Neuron, Vol. 39, 937–950, September 11, 2003, Copyright ©2003 by Cell Press

Sonic Hedgehog Is Required for Progenitor Cell Maintenance in Telencephalic Stem Cell Niches

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Summary

To directly test the requirement for hedgehog signaling in the telencephalon from early neurogenesis, we examined conditional null alleles of both the Sonic hedgehog and Smoothened genes. While the removal of Shh signaling in these animals resulted in only minor patterning abnormalities, the number of neural progenitors in both the postnatal subventricular zone and hippocampus was dramatically reduced. In the subventricular zone, this was partially attributable to a marked increase in programmed cell death. Consistent with Hedgehog signaling being required for the maintenance of stem cell niches in the adult brain, progenitors from the subventricular zone of floxed Smo animals formed significantly fewer neurospheres. The loss of hedgehog signaling also resulted in abnormalities in the dentate gyrus and olfactory bulb. Furthermore, stimulation of the hedgehog pathway in the mature brain resulted in elevated proliferation in telencephalic progenitors. These results suggest that hedgehog signaling is required to maintain progenitor cells in the postnatal telencephalon.

Introduction

Sonic hedgehog (Shh) is required for multiple aspects of development in a wide range of tissue types (reviewed in McMahon et al., 2003). In the mammalian CNS, Shh is essential for the establishment of the ventral pattern along the entire neuraxis, including the telencephalon (Echelard et al., 1993; Ericson et al., 1995; Marti et al., 1995; Chiang et al., 1996). In addition, Shh has been demonstrated to play a mitogenic role in the expansion of granule cell precursors during CNS development and when ectopically expressed in the developing spinal cord (Wechsler-Reya and Scott, 1999; Rowitch et al., 1999; Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999).

Analyses of *Shh* mutants have provided important insights into Shh's role in brain patterning. In *Shh* null mice, dorsoventral patterning, the specification of ventral cell populations, and general brain proliferation are all affected. Specifically, in these mutants the telencephalon is greatly dysmorphic and much reduced in size. In addition, the telencephalon of *Shh* mutants appears as a single fused vesicle that is strongly dorsalized, the only remnant of ventral patterning being the persistence of panventral telencephalic markers in a small region of the ventral midline (Chiang et al., 1996; Rallu et al., 2002). Furthermore, analysis of markers of oligodendrocyte differentiation suggests that this population is entirely absent in *Shh* mutants (Lu et al., 2000). Another striking feature of the *Shh* mutant phenotype is a 90% reduction in the size of the telencephalon and a disproportionate reduction in the diencephalon at birth, indicating that the early loss of Shh activity in the forebrain must affect both dorsal and ventral structures. Recent evidence suggests a Shh-mediated signaling relay may be responsible for Shh-dependent development in the dorsal diencephalon (Ishibashi and McMahon, 2002). Unfortunately, the perinatal lethality of *Shh* mutants has precluded any analysis of Shh's role in later aspects of forebrain neurogenesis.

In general agreement with the loss-of-function phenotype, gain-of-function approaches have demonstrated that misexpression of *Shh* in the embryonic telencephalon results in the expression of ectopic ventral markers (Kohtz et al., 1998; Gaiano et al., 1999; Gunhaga et al., 2000), abnormal proliferation (Gaiano et al., 1999), and the appearance of supernumerary oligodendrocytes (Nery et al., 2001). By contrast, in vitro and in vivo upregulation of Shh signaling before or after the initiation of neurogenesis results in pronounced hypertrophy of telencephalic regions (Kohtz et al., 1998; Gaiano et al., 1999; Rowitch et al., 1999; Dahmane et al., 2001). These different roles of Shh could reflect the timing of exposure of telencephalic tissues to Shh or the concentration of Shh they experience. Alternatively, they may indicate that throughout development different tissues have varying intrinsic competences in their response to hedgehog signaling.

Here we specifically address the later requirement for Shh signaling in the telencephalon. To achieve this, we have used genetic approaches to remove either the production of active Shh ligand or the ability of forebrain cells to respond to Shh signals subsequent to the initiation of early patterning. We have also used a pharmacological approach to activate Shh signaling in the adult brain. These studies suggest that hedgehog signaling in the postnatal telencephalon acts to both promote

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proliferation and maintain populations of neural progenitors. Hence, Shh signaling in the mammalian telencephalon may participate in the maintenance of a neural stem cell niche akin to the action of the hedgehog pathway in *Drosophila* ovaries and testes (Forbes et al., 1996a, 1996b; Zhang and Kalderon, 2000).

Results

In this study, we have used a conditional loss-of-function approach to examine the role of Shh signaling during telencephalic development after the initiation of neurogenesis. This was achieved through the use of a conditional *Shh* allele and a transgenic line in which the neural-specific *Nestin* promoter/enhancer directs the expression of *Cre* recombinase (N^{Cre}) in neural progenitor populations. The existence of two other hedgehog proteins (Echelard et al., 1993) raises the possibility that in addition to Shh these other ligands may also have a role in telencephalic development. Indeed, such a mechanism has been proposed to reconcile the finding that, while telencephalic tissue from *Shh* null animals can generate oligodendrocytes in vitro (Nery et al., 2001), cyclopamine-treated telencephalic tissue cannot (Tekki-Kessaris et al., 2001). To rule out the possibility of compensatory hedgehog signaling, we have also examined the phenotype generated by the removal of *Smo* gene function in N^{Cre} mice. The single mammalian *Smo* gene, like its *Drosophila* counterpart, is essential for transduction of all hedgehog signaling (Zhang et al., 2001).

Loss of Hedgehog Signaling in $Shh^{n/c};Nestin^{cre}$ or $Smo^{n/c};Nestin^{cre}$ Animals

Nestin, a ubiquitous marker of neural progenitors, is activated after the initiation of early patterning events in the mammalian neural tube (Zimmerman et al., 1994; Lendahl et al., 1990). To examine the distribution of cells in which *Cre* is expressed, the N^{cre} animals were crossed to a *R26R* line in which Cre-mediated recombination transcriptionally activates a *LacZ* reporter allele (Soriano, 1999; Graus-Porta et al., 2001). These results confirmed the complex spatial and temporal activation of the *Nestin* allele and demonstrated that most if not all telencephalic neural precursors had undergone a Cre-mediated recombination by E12.5 (Figure 1A; Graus-Porta et al., 2001). To evaluate the effectiveness of this transgene in the removal of *Shh* itself in $Shh^{n/c};N^{cre}$ embryos, we used an exon2-specific probe that recognizes the region of the *Shh* transcript that is deleted after Cre-mediated recombination. Expression of an active *Shh* transcript was greatly reduced by E10.5, as was Shh signaling in target cells in $Shh^{n/c};N^{cre}$ brains (Figures 1B and 1C and data not shown). Similarly, when $Smo^{n/c};N^{cre}$ embryos were examined for the expression of *Patched1* (*Ptch1)* and *Gli1*, whose expression provides a direct indication of Shh signaling (reviewed in Ingham and McMahon, 2001), we observed that Shh signaling was almost completely absent from its normal telencephalic domain by E12.5 in these animals (Figures 1D–1G). In a separate study, we have examined the phenotype resulting from earlier removal of hedgehog signaling within the telencephalon by examining $Smo^{n/c}$ mice where excision of the floxed allele was directed by Cre expression from the *Foxg1* loci (Hebert and McConnell, 2000). In these mice removal of *Smo* gene function begins at E8.5 and is complete by E10 (Hebert and McConnell, 2000; G.F., unpublished data). In contrast to $Smo^{n/c};N^{cre}$ brains, the telencephalon of $Smo^{n/c};Foxg1^{cre/+}$ mice is strongly dorsalized (G.F., unpublished data). This demonstrates that hedgehog's requirement in regionalization of the

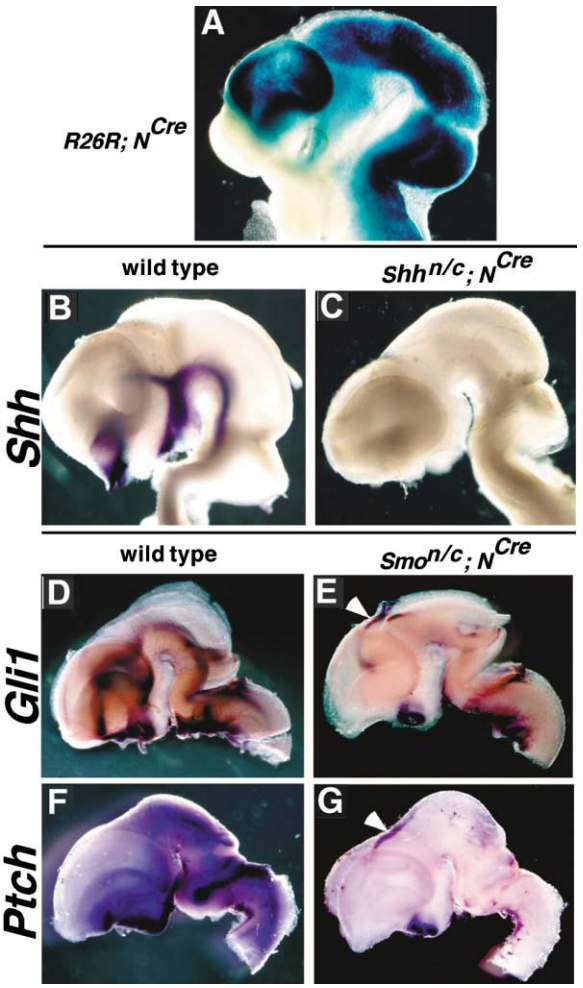

Figure 1. Shh Signaling Is Abolished in the Forebrain of $Shh^{n/c};N^{Cre}$ and $Smo^{n/c};N^{Cre}$ Embryos by E12.5

(A) A $R26R;N^{Cre}$ brain is shown. Here, β-galactosidase histochemistry provides an indirect assay of the extent of Cre activity in these animals. Note that the entire telencephalon appears to be blue. (B) and (C) show in situ hybridizations of wild-type (B) and $Shh^{n/c};N^{Cre}$ (C) animals using an exon2 *Shh* probe that recognizes the regions removed in Cre-mediated recombination. Note in (C) the absence of exon2-containing transcripts in telencephalic tissue, indicating the successful deletion of *Shh* exon2 in $Shh^{n/c};N^{Cre}$ animals by E12.5. In situ hybridization of targets of the Shh signaling pathway, *Gli1* (D and E), and *Ptch1* (F and G) on E12.5 wild-type (D and F) and $Smo^{n/c};N^{Cre}$ (E and G) embryo brains. Note that the expression of *Gli1* and *Ptch1* in the forebrain was completely abolished in the mutants (E and G). Instead, ectopic expression of *Gli1* and *Ptch1* was observed in the dorsal midline of the diencephalon of mutant embryos (arrowhead in [E] and [G]). This occurs due to the combined result of the removal of *Ptch1*-mediated inhibition in more ventral regions and the lack of Nestin-mediated Cre activity in the dorsal diencephalon.

brain precedes the ages studied in the present experiments.

