Temporal requirement for hedgehog signaling in ventral telencephalic patterning

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Summary

Hedgehog signaling is required for multiple aspects of brain development, including growth, the establishment of both dorsal and ventral midline patterning and the generation of specific cell types such as oligodendrocytes and interneurons. To identify more precisely when during development hedgehog signaling mediates these events, we directed the removal of hedgehog signaling within the brain by embryonic day 9 of development, using a *FoxG1^{Cre}* driver line to mediate the removal of a conditional smoothened null allele. We observed a loss of ventral telencephalic patterning that appears to result from an initial lack of specification of these structures rather than by changes in proliferation or cell death. A further consequence of the removal of smoothened in these mice is the near absence of both oligodendrocytes and interneurons. Surprisingly, the dorsal midline appears to be patterned normally in these mutants. Together with previous analyses, the present results demonstrate that hedgehog signaling in the period between E9.0 and E12 is essential for the patterning of ventral regions and the generation of cell types that are thought to largely arise from them.

Key words: Telencephalon, Sonic hedgehog, Patterning, Oligodendrocyte, Interneuron

Introduction

An understanding of the basic mechanisms for the generation of neural cell diversity is an essential first step in tracing the development of the mature mammalian CNS from its beginnings as a simple neuroepithelium. The role of hedgehog (Hh) signaling in neural development has been studied in the developing mammalian spinal cord, and it is now clear that Hh proteins are an essential part of ventral neural patterning throughout the entire neuraxis, including the telencephalon (Ericson et al., 1995; Roelink et al., 1995). Whereas some aspects of Hh signaling in the telencephalon resemble that in the spinal cord, in other respects, Hh function in this area appears distinct. The unique phenotypes associated with loss of Hh signaling in this region are illustrated by the clinically relevant forebrain disorder known as holoprosencephaly (HPE). In addition to a marked reduction in size, the holoprosencephalic brain is devoid of both ventral and dorsal midline structures, resulting in a single, undivided telencephalic vesicle (Muenke and Beachy, 2000; Wallis and Muenke, 1999). As such, this genetic disorder provides an excellent starting point towards understanding the central role for Hh signaling in the maturing telencephalon. One member of the Hh family, sonic hedgehog (*Shh*), has been implicated as a HPE candidate gene, as murine *Shh* knockouts reproduce all aspects of the HPE phenotype, except for the happlo-insufficient dosage dependence found in humans (Chiang et al., 1996; Corbin et al., 2003; Rallu et al., 2002).

The requirement of Shh ligand for ventral patterning is strikingly similar in the ventral telencephalon and all other levels of the ventral neuraxis – ventral cell fates are absolutely dependent on Shh, whereas more lateral fates can still be achieved in the absence of this ligand (Litingtung and Chiang, 2000; Pierani et al., 1999; Rallu et al., 2002). *Shh* null mice have morphologically abnormal cortical midlines observable as early as embryonic day 10.5 (E10.5) (Rallu et al., 2002). More detailed analysis, using both gain-of-function and conditional loss-of-function techniques, has also implicated *Shh* in specification of oligodendrocyte precursors as well as maintenance of postnatal telencephalic progenitor populations (Machold et al., 2003; Nery et al., 2001; Tekki-Kessaris et al., 2001). The means by which *Shh* is able to effect these disparate developmental events is unclear.

A logical place to examine the mechanistic differences that control these processes lies in the only known mediators of mammalian Hh signaling, the three Gli transcription factors (Bai et al., 2004). With regards to dorsoventral patterning and telencephalic size, only the repressor form of *Gli3* has been shown as yet to play a decisive role, suggesting that these different actions of Hh signaling are not mediated by different Gli transcription factors (Park et al., 2000; Rallu et al., 2002). An alternative possibility is that the functional consequences of Hh signaling vary over time (Gunhaga et al., 2000; Kohtz et al., 1998). The complex, rapidly changing patterns of *Shh* expression in and around responsive telencephalic tissue also suggests a model of temporally distinct signaling events.

We have recently shown using a conditional Smoothened

(*Smo*) allele that at least some aspects of Hh signaling can be attributed to distinct development time frames (Machold et al., 2003). Smoothened is an obligate cell-autonomous mediator of all Hh-signaling, hence all Hh-signaling is lost in *Smo* mutants (Zhang et al., 2001). Experiments that removed *Smo* gene function in the CNS by E12 left forebrain patterning unperturbed, while to differing degrees affecting oligodendrogenesis and postnatal progenitor survival to differing degrees (Machold et al., 2003). These data argue that Hh's control of intrinsic patterning molecules is complete and set by this point in the developing telencephalon. The exploration of earlier windows of Hh signaling in the telencephalon offers the potential to further reconstruct the spatial and temporal requirement for Hh in the rapidly developing neuroepithelium.

In these experiments, we have ablated *Smo* function by E9 in the mouse telencephalon. We see gross ventral patterning defects accompanied by complete dorsalization of the telencephalon. Consequently, we also see near complete loss of two telencephalic populations that are largely ventrally derived, interneurons and oligodendrocytes. Our data highlights a window of telencephalic Hh signaling that is crucial for the establishment of ventral patterning.

Materials and methods

Genotyping of conditional Smoothened and *Foxg1^{Cre}* alleles

PCR was used to genotype both the conditional *Smo* and *Foxg1^{Cre}* alleles. The following primers were used for the *Smo* allele: smo wt 5′ (5′-cca ctg cga gcc ttt gcg cta c) and smo wt 3′ (5′-ccc atc acc tcc gcg tcg ca) amplify a 180-bp fragment of the wild-type *Smo* locus; smo conditional 5′ (5′-atg gcc gct ggc cgc ccc gtg) and smo conditional 3′ (5′-ggc gct acc ggt gga tgt gg) amplify a 600-bp fragment present only in the unrecombined conditional allele; smo rec 5′ (5′-ggc ctg cgc tgc tca aca tgg) and smo rec 3′ (5′-cca tca cgt cga act cct ggc) amplify a 350-bp fragment present in the recombined conditional allele. The following primers were used for the *Foxg1^{Cre}* allele: Cre 5′ (5′-taa aga tat ctc acg tac tga cgg tg) and Cre 3′ (5′-tct ctg acc aga gtc atc ctt agc) amplify a 300-bp fragment within the Cre recombinase.

Tissue preparation, histology and in situ hybridization

E9.5, E10.5 and E12.5 embryos were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA) for 2-3 hours at 4°C. E18.5 embryos were transcardially perfused and post-fixed for 2-3 hours. Embryos were then washed in phosphate buffered saline (PBS) and cryoprotected in 30% sucrose in PBS. Tissues were embedded in TissueTek, frozen on dry ice, and sectioned serially at 18 μm for in situ hybridization and immunohistochemical analysis. Section in situ hybridizations were performed as previously described using non-radioactive DIG-labeled probes (Wilkinson and Nieto, 1993). The cDNA probes used included *Gli1*, *Foxg1*, *Nkx2.1*, *Gsh2*, *Pax6*, *Emx2*, *Ngn2*, *Mash1*, *Dlx2*, *Lhx6*, *GAD67*, *Olig2* and *Pdgfra*.

