

ARTICLE

BMP Ligands Act Redundantly to Pattern the Dorsal Telencephalic Midline

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Summary: The embryonic telencephalon is patterned into several areas that give rise to functionally distinct structures in the adult forebrain. Previous studies have shown that BMP4 and BMP2 can induce features characteristic of the telencephalic midline in cultured explants, suggesting that the normal role of BMP4 in the forebrain is to pattern the medial lateral axis of the telencephalon by promoting midline cell fates. To test this hypothesis directly in vivo, the *Bmp4* gene was efficiently disrupted in the telencephalon using a CRE/loxP approach. Analysis of *Bmp4*-deficient telencephalons fails to reveal a defect in patterning, cell proliferation, differentiation, or apoptosis. The absence of a phenotype in the *Bmp4*-deficient telencephalon along with recent genetic studies establishing a role for a BMP4 receptor, BMPRIA, in telencephalic midline development, demonstrate that loss of *Bmp4* function in the telencephalon can be compensated for by at least one other *Bmp* gene, the identity of which has not yet been determined. *genesis* 35:214–219, 2003.

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INTRODUCTION

During midgestation development in the mouse, the anterior end of the neural tube evaginates to form the bilaterally symmetrical telencephalic hemispheres. The telencephalon later develops into several functionally distinct structures of the adult cerebral hemispheres, such as the basal ganglia, cerebral cortex, hippocampus, and choroid plexus. How the areas of the telencephalon destined to form these adult structures become specified during development remains a poorly understood process.

The neuroepithelial progenitor cells of the telencephalon acquire positional identities along the anterior-posterior axis and the medial-lateral (M-L) axis (the original dorso-ventral axis). Positional identities are acquired prior to the arrival of thalamic afferents, as

evidenced by the restricted expression patterns of several genes (reviewed by Ragsdale and Grove, 2001). A number of *Bmp* genes, including *Bmp2*, 4, 5, 6, and 7, are expressed along the dorsal telencephalic midline, a potential signaling center for M-L patterning (Furuta *et al.*, 1997). In culture experiments, BMP2 and BMP4 promote telencephalic midline features, including the inhibition of proliferation, promotion of apoptosis, induction of the dorsal midline marker *Msx1*, and repression of more laterally expressed genes such as *Lhx2* and *Foxg1* (Furuta *et al.*, 1997; Monuki *et al.*, 2001). An essential role for BMP signaling in midline formation has been revealed by the conditional deletion of the BMP receptor gene *Bmpr1a* in the telencephalon, which leads to loss of the most dorsomedial cell type, the choroid plexus (Hébert *et al.*, 2002). Complementary experiments have shown that overexpression of a constitutively active BMPRIA leads to a transformation of part of the lateral telencephalon into choroid plexus (Panchision *et al.*, 2001). These results suggest that ligands for BMPRIA, such as BMP2 and 4, pattern the dorsal telencephalon by regulating the expression of genes and the fate of cells along the M-L axis. If BMPs act as morphogens to specify cell fates in a concentration-dependent fashion, then reducing the overall level of BMP signaling by deleting one of the *Bmp* genes could result in a positional shift in telencephalic cell fates, confirming the hypothesis that BMPs pattern the dorsal telencephalon.

The role that each BMP ligand plays in vivo in specifying cell fates along the M-L axis remains unexplored. Targeted disruption of genes encoding BMP ligands have so far not been informative as to their role in the devel-

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Table 1
Genotype of Recovered Embryos

	Bmp4 ^{+/+}	Bmp4 ^{lox/+^a}	Bmp4 ^{lox/lox}	Cre; Bmp4 ^{+/+}	Cre; Bmp4 ^{lox/+}	Cre; Bmp4 ^{lox/lox}
E12.5 ^b	—	35	28	—	31	24
E12.5	64	41	21	51	35	16
E14.5	21	16	4	27	16	3 ^c
P0	23	13	2	30	15	0
P1-P7	33	13	1	20	9	0

^a“Lox” is used as an abbreviation for “loxP-lacZ-neo”; in contrast to a previous report (Kulesa and Hogan, 2002), fewer heterozygotes, with or without Cre, survive until birth, perhaps due to a difference in strain background. Cre refers to Foxg1-Cre.

