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Dorsoventral patterning is established in the telencephalon of mutants lacking both Gli3 and Hedgehog signaling

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SUMMARY

Considerable data suggest that sonic hedgehog (Shh) is both necessary and sufficient for the specification of ventral pattern throughout the nervous system, including the telencephalon. We show that the regional markers induced by Shh in the E9.0 telencephalon are dependent on the dorsoventral and anteroposterior position of ectopic Shh expression. This suggests that by this point in development regional character in the telencephalon is established. To determine whether this prepattern is dependent on earlier Shh signaling, we examined the telencephalon in mice carrying either *Shh*- or *Gli3*-null mutant alleles. This analysis revealed that the expression of a subset of ventral telencephalic markers, including *Dlx2* and *Gsh2*, although greatly diminished, persist in *Shh*$^{-/-}$ mutants, and that these same markers were expanded in *Gli3*$^{-/-}$ mutants. To understand further the genetic interaction between Shh and Gli3, we examined *Shh/Gli3* and *Smoothened/Gli3* double homozygous mutants. Notably, in animals carrying either of these genetic backgrounds, genes such as *Gsh2* and *Dlx2*, which are expressed pan-ventrally, as well as *Nkx2.1*, which demarcates the ventral most aspect of the telencephalon, appear to be largely restored to their wild-type patterns of expression. These results suggest that normal patterning in the telencephalon depends on the ventral repression of Gli3 function by Shh and, conversely, on the dorsal repression of Shh signaling by Gli3. In addition these results support the idea that, in addition to hedgehog signaling, a Shh-independent pathways must act during development to pattern the telencephalon.

Key words: Telencephalon, Shh, Gli3, Mouse

INTRODUCTION

The forebrain arises from the most anterior part of the neural plate and is subsequently divided into the telencephalon and the diencephalon (Fishell, 1997; Rubenstein and Beachy, 1998; Shimamura et al., 1995). After neural tube closure (mouse E9), proliferation within the ventral regions of the telencephalon results in the emergence of three eminences: first, the medial ganglionic eminence (MGE) and later, the lateral and caudal ganglionic eminences (LGE and CGE, respectively) (Fishell, 1997) (S. Nery, G. F. and J. G. C., unpublished). Collectively, these eminences make up the anlage of much of the telencephalic basal ganglia. These structures are thought to give rise to the globus pallidus, the striatum, and large populations of oligodendrocytes and cortical interneurons (Wichterle et al., 2001) (S. Nery, G. F. and J. G. C., unpublished). All three eminences appear to be absent in sonic hedgehog (*Shh*) null mutants (Chiang et al., 1996) (J. G. C., unpublished).

Shh has been shown to play a fundamental role in the establishment of ventral identity throughout the neural tube (reviewed by Ericsson et al., 1995a; Litingtung and Chiang, 2000b; Patten and Placzek, 2000). As a result, much work has been performed to elucidate the mechanisms by which Shh generates cell diversity within the ventral neural tube, especially in the spinal cord. A considerable body of evidence favors the idea that Shh acts as a morphogen, forming a gradient within the ventral neural tube to which cells respond in a concentration-dependent manner (reviewed by Briscoe and Ericsson, 2001; Jessell, 2000). Collectively, in vitro explant studies using recombinant Shh (Ericsson et al., 1996; Ericsson et al., 1997; Roelink et al., 1995) and in vivo electroporation studies with *Shh* (Agarwala et al., 2001) or a dominant-negative form of patched (Briscoe et al., 2001) strongly support this hypothesis in the midbrain and spinal cord, respectively.

Although Shh is also clearly required in forebrain regions (Chiang et al., 1996; Ericsson et al., 1995b), to date less effort has been focused on its role in patterning the telencephalon. Similar to the spinal cord, embryos that lack *Shh* fail to form ventral telencephalic structures and to express corresponding markers, while dorsal marker expression is expanded ventrally (Chiang et al., 1996). Furthermore, ectopic expression of *Shh* is sufficient to induce ventral telencephalic markers in dorsal

regions of the telencephalon both in vitro (Ericsson et al., 1995b; Kohtz et al., 1998; Shimamura and Rubenstein, 1997) and in vivo (Barth and Wilson, 1995; Corbin et al., 2000; Gaiano et al., 1999; Hauptmann and Gerster, 1996). However, when and how Shh is required for ventral telencephalic patterning and whether it acts to differentially specify the MGE, the LGE and the CGE is not presently clear. Beginning around E9.0, Shh is expressed within the ventral telencephalon, primarily in the MGE, preoptic area and prospective amygdala (Nery et al., 2001; Sussel et al., 1999). Notably, the specific loss of *Shh* expression within the telencephalon, which occurs in *Nkx2.1* (*Titf1* – Mouse Genome Informatics) (Sussel et al., 1999) and *BF1* (*Foxg1* – Mouse Genome Informatics) (Huh et al., 1999) null mutants, does not recapitulate the phenotype observed in *Shh* mutants. This suggests that earlier expression of Shh, probably from non-neural tissues such as the dorsal foregut (i.e. prechordal plate) (Shimamura and Rubenstein, 1997) or Hensen's node (Gunhaga et al., 2000), must be responsible for the more extensive telencephalic defects seen in *Shh* mutants.

The Ci/Gli family of zinc-finger transcription factors has been implicated as required transducers of the hedgehog signaling pathway (reviewed by Aza-Blanc and Kornberg, 1999; Ruiz i Altaba et al., 2002). Gli proteins, like Ci, display both activator and repressor activities that are regulated post-translationally (Aza-Blanc et al., 1997; Ingham and McMahon, 2001). Different regions of the Gli proteins encode distinct functions: N-terminal regions encode a repressor function, whereas C-terminal regions are required for positive activity. Analysis of mice mutants for each of the three murine Gli genes has shown that *Gli3* is the only Gli gene that plays a significant role in patterning the telencephalon. Specifically, mice that lack *Gli1* and *Gli2* show minor defects in telencephalic patterning (Park et al., 2000), whereas *Gli3* mutant mice have strong abnormalities in the dorsal telencephalon (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). Gli3 protein appears to function primarily as a repressor (reviewed by Ingham and McMahon, 2001) and its activity seems to be negatively regulated by Shh (Marigo et al., 1996; Wang et al., 2000). Shh signaling, both in vitro and in the limb, has been shown to inhibit Gli3 processing into an N-terminal fragment that carries a repressor activity. In agreement with this, analysis of mice lacking both *Shh* and *Gli3* revealed that, in the spinal cord, Shh is required to inhibit Gli3 function in order to form motoneurons (Litingtung and Chiang, 2000a).

In the present study, we have used both gain- and loss-of-function methods to study the importance of hedgehog signaling in general and Shh in particular in the establishment of dorsoventral patterning in the telencephalon. We find that virally mediated ectopic sonic hedgehog expression induces different regional markers, depending on the dorsoventral or anteroposterior position of the infection. Conversely, some aspects of ventral telencephalic patterning persist in the absence of Shh. In addition, we observe that dorsoventral patterning in the telencephalon appears to be largely restored in *Shh/Gli3* and *Smo/Gli3* double homozygous mutants. Our data reveal that Shh and Gli3 antagonize each other's function in patterning the telencephalon. Furthermore, our findings suggest that a hedgehog-independent mechanism, such as possibly the BMP-signaling pathway (Liem et al., 2000), is capable of acting in parallel to Shh in establishing dorsoventral pattern in the telencephalon.

MATERIALS AND METHODS

Animals, virus preparation and injections

Mice used in these studies were maintained according to protocols approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. For staging of embryos, noon on the day the vaginal plug was considered as embryonic day 0.5 (E0.5). Virus preparation and ultrasound surgery were both performed as previously described (Gaiano et al., 1999). Stocks of Shh-expressing or ActSmo-expressing viruses were injected at titers of 5×10^7 and 5×10^8 cfu/ml respectively. Mouse embryos were injected from E8.5 to E10.5 and sacrificed 4 days later (E12.5 to E14.5). Viral expression usually begins 12 hours after infection (E9.0 at the earliest in our case).

Genotyping of mutant mice

Shh (Chiang et al., 1996) and *Gli3* (*Xt^J* from Jackson Laboratory) mutant mice were maintained on a C57/Bl6 and C3HeB/FeJ background, respectively. Different mutant combinations were generated by intercrossing *Shh$^{+/-}$;Gli3$^{+/-}$* or *Smo$^{+/-}$;Gli3$^{+/-}$* heterozygous animals. Embryos were genotyped by PCR as described previously (Litingtung and Chiang, 2000a; Maynard et al., 2002; Zhang et al., 2001).

Staining of tissue sections

Whole heads or embryos were fixed at 4°C for 1-4 hours in 4% paraformaldehyde, then cryoprotected overnight in 30% sucrose in PBS, embedded in HistoPrep (Fisher Scientific) and frozen. All tissue was sectioned serially at 12 to 20 μm and processed for immunohistochemistry or RNA in situ hybridization.

Infected cells were identified by the expression of the alkaline phosphatase reporter (PLAP) as described previously (Gaiano et al., 1999). Analysis of gene induction was performed by RNA in situ hybridization on adjacent 20 μm sections. Note that the thickness of the sections and the incompatibility of histochemistry and in situ hybridization on the same sections sometimes made it hard to correlate the infected cells with the cells that display ectopic expression (e.g. Fig. 2J-K).

