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Region-specific requirement for cholesterol modification of sonic hedgehog in patterning the telencephalon and spinal cord

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Sonic hedgehog (Shh) secreted from the axial signaling centers of the notochord and prechordal plate functions as a morphogen in dorsoventral patterning of the neural tube. Active Shh is uniquely cholesterol-modified and the hydrophobic nature of cholesterol suggests that it might regulate Shh spreading in the neural tube. Here, we examined the capacity of Shh lacking the cholesterol moiety (ShhN) to pattern different cell types in the telencephalon and spinal cord. In mice expressing ShhN, we detected low-level ShhN in the prechordal plate and notochord, consistent with the notion that ShhN can rapidly spread from its site of synthesis. Surprisingly, we found that low-level ShhN can elicit the generation of a full spectrum of ventral cell types in the spinal cord, whereas ventral neuronal specification and ganglionic eminence development in the $Shh^{N/-}$ telencephalon were severely impaired, suggesting that telencephalic patterning is more sensitive to alterations in local Shh concentration and spreading. In agreement, we observed induction of Shh pathway activity and expression of ventral markers at ectopic sites in the dorsal telencephalon indicative of long-range ShhN activity. Our findings indicate an essential role for the cholesterol moiety in restricting Shh dilution and deregulated spread for patterning the telencephalon. We propose that the differential effect of ShhN in patterning the spinal cord versus telencephalon may be attributed to regional differences in the maintenance of *Shh* expression in the ventral neuroepithelium and differences in dorsal tissue responsiveness to deregulated Shh spreading behavior.

KEY WORDS: Sonic hedgehog, Lipid modification, Telencephalon, Cholesterol, Patterning, Mouse

INTRODUCTION

The appearance of distinct neuronal cell types at defined positions in the ventral neural tube is dependent on inductive signaling mediated by sonic hedgehog (Shh) (Agarwala et al., 2001; Dale et al., 1997; Fuccillo et al., 2006; Jessell, 2000; Sur and Rubenstein, 2005). In mice lacking Shh, most ventral neuronal cell types are lost along the rostral-caudal axis of the neural tube, whereas dorsal structures are expanded ventrally (Chiang et al., 1996; Rallu et al., 2002). It is thought that neurons generated in progressively more ventral regions in the spinal cord require correspondingly higher levels of Shh for their induction (Briscoe and Ericson, 2001; Jessell, 2000). In the basal ganglion of the telencephalon, the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE), which give rise to the globus pallidus and striatum, respectively, are generated sequentially in response to Hedgehog (Hh) signaling (Ericson et al., 1995; Kohtz et al., 1998; Shimamura and Rubenstein, 1997).

Although Shh can elicit diverse cell fates in the neural tube, much less is known about the factors that regulate Shh range and activity gradient. Active Shh is dually lipid-modified (ShhNp), with palmitic acid at its N-terminus and cholesterol at its C-terminus (Mann and Beachy, 2004). The hydrophobic nature of lipids indicates that there might be a dedicated release mechanism for Shh. Disp1, a multi-pass transmembrane protein, appears to fulfil this mechanistic requirement (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002), as its function is necessary only for lipidated Shh secretion (Li et al., 2006; Tian et al., 2005). Lipid-modified Shh has also been shown to form a large multimeric complex in vitro (Chen et al., 2004a; Zeng et al., 2001). It has been suggested that the lipid moieties are embedded in the core of these complexes, analogous to micelles, thus facilitating Shh interaction with – and its spreading across – the extracellular matrix (Zeng et al., 2001). The biological function of lipid moieties in regulating Shh function has been investigated recently. Misexpression studies in rats indicated that palmitoylation is required for Shh to induce ectopic ventral cell fates in the dorsal telencephalon (Kohtz et al., 2001), suggesting that palmitoylation is required for Hh activity. Indeed, mice deficient in an enzyme that catalyzes palmitoylation, or carrying a variant of Shh that is incapable of being palmitoylated, showed developmental defects similar to those of loss of Shh function (Chen et al., 2004a).

In contrast to palmitoylation, lack of cholesterol modification (ShhN) does not appear to significantly affect the intrinsic potency of Shh. It has been shown that ShhN isolated from tissue culture cells can induce ectopic ventral cell types in neural explants with comparable or higher efficiency than ShhNp at similar concentration (Feng et al., 2004). Instead, the cholesterol moiety appears to affect the range of Hh spreading in the target field. Recent mosaic studies with *Drosophila* wing imaginal discs showed that Hh lacking cholesterol (Hh-N) has an extended range of spreading and for inducing ectopic low-threshold Hh target genes, although the expression of high-threshold target genes near the source was reduced (Callejo et al., 2006; Dawber et al., 2005; Gallet et al., 2006). However, another report indicated that wing disc clones that expressed Hh-N were unable to elicit low-threshold Hh target genes at a distance (Gallet et al., 2006). These differences might be associated with different levels of Shh that are expressed at the sites of synthesis (Guerrero and Chiang, 2006).

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Previous efforts to understand the role of cholesterol modification concluded that the cholesterol moiety is required for long-range spreading and signaling in the mouse limb buds (Lewis et al., 2001). Accordingly, it has been proposed that cholesterol modification is required for the formation of Shh multimers involved in long-range signaling and proper vertebrate forebrain patterning (Feng et al., 2004). However, a more recent study suggested that ShhN retained some paracrine activity because it was capable of inducing several ventral neuronal cell types in the spinal cord (Tian et al., 2005). Given this controversy, the role of the cholesterol moiety in Shh function has been re-assessed recently in the limb bud (Li et al., 2006). That study found that cholesterol modification is required to restrict the spread of Shh across the anteroposterior axis of the limb bud, similar to Hh-N function in *Drosophila*. The extended range of ShhN movement across the anteroposterior axis of the limb bud also resulted in an appreciable reduction of local ShhN levels, suggesting that the cholesterol moiety not only regulates the range, but also the shape of the Shh morphogen gradient.

In this study, we utilized mice exclusively expressing the ShhN form of Shh to address the function of the cholesterol moiety in patterning the spinal cord and telencephalon. We found that dorsoventral patterning of the telencephalon in embryos lacking cholesterol modification of Shh was severely compromised, as indicated by the lack of MGE progenitors and the appearance of widespread ectopic LGE progenitors in the dorsal telencephalon. By contrast, patterning of the ventral spinal cord was largely unaffected and no ectopic expression of ventral cell fates at dorsal sites was observed in $Shh^{N/-}$ embryos. These findings suggest that patterning of the telencephalon is highly sensitive to alterations in Shh spreading behavior.

MATERIALS AND METHODS

Mice

To generate conditional $Shh^{flox/+}$ mice, loxP sequences were inserted flanking the *Shh* processing domain, which is situated in exon 3 (Li et al., 2006). The resulting mice containing the Shh^{flox} (unrecombined) allele were maintained as heterozygotes, because homozygotes die soon after birth (Li et al., 2006). The Shh^{flox} allele has reduced capacity to generate lipid-modified Shh (ShhNp) owing to interference of the loxP site with the Shh autocatalytic processing reaction (Li et al., 2006) (data not shown), thus it should be considered hypomorphic. Embryos uniquely expressing Shh without cholesterol modification were generated by mating $Shh^{flox/+}$ animals with the $Shh^{+/-}$*;Sox2-Cre* deleter strain as previously described (Li et al., 2006). $Shh^{N/+}$*;Disp1*$^{-/-}$ embryos were obtained from crosses between $Shh^{flox/+}$*;Disp1*$^{+/-}$ and *Sox2-Cre;Disp1*$^{+/-}$ mice. *Disp1*$^{+/-}$ mice have been described (Ma et al., 2002). *Sox2-Cre* mice (Hayashi et al., 2002) and *Ptch1-lacZ* mice (Goodrich et al., 1997) were obtained from the Jackson Laboratory. All control embryos were either $Shh^{+/+}$ *or* $Shh^{+/-}$; we have not observed any differences in the development or Shh staining patterns of these embryos (see Fig. S3 in the supplementary material).

Immunohistochemistry and western blot analysis

Immunohistochemistry analyses were performed on tissue sections collected from OCT- or paraffin-embedded embryos as previously described (Li et al., 2006). The primary antibodies used were rabbit anti-Foxa2 (Li et al., 2007) (1:100), mouse anti-Nkx2.2 (DSHB, 1:2), mouse anti-Isl1/2 (DSHB, 1:10), sheep anti-Chx10 (Exalpha Biologicals, 1:50), mouse anti-En1 (DSHB, 1:5), mouse anti-Pax7 (DSHB, 1:2), mouse anti-Pax6, (DSHB, 1:1), mouse anti-Nkx2.1 (Neomarkers, 1:100), rabbit anti-Nkx6.1 (1:3000, gift of Christer Betsholz, Karolinska Institute, Stockholm, Sweden), rabbit anti-Olig2 (1:3000, gift of Hirohide Takebayashi, Kyoto University, Yoshida, Japan), mouse anti-Mash1 (1:100, gift of Jane Johnson, University of Texas Southwestern Medical Center, Dallas, TX, USA), rabbit anti-Gsh2 (1:500, gift of Kenneth Campbell, University of Cincinnati, Cincinnati, OH, USA), mouse anti-Tuj1 (Sigma, 1:400), goat anti-Shh (Santa Cruz Biotechnology, 1:1000). For western analysis, protein lysate samples (200 μg each), collected from E15.5 whole brains, were resolved on 6% SDS-polyacrylamide gels. Gli3-190 and Gli3R species were detected using a Gli3 N-terminal-specific antibody as previously described (Litingtung et al., 2002).