Interestingly in $Smo^{n/c};N^{cre}$ mutants, we observed ectopic activation of *Ptch1* and *Gli1* in the dorsal diencephalon, in an area where Shh signaling is preserved in mutant animals as a result of the absence of Nestin-mediated Cre expression in this region (Figures 1E and 1G). This reflects a dramatically increased range of Shh action in the absence of Ptch1-mediated inhibition of Shh movement, as observed in other systems (e.g., Chen and Struhl, 1996; Burke et al., 1999; Long et al., 2001). Given the similarities in the loss of *Ptch1* and *Gli1* in $Shh^{n/c};N^{cre}$ and $Smo^{n/c};N^{cre}$ animals and the observation that morphologically the phenotypes of these alleles were indistinguishable, we focused our more detailed analysis on $Smo^{n/c};N^{cre}$ animals.

Telencephalic Patterning at E12.5 in $Smo^{n/c};N^{cre}$ Mice Appeared Largely Normal

In *Shh* null mutants, most aspects of ventral patterning in the telencephalon are abrogated (Chiang et al., 1996; Rallu et al., 2002). By contrast, analysis of both telencephalic morphology and the distribution of regional markers indicated that patterning appeared to be relatively unperturbed in $Smo^{n/c};N^{cre}$ animals at E12.5. The distribution of dorsally expressed markers such as Pax6 and *Ngn2* was indistinguishable in mutant and wild-type animals (Figures 2A and 2B and data not shown). Similarly, the expression domain of *Gsh2* and *Dlx2* (Figures 2A and 2B and data not shown), which demarcate the entire ventral telencephalon (Corbin et al., 2000; Yun et al., 2001; Toresson et al., 2000), was unchanged in distribution, although in some cases levels of expression were moderately reduced (data not shown).

As might be expected from studies to date, it was the ventral-most structure in the telencephalon, the medial ganglionic eminence (MGE), that was most affected in these mutants. In $Smo^{n/c};N^{cre}$ embryos, the MGE was reduced in size by 50% and was often fused with the LGE, the structure immediately lateral to the MGE (compare Figures 2E and 2H), a phenotype resembling that observed in *Nkx2.1* mutants. However, the expressivity of this phenotype varied (compare Figures 2H and 2L). Notably, even in the most severely affected mutants, the remaining *Nkx2.1* expression had a normal distribution (Figures 2C–2E and 2F–2H). Furthermore, the MGE in $Smo^{n/c};N^{cre}$ brains maintained a normal pattern of gene expression, as shown by the persistence of the normal domain of *Lhx6* expression (data not shown). This suggests that the defect observed at E12.5 in the $Smo^{n/c};N^{cre}$ telencephalon resulted from a failure in the expansion or survival of MGE progenitors within this region rather than a misspecification of their regional identity. One population that was affected in these mutants was oligodendrocyte precursors, as reflected by the loss or reduction (depending on the anteroposterior level examined) of the preoligodendrocyte marker *Sox10* (Figures 2I and 2L). The underlying cause of the reduced MGE size and decreased oligodendrocyte precursor numbers in affected mutants is unclear. We observed no obvious differences in the levels of proliferation or cell survival within the MGE of wild-type versus mutant embryos at E12.5 (Figure 2J, 2K, 2M, and 2N).

In addition to oligodendrocytes, interneurons are the other major population that arises from the MGE (Anderson et al., 1997; Wichterle et al., 2001). Consistent with the observation that *Dlx2* expression is unchanged in these mutants (data not shown), we detected no proportional decrease in the numbers of GABAergic cells in the cortex, hippocampus, or striatum (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/39/6/937/DC1).

The Expression of *Shh*, *Ptch1*, and *Gli1* in Postnatal Telencephalon

In order to explore which populations in the postnatal (P15) brain might be affected by the loss of hedgehog signaling, we examined the distribution of Shh and its transcriptional targets *Ptch1* and *Gli1* (reviewed in Ingham and McMahon, 2001). Immunostaining using a Shh-specific antibody revealed that Shh is broadly distributed in the P15 telencephalon (Figures 3A and 3B). The most prominent areas of Shh immunoreactivity are in the ventral telencephalon where embryonic *Shh* expression is observed. Specifically, we see Shh-positive cells within the ventral accumbens, entorhinal cortex, and ventral septum, along the path transited by cells in the rostral migratory stream. Furthermore, within layer 3 of the cortex and along the corpus callosum we also observe Shh immunoreactivity (asterisks in Figure 3A and data not shown). Finally, a population of axons in the medial septum is also immunopositive for Shh (arrow in Figure 3A). This observation is consistent with a recent report from the Schaffer laboratory (Lai et al., 2003), which reported that the septal (i.e., fimbria) fibers projecting to the hippocampus are a source of Shh within this structure. Further supporting the observations of Lai et al. (2003), we observe Shh staining in the white matter tracks in the CA3 and hilus regions of the hippocampus (arrows in Figure 3B). Examination of mice that are heterozygous for a LacZ reporter under the control of the endogenous Shh promoter displayed a similar pattern of cellular Shh expression (Figures 3C and 3D). In addition, they revealed the existence of Shh-expressing cells within the hilus of the dentate gyrus, adjacent to the hippocampal stem cell population (arrowhead in Figure 3D). As this staining was not evident in antibody visualization of Shh, it seems likely that these cells only express low levels of *Shh* and require the enzymatic amplification provided by LacZ staining to visualize. The expression of LacZ directed through either the *Ptch1* or *Gli1* loci was generally consistent in indicating in which populations the hedgehog pathway was activated. The expression of LacZ directed by the *Ptch1* loci was broader than that seen from the *Gli1* loci. The observation that *Gli1* expression more closely matched sources of endogenous Shh suggests that *Gli1* is the more accurate readout of Shh activation. Furthermore, embryonic studies indicate that all *Gli1* expression is Shh dependent, whereas *Ptch1*, the Shh receptor, is expressed at basal levels in the absence of any direct Hedgehog signaling input (Bai et al., 2002).

Gli1-LacZ and *Ptch1-LacZ* expression was observed in regions where proliferation persisted in the telencephalon (Figures 3G, 3J, 3L, and 3O). Specifically, within the SVZ, rostral migratory stream, and subcallosal region,

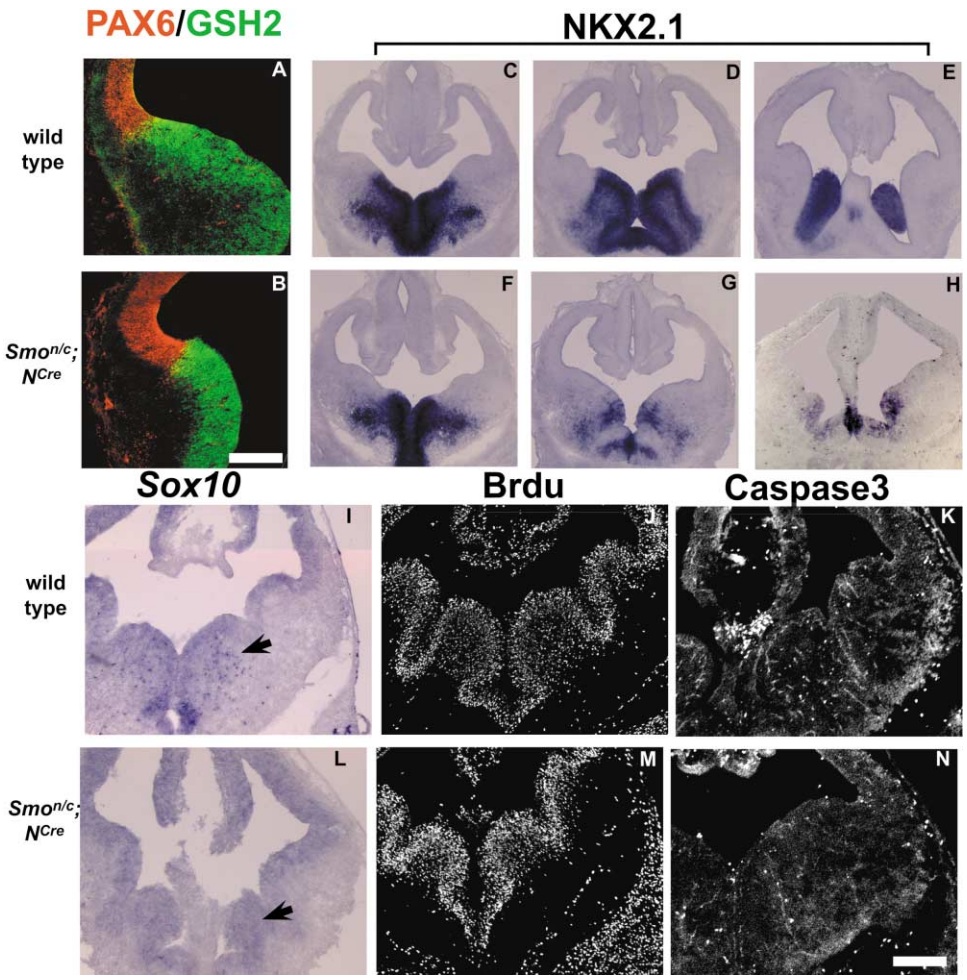

Figure 2. Telencephalic Regional Patterning Is Maintained in *Smo$^{n/c}$;N^{Cre}* Animals at E12.5

