Ectopic sonic hedgehog signaling impairs telencephalic dorsal midline development: implication for human holoprosencephaly

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Holoprosencephaly (HPE) is the most common developmental anomaly of the human forebrain, and in its severe form, the cerebral hemispheres fail to completely separate into two distinct halves. Although disruption of ventral forebrain induction is thought to underlie most HPE cases, a subset of HPE patients exhibits preferential dysgenesis of forebrain dorsal midline structures with unknown etiology. In this study, we show that Sonic hedgehog (Shh) lacking cholesterol moiety in one allele (ShhN/−) in mice can elicit ectopic Shh signaling in early telencephalon to induce ventral progenitor marker expression in the cortical region and impair telencephalic dorsal midline development. Prolonged ectopic ShhN signaling impaired Bmp and Wnt signaling from the dorsal patterning center through upregulation of Fgf8, leading to augmented cell proliferation, decreased cell death and impaired roof plate morphogenesis. Accordingly, ShhN/− mutant telencephalic dorsal midline structures, including cortical hem, hippocampus and choroid plexus, either failed to form or were hypoplastic. Strikingly, ShhN/− mutants displayed a spectrum of phenotypic features such as failure of anterior cerebral hemisphere to divide, hydrocephalus and cleft palate which have been observed in a human patient with milder HPE predicted to produce SHHN protein due to a truncation mutation in one SHH allele. We propose that elevated ectopic Shh signaling can impair dorsal telencephalic midline morphogenesis, and lead to non-cleavage of midline structures mimicking human HPE with dorsal midline defects.

INTRODUCTION

The telencephalon, the most anterior portion of the developing neural tube, can be generalized into two subdivisions, the pallium and subpallium. Secreted molecules generated by patterning centers extending from the embryonic telencephalic midline induce and maintain the molecular identities of each subdivision (1–3). Within the subpallium, development of the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE), which largely make up the anlage of the basal ganglia, requires Shh secreted initially from the prechordal plate and subsequently from the ventral telencephalic midline (4–6). Loss of Shh signaling in these ventral patterning centers leads to impaired ganglionic eminence development at the expense of dorsal cell types (6), whereas ectopic Shh signaling in the cortex can induce ventral telencephalic marker gene expressions (7,8). The roof plate is a dorsal patterning center that organizes the development of the dorsal and medium pallium, including the hippocampus and choroid plexus (9). The BMP subfamily of the TGFβ superfamily plays a critical role in patterning the dorsal neural tube (10). Multiple Bmp genes are expressed in the roof plate of the telencephalon (11) and mice deficient in type I Bmp receptor BmprIa or with ablation of Gdf7 (Bmp12)-expressing cells display abnormal choroid plexus development (12,13), consistent with a primary role of Bmp’s in dorsal telencephalic patterning. The Wnt-rich cortical hem lying dorsal to the choroid plexus and ventral to the hippocampus has been proposed to provide patterning cues to both the developing hippocampus and choroid plexus (14). Targeted inactivation of Wnt3a, or the downstream Wnt signaling co-factor Lef-1, severely impairs hippocampal development (15,16), whereas early inactivation of β-catenin, a key mediator of...
canonical Wnt signaling in the telencephalon, leads to disrupted growth of the hippocampus and choroid plexus (17). The current model suggests that Fgf8 expressed in the anterior margin of the telencephalon functions to coordinate the cross-regulation between dorsal and ventral patterning centers. Specifically, ventrally derived Shh promotes and maintains the proper dose of Fgf8 signaling, which in turn maintains a balance of dorsally derived Bmp and Wnt signaling to modulate telencephalic outgrowth and patterning (2,18).

Holoprosencephaly (HPE) is the most common developmental anomaly of the human forebrain, and in its severe form, the cerebral hemispheres fail to separate into two distinct halves (19–21). It is thought that disturbance of ventral forebrain induction underlies the disruption of hemispheric bifurcation. Consistent with this model, human patient studies have identified a battery of mutations involved in the SHH and NODAL signaling pathways, both of which are required for proper ventral forebrain patterning (21,22). However, these studies do not explain the observation that some HPE patients suffer from failure of hemispheric division, yet have relatively normal ventral brain structures, notably those with a condition known as Middle Interhemispheric Holoprosencephaly (MIH), a recognizable variant of HPE with differing clinical prognosis (23–26). Since mutation in human ZIC2, a zinc finger protein homologous to Drosophila odd-paired, has been identified in one MIH patient and knockdown of mouse Zic2 expression leads to deregulation of roof plate cell proliferation and apoptosis (27,28), ZIC2 mutations have been generally associated with the MIH syndrome (23). However, predominantly, ZIC2 mutations are associated with classical HPE (27), and given the heterogeneity of MIH and the lack of Zic2 mutations in many MIH patients, it is unlikely that ZIC2 mutations underlie the etiology of MIH in many patients.

