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Mutations in the BMP pathway in mice support the existence of two molecular classes of holoprosencephaly

Marie Fernandes[1], Grigoriy Gutin[1], Heather Alcorn[2], Susan K. McConnell[3] and Jean M. Hébert[1,*]

Holoprosencephaly (HPE) is a devastating forebrain abnormality with a range of morphological defects characterized by loss of midline tissue. In the telencephalon, the embryonic precursor of the cerebral hemispheres, specialized cell types form a midline that separates the hemispheres. In the present study, deletion of the BMP receptor genes, *Bmpr1b* and *Bmpr1a*, in the mouse telencephalon results in a loss of all dorsal midline cell types without affecting the specification of cortical and ventral precursors. In the holoprosencephalic *Shh*$^{-/-}$ mutant, by contrast, ventral patterning is disrupted, whereas the dorsal midline initially forms. This suggests that two separate developmental mechanisms can underlie the ontogeny of HPE. The *Bmpr1a*;*Bmpr1b* mutant provides a model for a subclass of HPE in humans: midline inter-hemispheric HPE.

KEY WORDS: Telencephalon, Dorsal midline, Choroid plexus, Cortical hem, Holoprosencephaly, BMP, SHH

INTRODUCTION

The cerebral hemispheres, the seat of higher cognitive function in mammals, develop from the embryonic telencephalon. The telencephalon becomes divided into morphologically distinct left and right hemispheres shortly after neural tube closure. Dorsally, the hemispheres are separated by two midline cell types: the choroid plexus, which produces the cerebrospinal fluid, and the cortical hem, which lies between the choroid plexus and hippocampal primordium. The molecular pathways that generate these midline cell types are poorly understood.

Holoprosencephaly (HPE), the most common congenital forebrain defect in humans (Wallis and Muenke, 2000; Muenke and Beachy, 2000), is characterized by a loss of midline tissue. The large spectrum of phenotypic severity and midline areas affected in inherited cases of human HPE are consistent with a complexity of genetic pathways regulating midline formation (Nanni et al., 1999; Ming and Muenke, 2002). In only a minority of HPE cases have mutations at known genetic loci been identified (Wallis and Muenke, 2000; Muenke and Beachy, 2000). In most identified cases, the mutated genes normally function in the ventral telencephalon. Disruption of the ventrally acting SHH or Nodal signaling pathways leads to HPE (Wallis and Muenke, 2000; Muenke and Beachy, 2000; Hayhurst and McConnell, 2003) and although not yet linked to HPE in humans, loss-of-function mutations in the mouse genes *Cdo* (*Cdon* – Mouse Genome Informatics; which encodes a multifunctional transmembrane protein), *Lrp2* (a low-density lipoprotein-related receptor) and *Chrd* and *Nog* (secreted BMP antagonists), exhibit loss of SHH expression and HPE (Anderson et al., 2002; Zhang et al., 2006; Spoelgen et al., 2005).

Of the genes known to cause HPE in mice, only mutations in *Lrp2*, *Chrd*;*Nog*, *Zic2* and *Fgf8* have been reported to display clear alterations in dorsal midline development (Anderson et al., 2002; Spoelgen et al., 2005; Nagai et al., 2000; Storm et al., 2006). Whereas *Lrp2* and *Chrd*;*Nog* mutants exhibit excessive dorsal midline features such as BMP expression and apoptosis (Furuta et al., 1997; Anderson et al., 2002; Spoelgen et al., 2005), the dorsal midline appears to be lost in the holoprosencephalic *Zic2* and *Fgf8* hypomorphic mutants (Nagai et al., 2000; Storm et al., 2006). *ZIC2* is also the only locus implicated in a subclass of HPE in humans, the midline inter-hemispheric HPE (MIH), which is associated with impaired dorsal midline formation, whereas in other classes of HPE the ventral medial area is primarily affected (Barkovich and Quint, 1993; Simon et al., 2002; Marcorelles et al., 2002; Nagai et al., 2000). Note that most *ZIC2* mutations also affect ventral midline formation (Brown et al., 2001).

Bmp2, *4*, *5*, *6* and *7* are expressed in the dorsal midline, prior to separation of the hemispheres (Furuta et al., 1997). The receptors are expressed broadly in telencephalic precursor cells. BMP4-soaked beads placed on explants of lateral telencephalon induce midline features (apoptosis and *Msx1* expression) and inhibit lateral features [proliferation and *Foxg1* expression (Furuta et al., 1997)]. BMP signaling is also crucial in specifying choroid plexus development (Panchision et al., 2001; Hebert et al., 2002). These findings have led to the hypothesis that BMP signaling is required to form the dorsal midline.