Analysis of cell proliferation and apoptosis

5-Bromodeoxyuridine (BrdU) in vivo labeling and TUNEL analysis were performed as previously described (Litingtung et al., 1998). Proliferative and apoptotic cell countings were carried out with at least five sections from three different embryos for each genotype. To quantify BrdU- or TUNEL-positive cells, coronal sections of the telencephalic neuroepithelium were subdivided into three regions: ventral (v), dorsal (d) and dorsal midline (dm). v is defined as the region of neuroepithelial cells ventral to the dorsal edge of the morphologically distinct LGE (see Fig. 2, arrowhead). The dm region consists of 200 neuroepithelial cells situated at the center of the dorsal midline and its immediate neighbors. The d region is located between regions v and dm. The percentage of BrdU-positive cells was determined by counting these in segments of 200 cells within the designated region of the telencephalon and dividing by the total number of cells in the segment. All sections were counterstained with the nuclear dye DAPI to highlight the total number of cells. At least five segments in each region were counted to generate data for statistical comparison.

X-Gal staining and transcript detection

X-Gal staining was performed according to standard protocols (Hogan et al., 1994). Whole-mount and section in situ hybridizations were performed as described (Li et al., 2004). The following cDNAs were used as templates for synthesizing digoxygenin-labeled riboprobes: *Shh* (Li et al., 2006), *Ptch1* (Goodrich et al., 1996), *Gli3* (Li et al., 2004) and *Nkx2.1* (Lazzaro et al., 1991).

Statistics

To assess differences among groups, statistical analyses were performed using a one-way analysis of variance (ANOVA) with Excel (Microsoft) and significance accepted at $P<0.05$. Results are presented as mean±s.e.m.

RESULTS

Absence of morphologically distinct medial and lateral ganglionic eminences in the $Shh^{N/-}$ telencephalon

To investigate the effect of ShhN on forebrain development, sections of control and $Shh^{N/-}$ mutant embryos in the telencephalic region were stained with Hematoxylin and Eosin and analyzed for morphological differences. At E9.5, we did not detect any apparent external morphological abnormalities in the brains of $Shh^{N/-}$ and control embryos (data not shown). At E10.5, however, $Shh^{N/-}$ mutant forebrain exhibited several distinct malformations, including enlarged brain ventricles and narrowed frontal nasal processes (Fig. 1, compare C,D with the control in A,B). At E10.5, the MGE is evident as a neuroepithelial eminence protruding into the lateral ventricles of control embryo telencephalon; the MGE is also a proliferative zone that later gives rise to the globus pallidus (Fig. 1B,H). Strikingly, the $Shh^{N/-}$ telencephalon failed to form MGE, but instead developed a flattened neuroepithelium (Fig. 1D,J). The LGE, a second morphologically distinct ventral structure, develops between E11.5 and E12.5 in control embryos and is situated between the cortex and MGE (Fig. 1H). No LGE bulge was observed in the E12.5 $Shh^{N/-}$ telencephalon (Fig. 1J). The loss of morphologically distinct ganglionic eminences is indicative of impaired ventral patterning in the $Shh^{N/-}$ telencephalon. Notably, the $Shh^{-/-}$ telencephalon also did not display any ventral structures (Fig. 1E,F,K,L). In addition to ventral defects, dorsal telencephalic midline structures such as the hippocampus primodium (HP) and choroid plexus (CP) were also malformed in the $Shh^{N/-}$ mutant (Fig. 1, compare B,H with D,J).

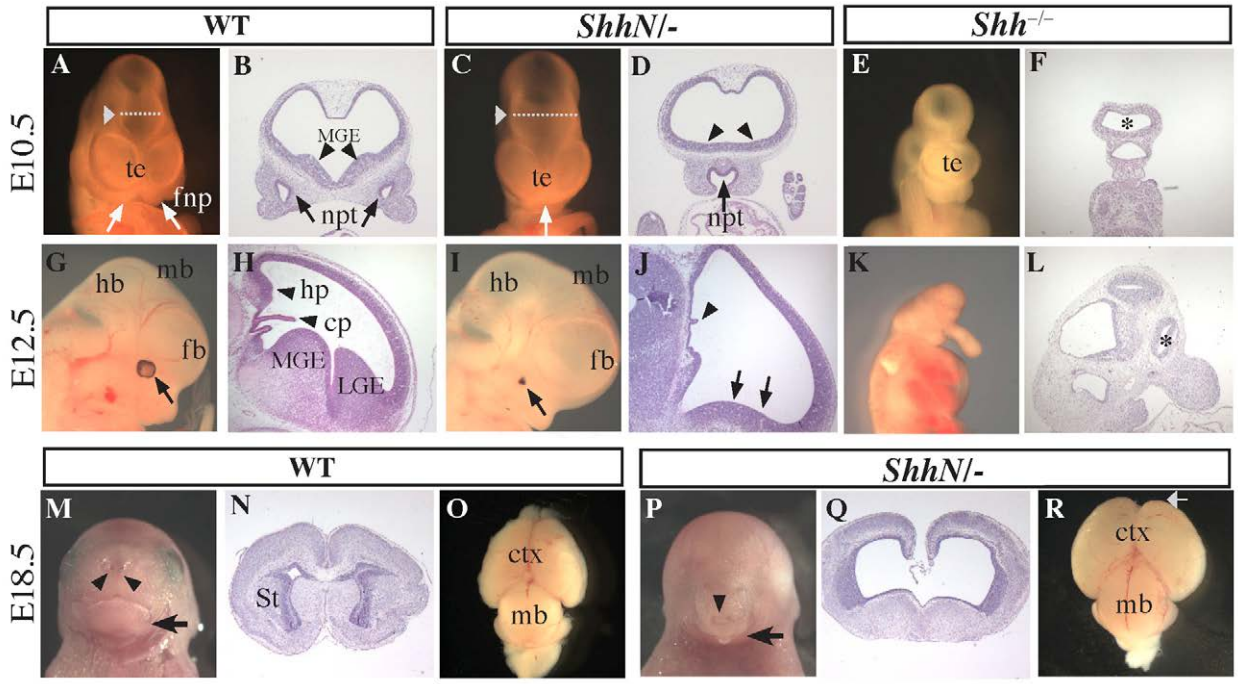

Fig. 1. *Shh*$^{N/-}$ mutant forebrain displays HPE-like phenotypes. (**A-F**) Frontal view of control (A,B), *Shh*$^{N/-}$ (C,D) and *Shh*$^{-/-}$ (E,F) mouse forebrains at E10.5. B,D,F are Hematoxylin and Eosin-stained coronal sections. *Shh*$^{N/-}$ telencephalon shows enlarged forebrain ventricles (A,C, arrowheads) and lacks MGE bulge (B,D, arrowheads). Additionally, the frontal nasal process (fnp) is closely positioned in the *Shh*$^{N/-}$ mutant (compare arrows in A and C) and displays a single nasal pit (npt, arrows in B,D). Note that the ventricles of the *Shh*$^{N/-}$ telencephalon are distinctly different from those of *Shh*$^{-/-}$, which are collapsed into a small ventricle (asterisk in F,L). (**G-L**) Sagittal view of control (G,H), *Shh*$^{N/-}$ (I,J) and *Shh*$^{-/-}$ (K,L) embryos at E12.5. H,J,L are Hematoxylin and Eosin-stained sections. Enlarged forebrain (fb), midbrain (mb) and hindbrain (hb) ventricles in *Shh*$^{N/-}$ are clearly evident (G,I). In addition to MGE, other morphologically distinct structures such as LGE and the choroid plexus (cp) are absent or defective in the *Shh*$^{N/-}$ telencephalon (arrows and arrowheads in H,J). (**M-R**) E18.5 control and *Shh*$^{N/-}$ embryos showing frontal (M,P), coronal (N,Q) and dorsal (O,R) views. Note hypoplasia of the lower jaw (P, arrow), single nostril (P, arrowhead), absence of olfactory bulb (R, arrow) and enlarged cerebral cortex (ctx) and midbrain (R) in *Shh*$^{N/-}$ mutants. MGE, medial ganglionic eminence; HP, hippocampus primordium; LGE, lateral ganglionic eminence; te, telencephalon; St, striatum.

Interestingly, the widely separated telencephalic ventricles in *Shh*$^{N/-}$ clearly differed from that of the *Shh*$^{-/-}$ embryo, in which the telencephalic ventricles were fused (Fig. 1, compare C with E), suggestive of an altered Shh signaling gradient in *Shh*$^{N/-}$ forebrain rather than a loss of signaling activity. Analysis of E18.5 *Shh*$^{N/-}$ embryos revealed several characteristics of the holoprosencephaly (HPE)-like phenotype including defective eye formation, hypoplastic mid-facial structures, a single centrally located nostril and the absence of olfactory bulb in the forebrain (Fig. 1M-R).

Increased apoptosis, proliferation and reduced differentiation in the *Shh*$^{N/-}$ telencephalon

To determine whether altered cell apoptosis and/or proliferation might contribute to the defective development of ganglionic eminences in *Shh*$^{N/-}$ mutants, we performed the TUNEL cell death and BrdU proliferation assays. Analysis of E11.5 embryos indicated that cell death in the Mash1-positive (Ascl1 – Mouse Genome Informatics) LGE domain and the cortex of the neuroepithelial layer and its surrounding mesenchyme was increased in the *Shh*$^{N/-}$ telencephalon compared with the control (Fig. 2A,B,D,E). Intriguingly, the percentage of BrdU-positive cells in the proliferative zones of the LGE (Fig. 2H,K; 42±3.7 versus 58±1.8, P<0.001) and the cortex (Fig. 2I,L; 50±1.8 versus 66±4.7, P<0.001) were moderately increased in the *Shh*$^{N/-}$ telencephalon, raising the possibility that this increase in cell proliferation may be attributed to exposure of neural progenitors to low-level, long-range Shh signaling.

