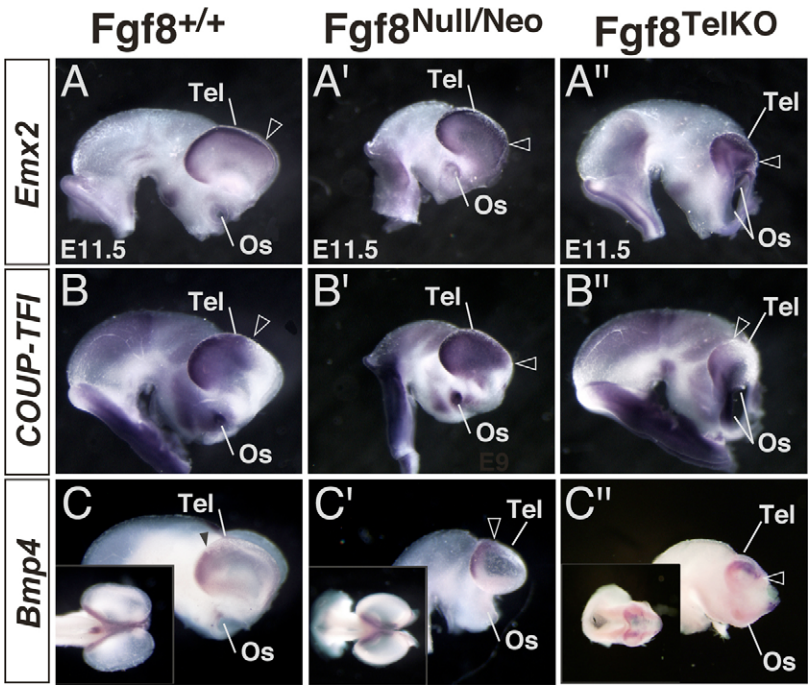

Fig. 7. Rostrocaudal gradients of *Emx2* and *COUP-TF1* expression are modified in *Fgf8* mutant embryos. Whole-mount in situ hybridization on dissected E11.5 rostral neural tube showing *Emx2* (**A-A″**), *COUP-TFI* (**B-B″**) and *Bmp4* (**C-C″**) expression. Lateral views are presented. In C-C″ a box in the lower left corner shows an additional dorsal view. In wild-type embryos, the *Emx2*-high-expression domain is detected in the caudomedial telencephalon (arrowhead in A). In both *Fgf8*$^{Null/Neo}$and *Fgf8*^{TelKO} embryos, the *Emx2*-high-expression domain is expanded laterally and rostrally (arrowhead in A′,A″). In wild-type embryos, the *COUP-TF1*-high-expression domain is restricted to caudolateral telencephalic regions (arrowhead in B). In *Fgf8*$^{Null/Neo}$embryos, the *COUP-TF1*-high-expression domain expands rostrally (arrowhead in B′) whereas it remains in a caudolateral position in *Fgf8*^{TelKO} mutants (arrowhead in B″). Such a differential effect on *COUP-TFI* expression correlates with the difference in *Bmp4* expression in the two *Fgf8* mutants. Indeed, in *Fgf8*$^{Null/Neo}$ embryos, *Bmp4* expression is the same as in wild-type embryos (restricted to dorsal midline tissues; arrowhead in C,C′). By contrast, *Bmp4* expression expands in the telencephalic vesicles of *Fgf8*^{TelKO} mutant embryos (arrowhead in C″). Os, optic stalk; Tel, telencephalon.

gradient (reviewed by O'Leary and Nakamgawa, 2002; Grove and Fukuchi-Shimogori, 2003; Muzio and Mallamaci, 2003; Garel and Rubenstein, 2004), suggesting that *COUP-TF1* expression may be repressed by dorsal patterning signals such as BMPs and/or WNTs. Previous work showed that *Bmp4* expression is reduced in the rostral midline of $Fgf8^{Null/Neo}$ mutants at E10.5, but is increased in this region in $Fgf8^{TelKO}$ mutants (Storm et al., 2003). We therefore examined the expression of *Bmp4* in *Fgf8* mutants at E11.5.

Our results revealed that different doses of *Fgf8* produce distinct alterations of rostrocaudal and mediolateral patterning in the telencephalic primordium. *Bmp4* expression in the wild-type telencephalon at E11.5 was restricted to dorsal paramedian tissues, where it showed a caudal-to-rostral gradient (Fig. 7C). In $Fgf8^{Null/Neo}$ mutants, the caudal expression of *Bmp4* appeared normal, whereas rostrally its expression appeared to be reduced (Fig. 7C′). By contrast, in the $Fgf8^{TelKO}$ mutant, *Bmp4* was more broadly expressed in the dorsal telencephalic vesicles (Fig. 7C″). The differential effects of the two *Fgf8* genotypes on *Emx2* and *COUP-TF1* expression may be mediated by the divergent effects on levels of *Bmp4* expression.

