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 (Mariana 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 TGF 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; Shamamulingam 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), Fgf8low (exons 2 and 3 are present but can be deleted by Cre-mediated recombination), Fgf8neo (~40% normal expression), Fgf8null (exons 2 and 3 are deleted) (Meyers et al., 1998). Mice lacking Fgf8 (Fgf8null) die during gastrulation (Sun et al., 1999), whereas telencephalic conditional Fgf8nullcre nulls (Fgf8low/low/foxcre recombinated using Foxg1-Cre), Fgf8nullneo and Fgf8neo/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 (Fgf8Null/Neom) or increases (Fgf8TelKO) in Bmp4 expression and apoptosis.

The previous studies concentrated on the phenotype of the Fgf8TelKO and Fgf8Null/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 Fgf8TelKO and Fgf8Null/Neo phenotypes. Therefore, here we report studies of Fgf8 dose-dependent effects on neural plate and early post-neuralization 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. Fgf8Null/Neo and Fgf8TelKO mice were crossed to produce Fgf8Null/Neo embryos. Fgf8Null/Neom and Foxg1-CreFgf8Null/Neom mice were crossed to produce Fgf8Null/Neom;Foxg1-Cre(Fgf8TelKO) 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 Fgf8Null/Neom 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 10-15 μm cryostat sections as described previously (Yun et al., 2001). Rabbit anti-phosphohistone-3 (PH3) 1/4000 (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 10-15 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 Fgf8Null/Neo hypomorphic and Fgf8TelKO mutants

Comparative analysis of Cresyl Violet-stained horizontal sections prepared from E14.5 wild-type, Fgf8Null/Neo and Fgf8TelKO 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 Fgf8Null/Neo mutants, whereas no clear phenotypic variation was observed in Fgf8TelKO mutants. In all mutants, rostral and rostroventral structures showed the most profound alterations. Both Fgf8Null/Neo and Fgf8TelKO 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 Fgf8TelKO mutants. The rostral midline of the mutants differed: the Fgf8Null/Neo midline was thicker than that in Fgf8TelKO 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 Fgf8Null/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 Fgf8Null/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 Fgf8TelKO 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
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’/H11032,E/H11033, Fig. 4) no differences were detected between the genotypes. In the rostroventral telencephalon (box 2; Fig. 3E,E’/H11032,E/H11033, Fig. 4), wild-type embryos had roughly a twofold higher mitotic index than either mutant (Fig. 3E,E’/H11032,E/H11033 and Fig. 4; Table 1).

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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 Fgf8Null/Neo mutants but was maintained in Fgf8TelKO 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’,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 Fgf8Null/Neo mutants (Fig. 5D’). In contrast, in Fgf8TelKO 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 Fgf8TelKO mutants exhibited distinct patterns of Msx1 and Sp8 expression. These changes reflect the complexity of regulatory interactions in the dorsal midline.

**Fgf8Null/Neo and Fgf8TelKO mutants exhibit distinct patterns of apoptosis in the rostral midline**

Alterations in the expression of Fgf8 are associated with cell death (Trump 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 Fgf8Null/Neo mutants (Fig. 5E’, Fig. 6A’-E’), whereas Fgf8TelKO 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

![Fig. 2. Analysis of neural plate patterning in Fgf8Null/Neo embryos.](Image)

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 E,F’). Foxg1 expression is not detected in the mutant pharyngeal region (arrows in E,E’).

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

<table>
<thead>
<tr>
<th>Marker assayed</th>
<th>Genotype</th>
<th>Number of embryos</th>
<th>Box 1</th>
<th>Box 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH3 Wild type</td>
<td>3</td>
<td>1.4±0.2</td>
<td>26±5.7</td>
<td></td>
</tr>
<tr>
<td>PH3 Null/Neo</td>
<td>2</td>
<td>1.5±0.7 (0.37)</td>
<td>12±4.6 [0.016]</td>
<td></td>
</tr>
<tr>
<td>PH3 TelKO</td>
<td>2</td>
<td>1.7±0.6 (0.39)</td>
<td>13±3.7 [0.003]</td>
<td></td>
</tr>
<tr>
<td>TUNEL Wild type</td>
<td>3</td>
<td>5±1.2</td>
<td>2.5±1.3</td>
<td></td>
</tr>
<tr>
<td>TUNEL Null/Neo</td>
<td>2</td>
<td>1.6±0.9 (0.005)</td>
<td>38±18 [0.005]</td>
<td></td>
</tr>
<tr>
<td>TUNEL TelKO</td>
<td>2</td>
<td>5.5±1.2 (0.37)</td>
<td>38±20 [0.001]</td>
<td></td>
</tr>
</tbody>
</table>