Immunofluorescence was performed as described previously (Corbin et al., 2000). The following primary antibodies were used: mouse anti-Pax6 (1:1000, gift of A. Kawakami) and rabbit anti-Gsh2 (1:3500, gift of K. Campbell).

In situ hybridization was performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Wilkinson and Nieto, 1993) using non-radioactive digoxigenin-labeled probes for *Nkx2.1* (Shimamura et al., 1995), *Dlx2* (Porteus et al., 1991), *Gsh2* (Hsieh-Li et al., 1995), *Mash1* (*Ascl1* – Mouse Genome Informatics) (Guillemot and Joyner, 1993), *Gli1*, *Gli3* (Hui et al., 1994) and *Ptch* (Goodrich et al., 1997).

Dlx2 mRNA expression was also determined by X-Gal staining of embryos from a strain of mice in which the *taulacZ* gene has been targeted to the *Dlx2* locus (Corbin et al., 2000) (S. Nery, G. F. and J. G. C., unpublished). X-Gal staining was performed as described previously (Corbin et al., 2000).

RESULTS

The competence of the E9.0 telencephalon to respond to Shh signaling is spatially restricted

To better understand the mechanisms by which Shh patterns the telencephalon, we chose to investigate the effects of ectopic Shh signaling in vivo. We used a gain-of-function approach that combines high-titer retroviral vectors and ultrasound imaging (Gaiano et al., 1999). Retroviruses expressing the full-

length Shh protein were injected into the embryonic forebrain at different stages of development, the earliest of which (mouse E8.5) preceded the establishment of overt dorsoventral patterning in the telencephalon. The injection of Shh virus at these early time points had a dramatic effect on the morphology of the brains, with the vesicles being substantially enlarged compared with wild-type embryos (Fig. 1E,F). These morphological defects may be the result of an abnormal proliferation induced by Shh. Indeed, the role of Shh in cell proliferation within the nervous system has been shown in the cerebellum (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999), the retina (Jensen and Wallace, 1997) and the dorsal neural tube (Rowitch et al., 1999). The morphological abnormalities in Shh-infected brains made any analysis of dorsoventral patterning in these animals difficult. Thus, we constructed a retrovirus encoding a constitutively active form of the Smoothened protein (ActSmo) (Hynes et al., 2000; Xie et al., 1998). Smoothened is a transmembrane protein that, in the absence of Shh, is repressed by the Shh receptor Patched (Ptch) (reviewed by Ingham and McMahon, 2001; Kalderon, 2000; Murone et al., 1999). This repression is thought to be relieved by the binding of Shh to Patched, allowing Smoothened to activate downstream elements of the Shh signaling pathway cell-autonomously. Activated Smoothened has been shown to mimic the patterning effects of ectopic Shh in the dorsal neural tube (Hynes et al., 2000). Furthermore, the activity of ActSmo is likely to be cell-autonomous (Hynes et al., 2000), allowing us to study the effects of ectopic hedgehog signaling without perturbing the local environment of the infected cells. We found that infections with a virus expressing ActSmo did not result in the gross morphological deformations observed with Shh (Fig. 1C,D) but was indistinguishable from Shh in its ability to alter regional gene expression.

ActSmo- and Shh-expressing viruses were injected at various times ranging from E8.5 to E10.5 and embryos were sacrificed 4 days later for analysis (E12.5 to E14.5). Notably, we observed that the ectopic expression of ActSmo (or Shh itself) resulted in the same modifications of telencephalic patterning regardless of the age of the infection (data not shown). This suggests that the competence of the telencephalic tissue to respond to ectopic induction of the Shh pathway is not temporally regulated within this window of embryonic development.

Ventral patterning by Shh in the spinal cord is in part mediated through the inhibition of dorsally expressed transcription factors (Briscoe et al., 2000; Ericsson et al., 1997). Similarly in the telencephalon, in vitro exposure to Shh can inhibit the expression of dorsal markers (Kohtz et al., 1998). Moreover, dorsal gene expression expands ventrally in the absence of Shh (Chiang et al., 1996). We found that the expression of *Pax*6, a dorsal telencephalic marker, is indeed repressed in the infected cells (Fig. 2A-C), in accordance with previous results in spinal cord (Ericsson et al., 1996). A similar result was obtained for the dorsally expressed proneural genes such as *Ngn2*, *Math2* and *NeuroD* (data not shown).

We next sought to determine the extent to which ventral markers characteristic of the MGE (*Nkx2.1*) and MGE/LGE (pan-ventral genes *Gsh2*, *Dlx2*) could be induced ectopically by virally mediated activation of Shh signaling. We observed that the identity of the markers induced, and therefore the cell types, was mainly dependent on the position of the infection. Along the dorsoventral axis, the induction of *Nkx2.1* expression in response to ectopic Shh signaling was restricted to the LGE and lateral neocortex (Fig. 2G,H), whereas *Gsh2* and *Dlx2* could be induced throughout the neocortex (Fig. 2I,J and data not shown). Interestingly, we noted that the induction of pan-ventral gene expression (*Gsh2*, *Dlx2*) was weaker in the medial regions of the telencephalon (Fig. 2I,J). Sagittal sections of ActSmo infected brains revealed the rostrocaudal extent of ventral gene induction: while *Nkx2.1* induction was restricted to the LGE and septum (Fig. 2D,F and data not shown), pan-ventral gene induction within the cortex was only excluded from the more posterior regions (Fig. 2D,E). These differences could be accounted for by regional variations in the ability of the telencephalon to activate downstream components of the Shh pathway. To address this issue we analyzed patched (*Ptch)* and *Gli1* gene expression, which are believed to be generic downstream response genes in the Shh signaling pathway. We found that the expression of either of these genes was upregulated at all positions along the dorsoventral or anteroposterior axis (Fig. 2K-O). Based on these criteria, this suggests that all regions of the telencephalon are equally competent to respond to Shh signaling.

These gain-of-function experiments show that regional identity can be altered by Shh signaling through the early phases of neurogenesis. However, we found that this plasticity is somewhat limited: the ventral identities that Shh and ActSmo can induce are dependent on the dorsoventral or anteroposterior position of the mis-expression. This suggests that an intrinsic pattern, which is either Shh dependent or independent, has been established within the telencephalon by

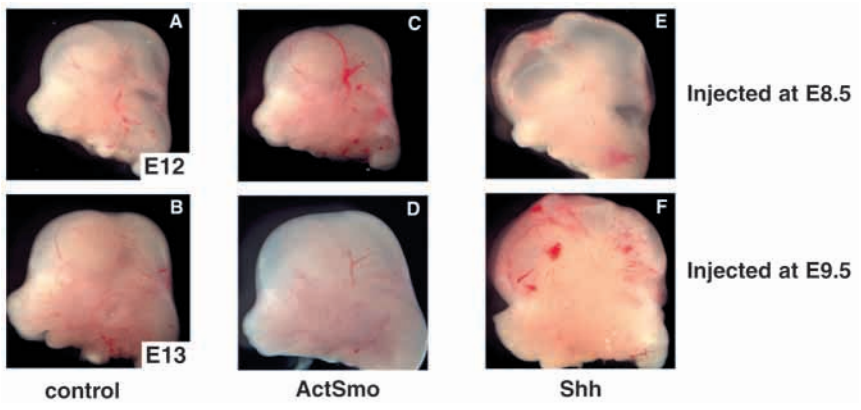

Fig. 1. Differential effects of Shh- or ActSmo-expressing retroviruses on brain morphology. Animals were infected in utero with control virus (A,B) and viruses expressing ActSmo (C,D) or Shh (E,F). Injections were performed at E8.5 (A,C,E) or E9.5 (B,D,F) and analyzed 4 days later (E12.5 or E13.5, respectively). Embryos injected with Shh virus showed a dramatic increase in the size of their heads, with substantially enlarged telencephalic vesicles (E,F). Despite the fact that the ActSmo virus was injected at a 10-fold greater titer than Shh virus, heads from these embryos appeared to have largely normal morphology (C,D).

the time the Shh pathway is activated ectopically (E9.0 at the earliest, i.e. 12 hours after infection).