Gsh2 and *Pax6* expression (A and B) appear equivalent in mutant animals compared to heterozygotes (data not shown) or wild-type littermates. In situ hybridization for *Nkx2.1* expression at three progressively anterior levels of the telencephalon in wild-type (C–E) and *Smo$^{n/c}$;N^{Cre}* animals (F–H) demonstrate that the expression of *Nkx2.1* is markedly diminished in the telencephalon of some *Smo$^{n/c}$;N^{Cre}* mutants, being almost completely absent at anterior (H) and reduced at posterior telencephalic levels (F and G). Reflecting the variable expressivity in these mutants, the size of the MGE among *Smo$^{n/c}$;N^{Cre}* mutants differed considerably. While the MGE in the mutant specimen shown in (F)–(H) is markedly reduced in size compared to wild-type animals, this structure appeared almost normal in other animals of the same genotype (e.g., [L]–[N]). Examination of oligodendrogenesis in E12 *Smo$^{n/c}$;N^{Cre}* mutants demonstrated that, even in mutants where the MGE appeared morphologically normal, the expression of the oligodendrocyte precursor marker *Sox10* was absent at the level shown (arrowheads in [I] and [L]) and considerably reduced at more posterior levels (data not shown). Even in cases where the MGE was reduced in size, the pattern of BrDU incorporation was indistinguishable from wild-type animals (J and M). Similarly, the level of apoptosis as indicated by Caspase3 (K and N) or TUNEL (not shown) staining was equivalent in *Smo$^{n/c}$;N^{Cre}* mutants compared to either heterozygotes or wild-type littermates. The scale bar in (N) represents 500 μm in (C)–(H) and approximately 200 μm in (I)–(N). The scale bar in (B) represents 100 μm in (A) and (B).

LacZ expression directed from both the *Gli1* and *Ptch1* loci was detectable (Figures 3G, 3J, 3L, and 3O). Comparison of the distribution of LacZ- and Brdu-positive cells suggests that a subpopulation of actively proliferating cells in the SVZ and subcallosal regions (Figure 3H, 3I, and 3L–3N) as well as most in the dentate gyrus (Figures 3J, 3K, 3O, and 3P) were double labeled. In contrast to a recent report using Ptch1 antibody that observed high levels of Ptch1 immunoreactivity throughout the hippocampus (Lai et al., 2003), we only observe significant upregulation of LacZ expression in *Ptch1$^{LacZ/+}$* or *Gli1$^{LacZ/+}$* mice in the dentate gyrus (Figures 3J, 3K, 3O, and 3P). As the Ptch1 antiserum used in the Lai et al. (2003) study has not been characterized for its specificity in immunocytochemistry, we believe that our findings better reflect the normal distribution of Ptch1 in the hippocampus.

The P15 Cortex, Hippocampus, and Olfactory Bulb Were Abnormal in *Smo$^{n/c}$;Nestin^{cre}* Mice

Shh$^{n/c}$;N^{cre} and *Smo$^{n/c}$;N^{cre}* were born in normal Mendelian numbers. Although most pups died within 10 days of birth, approximately 10% of these mice were able to survive for up to 19 days postnatally. Those that did survive to P19 showed clear motor dysfunction, a likely result of defects in the cerebellum that will be addressed in detail elsewhere (A.P.M., unpublished data). Among the *Shh$^{n/c}$;N^{cre}* and *Smo$^{n/c}$;N^{cre}* mice that survived until

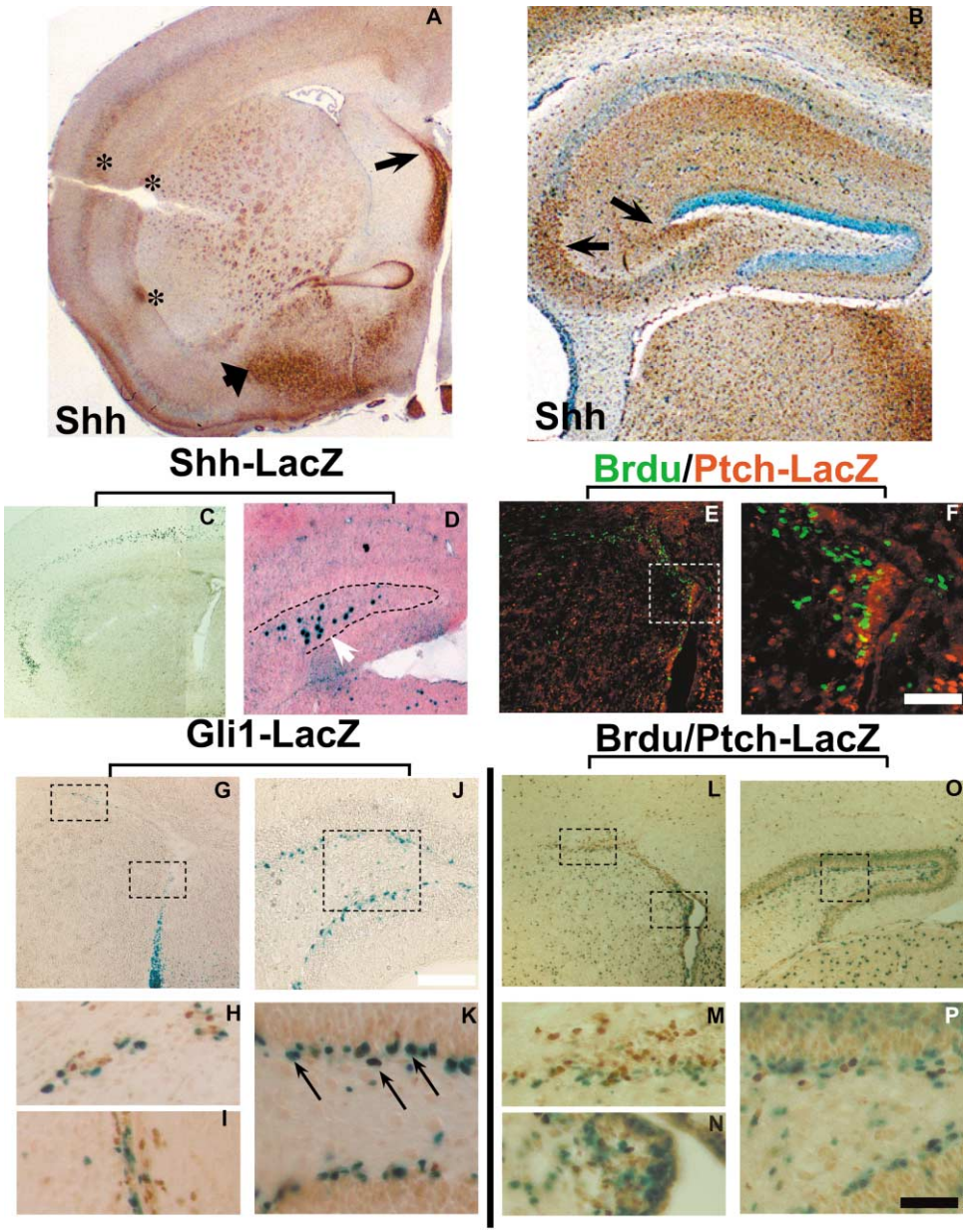

Figure 3. Postnatal Expression of Shh, *Gli1*, and *Ptch1* in Proliferating and Postmitotic Telencephalon

(A) and (B) show immunocytochemical localization of Shh in the adult telencephalon. Arrowhead in (A) shows the expression of Shh in the ventral accumbens, entorhinal cortex, and ventral septum. Arrow in (A) shows axonal labeling in the medial septum. Asterisks in (A) show expression of Shh beneath the corpus callosum (lower two) and in layer 3 of the cortex (upper asterisk). Arrows in (B) show the Shh expression in the CA3 and hilus regions of the hippocampus (this Shh presumably originates from the efferent fimbria fibers, including the medial septal fibers shown in [A]). (C) illustrates that a similar pattern of LacZ expression is observed in $Shh^{LacZ/+}$ animals as seen with immunocytochemistry. (D) shows that LacZ-positive cells (arrowhead) are observed in the hilus of the hippocampus in $Shh^{LacZ/+}$ animals (dashed line indicates the apical surface of the dentate gyrus). (E) is a photomicrograph of the striatum and SVZ of a P15 $Ptch1^{LacZ/+}$ mouse double labeled for BrdU (green) and LacZ (red). (F) is an enlargement of the boxed region in (E). In these animals, only a few cells in the SVZ are double labeled. (G) and (J) show the striatum and associated SVZ (G) and the hippocampus (J) of P15 $Gli1^{LacZ/+}$ mice. (L) and (O) show the striatum and associated SVZ (L) and the hippocampus (O) of P15 $Ptch1^{LacZ/+}$ mice double labeled for LacZ (green) and BrDU (brown). (H), (I), and (K) show enlargements of the double labeling for LacZ and BrDU in adjacent sections from those shown in (G) or (J), respectively, from the approximate areas shown in (G) or (J). (H) is the area shown in the upper box and (I) is the lower box of (G), while (K) is the area shown in the box in (J). (M), (N), and (P) show enlargements of the double labeling for LacZ and BrDU in sections shown in (L) or (O), respectively. (M) is the area shown in the upper box and (N) is the lower box of (L), while (P) is the area shown in the box in (O). The small rip in the tissue in (A) is the unfortunate consequence of the dehydration step necessary for optimal Shh immunostaining. The scale bar in (F) represents 750 μm in (A), 200 μm in (B), 2000 μm in (C) and (E), 150 μm in (D) and (F). The scale bar in (P) represents 1500 μm in (G) and (L), 200 μm in (J) and (O), and 75 μm in (H), (I), (K), (M), (N), and (P).