MATERIALS AND METHODS

Mice

Breeding and genotyping of *Foxg1*^{Cre}, *Bmpr1a*$^{fx/fx}$ and *Bmpr1b*$^{-/-}$ mice have been described previously (Hebert and McConnell, 2000; Mishina et al., 2002). Control embryos were *Foxg1*$^{Cre/+}$;*Bmpr1a*$^{fx/+}$;*Bmpr1b*$^{-/+}$.

RNA in situ hybridization, TUNEL and immunolabeling

RNA in situ hybridization, TUNEL and immunolabeling were carried out on 10-12 μm cryosections from fresh frozen embryos. For RNA situ analysis, sections were hybridized with ^{35}S-labeled RNA probes as previously described (Frantz et al., 1994). Plasmids were used to make probes: *Bmp4* (Jones et al., 1991), *Fgf8* (Crossley and Martin, 1995), *Foxg1* (Tao and Lai, 1992), *Lhx2* (Rodriguez-Esteban et al., 1998), *Msx1* (A. W. Helms), *Nkx2.1* (Shimamura et al., 1995), *Pax6* (Walther and Gruss, 1991), Wnts (Grove et al., 1998), *Gsh2* (*Gsx2* – Mouse Genome Informatics) (Hsieh-Li et al., 1995), *Sfrp2* (Rattner et al., 1997), *Ttr* (gift of W. S. Blaner, Columbia University, NY, USA), *Rfx4-v3* (Blackshear et al., 2003), *Zic2* (gift of M. Hayhurst, Stanford University, CA, USA), *Actr1* (ATTC, Image Clone 5336738). TUNEL analysis was performed according to the manufacturer's specifications (Roche, catalog #2156792). Immunolabeling

[1]Departments of Neuroscience and Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA. [2]Developmental Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA. [3]Department of Biological Sciences, Stanford University, Stanford, CA, USA.

*Author for correspondence (e-mail: jhebert@aecom.yu.edu)

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for phosphohistone H3 was performed with a polyclonal (Upstate, 1:200) and a Texas Red-conjugated secondary antibody. Cell counts and data analysis were performed as previously described (Gutin et al., 2006).

RESULTS AND DISCUSSION

The *Bmpr1a;Bmpr1b* double mutant is holoprosencephalic

To test the role of BMP signaling in telencephalon development, mouse embryos lacking both *Bmpr1a* and *Bmpr1b* were generated using the cross illustrated in Fig. 1A. Double-mutant embryos were obtained until E11.5 without signs of necrosis. However, at this age, the expected ratio of double mutants (6.25%) was not obtained (3.5% of 202 embryos), indicating some lethality. The dorsomedial area of the E11.5 double-mutant telencephalon appeared rounded and less defined, whereas a midline remained visible rostrally. Severe reduction of the eyes was also observed (Fig. 1C). These phenotypes are 100% penetrant with essentially invariable severity. RNA in situ hybridization analysis for the deleted segment of *Bmpr1a*, as previously performed (Hébert et al., 2002), revealed that expression begins to disappear at E8.5 (10 somites) and is absent by E9.5 in the telencephalon, whereas expression is unaffected in other tissues, such as the head mesenchyme, foregut and heart (see Fig. S1 in the supplementary material).

At E10.5 and E11.5, although a midline still formed rostrally in the mutant, it did not show the characteristic thinning observed in controls (Fig. 1D-E′,H,I). More posteriorly in controls, the choroid plaque was identifiable as a thin neuroepithelium protruding inward from the dorsal midline creating the medial walls that separate the ventricles, whereas in the double mutant this thin inward protrusion failed to form, resulting in HPE (Fig. 1F-G′,J,K). The worsening phenotype at E11.5 indicates that the phenotype observed at E10.5 is not due to a delay in midline development. Even more posteriorly, the diencephalon of the mutant was rounded and displaced, presumably owing to the lack of an invaginated dorsal telencephalic midline in which to intercalate (data not shown). These results indicate that disrupting BMP signaling prevents the formation of a morphologically defined dorsal midline and induces HPE.

