Mouse models of holoprosencephaly

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Purpose of review

Holoprosencephaly (HPE) is the most common anomaly of forebrain development in humans. The pathogenesis of HPE results in a failure of the brain hemispheres to separate during early development. Here we review experimental models of HPE in which some of the genes known to cause HPE in humans have been disrupted in the mouse.

Recent findings

To date, mutations that cause HPE have been identified in seven genes. Three of these genes encode members of the Sonic hedgehog (SHH) signaling pathway, which regulates the development of ventral structures throughout the neuraxis. Two other HPE mutations affect signaling by Nodal ligands, which also play important roles in neural patterning. The roles of the two other known HPE genes are not yet clear. Analysis of genetically altered mice has revealed that mutations in other members of the SHH and Nodal signaling pathways also result in HPE phenotypes.

Summary

Studies of HPE in the mouse have provided a framework for understanding key developmental events in human brain development and may provide new candidate genes for human HPE. Despite this progress, fundamental mysteries remain about how molecules that pattern ventral brain regions ultimately disrupt the formation of the cerebral hemispheres in dorsal regions.

Keywords

brain patterning, forebrain, holoprosencephaly, Nodal, Sonic hedgehog

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Abbreviations

BMP bone morphogenetic protein
CNS central nervous system
SHH Sonic hedgehog

1350-7540

Introduction

Holoprosencephaly is a devastating defect of brain development that results from mutations in genes required for the normal formation of the forebrain. During brain development, the rostral neural tube, which begins as a single fluid-filled chamber, bifurcates into the two cerebral hemispheres. Holoprosencephaly disrupts this bifurcation and results in a fusion of the two hemispheres. Studies of genetic defects in human patients have identified at least seven distinct genes that are mutated in holoprosencephaly; most of these are involved in known genetic pathways that govern the development of ventral brain structures. These two pathways involve the signaling molecules Sonic hedgehog (SHH) and Nodal, both of which can induce ventral cell fates in a mirror-symmetrical manner on either side of the midline. Although mutations in genes required for ventral midline development go far in explaining some of the defects in holoprosencephaly, fundamental mysteries remain about why and how failures in dorsal midline formation also occur in this disorder. Here we examine the molecular underpinnings of midline development as revealed by genetic mutations in the mouse. These studies collectively suggest that the disruption of pathways for ventral brain development results in anomalies consistent with human holoprosencephaly, including a failure in dorsal midline formation and the fusion of the two hemispheres.

Neural and genetic anomalies in holoprosencephaly

In the most severe cases of holoprosencephaly (alobar holoprosencephaly), hemispheric bifurcation fails to occur and the rostral brain forms a single holosphere, at times including the formation of a single cyclopic eye [1]. Within the telencephalon, both dorsal and ventral structures are fused and lack an obvious midline. In a less severe form of holoprosencephaly (semilobar holoprosencephaly), the anterior hemispheres fail to cleave but the posterior hemispheres are separated. In the most mild form (lobar holoprosencephaly), reasonably good hemispheric separation occurs but there is hypoplasia of the anterior cortical structures and variable cleavage of the basal ganglia and thalamic nuclei [1].

Holoprosencephaly has both environmental and genetic causes. For example, ingestion of the teratogen cyclopamine causes holoprosencephaly by disrupting signaling through the SHH pathway [2], which patterns the ventral midline throughout the central nervous system (CNS) [3]. Genetic studies have identified at least 12 loci

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associated with familial or sporadic forms of holoprosencephaly, and seven of these loci have been definitively identified as specific genes [4]. Three of the known genes encode members of the SHH signaling pathway, which regulates ventral development in both the forebrain and the spinal cord [3,5–9]. Human mutations have been uncovered in *Shh*, which encodes a secreted signaling ligand localized at early stages to ventral domains in the developing neural tube; *Patched* (*Ptc*) [10], which encodes a receptor for SHH, and *DispatchedA* (*DispA*) [11•], which is required for the release and extracellular accumulation of Hedgehog (HH) proteins. Two additional holoprosencephaly mutations implicate the Nodal signaling pathway, which plays an important role in neural patterning. The transcriptional co-repressor tumor growth interacting factor (*TGIF*) [12] represses the activity of SMAD transcription factors, which are activated by Nodal signaling, and *Tdgf1/Cripto* encodes a membrane-associated protein that serves as a co-receptor for Nodal signaling. The two other known holoprosencephaly genes do not play an obvious role in either of the above pathways. *Six3* encodes a homeodomain transcription factor expressed in the ventral forebrain [13], and *Zic2* encodes a zinc-finger transcription factor homologous to *odd-paired* in *Drosophila* [14].