^bEmbryos in this row were obtained by crossing Foxg1-Cre;Bmp4^{lox/+} males with females homozygous for Bmp4^{lox}, whereas embryos in all other rows were obtained with heterozygous females.

^cThese three mutant embryos were necrotic.

oping telencephalon because either the mutations cause embryonic lethality prior to telencephalic development, as for disruptions of *Bmp4* and *Bmp2* (Winnier *et al.*, 1995; Zhang and Bradley, 1996), or show no phenotype due to possible functional overlap, as for disruptions of *Bmp5*, 6, and 7 (Kingsley *et al.*, 1992; Dudley *et al.*, 1995; Luo *et al.*, 1995; Solloway *et al.*, 1998).

We have previously described a CRE/*loxP* conditional genetic approach for knocking out genes in the telencephalon using the Foxg1-Cre mouse line (Hébert and McConnell, 2000). As a first step in determining the role of individual BMP ligands *in vivo*, we have conditionally disrupted *Bmp4* in the telencephalon by crossing mice that carry a floxed allele of *Bmp4*, *Bmp4*^{loxP-lacZ-neo} (Kulesa and Hogan, 2002), to Foxg1-Cre mice. Embryos that are homozygous for *Bmp4*^{loxP-lacZ-neo} and carry the *Foxg1-Cre* allele, henceforth referred to as mutant embryos, do not survive to term and die between E12.5–E14.5 (Table 1). Mice that are homozygous for *Bmp4*^{loxP-lacZ-neo} but do not carry the *Foxg1-Cre* allele are also lost in significant numbers (Table 1), consistent with the hypomorphic nature of the *Bmp4*^{loxP-lacZ-neo} allele (Kulesa and Hogan, 2002). Mutant embryos are severely deficient in eye development (Fig. 2a), which is also observed in the hypomorphic *Bmp4*^{loxP-lacZ-neo/loxP-lacZ-neo} mice in the absence of any *cre* allele (Kulesa and Hogan, 2002). A more extensive study of the role of *Bmp4* in eye development will be described elsewhere. In addition, prior to the onset of necrosis, mutant embryos exhibit abnormal face development with recessed jaws and snout (Fig. 2a). Interestingly, Foxg1-Cre;Bmpr1a^{fx/null} embryos, in which *Bmpr1a* is efficiently deleted from the telencephalon, show similar facial abnormalities and die before birth (Hébert *et al.*, 2002), suggesting that BMP4 acts through BMPRIA both in the branchial arches that form the face and in a tissue essential for prenatal survival such as the heart or vasculature. The facial defects in both the *Bmpr1a* and *Bmp4* mutants are likely to be the result of recombination in the branchial arches, which is expected due to *Foxg1* expression in this tissue (Tao and Lai, 1992; Hatini *et al.*, 1994; Hébert and McConnell, 2000). However, the prenatal lethality of these mutants was not predicted and could either be due to recombination in tissues that do not normally express *Foxg1*, as previously observed (Hébert and McConnell,

2000), or in the case of *Bmp4*, due to the hypomorphic nature of the floxed allele, which is partially disrupted by the presence of a *neomycin* expression cassette (Kulesa and Hogan, 2002). Other than the eye and face defects, mutant embryos appeared grossly normal. Only embryos that showed no signs of necrosis were used for the analysis presented here.

Upon CRE-mediated recombination of the *Bmp4*^{loxP-lacZ-neo} allele, a *lacZ* open reading frame replaces the *Bmp4* open reading frame leading to expression of *lacZ* in a pattern that mimics that of *Bmp4* (Kulesa and Hogan, 2002). If recombination occurs efficiently in Foxg1-Cre; *Bmp4*^{loxP-lacZ-neo} embryos, then β-Gal activity should be observed wherever CRE activity overlaps normal *Bmp4* expression, namely, within the dorsal telencephalic midline. X-gal staining of mutant embryos reveals that the *Bmp4*^{loxP-lacZ-neo} allele is in fact efficiently recombined in the telencephalon of mutant embryos. Whole-mount staining reveals extensive β-Gal activity along the dorsal telencephalic midline of E12.5 embryos that carry the *Foxg1-Cre* allele and are either heterozygous or homozygous for the *Bmp4*^{loxP-lacZ-neo} allele (Fig. 1a). Coronal sections through the midline region demonstrate that the vast majority, if not all, of the midline choroid plexus and cortical hem cells, which normally express *Bmp4*, are recombined (Fig. 1b). Moreover, *Bmp4* mRNA can no longer be detected in the E12.5 mutant telencephalon by RNA *in situ* hybridization (Fig. 1c–d'). Therefore, *Bmp4* expression is likely to have been completely eliminated in the mutant telencephalon prior to E12.5, consistent with observations of other floxed alleles in *Foxg1-Cre* mice where recombination starts at ~E8.5 and is complete between E9 and E10 in the rostral prosencephalon (Hébert and McConnell, 2000; Hébert *et al.*, 2002). It should be noted that even though *Foxg1* is not expressed in the dorsal telencephalic midline from E11.5 on, ablation of *Bmp4* still occurs in the midline of mutants due to earlier transient *Foxg1* expression throughout this region (Hébert and McConnell, 2000).