We have previously generated mice expressing non-cholesterol-modified Shh (ShhN) and demonstrated that ShhN can spread far from its site of synthesis to elicit ectopic Shh pathway activation in the limb bud (29). In this study, we show that persistent ectopic ShhN signaling in the dorsal telencephalon severely alters Bmp and Wnt signaling from dorsal patterning centers that are crucial for proper dorsal midline development, resulting in altered behavior of roof plate cells and impaired roof plate invagination. Hence, the cortical hem does not form and development of the choroid plexus and hippocampus is severely disrupted. Strikingly, we found that ShhN/+ mice exhibited a spectrum of phenotypic features such as partial division of cerebral hemispheres, hydrocephalus and cleft palate (CLP) while their external craniofacial features were largely unaffected. All of these characteristics were observed in a human patient with milder HPE and predicted to produce SHHN protein due to a truncation mutation in one SHH allele. Thus, our results suggest a novel mechanism by which ectopic Shh signaling impairs dorsal forebrain development, resulting in non-cleavage of midline structures. In addition, our study may shed light on the molecular pathogenesis of MIH, a variant of HPE that preferentially shows disruption of telencephalic dorsal midline structures.

RESULTS

ShhN/+ telencephalon displays early morphological defects

Starting from E10.5, ShhN/+ embryos exhibited several distinct morphological differences when compared with wild-type, such as enlarged brain ventricles and widely separated telencephalic ventricles (Fig. 1A, A’). H&E-stained coronal sections of E10.5 telencephalon revealed that ShhN/+ dorsal midline failed to invaginate, which is apparent in wild-type. In contrast, development of the ventral MGE, was morphologically comparable at this stage between wild-type and ShhN/+ (Fig. 1B, B’). At E12.5, ShhN/+ telencephalon lacked the characteristic thickened hippocampal neuroepithelium found in the dorsal midline (Fig. 1D, D’). In addition to MGE and LGE at their respective locations, the appearance of a third eminence with LGE character was evident in the mutant (labeled as double-asterisks in Fig. 1D’, see below).

ShhN/+ telencephalic roof plate displays increased proliferation and reduced apoptosis

The invagination and remodeling of the telencephalic dorsal midline is normally accompanied by reduced roof plate cell proliferation and increased cell death (11,28). To determine whether these cellular properties are associated with the failure of dorsal midline down-growth in ShhN/+, we performed cell proliferation and apoptosis analysis of the dorsal

Figure 1. Shhn/+ telencephalon displays early dorsal midline defects (A, A’). Dorsal–frontal views of E10.5 wild-type (A) and Shhn/+ (A’) embryos. Note the widely separated telencephalic ventricles by expanded dorsal midline tissue in Shhn/+ (B, B’). Coronal sections of E10.5 telencephalon show defective down-growth of Shhn/+ dorsal midline (arrowhead in B) compared with wild-type (arrowhead in B’). (C, C’) Lateral views of E12.5 embryos reveal enlarged brain ventricles in Shhn/+ mutants (C), indicative of hydrocephalus. (D, D’) Coronal sections of E12.5 wild-type (D) and Shhn/+ (D’) telencephalon. In addition to persistent defects in dorsal midline invagination, the presence of a third eminence (labeled as double-asterisks) positioned between the second ventral eminence (labeled as asterisks) and cortical region is also evident in Shhn/+ telencephalon (asterisk in D’). (E, E’, G, G, I, I, H) Roof plate cell proliferation analysis. Proliferation index shown in (M) is the percentage of BrdU-positive cell numbers over total cell numbers highlighted by DAPI staining. Dorsal midline cell proliferation index was comparable between wild-type (E) and Shhn/+ (E’) at E9.5. Compared with surrounding cortical region, cell proliferation is reduced in the roof region of E10.0 wild-type (G, data not shown). Shhn/+ roof plate cells show about 1.8-fold higher proliferation index when compared with wild-type control (G, M). Similarly, enhanced roof plate cell proliferation was found in Shhn/+ at E10.5 (I, I’, M). White bracket denotes a central region of the roof plate. (F, F’, H, H’, J, J’, N) Roof plate cell death analysis. Cell death index shown in (N) is the percentage of TUNEL-positive cell numbers over total cell numbers highlighted by DAPI staining. Roof plate cells normally display high apoptotic activity, whereas very little apoptosis is observed in other telencephalic regions (F, H, J and data not shown). However, most Shhn/+ roof plate cells fail to undergo apoptosis and show significantly reduced cell death index when compared with wild-type at E9.5, E10.0 and E10.5 (F, H’, J, J’, N). (K, K, L, L’) Yellow spots observed in (H, J, J’) were auto-fluorescent blood cells. Expression of Lhx5 protein, a roof plate progenitor cell marker, is significantly expanded in E10.0 Shhn/+ dorsal midline (K, O) when compared with wild-type (K, O). Similarly Lhx5 mRNA expression domain is also significantly expanded in the mutant at E13.5 (compare L and L’).
midline neuroepithelium. At E9.5, a stage prior to dorsal midline invagination, we detected prominent apoptotic activity at the most dorsal-medial tissue in wild-type telencephalon. In contrast, apoptosis was essentially eliminated in ShhN+/ dorsal midline (Fig. 1F, F'), although cell proliferation was not significantly different from wild-type in this region (Fig. 1E, E'). At E10, differential proliferation was observed in wild-type dorsal telencephalon with reduced proliferation in the roof plate region compared with adjacent neuroepithelium. However, in ShhN+, cell proliferation in the roof region was comparable to adjacent tissues and significantly higher when compared with wild-type (ShhN+ roof cell proliferation index was approximately 1.8-fold higher) (Fig. 1G, G', M). Similarly, significantly reduced apoptosis was detected in the ShhN+ roof region (more than 7-fold lower) compared with wild-type (Fig. 1H, H', N). The augmented proliferation and reduced apoptosis in the mutant dorsal midline persisted at E10.5 (Fig. 1I, I', J, J', M, N). Lhx5, a member of the LIM homeobox gene family encoding a transcription factor, is normally expressed in the roof plate progenitors, but not in the choroid plexus or cortical hem (30,31). We found that the Lhx5-expressing domain expanded in ShhN+ dorsal telencephalic region as early as E10 with more than 2-fold increase in cell number, which persisted to E13.5, indicating a significant expansion of roof plate progenitor cell population (Fig. 1K, K', L, L', O; Supplementary Material, Fig. S1). We conclude that defective dorsal midline development in ShhN/+ is attributed to increased cell proliferation and reduced cell death in the roof plate region, resulting in expansion of the roof plate progenitor pool. These defects may also lead to failure of tissue invagination.