Sonic hedgehog and ventral brain development

Since the initial molecular identification of SHH in 1993, the signaling pathway by which SHH influences cell fate decisions has been well studied (Fig. 1). In the absence of ligand, the transmembrane protein Smoothened (SMO) is inhibited by the SHH receptor, Patched (PTC). Binding of SHH to PTC relieves this inhibition and initiates a rather complex series of intracellular events that converts GLI proteins into transcriptional activators [15]. SHH plays a well-characterized role in inducing ventral neuronal cell types along the entire rostrocaudal extent of the neuraxis, including the forebrain [8,16–19]. Below we review the phenotype of *Shh* mutant mice in the context of the developmental abnormalities seen in human holoprosencephaly. We then compare the *Shh* mutant phenotype with those found in mutations of other members of the SHH signaling pathway.

Mice bearing null mutations in *Shh* die shortly after birth, but before this time they develop defects resembling those in human holoprosencephaly cases involving *Shh* mutations. The loss of *Shh* function in mouse embryos is associated with a loss of ventral structures throughout the neural axis, including the ventral forebrain, as assessed by *Nkx2.1* expression [20,21••] (Fig. 2). As in the mutant spinal cord, dorsal patterns of gene expression in the *Shh*-deficient forebrain expand to encompass regions normally fated to become ventral: for example, the entire brain vesicle expresses the dorsal telencephalic markers *Emx1* [3] and *Otx1* [21••], which are normally excluded from ventral domains, and *Pax6* and *Otx2* expand into the frontoventral regions, which normally show only low levels of expression [21••]. Surprisingly, some markers normally expressed in lateral regions of the ventral telencephalon persist in *Shh* mutant animals. Both *Dlx2* and *Gsh2* are expressed in the mutant brain, but their patterns suggest that lateral structures have been displaced ventrally to encompass the midline [22••] (Fig. 2).

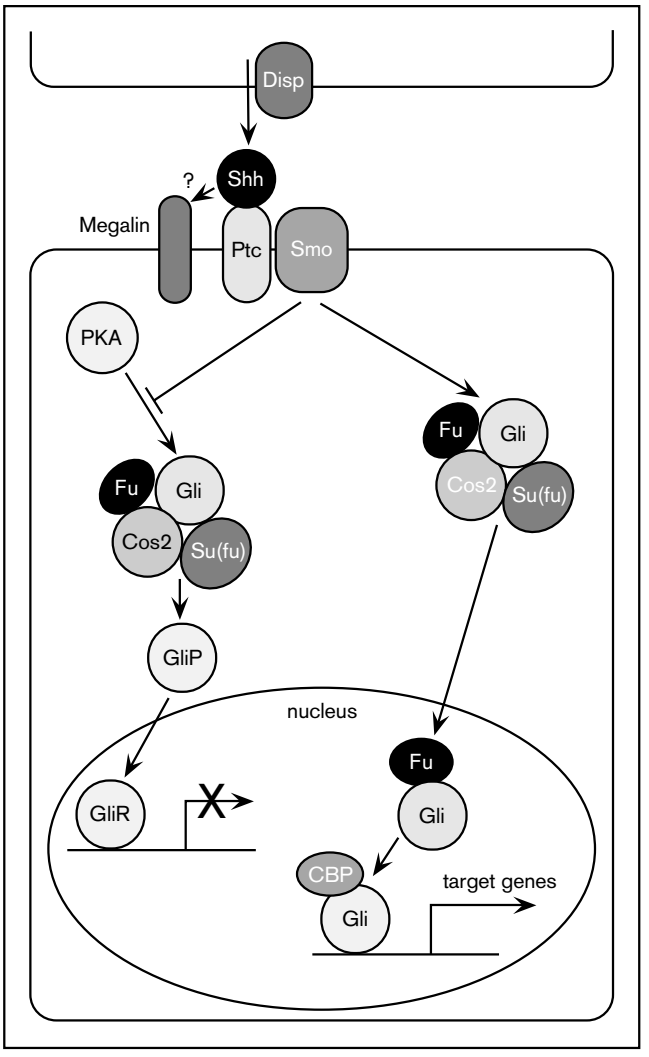
Mice lacking *Shh* also develop cyclopia with an overlying proboscis, characteristic of severe holoprosencephaly [3]. The optic vesicle of *Shh* mutants is a single and continuous structure from its earliest appearance [3], suggesting that SHH plays a critical role in dividing a single eye field into two. During early development, *Pax6* expression normally delineates a continuous region in the ventral diencephalon that is subsequently split into two regions, each of which gives rise to an optic vesicle. Removal of the prechordal plate (which expresses SHH) results in the maintenance of a single domain of *Pax* expression, whereas transplantation of this tissue is sufficient to suppress *Pax6* [23]. In *Shh* mutant embryos, *Pax6* is expressed in a continuous domain extending across the ventral midline of the neural tube [3], suggesting a failure in the normal bifurcation of the ventral eye field.

