

# Sonic hedgehog maintains the identity of cortical interneuron progenitors in the ventral telencephalon

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## Summary

Fate determination in the mammalian forebrain, where mature phenotypes are often not achieved until postnatal stages of development, has been an elusive topic of study despite its relevance to neuropsychiatric disease. In the ventral telencephalon, major subgroups of cerebral cortical interneurons originate in the medial ganglionic eminence (MGE), where the signaling molecule sonic hedgehog (Shh) continues to be expressed during the period of neurogenesis. To examine whether Shh regulates cortical interneuron specification, we studied mice harboring conditional mutations in Shh within the neural tube. At embryonic day 12.5, NestinCre:Shh<sup>F1/F1</sup> mutants have a relatively normal index of S-phase cells in the MGE, but many of these cells do not co-express the interneuron fate-determining gene Nkx2.1. This effect is reproduced by inhibiting Shh signaling in slice cultures, and the effect can be rescued in NestinCre:Shh<sup>F1/F1</sup> slices by the addition of

exogenous Shh. By culturing MGE progenitors on a cortical feeder layer, cell fate analyses suggest that Shh signaling maintains Nkx2.1 expression and cortical interneuron fate determination by MGE progenitors. These results are corroborated by the examination of NestinCre:Shh<sup>F1/F1</sup> cortex at postnatal day 12, in which there is a dramatic reduction in cell profiles that express somatostatin or parvalbumin. By contrast, analyses of Dlx5/6Cre:Smoothened<sup>F1/F1</sup> mutant mice suggest that cell-autonomous hedgehog signaling is not crucial to the migration or differentiation of most cortical interneurons. These results combine *in vitro* and *ex vivo* analyses to link embryonic abnormalities in Shh signaling to postnatal alterations in cortical interneuron composition.

Key words: Nkx2.1, Parvalbumin, Somatostatin, Conditional knockout, Smoothened

## Introduction

Cell fate determination in the cerebral cortex is a crucial field of study due to its relevance to mental and neurological illnesses. GABAergic interneurons, which comprise 20–30% of cortical neurons, modulate cortical output (Krimer and Goldman-Rakic, 2001) and regulate the ‘critical period’ refinement of cortical connectivity (Hensch et al., 1998). Fate-mapping experiments *in vivo* (Wichterle et al., 1999; Wichterle et al., 2001; Anderson et al., 2002) and *in vitro* (Lavdas et al., 1999; Wichterle et al., 1999; Anderson et al., 2001) have suggested that the medial ganglionic eminence (MGE) is the primary source of cortical interneurons in rodents.

Recently, we demonstrated the existence of regional specificity for the genesis of cortical interneuron subgroups (Xu et al., 2004). Parvalbumin (Pv; Pvalb – Mouse Genome Informatics)- and somatostatin (Som; Sst – Mouse Genome Informatics)-expressing interneuron subgroups require the transcription factor Nkx2.1 for their specification, and originate primarily in the MGE. Calretinin-expressing subgroups are not Nkx2.1 dependent and appear to originate primarily in the dorsal, non-Nkx2.1-expressing region of the caudal ganglionic eminence (CGE).

Despite recent advances in our understanding of the origins and regulation of cortical interneuron migrations (Marin et al.,

2001; Marin et al., 2003; Alifragis et al., 2004), apart from the dependence on Nkx2.1, little is known about the specification of interneuron fate. One factor that appears to function upstream of Nkx2.1 is sonic hedgehog (Shh). In the telencephalon, Shh is initially secreted from the prechordal mesoderm; it then induces its own expression in the overlying neural tube, in the proliferative zone of the preoptic area and in the mantle region of the MGE (Ericson et al., 1995; Shimamura et al., 1995). Shh permits the initial induction of transcription factors with ventrally restricted patterns of expression, including *Dlx1*, *Dlx2*, *Mash1* and *Nkx2.1* (Kohtz et al., 1998; Gunhaga et al., 2000), and it is normally required for the formation of the ventral telencephalon (Chiang et al., 1996; Rallu et al., 2002).

Recently, an analysis of the role of Shh signaling on the initial patterning of the telencephalon was made using a FoxG1-Cre:Smoothened<sup>F1/F1</sup> conditional knockout, which loses Shh signaling by about embryonic day (E) 9 (Fuccillo et al., 2004). In these embryos, initial patterning of the ventral telencephalon fails, such that ventrally derived cell types, including cortical interneurons, are absent. However, the functions of later telencephalic Shh expression during the age range of neurogenesis are less clear, although, throughout the neuroaxis, Shh support of progenitor proliferation maintains

multipotent progenitor ‘niches’ (Britto et al., 2002; Machold et al., 2003). In vitro studies suggest that Shh signaling can direct telencephalic progenitors towards a GABAergic phenotype (Yung et al., 2002; Gulacsi and Lillien, 2003; Watanabe et al., 2005), although the downstream effectors of this process remain unclear.