ShhN+ telencephalon shows expansion of the basal ganglionic eminences

To examine dorsoventral patterning in ShhN+ telencephalon, we performed molecular marker analyses at stages when the telencephalon displays distinct regional patterns. Pax6, a homeobox gene that represses Nkx expression and is itself repressed by Shh signaling (32), is uniformly expressed in the cortical region of the telencephalon. However, at E12.5, Pax6 expression showed non-uniform and a more dispersed pattern in ShhN+ embryos (Fig. 2A, A'). By E13.5, the reduction in Pax6 expression domain became most evident (Fig. 2H, H'). Additional dorsal pallium markers, Ngn2 and Emx2, showed similarly reduced expression domain and intensity, indicative of severe dorsal patterning defects. In contrast to the loss of cortical progenitors, we observed dorsal expansion of markers indicative of ventral telencephalic progenitors in ShhN+. The expression of Nkx2.1 is normally restricted to the MGE domains (Fig. 2B), but in ShhN+/ Nkx2.1-positive cells expanded to the ventral portion of the second ganglionic eminence where LGE normally forms (arrowheads in Fig. 2B). This observation is consistent with the expanded Shh expression domain up to the second eminence (see below). Mash1, which encodes a pro-neural basic helix-loop-helix transcription factor, is expressed in the pan-ventral telencephalic region, including both MGE and LGE (33–35). Previous studies have shown that exogenous Shh is capable of eliciting ectopic LGE markers including Mash1 expression in naïve telencephalic explants (8,36). We found that Mash1 expression expanded dorsally encompassing the ectopic third eminence and scattered sites within the cortical region of ShhN+/ telencephalon (Fig. 2C, C', D, E1, E2). Notably, a significant portion of ectopic Mash1-expressing cells also expressed Gsh2 (Fig. 2F1, F2), an Shh-dependent inducible transcription factor normally expressed in the MGE and LGE (37). This is in contrast to wild-type where co-expressing cells were restricted to the basal ganglionic eminences (Fig. 2F). Since Isl1 is expressed in differentiating neurons within the subventricular zone and mantle zone of the LGE (38), we further performed Isl1 immunostaining (Fig. 2G, G1, G2), showing that ShhN+ cortical region also contained differentiated neurons of LGE identity. Taken together, we conclude that the LGE and, to a lesser extent, MGE are expanded at the expense of the cortical domain in ShhN+/ telencephalon.

The development of hippocampus, choroid plexus and cortical hem is defective in ShhN+ telencephalon

The observation that ShhN+ telencephalon failed to invaginate and lacked morphologically distinguishable dorsal midline suggests that the generation of midline-derived structures is affected. To determine the extent of dorsal midline morphogenesis defects, regional marker analyses were performed at E13.5 when dorsal midline structures and their molecular identities are well established. Previous studies demonstrated that Bmp4 and Bmp7 expressions normally mark the choroid plexus (11,39). We found that expression of these two Bmp genes in ShhN+/ dorsal telencephalic epithelium was barely detectable, while persistent Bmp7 expression was found in the dorsal medial mesenchyme (Fig. 3A, A', B, B'). Also, ShhN+/ exhibited no expression of definitive choroid plexus marker transthyretin (Ttr) (Fig. 3C, C'), suggesting a failure of choroid plexus specification at this stage. While the expression of Wnt2b normally marks the Wnt-rich cortical hem (15,39), its transcript was not detectable in ShhN/+ mutants (Fig. 3D, D'). Wnt5a expression, which encompasses the cortical hem and mesenchyme underneath the hippocampus and distal cortex, was also severely affected in ShhN+/ mutants (Fig. 3E, E'). Similarly, expression of the Wnt pathway component Lef1 diminished considerably in ShhN+/ mutants, in contrast to its normal expression in the choroid plexus and hippocampus (Fig. 3F, F'). Taken together, we conclude that the development of the hippocampal primordium, cortical hem and choroid plexus is significantly compromised in ShhN/+ mutants.