Sonic hedgehog and dorsal brain development

In the forebrains of *Shh* mutant mice, the formation of the dorsal midline is grossly disrupted [3]. During normal development, cells at the dorsal midline invaginate and divide the brain into left and right hemispheres. This invagination is accompanied by the formation of midline structures, including the choroid plexus and hippocampus. Midline structures fail to form in *Shh* mutant mice, and *Emx1*, which is normally excluded from the dorsal midline region, is expressed uniformly across the brain's dorsal surface [3].

How a ventrally localized signaling molecule, SHH, affects the development of cells located at a distance within the dorsal midline presents an intriguing mystery. Within the spinal cord, SHH acts in a concentration-dependent manner to induce distinct fates over relatively short distances [24]. *Shh* mutant mice show no obvious defects in the dorsal regions of the spinal cord, consistent with a local, ventral requirement for this signaling molecule. In contrast, *Shh* is required for normal dorsal midline development in the forebrain. It is possible that in the anterior neural tube, SHH plays a variety of inductive and regulatory roles that affect the establishment or maintenance of other local organizing

Figure 1. A simplified summary of SHH signaling



The release and extracellular accumulation of SHH is regulated by DispatchedA (Disp). In the absence of ligand, the activity of SMO is inhibited by PTC. Binding of SHH to PTC relieves this inhibition and leads to the conversion of GLI proteins into transcriptional activators. In the forebrain, GLI1 acts exclusively as an activator, whereas GLI2 can show a switch from repression to activation; however, telencephalic development is relatively normal in mice lacking both *Gli1* and *Gli2* [18]. GLI3 serves primarily as a repressor of SHH function, and forebrain development is largely rescued in *Shh; Gli3* double knockout mice. SHH can also bind to Megalin, but the role of this binding has yet to be elucidated fully.
This schematic is based on Fig. 4 from Litingtung and Chiang [19], reproduced with permission.

centers. For example, the loss of *Shh* leads to a failure of anterior neural cells to express the potent morphogen FGF8 [21••], which may itself help to pattern anterior midline structures [25].

Surprisingly little is known about the molecular mechanisms that pattern the dorsal midline and partition the forebrain into two separate cerebral hemispheres. Cells in the dorsal midline region express bone morphogenetic protein (BMP) and Wnt ligands, which are thought to organize dorsal structures [25]. In particular, BMP ligands induce the expression of midline markers, suppress proliferation, and increase levels of cell death, all of which should play important roles in creating distinct hemispheres [26]. Fishell and colleagues (personal communication) have evidence that *Bmp* and *Wnt* gene expression is disrupted in *Shh*-deficient mice, consistent with the absence of a dorsal midline. However, *Msx1* (a downstream target of BMPs) shows an expanded expression domain in *Shh* mutants, and the expression of *Bmp2* and *Bmp7* also show a moderate expansion [21••]. Indeed, the process of hemispheric bifurcation appears to be more complicated than previously imagined; in contrast to *Shh* mutants, the cerebral hemispheres separate normally in mice bearing conditional mutations in *Bmp4* [27] or a major BMP receptor *Bmpr1a* [28••], as well as in *Wnt3a* mutant mice [29]. The mechanisms by which *Shh* regulates hemispheric partitioning thus remain foggy at best.