Reduced expression of *Shh* and *Nkx2.1* in the rostroventral telencephalon

As both the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants showed an expansion of caudal molecular properties (i.e. *Emx2* and *COUP-TFI* expression) into the rostral telencephalon, we investigated whether other aspects of rostral telencephalic patterning were disrupted.

Fate maps of the anterior neural plate show that the primordia of subcortical (subpallial) structures are rostral to the primordia of cortical (pallial) structures (Cobos-Sillero et al., 2001). During neurulation, the rostroventral region of the telencephalon expresses markers that are characteristic of the ventral neural tube, beginning with the expression of *Nkx2.1* (*Titf1* – Mouse Genome Informatics) and subsequently with expression of *Shh* (Crossley et al., 2001). During this patterning phase of the subpallium, the expression pattern of *Fgf8* is highly dynamic, including its extension through the chiasmatic region into the optic stalks, followed by splitting into separate domains (Crossley et al., 2001).

The expression of *Shh* in the rostroventral telencephalon is closely intertwined with that of *Fgf8*. SHH function is required to maintain *Fgf8* expression (Ohkubo et al., 2002), and in zebrafish, a reduction in the expression of both *Fgf3* and *Fgf8* results in decreased hypothalamic expression of *Shh* (Walshe and Mason, 2003). We therefore examined *Shh* expression in $Fgf8^{Null/Neo}$ mutants at E9.5, early during telencephalic regionalization (Fig. 8B,B′). *Shh* expression in the subpallium (preoptic and anterior entopeduncular areas) was greatly reduced in $Fgf8^{Null/Neo}$ brains. Although a few scattered clusters of Shh^{+} cells were visible in the rostroventral telencephalon, morphogenesis of the AEP/MGE was severely disrupted (arrow in Fig. 8B,B′). Furthermore, *Shh* expression at the base of the optic stalks and along the lamina terminalis was both wider and more intense.

Nkx2.1 function is required for the induction of telencephalic *Shh* expression (Sussel et al., 1999). Therefore, we examined *Nkx2.1* expression in the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants at E10. In $Fgf8^{Null/Neo}$ mutants, *Nkx2.1* expression in the subpallium was clearly reduced, and the $Fgf8^{TelKO}$ mutant lacked *Nkx2.1* expression in the telencephalon (Fig. 8A,A′,A″). These results suggest that FGF8 function is required for induction of basal telencephalic characteristics.

Patterning and differentiation defects of the subpallium at E12.5

Given the reduction in *Nkx2.1* expression in $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants, we examined the patterning and differentiation in the subpallium using in situ hybridization on coronal sections from E12.5 embryos (Fig. 9). Both $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants exhibited a loss of rostral subcortical structures, including the septum, lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE; Fig. 9).

Consistent with the loss of subcortical morphology observed at E10, the telencephalic expression of *Nkx2.1* and *Shh* (which are required for subcortical development) was greatly reduced ($Fgf8^{Null/Neo}$) or lost ($Fgf8^{TelKO}$) at E12.5 (Fig. 9C-D″). The small zone of telencephalic *Nkx2.1* expression that remained in $Fgf8^{Null/Neo}$ embryos at E10 (Fig. 8A,A′) revealed that not all subcortical molecular features were eliminated in these mutants. Indeed, the medial part of the rostroventral telencephalon continued to express both *Dlx2* and *Dlx5* (Fig. 9E′,F′). These homeobox genes are expressed in most of the subcortical telencephalon of control mice; *Dlx2* is expressed primarily in progenitors, whereas *Dlx5* is expressed in late progenitors and in subsets of postmitotic neurons (Fig. 9E,F) (Eisenstat et al., 1999). The expression of *Dlx2* and *Dlx5* in the $Fgf8^{Null/Neo}$ mutant suggests that subpallial neurogenesis in at least one subcortical region is maintained. There is a small region expressing *Dlx2* in the rostroventral telencephalon of the $Fgf8^{TelKO}$ mutants (Fig. 9E″).