Immunohistochemistry

The following antibodies were used: mouse anti-TuJ1 (1:1000, Covance), rabbit anti-Caspase3 (1:200, Cell Signaling), mouse anti-BrdU (1:100, Becton Dickinson), rabbit anti-Calbindin (1:5000, Swant), rabbit anti-GABA (1:1000, Sigma), mouse anti-GalC (1:10, Chemicon), mouse anti-O4 (1:10, Chemicon). Triton X-100 was not used when O4 was the primary antibody. Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and were raised in both donkey and goat.

Oligodendrocyte culturing

E16.5 cortical culturing experiments were performed as previously described, except that the culturing media had no mitoC added (Nery et al., 2001).

Results

In this study, we have employed a conditional loss-of-function approach to study the role of Hh signaling before the initiation of neurogenesis in the developing telencephalon. The ability of neural progenitors to respond to secreted Hh proteins is entirely dependent on the membrane-spanning Smoothened protein, which drives the intracellular portion of the Hh signaling cascade (Ingham and McMahon, 2001). We have taken advantage of a conditional *Smo* allele, where the first exon of the *Smo* gene is surrounded by loxP sites, to cell-autonomously remove Hh responsiveness in telencephalic progenitors (Long et al., 2001). *Smo* removal at early stages of telencephalic development was achieved through the use of Cre recombinase under the regulatory control of the *Foxg1* locus (Hebert and McConnell, 2000). With this allele, Cre recombinase is strongly expressed throughout the telencephalon prior to neurogenesis.

Removal of telencephalic Hh responsiveness

To more precisely visualize the timing and extent of *Smo* recombination in telencephalic progenitors, we crossed the *Foxg1^{Cre}* mice with the R26R ROSA reporter strain, in which a floxed *Neo* cassette interrupts the ubiquitous expression of the *lacZ* gene (Soriano, 1999). *Foxg1^{Cre}*-mediated recombination is present in the E7.5 pre-somitic embryo in the rostral part of the neural plate (Fig. 1A). Recombination at this stage appeared mosaic, with β-gal-positive cells dispersed throughout the medio-lateral extent of the anterior neural plate. During the stages of embryonic turning (Fig. 1B), dynamic changes of Cre recombinase expression were observed. At 10 somites (~E8.25) recombination was complete in the anterior neural ridge and had initiated in the ventral telencephalon. By the 16-somite stage, recombination appeared complete throughout the telencephalon as judged by ROSA reporter analysis. All progenitors along the entire A-P extent of the telencephalon displayed *Cre*-mediated recombination, as evidenced by β-gal staining extending from the telencephalic-diencephalic border to the floor of the ventral diencephalon (Fig. 1C). In addition to the telencephalon expression, mosaic Cre was found at the mid-hindbrain junction and the branchial arches, two domains of known *Foxg1* expression (Hatini et al., 1999; Hebert and McConnell, 2000). A low level of mosaic Cre expression was detected throughout the rest of the embryo and is attributable to mouse strain-specific ectopic Cre expression from the *Foxg1^{Cre}* transgene (data not shown) (Hebert and McConnell, 2000). To functionally assess the degree of *Smo* removal, we examined the expression of *Gli1* transcripts, a sensitive indicator of Hh signaling events (Bai et al., 2002). At both E9 and E10.5 in the conditional knockout there was no detectable *Gli1* expression in the telencephalon, a clear indication that all of Hh signaling had been abrogated (Bai et al., 2004) (see Fig. S1A,B in supplementary material, Fig. 1D,E). Genetic removal of Hh-responsiveness prior to the onset of neurogenesis in *Smo$^{c/-}$;Foxg1^{Cre}* mice generated a severe telencephalic phenotype (Fig. 1F). At E18.5,

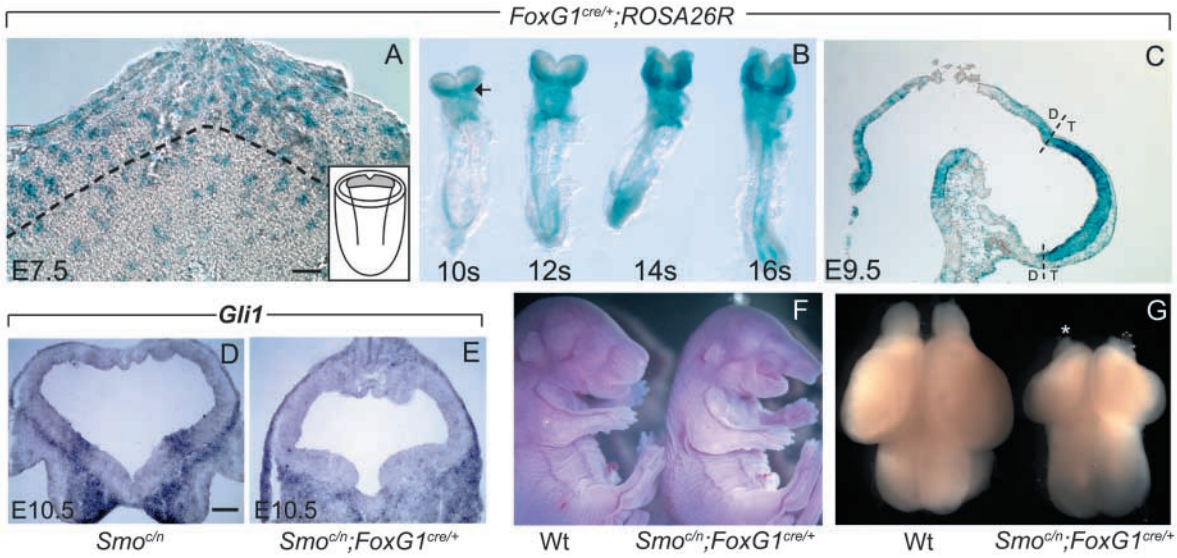

Fig. 1. *Foxg1*^{Cre} removes the Hedgehog responsiveness of telencephalic progenitors in conditional *Smo* mice. (A-C) β-gal expression of *Foxg1*^{Cre}*;ROSA26R* mice shows the extent of Cre-mediated recombination at E7.5 (A), 10-16 somite stage (B), and E9.5 (C). Note that A is a flattened dissection of the anterior neural plate as diagrammed in the inset, with the anterior neural ridge at the top and the broken line representing the approximate telencephalic primordium. The arrow in B is marking the *lacZ* staining in the anterior neural ridge. (C) *Foxg1*^{Cre}-mediated recombination extends throughout the anterior-posterior extent of the E9.5 telencephalon. (D,E) Coronal section of an E10.5 *Smo*$^{c/–}$ (D) and *Smo*$^{c/–}$*;Foxg1*^{Cre} mutant (E) telencephalon showing the absence of *Gli1* transcripts in the latter, a sensitive readout of active Hedgehog signaling. (F) Comparison of E18.5 wild-type and *Smo*$^{c/–}$*;Foxg1*^{Cre} mutant embryos. (G) Wholemount dorsal view of wild-type and *Smo*$^{c/–}$*;Foxg1*^{Cre} mutant brains shows severe size reduction of the telencephalic vesicles and olfactory bulbs. The rudimentary olfactory bulb of the mutants is marked with an asterisk. Scale bars: 100 μm in A; 200 μm in D,E.