We next determined whether the capacity for neural progenitor cells to proceed with differentiation is altered in *Shh*$^{N/-}$ mutant telencephalon. We stained *Shh*$^{N/-}$ and control sections with an antibody against neuronal class III tubulin (Tuj1; Tubb3 – Mouse Genome Informatics) that marks all differentiated neurons. At E11.5, Tuj1-positive cells normally consisted of a thick layer of differentiated neurons within the subventricular zone and mantle zone of MGE and LGE (Fig. 2O). However, in *Shh*$^{N/-}$ mutants, we observed significant reduction in the population of differentiating neurons within the presumptive LGE domain (Fig. 2T). There was no obvious difference in differentiation in the cortex and dorsal midline region between control and *Shh*$^{N/-}$ embryos at this stage (Fig. 2P,Q,U,V). Similarly, Isl1, a ventral-specific differentiation marker, also showed significant reduction in the differentiation capacity of *Shh*$^{N/-}$ ventral neural epithelium (Fig. 2R,S,W,X). Collectively, these observations suggest that increased apoptosis and reduced neuronal differentiation contribute to the defective development of LGE in *Shh*$^{N/-}$ mutants.

In addition to basal ganglions, dorsal midline derivatives such as the secretory epithelial cells of the choroid plexus and hippocampus primodium were also affected in *Shh*$^{N/-}$ (Fig. 1H,J). The rapid invagination and remodeling of the dorsal midline neuroepithelium is normally accompanied by reduced proliferation and increased cell death (Furuta et al., 1997; Nagai et al., 2000). Although cell death still occurred in the *Shh*$^{N/-}$ dorsal roof (Fig. 2C,F; 41±5.7 versus 44±6.9, P=0.48), we observed a near 100% increase in cell

proliferation in the roof and medial wall of the dorsal midline (Fig. 2J,M, arrowheads; 34.4±1 versus 72±3, $P<0.001$), suggesting that ShhN might have long-range effects on dorsal midline development (see below). Taken together, these results indicate that the cholesterol moiety of Shh is required for normal development of both dorsal and ventral telencephalic structures.

Absence of MGE progenitors in the *Shh*$^{N/-}$ telencephalon

To gain further insight into the effect of ShhN on telencephalic patterning, we performed molecular characterization by examining markers that are regulated by Shh signaling. *Nkx2.1* (*Titf1* – Mouse Genome Informatics) is a homeobox gene whose expression in the mouse is first detectable in the basal telencephalic neuroepithelium at the three-somite stage, and subsequently localizes to a morphologically distinct region, the MGE (Shimamura et al., 1995). At E12.5, Nkx2.1 was uniformly expressed in the MGE of the control embryo, whereas no Nkx2.1 expression could be detected in the *Shh*$^{N/-}$ mutant telencephalon, indicating severe loss of MGE identity (Fig. 3A,B). *Mash1*, which encodes a neural basic helix-loop-helix transcription factor, is expressed in the pan-ventral telencephalic region, including the MGE and LGE (Casarosa et al., 1999; Horton et al., 1999; Lo et al., 1991). Mash1 expression in these regions is regulated by Hh signaling and marks a transient population of committed neural precursors before differentiation (Fuccillo et al., 2004). Although Mash1 expression was present in both MGE and LGE of control embryos, Mash1-positive cells encompassed a relatively large ventral portion of the *Shh*$^{N/-}$ mutant telencephalon (Fig. 3E,F), indicating that LGE progenitor cells were present despite the lack of development of a morphologically distinct LGE structure. Pax6 expression is normally confined to the dorsal cortical region abutting the LGE boundary (Fig. 3I). We observed prominent expansion of Pax6 expression in the ventral neuroepithelium, with reduced levels in the presumptive LGE domain (Fig. 3J, arrowheads). The expanded Pax6 expression domain was observed in the *Shh*$^{-/-}$ mutant telencephalon, which often collapsed at this stage with no expression of ventral markers such as Mash1 and Nkx2.1 (Fig. 3D,H,L). Although these results point to a requirement for the cholesterol moiety of Shh in patterning the ventral telencephalon, the possibility remains that the expression level of the *ShhN* allele is not as high as the wild-type allele when it is expressed alone in *Shh*$^{N/-}$ embryos, thus generating haploinsufficiency. Because it is not possible to generate *ShhN* homozygous mutants (see Materials and methods), we compared the patterning capacity of the *Shh*^{flox} allele, in which the *ShhN* allele is generated upon Cre-mediated deletion of the sequence encoding the C-terminal autoprocessing domain. With the exception of the cholesterol moiety, the proteins generated from these two alleles are the same. However, the *Shh*^{flox} allele is hypomorphic in that it generates a lower level of bioactive lipid-modified ShhNp owing to loxP sequence interference with autoprocessing of the Shh precursor (Li et al., 2006) (data not shown). Thus, *Shh*$^{flox/-}$ embryos died soon after birth with multiple defects, including cleft secondary palate and distorted eye formation (see Fig. S1A-E in the supplementary material), characteristics of impaired Shh signaling (Rice et al., 2004; Zhang and Yang, 2001). However, dorsoventral patterning of

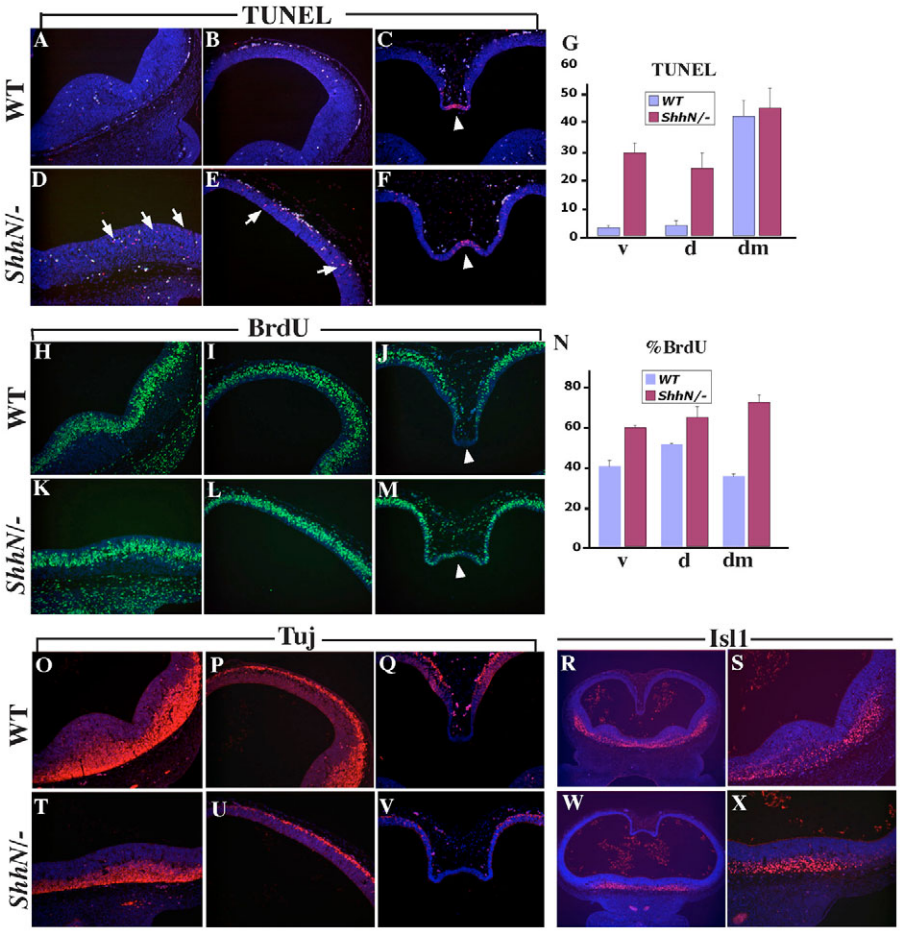

Fig. 2. Cell proliferation, apoptosis and differentiation in the *Shh*$^{N/-}$ telencephalon. (**A-F**) TUNEL analysis of sections from E11.5 wild-type (A-C) and *Shh*$^{N/-}$ (D-F) mouse embryos at the level of ventral (A,D), dorsal (B,E) and dorsal midline (C,F). (**G**) Bar chart showing that there are more apoptotic cells in ventral and dorsal regions of the *Shh*$^{N/-}$ telencephalon (D,E, arrows). The number of apoptotic cells in the dorsal midline is comparable between *Shh*$^{N/-}$ and control (C,F, arrowheads).
(**H-M**) Coronal sections of E11.5 control (H-J) and *Shh*$^{N/-}$ (K-M) telencephalon labeled with BrdU antibody. (**N**) Bar chart showing that there is a statistically significant increase in the percentage of BrdU-positive cells in ventral (v), dorsal (d) and dorsal midline (dm) of *Shh*$^{N/-}$ telencephalic neuroepithelium as compared with controls.
(**O-X**) Immunofluorescence of E11.5 control (O-S) and *Shh*$^{N/-}$ (T-X) telencephalic sections labeled with Tuj (O-Q,T-V) and Isl1 (R,S,W,X) antibodies to highlight neuronal differentiation. Note that differentiation in the ventral region is visibly reduced in *Shh*$^{N/-}$ neuroepithelium (compare O,R,S with T,W,X). v, Mash1^{+} ventral region; d, Pax6^{+} dorsal region; dm, dorsal midline.