P15, gross inspection of the telencephalon did not reveal any obvious phenotype other than enlarged telencephalic ventricles and a 30% reduction in brain size (Figures 4A and 4B). Nonetheless, analysis of coronal sections from these mice revealed a number of specific defects. Aside from the reduction in brain mass, the general organization of postmitotic tissues appeared relatively normal. By contrast, analysis of the proliferative zones in *Smo$^{n/c}$;N^{cre}* animals revealed marked abnormalities in both the size of these regions and the appearance of the progenitors contained within them (Figures 4A–4D). The three regions in which neurogenesis normally persists postnatally are the SVZ, its associated rostral migratory stream (RMS), and the dentate gyrus of the hippocampus (Figure 4; reviewed in Gage et al., 1998; Alvarez-Buylla and Garcia-Verdugo, 2002). Each of these regions were reduced in size in *Smo$^{n/c}$;N^{cre}* animals and contained reduced numbers of cells (compare Figure 4C versus 4D, 4G versus 4H, and 4I versus 4J). In the adult hippocampus, the dentate gyrus normally contains proliferating cells (Eriksson et al., 1998). In this region, we observed a specific reduction in the number of granule cells relative to other regions of the hippocampus, and those remaining granule cells were abnormal (Figure 4G versus 4H). Similarly, the olfactory bulb of these animals, an area that is continually supplied by nascent neurons from the SVZ (Doetsch et al., 1999), was abnormally small and exhibited a disorganized cytoarchitecture. Furthermore, granule cells within the olfactory bulb were greatly decreased in number compared to wild-type siblings (Figure 4I versus 4J).

The Generation of Oligodendrocytes Was Compromised in *Smo$^{n/+}$;N^{cre}* Animals

Given the embryonic loss of oligodendrocyte precursors (Figures 2I and 2L) and the fact that the postnatal SVZ is also a source of oligodendrocytes (Levison and Goldman, 1993; Goldman, 1995), we investigated whether this population was compromised in P15 *Smo$^{n/c}$;N^{cre}* mutants. Consistent with a deficiency in oligodendrogenesis, in P15 *Smo$^{n/c}$;N^{cre}* mutant animals both the corpus callosum and anterior commissure were dramatically reduced, and axon tracts were defasciculated (Supplemental Figure S2A–S2D at http://www.neuron.org/cgi/content/full/39/6/937/DC1). Furthermore, we observed an obvious reduction of the mature oligodendrocyte marker myelin basic protein (MBP) at P15 (Supplemental Figure S2E–S2H; Bansal et al., 1989; Braun et al., 1988). Quantitation of oligodendrocyte numbers in these animals revealed a 30% decrease in myelination, in addition to the general reduction in brain mass reported above (Supplemental Figure S2J).

Reduced Numbers of Proliferating Cells and Increased Cell Death in the Perinatal Telencephalic Progenitor Zones of *Smo$^{n/c}$;N^{cre}* Animals

To investigate the underlying cause of the abnormalities we observed in the size and morphology of cells within the olfactory bulb, SVZ, and hippocampus, we investigated the pattern of proliferation in these regions in *Smo$^{n/c}$;N^{cre}* animals. P15 *Smo$^{n/c}$;N^{cre}* mice and heterozygous littermate controls were injected with BrdU to examine cell proliferation within telencephalic progenitor

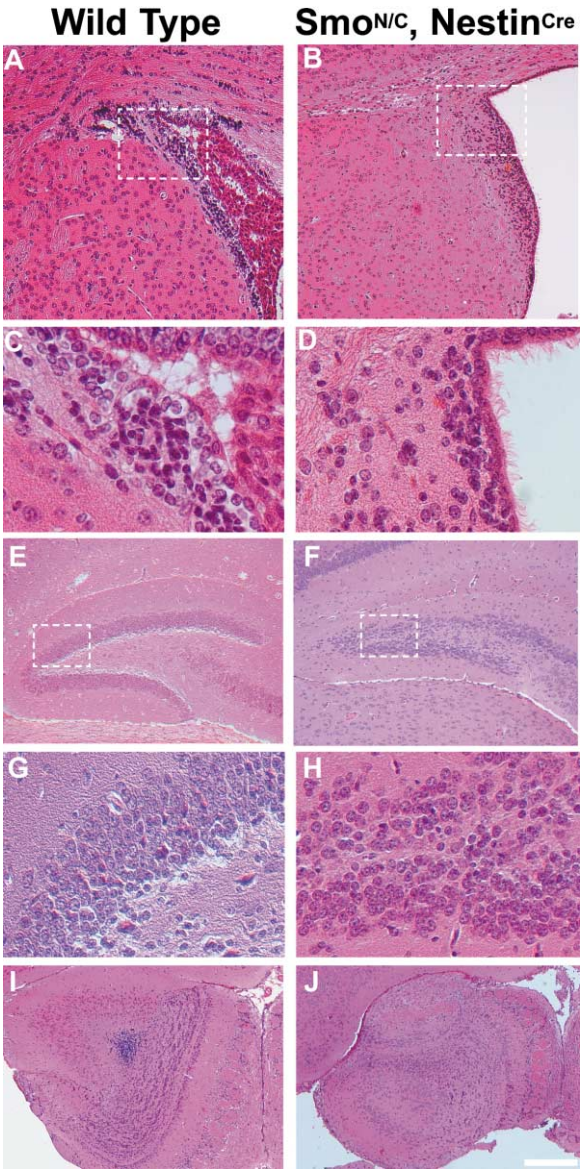

Figure 4. Reduction in the SVZ and in Granule Cells in the Dentate Gyrus and Olfactory Bulb of P15 *Smo$^{n/c}$;N^{Cre}* Brains

P15 wild-type (A and C) and *Smo$^{n/c}$;N^{Cre}* brains (B and D). *Smo$^{n/c}$;N^{Cre}* brains are moderately reduced in size and have enlarged telencephalic ventricles. (C) and (D) show higher-power views of the SVZ regions shown in (A) and (B), respectively. Note the disorganization and sparseness of cells in the *Smo$^{n/c}$;NCre* SVZ (D) compared to wild-type controls (C). (E)–(H) show low-power (E and F) and high-power (G and H) views of the hippocampus of wild-type (E and G) and *Smo$^{n/c}$;N^{Cre}* (F and H) animals. Note the marked reduction in the size and number of granule cells in the dentate gyrus of *Smo$^{n/c}$;N^{Cre}* brains (F and H). (I) and (J) show granule cells in the P15 olfactory bulb of wild-type (I) and *Smo$^{n/c}$;N^{Cre}* (J) animals. As in the hippocampus, there is also a reduction in the size and number of granule cells in the granule cell layer of the olfactory bulb. The scale bar in (J) represents 200 μm in (E), (F), (I), and (J); 100 μm in (A) and (B); and 25 μm in (C), (D), (G), and (H).

domains. In all three regions of active DNA replication, the numbers of BrdU-incorporating cells were significantly reduced in *Smo$^{n/c}$;N^{cre}* animals (Figures 5A–5G, $p > 0.001$), suggesting that either proliferation within

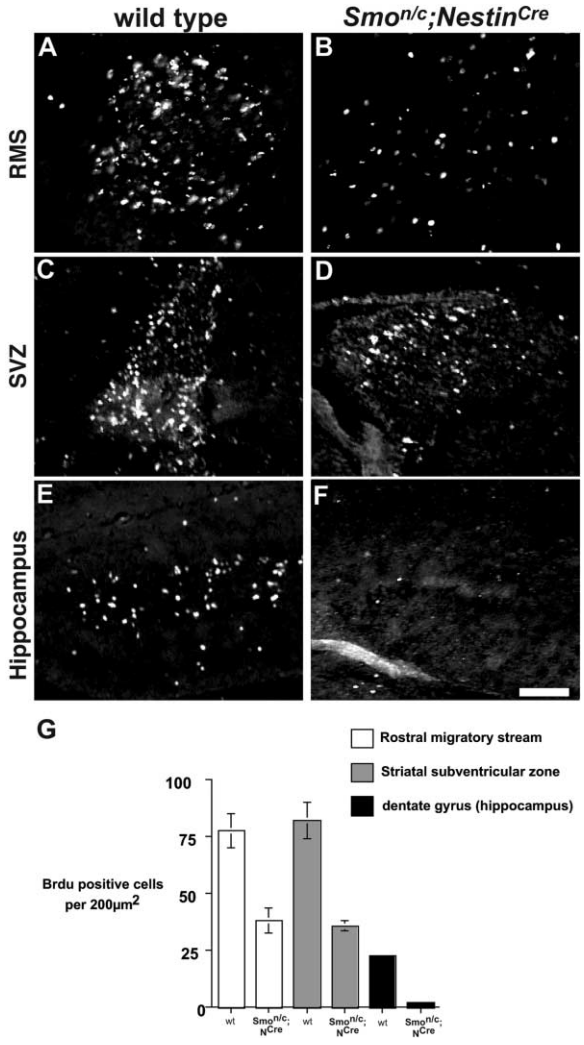

Figure 5. P15 *Smo$^{n/c}$;N^{Cre}* Animals Have Reduced BrdU Incorporation in the Olfactory Bulb, SVZ, and Dentate Gyrus