ShhN elicits ectopic signaling throughout ShhN+ telencephalic neuroepithelium

In order to assess Shh signaling in wild-type and ShhN+ telencephalon, we utilized Patched-LacZ mice, in which β-galactosidase activity indicates Shh signaling activity (40). At E10.5, coronal sections through the MGE region revealed that while Shh signaling activity was confined within the MGE of wild-type telencephalon, ShhN could evoke signaling activity dorsal to the morphological boundary of MGE in ShhN+/ mutants (Fig. 4D, D'). However, we could not
detect expanded ShhN protein by immunohistochemistry possibly due to a level that is below detection threshold (Fig. 4B, B'). By E13.5, ectopic ShhN signaling activity was detected throughout the ShhN/+ telencephalic neuroepithelium in contrast with Shh signaling being restricted to the ventral telencephalon in wild-type (Fig. 4E, E'). In addition, we found that the domain of Shh transcript in the ventral telencephalic region and zona limitans intrathalamica (zli) at E10.5 appears to be slightly expanded in ShhN/+ (Fig. 4A, A'). This expansion becomes more evident at E13.5 when...
we found a small number of Shh-expressing cells extending beyond the morphological boundary of the MGE into the ventral portion of the adjacent eminence (Fig. 4C, C ). This consistent with expanded Nkx2.1 expression (Fig. 2B`). Another indicator of Shh pathway activation is the accumulation of the zinc finger transcription factor, Gli3, by inhibiting the formation of its repressor forms (41,42). Therefore, we determined the relative amount of Gli3 repressor (Gli3R) to Gli3 full length (Gli3−190) as another readout of Shh signaling. Consistent with widespread Ptch1 reporter expression, we observed more than 30% reduction in Gli3R/Gli3−190 ratio in E13.5 whole brain extracts of ShhN/+ mutants when compared with wild-type (Fig. 4F). Taken together, with the ectopic ShhN expression domain encompassed the entire anterior telencephalon of ShhN/+ telencephalic neuroepithelium is subject to globally expanded SkhN signaling along the dorsal–ventral axis.

**Ectopic ShhN signaling impairs dorsal telencephalic Bmp and Wnt signaling through upregulation of fgf8**

In order to gain insight into the mechanism by which ectopically enhanced ShhN signaling affects dorsal telencephalic patterning, we examined the expression of multiple essential components of the Fgf, Bmp and Wnt signaling pathways that work in concert to regulate regional identities and growth (2). Fgf proteins such as Fgf8, Fgf17, Fgf18 and Fgf15, from the anterior telencephalon, have been shown to function as anterior patterning molecules (43–46). Among these growth factors, the role of Fgf8 has been extensively studied and it has been shown that Shh signaling is required to maintain robust Fgf8 expression (47,48). We therefore asked whether Fgf8 expression is altered in ShhN/+ telencephalon. As expected, we observed an expansion of Fgf8 expression domain in the anterior forebrain of ShhN/+ mutants as early as E9.5 (Fig. 5A, A`). At E10.5, the Fgf8 expression domain encompassed the entire anterior telencephalic midline tissue in ShhN/+ and even extended into the anterior diencephalon (Fig. 5B, B`).

One critical function of Fgf8 in the anterior neural ridge is to restrict Bmp signaling expression to the dorsal midline (18). Bmp signaling, in turn, is thought to promote localized cell death and the differentiation of a dorsal midline derivative, the choroid plexus, from roof plate progenitors (11,13). Thus, defective Bmp signaling could explain some of the midline defects observed in ShhN/+ telencephalon despite expansion of roof plate progenitors. To test this possibility, we compared Bmp4 expression and Bmp signaling activity in early wild-type and ShhN/+ telencephalon. Bmp4 expression was monitored using a sensitive lacZ reporter driven by the endogenous Bmp4 promoter (49). At E9.5, a stage at which there was no evident morphological dorsal midline defect in ShhN/+ (Fig. 1E, E`, F, F`), a reduction in Bmp4-expressing domain was already apparent in the dorsal telencephalic midline (arrowhead in Fig. 5C, C`). This reduction of Bmp4 expression appeared to correlate with the lack of cell death at this stage (Fig. 1F`). By E10.5, the Bmp4 reduction was more pronounced and extended to the rostral diencephalon (Fig. 5D, D`). Similarly, in situ analysis of Bmp4 transcript also showed barely detectable level in the dorsal–medial telencephalon of ShhN/+ mutants, in contrast to its robust expression in wild-type (Fig. 5E, E`). Concomitantly, the expression of Msx1, which correlates with sites of elevated Bmp signaling (5,11), was also significantly reduced in ShhN/+ mutants (Fig. 5F, F`). Since it has been shown that nuclear accumulation of phosphorylated forms of Smad1, 5, 8 proteins (pSmads) is an indication of active Bmp signaling (50), we also determined pSmads expression by immunostaining. At E10.5, we observed significant accumulation of nuclear pSmads in the neuroepithelium of wild-type hippocampal primordium (white arrows in Fig. 5G) in contrast to its low level in the roof plate region. However, we did not detect high level of pSmads in ShhN/+ dorsal telencephalic neuroepithelium, suggesting a severe loss of Bmp signaling. Notably, ShhN/+ dorsal telencephalic mesenchymal cells also showed significantly reduced pSmads staining (Fig. 5G, G`, data not shown).
together, these findings indicate that deficient Bmp signaling at the dorsal midline likely accounts for the reduced cell death and defective choroid plexus formation in ShhN/þ telencephalon.

The cortical hem, a developmentally transient structure positioned ventral to the hippocampus and dorsal to the telencephalic choroid plexus, is a source of multiple Wnt proteins that have also been implicated in dorsal midline development (15,39). Expanding the Fgf8 expression domain by in utero electroporation suppressed the expression of Wnt2b and Wnt5a, which demarcate Wnt-rich cortical hem, are significantly reduced in ShhN/+. (F, F’) A hippocampal precursor marker Lef1 is similarly reduced in ShhN/+. The absence of in situ hybridization signals is not due to mRNA degradation in ShhN/þ sections as their expressions are clearly present in other areas in the same section (e.g. strong Bmp4 expression were present in the ShhN/þ palatal epithelium, data not shown).