Smo$^{c/–}$*;Foxg1*^{Cre} mice had smaller telencephalic vesicles than wild-type controls and a large hole in the ventral telencephalon (Fig. 1G and data not shown). *Smo*$^{c/–}$*;Foxg1*^{Cre} olfactory bulbs were also reduced in size. The appearance of holoprosencephalic facial defects, including a rudimentary proboscis and centrally-displaced eyes, was most probably because of a disruption of Hh signaling in the facial ectoderm and branchial arches, two other sites of *Foxg1*^{Cre}-mediated *Smo* excision (Fig. 1F).

The profound telencephalic phenotype displayed by the *Smo*$^{c/–}$*;Foxg1*^{Cre} mutants could be explained by changes in progenitor cell proliferation, cell death, or patterning. Abnormalities in cell proliferation were explored by studying BrdU incorporation at E10.5, approximately one day after complete *Smo* removal. By E10.5, the telencephalic morphology of *Smo*$^{c/–}$*;Foxg1*^{Cre} mutants was already distinguishable from wild-type littermates by a marked reduction in size. However, BrdU incorporation appeared unaltered within the proliferating neuroepithelium of mutant genotypes as compared with wild-type controls (Fig. 2A,B). We next examined potential abnormalities in cell death by cleaved Caspase3 antibody staining. *Smo*$^{c/–}$*;Foxg1*^{Cre} mutants showed a significant increase in both ventral and dorsal cell death as compared with wild-type controls (compare Fig. 2C and 2D), with a larger portion of the death occurring in the lateral cortex. Cell death in both E9.5 and E10.5 mutants appeared to increase in the cortex more posteriorly (data not shown). By E12.5, cell death was occurring throughout the dorsal-ventral extent of the mutant telencephalon, with the exception of the developing hippocampal primordium and choroid plexus (Fig. 2F). Taken together, these data suggest that removal of Hh signaling in telencephalic progenitors resulted in a general increase in cell death. However, the distribution of cell death is not predominantly ventral, as might be expected from the severity of the gross ventral phenotype.

Earliest ventral patterning is never established

The lack of evidence for either profound alterations in cell proliferation or massive regionally localized apoptosis encouraged us to further examine neural differentiation and patterning defects to explain the *Smo*$^{c/–}$*;Foxg1*^{Cre} mutant phenotype. TuJ1 staining was employed to label cells that have exited the ventricular zone and adopted a more differentiated neuronal fate. At E10.5, wild-type sections showed the beginnings of ventral neurogenesis, with pockets of TuJ1-positive cells appearing beneath the developing ventral ganglionic eminences (Fig. 2C). Ventrally located TuJ1-positive neurons were completely absent from the E10.5 mutant telencephalon (Fig. 2D). The absence of these early ventrally derived TuJ1-positive populations indicated that early telencephalic patterning was perturbed. Note that at E12.5, although the expression of TuJ1 in ventral regions is reduced compared with wild-type controls, the pattern of expression resembles that seen in the wild-type lateral ganglionic eminence (LGE) rather than that observed in the cortex (Fig. 2E,F). We analyzed the expression of *Nkx2.1*, *Gsh2*, and *Pax6* at E10, a stage when these transcription factors demarcate the medial, lateral, and dorsal telencephalic progenitor zones, respectively. *Nkx2.1* expression is initiated in the diencephalon at 1-3 somites and extends into the ventral telencephalon by the 13-somite stage (E9), forming a boundary with the Pax6-positive cortical primordium (see Fig. S1E in supplementary

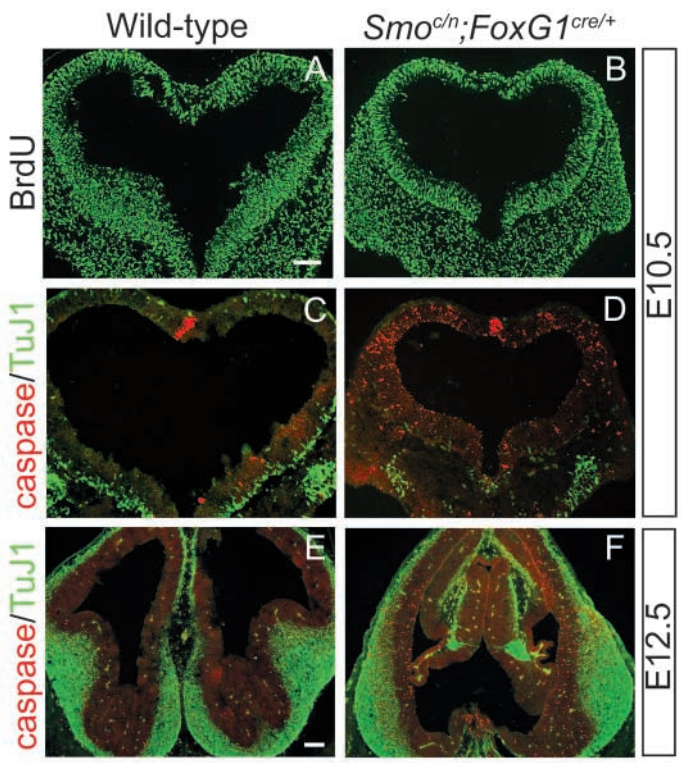

Fig. 2. Altered levels of cell death in *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants. (A-D) E10.5 coronal sections of the telencephalon stained with antibodies against BrdU (A,B) or caspase and TuJ1 (C,D). *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants have a general increase in cell death at E10.5 and an absence of the earliest differentiating neurons (D) as compared with wild-type littermates (C). (E,F) E12.5 coronal sections of the telencephalon stained with antibodies against caspase and TuJ1. *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants show continued widespread cell death with the exception of the dorsal cortical midline (F). Note that the pattern of TuJ1 expression in mutant embryos (F) resembles that seen in wild-type LGE (E). Scale bars: 200 μm in A-D; 300 μm in E,F.

material) (Corbin et al., 2003; Shimamura et al., 1995). At E10, *Gsh2* expression is first observed between ventral *Nkx2.1* and dorsal *Pax6* domains (Fig. 3C,E,G) (Corbin et al., 2003). In situ hybridization analysis of *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants showed that *Nkx2.1* expression was absent from the E10 (28 somites) ventral telencephalon (Fig. 3F). Residual ventral *Nkx2.1* expression in mutants was of diencephalic origin, as judged by the absence of *Foxg1* staining, a marker of telencephalic versus diencephalic fate (compare Fig. 3B with 3F) (Hatini et al., 1994). *Nkx2.1* expression was likewise absent from 14-17 somite mutant embryos, the earliest known time point of telencephalic *Nkx2.1* expression (see Fig. S2B,D in supplementary material). *Gsh2* expression was also absent from the mutant telencephalon (Fig. 3H) whereas *Pax6* was expanded through the entire dorsal-ventral extent of the proliferating neuroepithelium (Fig. 3D). The early patterning defects of the *Smo*$^{c/-}$;*Foxg1*^{Cre} mutant are reminiscent of the *Shh* null phenotype (Corbin et al., 2003; Rallu et al., 2002). The loss of *Nkx2.1* expression and the concomitant *Pax6* expansion highlight the differential regulation of these genes by Hh signaling. *Gsh2* expression, which is absent from *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants is often present in the ventral midline of *Shh* mutants (Rallu et al., 2002). There are two possible explanations for this discrepancy - residual ventral patterning is established by the redundant function of the two other Hh ligands or a ligand-independent activity of *Smo* exists. This aside, the lack of early ventral patterning markers shows that blocking Hh-responsiveness in the telencephalic neuroepithelium by E9 prevents the correct establishment of early ventral and lateral telencephalic character.