(A)–(F) show BrdU-incorporating cells in the rostral migratory stream (A and B), SVZ (C and D), and dentate gyrus (E and F) of wild-type (A, C, and E) and *Smo$^{n/c}$;N^{Cre}* (B, D, and F) animals at E18.5. Data are quantitated in (G). Note that both qualitative (A–F) and quantitative (G) analysis suggests that the numbers of proliferating cells in all three of these progenitor zones are reduced in *Smo$^{n/c}$;N^{Cre}* animals. The scale bar in (F) represents 200 μm in (A)–(F).

these precursor populations was reduced or there was a direct loss of cells within these populations that secondarily results in decreased numbers of proliferating progenitors. To differentiate between these possibilities, we examined the telencephalon for direct evidence of cell death using Caspase3 and TUNEL staining (Figure 6). Caspase3 is a protein directly involved in programmed cell death (reviewed in Budihardjo et al., 1999), while TUNEL staining reveals the presence of DNA cleavage that characterizes apoptosis (reviewed in Roth and D'Sa, 2001). Caspase3 and TUNEL staining within the SVZ at E18.5 (data not shown) and P15 revealed the presence of large numbers of apoptotic cells (Figure 6). As oligodendrogenesis persists postnatally, we examined whether there was increased cell death in myelinated areas such as the corpus callosum. Double labeling with the mature oligodendrocyte marker CNPase demonstrated that myelinated corpus callosum in P15 telencephalon was decreased in size (Figures 6G and 6H, see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/39/6/937/DC1 for a more detailed analysis). The generation of postnatal oligodendrocytes is likely a result of both the embryonic decrease in oligogendrogenesis (Figures 2I and 2L) and the enhanced apoptosis in the SVZ of these mutants. In contrast, despite the abnormal appearance of cells in the hippocampus of *Smo$^{n/c}$;N^{cre}* animals (Figures 4E–4H) and the reduction in proliferating cells (Figures 5E–5G), no similar indications of apoptosis were observed in the dentate gyrus (data not shown). Together these results suggest that the decreased proliferation in the hippocampus may be attributable to Shh's role as a mitogen in this region. By contrast, while Shh may also promote proliferation in the SVZ, it also appears to be required for the trophic support of progenitors.

Hh Agonist Increases the Proliferation and Gli1 Expression in the SVZ and Dentate Gyrus

To further determine whether the Shh pathway is involved in cell proliferation in the postnatal SVZ and dentate gyrus, we utilized a recently described small-molecule activator of the Shh signaling pathway (Hh agonist; Frank-Kamenetsky et al., 2002). This agonist is known to upregulate Hedgehog signaling in hedgehog-responsive tissue through what appears to be its interactions with Smoothened (Frank-Kamenetsky et al., 2002). We first demonstrated that administration of Hh agonist to mice activated the Shh pathway in the adult brain, as shown by increased *Gli1* expression (Figure 7). Wild-type and *Gli1$^{LacZ/+}$* mice were then treated with Hh agonist for either 3 or 1 day, respectively. Two hours prior to sacrifice, animals were given a pulse of Brdu. In these animals, the numbers of Brdu-immunoreactive cells in wild-type mice and LacZ-positive cells in *Gli1$^{LacZ/+}$* mice were increased in both the SVZ and dentate gyrus following Hh agonist treatment (Figure 7). Together these results demonstrate that the hedgehog pathway remains important and stimulates progenitor cell proliferation in the adult brain.

Progenitors from the SVZ of *Smo$^{n/c}$;N^{cre}* Mice Show Reduced Potential to Generate Neurospheres

To directly examine Shh's requirement for the maintenance of telencephalic stem cells, we dissected tissues from the SVZ to examine the capacity of these cells to form neurospheres in vitro. The neurosphere assay was developed by Reynolds and Weiss (1992) to test for the presence of stem cell populations in the CNS. In this assay, neural cells are dissociated, plated at low density, and examined for their ability to proliferate in clusters of clonally related cells. Pluripotency of progenitors within these spheres can be demonstrated by dissociating these clusters and allowing the cells to attach and differentiate. However, if attachment is prevented, these cells can be passaged in the presence of FGF or EGF into secondary neurospheres. If Shh is required for stem cell maintenance, one would predict that *Smo$^{n/c}$;N^{cre}* progen-

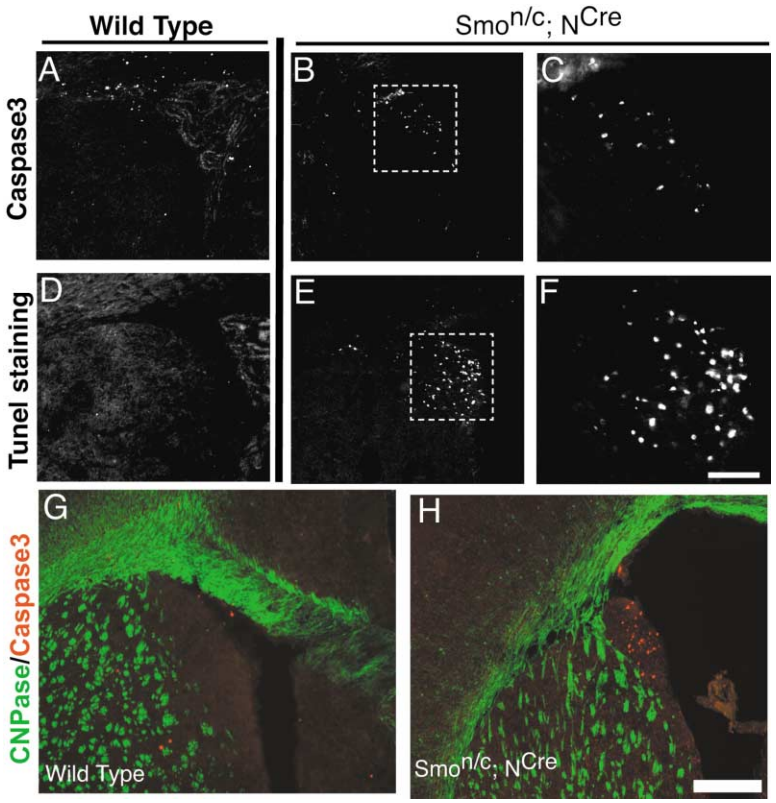

Figure 6. Apoptosis in the SVZ of *Smo*$^{n/c}$;*N*^{Cre} Brains at P15

(A)–(F) show Caspase3 (A–C) and TUNEL (D–F) staining in P15 wild-type (A and D) and *Smo*$^{n/c}$;*N*^{Cre} (B, C, E, and F) telencephalon. While numerous Caspase3- (B and C) and TUNEL-positive (E and F) cells were observed in the SVZ of *Smo*$^{n/c}$;*N*^{Cre} mice, none were observed in wild-type animals (A and D). (C) and (F) show higher-powered views of the areas shown in (B) and (E), respectively. (G) and (H) show double labeling for CNPase (in green) and Caspase3 (in red), demonstrating that the cell death in P15 telencephalon is confined to the SVZ and does not occur in mature oligodendrocytes. The scale bar in (H) represents 100 μm in (C) and (F); 150 μm in (G) and (H); and 200 μm in (A), (B), (D), and (E).

itors from the SVZ would yield fewer neurospheres but the potential, size, and ability to passage individual neurospheres would be largely unaffected.

We cultured SVZ progenitors from wild-type or mutant animals in the presence of EGF or FGF. PCR genotyping of the resultant spheres confirmed that *Smo*$^{n/c}$;*N*^{cre} neurospheres had undergone complete recombination and were therefore null for *Smo* activity (Figures 8A and 8B). In both EGF and FGF supplemented cultures, we observed that the loss of *Smo* function resulted in a dramatic reduction in the numbers of neurospheres formed from the SVZ (Figure 8C). However, the size range of the neurospheres was indistinguishable regardless of whether SVZ progenitors were isolated from wild-type or *Smo*$^{n/c}$;*N*^{Cre} brains (Figure 8D). Furthermore, the ability of neurospheres to differentiate into neurons, astrocytes, or oligodendrocytes and to be passaged clonally into secondary neurospheres was equivalent (data not shown, Figure 8D). These results suggest that hedgehog signaling is required for the maintenance of neural stem cells in the SVZ. We attempted to determine whether the addition of Shh was sufficient to promote SVZ neural progenitor survival by culturing progenitors in the presence of Shh for several days prior to the addition of mitogen (EGF and FGF). However, Shh did not promote either the survival or expansion of the neural progenitor population in vitro (Figure 8E). These results are in contrast to those recently reported by Lai et al. (2003) in their study of hippocampal stem cells and argue that the requirement for Shh in the hippocampus versus the SVZ stem cell populations may differ.