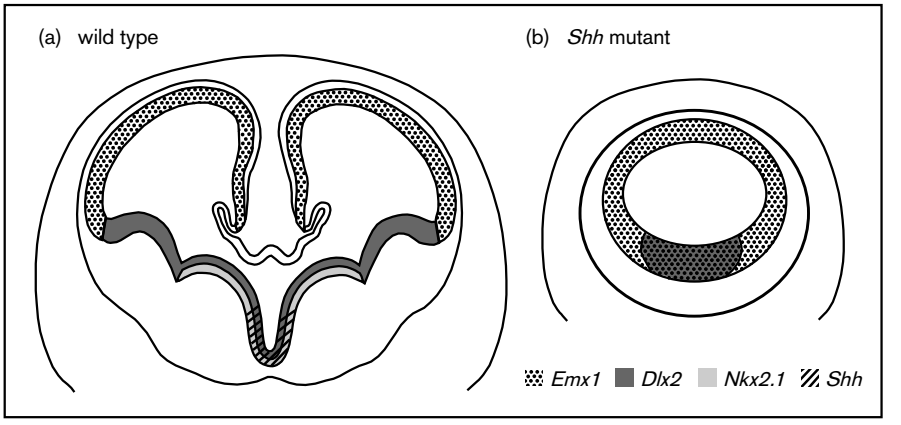
The most direct link between SHH signaling and dorsal patterning is found in the antagonistic relationship between SHH and the dorsally expressed transcriptional regulator GLI3. GLI proteins are zinc-finger transcription factors that serve either as repressors or activators of Hedgehog target genes [30]. Although three *Gli* genes are expressed in the developing brain, telencephalic midline formation does occur in mice bearing homozygous mutations in both *Gli1* and *Gli2* [18]. These data suggest that the primary mediator of SHH signaling in the telencephalon must be GLI3, a potent repressor of SHH signaling. However, although much of the dorsal telencephalon is ventralized in *Gli3* mutant mice and the midline expression of several *Bmp* genes is lost, two cerebral hemispheres do still form [18,22••,31]. Interestingly, dorsal–ventral patterning is largely rescued in mice that bear null mutations in both *Shh* and *Gli3* [22••], suggesting that the main role of SHH in neural patterning may be to suppress the ventral expression of *Gli3* and thus enable ventral phenotypes to develop.

The Sonic hedgehog signaling pathway and holoprosencephaly

Given that disruption of *Shh* results in severe holoprosencephaly in the mouse, it is not surprising that other components of the SHH signaling pathway are also required for normal forebrain development. However, the peculiar mechanisms that underlie SHH signaling predict that not all loss-of-function mutations in members of the SHH pathway should result in holoprosencephaly. For example, the SHH receptor PTC normally functions to repress signaling through SMO; repression is relieved when SHH binds to PTC (Fig. 1). The loss of PTC function results in a

Figure 2. A summary of gene expression patterns in (a) wild-type mice and (b) *Shh* mutant mice

The expression of dorsal markers such as *Emx1* is expanded both dorsally and ventrally in *Shh* mutants, which show no signs of midline development. Other dorsal markers such as *Otx1*, *Otx2*, and *Pax6* also show a ventral expansion (not shown). Whereas the expression of some ventral markers (such as *Nkx2.1*) is lost in *Shh* mutants, others such as *Gsh2* (not shown) and *DLx2* are retained.