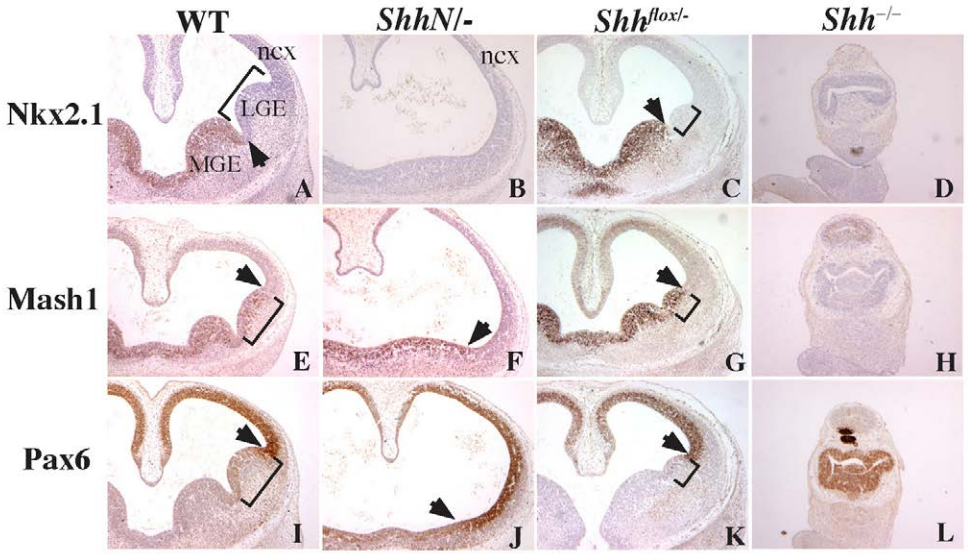

Fig. 3. Absence of MGE progenitors in the mouse *Shh$^{N/-}$* telencephalon. (**A-D**) Nkx2.1 expression in control (A), *Shh$^{N/-}$* (B), *Shh$^{flox/-}$* (C) and *Shh$^{-/-}$* (D) telencephalons. Nkx2.1 is an MGE marker (arrow in A points to the boundary of the Nkx2.1 expression domain) and its expression is maintained in *Shh$^{flox/-}$*, but lost in *Shh$^{N/-}$* and *Shh$^{-/-}$*, telencephalons. (**E-H**) Mash1 expression in control (E), *Shh$^{N/-}$* (F), *Shh$^{flox/-}$* (G) and *Shh$^{-/-}$* (H) telencephalons. Mash1 expression delineates the ventral region of the control telencephalon, encompassing the MGE and LGE. Mash1 expression was reduced in the LGE domain of *Shh$^{flox/-}$* as compared with the control. Mash1 expression was maintained in the ventral *Shh$^{N/-}$* telencephalon (arrows in E and F point to the boundary of the Mash1 expression domain). No Mash1-positive cells were found in *Shh$^{-/-}$* telencephalon at this stage. (**I-L**) Pax6 expression in the telencephalon of control (I), *Shh$^{N/-}$* (J) *Shh$^{flox/-}$* (K) and *Shh$^{-/-}$* (L) embryos. Pax6 normally marks the dorsal telencephalon with the exception of the distal dorsal midline. Note that weak Pax6 expression extends to the ventral region in the *Shh$^{N/-}$* telencephalon (arrows in I and J point to the presumptive boundary between neocortex and LGE). *Shh$^{flox/-}$* showed comparable Pax6 expression to that of the control, whereas the *Shh$^{-/-}$* telencephalon was completely dorsalized at this stage. Brackets indicate LGE domains. ncx, neocortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence.

the telencephalon was maintained in hypomorphic *Shh$^{flox/-}$* mutants, although a reduction of the LGE domain was evident (Fig. 3C,G,K). Therefore, the severe patterning defect in *Shh$^{N/-}$* is primarily attributed to failure of cholesterol modification rather than haploinsufficiency, which is also consistent with the ectopic activation of Shh pathway activity observed in the dorsal telencephalon (see below).

Widespread activation of ventral LGE marker expression in the *Shh$^{N/-}$* telencephalic neuroepithelium

As noted above, we observed increased cell proliferation in the *Shh$^{N/-}$* telencephalon at E11.5, suggestive of long-range spreading of ShhN. Indeed, we detected ectopic expression of Shh-responsive genes that mark LGE neurons in the dorsal *Shh$^{N/-}$* telencephalon beginning at E13.5. Specifically, we found that Gsh2, a homeobox protein, was ectopically expressed in the dorsal midline region at E13.5 in the *Shh$^{N/-}$* telencephalon (Fig. 4C,Ca), as compared with its normally restricted expression in the ventricular zone of control MGE and LGE (Fig. 4A,Aa). Similarly, Mash1 was also ectopically expressed at the dorsal midline (Fig. 4B,D). By E15.5, expression of Gsh2 and Mash1 was detected in the ventricular zone of the striatum, a derivative of the LGE, but not in the cortical region (Fig. 4E-F). However, in *Shh$^{N/-}$* their expression was prominent along the dorsoventral axis of the telencephalic neuroepithelium (Fig. 4G-Hc). We found that the expression of *Gli3*, which encodes a zinc-finger transcriptional repressor in the absence of Shh signaling, was selectively excluded from the dorsal midline in *Shh$^{N/-}$* as well as control telencephalon (Fig. 4I-Ja). This observation is consistent with the finding that dorsal midline cells were more sensitive to the long-range patterning effect of ShhN than the lateral cortex where *Gli3* is expressed.

The emergence of ectopic LGE marker expression and defective midline dorsal structures at late gestation in *Shh$^{N/-}$* are consistent with the notion that the dorsal telencephalon might be exposed to low levels of ectopic ShhN signal. Indeed, we detected low but consistent Shh pathway activation in the dorsal telencephalon of *Shh$^{N/-}$* mutants as indicated by sensitive *Ptch1-lacZ* reporter expression (Fig. 5B), whereas no dorsal Shh signaling was found in a comparable region of the control embryo (Fig. 5A). Because Shh pathway activation leads to the accumulation of full-length Gli3 (Gli3-190) through the inhibition of Gli3 repressor (Gli3R) formation (Litingtung et al., 2002; Wang et al., 2000), we determined the relative amount of Gli3R to Gli3-190 as another readout of Shh signaling. Consistent with widespread Ptch1 reporter expression, we observed as much as a 50% reduction in the Gli3R/Gli3-190 ratio in E15.5 whole-brain extracts of *Shh$^{N/-}$* mutants as compared with controls (Fig. 5C,D). Ectopic Shh signaling is not the result of aberrant ectopic Shh expression, as we did not observe Shh-expressing cells or their descendents in the dorsal telencephalon using the *Shh-Cre* transgenic line that we described previously (Li et al., 2006) (data not shown). Thus, it appears that the cumulative effect of long-range ectopic ShhN signaling resulted in the induction of ventral marker expression in the dorsal neural progenitor population.

The cholesterol moiety is required for progenitor cell expansion but not subtype specification in the spinal cord

In order to determine whether specification of ventral cell types was similarly compromised in *Shh$^{N/-}$* mutant spinal cord, as in the telencephalon, we analyzed the expression patterns of several homeodomain transcription factors that identify different cell types including neuronal progenitors in the spinal cord (Briscoe and Ericson, 2001; Jessell, 2000). Pax7 is a class II factor that is repressed by Shh and is expressed in all dorsal progenitor domains, whereas Nkx6.1 (Nkx6-1 – Mouse Genome Informatics) is a class I factor that is induced by Shh and expressed in the broad ventral progenitor domains of V2 (p2), V3 (p3) interneurons and motor neurons (pMN). Similarly, Olig2 and Nkx2.2 (Nkx2-2 – Mouse Genome Informatics) are class I factors that are selectively expressed in the pMN and p3 progenitors, respectively. In *Shh$^{N/-}$*

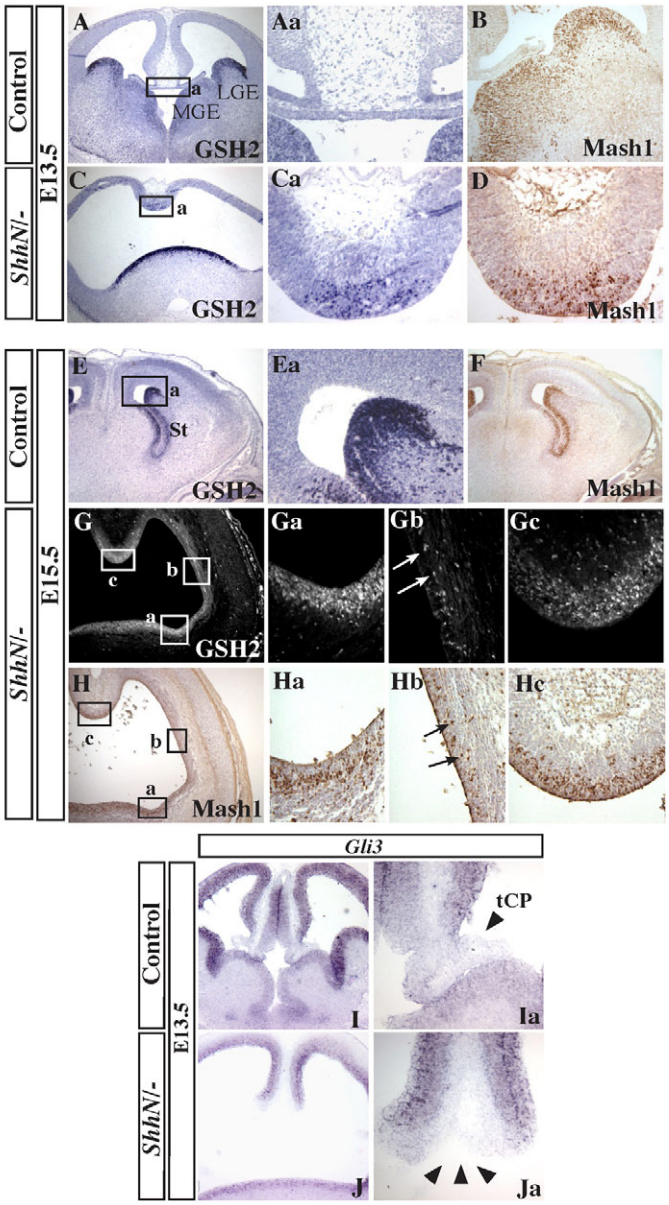

Fig. 4. Global appearance of LGE fate in *Shh*$^{N/-}$ telencephalic neuroepithelium at later developmental stages. (**A-D**) E13.5 control (A-B) and *Shh*$^{N/-}$ (C-D) mouse coronal sections immunostained with LGE marker, Gsh2 or Mash1 as indicated. Aa and Ca are higher magnifications of the boxed regions in A and C, respectively. (**E-Hc**) E15.5 control (E-F) and *Shh*$^{N/-}$ (G-Gc,H-Hc) coronal sections stained with Gsh2 or Mash1 antibody as indicated. Ea-c, Ga-c and Ha-c are higher magnifications of the boxed regions in E, G and H. At E13.5, ectopic Gsh2 or Mash1 expression in *Shh*$^{N/-}$ is restricted to the dorsal midline (Ca,D). By E15.5, ectopic LGE marker expression was also detected in the neocortex region (Gb,Hb, arrows). (**I-Ja**) *Gli3* expression in E13.5 control and *Shh*$^{N/-}$ telencephalon. (I,Ia) Strong *Gli3* expression was detected in control telencephalic cortex and LGE, weak expression was present in MGE, and no *Gli3* expression was detected in cortical hem and developing choroid plexus. (J,Ja) *Gli3* expression was prominent along the dorsoventral axis of the *Shh*$^{N/-}$ telencephalon, but selectively absent in the dorsal midline. MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; ncx, neocortex; st, striatum; tCP, telencephalic choroid plexus.