Discussion

We have used multiple strategies to remove or activate Shh signaling in the telencephalon to determine its later role in these regions. Our results suggest that subsequent to E12.5 the primary requirements for Shh signaling in the telencephalon are for oligodendrogenesis and the maintenance of adult neural progenitor populations. In contrast to *Shh*$^{-/-}$ null mutants (Chiang et al., 1996; Rallu et al., 2002), the only obvious telencephalic patterning abnormalities observed in these animals were a variably penetrant decrease in the size of the MGE and a deficit in the production of early oligodendrocyte precursors. Apart from these defects, our results suggest that hedgehog signaling in the telencephalon has no prominent role during embryonic neurogenesis (E12–E18). Despite the lack of an embryonic patterning or neurogenic phenotype, both *Shh*$^{n/c}$;*N*^{cre} and *Smo*$^{n/c}$;*N*^{cre} mice revealed a later function for hedgehog signaling. Previous work suggests that oligodendrocytes and granule neurons are two cell types that arise postnatally (Levison and Goldman, 1993; Lois and Alvarez-Buylla, 1993), and we observe that the loss of Shh signaling has a dramatic effect on both these populations. Specifically, the number of proliferating cells that give rise to these cell types was markedly decreased in both the hippocampal dentate gyrus and the SVZ of *Smo*$^{n/c}$;*N*^{cre} animals. In addition, the loss of Shh signaling results in SVZ cells undergoing programmed cell death. Conversely, pharmacological activation of Shh signaling in postnatal mice increases the number of proliferating

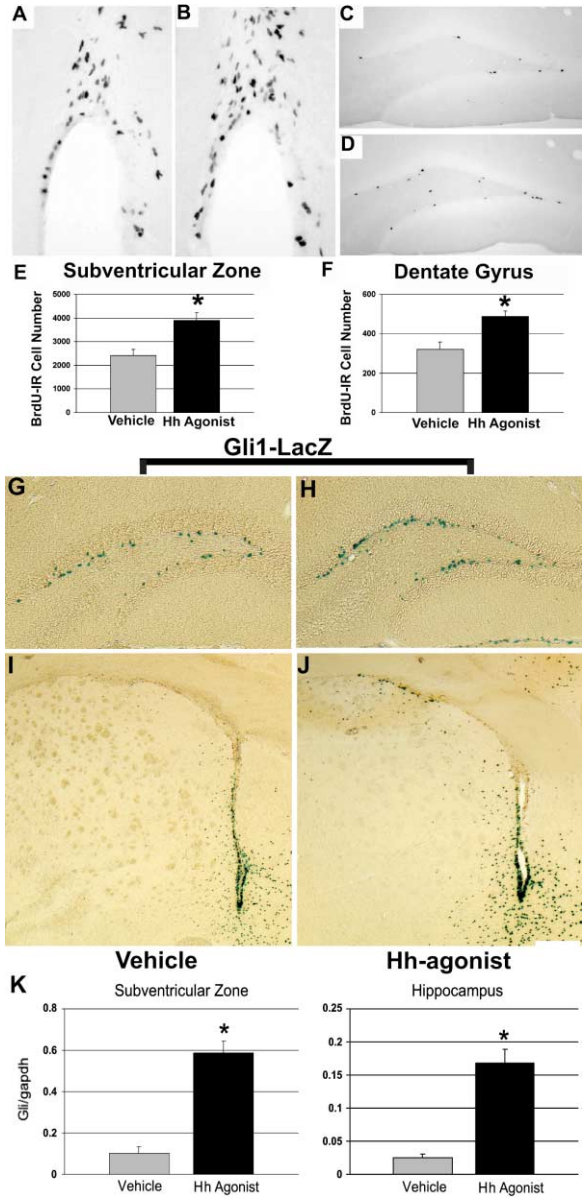

Figure 7. Oral Administration of Hh Agonist Increased Numbers of BrdU-Immunoreactive Cells and *Gli1* Expression in the Adult Mouse SVZ and Hippocampal Dentate Gyrus

(A)–(D) show photomicrographs showing BrdU-immunoreactive (IR) nuclei in coronal sections through the rostral SVZ (A and B) and hippocampal dentate gyrus (C and D) in adult mice. Increased numbers of BrdU-positive cells are seen in mice treated daily with Hh agonist for 3 days (20 mg/kg, [B] and [D]) in both brains regions as compared to vehicle-treated controls (A and C). (E) and (F) show graphs quantifying the number of BrdU-positive cells in the SVZ and dentate gyrus in mice treated with Hh agonist and vehicle. Data are expressed as mean ± SEM. * indicates significant difference between groups, $p < 0.05$. (G)–(J) show LacZ staining in the dentate gyrus (G and H) and SVZ (I and J) of $Gli1^{LacZ/+}$ mice oral dosed with vehicle (G and I) or Hh agonist (H and J) for 1 day prior to sacrifice. (K) shows quantitative induction of *Gli1* mRNA, as measured by quantitative RT-PCR, in both the SVZ (left) and hippocampus (right) following daily administration of Hh agonist (20 mg/kg) for 3 days as compared to vehicle treatment. Data are expressed as mean ± SEM. * indicates significant difference between groups, $p < 0.05$.

cells in the hippocampus and SVZ and upregulates the levels of *Gli1* in these populations. Our in vitro analysis as well as recent findings from the Schaffer laboratory also support the idea that Shh functions to maintain these progenitors (Lai et al., 2003). Together, our results support a model in which Shh signaling in the adult telencephalon is critical for maintaining stem cell niches in the forebrain.

The Role of Shh Signaling in Mitosis versus Maintenance of Neural Progenitors

Several lines of evidence support a role for Shh regulating progenitor cell proliferation in the CNS. Besides the reduction in cell types due to ventral patterning defects (Rallu et al., 2002), *Shh* null animals have a marked decrease in the size of the brain (Chiang et al., 1996). Conversely, *Shh* gain-of-function studies have demonstrated both in vitro and in vivo that ectopic exposure of the telencephalon and other areas of the neural tube to Shh results in hypertrophy (Gaiano et al., 1999; Rowitch et al., 1999). Analysis by a number of groups examining the cerebellum indicates that Shh emanating from Purkinje cells mediates the proliferation of granule precursors in the external granule cell layer (Wechsler-Reya and Scott, 1999; Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; A.P.M., unpublished data). Further, human disorders resulting in an increase in Shh signaling, as a result of *Ptch1* heterozygosity or activation of Shh signaling in target cells, results in the formation of cerebellar medulloblastomas (reviewed in Ingham, 1998). Finally, Shh has been proposed to regulate proliferation in dorsal CNS structures during both late embryogenesis and adulthood (Dahmane et al., 2001).

Our data suggests that Shh signaling has only modest effects on the general growth of ventral telencephalic regions after E12.5. In contrast, it is essential in the dentate gyrus and the olfactory bulb, regions in which neurogenesis continues in the adult. Specifically, $Smo^{n/c};N^{cre}$ mice displayed a marked decrease in the levels of proliferation in both these areas. In addition, in the SVZ, apoptotic cell death is dramatically increased. Recent work by Lai et al. is consistent with these findings. They report that within the hippocampus Shh signaling acts as a mitogen for adult progenitors residing in the dentate gyrus. While our data support the contention that Shh is required for the maintenance of hippocampal and SVZ progenitors, it is less clear that within the SVZ Shh acts directly as a mitogen. Although both the SVZ and dentate gyrus of $Smo^{n/c};N^{cre}$ mutants appear to have fewer progenitors, Shh alone was unable to support neurosphere formation or expansion from either wild-type or $Smo^{n/c};N^{cre}$ SVZ progenitors (data not shown), whereas Shh appears to be able to substitute for FGF in the expansion of dentate gyrus precursors in rat (Lai et al., 2003). Similarly, while almost all hippocampal cells labeled by a Brdu pulse 2 hr prior to sacrifice at P15 expressed *Gli1*, only a small subpopulation of similarly labeled cells in the SVZ express this direct target of Shh signaling. Nonetheless, in both the SVZ and hippocampus, increasing levels of Shh signaling increase cell proliferation. Hence, it will require further work to determine the precise actions of hedgehog signaling in maintaining telencephalic progenitor regions.

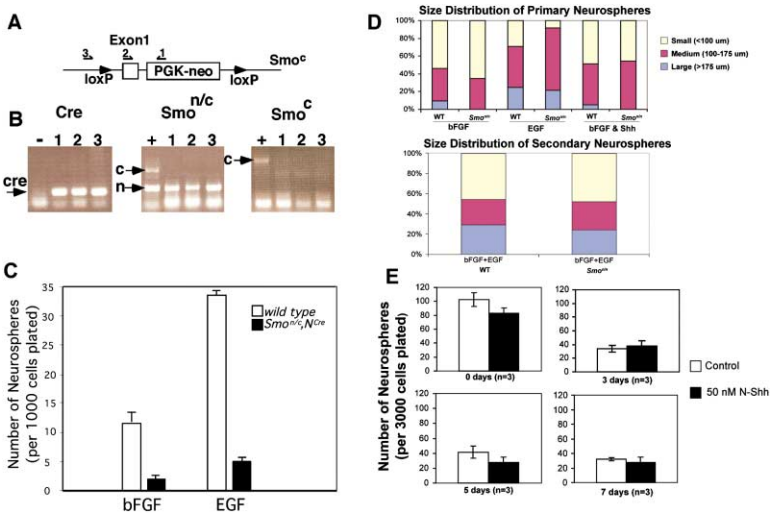

Figure 8. The P15 SVZ of *Smo*$^{n/c}$;*N*^{Cre} Animals Has Reduced Numbers of Neural Stem Cells

(A) shows a diagrammatic representation of the conditional *Smo*^{c} locus and position and specificity of the PCR primers used to assess recombination of the conditionally null *Smo* locus. (B) shows neurospheres from three independent *Smo*$^{n/c}$;*N*^{Cre} mutant donors (1, 2, and 3) that were subjected to PCR. *Smo*$^{n/c}$ animals served as a control for the absence of Cre activity (− in left panel) and for the presence of *Smo*^{c} and *Smo*^{n} alleles in *Smo*$^{n/c}$ or *Smo*$^{c/+}$ PCR (+ in middle or right panel). The presence of the Cre transgene was determined with a specific PCR reaction that produced a 354 bp product (arrow in left panel). To confirm the absence of the conditional allele, two independent PCR reactions were carried out. One PCR amplifies both the conditional (c; ~1000 bp) and null (n; 380 bp) allele (middle panel) using oligonucleotides 1 and 3 (marked by half arrow), and the other (right panel) amplifies only the conditional allele (600 bp) using oligonucleotides 1 and 2. + and − are the positive and negative controls for each PCR reaction, respectively. The null allele from the *Smo*^{n} locus is amplified in the primer 1 and 3 PCR and comigrates with the null allele generated by Cre-mediated recombination of the *Smo*^{c} allele. (C) shows quantitation of the numbers of neurospheres generated from cells derived from either wild-type and *Smo*$^{n/c}$;*N*^{Cre} donors. The number of neurospheres formed per 1000 cells was dramatically reduced when the donor cells lacked *Smo* gene activity. (D) shows that we observed no statistically significant differences in the percentages of large, medium, and small primary (top panel) and secondary (bottom panel) neurospheres derived from *Smo*$^{n/n}$ and wild-type neural progenitors grown for 7 days in vitro (n = 3 mice for each experiment) regardless of whether these spheres were grown in the presence of bFGF, EGF, or bFGF and Shh. (E) shows neural progenitors cultured in the presence or absence of 50 nM N-Shh for 0, 3, 5, or 7 days prior to the addition of mitogen (EGF and FGF). Quantitation of the number of neurospheres that formed 7 days in vitro after growth factor addition is shown below for each time point. Notice that there is no statistical difference in the number of neurospheres that formed from neural progenitors cultured in the presence versus absence of N-Shh.