In situ hybridization analyses showed that, while Wnt2b, Wnt3a, Wnt5a delineated the Wnt-rich hem of wild-type dorsal midline at E10.5 and E11.5, their expressions were markedly diminished in ShhN/þ mutants (Fig. 5H, H’, J, J’, K, K’). Notably, comparable expression of Wnt7a that is normally expressed at the lateral and dorsal cortex (39), but not the cortical hem, was observed in wild-type and ShhN/þ (data not shown), indicating a specific loss of Wnt genes in the dorsal midline region. The severe loss of Wnt signaling activity in ShhN/þ dorsal telencephalon was confirmed using TOPGAL reporter mice, which express β-galactosidase under the control of multimerized LEF/TCF consensus binding sites (Fig. 5I, I’, L, L’). The absence of apparent Wnt signaling activity may account for the severely defective hippocampal development in ShhN/þ mutants.

Taken together, we conclude that ectopic ShhN signaling in the dorsal telencephalic region specifically impaired Bmp and Wnt signaling from dorsal patterning centers, likely via upregulation of Fgf8 expression.
**ShhN/+/** mice display a spectrum of phenotypic features mimicking the human HPE

Next, we characterized the **ShhN/+** forebrain and craniofacial defects at perinatal stages. Surprisingly, **ShhN/+** mice showed relatively normal external craniofacial morphological defects, although a bulging cranium, which is generally associated with hydrocephalus (51), was clearly evident in more than 10 embryos examined (Fig. 6A, A'). Gross analysis of brains indicated an enlarged forebrain (and midbrain) and lack of olfactory bulbs in mutants (Fig. 6B, B'). H&E staining of coronal sections revealed that the enlarged ventral eminences in **ShhN/+** were well separated (Fig. 6C, C'), whereas the dorsal midline of **ShhN/+** telencephalon was severely hypoplastic and lacked conjunction of corpus callosum and septum that normally separated anterior cerebral hemispheres.

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**Figure 4.** Widespread activation of Shh signaling in **ShhN/+** telencephalon (**A, A', B, B', C, C'**). **Shh** transcript and **Shh** protein expression in wild-type (**A–C**) and **ShhN/+** (**A’–C’**). Whole-mount in situ staining of Shh reveals a generally similar pattern between wild-type and **ShhN/+**; however, a modest expansion of **Shh** expression in MGE and zona limitans intrathalamica is observed in **ShhN/+** when compared with wild-type (arrows in **A** and **A'**). Consistently, **Shh**-expressing domain extends into the ventral portion of the second ventral eminence in **ShhN/+** (**C’**), while **Shh** transcript signal is confined within the MGE domain in wild-type (**C**). (**B and B’**) **Shh** protein signal is detected mainly within the MGE region in both wild-type and **ShhN/+**. At E10.5, **Shh** signaling activity, as evidenced by **Ptch1-LacZ** expression, is confined within the MGE region in wild-type (**D**), but is extended in **ShhN/+** (**D’**). At E13.5, **Ptch1-LacZ** expression is strongly expressed in the MGE and only weakly in the LGE (**E**), but in **ShhN/+**, the weak LGE expression extends to the entire cortical region including dorsal midline (**E’**). (**F**) Immunoblotting of whole brain protein extracts from E13.5 wild-type and **ShhN/+** followed by incubation with Gli3-specific antibody recognizing Gli3 full-length (Gli3190) and repressor forms (Gli3R); Gli3−/− brain extract is used as negative control. Histogram shows relative Gli3R/Gli3190 ratio.
Dorsal interhemispheric cyst-like structure was occasionally observed in ShhN+/ telencephalon (arrow in Fig. 6C). In addition, the dorsal telencephalon of ShhN+/ mutants lacked middle anterior commissure and characteristic choroid plexuses and hippocampal structures seen in wild-types (Fig. 6E). Nevertheless, a small region of simplified epithelium with Ttr expression in the dorsal midline of the anterior forebrain in the newborn mutant was observed (Fig. 6F). Since Ttr expression was absent in E13.5 ShhhN+/ mutants, this observation suggests either delayed/defective choroidal fate specification or a diencephalic origin of the choroid plexus. Regardless, the presence of low but persistent level of Bmp signaling in the roof region (shown as residual Bmp4 and Bmp7 expressions at E10.5 in Fig. 5E, E' and at E13.5 in Fig. 3A, A', B, B') likely contributed to the reduced choroidal fate in the mutant. Similarly, the development of the hippocampus was also significantly compromised. The expression of Prox1 marks the characteristic V-shape dentate gyrus of the hippocampus; we observed few and scattered Prox1+ cells at the distal cortical neuroepithelium in ShhN+/ cells (Fig. 6G), consistent with significantly reduced Lef1 expression in the hippocampal precursors (Fig. 3F).