Smo$^{c/-}$;*Foxg1*^{Cre} mutants show complete dorsalization

We next explored what effects the absence of this early Hh-directed patterning would have on subsequent events in telencephalic development. In E12.5 coronal sections, the *Smo*$^{c/-}$;*Foxg1*^{Cre} mutant brains were morphologically abnormal as judged by complete lack of all three ventral ganglionic

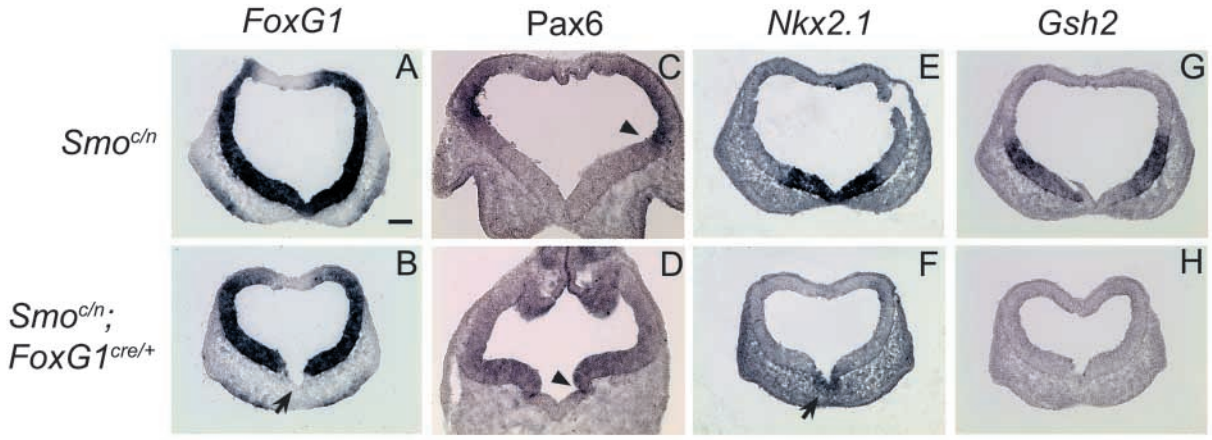

Fig. 3. Early ventral patterning is never established in *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants. (A-H) E10 coronal sections of the telencephalon stained by in situ hybridization with probes against *Foxg1* (A,B), *Pax6* (C,D), *Nkx2.1* (E,F) and *Gsh2* (G,H). The expression of *Pax6* (C), which is normally restricted to the dorsal telencephalon (arrowhead at dorsal-ventral boundary), is found throughout the *Smo*$^{c/-}$;*Foxg1*^{Cre} mutant telencephalon (D). The wild-type ventral expression of *Nkx2.1* (E) is absent from the mutant, with the exception of a small region of *Nkx2.1* staining (F, arrow) that is of diencephalic origin (compare with arrow in B). *Gsh2*, which marks the intermediate telencephalic ventricular zone at this stage (G), is never observed in *Smo*$^{c/-}$;*Foxg1*^{Cre} mutants (H). Scale bar in A: 200 μm for A-H.

eminences (compare Fig. 4A with 4B). The cortex appeared to extend ventrally until the limits of a structure with some diencephalic character (asterisk in Fig. 4B), confirmed both by the absence of telencephalic-specific *Foxg1* staining and the presence of the diencephalic marker, *Dbx1* (Fig. 4B and data not shown) (Lu et al., 1992). Neurogenin 2 (*Ngn2*; *Neurog2* – Mouse Genome Informatics), a bHLH proneural gene shown to be essential for dorsal neuronal identity, normally has a cortical expression pattern that terminates sharply at the cortico-striatal boundary (Fig. 4C) (Fode et al., 2000). Consistent with the aforementioned ventral expansion of the early dorsal *Pax6* domain, *Ngn2* expression by in situ hybridization is observed throughout the telencephalic ventricular zone in $Smo^{c/-};Foxg1^{Cre}$ mutants (Fig. 4D). Similarly, *Emx2*, a homeodomain transcription factor implicated in development of the archipallium, also has its cortico-striatal expression boundary expanded ventrally towards the underlying diencephalon in mutant brains (compare Fig. 4E with 4F) (Pellegrini et al., 1996). It appears that cortical development progresses normally throughout these expanded dorsal regions, as indicated by the normal expression of the layer V cortical marker Er81 within the mutant cortex (data not shown).

The appearance ventrally of a small TuJ1-positive mantle, despite the absence of morphologically apparent ventral eminences encouraged us to explore a possible recovery of later aspects of ventral patterning. *Nkx2.1*, which marks the entire extent of the E12.5 medial ganglionic eminence (MGE) and the anterior-most domain of the caudal ganglionic eminence (CGE), is present only in the diencephalon of $Smo^{c/-};Foxg1^{Cre}$ mutants (Fig. 4G,H). The expression of *Gsh2* normally expands from its initiation at E10 to cover much of the ventral telencephalon by E12.5, forming a sharp cross-repressive boundary with *Pax6* at the dorsal-ventral border (Fig. 4I) (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). $Smo^{c/-};Foxg1^{Cre}$ mutants show no *Gsh2* expression, arguing against a delayed rescue of the earlier phenotype (Fig. 4J). In addition, expression of the proneural gene *Mash1* in mutants is limited only to the ventral diencephalon and part of the cortical hem, a region where *Mash1* is expressed by SVZ cells en route to the developing dentate gyrus (Pleasure et al., 2000b). Taken together, this data suggests that there is no later Hh-independent recovery from the initial absence of early ventral telencephalic patterning.

In marked contrast to the absence of ventral telencephalic patterning, the dorsal cortical midline of $Smo^{c/-};Foxg1^{Cre}$ mutants appears well preserved. This is quite surprising in light of the *Shh* null phenotype, in which both ventral and dorsal midline structures are affected (Chiang et al., 1996). Marker analysis of this region confirms the presence of all normal dorsal midline signaling centers (data not shown), confirming the morphological prediction that this structure has formed normally in $Smo^{c/-};Foxg1^{Cre}$ mutants. This implies that Hh signaling is required before E9 in patterning the dorsal cortical midline.