Pan-ventral gene expression is maintained in *Shh* mutants

Previous analysis of *Shh*-null animals revealed that, in addition to the lack of any obvious ventral telencephalic structures, the domain of dorsal gene expression spreads ventrally to include the whole telencephalon (Chiang et al., 1996; Pabst et al., 2000). However, when we investigated the expression of pan-ventral genes in these mutants, we were surprised to observe that some ventral telencephalic patterning remains in the absence of Shh (Fig. 3). *Dlx2* expression was observed in the remaining telencephalon of E10.5 to E12.5 *Shh* mutants as visualized by in situ hybridization or by using a *Dlx2*^{taulacZ} allele (Fig. 3A-D). Similarly, *Gsh2* expression (another pan-ventral gene) persisted in *Shh* mutants (Fig. 3E,F) (H. Toresson and K. Campbell, personal communication). Notably, in these mutants the domain of expression of both *Dlx2* and *Gsh2* was restricted to a region adjacent to the ventral midline. As *Shh* mutants are extremely dysmorphic, it is impossible to know with certainty which ventral telencephalic domains persist in these mutants. Nonetheless, the fact that *Nkx2.1* expression was missing in *Shh*$^{-/-}$ embryos (data not shown) (Pabst et al., 2000) while *Dlx2* and *Gsh2* expression persisted suggests that the most ventral domain (i.e. MGE) is absent in *Shh*$^{-/-}$ mutants, while the lateral regions (i.e. LGE) are displaced ventrally. In this regard, this result is likely to be analogous to that in spinal cord, where the laterally positioned V0 and V1 interneurons, which are characteristic of lateral cell fates, persist in Shh$^{-/-}$

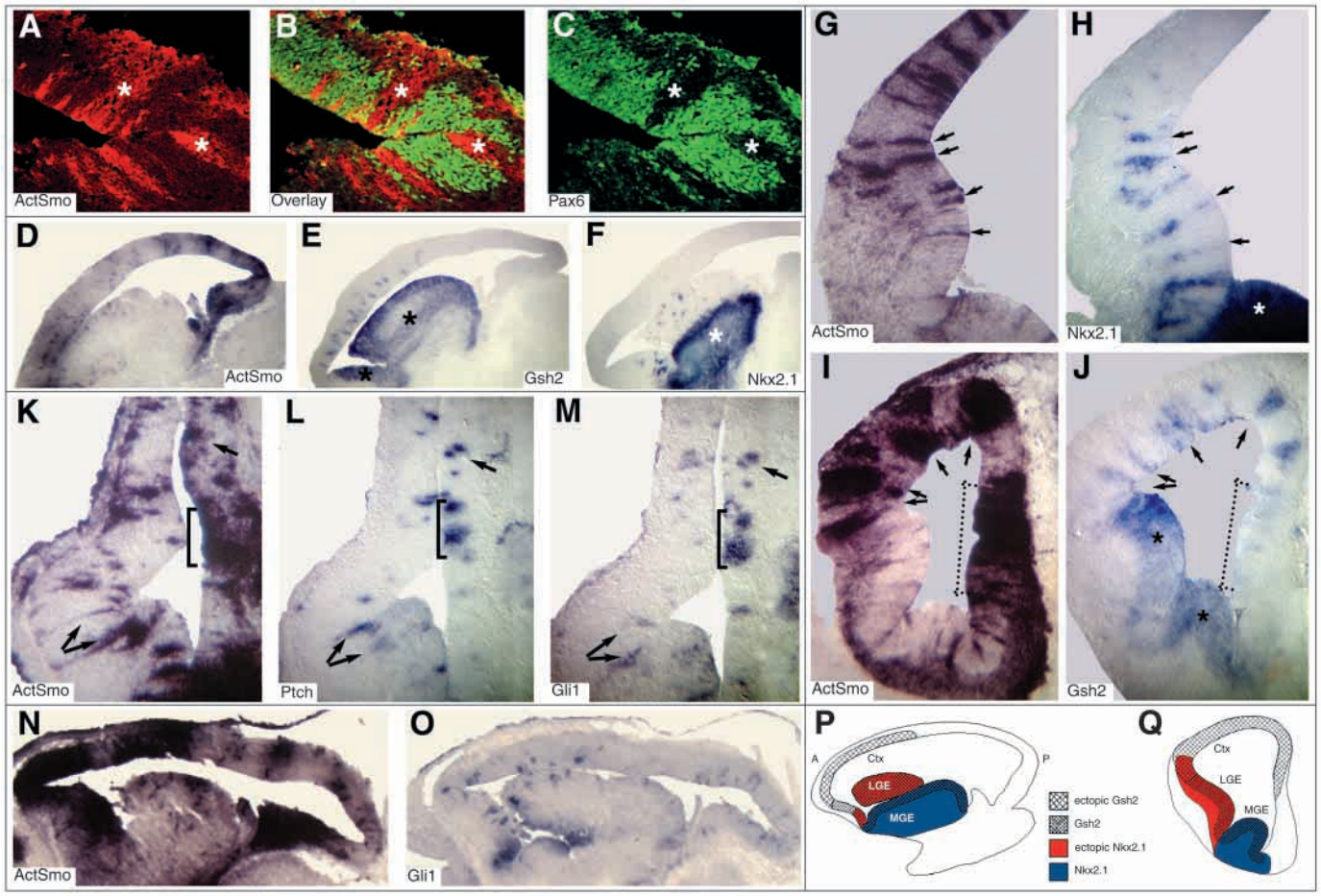

Fig. 2. Effects of ectopic Shh signaling on regional patterning in the telencephalon. Embryos were injected with ActSmo-expressing virus at E9.5 and analyzed at E14.5. Histochemical staining for PLAP reveals the location of virally infected cells co-expressing ActSmo (A,D,G,I,K,N). Adjacent sections were assayed for regionally expressed homeodomain genes (A-J) or for the expression of downstream targets of Shh signaling pathway (K-O). (A-C) Confocal immunohistochemistry for PLAP (A) and Pax6 (C) proteins within the lateral neocortex shows a repression of Pax6 expression in clusters of cells infected by ActSmo (asterisks). (G-J) Coronal sections of ActSmo-infected brains stained for ventral genes expression. Ectopic *Nkx2.1* expression is restricted to the LGE and lateral neocortex (H, arrows), whereas *Gsh2* expression can be induced throughout the neocortex (J, arrows). Dotted bracket illustrates weaker levels of induction within the medial neocortex. Sagittal sections (D-F) reveal a similar restriction along the rostrocaudal extent of the telencephalon. Asterisks in D-J represent the sites of endogenous expression. (K-O) Ectopic expression of ActSmo induces the upregulation of *Gli1* and *Ptch* expression all along the dorsoventral (L,M, arrows and bracket) or anteroposterior (O) axes, showing that the telencephalon has the competency to respond to Shh signaling at this age. The mismatch between areas of viral infection and *Ptch* or *Gli1* induction reflects the fact that these are visualized in adjacent sections. This is unavoidable because of the incompatibility of PLAP histochemistry and in situ hybridization. (P,Q) Schematic representations of a sagittal (P) and coronal (Q) section showing *Nkx2.1* and *Gsh2* endogenous domains of expression as well as the regions where their expression can be induced by Shh signaling.

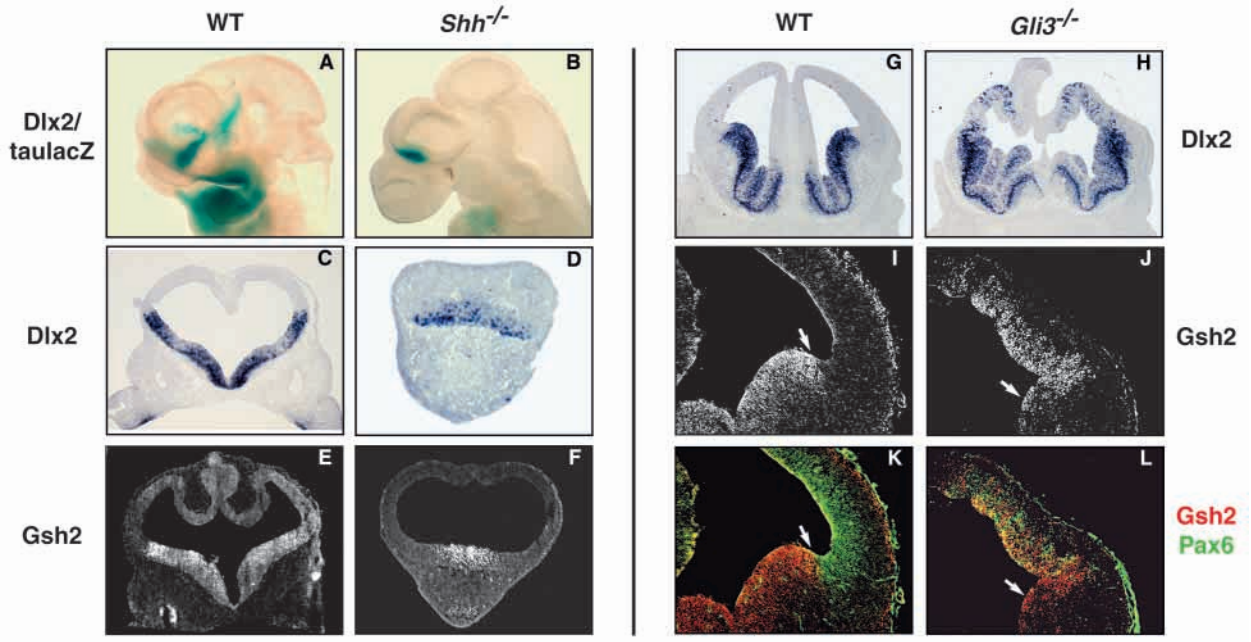

Fig. 3. *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ mutants show opposite telencephalic phenotypes. Pan-ventral gene expression was analyzed in E10.5 *Shh*$^{-/-}$ (A-F) and E12.5 *Gli3*$^{-/-}$ (G-L) mutants. *Dlx2* and *Gsh2* expression is maintained but displaced ventrally in *Shh*$^{-/-}$ embryos (B,D,F). By contrast, *Dlx2* and *Gsh2* expression expands dorsally in *Gli3*$^{-/-}$ mutants (H,J,L). (K,L): Gsh2 and Pax6 expression domains abut at the corticostriatal boundary in wild-type animals (K, arrow). This boundary is affected in *Gli3*$^{-/-}$ embryos (L), with *Gsh2* expression expanding dorsally. *Dlx2* expression was assayed either by X-gal staining on whole-mount embryos (A,B) or by in situ hybridization on coronal sections (C,D,G,H). Gsh2 expression was assayed by immunofluorescence against the Gsh2 protein (E,F,I-L).

embryos but are displaced toward the midline (Pierani et al., 1999). The observation that ventral gene expression can occur in the absence of Shh suggests the existence of a Shh-independent pathway. This prompted us to examine other genes that could play a role in establishing dorsoventral patterning in the telencephalon.