In contrast to a monoventricle in the anterior telencephalon, the ventricles in the posterior telencephalon are clearly separated by an enlarged thalamus (Fig. 6D).
thalamus was confirmed with Prox1 staining which is also expressed in the ventral–lateral thalamic region (Supplementary Material, Fig. S2). Although external craniofacial morphology appeared relatively unaffected, ShhN/+ mutants developed CLP. The palatal shelves failed to fuse in ShhN/+ at E15.5 (Fig. 6H, H′), leading to a cleft secondary palate in newborn pups (Fig. 6I, I′, J, J′). Strikingly, some of the phenotypic features including partial separation of the forebrain ventricles, hydrocephalus and CLP are similar to those found in a human patient with milder HPE carrying a truncation mutation (CAG → TAG, Q209X) in one SHH allele that was predicted to produce SHHN protein due to failure to catalyze cholesterol transfer; the other SHH allele was normal, thus indicating that the genotype of this patient is SHHN/+ (52).

**DISCUSSION**

HPE is the most common developmental anomaly of the human forebrain (21). Considerable amount of effort has been exerted to understand the pathogenesis of HPE, and several animal models that are generally associated with loss of Shh signaling have been established, showing severely disrupted ventral forebrain as a phenotypic hallmark. Our finding that ShhN/+ mice exhibit many aspects of forebrain defects observed in a human patient with milder HPE carrying a truncation mutation (CAG → TAG, Q209X) in one SHH allele that was predicted to produce SHHN protein due to failure to catalyze cholesterol transfer; the other SHH allele was normal, thus indicating that the genotype of this patient is SHHN/+ (52).

**Ectopic Shh signaling impairs dorsal organizing center function**

Early telencephalic regionalization is mediated by diffusible morphogenetic ligands and graded transcription factor expressions (1–3). It has long been established that Shh signaling is essential for ventral telencephalic patterning (4). Recent studies indicate that this requirement appears to be mediated through Fgf signaling, as deletion of Fgf8 or multiple Fgf receptors in the telencephalon leads to loss of ventral cell fates analogous to that of Shh−/− mutants (18,53). Thus, the expansion of Fgf8 expression to the dorsal midline in ShhN/+ mutants is consistent with long-range activation of Shh signaling. Because Fgf signaling normally promotes cell survival by restricting Bmp4 expression in the dorsal midline (11,54), the decrease in cell death in ShhN/+ is in agreement with significantly reduced Bmp signaling. In addition, impaired choroid plexus formation can also be attributed to defective Bmp signaling as Bmpr1a gain- and loss-of-function studies reveal that Bmpr1a is required for promoting choroidal fate by antagonizing cortical specification (13). The hippocampus is another midline-derived structure that is defective in ShhN/+ telencephalon. Studies of Wnt3a or Lef1 mutants have demonstrated that high level of canonical Wnt signaling close to the hem is vital for normal hippocampus development (15,16,55). The ability of ectopic Fgf8 to suppress Wnt2b, Wnt3a and Wnt5a expressions at the cortical hem (14) is also consistent with hippocampus hypoplasia observed in ShhN/+ telencephalon. While altered dorsal patterning centers in ShhN/+ can be logically connected to the expanded Fgf8 expression via ectopic Shh signaling, considering the prominent role of Shh in promoting neuroepithelial cell proliferation (56,57), it remains possible that Shh signaling can also directly contribute to increase cell proliferation and reduced cell death observed at the dorsal midline, resulting in defective roof plate progenitor cell differentiation, and subsequently dampening roof signaling activities (Fig. 7).

**ShhN/+ as a novel mouse model of human HPE**

HPE is characterized by a spectrum of brain malformations in which the bilateral cerebral hemispheres fail to separate along the midline. One of the principle underlying mechanisms of HPE pathogenesis is the disruption of basal forebrain patterning associated with impaired Shh signaling during early embryogenesis (22). Indeed, molecular genetic analyses have
Figure 6. ShhN+/+ mice mimic human HPE phenotypes. (A, A') Wild-type and ShhN+/+, both showing relatively normal external craniofacial morphologies except bulging forehead characteristic of hydrocephalus in mutant (white bracket in A'). (B, B') Gross analysis of brains indicating lack of olfactory bulbs in mutant. (C, C’, D, D’) Coronal sections of wild-type and ShhN+/+ telencephalons shown in (B) and (B’). In the rostral telencephalon, the corpus callosum (CC) and septum (s) that normally connect with each other to divide the telencephalic lobes into two lateral ventricles are either missing or defective in the mutant resulting in a single ventricle (compare C and C’). The mutant dorsal midline is severely hypoplastic with simplified epithelium and cyst-like structure (arrow). Note the presence of normally separated ventral eminences in ShhN+/+. (C’). Caudally, ShhN+/+ telencephalon lacks well-formed U-shaped hippocampus and the ventricles are separated by enlarged thalamus (D’). (E, E’) Brain cross-sections further show that the mutant lacks dorsal midline fissure, telencephalic choroid plexuses (CP) and anterior commissure (AC), while the separation of the thalamus (TH) is not affected. The simplified epithelium at the dorsal midline in ShhN+/+ exhibits Ttr expression (F’), similar to the normal telencephalic choroid plexus in wild-type (F). (G) and (G’) were zoomed-in view of boxed-area shown in (D) and (D’). While Prox1+ cells mark the characteristic V-shape dentate gyrus of the wild-type hippocampus (G), few and scattered Prox1+ cells are found at the most distal ShhN+/+ cortical neuroepithelium (G’). (H, H’, I, I’, J, J’) ShhN+/+ mutants exhibit cleft secondary palate (CLP). E15.5 coronal section through the palatal region shows failure of palatal fusion in ShhN+/+ (compare H to H’). A direct view of the secondary palate region with mandible removed shows clef palate in ShhN+/+ (compare I to I’). Skeletal staining further shows the presence of widely separated palatal shelves (I’), allowing direct view of the presphenoid bone (psp) underneath the otherwise fused palatine shelves. Note that the maxillary shelves are not fully mineralized in the mutant (asterisks) (J).