Patterning and differentiation defects of the pallium at E12.5

The $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants generated different types of tissues in the rostrodorsal telencephalon. In $Fgf8^{Null/Neo}$ mutants, a thickened neuroepithelium that produced *Tbr1*-expressing neurons

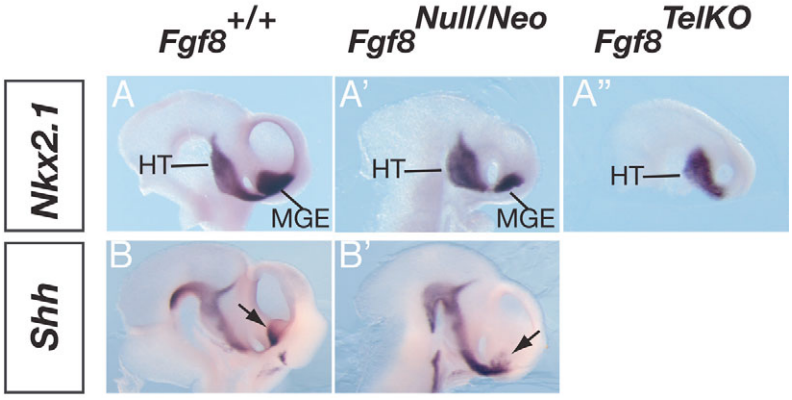

Fig. 8. $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants have progressive reduction of telencephalic *Nkx2.1* expression. Whole-mount in situ hybridization on dissected E10 rostral neural tube using probes for *Nkx2.1* (**A**,**A′**,**A″**) and *Shh* (**B**,**B′**) shows that expression of both genes is reduced in the telencephalic domains of $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ embryos. *Shh* expression in the telencephalon of the $Fgf8^{TelKO}$ mutant was not assessed because previous work demonstrated that loss of *Nkx2.1* function in the telencephalon prevents *Shh* induction in the telencephalon (Sussel et al., 1999). Arrows in B,B′ indicate Shh expression in the basal telencephalon. HT, hypothalamus; MGE, medial ganglionic eminence.

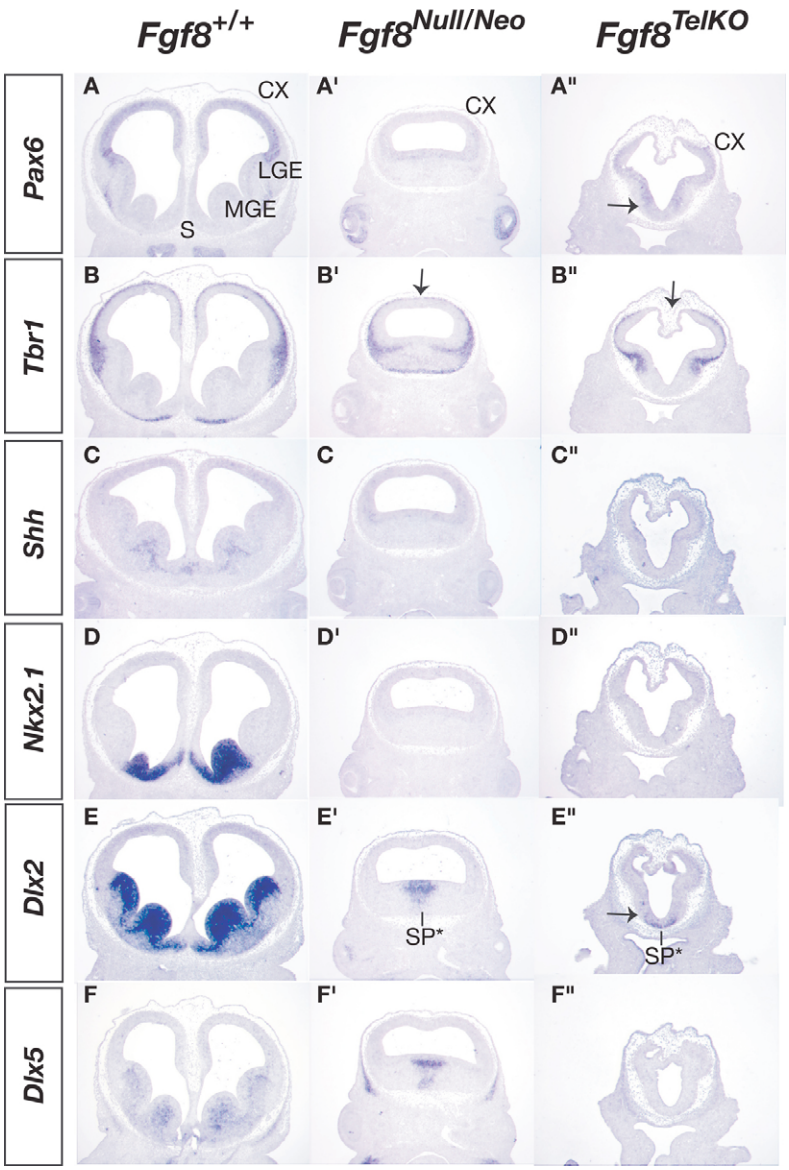

Fig. 9. *Fgf8* mutants lose ventral molecular properties in the telencephalon. In situ hybridization on coronal sections from E12.5 wild type, $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ embryos showing *Pax6* (**A-A″**), *Tbr1* (**B-B″**), *Shh* (**C-C″**), *Nkx2.1* (**D-D″**), *Dlx2* (**E-E″**) and *Dlx5* (**F-F″**) expression. Horizontal arrows in A′ and E″ indicate the approximate pallial-subpallial boundary in the $Fgf8^{TelKO}$ mutant. Also note the divergent morphology of the dorsal midline tissues in the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants (vertical arrows in B′,B″). CX, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; S, septum; SP*, unspecified subpallium.