Dorsal midline cell types fail to form in the *Bmpr1a;Bmpr1b* double mutant

The telencephalic dorsal midline is composed of the choroid plexus and the cortical hem. The choroid plexus is marked at E11.5 and onward by the expression of *Ttr*. In the double mutant, no *Ttr*-positive cells were detected anywhere along the rostrocaudal axis of the telencephalic dorsal midline (Fig. 2A,B). From E10/E10.5 onward, *Wnt2b* and *Wnt3a* were strongly expressed in the cortical hem (Grove et al., 1998; Lee et al., 2000). In the double mutant at E10.5 and E11.5, expression of these genes was greatly diminished or absent throughout the rostrocaudal axis (Fig. 2D and data not shown). Moreover, the choroid plaque and cortical hem normally express *Msx1* at E9.5 and 10.5. In the mutant, however, *Msx1* expression was lost (Fig. 2F and see Fig. S4 in the supplementary material). These results demonstrate that BMP signaling is required for formation of the choroid plexus and cortical hem.

Other markers that are also expressed in the medial area of the dorsal telencephalon were, however, still readily detected in *Bmpr1a*;*Bmpr1b* mutants. *Bmp4* and *Wnt8b*, whose expression at E10.5 normally extends beyond the choroid plaque and cortical hem into the adjacent neuroepithelium, were still expressed in the double mutant, although the length of the expression domain appeared diminished, presumably owing to the loss of midline cell types (Fig. 2G,H and data not shown). Interestingly, *Zic2*, a gene implicated in forms of human and mouse HPE in which the dorsal midline is lost (Nagai et al., 2000), was expressed in double mutants (Fig. 2I,J), demonstrating that BMP signaling is not required for the maintenance of *Zic2* expression.

From E9.5 onward, the midline is marked by high levels of apoptosis. In control E10.5 brains, TUNEL labeling was clearly visible at the dorsal midline, whereas in the double mutant no midline apoptosis was observed (Fig. 2K,L). This demonstrates that BMPR signaling is required in vivo for midline apoptosis in the telencephalon. Surprisingly, the double mutant also exhibited a loss of apoptosis in the rostroventral area of the midline. One potential explanation is that the strong dorsal expression of