identified numerous SHH mutations in HPE patients, including a Q209X truncation mutation in one SHH allele that is predicted to produce SHHN protein (52). The Q209X patient was previously diagnosed with semi-lobar HPE, a milder form of HPE in which the brain is divided with two distinct hemispheres only in the caudal but not rostral region of the telencephalon (19–21). This patient also exhibited hydrocephalus and CLP; all of these phenotypic features are reproduced in the ShhN+/+ mutant embryos. However, unlike ‘classical’ HPE, Shh signaling is ectopically activated and the basal forebrain is well separated in ShhN+/+ mutants. Molecular analysis further revealed that the lack of regional cerebral hemispheric separation in ShhN+/+ mutants is largely attributed to the disruption of dorsal midline signaling center. In this context, it is also interesting to note that dorsal midline defects have been reported in some patients with semi-lobar HPE (58). These observations suggest a novel mechanism by which ectopic Shh signaling could be associated with milder HPE.

Recent brain images and genetic studies in human have implicated an association of impaired embryonic roof plate development with MIH, a peculiar subtype of HPE, displaying non-cleavage of the cerebral hemisphere in the posterior frontal and parietal region. Notably, ShhN+/+ mutants share several features observed in MIH patients, including: (i) largely normal craniofacial structures; (ii) frequent agenesis of the dorsal corpus callosum while ventral forebrain nuclei are generally separated; (iii) midline third ventricle is separated by hypothalamus at the posterior frontal lobe; (iv) dysgenesis of dorsal midline-derived structures including absence or reduced choroid plexus and hypoplastic hippocampus (23,25,26). We are only beginning to understand the molecular pathogenesis of MIH. The mechanism underlying the failure of hemispheric bifurcation in MIH appears to be different from that of classical HPE (22,23). In MIH, the defective functions of genetic factors impair roof plate development, whereas in classical HPE the floor plate is mostly affected. To date, the only gene implicated in MIH pathogenesis has been linked to mutations in ZIC2, which encodes a zinc finger protein that is homologous to Drosophila odd-paired (27). Reduced Zic2 expression in mice results in
failure of roof plate invagination and fusion of cerebral hemispheres with relatively normal ganglionic eminence development (28). Indeed, it has been hypothesized that haplo-insufficiency in ZIC2 underlies MIH in humans, and the absence of craniofacial malformation, which is normally observed in mutants with loss of Shh signaling (23,27), argued that this anomaly may arise by an SHH-independent pathway. However, predominant ZIC2 mutations are

Figure 7. Proposed mechanism of dorsal midline dysgenesis and HPE mediated by ectopic Shh signaling. (A) Schematic of predicted protein products from mouse ShhN and human Q209X alleles. The mature Hh is synthesized as a precursor protein that undergoes a series of post-translational modifications, leading to covalent attachment of a cholesterol moiety at its carboxyl-terminus (Glycine 199) and palmitic acid at its N-terminal (72). In ShhN as well as SHH-Q209X, cholesterol modification does not occur due to deletion of the C-terminal processing domain of the precursor protein. (B) During normal development, Bmp and Wnt signals generated from the roof plate (RP) and its neighboring neuroepithelium are thought to pattern the dorsal midline structures such as choroid plexus, cortical hem and hippocampus (1). Shh secreted from the ventral telencephalic midline is required to maintain Fgf8 expression in the anterio-ventral midline (anterior neural ridge, ANR). In turn Fgf8 restricts Bmp and Wnt signals to the dorsal midline (2). In ShhN/+ mutants, widespread activation of Shh signaling activity leads to expansion of Fgf8 expression to the dorsal midline, which in turn downregulates Bmp and Wnt signaling. In addition, long-range ShhN signaling may also directly promote cell proliferation and reduce cell death at the dorsal midline.
associated with classical HPE (27,59). The fact that Zic2 ablation in mice does not faithfully reproduce MIH phenotypes (28), indicates that the molecular pathogenesis of MIH could be heterogeneous. Bmp and Wnt ligands derived from the roof region are thought to organize dorsal structures. Roof plate cell ablation leads to loss of dorsal midline-derived structures as well as associated Bmp signaling and thus, it has been proposed that Bmps expressed in the roof plate are candidate genes for MIH (60). However, fate-mapping studies indicate that cells of the choroid plexus and Wnt-rich cortical hem are descendents of roof plate progenitors (12). Therefore, defective dorsal midline development could arise secondary to the deletion of roof plate cell population rather than a consequence of reduced Bmp signaling. Furthermore, studies of Gdf7 (Bmp12) and telencephalon-specific conditional Bmp4 and Bmpr1a mutants implicate a role of Bmp signaling primarily in choroid plexus specification and differentiation (12,13,61). Therefore, it remains an open question as to whether and how impaired Bmp signaling affects the intricate signaling network involved in dorsal telencephalic patterning that result in severe defects in multiple midline structures such as the choroid plexus, cortical hem and hippocampus observed in roof plate-depleted mutants. Our study demonstrates that reduced Bmp signaling coupled with impaired Wnt signaling appear to synergistically affect dorsal midline development. Thus, reduction in hem-enriched Wnt or its pathway activity should be considered as an integral part of MIH pathogenesis.