at the dorsal midline was apparent (Fig. 9B′, arrow), whereas $Fgf8^{TelKO}$ mutants produced a thin, *Tbr1*-negative midline that resembled choroid plexus tissues (Fig. 9B″, arrow). *Tbr1* encodes a T-box transcription factor expressed in postmitotic pallial (cortical) neurons (Hevner et al., 2001).

We observed a ventral expansion of cortical molecular properties in *Fgf8* mutant mice based on the expression of genes that mark cortical progenitors (*Pax6*) and postmitotic cells (*Tbr1*) at E12.5 (Fig. 9 and data not shown). *Pax6* is normally expressed in a low dorsal-high ventral gradient in cortical progenitors (Toresson et al., 2000; Yun et al., 2001); it is also expressed at low levels in LGE, but not MGE progenitors (Sussel et al., 1999; Stoykova et al., 2000). In $Fgf8^{Null/Neo}$ mutants, *Pax6* expression in the progenitor zone extended throughout nearly the entire telencephalon (Fig. 9A,A′). *Pax6* was expressed in $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants in a ventrodorsal gradient that nearly extended throughout the entire telencephalon, consistent with the loss of subpallial structures. Unlike controls, however, both $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants showed reduced *Pax6* expression in rostral telencephalic regions (Fig. 9A,A′,A″).

Tbr1 continued to be expressed in both *Fgf8* mutants, and its domain of expression extended into morphologically ‘ventral’ parts of the rostral telencephalon. This suggests that ventral cortical structures such as the lateral and ventral pallium are produced in these domains (Fig. 9B,B′,B″).

DISCUSSION

The expression of *Fgf8* at the rostral limit of the telencephalon has profound and complex roles in most aspects of telencephalic patterning and morphogenesis. We have performed an in depth analysis of the patterning of the rostral neural plate and early forebrain in $Fgf8^{Null/Neo}$ (severely hypomorphic) and $Fgf8^{TelKO}$ (conditional null) mouse mutants, focusing on regional specification, proliferation, apoptosis and cross regulation between the rostral, dorsal and ventral patterning centers. Molecular analysis of the anterior neural plate and early neural tube showed that a reduction in *Fgf8* expression does not prevent the induction or maintenance of key regulators of prosencephalic (*Six3*) or telencephalic (*Foxg1*) identity (Figs 2, 3). However, at least some *Fgf8* expression persists in the anterior neural ridge of both $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants;

thus further studies in which all *Fgf8* expression is blocked in this region are needed to establish definitively whether *Fgf8* is required for the specification of the anterior prosencephalon (including the telencephalon). The rostral expansion of *Emx2*, *Otx2* and *Wnt8b* in the *Fgf8* mutants that we observed shows that reduced *Fgf8* dose results in the molecular caudalization of the anterior prosencephalon (Figs 2, 3, 5, 7). These observations are consistent with the known ability of FGF8 to function as a repressor of *Emx2*, *Otx2* and *Wnt3a* expression at later developmental stages and in different parts of the embryonic brain (Martinez et al., 1999; Crossley et al., 2001; Garel et al., 2003; Fukuchi-Shimogori and Grove, 2003; Shimogori et al., 2004).

There is currently some controversy about the respective roles for *Fgf8* and *Emx2* in contributing to patterning of the neocortex. Whereas *Fgf8* clearly has a central role in regulating the level of *Emx2* expression in progenitor cells of the rostral cortex (and thereby contributes to cortical patterning), there is very strong evidence that *Emx2* has an autonomous function in specifying the regional fate of progenitors in the caudal cortex (Hamasaki et al., 2004; Muzio et al., 2005). Therefore, it is our view that although *Fgf8* and *Emx2* contribute to regulating each other's expression, each gene also contributes to patterning of the neocortex, and other embryonic tissues, through additional pathways. For instance, *Emx2* has recently been shown to positively regulate WNT signaling, particularly in caudodorsal parts of the cortex (Muzio et al., 2005); these are regions where *Fgf8* expression is very low or not present.