Comparison of *Shh*$^{n/c}$;*N*^{cre} and *Smo*$^{n/c}$;*N*^{cre} Mutants to *Nkx2.1* Null Animals

Both *Shh*$^{n/c}$;*N*^{cre} and *Smo*$^{n/c}$;*N*^{cre} animals had telencephalic defects that morphologically resembled those seen in *Nkx2.1* null animals. Most notably, the MGE was markedly reduced in size. However, whereas this phenotype reflects an MGE to LGE transformation in *Nkx2.1* mutants (Sussel et al., 1998), the MGE was specified normally following late removal of Shh signaling, as evidenced by persistence of *Lhx6* in *Smo*$^{n/c}$;*N*^{cre} animals (data not shown). Further, although *Nkx2.1* mutants showed a dramatic decrease in the numbers of cortical GABAergic interneurons, their numbers were not proportionally decreased in *Smo*$^{n/c}$;*N*^{cre} mutant brains. Most likely, the loss of cortical GABAergic interneurons in *Nkx2.1* mutants reflects the transformation of the MGE into an LGE rather than a loss of Shh signaling per se in *Nkx2.1* mutants. Indeed, while the expression of Shh within the telencephalon itself is lost in *Nkx2.1* mutants (Sussel et al., 1998; Nery et al., 2001), the earlier nonneural expression of Shh required for ventral patterning is unaffected.

Notably, we have previously shown that the generation of oligodendrocytes is compromised in *Nkx2.1* mutants (Nery et al., 2001, see also Tekki-Kessaris et al., 2001) and that this phenotype can be partially rescued by virally mediated Shh expression (Nery et al., 2001). Consistent with this result, we observe that the production of oligodendrocyte progenitors is compromised in *Smo*$^{n/c}$;*N*^{cre} mice. These findings lend further support to the idea that Shh's primary function during telencephalic neurogenesis is to support the generation of oligodendrocytes but, as noted below, does not obviate a later role for Shh in oligodendrocytes that are generated later in the pallium (Gorski et al., 2002).

The Role of Hedgehog Signaling in Oligodendrogenesis

Recent studies have argued that the generation of oligodendrocytes in the forebrain depends on hedgehog signaling from E12.5 onward (Nery et al., 2001; Tekki-Kessaris et al., 2001; Alberta et al., 2001). The present results are consistent with this hypothesis. However, our findings suggest that the roles of Shh during embryonic oligodendrogenesis and in the adult are distinct. Consistent with this idea, while oligodendrocytes are thought to arise from ventral regions during embryogenesis (Nery et al., 2001), recent work has demonstrated that a majority of oligodendrocytes in the corpus callosum and fimbria originate from the cortex (Gorski et al., 2002). Embryonically we observe that oligodendrocyte precursors in the MGE are greatly diminished in number. This suggests that during embryogenesis Shh may be acting to specify oligodendrocyte progenitors. By contrast, the cortical origin of many oligodendrocytes combined with the reduction in the corpus callosum seen in *Smo*$^{n/c}$;*N*^{Cre} suggests that hedgehog signaling is required for the generation of pallial derived oligodendrocytes generated later in development. Further study will be required to understand the function of hedgehog signaling in this population.

More generally, recent work has raised questions as to the absolute requirement for Shh in the generation of oligodendrocytes in the telencephalon (Nery et al., 2001). In support of Shh signaling being essential for the generation of oligodendrocytes, in vitro blockade of

Shh signaling using pharmacological means completely blocks oligodendrocyte formation. The application of cyclopamine, a general inhibitor of the hedgehog signaling pathway, to dissociated telencephalic cells isolated at E10.5 and cultured for 8 days in vitro prevents oligodendrocyte formation (Tekki-Kessaris et al., 2001). However, *Shh* null mutants generate oligodendrocytes (Nery et al., 2001), implying that other hedgehog proteins (Dhh or Ihh) may compensate. In our study, oligodendrocytes formed but in reduced numbers following the removal of Smo activity by E12.5. Thus, if there is an absolute requirement for hedgehog signaling in the specification of oligodendrocyte precursors, this must occur during the early patterning phases of development. The analysis of *Smo* mutant cells in chimeras generated between *Smo* and wild-type cells may resolve this issue.

By birth, neurogenesis in the telencephalon becomes restricted to a few specialized regions adjacent to the ependyma, in the SVZ and dentate gyrus (Gage et al., 1998; Alvarez-Buylla and Garcia-Verdugo, 2002). These areas become specialized stem cell niches akin to those in the gonads, skin, and gut (Parisi and Lin, 1998). Notably, in *Drosophila*, hedgehog signaling regulates the stem cell property of cells that give rise to somatic cells in the ovary and germline in males (Forbes et al., 1996a, 1996b; Tworoger et al., 1999; Zhang and Kalderon, 2000; King et al., 2001). Taken together with our findings, this suggests that hedgehog signaling is intimately involved in the maintenance of stem cell niches across widely divergent species and cell types. Of immediate interest will be to determine the precise somatic cells within the telencephalic SVZ and dentate gyrus that produce Shh through to adulthood and to further explore the in vivo relationship between the cells producing the Shh ligand and the neural cells that respond to the signaling input. Finally, it may be possible to take advantage of the continued importance of Shh signaling in adult progenitor zones, to develop new therapeutic approaches for neural disorders. We have shown that activation of the Shh signaling pathway in situ, using an orally administered Hh agonist, leads to proliferation of endogenous progenitor cells. If these cells can be recruited to repopulate areas of damage or cell loss in the adult brain, this strategy may allow the body's own developmental pathways and resident progenitor cells to be utilized for regenerative therapies.

Experimental Procedures

Generation and Breeding of Floxed *Shh* and Floxed *Smo* Mice

For a description of the conditional Shh^{c} and Smo^{c} alleles, recombination at this loci, and genotyping procedures, see Lewis et al. (2001) and Long et al. (2001). To remove *Shh* or *Smo* activity from neural precursors, $Nestin^{Cre}$ (N^{Cre}) (Graus-Porta et al., 2001) animals were first intercrossed to mice carrying the *Shh* null (Shh^{n}; St-Jacques et al., 1998) or *Smo* null (Smo^{n}; Zhang et al., 2001) alleles to generate $Shh^{n/+};N^{Cre}$ or $Smo^{n/+};N^{Cre}$ males. These were then mated to either $Shh^{c/c}$ or $Smo^{c/c}$ females to generate embryos or pups of the informative genotypes $Shh^{n/c};N^{cre}$ and $Smo^{n/c};N^{cre}$ (approximately 25% of pups in litters). The N^{Cre} allele used for directing removal of *Shh* or *Smo* expresses the Cre recombinase from the neural-specific enhancer of the nestin promoter (Tronche et al., 1999). This nestin-Cre allele induces efficient and widespread recombination in precursors of neurons and glia starting around embryonic day E10.5, consistent with the known properties of the neural-specific enhancer of the nestin promoter (Zimmerman et al., 1994; Graus-Porta et al., 2001). Conversely, this transgene does not result in Cre expression in nonneural sources of Shh signaling, such as the limb and notochord.

Generation and Usage of $Shh^{ires\text{-}LacZ};Ptch1^{LacZ}$ and $Gli1^{LacZ}$ Reporter Strains

Heterozygous $Shh^{ires\text{-}LacZ}$ (gift of Andrea S. Kottman and Thomas Jessell; A.P.M., unpublished data), $Ptch1^{LacZ}$ (Jackson Labs; Goodrich et al., 1997), and $Gli1^{LacZ}$ (gift of A. Joyner; Bai et al., 2002) were collected at E18 and P15. Two hours prior to sacrifice, either the pregnant mother or the postnatal pup was injected with a pulse of BrDU. Animals were fixed with 4% paraformaldhyde, sectioned, and histochemically stained for β-galactosidase activity. In some cases, animals were then processed to visualize cells that incorporated BrDU using immunofluorescence or Dab as a histochemical substrate.

Tissue Preparation and Histology

E12.5, E18.5, and P15 embryos were fixed overnight in 4% paraformaldehyde at 4°C, rinsed in PBS, dehydrated in methanol, and stored at −20°C prior to whole-mount in situ hybridization. For section in situ hybridization, heads of embryos or postnatal pups were fixed by immersion in 4% paraformaldehyde (PFA) for 2–3 hr at 4°C. Then all samples were cryoprotected in 30% sucrose in PBS, embedded in Histoprep (Fisher Scientific), and frozen. All tissue was serially sectioned at 20 μm prior to RNA in situ hybriziation or immunofluorescence analysis.