The absence of cortical interneurons

The profound early ventral patterning defects present in

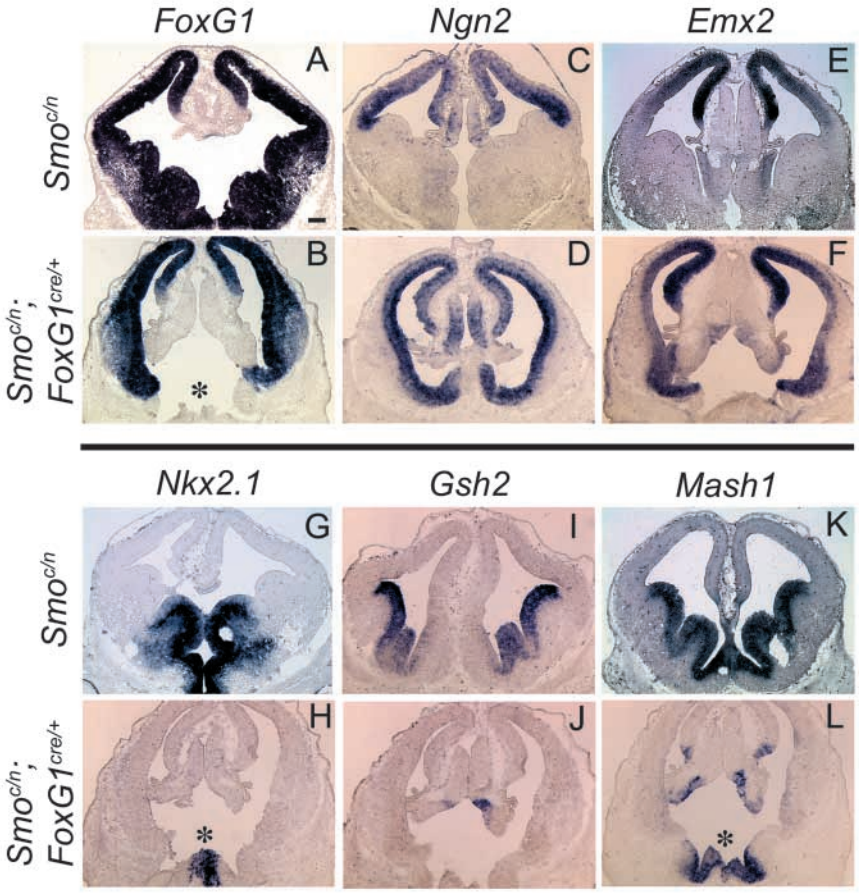

Fig. 4. $Smo^{c/-};Foxg1^{Cre}$ mutants show complete dorsalization. (A-L) E12.5 coronal sections of the wild-type (A,C,E,G,I,K) and $Smo^{c/-};Foxg1^{Cre}$ mutant (B,D,F,H,J,L) telencephalon stained by in situ hybridization. *Foxg1* staining of the mutant telencephalon shows the morphological absence of all three ventral eminences (B). Both *Ngn2* and *Emx2*, which are normally restricted to the developing cortical fields (C,E), are expanded throughout the entire ventral extent of the mutant telencephalon (D,F). *Nkx2.1*, *Gsh2* and *Mash1* are all expressed within overlapping domains of the ventral telencephalon (G,I,K). These three markers are absent from the $Smo^{c/-};Foxg1^{Cre}$ mutant telencephalon (H,J,L). Note that the ventral staining for *Nkx2.1* and *Mash1* is not telencephalic (asterisks, compare with asterisk in B). Scale bars: 300 μm in A-L.

Smo$^{c/–}$;Foxg1^{Cre} mutants enabled us to examine the importance of Hh signaling in populations that have significant ventral contributions, namely cortical interneurons and oligodendrocytes. A large body of work including genetic analysis, fate-mapping and transplantation studies indicates that most, if not all cortical interneurons are derived from the ventral ganglionic eminences (Anderson et al., 1997; Nery et al., 2002; Pleasure et al., 2000a; Wichterle et al., 2001). In light of the complete absence of ventral patterning in the *Smo$^{c/–}$;Foxg1^{Cre}* mutants, we decided to examine whether cortical interneurons could still be generated prenatally by some Hh-independent process. The perinatal lethality of our mutants precludes analysis of specific mature interneuron phenotypes in an in vivo context. However, a great deal can be learned by correlating early expression of genes important in acquisition of interneuron character with more general perinatal markers of the GABAergic inhibitory interneurons class. To this end, we first analyzed the telencephalic expression of several genes essential for the proper maturation of interneurons. *Dlx2* is part of an evolutionarily conserved gene cluster that is widely expressed throughout the basal telencephalon and has been implicated in the tangential migration of ventrally derived interneurons (Anderson et al., 1997). In E12.5 wild-type brains, *Dlx2* expression is observed in all three ventral eminences (Fig. 5A). As holds true for all observed ventral patterning genes, *Dlx2* expression is completely absent from the *Smo$^{c/–}$;Foxg1^{Cre}* mutant telencephalon (Fig. 5B). *Lhx6* is a LIM homeodomain transcription factor that is expressed in cells migrating tangentially from the MGE to the cortex, a population that is thought to consist largely of interneurons (Fig. 5C) (Lavdas et al., 1999). Like *Dlx2*, *Lhx6* is also absent from all ventral domains of *Smo$^{c/–}$;Foxg1^{Cre}* mutants (Fig. 5D). The loss of *Lhx6* and *Dlx2* in *Smo$^{c/–}$;Foxg1^{Cre}* mutants does not preclude the existence of normal interneuron progenitor pools but rather implies that any interneurons that could still be generated would probably have defects in tangential migration. We therefore examined *GAD67* in situ hybridization staining to better assess the state of interneuron progenitor development. *GAD67* (*Gad1* – Mouse Genome Informatics) is a gene that codes for an isoform of glutamic acid decarboxylase, a rate-limiting enzyme in the synthesis of the inhibitory neurotransmitter, GABA. Wild-type *GAD67* staining is found in the mantle of all three eminences as well as in the midline septum, whereas in *Smo$^{c/–}$;Foxg1^{Cre}* mutants, only ventral diencephalic *GAD67* staining can be observed (compare Fig. 5E with 5F).