mutants, all ventral cell types were generated at defined locations (Fig. 6), in agreement with previously reported ShhN function in the spinal cord (Tian et al., 2005). The number of floorplate cells labeled by Foxa2 was comparable between control and *Shh*$^{N/-}$ embryos (Fig. 6C,D,N). However, there was an overall reduction in the total number of ventral neuronal progenitor domains and corresponding Chx10^{+} (V2, P=0.001), En1^{+} (V1, P=0.001) and Isl1^{+} (MN, P=0.007) neuronal subtypes that correlated with the reduced neural tube size in *Shh*$^{N/-}$ embryos (Fig. 6N,O). Taking into account the difference in neural tube size at E10.5 (the control spinal cord was about 44% larger than that of *Shh*$^{N/-}$; see Fig. S2J in the supplementary material), the Nkx6.1^{+} (p3, pMN and p2) progenitor domains in *Shh*$^{N/-}$ were slightly reduced as compared with the control (Fig. 6M, 35±3% versus 31±3%, P=0.05). Notably, there was an apparent expansion of the Pax7^{+} progenitor domain (Fig. 6M, 49±2% versus 63±2%, P<0.001) and concomitant reduction of p0+p1 (V0 and V1 progenitors) progenitor domains (Fig. 6M, 16±2% versus 6.2±1%, P<0.001). The expansion of the Pax7^{+} domain is not a consequence of maintaining dorsal tissue mass as a result of reduced neural tube size, as ventral expansion of dorsal progenitors was still observed at E9.5 when *Shh*$^{N/-}$ spinal cord exhibited a size comparable to the control (see Fig. S2A-H in the supplementary material). These results indicate that although neuronal subtypes are specified and patterned in *Shh*$^{N/-}$ spinal cord, the cholesterol moiety of Shh is, nevertheless, required for normal expansion of ventral progenitors, particularly p0+p1 progenitor populations.

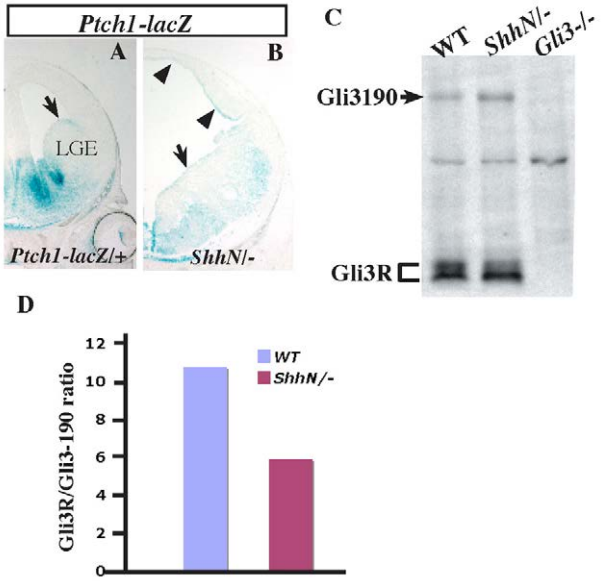

Fig. 5. Ectopic activation of Shh signaling in the dorsal region of the *Shh*$^{N/-}$ telencephalon. (**A**,**B**) E14.5 *Ptch1-lacZ* (A) and *Shh*$^{N/-}$;*Ptch1-lacZ* mouse coronal sections stained with X-Gal to highlight sites of Shh signaling in the telencephalon. In control embryos, *lacZ* expresssion is restricted to the basal ganglions, with weaker *lacZ* expression in the LGE domain (A, arrow). In *Shh*$^{N/-}$ embryos, weak *lacZ* expression is additionally detected at many sites in the dorsal telencephalon (B, arrowheads), consistent with ectopic LGE marker expression. (**C**) Immunoblotting of whole-brain protein extracts followed by incubation with a Gli3-specific antibody recognizing full-length (Gli3-190) and repressor forms (Gli3R) of Gli3. The Gli3R/Gli3-190 ratio is reduced in the E15.5 *Shh*$^{N/-}$ brain. (**D**) Bar chart showing Gli3R/Gli3-190 ratio.

ShhN protein does not accumulate at its site of synthesis

We next examined ShhN protein distribution in the telencephalon and spinal cord at stages when ventral neural progenitors were being specified. In the developing forebrain, *Shh* expression is first detected in the dorsal foregut endoderm (also referred to as the prechordal plate) underlying the future telencephalic neuroepithelium, and the ventral neuroepithelial expression of *Shh* does not occur until the eight-somite stage (Echelard et al., 1993; Shimamura and Rubenstein, 1997) (Fig. 7A,C). In $Shh^{N/-}$ embryos, *Shh* RNA expression in the dorsal foregut was maintained (Fig. 7E, arrow), but the level of ShhN protein was significantly reduced (Fig. 7G, arrow). Accordingly, *Shh* RNA expression in the overlying neuroepithelium was almost undetectable (Fig. 7E,G, arrowheads). By E9.5, *Shh* expression was completely abolished, although its expression in other regions of the brain was maintained (Fig. 7B,F). Similarly, we failed to detect ShhN protein in $Shh^{N/-}$ ventral telencephalon, whereas Shh protein was always observed in the midline of control embryos (Fig. 7D,H). As expected, Nkx2.1 expression, which overlapped with Shh expression in the ventral telencephalon, was not observed (Fig. 7I,M). The expression of *Ptch1*, a downstream target of Shh signaling (Goodrich et al., 1996), was also severely compromised in this region (Fig. 7J,N). In contrast to the telencephalon, *Shh* transcript and ShhN protein expression in the floorplate and notochord at E10.5 were clearly detectable, but the ShhN protein level was significantly reduced (Fig. 7K,L,O,P). Interestingly, ShhN protein predominantly localized to the apical membrane of floorplate cells (arrowheads in Fig. 7P), whereas ShhNp protein localized to both apical and basolateral membranes. This is in agreement with a recent study in *Drosophila* in which Hh-N accumulated only at the apical

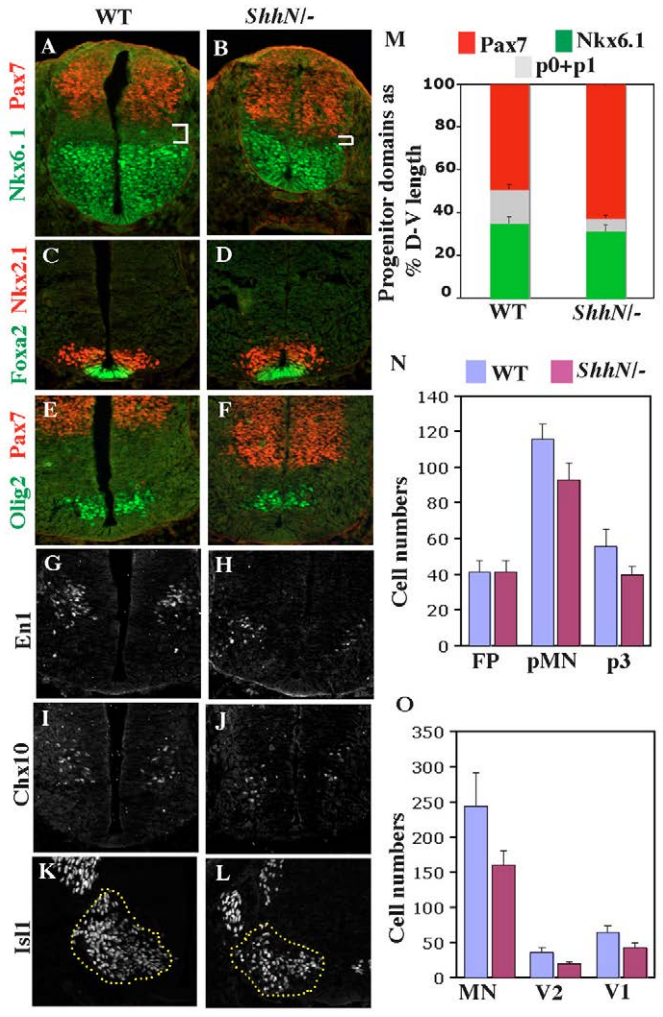

Fig. 6. Dorsoventral patterning is largely unperturbed in $Shh^{N/-}$ spinal cord. (**A-L**) Cross-sections of E10.5 control (A,C,E,G,I,K) and $Shh^{N/-}$ (B,D,F,H,J,L) mouse spinal cord stained with the indicated markers of neural progenitors and differentiated neurons at the branchial level. Nkx6.1 is expressed in the broad ventral domains encompassing the p3, pMN and p2 progenitor domains. Olig2 is selectively expressed in the pMN progenitor domain. Pax7 is expressed in all dorsal progenitor domains. The domain that is negative for Pax7 and Nkx6.1 represents the p0 and p1 progenitor domains (brackets in A,B). (**M**) Quantification of Pax7, Nkx6.1 and p0+p1 progenitor domain size as a percentage of total dorsoventral (D-V) length of the neural tube. (**N**,**O**) Quantification of total numbers of cells in the FP (Foxa2$^+$), pMN (Olig2$^+$), p3 (Nkx2.2$^+$), MN (Isl1$^+$), V2 (Chx10$^+$) and V1 (En1$^+$) domains in the wild type and $Shh^{N/-}$.