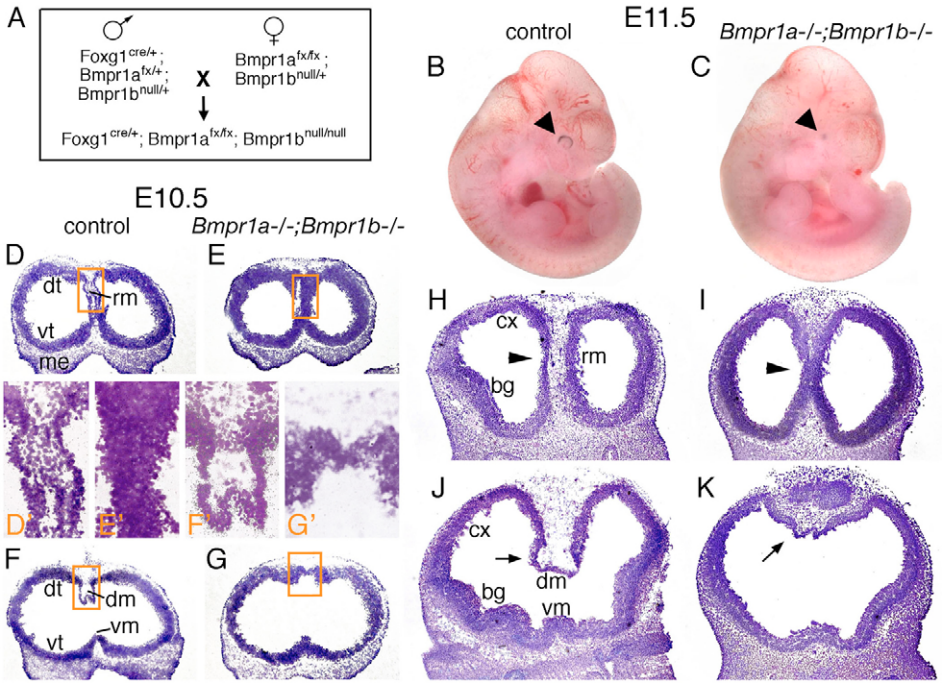

Fig. 1. The *Bmpr1a;Bmpr1b* double-mutant mouse is holoprosencephalic. (**A**) The genetic cross that generates the double mutant. (**B**,**C**) E11.5 double-mutant and control littermates. Note the small eye in the mutant (arrowhead). (**D-K**) Cresyl Violet staining of E10.5 (D-G) and E11.5 (H-K) control and mutant telencephalons from rostral (D,E,H,I) and more posterior coronal sections (F,G,J,K). D′ and E′ are magnifications of the rostral midline (boxed) in D and E, respectively, showing a thick neuroepithelium in the mutant. F′ and G′ are magnifications of the dorsomedial regions (boxed) in F and G, respectively, showing no invagination in the mutant. Arrowheads (H,I) point to the rostral midline, which is thicker in the mutant (I). Arrows (J,K) indicate the missing dorsal midline in the mutant (K). bg, basal ganglia; cx, cerebral cortex; dm, dorsal midline; dt, dorsal telencephalon; me, facial mesoderm; rm, rostral midline; vm, ventral midline; vt, ventral telencephalon.

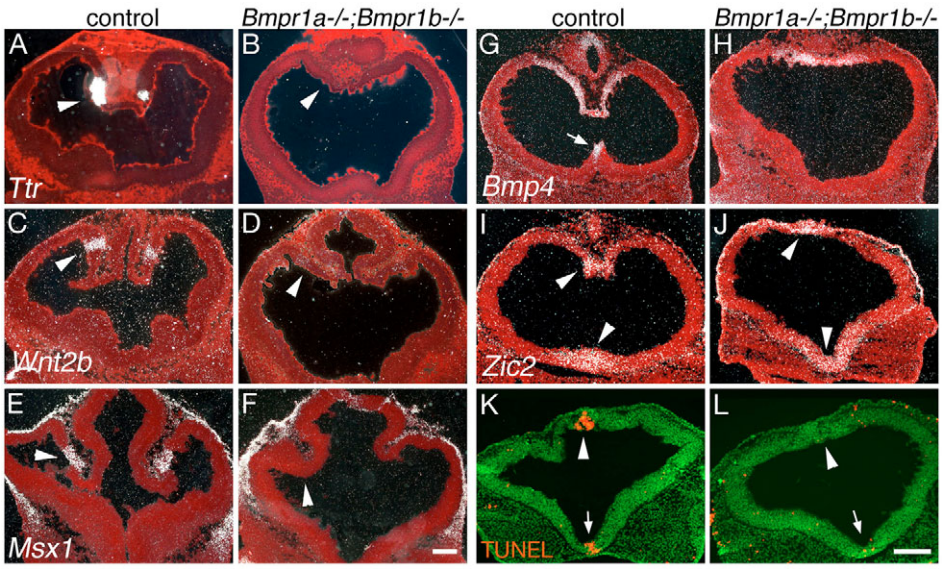

Fig. 2. The dorsal midline fails to develop in *Bmpr1a;Bmpr1b* double-mutant mice. Expression analysis using radioactive RNA in situ hybridization on E11.5 (**A-F**) and E10.5 (**G-J**) coronal sections. (A-F) The mutant lacks expression (arrowheads) of markers for: the choroid plexus, *Ttr* (A,B); the cortical hem, *Wnt2b* (C,D); and for both choroid plexus and hem, *Msx1* (E,F). (G-J) The expression of *Bmp4* in the dorsal midline and adjacent neuroepithelium is maintained in the mutant (G,H), although the domain of expression is reduced. Note the rostroventral midline expression of *Bmp4* (arrow in G). The ventral and dorsal midline expression of *Zic2* is maintained in the mutant (arrowheads in I,J). (**K**,**L**) TUNEL staining for apoptotic cells is absent at the dorsal (arrowheads) and ventral (arrows) midline in the E10.5 *Bmpr1a;Bmpr1b* mutant. Scale bars: 150 μm.

certain Bmp genes, such as *Bmp4* and *Bmp5*, extends far enough through the rostral midline to act ventrally at E10.5 (Fig. 2G, arrow, and data not shown).