An intriguingly consistent phenotype of ShhN/+ mice that parallels the phenotype observed in some human HPE patients, including MIH, is the CLP. Studies on Fgf10−/−, Fgfr2b−/− and Shh−/− conditional mutant mice, all of which exhibit CLP, demonstrate that palatal epithelial Shh expression is a downstream target of Fgf10 expressed in the palatal mesenchyme (62). Thus, loss of Shh signaling is generally associated with induction of CLP. However, ShhN/+ mice exhibit Shh gain-of-function signaling in many developmental contexts such as in the telencephalon, spinal cord and limb bud (29) (this study and data not shown). Thus, the notion that Shh gain-of-function could also lead to CLP is particularly interesting. Recent investigation into Insig1 and Insig2 knockout mice also postulates that excess Shh signaling may cause CLP (63). Furthermore, hydrocephalus and CLP have been reported in several patients with a duplication of distal chromosome 7q, which contains the SHH gene locus, raising the possibility that over-expression of SHH may contribute to these craniofacial defects (64). Consistent with this notion, approximately 5% of Gorlin syndrome patients with PTCH1 mutations, in which Shh pathway activity is elevated, develop CLP (65). Further studies are required to determine the mechanism by which dysregulation of Shh signaling in ShhN/+ leads to CLP formation. Similarly, how dysregulation of Shh signaling leads to absence of olfactory bulb in ShhN/+ mutants remains to be determined. However, olfactory bulb aplasia has been observed in Gli3 loss-of-function mutants (66–68), suggesting a causative link between loss of Gli3 repressor activity and failure of olfactory bulb development. Interestingly, olfactory bulb hypoplasia has also been reported in MIH patients (23).

MATERIALS AND METHODS

Animals

Generation of embryos expressing ShhN was carried out by mating Shhfox/−/+ animals with Sox2-Cre deleter strain as previously described (29). Sox2-Cre (69), TOPGAL (70) and Ptc1lac−/+ mice (40) were obtained from the Jackson Laboratory. Bmp4lac−/+ mice were kindly provided by Brigid Hogan. The perinatal death of Shhfox/fox, Shhn/+ and Shhn/− mice precludes analysis of Shhn/− phenotype. Three to six embryos from wild-type and Shhn/+ were used for each morphological/molecular analysis shown in each figure.

Immunohistochemistry and western analysis

All immunohistochemistry analyses were performed on tissue sections collected from OCT- or paraffin-embedded embryos as previously described (29). The primary antibodies were mouse anti-Pax6, (DSHB, 1:1), mouse anti-Nkx2.1, (Neomarkers, 1:100), mouse anti-Mash1 (gift of Jane Johnson, 1:100), goat anti-Shh (Santa Cruz Biotechnology, 1:1000), rabbit anti-phospho-Smad1/5/8 (gift of Tom Jessell and Ed Lauffer, 1:1000), rabbit anti-Gsh2 (Kenneth Campbell, 1:1000), rabbit anti-Prox1 (Axxora, 1:500), mouse anti-Lhx1/5 (DSHB, 1:10). Since Lhx1 is not expressed in the telencephalon at early stages (71), the staining pattern shown in Figure 1 revealed Lhx5 expression pattern. For western analysis, protein lysate samples, 200 μg each, collected from E13.5 whole brains, were resourced on 6% SDS–polyacrylamide gels. Gli3−190 and Gli3R species were detected using a Gli3 N-terminal specific antibody as described (42).

Analysis of cell proliferation and apoptosis

BrdU (5-BromodeoxyUridine) in vivo labeling and TUNEL analysis were performed as previously described (71). For statistical analysis, three embryos from either wild-type or Shhn/+ were used to perform BrdU and TUNEL analyses at each developmental stage. At least five stained sections were randomly selected and counted for each genotype to generate the proliferation and cell death indices shown in Figure 1. To assess differences among groups, statistical analyses were performed using a one-way analysis of variance (ANOVA) with Microsoft Excel (Microsoft Corporation) and significance accepted at P < 0.05. Results are presented as mean ± SEM.

X-gal staining and transcript detection

X-gal staining for β-galactosidase was performed according to standard protocol. Whole-mount and section in situ hybridizations were performed as described (71). The following cDNAs were used as templates for synthesizing digoxigenin-labeled riboprobes: Shh, Lhx5 (H. Westphal, NIH), Bmp4 (S. Lee, Johns Hopkins), Bmp7 (E. Robertson, University of Oxford), Msx1 (R. Mason, University of North Carolina), Wnt3a (Image 426103), Wnt3a (A. McMahon, Harvard University), Wnt2b (L. Zakin, Pasteur Institute), Lef1 (R. Grossehedl, University of California San Francisco), Fgf8 (G. Martin, University of California San Francisco), Foxg1 (E. Lai, Cornell University), Emx2 (A. Simeone,
European School of Molecular Medicine, Tr (EST#1078224, ATCC).

Skeletal preparation
Cartilage and bones were stained with Alcian blue and Alizarin red as described (29).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. The authors declare that they have no financial interests.

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