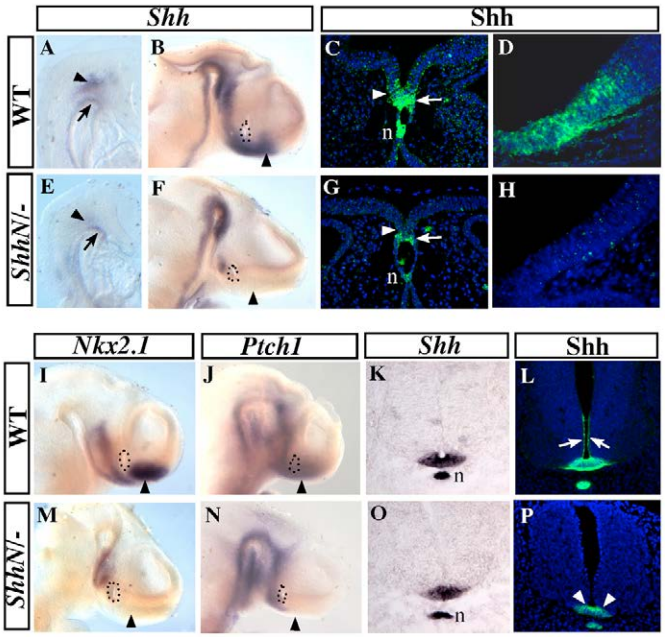

Fig. 7. Expression of Shh RNA, protein and target genes in $Shh^{N/-}$ neural tube. (**A-H**) Comparison of Shh RNA and protein expression in control and $Shh^{N/-}$ mouse forebrains. At the nine-somite stage, *Shh* expression can be detected in the forebrain neuroepithelium (A,E, arrowheads) and the underlying dorsal foregut endoderm (arrows). Note that *Shh* RNA expression is selectively lost in $Shh^{N/-}$ telencephalon (E,F, arrowheads). Similarly, Shh protein is also undetectable in the ventral $Shh^{N/-}$ telencephalon (H, arrowhead in G), and protein expression in the dorsal foregut is significantly reduced at the eight-somite stage (G, arrow). The dotted line (B,F) encloses optic vesicles. (**I**,**J**,**M**,**N**) *Nkx2.1* and *Ptch1* expression in control and $Shh^{N/-}$ forebrain at E9.5. The dotted line encloses optic vesicles. (**K**,**L**,**O**,**P**) Comparison of expression of Shh RNA and protein in control and $Shh^{N/-}$ spinal cord at E10.5. Shh protein is detected in both the basal and apical surfaces of the floorplate as well as in ependymal cells that line the central canal of the neural tube (L, arrows). However, in $Shh^{N/-}$ embryos, Shh protein resides exclusively at the apical compartment of floorplate cells (arrowheads in P) and does not accumulate at the ependymal cell surface. n, notochord.

membrane (Callejo et al., 2006). Intriguingly, we found that ShhNp protein was distributed along the apical cell membrane of ependymal cells (Fig. 7L, arrows), which line the central canal of the spinal cord. By contrast, we never observed ependymal localization of ShhN in the mutant spinal cord. It is possible that this ependymal cell layer represents an active site where ShhNp is normally concentrated and functions to properly expand ventral progenitor populations. Thus, the results suggest that ShhN has a propensity to spread away from its site of synthesis at the expense of its local concentration, similar to the situation observed in the limb bud (Li et al., 2006). As Shh expression in the ventral telencephalon is induced by the underlying prechordal plate (Ericson et al., 1995; Shimamura and Rubenstein, 1997), the ventral patterning defects observed in the $Shh^{N/-}$ telencephalon are likely to be attributable to the inability of telencephalic neuroepithelium to establish *Shh* expression in response to altered Shh spreading behavior in the prechordal plate.

ShhN rescues spinal cord, but not telencephalon, patterning in $Disp1^{-/-};Shh^{N/+}$ embryos

To confirm that the differential effects of ShhN on the development of the telencephalon and spinal cord were primarily mediated by paracrine signaling, we examined the patterning effects of ShhN in a $Disp1^{-/-}$ background, in which all Shh paracrine activities are elicited by ShhN because ShhNp is retained in Shh-producing cells. $Disp1^{-/-}$ single mutants normally die at or prior to E9.5 with a phenotype resembling $Smo^{-/-}$ embryos (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). However, the gross morphology of $Disp1^{-/-};Shh^{N/+}$ embryos largely resembled that of $Shh^{N/-}$ embryos, with enlarged brain ventricles, closely positioned frontal nasal process and defective eye formation (Fig. 8B, data not shown). As expected, we observed unperturbed dorsoventral neuronal patterning in E10.5 $Disp1^{-/-};Shh^{N/+}$ spinal cord, as demonstrated by well-defined dorsal Pax7 and ventral Nkx6.1 marker expression (Fig. 8G). Notably, we found an expanded pool of floorplate cells in $Disp1^{-/-};Shh^{N/+}$ spinal cord that often co-expressed the V3 interneuron marker Nkx2.2 and the floorplate marker Foxa2 (Fig. 8H-J). This is consistent with the notion that ShhN protein is capable of inducing floorplate formation and ShhNp cannot travel beyond the floorplate in the absence of Disp1 function, resulting in an elevated local concentration of Shh in the floorplate region (Fig. 8F, note how the robust floorplate Shh staining and the absence of ependymal Shh staining differ from that of control and $Shh^{N/-}$ embryos).

By contrast, the $Disp1^{-/-};Shh^{N/+}$ telencephalon showed patterning defects similar to that of $Shh^{N/-}$ embryos at E12.5, with the absence of MGE neurons and ventral expansion of the dorsal marker Pax6 (Fig. 8C-E), commensurate with the absence of Shh protein in the telencephalon (Fig. 8A). The above results support the notion that there is a more stringent requirement for cholesterol-modified Shh in patterning the ventral telencephalon than the spinal cord.

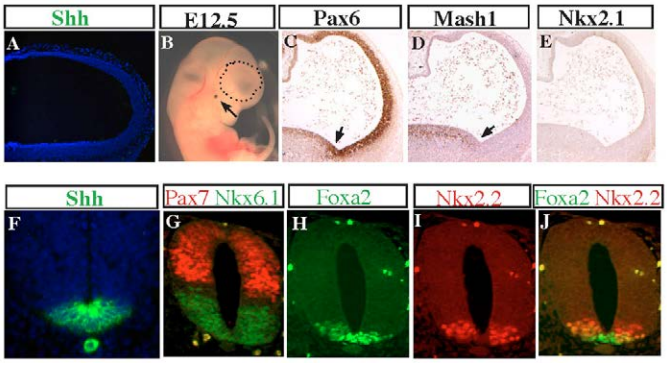

Fig. 8. ShhN rescues patterning defects in the spinal cord but not in the telencephalon of $Disp1^{-/-}$ mouse embryos. (**A-E**) The $Disp1^{-/-};Shh^{N/+}$ mutant telencephalon shows characteristics similar to those of the $Shh^{N/-}$ telencephalon, including absence of Shh (A) and Nkx2.1 (E) expression, enlarged forebrain ventricles (B, the region enclosed by the dotted line), reduced eye (B, arrow), ventrally extended Pax6 expression (C) and presence of Mash 1 expression (D). Arrows in C and D mark the presumptive boundary between LGE and cortex. (**F**) Robust Shh protein signal is visualized in notochord and floorplate. Note the absence of Shh protein staining in ependymal cells. (**G-J**) Dorsoventral patterning is established in $Disp1^{-/-};Shh^{N/+}$ mutant spinal cord, as demonstrated by defined expression domains of Pax7 and Nkx6.1 (G). Note the presence of V3 interneuron as shown by Nkx2.2 staining (I) and the expanded pool of floorplate cells (H).

DISCUSSION

To our knowledge, Shh is the only metazoan protein that has been shown to be covalently modified by a cholesterol moiety. To fully appreciate the biological function of this post-translational event, it is important to understand not only the role of cholesterol in regulating Shh protein trafficking, but also the tissue-specific requirement for this cholesterol adduct. This study indicates that cholesterol modification of Shh is required for patterning the telencephalon. In $Shh^{N/-}$ telencephalon, induction of ventral and dorsal anatomical structures and their corresponding cellular and molecular identities were significantly impaired. By contrast, Shh activity is not required for the generation of different ventral neuronal cell types in the spinal cord, although it is required for the full expansion of ventral progenitor domains. Thus, alterations in Shh local concentration and spreading behavior had more profound effects on the development of the telencephalon than of the spinal cord.

Enlarged brain ventricles in the $Shh^{N/-}$ mutant

One of the characteristic features in early development of the vertebrate brain is the appearance of brain ventricles. Generating a uniform and coordinated outgrowth of neuroepithelium from the neural tube involves balanced cell proliferation, apoptosis and differentiation. Previous studies provided evidence that Shh is an essential player to initiate and maintain ventricle expansion (Britto et al., 2002). Ablating Shh signaling by removing notochord resulted in elevated apoptosis and significantly reduced cell proliferation throughout the neuroepithelium of the developing avian midbrain and forebrain (Britto et al., 2002). This growth retardation resulted in a collapse of the telencephalon and metencephalon ventricles. Furthermore, gradual blockade of Shh signaling by increasing amounts of cyclopamine led to progressive brain collapse associated with defective cell proliferation and massive apoptosis (Cooper et al., 1998). $Shh^{-/-}$ forebrain development was also severely affected and it developed into one fused ventricle (Chiang et al., 1996). In contrast to reduced or collapsed brain ventricles associated with a reduction or absence of Shh signaling, $Shh^{N/-}$ embryos showed enlarged forebrain ventricles, which were more evident at later stages of development when ectopic activation of Shh signaling was detectable. This observation suggested that the cholesterol moiety of Shh plays a key role in maintaining forebrain ventricle expansion by regulating the range of Shh spreading.