Evidence that FGF8 controls the size of the telencephalon by regulating *Foxg1* expression

A progressive reduction of *Fgf8* gene dose in the $Fgf8^{Neo/Neo}$, $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants leads to progressive hypoplasia of the telencephalon. Whereas telencephalic size in $Fgf8^{Neo/Neo}$ mutants is nearly normal (Garel et al., 2003), the rostral-caudal dimension of the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ telencephalon at E11.5 was ~75% and ~50% of wild-type, respectively (Fig. 7). Reduced telencephalon size may be a consequence of alterations in proliferation and cell death. The mitotic index of the E9.0 $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ rostroventral telencephalon was reduced (Figs 3, 4; Table 1), suggesting that the telencephalic hypoplasia is due, at least in part, to reduced cell proliferation. We hypothesize that the reduction is mediated by the diminished expression of the winged-helix transcription factor *Foxg1* (Fig. 3). Previous reports have suggested that FGF8 is a positive regulator of *Foxg1* expression (Shimamura and Rubenstein, 1997; Ye et al., 1998). *Foxg1* promotes telencephalic cell proliferation (Xuan et al., 1995; Hardcastle and Papalopulu, 2000; Hanashima et al., 2002; Martynoga et al., 2005) through repressing SMAD signaling (Dou et al., 1999; Dou et al., 2000; Seoane et al., 2004). Thus we suspect that alterations in *Foxg1* contribute to the reduced telencephalic size in *Fgf8* mutants. In addition, the domain of strong *Six3* expression in the prosencephalon appeared to be reduced in mutants at E9.0 (Fig. 3); *Six3* is also implicated in repressing SMAD signaling (Gestri et al., 2005), and therefore may also regulate cell proliferation.

In addition to decreased cell proliferation, an increase in cell death was detected. The TUNEL assay showed roughly a 10-fold increased signal in the rostroventral telencephalon of both mutants compared to control embryos (Figs 5, 6; Table 1); the magnitude of this effect suggests that apoptosis may play a greater role in causing the telencephalic hypoplasia than decreased cell proliferation.

Interactions between the rostral and dorsal patterning centers control dorsal midline development

Altering the dose of *Fgf8* resulted in distinct effects on the development of the rostrodorsal midline. Previously, we reported reduced cell death in the telencephalic midline of $Fgf8^{Null/Neo}$ mutants, and increased cell death in the midline of $Fgf8^{TelKO}$ mutants at E10.5; both phenotypes were positively correlated with *Bmp4* expression (Storm et al., 2003). In the present study we found that one day earlier, at E9.5, the telencephalic midline of $Fgf8^{Null/Neo}$ mutants contained a reduced number of $TUNEL^+$ cells relative to wild-type, whereas the rostrodorsal midline of the $Fgf8^{TelKO}$ mutants resembled that of wild-type embryos (Figs 5, 6; Table 1). Although we did not detect *Bmp4* expression in either mutant at E9.5 (Fig. 5), there were changes in the expression of *Msx1*. *Msx1* is a pro-apoptotic homeobox gene expressed in the dorsal midline of the neural tube (Bach et al., 2003) that can be induced in the neuroepithelium by BMP signaling (Shimamura and Rubenstein, 1997). We found that *Msx1* expression was not detectable in $Fgf8^{Null/Neo}$ mutants and maintained in $Fgf8^{TelKO}$ embryos; thus its expression pattern was positively correlated with midline apoptosis. By E11.5, *Bmp4* expression in the $Fgf8^{Null/Neo}$ mutant was restricted to caudal parts of the telencephalon, whereas it was broadly expressed in the $Fgf8^{TelKO}$ mutant (Fig. 7).

These divergent effects of the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutations on midline cell death and *Bmp4*/*Msx1* expression were correlated with distinct patterns of histogenesis at the rostrodorsal midline on E12.5 (Fig. 9). Whereas the $Fgf8^{Null/Neo}$ mutant had a thickened, holoprosencephalic midline with molecular features typical of cortex ($Tbr1^+$) (Fig. 9), the dorsal midline of the $Fgf8^{TelKO}$ mutant was thin and appeared choroid plexus-like, consistent with the known roles of BMP signaling in choroid plexus development (Hebert et al., 2002). In contrast, the dorsal midline of $Fgf8^{Neo/Neo}$ (mild hypomorph) mutants appeared grossly normal, although subtle defects were suggested by the failure of the corpus callosum to form (Huffman et al., 2004). Thus, different levels of *Fgf8* expression create a spectrum of dorsal midline defects that are correlated with alterations in *Bmp4* and *Msx1* expression, genes that regulate dorsal midline development (Liu et al., 2004; Ramos et al., 2004; Hebert et al., 2002). Of course, other genes whose expression is misregulated in *Fgf8* mutants, such as *Sp8*, may also contribute to these dorsal midline phenotypes.