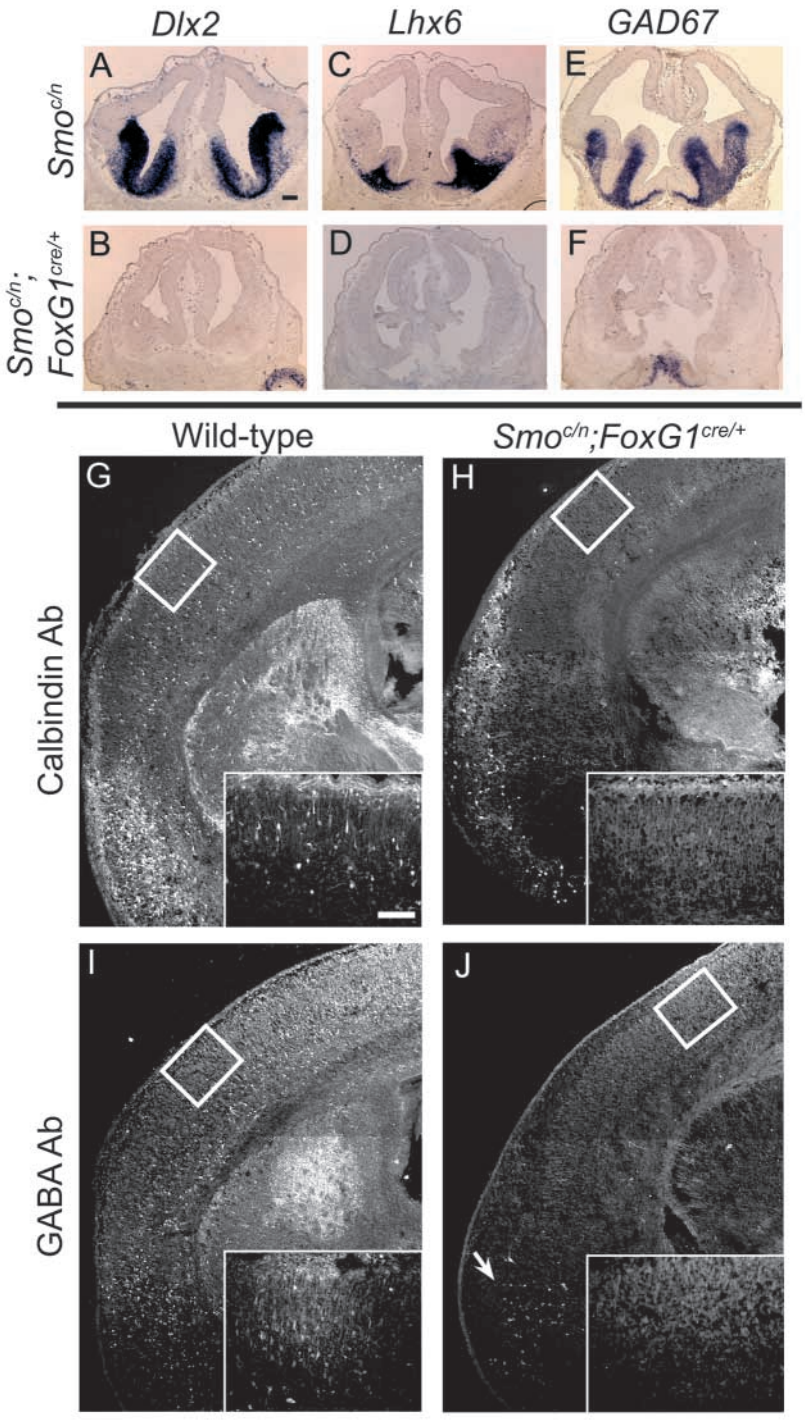

Fig. 5. Most cortical interneuron precursors are dependent on early Hedgehog signaling. (A-F) Coronal sections of an E12.5 wild-type (A,C,E) and *Smo$^{c/–}$;Foxg1^{Cre}* mutant (B,D,F) telencephalon, stained by in situ hybridization. The *Dlx2*, *Lhx6* and *GAD67* genes have been implicated in various aspects of interneuron development, and are expressed strongly in the basal E12.5 telencephalon (A,C,E). These genes are absent from the *Smo$^{c/–}$;Foxg1^{Cre}* mutant telencephalon (B,D,F). (G-J) Coronal sections of an E18.5 wild-type (G,I) and *Smo$^{c/–}$;Foxg1^{Cre}* mutant telencephalon (H,J), stained with antibodies against Calbindin and GABA. Both Calbindin and GABA are expressed by cortical interneurons throughout the layers of the wild-type cortical plate (G,I, see insets). In *Smo$^{c/–}$;Foxg1^{Cre}* mutants, Calbindin and GABA staining is absent from the cortical plate and is restricted to a ventral-lateral region of the cortex (H,J, see insets). Scale bars: 300 μm in A-F; 500 μm in G-J for low-power field, and 125 μm for high-power inset.

At E18.5, antibodies to calbindin label a subpopulation of tangentially migrating interneurons in the developing cortical plate (Fig. 5G) (Anderson et al., 1997). *Smo$^{c/-}$;Foxg1^{Cre}* mutants lack calbindin immunoreactivity with the exception of the ventral-lateral-most domain of the cortex (Fig. 5H). Calbindin immunoreactivity can be compared with GABA antibody staining to discern true GABAergic cortical interneurons. As is found for calbindin staining, the widespread GABA immunoreactivity of the E18.5 wild-type cortex is reduced to a small GABA-positive region in the ventral-lateral cortex (compare Fig. 5I with 5J). These results imply that despite the large diversity of mature cortical interneurons, their embryonic precursors virtually all depend on Hh signaling.

Oligodendrocyte formation is severely perturbed in *Smo$^{c/-}$;Foxg1^{Cre}* mutants

The ventral telencephalon has also been shown to be an important source of oligodendrocyte precursors (OPCs) that undergo tangential migration into the cortex. The association of OPC markers with regions of *Shh* expression and in vivo *Shh* gain and loss of function studies have intimately linked the Hh pathway and oligodendrogenesis (Nery et al., 2001; Olivier et al., 2001; Tekki-Kessaris et al., 2001). The *Smo$^{c/-}$;Foxg1^{Cre}* mutants provide a genetic approach to assessing OPC formation in progenitor cells that are insensitive to all Hh ligands. Analysis of the expression of embryonic OPC markers was combined with in vitro primary culture assays to determine the potential of OPC in *Smo$^{c/-}$;Foxg1^{Cre}* mutants. The bHLH transcription factor *Olig2* is proposed to have a role in telencephalic OPC generation, based both on its ventral expression pattern as well as its known function as a motor neuron to oligodendrocyte switch in the spinal cord (Fig. 6A) (Zhou et al., 2001). The normal ventral *Olig2* expression, found in a high medial to low lateral pattern, is completely absent from *Smo$^{c/-}$;Foxg1^{Cre}* mutants at E12.5 (Fig. 6B). *Pdgfra*, which seems to mark OPC before their migration in the developing spinal cord, is normally expressed in a punctate array in the anterior entopeduncular area of the telencephalon and later in the ventral eminences (Fig. 6C) (Nery et al., 2001; Tekki-Kessaris et al., 2001). As judged by *Pdgfra* in situ staining, there were no OPC remaining in the E12.5 mutant ventral telencephalon (Fig. 6D). A more direct in vivo investigation of mature oligodendrocyte populations was impossible, because of the perinatal lethality of the *Smo$^{c/-}$;Foxg1^{Cre}* mutants. Consequently, an in vitro cortical culture system was employed to examine whether the lack of early OPC markers correlated with a decrease in the formation of more mature oligodendrocyte populations. Cortical cultures were made from E16.5 disassociated cortex of wild-type and mutant mice and were subsequently grown for four days before being fixed and stained with various markers of oligodendrocyte maturation. Immunoreactivity to O4 antibody, which labels O-antigens on differentiated oligodendrocytes, was normalized to the number of total cells in culture, as ascertained by DAPI and TuJ1 staining (Fig. 6E,F). Wild-type cortical cultures produced 25-fold more oligodendrocytes than *Smo$^{c/-}$;Foxg1^{Cre}* mutant cultures, which yielded virtually no O4-positive cells (Fig. 6G).

Discussion

Shh null mutants have highly abnormal telencephalic development. Indeed, by birth, the single fused vesicle that represents the remnant of the telencephalon is impossible to positively identify without the use of telencephalic-specific marker expression (Corbin et al., 2003; Rallu et al., 2002). Hence, although Hh signaling is clearly central to patterning of this region, distinguishing the specific requirement for Hh signaling within this structure from the cumulative and more widespread failure in embryonic development is difficult.