constitutive activation of SHH/SMO signaling [31]; consistent with this, *Ptc1* null mutant mice show expanded ventral tissues [31]. Conversely, mutations that enhance PTC activity should decrease SHH activity and result in a holoprosencephaly phenotype. Indeed, missense mutations in *PTC* that cause holoprosencephaly in humans [10] are predicted to alter extracellular domains involved in SHH binding or intracellular regions that convert the PTC protein to an inactive state when SHH is present. Mice bearing such mutations in the forebrain have not yet been generated. The loss of SMO function, in contrast, should resemble the loss of SHH. Indeed, *Smo* knockout mice exhibit cyclopia and holoprosencephaly [32], but the brains of these mice have not been examined thoroughly, nor have human holoprosencephaly mutations been identified in this gene. The mutant mice die by embryonic day 9.5, consistent with a role for SMO in transmitting signals through multiple members of the Hedgehog family and not just SHH itself.

Several human holoprosencephaly mutations have been identified recently in the *DispA* gene [11•]. DISPA regulates SHH signaling by increasing the amount of the cleaved, active form of SHH protein available to neighboring cells [11•]. *DispA* mutant mice die by E9.5, but mutant embryos display midline defects and a fused eye field similar to defects seen in *Shh* and *Smo* knockout mice at early embryonic stages [11•].

A newly identified member of the SHH signaling pathway implicated in holoprosencephaly in mice is Megalin, a member of the low-density lipoprotein receptor family that binds SHH, and may serve either as a co-receptor for SHH or a regulator of SHH endocytosis and intercellular movement [33]. Mouse embryos deficient in *megalin* exhibit fused cerebral hemispheres [34], as expected if megalin is required for SHH signaling. To date no human holoprosencephaly mutations in *megalin* have been identified.

The Nodal signaling pathway and holoprosencephaly

Nodal signals play a role in many developmental processes, including mesoderm formation, specifying the anterior–posterior and left–right axes, and patterning the nervous system [35,36••,37,38]. Although no human holoprosencephaly mutations in *nodal* have yet been identified, mutations in both mouse and zebrafish suggest a role for Nodal signaling in the formation of ventral brain structures. *Nodal* mutant mice show severe holoprosencephaly [35]. This phenotype probably reflects an essential role for Nodal in establishing a domain of *Shh* expression in the rostral regions of the early embryo, because *Nodal* mutant mice fail to express *Shh* in areas anterior to the hindbrain. In addition, the prechordal plate, where *Shh* is normally expressed, is missing [35]. Similarly, *Shh* expression in the zebrafish forebrain depends on Nodal activity [36••], and mutations in the Nodal gene *cyclops* result in a fused telencephalic vesicle and cyclopia, presumably mediated by defects in the SHH pathway. Mice that are heterozygous for mutations in *nodal* and also heterozygous or homozygous for mutations in other genes (*Smad2* or *ActRIIA*) likely to act in the Nodal pathway also exhibit variable cyclopic phenotypes [38,39].

The activity of Nodal signals can be regulated extracellularly by EGF-CFC co-factors, which include proteins encoded by the zebrafish *one-eyed pinhead* (*oep*) gene and the murine and human genes *Tdgf1* (*Cripto*) and *Cfc1* (*Cryptic*). EGF–CFC proteins enhance the binding of Nodal proteins to type I and type II activin-like receptors, and thereby facilitate Nodal signaling. *Tdgf1/Cripto* is a recently characterized human holoprosencephaly gene [40]. In the mouse, mutations in *Cripto*

lead to embryonic lethality by day 11.5 [41]; unfortunately, head development was not examined. However, mutation of the *Cripto* homolog *oep* in zebrafish leads to holoprosencephaly and cyclopia, consistent with a role for Nodal signaling in the early ventralization of the head region.

In each of the mutations discussed above, deficits in Nodal signaling appear to cause holoprosencephaly through a failure to induce *Shh* expression in the head. It is therefore surprising to find that in humans, missense mutations in *TGIF* cause holoprosencephaly [12]. TGIF is a transcription factor that inhibits signaling through Nodal/transforming growth factor beta pathways by blocking the action of SMAD proteins [42,43]. A loss of TGIF function would therefore be expected to enhance Nodal signaling; however, the phenotypes that result from mutations resemble those seen with a loss of Nodal function. No studies of brain development in *TGIF* mutant mice have yet been reported. However, we speculate that the requirement for *TGIF* in humans may not reflect its regulation of Nodal signaling, but rather might reflect a distinct role in inhibiting signals mediated by retinoic acid, which itself can inhibit *Shh* expression.