Shh has been shown to be required to antagonize Gli3 function in ventral regions of the spinal cord (Litingtung and Chiang, 2000a). In the telencephalon, *Gli3* is expressed at high levels in dorsal and lateral regions (cortex and LGE) and at low levels ventrally (in the MGE). Interestingly, *extra-toes* mice (*Xt*^{J}), which carry a deletion encompassing the *Gli3* gene (Buscher and Ruther, 1998; Hui and Joyner, 1993; Schimmang et al., 1992; Vortkamp et al., 1992), show defects opposite to those observed in *Shh* mutants. In *Gli3* mutants the morphology of the forebrain is perturbed dorsally: the cortex is highly reduced in size, and the hippocampus and the choroid plexus are absent (Grove et al., 1998; Theil et al., 1999; Tole et al., 2000). Furthermore, the expression of a number of pan-ventral genes, such as, *Dlx2* (Fig. 3G-H) (Tole et al., 2000), *Gsh2* (Fig. 3I-J) and the bHLH gene *Mash1* (data not shown), spreads precociously into cortical areas. Even though ventral markers were ectopically expressed, the cortex of *Gli3* mutants retained its dorsal character, as shown by the persistence of dorsal gene expression, including *Pax6* (Fig. 3K,L) (Tole et al., 2000). Interestingly, Gsh2 and Pax6 proteins appeared to be co-expressed in the cortex of *Gli3* mutants, as shown by double immunostaining (Fig. 3L). *Gsh2* and *Pax6* expression domains generally abut at the boundary between the cortex and the LGE (i.e. the corticostriatal boundary) (Fig. 3K) and these proteins are normally mutually repressive (Toresson et al., 2000; Yun et al., 2001). Our results showed that *Gsh2* expression no longer respects this boundary in the telencephalon of *Gli3* mutants, suggesting that the repression of Gsh2 by Pax6 is dependent on Gli3.

Partial rescue of dorsoventral patterning in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants

In order to analyze the extent to which dorsoventral patterning can occur in the absence of *Shh* and *Gli3*, we analyzed the telencephalic phenotype of Shh/Gli3 compound mutants. Interestingly, *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos displayed a remarkable rescue of brain morphology when compared with *Shh*$^{-/-}$ embryos. Although the telencephalon of *Shh*$^{-/-}$ embryos at E12.5 was greatly reduced in size and composed of a single vesicle fused at the midline (Fig. 4B,B′) (Chiang et al., 1996), *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos had a much bigger telencephalon in which two vesicles were clearly discernable (Fig. 4C,C′, arrows). Moreover, these embryos had a reduced proboscis (arrowheads) and two relatively well-formed eyes, albeit fused together and located at the midline (Fig. 4D-S, arrows).

As these embryos showed a significant morphological rescue of the *Shh*-null phenotype, we analyzed in detail the status of dorsoventral patterning in the telencephalon. We observed that *Dlx2* expression, which was shifted either ventrally or dorsally in *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ mutants, respectively, was restored in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos to a level and distribution which closely resembled the wild-type pattern (Fig. 4D,E). A similar rescue was observed for other pan-ventral genes such as *Gsh2* and *Mash1* (Fig. 4J-M). The restoration of the expression of pan-ventral genes to their normal regions was further demonstrated by the existence of a sharp boundary between the

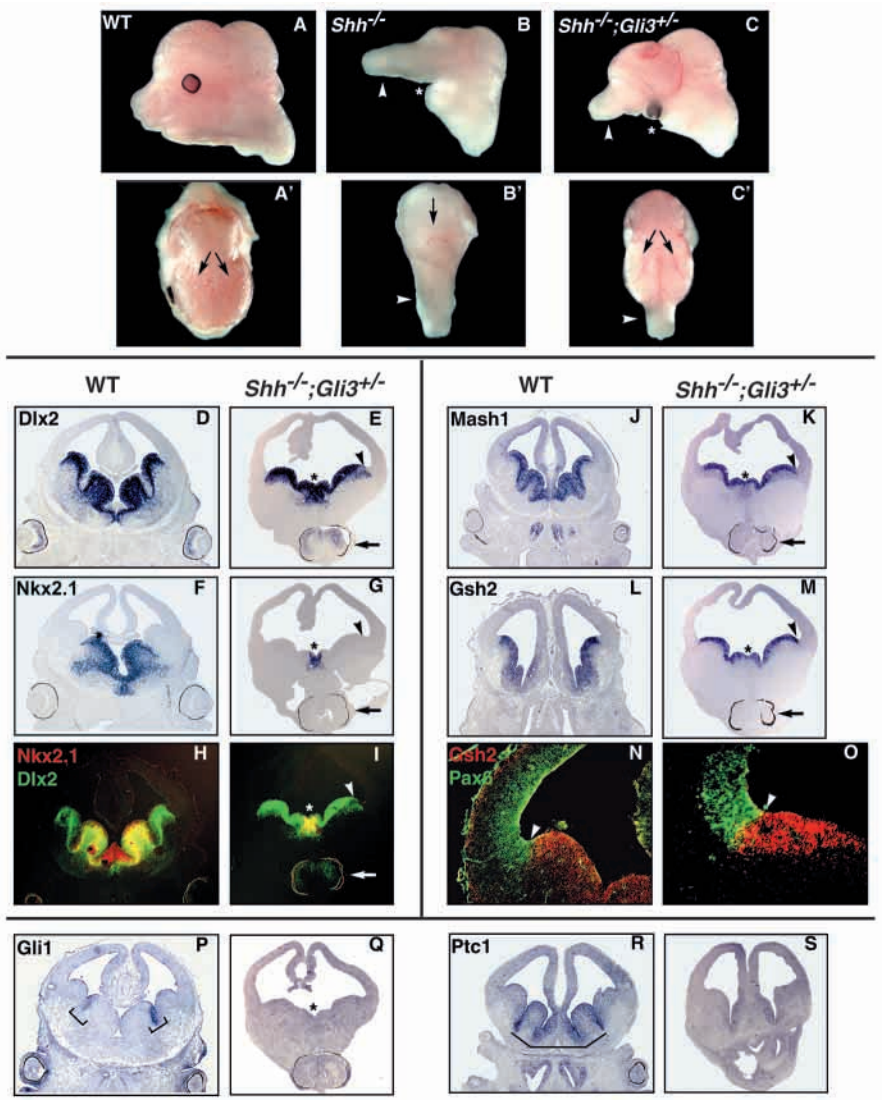

Fig. 4. Dorsoventral patterning of the telencephalon is largely restored in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants. Lateral (A-C) and dorsal (A′-C′) views of the heads from wild-type, *Shh*$^{-/-}$ and *Shh*$^{-/-}$;*Gli3*$^{+/-}$ E12.5 embryos, showing the extent of the phenotypic rescue. The telencephalon of *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos is formed of two paired vesicles (arrows), in sharp contrast to the phenotype of *Shh*$^{-/-}$ embryos. Note that the eye phenotype is also partially rescued as indicated by the presence of two distinct eyes, albeit fused at the midline and the proboscis observed in *Shh*$^{-/-}$ animals is reduced in size (asterisks in B,C; arrows in E,G,K,M; arrowheads in B-C′). (D-O) Coronal sections of wild-type and *Shh*$^{-/-}$;*Gli3*$^{+/-}$ E12.5 embryos assayed for various region-specific homeobox gene expression. *Dlx2* (D,E), *Mash1* (J,K) and *Gsh2* (L,M) expression in the mutants closely resembles that in wild-type embryos, showing that ventrolateral patterning is established normally in this context. Double immunohistochemistry for Gsh2 and Pax6 further demonstrate that LGE- and cortex-like structures are properly established in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ animals (N,O). Arrowheads delineate the boundary between the LGE and the cortex. In the ventral midline, a small region of *Nkx2.1* expression is observed in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos (G,I, asterisks). Overlay of *Nkx2.1* (red) and *Dlx2* (green) RNA in situ hybridization from adjacent sections using Adobe Photoshop 4, shows the nested pattern of *Nkx2.1* within a broader *Dlx2* domain in both wild-type and mutant embryos (H,I). (P-S) Coronal sections of wild-type and *Shh*$^{-/-}$;*Gli3*$^{+/-}$ E12.5 embryos assayed for *Gli1* (P,Q) and *Ptch* (R,S) expression, showing that the Shh pathway is not active. Brackets in P,R indicate the extent of *Gli1* (P) or *Ptch* (R) expression.