One way in which BMPs have been postulated to pattern the telencephalon is by inhibiting the proliferation of progenitor cells and promoting either differentiation or apoptosis (Furuta *et al.*, 1997; Li *et al.*, 1998; Mehler *et al.*, 2000). In support of this hypothesis, BMP signaling through *Bmpr1a* is required for the inhibition

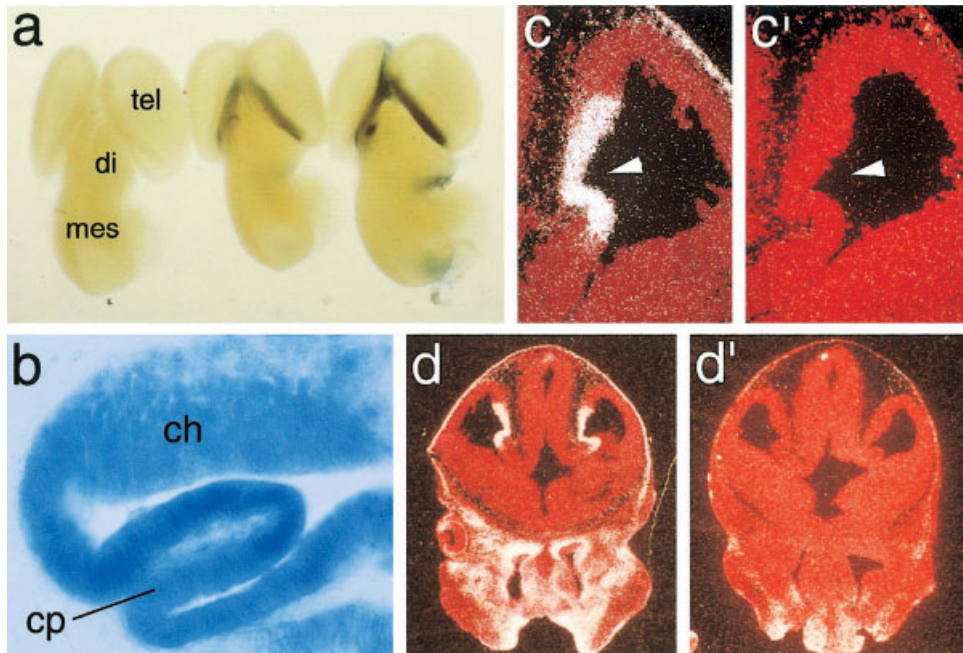


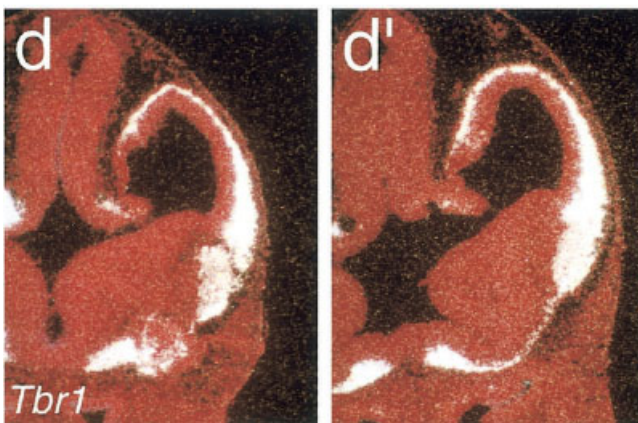
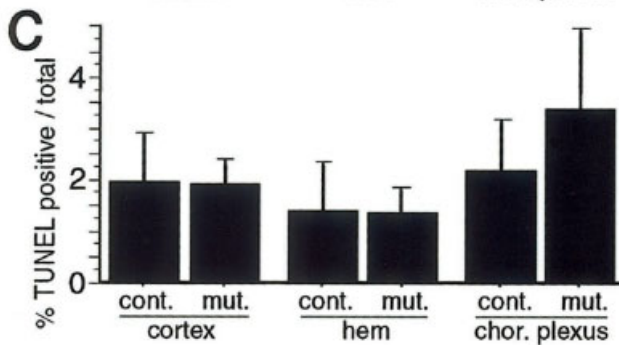
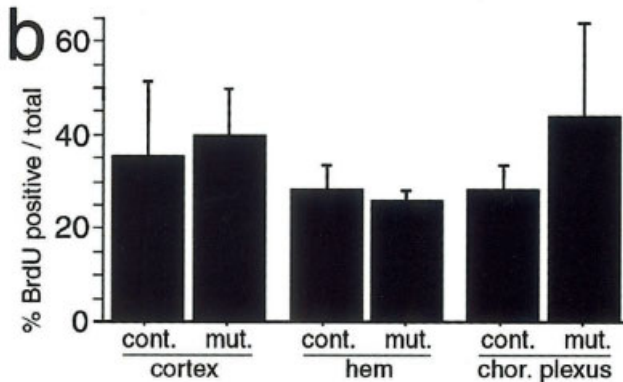
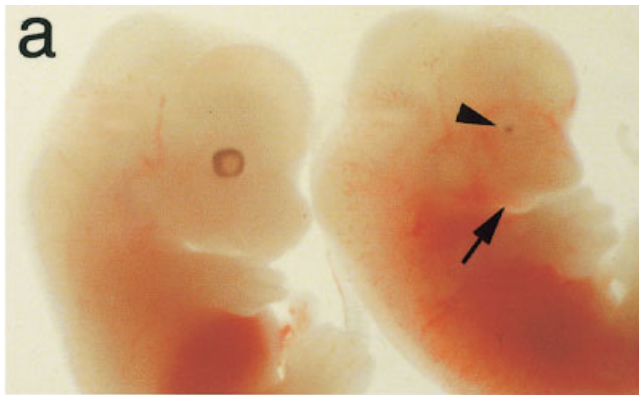
FIG. 1. Recombination of the *Bmp4*^{loxP-lacZ-neo} allele occurs efficiently in the telencephalon of E12.5 embryos that carry the *Foxg1-Cre* allele. **a:** X-gal staining shown in blue reveals extensive CRE-mediated recombination of *Bmp4*^{loxP-lacZ-neo} in the dorsal telencephalic midline of the heterozygous (middle) and homozygous (right), but not control (left), brains. **b:** Thin sections illustrate that the vast majority of cells, if not all the cells, in the choroid plexus (cp) and cortical hem (ch) of the dorsal midline are recombined. In situ RNA hybridization of the control (**c,d**) and mutant (**c',d'**) head with a probe for *Bmp4*; *Bmp4* expression is lost from the dorsal telencephalic midline (**c,c'** arrowheads) as well as from the skin surrounding the head, the olfactory epithelium, and part of the branchial arch derivatives in the face (**d,d'**), as expected given the normal expression pattern of *cre* in *Foxg1-Cre* mice (Hébert and McConnell, 2000).