Previous reports have suggested that BMPs inhibit the proliferation of neural progenitors in vitro (Furuta et al., 1997; Mehler et al., 2000). *Bmpr1a* and *Bmpr1b* have also been hypothesized to have opposing functions, with *Bmpr1a* promoting and *Bmpr1b* inhibiting telencephalic precursor cell proliferation (Panchision et al., 2001). In the E10.5 *Bmpr1a;Bmpr1b* double mutant, no significant changes in the percentage of mitotic (phosphohistone H3-positive) cells were observed in medial or lateral areas (for pooled areas: control, 5.6±1.5% s.d.; mutant, 5.9±1.2%; P=0.6), suggesting that at least at this age, BMP signaling is not required to regulate proliferation. Overall, the phenotypes observed demonstrate functional overlap between *Bmpr1a* and *Bmpr1b* in dorsal midline development.

Overexpression of a constitutively active BMPR1A transforms cortical precursors into choroid plexus cells (Panchision et al., 2001), suggesting that BMP signaling has the potential to induce the choroid plexus cell fate. Deletion of *Bmpr1a*, although demonstrating a requirement for BMP signaling in choroid plexus development, failed to distinguish between a requirement to induce precursor cells to adopt a choroid plaque fate or to promote the differentiation of already specified precursors (Hebert et al., 2002). However, in the *Bmpr1a;Bmpr1b* double mutant, expression of *Msx1*, a marker for both choroid plaque and cortical hem precursors, was absent (Fig. 2E,F), consistent with a failure to induce these cell types. The alternative possibilities that BMP signaling is required to maintain proliferation or survival of specified precursors are less likely because no change in these processes was observed in the double mutant.

Cortical patterning is grossly normal in the double mutant

Compelling evidence suggests that BMPs pattern the cortical neuroepithelium (Monuki et al., 2001; Shimogori et al., 2004; Hirabayashi et al., 2004; Crossley et al., 2001; Ohkubo et al., 2002; Cheng et al., 2006; Theil et al., 2002). To address this

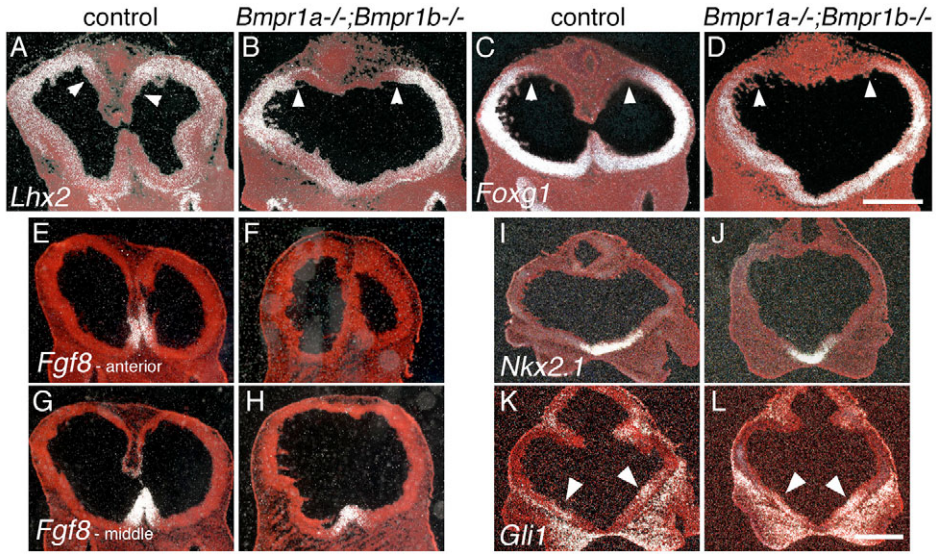

Fig. 3. Cortical and ventral patterning is preserved in *Bmpr1a;Bmpr1b* mutant mice. RNA in situ hybridization on E10.5 coronal sections. (**A-D**) The domains of expression for *Lhx2* (A,B) and *Foxg1* (C,D) are unchanged in the mutant and remain excluded from the dorsomedial area (arrowheads in A-D). (**E-H**) *Fgf8* expression is absent from the most anterior region along the rostral midline (E,F), but is maintained ventrally, as seen in more posterior sections (G,H). (**I-L**) The ventromedial markers *Nkx2.1* (I,J) and *Gli1* (K,L) are expressed in the mutant, suggesting that ventral cell fate is unaffected and that SHH signaling is active. Scale bars: 275 μm.