Gsh2 and *Pax6* expression domains, similar to what is observed at the corticostriatal boundary of wild-type brains (Fig. 4N,O). Notably, while the telencephalic morphology of the *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos was affected, a thickening in the lateral wall of the telencephalon was observed ventrally to the *Pax6* expression domain in a region where pan-ventral genes were strongly expressed (Fig. 4D-S; arrowheads). Thus, it seems that the LGE is properly specified in these mutants.

To further investigate to what extent dorsoventral patterning was restored in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos, we looked at the expression of the homeobox gene *Nkx2.1*, which is missing in *Shh*$^{-/-}$ embryos. Surprisingly, we observed that low levels of *Nkx2.1* expression were rescued in these embryos (Fig. 4F,G). In these animals *Nkx2.1* expression was specifically located in the most ventral part of the telencephalon and was nested within the domains of expression of *Dlx2* (Fig. 3H,I), *Gsh2* and *Mash1*. Thus, although not very prominent, it appeared that a small MGE-like structure can also form in these embryos in absence of Shh (Fig. 4D-S, asterisks). The high levels of recovery observed in *Shh* mutants where only one copy of *Gli3* gene is removed prompted us to investigate the extent of recovery in animals lacking both *Shh* and *Gli3* gene function.

Dorsoventral patterning is largely restored in the telencephalon of *Shh/Gli3* double mutants

Telencephalic analysis of *Shh/Gli3* double homozygous mutants was extremely difficult as a result of a high rate of exencephaly. Most of the *Shh/Gli3* double homozygous mutants analyzed were exencephalic (11/12=91%): the neural tube failed to close at various levels of the forebrain or midbrain and a massive overgrowth of the tissue disrupted telencephalic morphology. We found that *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos had a higher proportion of exencephaly than did *Gli3*$^{-/-}$ (2/12=16.6%) or *Gli3*$^{-/-}$;*Shh*$^{+/-}$ (10/32=31%) embryos. Although exencephalic mutants could not be analyzed for dorsal patterning defects, ventral patterning was analyzed in parallel to non-exencephalic mutants. As the exencephalic defects were more severe at E12.5 than at E10.5, we chose to analyze the double homozygous mutants at E10.5.

In a *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutant embryo that was not exencephalic, the telencephalon appeared to have a relatively normal morphology, although dorsal midline structures were missing (Fig. 5; arrows). The level and distribution of expression of pan-ventral genes such as *Dlx2* and *Gsh2* was similar in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos and in their wild-type

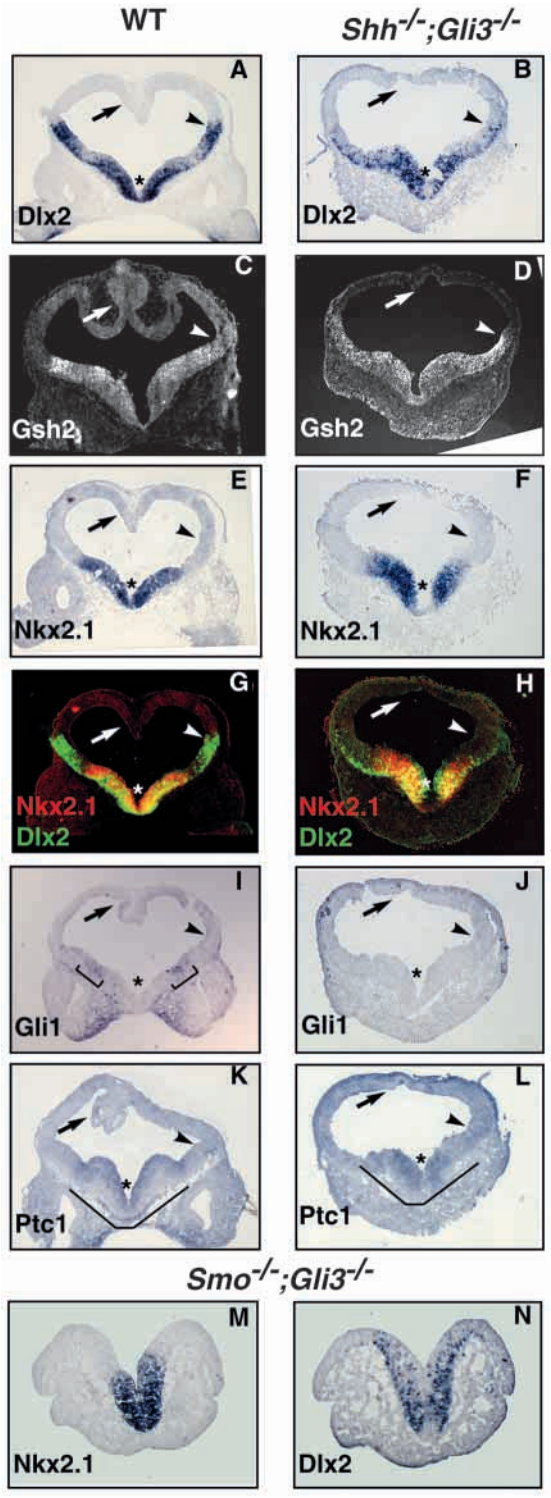

Fig. 5. Dorsoventral patterning is established properly in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ double mutants. (A-L) Coronal sections of wild-type and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ E10.5 embryos. The telencephalic morphology appears relatively normal in the mutants, except for the loss of dorsal midline structures (arrows). (A-L) Analysis of various homeodomain genes expression. *Dlx2* expression assayed by RNA in situ hybridization (A,B) and Gsh2 expression assayed by immunofluorescence (C,D) show that normal ventrolateral patterning is established in the double homozygous mutants. Arrowheads delineate the boundary between lateral and dorsal regions in both wild-type and mutant embryos. *Nkx2.1* expression also appears normal (E,F). Overlay of *Nkx2.1* (red) and *Dlx2* (green) RNA in situ hybridization on adjacent sections using Adobe Photoshop 4 (G,H) shows a similar nested pattern of expression for these two genes in wild-type and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double homozygous embryos. This reveals the existence of ventral and lateral structures similar to the MGE and LGE in the wild-type embryos (asterisk and arrowhead, respectively). *Gli1* expression is absent in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (I,J), showing that the Shh pathway is not active. Brackets in I indicate *Gli1* expression. (K,L) *Ptch* is expressed at low levels in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, with higher expression in ventral areas (bracket), similar to wild-type embryos. (M,N) Analysis of ventral genes expression in *Smo*$^{-/-}$;*Gli3*$^{-/-}$ E10.5 embryos. As a result of exencephaly, the dorsal telencephalic structures are disrupted. Despite this, the ventral structures can still be analyzed. *Nkx2.1* and *Dlx2* are expressed in ventral areas of the telencephalon. Despite the exencephalic morphology of the mutants ventral pattern appears unperturbed, as evidenced by the presence of *Nkx2.1* expression nested within the broader *Dlx2* domain. This suggests that MGE and LGE cell fates are specified properly in *Smo*$^{-/-}$;*Gli3*$^{-/-}$ double mutants.

littermates (Fig. 5A-D). Remarkably, in contrast to *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$ embryos, double homozygous mutants had a complete rescue of *Nkx2.1* expression (Fig. 5E,F). Similar to wild-type animals, *Nkx2.1* expression was observed in an area nested within the wider expression domain of *Dlx2* (Fig. 5G,H, asterisk) and *Gsh2* (Fig. 5C,D). This nested expression pattern suggested that both the MGE and the LGE were specified in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants. However, in the absence of a more refined analysis of gene expression patterns within the ventral telencephalon, we cannot exclude the possibility that some aspects of dorsoventral patterning (such as the ventral midline) are not restored in these mutants. Nonetheless, our data suggest that a key role of Shh in patterning the ventral telencephalon is to inhibit *Gli3* function, thereby indirectly allowing ventral gene expression. Complementary to the restoration of ventral patterning defects observed in *Shh*$^{-/-}$ mutants, the dorsal telencephalic defects present in *Gli3*$^{-/-}$ mutants were also rescued in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants. This argues for a requirement for Gli3 in antagonizing Shh function in the dorsal telencephalon, a role apparently not played by Gli3 in the spinal cord (Litingtung and Chiang, 2001a).

The persistence of dorsoventral patterning in the absence of *Shh* and *Gli3* gene function could be the result of compensation by other members of the hedgehog family or the actions of a hedgehog-independent pathway. To differentiate between these possibilities, we analyzed ventral telencephalic gene expression in *Smoothened/Gli3* double mutants, in which all hedgehog signaling is abrogated (Zhang et al., 2001). Although, as in the *Shh/Gli3* mutants, these animals suffer from a high rate of exencephaly, ventral aspects of the *Smo/Gli3* telencephalon can still be analyzed. In these mutants both pan-ventral markers such as *Dlx2*, as well as the MGE-specific marker *Nkx2.1* were expressed in their normal distribution (Fig. 5M,N). Furthermore, the fact that *Nkx2.1* expression was nested within the *Dlx2* expression domain suggested that ventral patterning is largely rescued in these compound mutants. Thus, our results reveal that, with the exception of the dorsal midline, a hedgehog-independent signaling pathway is able to establish at least basic elements of dorsoventral patterning in the telencephalon in the absence of both *Gli3* and *Shh or Smo* gene function.