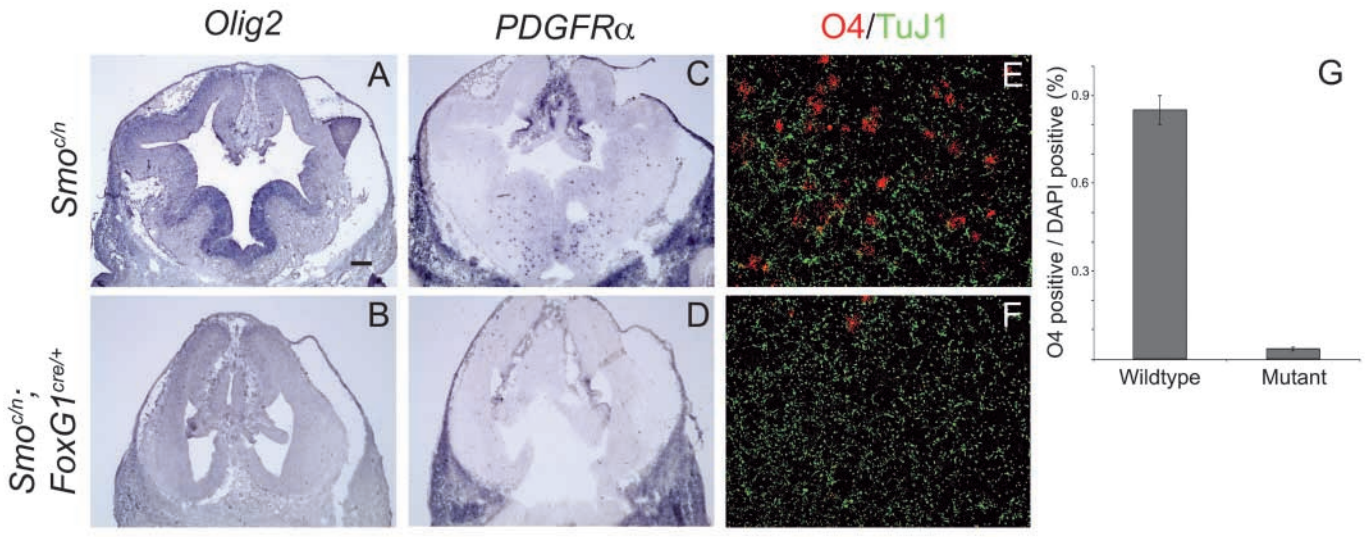

Fig. 6. Oligodendrocyte development is severely perturbed in the *Smo$^{c/-}$;Foxg1^{Cre}* mutant. (A-D) Coronal sections of an E12.5 wild-type (A,C) and *Smo$^{c/-}$;Foxg1^{Cre}* mutant (B,D) telencephalon. Expression of *Olig2* and *Pdgfra* (A,C), both markers of oligodendrocyte precursors, was absent from the mutant telencephalon (B,D). Note that the anterior entopeduncular area, where markers of oligodendrocyte precursors are typically first seen, is not present in *Smo$^{c/-}$;Foxg1^{Cre}* mutants. (E,F) Representative low-power fields of cortical cultures derived from E16.5 wild-type (E) and mutant (F) cortex, stained four days later with O4, TuJ1 and DAPI. *Smo$^{c/-}$;Foxg1^{Cre}* mutant cultures show a 25-fold reduction in O4-positive oligodendrocyte precursors (G). Scale bars: 300 μm in A-D.

Moreover, the precise temporal role of various aspects of Hh signaling in telencephalic development has been posited to begin as early as the beginning of gastrulation (Gunhaga et al., 2000) and continue on through to postnatal neurogenesis (Dahmane et al., 2001; Machold et al., 2003). In this study, we demonstrate that the complete loss of Hh signaling by E9 results in a failure of ventral telencephalic patterning. As a consequence, ventrally derived migratory populations, including both oligodendrocytes and interneurons, are severely compromised. Previously we have shown the abrogation of telencephalic Hh-responsiveness by E12 greatly perturbs the postnatal neurogenic populations of the subventricular zone and the hippocampus, but leaves patterning of ventral progenitor zones unchanged (Machold et al., 2003). Hence, we demonstrate that the period between E9 and E12 is a crucial time window in the telencephalon for Hh's assignment of ventral identity.

Ventral patterning is never established in *Smo$^{c/-}$;Foxg1^{Cre}* mice

The most striking phenotype seen in *Smo$^{c/-}$;Foxg1^{Cre}* mice is the complete absence of ventral telencephalic markers (Fig. 3), accompanied by an expansion of dorsal markers (Fig. 4). Moreover, rather than being lost after transient expression, the earliest markers of ventral telencephalic fate are never established in *Smo$^{c/-}$;Foxg1^{Cre}* mutants. This dramatic phenotype could be attributed either to decreases in ventral progenitor cell proliferation, localized increases in ventral cell death, regional fate transformation or a lack of proper specification. Cell proliferation levels are largely unaltered in E10.5 *Smo$^{c/-}$;Foxg1^{Cre}* mutants, suggesting that Hh signals at this early time period may not greatly alter progenitor proliferation rates (Fig. 2). Analysis of *Smo$^{c/-}$;Foxg1^{Cre}* mice at E9.5 and at E10.5 show marked increase in cell death compared with wild-type controls. A fairly widespread pattern of cell death at E9.5 gave way to a more dorsally localized focus of apoptosis by E10.5. However, at no time investigated is there evidence of a massive, ventral-specific apoptosis that could have ablated the entire population of early ventral telencephalic progenitors. These observations suggest that either a failure in the specification of ventral cell types or a dorsal to ventral fate transformation has occurred in the telencephalon of *Smo$^{c/-}$;Foxg1^{Cre}* mice. In support of the latter, the ventral regions of the mutant telencephalon, although expressing cortical markers maintain a pattern of neurogenesis that resembles that seen in the wild-type LGE (Fig. 2E,F). Regardless, it seems likely that these two alternatives represent different sides of the same patterning process (Ericson et al., 1996). As has been demonstrated in the spinal cord, telencephalic Hh signaling is probably first required to create a region where dorsal patterning genes are excluded. Within this domain, Hh signaling can specify progenitor domains by a derepression mechanism, most likely through the modulation of levels of the Gli3 repressor protein (Litingtung and Chiang, 2000).

To investigate whether ventral telencephalic patterning requires direct Hh signaling or acts through some relay mechanism of non-telencephalic Hh, we analyzed *Smo* null chimeras. We observe that in *Smo* null chimeras, null cells are largely excluded from the ventral-most territory (Supp. 2A). When *Smo* null cells are present in this territory, they fail to express pan-ventral telencephalic markers (Supp. 2A-D). These results are consistent with the loss of ventral patterning resulting from failure of direct Hh specification within ventral progenitors.

The early lack of ventral specification seen in *Smo$^{c/-}$;Foxg1^{Cre}* mutants has profound consequences for the remainder of telencephalic development. There is no recovery of later ventral progenitor fates through a *Smo*-independent patterning mechanism. By all markers examined, *Smo$^{c/-}$;Foxg1^{Cre}* mutants are devoid of all aspects of ventral telencephalic character at E12.5 (Fig. 4). Thus, it seems that telencephalic progenitors require Hh signaling around E9 to specify ventral telencephalic fates. In conjunction with previous studies from our lab that show grossly normal ventral patterning in *Smo$^{c/-}$;Nestin^{Cre}* mutants, it would seem that the time period between E9 and E12 is critical for specifying ventral character. It is important to clarify that these timepoints are conservative estimates of when complete removal of Hh signaling is achieved. The *Foxg1^{Cre}* driver is active in prospective ventral telencephalon as early as E7 and thus creates a gradual loss of Hh responsiveness beginning at this timepoint. Likewise, *Nestin^{Cre}*-mediated recombination begins ventrally at E9.5 and completes removal of Hh signaling by E12. In addition, both Cre drivers have a ventral to dorsal sequence of activation that mimics the neurogenic gradient (Fig. 1). Therefore, this E9-E12 window could in reality be shifted at least a day earlier. It also remains possible that initial aspects of Hh-dependent ventral patterning could take place at gastrulation, as has been suggested by in vitro explant work in the chick telencephalon (Gunhaga et al., 2000). Although in principle this could be stringently addressed using Cre-driver lines with earlier patterns of telencephalic expression, such reagents are not yet available.