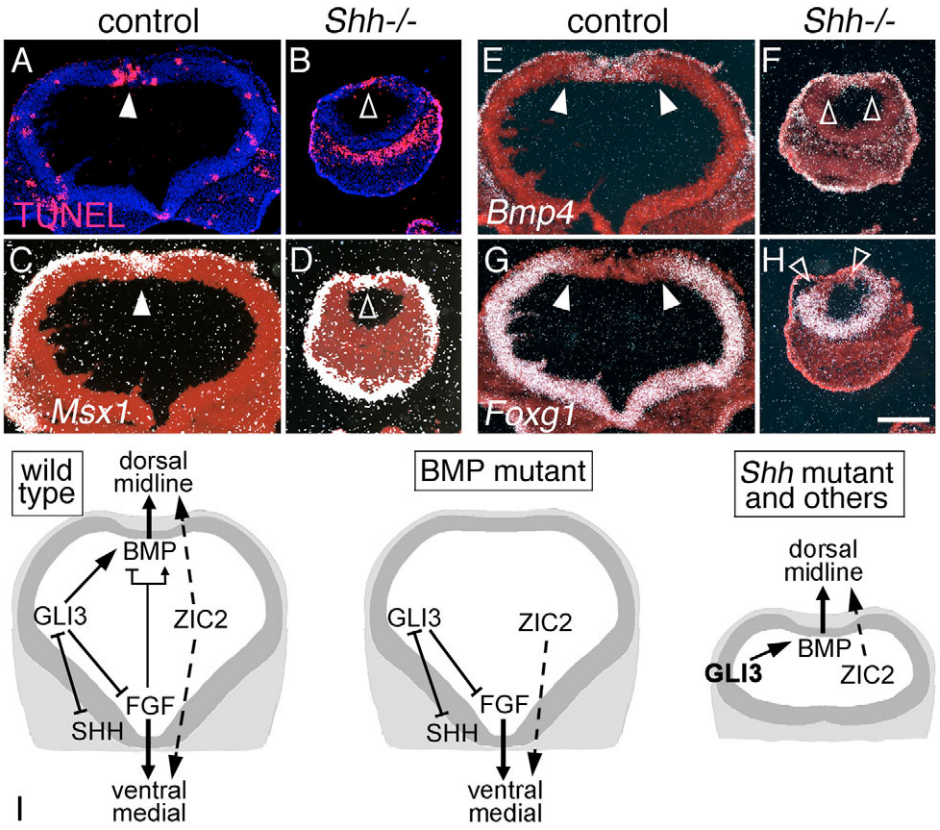

Fig. 4. The midline phenotype in the *Shh* mutant differs from that in the *Bmpr1a;Bmpr1b* mutant mouse. TUNEL analysis (**A**,**B**) and RNA in situ hybridization (**C-H**) on E10.5 coronal sections. (A,B) TUNEL-positive cells are detected at the most dorsomedial neuroepithelium in the *Shh* mutant (B, arrowhead), where apoptosis occurs in control embryos (A, arrowhead). (C-H) The dorsal midline markers *Msx1* (C,D) and *Bmp4* (E,F) are expressed in the *Shh* mutant (arrowheads) and *Foxg1* remains excluded from that area (H, arrowheads). Scale bar: 200 μm. (**I**) Model illustrating distinct mechanisms of HPE. In the wild type (left), BMPs acting downstream of GLI3 (Grove et al., 1998; Kuschel et al., 2003; Theil et al., 1999) and ZIC2 are both required for the formation of the dorsal midline (ZIC2 is also required ventrally). Note that *Fgf8* may also regulate dorsal midline development (Crossley et al., 2001), although only reduction, not loss, of *Fgf8* expression leads to dorsal HPE (Storm et al., 2006). SHH acts indirectly to generate ventral cells by antagonizing GLI3, which in turn relieves the repression of Fgf expression (Aoto et al., 2002; Gutin et al., 2006; Rallu et al., 2002). In the Bmpr double mutant (middle), the dorsal midline fails to form despite the maintenance of *Zic2* expression. However, ventromedial cell fates are not affected, as is also the case in the MIH subclass of HPE. In the *Shh* mutant and other mutants in which SHH signaling is disrupted (right), dorsal midline features are initiated but ventromedial cells are lost.