To determine whether the restoration of dorsoventral

patterning in *Shh*$^{-/-}$ mutants when *Gli3* is removed (*Shh*$^{-/-}$;*Gli3*$^{-/-}$) was due to an activation of downstream elements of the Shh pathway independent of Shh protein, we examined the levels of expression of *Gli1* and *Ptch*, two targets of Shh signaling. *Gli1* and *Ptch* are normally expressed in the ventral telencephalon in close proximity to the sites of Shh expression (Platt et al., 1997). *Gli1* expression in the telencephalon is restricted to a small region at the border of the MGE and the LGE (Fig. 4P, Fig. 5I; brackets). As in *Shh*$^{-/-}$ mutants (data not shown) this expression was absent in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (Fig. 4Q, Fig. 5J), consistent with the suggestion that *Gli1* activation is strictly dependent on Shh. In wild-type animals *Ptch* is expressed in a broader region than *Gli1*, encompassing the entire extent of the MGE (Fig. 4R, Fig. 5K; brackets). Whereas *Ptch* expression is absent in *Shh*$^{-/-}$ and *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants (data not shown and Fig. 4S), low levels of *Ptch* expression were detected throughout the telencephalon in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, with higher levels ventrally (Fig. 5L, brackets). Higher levels of expression ventrally were also observed in double homozygous mutants that were exencephalic (data not shown). This suggests that *Ptch* expression is dependent on the removal of Gli3 repressor activity in ventral areas and that the loss of *Ptc1* expression observed in *Shh*$^{-/-}$ mutants may be due to a lack of inhibition of Gli3 function.

DISCUSSION

In this paper, we present evidence for the existence of a pathway that can establish elements of dorsoventral patterning in the telencephalon in the absence of both hedgehog signaling and *Gli3* gene function. We find that ectopic activation of the Shh pathway at E8.5/E9.0 results in ventralization of the telencephalon. Importantly, it appears that the ventral identity induced upon ectopic activation of the Shh pathway is determined by the dorsoventral position of Shh misexpression. Second, we find that some remnants of ventral gene expression persist in *Shh*$^{-/-}$ mutant animals and, in a complementary fashion, these same ventral genes are expanded in *Gli3*$^{-/-}$ mutants. Third, analysis of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ mutants shows that aspects of dorsoventral pattern are established in the telencephalon relatively normally in the absence of hedgehog signaling and *Gli3* gene function. Nevertheless, we cannot rule out that a more refined analysis of ventral patterning in the telencephalon in these compound mutants will not reveal further defects. Regardless of this caveat, this analysis suggests that the primary role of Shh in the telencephalon is to repress Gli3 function ventrally, and that the primary requirement for Gli3 is to repress Shh function in dorsal areas. The notable exception to this relationship is in the dorsal midline, which is disrupted in *Shh*, *Gli3* and *Shh/Gli3* double homozygous mutants. Indeed, the more penetrant exencephalic phenotype in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants argues that these genes may actually cooperate in this region.

Telencephalic competence to respond to Shh signaling

In the present study, we undertook an in vivo gain-of-function approach to address the role of Shh in telencephalic patterning. By infecting the mouse telencephalon at different time points (E8.5 to E10.5), we showed that the differential induction of MGE and LGE/MGE markers by Shh signaling was determined by the intrinsic character of the infected tissues, within both the dorsoventral and anteroposterior axes. Although it could be argued that the failure to induce *Nkx2.1* in dorsal regions of the telencephalon or *Gsh2* in dorsomedial telencephalon was the result of our viral vectors producing insufficient levels of Shh signaling, results from our previous studies argue against this (Kohtz et al., 1998). Indeed, *Nkx2.1* expression could not be induced in vitro by Shh in E11.5 rat dorsal telencephalic explants (E9.5 in mouse), even when they were exposed to extremely high levels of recombinant protein (i.e. 300 nM). Furthermore, the differential induction we observed with our viral vectors did not appear to be the result of a differential activation of the Shh pathway in dorsal and lateral regions, as *Gli1* and *Ptch* could be induced ectopically in the telencephalon, regardless of where or when the infection occurred. Thus, our gain-of-function data suggest that by E8.5/E9.0 the telencephalon has been patterned along both the dorsoventral and anteroposterior axis. However, these experiments cannot distinguish whether this apparent prepattern is the result of hedgehog independent signaling or patterning by Shh prior to E9.0. In support of the latter suggestion, work by the Edlund laboratory suggests that the telencephalic expression of *Nkx2.1* results from Shh signaling in the node (Gunhaga et al., 2000). Similarly, we found that rat explants at headfold stage (E9.5 in rat, E8.0 in mouse) could uniformly induce *Nkx2.1* expression in response to recombinant Shh protein (Kohtz et al., 1998), suggesting that the restriction observed in this paper may not exist in slightly younger embryos (before E9.0).

Even if Shh normally acts to prepattern the telencephalon prior to E9.0, our results show that some ventral pattern is established in the telencephalon in absence of Shh. Specifically, we found that *Dlx2* and *Gsh2* expression within the ventral telencephalon is reduced but not absent in *Shh*$^{-/-}$ mutant embryos. This result is reminiscent of the persistence of V0 and V1 cell fates in the spinal cord of *Shh*$^{-/-}$ mutants (Pierani et al., 1999). Given that in some contexts members of the hedgehog family can partially compensate for the loss of *Shh* (Pathi et al., 2001), perhaps the persistence of ventrolateral markers indicates the presence of other hedgehog ligands. Indeed, Indian and sonic hedgehog are both expressed in the early foregut (Bitgood and McMahon, 1995; Ingham and McMahon, 2001). Unfortunately, this question cannot be addressed directly as *Smo*$^{-/-}$ mutants die before any telencephalic patterning is molecularly evident (Zhang et al., 2001). However, based on the analysis of *Smo/Gli3* double homozygous mutants (see below), such compensation appears unlikely.

The regulation of ventral and lateral patterning in the telencephalon by Shh and Gli3.

To further investigate the cross-repressive interaction between Shh and Gli3 in the telencephalon, we analyzed several *Shh* and *Gli3* mutant combinations. The development of the telencephalon is grossly abnormal in *Shh*$^{-/-}$ (Chiang et al., 1996) and to a lesser extent in *Gli3*$^{-/-}$ mutants (Grove et al., 1998; Theil et al., 1999). We found that telencephalic morphology is largely restored in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants. Consistent with their rescued morphology, most ventrolateral

patterning is established in *Shh* mutants lacking a single copy of *Gli3* (*Shh*$^{-/-}$;*Gli3*$^{+/-}$). Furthermore, barring the loss of dorsal midline structures, mutants lacking both *Shh* and *Gli3* function possess sets of markers characteristic of MGE, LGE and cortex, showing that all these structures are at least partially specified in the absence of *Shh* and *Gli3*. The same result was obtained in *Smo*$^{-/-}$;*Gli3*$^{-/-}$ mutants in which all hedgehog signaling is abolished. Therefore, many aspects of dorsoventral telencephalic patterning can be established in the complete absence of hedgehog provided that *Gli3* gene function is also abolished.

Despite this, the interplay between Shh and Gli3 is crucial to the normal positioning of the different domains of ventral telencephalic gene expression. For example, our data suggests that MGE and LGE markers are differentially sensitive to the dose of Gli3 protein or activity. Although the expression of pan-ventral genes characteristic of the LGE is fully restored in *Shh*$^{-/-}$ animals with one copy of *Gli3* removed, the MGE marker *Nkx2.1* is only rescued in the complete absence of *Gli3* gene function. Consistent with this, unlike more broadly expressed ventral markers, *Nkx2.1* cannot be induced by ectopic Shh signaling in the cortex, where *Gli3* is highly expressed. Similarly, *Nkx2.1* expression does not spread dorsally in *Gli3*$^{-/-}$ mutants, whereas pan-ventral gene expression does. One explanation for these findings may be that *Gli2*, which is expressed in a pattern similar to *Gli3* in the telencephalon, possesses weaker repressor activity than *Gli3* and acts in a complementary fashion. In this scenario, *Gli2* prevents the expansion of *Nkx2.1* expression in *Gli3*$^{-/-}$ mutants but is insufficient to repress pan-ventral genes, such as *Dlx2* and *Mash1*. Indeed, the greater sensitivity of MGE markers to repression by Gli repressors may be one mechanism that underlies the differential specification of the MGE and LGE.