In Situ Hybridization

Section in situ hybrizations were carried out as described in Schaeren-Wiemers and Gerfin-Moser (1993) and Wilkinson and Nieto (1993) using nonradioactive DIG-labeled probes. The *cDNA* probes used were *Shh* (Echelard et al., 1993), *Gli1* (Kinzler et al., 1988), *Ptch1* (Goodrich et al., 1997), *Nkx2.1* (Kimura et al., 1996), *PDGFRα* (Mercola et al., 1990), *Gsh2* (Szucsik et al., 1997*), Dlx2* (Bulfone et al., 1993), *Ngn2* (Ma et al., 1999), *Sox10* (Zhou et al., 2001), and *Olig2* (Lu et al., 2000).

Immunohistochemistry

The following primary antibodies were used: rabbit α-Pax6 (American Research Products, Belmont, MA, 1:200), rabbit α-Gsh2 (Toresson et al., 2000; gift of K. Campbell), mouse α-TuJ1 (Berkeley Antibody Company, Richmond, CA, 1:200 on sections, 1:500 on neurospheres), rabbit α-GFAP (Accurate, Westbury, NY, 1:200 on neurospheres), rabbit α-GABA (Sigma, St. Louis, MO, 1:500), rabbit α-GAD7 (Sigma, 1:2000), rabbit α-MBP (SMI99 and SMI94, IgG2b, 1:1000, Sternberger Monoclonals Inc), rabbit α-Caspase3 (NEB, MA, 1:200), rabbit α-Shh (Ab80; Bumcrot et al., 1995; 1:200). Most secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and were raised in goat. Standard immunostaining procedures were used. Antibodies were diluted in PBS plus 1% normal bovine serum albumin plus 0.2% Triton X-100. TUNEL staining was performed using the ApopTag kit (Intergen Company, Purchase, NY).

Immunohistochemistry with Ab80

Brains from 15 days postpartum (dpp) and adult male mice were fixed in Sainte Marie's solution and processed for paraffin embedding and immunohistochemistry with anti-Shh antibody (Bumcrot et al., 1995), as described (Gritli-Linde et al., 2001). Other specimens were snap-frozen in liquid nitrogen-cooled isopentane and immersed in successive methanol baths at −80°C, −20°C, and +4°C for 3 days each. They were then embedded in paraffin and processed for immunohistochemistry as above.

Cell Dissociation, Isolation, and In Vitro Culturing

The telencephalic subventricular zone (SVZ) was dissected from P9–P14 wt, $Smo^{n/c};N^{Cre}$ embryos. The tissue to be dissociated was minced, washed twice in PBS with 1 mM EDTA, and incubated in papain (20 U/mL in EBSS, 1 mM cysteine, 0.5 mM EDTA) (Worthing-

ton) at 37°C for 45 min. Ovomucoid inhibitor and albumin (both 10 mg/mL) were used to inhibit papain and 100 U/mL DNase (Worthington) was included. Samples were triturated using a fire-polished Pasteur pipet, and cells were run over a buoyant-density gradient. Cells were washed twice with DMEM/F12 media, resuspended in DMEM/F12 (GIBCO), and run through a 40 μm cell strainer (Falcon) prior to sorting. The SVZ was dissected away from postmitotic tissues using Lumsden bioscissors. Cells were transferred to 6-well dishes and cultured at clonal density (<1–2 cells/μl) in DMEM/F12 and N2 supplements (Gibco), 2 mM glutamine (Gibco), and 2 μg/ml heparin (Sigma). When FGF2 and EGF (R&D Systems) were included in the media, these factors were added at 20 ng/ml and 10–20 ng/ml, respectively. When Shh (Bumcrot et al., 1995; Biogen) was included in the media, it was used at 50 nM. Primary neurospheres were isolated after 7–10 days of culture. These were then either differentiated or passaged to make secondary spheres. To promote differentiation, neurospheres were cultured for 5 days on poly-D-lysine coated LabTek II chamber slides (Nunc) in DMEM/F12, 2 mM L-glutamine, and penicillin/streptomycin with or without 50 nM Shh, and with or without 5% fetal bovine serum. Single spheres were dissociated and plated at clonal densities in 48-well plates.

Oral Treatment with Hh Agonist

Adult (8 weeks) male C57Bl/6 mice (Charles River) were housed and maintained on a 12/12 hr light/dark cycle (lights on at 6 am) and provided with ad libitum access to food and water. All experiments described here were reviewed and approved by the Curis Animal Care and Use Committee. Mice were treated for 3 days (once/day) with either vehicle (0.5% methyl cellulose, 0.2% Tween-80/dH2O, po; n = 10) or Hh agonist (20 mg/kg n = 10). Approximately 24 hr after the third dose, five mice/treatment group were injected with BrdU (0.1 ml/10 g b.w., ip of BrdU labeling reagent, Zymed Laboratories, Inc) and 2 hr later they were transcardially perfused with saline followed by 4% paraformaldehyde (pH 7.4; n = 5/treatment group). Brains were then removed, postfixed overnight, cryoprotected in 30% sucrose/PB (pH 7.4), frozen in cold isopentane, and tissue stored at −80°C until processing for BrdU immunohistochemistry. The remaining five animals/treatment group were euthanized by $C0_2$ overdose, brains rapidly removed, SVZ and hippocampal tissues isolated, and frozen on dry ice. For analysis of *Gli1* mRNA levels, tissues were homogenized and RNA isolated using TRIzol (Life Technologies, Inc).

In a second experiment, *Gli1*^{LacZ} heterozygous mice (8 weeks of age) were given a single oral dose of Hh agonist (20 mg/kg, n = 4 for each group; Frank-Kamenetsky et al., 2002) . The following day, mice were injected with BrdU 2 hr prior to sacrifice. Animals were sacrificed and processed for β-galactosidase histochemistry.

For BrdU analysis, every sixth coronal section (20 μm), beginning immediately behind the olfactory bulbs and continuing through the entire rostral-caudal extent of the hippocampus, was processed for BrdU immunohistochemistry. Sections were sequentially pretreated with 0.1 M glycine, 0.5% sodium borohydride and then denatured in 2 N HCl, at 40°C. Sections were rinsed well and then incubated in a blocking solution containing 5% normal donkey serum (NDS), 0.4% triton-X, and 1% hydrogen peroxide in PBS. Sections were then incubated in rat anti-BrdU (1:300; Harlan Sera-Labs) for 36–48 hr at 4°C. Sections were rinsed well and incubated in biotinylated rat secondary antibodies (1:2000, Jackson Laboratories), followed by avidin-biotin-horseradish peroxidase (ABC Elite, Vector Laboratories). Immunoreactivity was visualized by incubation in 0.04% diaminobenzidine (DAB) in Tris (pH 7.2) containing 0.1% nickel ammonium sulfate and activated by 0.02% hydrogen peroxide.

Numbers of BrdU-immunoreactive cells were counted in region-matched coronal sections through the SVZ and hippocampal dentate gyrus by an observer blind to the treatment of the animals. For the SVZ, immunoreactive cells were counted on one side of the brain, in eight sections (approximately 240 μm apart) through the rostral SVZ. For the DG, immunoreactive cells were counted on one side of the brain in ten matched sections (approximately 120 μm apart) through the entire rostral-caudal extent of the hippocampus. For both brain regions, cells were counted at 600× using an oil objective, and sections were sufficiently separated (120–240 μm) to ensure that cells were not counted twice. In both the SVZ and DG, immunoreactive cells were sometimes in small clusters. In these cases, only those BrdU-positive cells in which the entire nucleus outline was visible were counted. Cell counts from each brain region were totaled for each animal to give a total number of BrdU-positive cells/animal in the SVZ and DG (n = 5/treatment group). Group means were analyzed by two-tailed Student t test, and $p < 0.05$ was considered significant.

Acknowledgments

We thank Nicholas Gaiano for critical reading of this manuscript and help with in situ hybridizations, as well as Fred Gage for discussion of his recent findings concerning the role of Shh in the hippocampus and advice on hippocampal neuroprogenitor culture. We are also grateful to Ruediger Klein for the gift of *Nestin*^{Cre} mice and to Paula Lewis for analysis of *Shh* expression in *Shh*$^{n/c}$;*N*^{Cre} experiments. We also thank the following people for the gifts of mice, probes, or reagents: A. Joyner (*Gli1*$^{LacZ/+}$ mice), B. Vogelstein (*Gli1* probe), M. Scott (*Ptch1* probe), F. Guillemot *(Mash1; Ngn2* probes), S. Kimura (*Nkx2.1* probe), B. Richardson (*PDGFR*α probe), S. Potter (*Gsh2* probe), C. Stiles (*Olig2* probe), A. Kawakami (anti-Pax6), K. Campbell (anti-Gsh2), and D. Anderson (*Sox10* probe). We also acknowledge the contribution of Yuan Yuan Huang for her technical assistance and Joanne Gensert for her invaluable suggestions concerning our analysis of myelination. Work in G.F.'s laboratory was supported by a NIH grant (NS39007), a March of Dimes basic research grant, and a Children's Brain Tumor Foundation grant to G.F., by a NIH grant to J.G.C. (NS10962-01) and R.M. (1F32NS42525-01). Work in A.P.M.'s laboratory was supported by a grant from the NIH (NS 33642). A.G.L. is supported by the Swedish Medical Research Council grants 2789 and 14100. M.D.M. acknowledges support from the Clarke Fund and a gift from the Zewinski family.

Received: August 21, 2002
Revised: May 15, 2003
Accepted: July 17, 2003
Published: September 10, 2003

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