Temporal dependence of ectopic signaling mediated by ShhN

The ability of ShhN to enhance dorsal midline cell proliferation and elicit ectopic Shh-responsive gene expression in the dorsal telencephalon is consistent with the capacity of ShhN to spread over a long range. However, the induction of these genes can only be detected beginning at E13.5, suggesting that either the dorsal telencephalon is incompetent to receive the ShhN signal, or the long-range ShhN concentration is below the threshold for target gene expression at earlier stages. We favor the latter possibility, as previous neural explant studies showed that the competence to receive Shh signal in the dorsal telencephalon was diminished by E11.5 (Kohtz et al., 1998). Interpretation of a morphogen signal by responding cells is achieved by the signaling intensity, which is conveyed by the concentration of ligand and the duration of exposure (reviewed by Gurdon and Bourillot, 2001). Long-lasting residence of ligand-receptor signaling complex has been shown to lead to prolonged signaling events in activin-dependent TGF-β signaling (Jullien and Gurdon, 2005). Interestingly, the activated form of smoothened (Smo), a seven-pass transmembrane receptor that activates downstream effectors of the Shh pathway, is internalized in a Shh-dependent manner (Chen et al., 2004b). Recent time-course analysis suggested that spinal cord progenitor cells could integrate the level of signaling over time, providing evidence that the signaling duration is an important parameter, in addition to signaling strength, to control dorsoventral patterning of the spinal cord (Stamataki et al., 2005). Therefore, we speculate that prolonged exposure of the telencephalic neuroepithelium to ShhN has a cumulative influence on the mediation of the gradual deposition of activated Smo intracellularly, which might in turn lead to widespread expression of Shh-responsive genes such as *Gsh2* and *Mash1* in $Shh^{N/-}$ telencephalic neuroepithelium at later stages.

Regulation of long-range spreading of Shh by its cholesterol moiety in the neural tube

Although the mechanism of Shh spreading is not well understood, lipid-dependent Hh multimers have been proposed to regulate Hh spreading by interacting with extracellular matrix molecules such as heparan sulfate proteoglycans (HSPGs) (Callejo et al., 2006). Furthermore, hedgehog-interacting protein (Hhip), a membrane glycoprotein, can bind to Shh, thereby inhibiting its spread (Chuang et al., 2003; Chuang and McMahon, 1999). A recent mathematical model for Shh signaling dynamics predicted that the restrictive effect of these Shh-interacting molecules lowers the diffusion constant and concentrates the ligand near its secreting source (Saha and Schaffer, 2006). It is therefore possible that ShhN protein can escape the synergistic regulatory mechanisms exerted by Shh-interacting molecules, generating a high diffusion rate as predicted by the model (Saha and Schaffer, 2006). Given that *ShhN* transcript is undetectable in the ventral telencephalon and that ShhN would have to traverse across the neural epithelium – a relatively long distance – to activate Shh-responsive gene expression extending to the dorsal midline, we favor the idea that ShhN could spread into the subjacent cerebrospinal fluid (CSF) from its site of synthesis in the neural tube. This proposition is analogous to the situation in *Drosophila*, where Hh-N is expressed from peripodial cells of the wing imaginal disc and is proposed to be secreted into the lumen to activate low-threshold target genes as it accumulates near the apical surface (Gallet et al., 2006).

Diffusive molecules and proteins of the CSF can play crucial roles in regulating neuroepithelial cell behavior and it has been shown that CSF promotes neuroepithelial cell survival, proliferation and neurogenesis in mesencephalic explants (Gato et al., 2005; Parada et al., 2005). Recent studies have suggested that the Slit family of chemorepulsive proteins may be secreted into the CSF to generate a cilia-dependent concentration gradient necessary for vectorial migration of neuroblasts in the brain (Sawamoto et al., 2006). In this context, it is interesting to note that Shh-expressing Cos cells implanted within the lumen of the midbrain can rescue midbrain expansion defects generated by extirpation of the notochord, suggesting that the precise source of Shh signaling activity is not crucial for normal morphogenesis of early brain ventricles (Britto et al., 2002). Furthermore, we found that ventral spinal cord ependymal cells might be an important site at which ShhNp protein concentrates and/or through which it travels. Intriguingly, we found by immunostaining that ShhNp protein can be visualized along the apical side of the innermost ventricular zone neuroepithelial cells as early as E9.5. Although the significance of apical surface neuroepithelial expression is not clear, the fact that this pattern is absent in the ShhN mutant suggests that it might be required for expansion of ventral progenitor domains. Recent studies in the chick spinal cord have also reported similar apical surface neuroepithelial expression at the onset of oligodendrogenesis, suggesting a role of ShhNp in the expansion of oligodendrocyte precursors (Danesin et al., 2006). We envision two possible explanations for how Shh is localized to this region. First, a significant amount of Shh protein could travel in a planar direction through the innermost neuroepithelial cell layer, and subsequently spreads laterally into the ventricular zone to establish different neuronal progenitor identities. Alternatively, it is possible that ShhNp protein from the floorplate is secreted into the CSF, and ShhNp receptors that reside on the apical membrane of neuroepithelial cells function to concentrate and endocytose this ShhNp from the CSF. These two tentative explanations are not mutually exclusive. Megalin (Lrp2) is a member of the low-density lipoprotein (LDL) receptor-related protein family. Megalin function is essential for forebrain formation and its abundant expression at the apical surface of the CNS neuroepithelium at midgestation is very similar to ShhNp expression, notably at the apical surface of neuroepithelial cells (Assemat et al., 2005; Willnow et al., 1996). A mechanism has been proposed in which megalin is required to supply sufficient cholesterol to the rapidly dividing neuroepithelium before neural tube closure, possibly through the endocytosis of cholesterol-containing lipoprotein (Herz et al., 1997). Since it has been shown in tissue culture studies that megalin could function as an endocytic Shh receptor (McCarthy et al., 2002), it is possible that megalin has a direct role in propagating Shh signaling. Another endocytic receptor, cubilin (Cubn – Mouse Genome Informatics), an EGF-CUB protein, has a nearly identical apical surface neuroepithelial expression pattern to megalin (Assemat et al., 2005). Interestingly, a cubulin-related protein encoded by the zebrafish *scube2* gene has been shown to mediate Shh signaling in embryos (Hollway et al., 2006; Woods and Talbot, 2005). Therefore, it is possible that these two proteins act synergistically in promoting Shh signaling during mammalian development. It would be interesting to investigate the functional relationship of Shh, megalin and cubulin in ependymal cells and the existence of secreted Shh protein in the CSF.

Region-specific effects of ShhN in patterning the neural epithelium

Our study suggests that alteration of Shh spreading behavior has differential effects in patterning the telencephalon versus spinal cord. This is most likely to be attributable to regional differences in the generation and maintenance of *Shh* expression in the neuroepithelium.

In the spinal cord, the development of ventral progenitor cells is mediated by Shh secreted from the notochord and floorplate (Jessell, 2000). *Shh* expression in the floorplate is induced and maintained by the notochord, which is in close contact with the overlying spinal cord for an extended period of time. In the telencephalon, the development of basal ganglia appears to be mediated initially by Shh secreted from the prechordal plate (the dorsal foregut) and then by the ventral neuroepithelium (Ericson et al., 1995; Shimamura and Rubenstein, 1997). In contrast to the floorplate, *Shh* expression in the ventral telencephalic neuroepithelium becomes quickly independent of the influence of the underlying prechordal plate. Thus, a reduction in local Shh concentration in the prechordal plate and notochord, as shown in $Shh^{N/-}$ embryos, would have more profound effects on *Shh* expression and maintenance in the telencephalon than in the spinal cord.

An unexpected finding is that ShhN exerted no effect on dorsal progenitors, as there was no evidence of expansion or ectopic ventral progenitor cell fates in the dorsal region of the spinal cord. This observation is in contrast to findings in the telencephalon and limb buds (Li et al., 2006), where ectopic Shh pathway activation was detected far from the source of ShhN synthesis. There are at least two, non-mutually exclusive explanations for this difference. First, temporal responsiveness to ectopic ShhN might be different between dorsal telencephalon and dorsal spinal cord. Neural explant studies in rats indicated that E11.5 dorsal telencephalon (~E10.5 in mouse) remained competent to receive exogenous Shh stimulation (Kohtz et al., 1998), whereas E11 (~E10 in mouse) spinal cord did not demonstrate such competence (Charron et al., 2003). Likewise, the spinal cord might have lost competence to respond to ectopic ShhN by the time the signal reaches a threshold concentration necessary for cell fate specification. Alternatively, the concentration threshold required for ShhN to elicit ectopic patterning effects in the dorsal spinal cord is higher than for the dorsal telencephalon.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/11/2095/DC1

References

Agarwala, S., Sanders, T. A. and Ragsdale, C. W. (2001). Sonic hedgehog control of size and shape in midbrain pattern formation. *Science* **291**, 2147-2150.

Assemat, E., Chatelet, F., Chandellier, J., Commo, F., Cases, O., Verroust, P. and Kozyraki, R. (2005). Overlapping expression patterns of the multiligand endocytic receptors cubilin and megalin in the CNS, sensory organs and developing epithelia of the rodent embryo. *Gene Expr. Patterns* **6**, 69-78.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11**, 43-49.