Like *Bmp4*, *COUP-TF1* expression responded non-linearly in $Fgf8^{Null/Neo}$ and $Fgf8^{Neo/Neo}$ mutants. *COUP-TF1* expression expanded rostrally in $Fgf8^{Null/Neo}$ and $Fgf8^{Neo/Neo}$ mutants (Fig. 7) (Garel et al., 2003); however, its expression remained repressed in the rostral cortex of the $Fgf8^{TelKO}$ mutant (Fig. 7). We hypothesize that this may be caused by BMP4-mediated repression of *COUP-TF1*.

Whereas *Bmp4*, *Msx1* and *COUP-TF1* expression and cell death responded in a non-linear fashion in $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants, the expression of other genes was altered in graded manner. For example, the expression of *Wnt8b* and *Emx2* expanded into rostral regions of the telencephalon (Figs 3, 5), consistent with prior findings in $Fgf8^{Neo/Neo}$ mutants (Garel et al., 2003) and in electroporation studies in which the level of FGF signaling was modulated using expression of a secreted form of FGFR3 to reduce the extracellular concentrations of FGF ligands (Fukuchi-Shimogori and Grove, 2003; Shimogori et al., 2004). The correlation in the expansion of *Wnt8b* and *Emx2* may reflect the presence of WNT-regulated TCF binding sites in the *Emx2* enhancer (Theil et al., 2002).

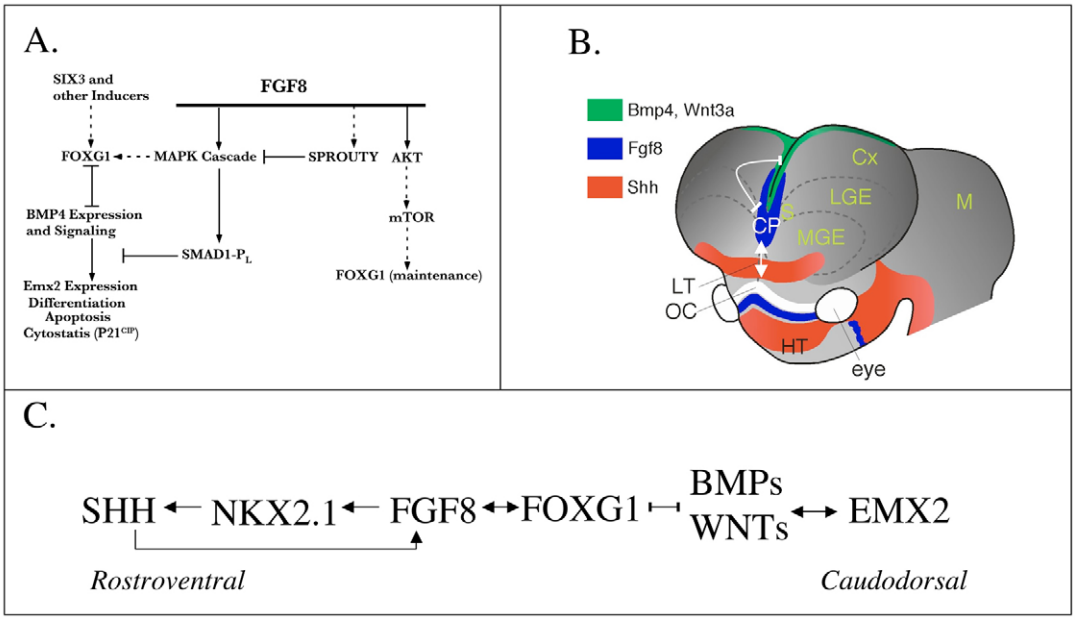

Fig. 10. Summary of proposed interactions between patterning centers. (**A**) Postulated signaling cascade downstream of FGF8. Not shown: FOXG1 also represses expression of WNT genes. Dotted lines indicate that the interaction is either indirect or potentially indirect. Note that *Foxg1* expression is maintained in part by mTOR (Hentges et al., 1999; Hentges et al., 2001), and that the MAPK cascade blocks BMP signaling through phosphorylation of the linker domain of SMAD (Kretzschmar et al., 1997; Pera et al., 2003). (**B**) Schema of a frontolateral view of the telencephalon showing the patterning centers as marked by expression of the genes indicated, the cross regulation between the *Fgf8* and *Bmp4*/*Wnt3a*-expressing centers, and the positive interactions between the *Fgf8* and *Shh*-expressing domains. (**C**) Postulated pathways interconnecting FGF, BMP, WNT and SHH signaling through the EMX2, FOXG1 and NKX2.1 transcription factors. Note that there is evidence that EMX2 positively regulates BMP/WNT signaling that in turn represses *Fgf8* expression (Shinozaki et al., 2004; Muzio et al., 2005; Shimogori et al., 2004). FGF8 signaling is required for induction of *Nkx2.1* expression in the telencephalon; we hypothesize that FGF signaling has a general role in ventral neural specification. Not described in this schema is the role that GLI3 plays in regulating the balance between forebrain signaling centers. GLI3 represses SHH-mediated effects on ventralization throughout the nervous system (reviewed by Ruiz et al., 2002). As in more caudal regions of the neural tube, the expression of ventral molecular features expands into dorsal structures within the *Gli3* mutant telencephalon (Tole et al., 2000; Rallu et al., 2002). *Gli3* mutants exhibit a reduction in BMP and WNT expression at the dorsal midline (Grove et al., 1998; Kuschel et al., 2003) and an expansion of *Fgf8* expression (Aoto et al., 2002; Kuschel et al., 2003), leading to the model that GLI3 plays a central role in mediating interactions between the telencephalic signaling centers (Aoto et al., 2002; Kuschel et al., 2003). However, since *Shh* expression is essentially eliminated from the telencephalon in the *Fgf8* mutants, it is not clear whether alterations in *Gli3* expression or function might contribute to their phenotypes. CP, commissural plate; Cx, cortex; HT, hypothalamus; LGE, lateral ganglionic eminence; LT, lamina terminalis; M, mesencephalon; MGE, medial ganglionic eminence; OC, optic chiasm; S, septum.