of midline cell proliferation (Hébert *et al.*, 2002). To investigate the possibility that *Bmp4* also plays an essential role in inhibiting progenitor cell proliferation, the number of cells that incorporated BrdU in control and *Bmp4*-deficient telencephalons was quantified. No difference in the rate of proliferation was observed between controls and mutants for the neocortex, the cortical hem, or the choroid plexus (Fig. 2b). To address the hypothesis that *Bmp4* regulates telencephalic apoptosis (Furuta *et al.*, 1997; Mehler *et al.*, 2000), the number of TUNEL-positive cells was quantified in mutants and controls. No difference in the rates of apoptosis could be distinguished between the control and mutant neocortex, cortical hem, or choroid plexus (Fig. 2c). Finally, the effect of loss of *Bmp4* on cell differentiation was addressed by examining the expression of a gene, *Tbr1*, which marks all early born telencephalic neurons (Bulfone *et al.*, 1995). Again, no apparent difference could be observed in neuronal differentiation, as assessed by the amount of *Tbr* staining or the width of the region marked by *Tbr* expression, between the control and mutant telencephalon (Fig. 2d,d'). Therefore, although BMP signaling through BMPRIA is required to regulate proliferation and differentiation at least in the midline (Hébert *et al.*, 2002), BMP4 itself is not required for this process.