hypothesis, we analyzed E10.5 and E11.5 double mutants for the expression domains of transcription factors that specify cortical fates: *Lhx2*, *Foxg1*, *Emx2* and *Pax6*. Surprisingly, the expression of these factors, normally present in cortical precursors and excluded or greatly reduced in the dorsal midline, was not affected in the *Bmpr1a*;*Bmpr1b* mutant (Fig. 3A-D and data not shown). This suggests either that BMP signaling does not regulate the expression of these genes or that there is another signal, possibly *Wnt8b* (see above) (Gunhaga et al., 2003), that compensates. In embryos in which the dorsal midline is ablated (and thus a primary source of both BMPs and WNTs is removed), the expression of *Emx2*, *Lhx2*, *Pax6* and *Ngn2* (*Neurog2*) is also maintained; although for *Emx2* and *Lhx2*, their normal gradients of expression are flattened (Cheng et al., 2006). Subtle changes in the slopes of expression gradients might not be detected with the RNA in situ approach used here. Expression of the *v3* variant of *Rfx4*, which marks the dorsal telencephalon and is essential for medial cortex and hem development (Blackshear et al., 2003), also appeared unperturbed in the double mutant (see Fig. S2 in the supplementary material).

It is also possible that BMP signaling through a more distantly related type I receptor or prior to recombination and loss of *Bmpr1a* (i.e. earlier than ~E8.5) is sufficient to convey grossly normal patterning to cortical regions. *Actr1*, which encodes a type I receptor with affinity for BMP2, 4, 6 and 7 (ten Dijke et al., 2003; Derynck and Zhang, 2003), was not expressed in the telencephalon until after E10.5 (see Fig. S3 in the supplementary material and data not shown). Nevertheless, the results presented here demonstrate that *Bmpr1a* and *Bmpr1b* are not required for maintaining the domains of expression of cortical transcription factors.

Ventral patterning is maintained in the *Bmpr1a;Bmpr1b* mutant

In most cases of HPE caused by a mutation in a known gene, ventral telencephalic development is greatly impaired in mice and humans (e.g. *Shh* mutants) (Muenke and Beachy, 2000; Hayhurst and McConnell, 2003; Chiang et al., 1996). However, expression of *Nkx2.1*, *Gli1* and *Fgf8*, which depends on SHH signaling (Ohkubo et al., 2002; Pabst et al., 2000; Park et al., 2000), and of *Shh*, *Gsh2* and *Sfrp2* was maintained in the E10.5 *Bmpr1a*;*Bmpr1b* double mutant, although *Fgf8* expression was lost in the rostral midline (Fig. 3E-L and data not shown). These results demonstrate that ventral cell specification is preserved in the *Bmpr1a*;*Bmpr1b* mutant, unlike in other mouse models for HPE (Muenke and Beachy, 2000; Hayhurst and McConnell, 2003).

The dorsal midline initially forms in the *Shh* mutant

Although some dorsal midline features have been reported in the *Shh* mutant (Ohkubo et al., 2002), a more detailed examination of the dorsal midline in these mutants is lacking. To examine the dorsal midline in *Shh* mutants, expression of *Msx1*, *Bmp5*, *Bmp4* and *Foxg1* was analyzed at E10.5. *Msx1*, *Bmp5* and *Bmp4* were still expressed in the dorsal midline, whereas *Foxg1* expression remained excluded

(Fig. 4C-H and data not shown). Moreover, apoptosis was maintained (Fig. 4A,B). This indicates that the initial formation of the dorsal midline occurs, unlike the HPE phenotype observed in the *Bmpr1a*;*Brmp1b* mutant. Moreover, the lack of hemispheric separation dorsally in the *Shh* mutant at E10.5 could at least in part be due to a failure of the expansion of lateral areas, as compared with control embryos (Fig. 4).

The present study demonstrates that mutations in the BMP pathway can lead to HPE with a lack of the dorsal midline. In contrast to the majority of HPE cases described in humans and mice, ventral patterning in *Bmpr1a*;*Bmpr1b* mutants is not affected and thus mimics the MIH subclass of HPE. These results support the existence of at least two developmental mechanisms leading to HPE: one involving loss of ventral medial cell types without a loss of early dorsal midline features, as in mutants in which the SHH pathway is disrupted; and a second involving a lack of dorsal midline development without impaired cell specification in the ventral telencephalon, as in the *Bmpr1a*;*Bmpr1b* mutant (Fig. 4I).

Note added in proof

Recent independent findings confirm the presence of early dorsal midline features in the $Shh^{-/-}$ mutant mice (Rash and Grove, 2007).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/21/3789/DC1

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