Previously we postulated that increases in FGF8 signaling repress the FOXG1-mediated repression of BMP4 signaling (Fig. 10) (Storm et al., 2003). Ongoing studies are aimed at testing whether sprouty genes, which encode FGF-induced repressors of FGF signaling (reviewed by Kim and Bar-Sagi, 2004), contribute to this effect. It will also be important to determine whether sprouty genes contribute to the opposing effects of the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutations on *Bmp4*/*Msx1* expression.

In principle some of the increase in *Bmp4* expression observed in $Fgf8^{TelKO}$ mutants may be caused by heterozygosity at the *Foxg1* locus (since these embryos carry a *Foxg1* allele that has been disrupted by insertion of *Cre*) (Hebert and McConnell, 1999). This is plausible for two reasons: (1) heterozygosity of *Foxg1* rescued the loss in *Bmp4* expression observed in $Fgf8^{Null/Neo}$ mutants (Storm et al., 2003); and (2) $Foxg1^{-/-}$ mutants ectopically express *Bmp4* (Dou et al., 1999; Muzio and Malamacci, 2005). However, to date, we have not observed a change in *Bmp4* expression in embryos bearing a single allele of *Foxg1-Cre*, therefore we conclude that the $Fgf8^{TelKO}$ phenotype largely reflects a loss of *Fgf8* expression.

Differences in the phenotypes of the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants might also be attributable to differences in the timing of reduced *Fgf8* expression. $Fgf8^{Null/Neo}$ mutants constitutively have reduced *Fgf8* expression in all tissues, whereas $Fgf8^{TelKO}$ mutants lack *Fgf8* expression primarily in the forebrain, beginning after rostral neural plate expression of *Foxg1* is initiated (~3 somite stage) (Shimamura and Rubenstein, 1997). In principle, reduced *Fgf8* expression during gastrulation in non-neural tissues could contribute to the phenotype of the $Fgf8^{Null/Neo}$ mutants, although we have not found evidence that a telencephalic phenotype arises by this mechanism.

Thus, several lines of evidence support the view that the *Fgf8*-expressing rostral patterning center has complex regulatory interactions with the dorsal patterning center (Fig. 10). First, *Fgf8* represses *Wnt3a* and *Wnt8b* expression (Fig. 5) (Shimogori et al., 2004). This has important implications for forebrain regionalization because WNT signaling is known to caudalize the prosencephalon (reviewed by Wilson and Houart, 2004) and is required for development of the hippocampal complex (caudodorsal telencephalic structures) (Galceran et al., 2000; Lee et al., 2000). Second, *Fgf8* dose has a more complex relationship with BMP expression (and probably signaling). Reducing the level of *Fgf8* initially lowers BMP expression, but further reductions lead to an increase in BMP expression. Third, BMP signaling is likely to negatively regulate *Fgf8* expression (Ohkubo et al., 2002; Shimogori

et al., 2004). We suggest that FGF8-mediated positive regulation of *Foxg1* expression plays a key role in maintaining the balance between FGF and BMP/WNT expression and signaling, since *Foxg1* is required to restrict *Bmp4*, *Wnt3a* and *Wnt8b* expression to the dorsal midline (Dou et al., 1999; Muzio and Mallamaci, 2005). This steady-state is further regulated by the BMP-mediated repression of *Foxg1* (Furuta et al., 1997; Ohkubo et al., 2002) and induction of *Msx1* (Furuta et al., 1997; Shimamura et al., 1997).