BMP4 is also hypothesized to pattern the telencephalon by regulating the expression of genes along the M-L axis (Furuta *et al.*, 1997; Monuki *et al.*, 2001). High levels

of BMP signaling are thought to induce *Msx1* (Furuta *et al.*, 1997), whereas lower levels are thought to induce the expression of the lateral marker *Lbx2* (Monuki *et al.*, 2001). Thus, reducing the overall level of BMP signaling by deleting a single component of the BMP pathway, such as BMP4, might be expected to lead to a shift in gene expression patterns and cell fates. However, at E12.5, a stage when the major telencephalic subdivisions along the M-L axis, including the ganglionic eminence, cerebral cortex, hippocampus, cortical hem, and choroid plexus, have just become morphologically distinguishable, the *Bmp4*-deficient telencephalon appears histologically normal. To further confirm this finding, we examined the expression patterns of genes that are regionally restricted along the M-L axis using RNA in situ hybridization analysis. No difference in the expression pattern of the ventral marker *Dlx2*, which marks both the medial and lateral ganglionic eminences, was observed between controls and mutants (Fig. 3f,f'). This is not surprising, given that the ventral telencephalon lies far from the source of BMP4 in the dorsal midline. *Lbx2* and *Foxg1* are two genes expressed throughout the telencephalon except in the dorsal midline. Expression of these two genes has been shown to be repressed by high levels of BMP4 in culture (Furuta *et al.*, 1997; Monuki *et al.*, 2001). However, loss of *Bmp4* does not lead to a derepression of these genes in the dorsal telencephalic midline (Fig. 3d-e'), nor does it lead to

derepression of other dorsal telencephalic markers normally excluded from the midline, such as *Ngn2* and *Emx1* (Fig. 3g,g', and data not shown).



Most significantly, the dorsal midline itself, which expresses the highest levels of *Bmp4* (Fig. 1; Furuta *et al.*, 1997) and where BMP signaling has been demonstrated to be essential for the formation of the most dorsomedial cell type, the choroid plexus (Hébert *et al.*, 2002), appears normal in the *Bmp4*-deficient telencephalon. *Ttr*, a gene specifically expressed in the choroid plexus, remains expressed at high levels in mutants (Fig. 3c,c'). In addition, *Msx1*, which marks both the choroid plexus region and the adjacent cortical hem, is expressed in its normal pattern within the dorsal telencephalic midline of mutants (Fig. 3a,a'), as is *Wnt2b*, which marks the cortical hem (Fig. 3b,b'). These results indicate that *Bmp4* on its own is essential neither for patterning the dorsal telencephalon, nor for the formation of the most dorsomedial cell type, the choroid plexus.

Previous experiments in which the nestin neural enhancer was used to drive expression of a constitutively active BMPRIA in the telencephalon suggested that activation of the BMP signaling pathway leads to a respecification of most of the dorsal telencephalon into midline telencephalon (Panchision *et al.*, 2001). Moreover, loss of the choroid plexus in the *Bmpr1a*-deficient telencephalon demonstrates that BMP signaling is at the very least required for formation of the most dorsomedial telencephalic cell type (Hébert *et al.*, 2002). However, here we show that loss of BMP4, a ligand for BMPRIA, has no effect on telencephalic patterning or development, indicating that another BMPRIA ligand must compensate for lack of BMP4. Although we do not know which ligand serves this role, BMP2 is the most likely suspect, for several reasons. BMP2 is the BMP family member most closely related to BMP4 (reviewed by Hogan, 1996; Massagué, 1996); it too can bind and signal through BMPRIA and it has similar effects to BMP4 on neural progenitor cells in culture (Furuta *et al.*, 1997; Monuki *et al.*, 2001). Generation of additional mutants in which several *Bmp* genes, such as *Bmp2* and *Bmp4*, are disrupted at once will further elucidate the precise mechanisms by which BMP signaling regulates telencephalic development.