Britto, J., Tannahill, D. and Keynes, R. (2002). A critical role for sonic hedgehog signaling in the early expansion of the developing brain. *Nat. Neurosci.* **5**, 103-110.

Callejo, A., Torroja, C., Quijada, L. and Guerrero, I. (2006). Hedgehog lipid modifications are required for Hedgehog stabilization in the extracellular matrix. *Development* **133**, 471-483.

Casarosa, S., Fode, C. and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525-534.

Caspary, T., Garcia-Garcia, M. J., Huangfu, D., Eggenschwiler, J. T., Wyler, M. R., Rakeman, A. S., Alcorn, H. L. and Anderson, K. V. (2002). Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. *Curr. Biol.* **12**, 1628-1632.

Charron, F., Stein, E., Jeong, J., McMahon, A. P. and Tessier-Lavigne, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* **113**, 11-23.

Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M. and Chuang, P. T. (2004a). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* **18**, 641-659.

Chen, W., Ren, X. R., Nelson, C. D., Barak, L. S., Chen, J. K., Beachy, P. A., de Sauvage, F. and Lefkowitz, R. J. (2004b). Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. *Science* **306**, 2257-2260.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-413.

Chuang, P. T. and McMahon, A. P. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621.

Chuang, P. T., Kawcak, T. and McMahon, A. P. (2003). Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. *Genes Dev.* **17**, 342-347.

Cooper, M. K., Porter, J. A., Young, K. E. and Beachy, P. A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603-1607.

Dale, J. K., Vesque, C., Lints, T. J., Sampath, T. K., Furley, A., Dodd, J. and Placzek, M. (1997). Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal mesoderm. *Cell* **90**, 257-269.

Danesin, C., Agius, E., Escalas, N., Ai, X., Emerson, C., Cochard, P. and Soula, C. (2006). Ventral neural progenitors switch toward an oligodendroglial fate in response to increased Sonic hedgehog (Shh) activity: involvement of sulfatase 1 in modulating Shh signaling in the ventral spinal cord. *J. Neurosci.* **26**, 5037-5048.

Dawber, R. J., Hebbes, S., Herpers, B., Docquier, F. and van den Heuvel, M. (2005). Differential range and activity of various forms of the Hedgehog protein. *BMC Dev. Biol.* **5**, 21.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.

Feng, J., White, B., Tyurina, O. V., Guner, B., Larson, T., Lee, H. Y., Karlstrom, R. O. and Kohtz, J. D. (2004). Synergistic and antagonistic roles of the Sonic hedgehog N- and C-terminal lipids. *Development* **131**, 4357-4370.

Fuccillo, M., Rallu, M., McMahon, A. P. and Fishell, G. (2004). Temporal requirement for hedgehog signaling in ventral telencephalic patterning. *Development* **131**, 5031-5040.

Fuccillo, M., Joyner, A. L. and Fishell, G. (2006). Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 772-783.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Gallet, A., Ruel, L., Staccini-Lavenant, L. and Therond, P. P. (2006). Cholesterol modification is necessary for controlled planar long-range activity of Hedgehog in Drosophila epithelia. *Development* **133**, 407-418.

Gato, A., Moro, J. A., Alonso, M. I., Bueno, D., De La Mano, A. and Martin, C. (2005). Embryonic cerebrospinal fluid regulates neuroepithelial survival, proliferation, and neurogenesis in chick embryos. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* **284**, 475-484.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.

Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.

Guerrero, I. and Chiang, C. (2006). A conserved mechanism of Hedgehog gradient formation by lipid modifications. *Trends Cell Biol.* **17**, 1-5.

Gurdon, J. B. and Bourillot, P. Y. (2001). Morphogen gradient interpretation. *Nature* **413**, 797-803.

Hayashi, S., Lewis, P., Pevny, L. and McMahon, A. P. (2002). Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Mech. Dev.* **119** Suppl. 1, S97-S101.

Herz, J., Willnow, T. E. and Farese, R. V., Jr (1997). Cholesterol, hedgehog and embryogenesis. *Nat. Genet.* **15**, 123-124.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Hollway, G. E., Maule, J., Gautier, P., Evans, T. M., Keenan, D. G., Lohs, C., Fischer, D., Wicking, C. and Currie, P. D. (2006). Scube2 mediates Hedgehog signalling in the zebrafish embryo. *Dev. Biol.* **294**, 104-118.

Horton, S., Meredith, A., Richardson, J. A. and Johnson, J. E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. *Mol. Cell. Neurosci.* **14**, 355-369.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.
Jullien, J. and Gurdon, J. (2005). Morphogen gradient interpretation by a regulated trafficking step during ligand-receptor transduction. *Genes Dev.* **19**, 2682-2694.
Kawakami, T., Kawcak, T., Li, Y. J., Zhang, W., Hu, Y. and Chuang, P. T. (2002). Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling. *Development* **129**, 5753-5765.
Kohtz, J. D., Baker, D. P., Corte, G. and Fishell, G. (1998). Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. *Development* **125**, 5079-5089.
Kohtz, J. D., Lee, H. Y., Gaiano, N., Segal, J., Ng, E., Larson, T., Baker, D. P., Garber, E. A., Williams, K. P. and Fishell, G. (2001). N-terminal fatty-acylation of sonic hedgehog enhances the induction of rodent ventral forebrain neurons. *Development* **128**, 2351-2363.
Lazzaro, D., Price, M., de Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113**, 1093-1104.
Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**, 599-612.
Li, Y., Zhang, H., Choi, S. C., Litingtung, Y. and Chiang, C. (2004). Sonic hedgehog signaling regulates Gli3 processing, mesenchymal proliferation, and differentiation during mouse lung organogenesis. *Dev. Biol.* **270**, 214-231.
Li, Y., Zhang, H., Litingtung, Y. and Chiang, C. (2006). Cholesterol modification restricts the spread of Shh gradient in the limb bud. *Proc. Natl. Acad. Sci. USA* **103**, 6548-6553.
Li, Y., Litingtung, Y., ten Dijke, P. and Chiang, C. (2007). Loss of Noggin-mediated Bmp7 antagonism in the pathogenesis of esophageal atresia. *Dev. Dyn.* **236**, 746-754.
Litingtung, Y., Lei, L., Westphal, H. and Chiang, C. (1998). Sonic hedgehog is essential to foregut development. *Nat. Genet.* **20**, 58-61.
Litingtung, Y., Dahn, R. D., Li, Y., Fallon, J. F. and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* **418**, 979-983.
Lo, L. C., Johnson, J. E., Wuenschell, C. W., Saito, T. and Anderson, D. J. (1991). Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev.* **5**, 1524-1537.
Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K. and Beachy, P. A. (2002). Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. *Cell* **111**, 63-75.
Mann, R. K. and Beachy, P. A. (2004). Novel lipid modifications of secreted protein signals. *Annu. Rev. Biochem.* **73**, 891-923.
McCarthy, R. A., Barth, J. L., Chintalapudi, M. R., Knaak, C. and Argraves, W. S. (2002). Megalin functions as an endocytic sonic hedgehog receptor. *J. Biol. Chem.* **277**, 25660-25667.
Nagai, T., Aruga, J., Minowa, O., Sugimoto, T., Ohno, Y., Noda, T. and Mikoshiba, K. (2000). Zic2 regulates the kinetics of neurulation. *Proc. Natl. Acad. Sci. USA* **97**, 1618-1623.
Parada, C., Martin, C., Alonso, M. I., Moro, J. A., Bueno, D. and Gato, A. (2005). Embryonic cerebrospinal fluid collaborates with the isthmic organizer to regulate mesencephalic gene expression. *J. Neurosci. Res.* **82**, 333-345.
Rallu, M., Machold, R., Gaiano, N., Corbin, J. G., McMahon, A. P. and Fishell, G. (2002). Dorsoventral patterning is established in the telencephalon of mutants lacking both Gli3 and Hedgehog signaling. *Development* **129**, 4963-4974.
Rice, R., Spencer-Dene, B., Connor, E. C., Gritli-Linde, A., McMahon, A. P., Dickson, C., Thesleff, I. and Rice, D. P. (2004). Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J. Clin. Invest.* **113**, 1692-1700.
Saha, K. and Schaffer, D. V. (2006). Signal dynamics in Sonic hedgehog tissue patterning. *Development* **133**, 889-900.
Sawamoto, K., Wichterle, H., Gonzalez-Perez, O., Cholfin, J. A., Yamada, M., Spassky, N., Murcia, N. S., Garcia-Verdugo, J. M., Marin, O., Rubenstein, J. L. et al. (2006). New neurons follow the flow of cerebrospinal fluid in the adult brain. *Science* **311**, 629-632.
Shimamura, K. and Rubenstein, J. L. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.
Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. R. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.
Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. (2005). A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. *Genes Dev.* **19**, 626-641.
Sur, M. and Rubenstein, J. L. (2005). Patterning and plasticity of the cerebral cortex. *Science* **310**, 805-810.
Tian, H., Jeong, J., Harfe, B. D., Tabin, C. J. and McMahon, A. P. (2005). Mouse Disp1 is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand. *Development* **132**, 133-142.
Wang, B., Fallon, J. F. and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423-434.
Willnow, T. E., Hilpert, J., Armstrong, S. A., Rohlmann, A., Hammer, R. E., Burns, D. K. and Herz, J. (1996). Defective forebrain development in mice lacking gp330/megalin. *Proc. Natl. Acad. Sci. USA* **93**, 8460-8464.
Woods, I. G. and Talbot, W. S. (2005). The you gene encodes an EGF-CUB protein essential for Hedgehog signaling in zebrafish. *PLoS Biol.* **3**, e66.
Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr, Schreiner, C. M. and Robbins, D. J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**, 716-720.
Zhang, X. M. and Yang, X. J. (2001). Temporal and spatial effects of Sonic hedgehog signaling in chick eye morphogenesis. *Dev. Biol.* **233**, 271-290.