Prenatal exposure to retinoic acid in mice and humans causes telencephalic malformations within the holoprosencephaly spectrum [44], and human *TGIF* mutations can resemble these phenotypes [45]. TGIF can bind a retinoid receptor response element and thereby inhibit retinoic acid signaling [46]. Mutations in TGIF could thus potentially lead to a loss of repressor function, consequently mimicking the effect of excessive retinoic acid exposure. Because high doses of retinoic acid can downregulate *Shh* expression [47], it is conceivable that *Shh* expression is disrupted after the mutation of *TGIF*. However, this hypothesis remains to be tested in knockout mice.

Six3 and Zic2: outliers in holoprosencephaly

Six3 is closely related to *Drosophila optix*, a member of the *Six/sine oculis* gene family [48]. In the mouse, early *Six3* expression demarcates the anterior border of the developing neural plate; later expression in the nervous system overlaps with that of *Pax6*, and is particularly prominent in the ventral eye field and in the optic recess, stalk, and vesicles [13]. No loss-of-function mutations for *Six3* have yet been reported in mice; however, experiments in which levels of *Six3* expression are reduced result in a failure of forebrain and eye development [49]. How *Six3* regulates these developmental events is not known. One possibility is that Six3 enables cells to become competent to SHH signals [50]; thus, the absence of Six3 may preclude SHH from acting ventrally.

Zic2 encodes a zinc finger transcription factor, which is a homolog of the Drosophila pair-rule gene *odd-paired* [51,52]. *Zic2* is unique among holoprosencephaly genes in that its expression is localized to the dorsal and ventral midline regions of the telencephalon, rather than predominantly in the ventral regions as for other identified holoprosencephaly genes. This raises the possibility that *Zic2* may serve as a key link between ventral signals in the telencephalon, such as SHH and Nodal, which somehow act at a distance to control the formation of dorsal structures.

Zic2 expression has been diminished in a partial loss-of-function or 'knockdown' (kd) mouse line [52]. These mice show a strong holoprosencephaly phenotype in which the cerebral hemispheres are fused, and structures derived from the dorsal midline of the forebrain are missing or reduced [52]. In contrast to wild-type embryos, in which the dorsal midline contains few mitotic cells and many dying cells, processes that contribute to midline invagination, there are more mitotic and fewer apoptotic cells in $Zic2^{kd/kd}$ mice [52]. In addition, the expression of *Wnt3a*, a roof plate marker, is reduced compared with wild-type animals [52]. However, unlike other mouse models for holoprosencephaly, *Zic2* mutant mice show normal ventral neural tube formation, as revealed by *Pax6* and *Shh* expression [52].

Remaining issues

Although mutational analysis in both human holoprosencephaly and in genetically engineered mice has focused on the SHH pathway, many questions remain. First, a known genetic locus has been identified for fewer than 20% of human cases of holoprosencephaly; one hopes that studies of mouse holoprosencephaly mutations will provide human geneticists with candidate genes for the majority of patients whose genetic lesions remain unknown. However, even for those patients with known mutations, huge gaps exist in our understanding of the mechanisms that underlie forebrain development. How defects in ventral brain patterning result in severe malformations of the dorsal brain regions remains a fundamental mystery that studies in the mouse may help to resolve. Furthermore, little is known about the developmental pathways followed by neurons and glial cells in the holoprosencephalic brain. Studies on gene expression in mice suggest that neurons in the dorsal regions adopt fates characteristic of neocortical regions, and that some aspects of ventral cell specification do occur. However, nothing is known about the neurotransmitter phenotypes, axonal connections, or functional properties of neurons in mice with holoprosencephaly. Further work on these models may thus not only identify new candidate genes, but may also provide an understanding of alterations in brain circuitry

that will suggest new treatments or interventions in human holoprosencephaly.

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