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.
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 Fgf8Null/Neo and Fgf8TelKO 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 Fgf8Null/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 Fgf8TelKO mutants (Fig. 6A″-E″), but these brains showed less apoptosis than the Fgf8Null/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 rostroventral 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), Fgf8Null/Neo (A'-E') and Fgf8TelKO (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 E9.5 Fgf8Null/Neo and Fgf8TelKO 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 Fgf8Null/Neo mutant. Msx1 expression is also not detectable in the olfactory placodes. Wnt8b expression is expanded rostrally in the Fgf8Null/Neo mutant and even more so in the Fgf8TelKO 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 Fgf8Null/Neo mutant, but is present in the midline of the Fgf8TelKO mutant (arrow points to midline in D-D'). TUNEL analysis on horizontal sections through the E9.0 forebrain labels apoptotic cells in wild-type, Fgf8Null/Neo and Fgf8TelKO 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 Fgf8Null/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. Fgf8TelKO 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.
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-TF1 (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′/H11032,A′/H11033). These data are consistent with observations made in mildly hypomorphic Fgf8 mutants (Fgf8Neo/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 Fgf8Null/Neo mutant embryos, but its rostral and dorsal expression did not expand in Fgf8TelKO mutants (Fig. 7B,B′/H11032,B′/H11033). 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 gradient.
gradient (reviewed by O’Leary and Nakamgawa, 2002; Grove and Fukuchi-Shimogori, 2003; Muzio and Mallamaci, 2003; Garel and Rubenstein, 2004), suggesting that COUP-TFI 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 Fgf8Null/Neo mutant at E10.5, but is increased in this region in Fgf8TelKO 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 Fgf8Null/Neo mutants, the caudal expression of Bmp4 appeared normal, whereas rostrally its expression appeared to be reduced (Fig. 7C’). By contrast, in the Fgf8TelKO 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-TFI 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 Fgf8Null/Neo and Fgf8TelKO 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-Silleró 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 (Tifl – 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 Fgf8Null/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 Fgf8Null/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 Fgf8Null/Neo and Fgf8TelKO mutants at E10. In Fgf8Null/Neo mutants, Nkx2.1 expression in the subpallium was clearly reduced, and the Fgf8TelKO 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 Fgf8Null/Neo and Fgf8TelKO mutants, we examined the patterning and differentiation in the subpallium using in situ hybridization on coronal sections from E12.5 embryos (Fig. 9). Both Fgf8Null/Neo and Fgf8TelKO 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 (Fgf8Null/Neo) or lost (Fgf8TelKO) at E12.5 (Fig. 9C-D”). The small zone of telencephalic Nkx2.1 expression that remained in Fgf8Null/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 Fgf8Null/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 Fgf8TelKO mutants (Fig. 9E’).

Patterning and differentiation defects of the pallium at E12.5

The Fgf8Null/Neo and Fgf8TelKO mutants generated different types of tissues in the rostrodorsal telencephalon. In Fgf8Null/Neo mutants, a thickened neuroepithelium that produced Tbr1-expressing neurons...
at the dorsal midline was apparent (Fig. 9B’, arrow), whereas Fgf8TelKO 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 Fgf8Null/Neo mutants, Pax6 expression in the progenitor zone extended throughout nearly the entire telencephalon (Fig. 9A,A’). Pax6 was expressed in Fgf8Null/Neo and Fgf8TelKO mutants in a ventrodorsal gradient that nearly extended throughout the entire telencephalon, consistent with the loss of subpallial structures. Unlike controls, however, both Fgf8Null/Neo and Fgf8TelKO 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 Fgf8Null/Neo (severely hypomorphic) and Fgf8TelKO (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 Fgf8Null/Neo and Fgf8TelKO 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 FGFR8 controls the size of the telencephalon by regulating Foxg1 expression