Cortical interneurons are virtually absent in *Smo$^{c/-}$;Foxg1^{Cre}* mice

The complete absence of ventral patterning throughout the development of *Smo$^{c/-}$;Foxg1^{Cre}* mutants provides an interesting context for investigating the importance of Hh signaling in the ventral progenitors that contribute to mature dorsal telencephalic cell types. A large body of evidence in mice has shown that the majority of interneurons are derived from the ventral telencephalon (Anderson et al., 1997; Wichterle et al., 2001; Nery et al., 2002). However, work in human embryonic cortical slices, in addition to early dorsal cortical culturing experiments hints at possible dorsal sources of interneurons (Letinic et al., 2002). Irrespective of their origin, it is presently unclear whether Hh is required by all interneuron precursors or if a Hh-independent subset of interneurons exists. Our work demonstrates that the vast majority of embryonically born interneuron precursors are Hh dependent. A small population of Hh-independent neurons having immunological profiles consistent with interneuron fate, seems to persist in the paleocortical region of the *Smo$^{c/-}$;Foxg1^{Cre}* mutant telencephalon (Fig. 5). Because of the perinatal lethality of these mutants, the mature class of these potential Hh-independent interneuron precursors remains unknown. It seems likely, however, that as all telencephalic *Dlx* expression is absent in *Smo$^{c/-}$;Foxg1^{Cre}* mutants, this population of Hh-independent interneurons would probably represent a *Dlx*-independent class of interneuron precursors.

Oligodendrogenesis requires hedgehog signaling between E9 and E12

Previous work from many labs has shown that Shh signaling is intimately linked with telencephalic oligodendrogenesis. The expression of early oligodendrocyte markers, such as *Pdgfra* and *plp/dm20*, is localized to the Shh-expressing region of the anterior hypothalamic neuroepithelium and anterior entopeduncular area. Loss of function data has argued both for and against Hh-independent oligodendrocyte formation (Nery et al., 2001; Tekki-Kessaris et al., 2001). In vivo analyses of *Nkx2.1* mutant mice, which lose telencephalic *Shh* expression, show a strong correlation between the loss of early OPC markers and the loss of Hh ligand. Strangely however, in vitro cortical cultures from *Shh* null mice are capable of generating oligodendrocytes (Nery et al., 2001; Tekki-Kessaris et al., 2001). Our results suggest that most, if not all oligodendrocyte precursors are Hh-dependent (Fig. 6). Marker analysis of oligodendrocyte precursors at E12.5 showed that these early populations were entirely absent from mutant forebrains. $Smo^{c/-};Foxg1^{Cre}$ cortical cultures, taken from E16.5 mice and grown four days in culture, give rise to few if any oligodendrocytes in comparison with wild-type controls. The apparent failure of OPC specification in $Smo^{c/-};Foxg1^{Cre}$ mutants is in contrast with the oligodendrocyte phenotype seen in *Shh* nulls, suggesting that the in vitro production of OPCs reflects the upregulation of other Hh ligands. Perhaps the greater severity of $Smo^{c/-};Foxg1^{Cre}$ as compared with $Shh^{-/-}$ mutants with regard to ventral patterning reflects a similar upregulation of Desert or Indian hedgehog in the latter.

With regard to oligodendrocyte and interneuron formation, a direct comparison of the phenotypes resulting from the $Nestin^{Cre}$- versus $Foxg1^{Cre}$-mediated removal of the conditional *Smo* is informative. Although both oligodendrocytes and interneurons were lost in the $Smo^{c/-};Foxg1^{Cre}$, both were clearly present in the $Smo^{c/-};Nestin^{Cre}$, albeit with some reduction in the oligodendrocyte population. This demonstrates that the same critical window needed for the establishment of ventral identity is the period required for the specification of these two cell types. It is tempting to suspect that the loss of cell specification is a direct result of the loss of patterning, implying that all interneuron and oligodendrocytes come from ventral origin. However, some caution should be taken with this interpretation. As $Smo^{c/-};Foxg1^{Cre}$ die at birth, one possibility is that there is a Hh-independent postnatal phase of oligodendrogenesis and perhaps interneuron production. The observation that Cre directed from the *Emx1* locus, a gene confined to dorsal domains throughout development, fate maps oligodendrocytes in the corpus callosum is consistent with this possibility (Gorski et al., 2002). Nonetheless, the exclusion of GABAergic cells from this same fate-mapping argues against a postnatal phase of dorsal interneuron production.

In striking contrast to the severe ventral phenotype observed, the dorsal cortical midline of $Smo^{c/-};Foxg1^{Cre}$ mutants appears to be unaffected. Consistent with morphological data, gene expression studies indicate that this region is unchanged in mutants (M.F. and G.F., unpublished). These results suggest that Hh-dependent patterning of the dorsal midline occurs earlier than E9, a prospect that we are currently exploring.

Our results have further elucidated temporal changes in the requirement for telencephalic Hh signaling. Specifically, we demonstrate that it is required at some time prior to E9 for dorsal patterning, then from E9-E12 for ventral telencephalic patterning. The $Smo^{c/-};Nestin^{Cre}$ mutants uncovered a third role for Hh signaling in maintenance of postnatal neural progenitors (Machold et al., 2003). As a consequence of removal of Hh signaling beginning at E9, interneurons and oligodendrocytes, two populations thought to be derived from ventral progenitor domains, are missing. In conjunction with data from the $Nestin^{Cre}$-mediated removal of Hh-responsiveness, we show that temporal division of Hh action is an important mechanism of Hh functional diversification in the mammalian telencephalon. Of the potential effectors of Hh, to date only *Gli3* gene function has been shown to have relevance to telencephalic patterning (Park et al., 2000; Rallu et al., 2002). Furthermore, unlike the spinal cord, where both activator and repressor forms of the Gli proteins are employed in generating diverse cell types, only the repressor form of Gli3 seems to be required in the forebrain (Bai et al., 2004; Rallu et al., 2002). With Hh signaling only serving to orchestrate the levels of this negative transcriptional regulator, the question of positive activators of ventral telencephalic patterning remains unanswered. Recent work has shown that in the spinal cord, Fgf and retinoid signaling may be direct activators of ventral patterning genes (Diez del Corral et al., 2003; Novitch et al., 2003). Both signaling pathways have been implicated in forebrain development, although neither has been directly associated with establishment of ventral patterning. Our data highlight an important window of ventral telencephalic development in which to look for the candidate signaling pathways that actively specify early ventral patterning, and the cell types later derived from these regions.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/20/5031/DC1

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