FIG. 2. Whereas facial defects occur in the E12.5 mutant, proliferation, apoptosis, and differentiation in the telencephalon are not affected by loss of *Bmp4*. **a:** Control (left) and mutant (right) embryos showing lack of eye formation (arrowhead) and abnormal face development (arrow). **b:** BrdU analysis reveals no significant difference in cell proliferation between control (cont.) and mutant (mut.) neocortex, cortical hem, and choroid plexus (error bars represent standard error). See Methods for details. **c:** TUNEL analysis reveals no significant difference between control and mutant neocortex, cortical hem, and choroid plexus. **d,d':** RNA in situ hybridization analysis using a probe against *Tbr1*, which marks all early born telencephalic neurons, shows no difference between the control (**d**) and mutant (**d'**) telencephalon.

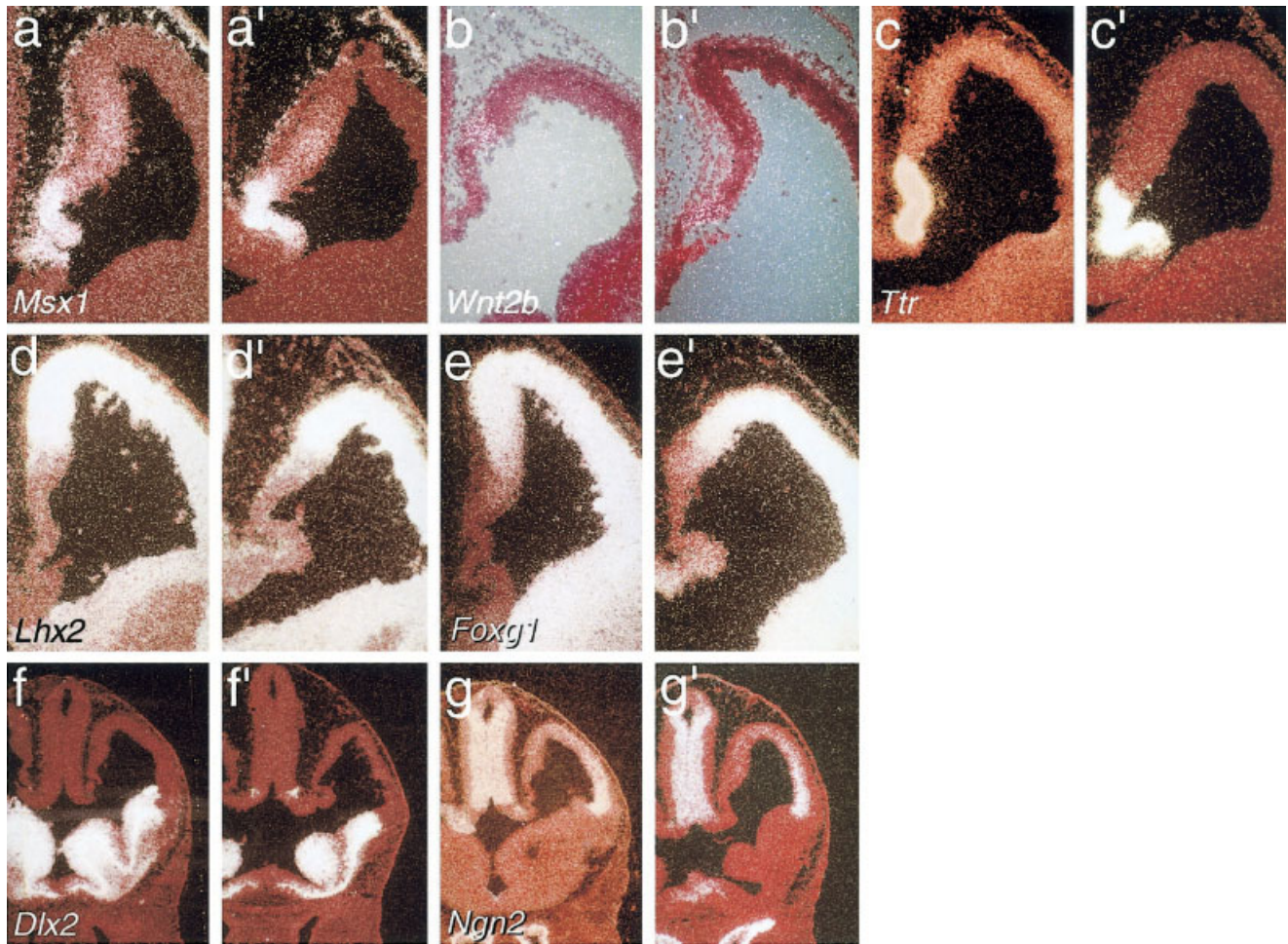


FIG. 3. Area-specific patterns of gene expression are normal in the *Bmp4*-deficient telencephalon. RNA in situ hybridization analysis was performed on E12.5 control (a–g) and mutant (a'–g') brains using probes for (a,a') *Msx1*, which marks the dorsal telencephalic midline, including the cortical hem and choroid plexus (b,b') *Wnt2b*, which marks the cortical hem (c,c') *Ttr*, which marks the choroid plexus (d,d') *Lhx2* and (e,e') *Foxg1*, which are both excluded from the dorsal telencephalic midline (f,f') *Dlx2*, which marks the ventral telencephalon, and (g,g') *Ngn2*, which marks the dorsal telencephalon excluding the midline.

MATERIALS AND METHODS

Maintenance of Mouse Lines

Mice were maintained in a mixed strain background and genotyped as described previously (Hébert and McConnell, 2000; Kulesa and Hogan, 2002). Because *Foxg1*-Cre mice were generated by targeting *Cre* to the *Foxg1* locus, creating a *Foxg1* null allele, only heterozygous *Foxg1*-Cre mice are used in the experiments described here. Mice that are heterozygous for *Foxg1* show no phenotype on their own (Xuan *et al.*, 1995; Hébert and McConnell, 2000; Hébert *et al.*, 2002; this report).

X-gal Staining

Whole brains were dissected from E12.5 embryos, stained whole with X-gal, and then plastic-embedded as previously described (Hébert and McConnell, 2000).