In the limb, the processing of Gli3 into an N-terminal repressor form is negatively regulated by Shh (Wang et al., 2000). As a result, Shh and the N-terminal Gli3 repressor are thought to form opposing gradients in the limb, akin to that suggested by our genetic analysis of the telencephalon. Our results also in many respects parallel the phenotype found in the spinal cord of *Shh/Gli3* double mutants (Litingtung and Chiang, 2000a). Similar to the telencephalon, ventrolateral cell fates in the spinal cord, including motoneurons and V0-V2 interneurons, were restored to wild-type levels in *Shh/Gli3* double homozygous mutants. However, unlike the telencephalon, the ventral-most fates fail to form in the spinal cord of these same animals (as indicated by the loss of *Nkx2.2* at the floorplate). Moreover, only partial rescue of ventrolateral genes expression was observed in the spinal cord of these mutants, while ventrolateral telencephalic gene expression appeared normal in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants. Furthermore, in the telencephalon the antagonism between Shh and Gli3 is reciprocal. By contrast, there is no evidence that Gli3 may antagonize Shh function in the spinal cord, as ventral gene expression does not expand dorsally in this tissue in the absence of *Gli3* gene function.

Evidence for a hedgehog independent pathway in patterning the ventral telencephalon

In light of the *Shh/Gli3* and *Smo/Gli3* double mutants phenotypes, it is worth considering how other signaling pathways might contribute to dorsoventral telencephalic patterning. It is possible that hedgehog-dependent and -independent pathways act in parallel and are functionally redundant, each able to specify ventral, lateral and dorsal cell fates in isolation. When hedgehog signaling is missing, as it is in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Smo*$^{-/-}$;*Gli3*$^{-/-}$ mutants, such redundancy would account for the observed rescue of dorsoventral patterning (Fig. 6). The use of two pathways that complement each other is reminiscent of the mechanism of anterior patterning in *Drosophila*. In flies, opposing anterior and posterior gradients specify the segmented body pattern. Maternal nanos protein acts as a repressor of hunchback in the posterior region, allowing abdominal patterning to occur; and the absence of both genes results in normal embryos (Hulskamp et al., 1989; Irish et al., 1989; Struhl, 1989). In parallel, bicoid specifies anterior patterning and hunchback can substitute for it in thorax and abdomen (Simpson-Brose et al., 1994; Wimmer et al., 2000).

Alternatively, we cannot formally exclude the possibility that the dorsoventral patterning of the telencephalon is initially established by a hedgehog-independent pathway and that the

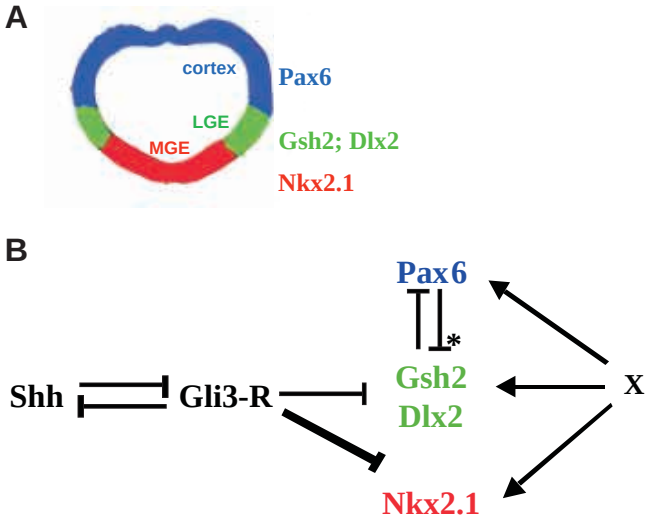

Fig. 6. Model of genetic interactions between *Shh* and *Gli3* in patterning the mouse telencephalon. (A) Schematic representation of a coronal section through an E10.5 mouse telencephalon, highlighting different domains along the dorsoventral axis. The prospective regions of the cortex (blue), the LGE (green) and the MGE (red) are shown. On the right, homeodomain genes are indicated that are expressed in these regions and have been shown to play an essential role in patterning the telencephalon (reviewed by Wilson and Rubenstein, 2000). Note that expression of *Gsh2* is only restricted to the lateral telencephalon for a short window of embryonic development (E10.0-E10.5) (Corbin et al., 2000). Ultimately *Gsh2* is expressed in both the LGE and MGE. (B) The results of the present study demonstrate that Shh is inducing ventral (*Nkx2.1*) and lateral (*Gsh2*, *Dlx2*) telencephalic patterning indirectly, through the inhibition of Gli3 repressor activity. Note that the regulation of *Nkx2.1* expression appears to be different from that of pan-ventral genes, as it is not affected in *Gli3*$^{-/-}$ single mutants (bold blunt arrow). Gsh2 expression and Pax6 expression at the corticostriatal boundary are repressed by each other. However, the repression of *Gsh2* expression by *Pax6* appears to be regulated by Gli3 (asterisk), as co-expression of these two genes is observed in the cortex of *Gli3*$^{-/-}$ mutants. Our data reveal the existence of an unknown hedgehog-independent pathway (X).

role of Shh in patterning the telencephalon is to subsequently maintain or refine the organization of the telencephalon. Perhaps Shh and Gli3, through their antagonistic interactions, act to stabilize dorsoventral telencephalic domains during mid-neurogenesis. This might occur by regulating the growth of the progenitor pools, by maintaining a pre-established pattern or by refining the regional boundaries between discrete territories, such as the MGE, LGE and cortex.

The identity of the signal(s) that lead to a normal dorsoventral patterning in the absence of *Shh* and *Gli3* remains unknown. One possibility is that the dorsoventral patterning observed in *Shh/Gli3* double mutants is the result of BMP signaling, which in spinal cord acts to induce dorsal and repress ventral gene expression. Recent studies in the spinal cord have also shown that BMPs antagonizes Shh signaling and that this inhibition may be crucial to the establishment of dorsal identities (Liem et al., 2000). It seems unlikely that the late dorsal expression of BMPs contributes to hedgehog-independent patterning as their expression requires Gli3 (Theil et al., 1999). However, earlier BMP7 expression in the prechordal plate or BMP4 expression in presomitic mesoderm might be playing a role (Dale et al., 1997; Dale et al., 1999), as might Nodal signaling (Rohr et al., 2001). Another alternative comes from studies in spinal cord that have implicated the retinoid signaling pathway in the generation of ventrolateral progenitors (Pierani et al., 1999). In the telencephalon, markers of retinoid synthesis are expressed in the LGE and the developing striatum (Li et al., 2000; Toresson et al., 1999) and retinoid signaling regulates striatal neuronal differentiation (Toresson et al., 1999). However, this expression occurs too late to play a role in the initial patterning of the telencephalon. If retinoids do act in the establishment of dorsoventral pattern in the telencephalon, a more likely source is the lateral cranial mesoderm, which expresses high levels of retinoids and is proximal to the telencephalon during the headfold stage ~E7.5/E8.0 (LaMantia et al., 1993). A third candidate is the Wnt pathway. Recent work has implicated an important role for Wnt inhibitors in the specification of the head (dickkopf) (Niehrs et al., 2001) as well as the telencephalon (*masterblind*) (Heisenberg et al., 2001; van de Water et al., 2001). Furthermore, a novel secreted Frizzled-related protein expressed in the anterior neural plate at the junction between neural and non-neural ectoderm is involved in promoting telencephalic development within the forebrain territory (Houart et al., 2002). Prior to neural tube closure, the dorsoventral axis of the telencephalon seems to be roughly translated into an anteroposterior axis (Shimamura et al., 1995). Thus, it is worth considering that, in addition to specifying the telencephalon as a whole, graded inhibition of Wnt signaling may also act to establish dorsoventral identity in the telencephalon.

Regardless of the pathway, it is clear that a complete appreciation of regional patterning within the telencephalon will require more than understanding the hedgehog signaling pathway. Determining the nature of these signals and how they act to complement the hedgehog pathway in patterning the telencephalon will no doubt prove interesting.

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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.

Aza-Blanc, P. and Kornberg, T. B. (1999). Ci: a complex transducer of the hedgehog signal. *Trends Genet.* **15**, 458-462.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

Briscoe, J., Chen, Y., Jessell, T. M. and Struhl, G. (2001). A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol. Cell* **7**, 1279-1291.

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

Briscoe, J., Pierani, A., Jessell, T. M. and Ericsson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101**, 435-445.

Buscher, D. and Ruther, U. (1998). Expression profile of Gli family members and Shh in normal and mutant mouse limb development. *Dev. Dyn.* **211**, 88-96.

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.

Corbin, J. G., Gaiano, N., Machold, R. P., Langston, A. and Fishell, G. (2000). The Gsh2 homeodomain gene controls multiple aspects of telencephalic development. *Development* **127**, 5007-5020.

Dahmane, N. and Ruiz-i-Altaba, A. (1999). Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* **126**, 3089-3100.

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.

Dale, K., Sattar, N., Heemskerk, J., Clarke, J. D., Placzek, M. and Dodd, J. (1999). Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin. *Development* **126**, 397-408.

Ericsson, J., Muhr, J., Jessell, T. M. and Edlund, T. (1995a). Sonic hedgehog: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Int. J. Dev. Biol.* **39**, 809-816.

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

Ericsson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericsson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Fishell, G. (1997). Regionalization in the mammalian telencephalon. *Curr. Opin. Neurobiol.* **7**, 62-69.

Gaiano, N., Kohtz, J. D., Turnbull, D. H. and Fishell, G. (1999). A method for rapid gain-of-function studies in the mouse embryonic nervous system. *Nat. Neurosci.* **2**, 812-819.