Interactions between the rostral and ventral patterning centers control subpallial development: evidence that FGF8 initiates ventral specification

The rostral patterning center is also essential for establishing the normal domains of *Nkx2.1* and *Shh* expression in the rostroventral telencephalon (Fig. 8A,A′,A″,B,B′, Fig. 9C,C′,C″,D,D′,D′). Reductions in *Fgf8* dose in the $Fgf8^{Null/Neo}$ and $Fgf8^{TelKO}$ mutants led to progressive reductions of *Shh*, *Nkx2.1*, *Dlx2* and *Dlx5* expression and ventral structures in the subpallial telencephalon. The residual subpallial structures seen in $Fgf8^{Null/Neo}$ embryos expressed *Dlx2* and *Dlx5* (Fig. 9E′,F′); ongoing studies are aimed at elucidating the histological identity of these structures, although we suspect that they may have a lateral ganglionic eminence/striatal phenotype based on their expression of low levels of *Pax6* in the ventricular zone, expression of *Dlx2* and *Dlx5*, and lack of *Nkx2.1* and *Tbr1* expression. Interestingly, *Shh* and *Nkx2.1* expression are maintained in the diencephalon in *Fgf8* mutant embryos, suggesting that another FGF gene may compensate for *Fgf8* in this region. It is possible that *Fgf10* expression in the ventral hypothalamus plays this role (Treier et al., 2001).

The failure of *Nkx2.1* induction in the $Fgf8^{TelKO}$ mutant telencephalon supports the hypothesis that FGF signaling may provide the initial step in telencephalic ventralization. Our data show that FGF8 signaling is essential for inducing ventral molecular (e.g. *Nkx2.1*) and histological properties within the rostral-most telencephalon. Likewise, in the endoderm, *Nkx2.1* induction is controlled by FGF signaling (Serls et al., 2005). It is possible that FGF signaling has a general role for inducing *Nkx2.1* expression, analogous to the role of receptor tyrosine kinase signaling in the induction of *vnd* (*Nkx* homologue) expression in the *Drosophila* embryonic central nervous system (von Ohlen and Doe, 2000). Indeed, such a role for FGF8 might explain the loss of ventral forebrain structures (hypothalamus) following expression of a dominant negative EPH receptor tyrosine kinase (Xu et al., 1996). Once *Nkx2.1* expression has been established, the expression of *Shh* is induced in the telencephalon (Sussel et al., 1999), suggesting that receptor tyrosine kinase signaling may play a general role in establishing ventral fates in the neural tube upstream of *Shh* function. This interpretation is consistent with a recent analysis of forebrain phenotypes in FGFR conditional mutants (G. Gutin, M. Fernandes, K. Yu, D. Ornitz, S. K. McConnell and J.M.H., unpublished results).

Comparison between *Fgf8* function in mouse and zebrafish

In zebrafish, *Fgf8* function is compensated by *Fgf3* (Walshe and Mason, 2003), whereas in mouse, *Fgf3* has not been associated with forebrain development. By Contrast, in mouse *Fgf15, Fgf17* and *Fgf18* expression overlap with *Fgf8* (Maruoka et al., 1998; Bachler and Neubuser, 2001; Gimeno et al., 2003); interactions between *Fgf8* and these other FGF genes remains to be demonstrated. In zebrafish, reduced expression of either *Fgf8* or *Fgf3* results in reduced expression of subpallial genes (e.g. *dlx2*); these defects are consistent with the demonstrated role of FGF/MAPK signaling in zebrafish subpallial development (Shinya et al., 2001). Furthermore, reductions in FGF dose in zebrafish affect development of the telencephalic midline, resulting in commissural defects (Shanmugalingam et al., 2000), as has been also noted in mouse *Fgf8* mutants (Huffman et al., 2004) (data not shown).

In addition to the well-described functions of *Fgf3* and *Fgf8* at the zebrafish midbrain/hindbrain organizer (Walshe et al., 2002; Jaszai et al., 2003; Wiellette and Sive, 2004), these genes have roles in zebrafish retinal and diencephalic development (Walshe and Mason, 2003; Martinez-Morales et al., 2005). We have also observed phenotypes in these tissues in the *Fgf8* mutants (unpublished), but a detailed analysis remains to be performed.

Concluding remarks

The growth, regional specification and morphogenesis of the telencephalon show a profound sensitivity to *Fgf8* gene dose. Furthermore, cross-regulation between the rostral (FGF), dorsal (BMP; WNT) and ventral (SHH) patterning centers plays an essential role in patterning the early telencephalon (Fig. 10). Modulation of the cross-regulation has the potential to control the relative size of structures whose morphogenesis is controlled by a given patterning center. For instance, a reduction in FGF8 signaling reduces the ratio of the frontal to sensory regions of the neocortex (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003). Therefore, controlling the relative strength of a given patterning center may provide a fundamental mechanism to modify the relative sizes of brain subdivisions during evolution and in disease states.

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