RNA In Situ Hybridization Analysis

Frozen sections were prepared and hybridized to ³⁵S-labeled probes as previously described (Frantz *et al.*, 1994). A minimum of three mutant and three control embryos were analyzed for each probe at each age. Plasmids used to make probes were kindly provided by William Blaner (*Ttr*), Elizabeth Grove (*Wnt2b*), John Rubenstein (*Dlx2*, *Tbr1*), and Juan Botas (*Lhx2*).

BrdU and TUNEL Analysis

Females pregnant with E12.5 embryos received an intraperitoneal injection with BrdU and were euthanized 1 h later. Embryos were collected, frozen in OCT, and sectioned on a cryostat. Fresh-frozen sections were used for either BrdU staining, as previously described (O'Rourke *et al.*, 1997), or for TUNEL analysis according to the manufacturer's specifications (Roche, Nutley, NJ, Cat. #2

156 792). Sections were counterstained with Syto11 to reveal cell nuclei. The fraction of BrdU- or TUNEL-positive cells was determined by counting the number of labeled cells in a radial segment spanning from the ventricular surface to the pial surface and dividing by the total number of Syto11+ cells in the segment. Segments were taken from the neocortex, cortical hem, and choroid plexus. One and two segments from each of three separate embryos were counted for each area for BrdU and TUNEL analyses, respectively. Approximately 400 and 1,300 cells were counted for each bar for BrdU and TUNEL, respectively. Expression of *cre* in mouse cells has previously been reported to increase the frequency of chromosomal abnormalities both in mice (Schmidt *et al.*, 2000) and in cultured cells (Loonstra *et al.*, 2001). In our studies, a higher rate of apoptosis was observed in the lateral and ventral telencephalon of embryos carrying only the *Foxg1-Cre* allele than of controls not carrying the *Foxg1-Cre* allele. However the increased rate of apoptosis due to CRE is not in itself sufficient to cause a phenotype. Embryos that carried the *Foxg1-Cre* allele were used as controls in the TUNEL and BrdU incorporation assays presented here, and all other experiments presented in this report included *Foxg1-Cre* embryos among the controls.

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