A progressive reduction of Fgf8 gene dose in the Fgf8Neo/Neo, Fgf8Null/Null and Fgf8TelKO mutants leads to progressive hypoplasia of the telencephalon. Whereas telencephalic size in Fgf8Null/Null mutants is nearly normal (Garel et al., 2003), the rostral-caudal dimension of the Fgf8Null/Null and Fgf8TelKO 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 Fgf8Null/Null and Fgf8TelKO 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 FGFR8 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 (Doherty 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 rostralmidline. Previously, we reported reduced cell death in the telencephalic midline of Fgf8Null/Neo mutants, and increased cell death in the midline of Fgf8TelKO 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 Fgf8Null/Neo mutants contained a reduced number of TUNEL+ cells relative to wild-type, whereas the rostrodorsal midline of the Fgf8TelKO 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 Mxi1. Mxi1 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 Mxi1 expression was not detectable in Fgf8Null/Neo and maintained in Fgf8TelKO embryos; thus its expression pattern was positively correlated with midline apoptosis. By E11.5, Bmp4 expression in the Fgf8Null/Neo mutant was restricted to caudal parts of the telencephalon, whereas it was broadly expressed in the Fgf8TelKO mutant (Fig. 7). These divergent effects of the Fgf8Null/Neo and Fgf8TelKO mutations on midline cell death and Bmp4/Mxi1 expression were correlated with distinct patterns of histogenesis at the rostralmidline on E12.5 (Fig. 9). Whereas the Fgf8Null/Neo mutant had a thickened, holoprosencephalic midline with molecular features typical of cortex (Tbr1) (Fig. 9), the dorsal midline of the Fgf8TelKO 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 Fgf8Neo/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 Mxi1 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-TFI expression responded non-linearly in Fgf8Null/Neo and Fgf8TelKO mutants. COUP-TFI expression expanded rostrally in Fgf8Null/Neo and Fgf8TelKO mutants (Fig. 7) (Garel et al., 2003); however, its expression remained repressed in the rostral cortex of the Fgf8TelKO mutant (Fig. 7). We hypothesize that this may be caused by BMP4-mediated repression of COUP-TFI.

Whereas Bmp4, Mxi1 and COUP-TFI expression and cell death responded in a non-linear fashion in Fgf8Null/Neo and Fgf8TelKO 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 Fgf8Null/Neo mutants (Garel et al., 2003) and in electroporation studies in which the level of FGFR 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).
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 Fgf8Null/Neo and Fgf8TelKO mutations on Bmp4/Msx1 expression.

In principle some of the increase in Bmp4 expression observed in Fgf8TelKO 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 Fgf8Null/Neo mutants (Storm et al., 2003); and (2) Foxg1-Cre 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 Fgf8TelKO phenotype largely reflects a loss of Fgf8 expression.

Differences in the phenotypes of the Fgf8Null/Neo and Fgf8TelKO mutants might also be attributable to differences in the timing of reduced Fgf8 expression. Fgf8Null/Neo mutants constitutively have reduced Fgf8 expression in all tissues, whereas Fgf8TelKO 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 Fgf8Null/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 (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.

**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 signaling centers (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 Glial mutant telencephalon (Tolle et al., 2000; Rallu et al., 2002). Glial 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 Glial3 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 Glial 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.

![Figure 10](image-url)
et al., 2004). We suggest that FGFR8-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, Wnt5a and Wnt10b expression to the dorsal midline (Dou et al., 1999; Muzzio 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 Mxcl1 (Furuta et al., 1997; Shimamura et al., 1997).

**Interactions between the rostral and ventral patterning centers control subpallial development: evidence that FGFR8 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 Fgfr8 dose in the Fgf8NullNeo and Fgfr8Gfko 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 Fgf8NullNeo 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 Fgfr8 mutant embryos, suggesting that another FGF gene may compensate for Fgfr8 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 Fgfr8Gfko 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 (Gutin, M. Fernandes, K. Yu, D. Ornitz, S. K. McConnell and J.M.H., unpublished results).

**Comparison between Fgfr8 function in mouse and zebrafish**

In zebrafish, Fgfr8 function is compensated by Fgfr3 (Walsh and Mason, 2003), whereas in mouse, Fgfr3 has not been associated with forebrain development. By Contrast, in mouse Fgfr15, Fgfr17 and Fgfr18 expression overlap with Fgfr8 (Maruoka et al., 1998; Bachler and Neubuser, 2001; Gimenno et al., 2003); interactions between Fgfr8 and these other FGF genes remains to be demonstrated. In zebrafish, reduced expression of either Fgfr8 or Fgfr3 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 Fgfr8 mutants (Huffman et al., 2004) (data not shown).

In addition to the well-described functions of Fgfr3 and Fgfr8 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 Fgfr8 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 Fgfr8 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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