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.

Grove, E. A., Tole, S., Limon, J., Yip, L. and Ragsdale, C. W. (1998). The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. *Development* **125**, 2315-2325.

Guillemot, F. and Joyner, A. L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. *Mech. Dev.* **42**, 171-185.

Gunhaga, L., Jessell, T. M. and Edlund, T. (2000). Sonic hedgehog signaling at gastrula stages specifies ventral telencephalic cells in the chick embryo. *Development* **127**, 3283-3293.

Hauptmann, G. and Gerster, T. (1996). Complex expression of the zp-50 pou gene in the embryonic zebrafish brain is altered by overexpression of sonic hedgehog. *Development* **122**, 1769-1780.

Heisenberg, C. P., Houart, C., Take-Uchi, M., Rauch, G. J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M. L., Geisler, R. et al. (2001). A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev.* **15**, 1427-1434.

Houart, C., Caneparo, L., Heisenberg, C., Barth, K., Take-Uchi, M. and Wilson, S. (2002). Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. *Neuron* **35**, 255-265.

Hsieh-Li, H. M., Witte, D. P., Szucsik, J. C., Weinstein, M., Li, H. and Potter, S. S. (1995). Gsh-2, a murine homeobox gene expressed in the developing brain. *Mech. Dev.* **50**, 177-186.

Huh, S., Hatini, V., Marcus, R. C., Li, S. C. and Lai, E. (1999). Dorsal-ventral patterning defects in the eye of BF-1-deficient mice associated with a restricted loss of shh expression. *Dev. Biol.* **211**, 53-63.

Hui, C. C. and Joyner, A. L. (1993). A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat. Genet.* **3**, 241-246.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-413.

Hulskamp, M., Schroder, C., Pfeifle, C., Jackle, H. and Tautz, D. (1989). Posterior segmentation of the Drosophila embryo in the absence of a maternal posterior organizer gene. *Nature* **338**, 629-632.

Hynes, M., Ye, W., Wang, K., Stone, D., Murone, M., Sauvage, F. and Rosenthal, A. (2000). The seven-transmembrane receptor smoothened cell-autonomously induces multiple ventral cell types. *Nat. Neurosci.* **3**, 41-46.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Irish, V., Lehmann, R. and Akam, M. (1989). The Drosophila posterior-group gene nanos functions by repressing hunchback activity. *Nature* **338**, 646-648.

Jensen, A. M. and Wallace, V. A. (1997). Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* **124**, 363-371.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Kalderon, D. (2000). Transducing the hedgehog signal. *Cell* **103**, 371-374.

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.

LaMantia, A. S., Colbert, M. C. and Linney, E. (1993). Retinoic acid induction and regional differentiation prefigure olfactory pathway formation in the mammalian forebrain. *Neuron* **10**, 1035-1048.

Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A. and Drager, U. C. (2000). A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech. Dev.* **95**, 283-289.

Liem, K. F., Jr, Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-4866.

Litingtung, Y. and Chiang, C. (2000a). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat. Neurosci.* **3**, 979-985.

Litingtung, Y. and Chiang, C. (2000b). Control of Shh activity and signaling in the neural tube. *Dev. Dyn.* **219**, 143-154.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. *Dev. Biol.* **180**, 273-283.

Maynard, T. M., Jain, M. D., Balmer, C. W. and LaMantia, A. S. (2002). High-resolution mapping of the Gli3 mutation extra-toes reveals a 51.5-kb deletion. *Mamm. Genome* **13**, 58-61.

Murone, M., Rosenthal, A. and de Sauvage, F. J. (1999). Hedgehog signal transduction: from flies to vertebrates. *Exp. Cell Res.* **253**, 25-33.

Nery, S., Wichterle, H. and Fishell, G. (2001). Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain. *Development* **128**, 527-540.

Niehrs, C., Kazanskaya, O., Wu, W. and Glinka, A. (2001). Dickkopf1 and the Spemann-Mangold head organizer. *Int. J. Dev. Biol.* **45**, 237-240.

Pabst, O., Herbrand, H., Takuma, N. and Arnold, H. H. (2000). NKX2 gene expression in neuroectoderm but not in mesendodermally derived structures depends on sonic hedgehog in mouse embryos. *Dev. Genes Evol.* **210**, 47-50.

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., Nakashima, M. and Joyner, A. L. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* **127**, 1593-1605.

Pathi, S., Pagan-Westphal, S., Baker, D. P., Garber, E. A., Rayhorn, P., Bumcrot, D., Tabin, C. J., Blake Pepinsky, R. and Williams, K. P. (2001). Comparative biological responses to human Sonic, Indian, and Desert hedgehog. *Mech. Dev.* **106**, 107-117.

Patten, I. and Placzek, M. (2000). The role of Sonic hedgehog in neural tube patterning. *Cell Mol. Life Sci.* **57**, 1695-1708.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915.

Platt, K. A., Michaud, J. and Joyner, A. L. (1997). Expression of the mouse Gli and Ptc genes is adjacent to embryonic sources of hedgehog signals suggesting a conservation of pathways between flies and mice. *Mech. Dev.* **62**, 121-135.

Porteus, M. H., Bulfone, A., Ciaranello, R. D. and Rubenstein, J. L. (1991). Isolation and characterization of a novel cDNA clone encoding a homeodomain that is developmentally regulated in the ventral forebrain. *Neuron* **7**, 221-229.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rohr, K. B., Barth, K. A., Varga, Z. M. and Wilson, S. W. (2001). The nodal pathway acts upstream of hedgehog signaling to specify ventral telencephalic identity. *Neuron* **29**, 341-351.

Rowitch, D. H., St-Jacques, B., Lee, S. M., Flax, J. D., Snyder, E. Y. and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. *J. Neurosci.* **19**, 8954-8965.

Rubenstein, J. L. and Beachy, P. A. (1998). Patterning of the embryonic forebrain. *Curr. Opin. Neurobiol.* **8**, 18-26.

Ruiz i Altaba, A., Palma, V. and Dahmane, N. (2002). Hedgehog-Gli signaling and the growth of the brain. *Nat. Rev. Neurosci.* **3**, 24-33.

Schaeren-Wiemers, N. and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* **100**, 431-440.

Schimmang, T., Lemaistre, M., Vortkamp, A. and Ruther, U. (1992). Expression of the zinc finger gene Gli3 is affected in the morphogenetic mouse mutant extra-toes (Xt). *Development* **116**, 799-804.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* **121**, 3923-3933.

Shimamura, K. and Rubenstein, J. L. (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124**, 2709-2718.

Simpson-Brose, M., Treisman, J. and Desplan, C. (1994). Synergy between the hunchback and bicoid morphogens is required for anterior patterning in Drosophila. *Cell* **78**, 855-865.

Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in Drosophila embryos. *Nature* **338**, 741-744.

Sussel, L., Marin, O., Kimura, S. and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.

Theil, T., Alvarez-Bolado, G., Walter, A. and Ruther, U. (1999). Gli3 is required for Emx gene expression during dorsal telencephalon development. *Development* **126**, 3561-3571.

Tole, S., Ragsdale, C. W. and Grove, E. A. (2000). Dorsoventral patterning of the telencephalon is disrupted in the mouse mutant extra-toes(J). *Dev. Biol.* **217**, 254-265.

Toresson, H., Mata de Urquiza, A., Fagerstrom, C., Perlmann, T. and Campbell, K. (1999). Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation. *Development* **126**, 1317-1326.

Toresson, H., Potter, S. S. and Campbell, K. (2000). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. *Development* **127**, 4361-4371.

van de Water, S., van de Wetering, M., Joore, J., Esseling, J., Bink, R., Clevers, H. and Zivkovic, D. (2001). Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. *Development* **128**, 3877-3888.

Vortkamp, A., Franz, T., Gessler, M. and Grzeschik, K. H. (1992). Deletion of GLI3 supports the homology of the human Greig cephalopolysyndactyly syndrome (GCPS) and the mouse mutant extra toes (Xt). *Mamm. Genome* **3**, 461-463.

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.

Wechsler-Reya, R. J. and Scott, M. P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* **22**, 103-114.

Wichterle, H., Turnbull, D. H., Nery, S., Fishell, G. and Alvarez-Buylla, A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* **128**, 3759-3771.

Wilkinson, D. G. and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. *Methods Enzymol.* **225**, 361-373.

Wilson, S. W. and Rubenstein, J. L. (2000). Induction and dorsoventral patterning of the telencephalon. *Neuron* **28**, 641-651.

Wimmer, E. A., Carleton, A., Harjes, P., Turner, T. and Desplan, C. (2000). Bicoid-independent formation of thoracic segments in Drosophila. *Science* **287**, 2476-2479.

Xie, J., Murone, M., Luoh, S. M., Ryan, A., Gu, Q., Zhang, C., Bonifas, J. M., Lam, C. W., Hynes, M., Goddard, A. et al. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391**, 90-92.

Yun, K., Potter, S. and Rubenstein, J. L. (2001). Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. *Development* **128**, 193-205.

Zhang, X. M., Ramalho-Santos, M. and McMahon, A. P. (2001). Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. *Cell* **106**, 781-792.