In this paper, we provide evidence for a new function of Shh during the genesis of cortical interneurons, the maintenance of progenitor identity. Analyses of nestin-Cre (NsCre):*Shh*<sup>F1/F1</sup> mouse embryos at E12.5 reveal that there is a dramatic reduction in the proportion of S-phase progenitors that co-express Nkx2.1. Consequently, two Nkx2.1-dependent interneuron subgroups, those expressing Pv or Som, are greatly reduced in layers II to IV of the cortex at postnatal day 12. Several lines of evidence suggest that the postnatal loss of these interneuron subgroups primarily results from a loss of Shh signaling in the MGE, rather than from a later influence of Shh on interneuron migration, differentiation or survival. These findings suggest that Shh signaling during neurogenesis maintains cortical interneuron progenitor identity through its regulation of Nkx2.1 expression, and thus plays a crucial role in determining the relative composition of excitatory and inhibitory neurons of the cerebral cortex.

## Materials and methods

### Animals

Transgenic mice expressing Cre recombinase under the control of the nestin promoter [NsCre (Tronche et al., 1999), from the Jackson Laboratory] were crossed with a floxed Shh line (Jackson Laboratory) (Dassule et al., 2000) to generate NsCre:Shh<sup>F1/Wt</sup>. These mice were crossed with Shh<sup>F1/F1</sup> mice to generate conditional Shh mutants. In the analyses of these mice, both NsCre(-):Shh<sup>F1/Wt</sup> and NsCre(-):Shh<sup>F1/F1</sup> genotypes were considered to be ‘wild type’. Mice expressing Cre under the control of the Dlx5/Dlx6 promoter (Stenman et al., 2003a) (a gift from Kenny Campbell) were crossed with floxed smoothened mice (Jackson Laboratory) (Long et al., 2001) to generate Dlx5/6Cre:Smo<sup>F1/F1</sup> conditional mutants. All Cre-expressing, Rosa26 reporter (Soriano, 1999) and Z/EG reporter mice (Novak et al., 2000) were maintained on a C57BL/6J background. The GFP-expressing transgenic mice were maintained as previously described (Xu et al., 2004). Mice were maintained in accordance with the guidelines set by the author’s institution and the National Institutes of Health.

### Telencephalic slice cultures

Coronal telencephalic slices were generated and cultured as described (Xu et al., 2004). For cyclopamine experiments, 5 μM of cyclopamine dissolved in ethanol or an equivalent amount of ethanol (0.05% volume) was added to the medium. For the exogenous Shh experiments, recombinant modified N-terminal Shh protein (Shh-N; Curis) was diluted in Neurobasal/B27 (Nb/B27; Gibco) and used at a final concentration of 10 nM. Slices were maintained in vitro for 24–36 hours with no change of medium.

### BrdU labeling

For in vivo labeling of S-phase cells, one injection of BrdU (100 mg/kg, intraperitoneally) was made one hour prior to sacrificing the dam (unless indicated otherwise). For in vitro studies, BrdU (100 ng/ml) was added to the culture medium six hours prior to fixation or transplantation.

### Slice electroporation

Mouse patched 1 with a loop2 deletion [*mPtc1*<sup>Δloop2</sup>, a gift from Dr Gary Struhl (Briscoe et al., 2001)] was subcloned into *pCAGGS-mPtc1*<sup>Δloop2</sup>-IRES-GFP. The same vector without *mPtc1*<sup>Δloop2</sup> was used

as control. DNA was purified with the Endofree Plasmid Maxi Kit (Qiagen) and electroporated into the MGE region of E12.5 slices as described (Stuhmer et al., 2002a).

### Co-cultures of MGE-derived cells on cortical feeder cells

Primary cortical feeder cultures (100,000 cells per 36 mm<sup>2</sup> well of 16-well chamber slides, Lab-Tek) were prepared from the dissociated cortices of neonatal pups as described (Xu et al., 2004). For the cyclopamine treatment experiments, telencephalic slice cultures were made from pan-GFP expressing reporter mice (Hadjantonakis et al., 1998). Following one day in vitro (DIV), the periventricular proliferative zone of the MGE was dissected free, gently triturated and resuspended in Nb/B27 medium. Two thousand cells per well were added to cortical feeder cultures prepared 1 day earlier, and the cultures were maintained as described (Xu et al., 2004).

### In situ hybridization and immunohistochemistry

Postnatal mice were perfused with 4% paraformaldehyde (PFA) and processed for cryosectioning (Xu et al., 2004). Cryostat sections were obtained at 12 μm thickness for immunohistochemistry, and at 12 or 20 μm for in situ hybridization using digoxigenin-labeled riboprobes (Wilkinson and Nieto, 1993; Schaeren-Wiemers and Gerfin-Moser 1993). *cDNA* probes used were *Shh* (BC063087 from Open Biosystems), *Gli1* (Kinzler et al., 1988), *Nkx2.1* (Kimura et al., 1996), *Nkx6.2* (gift from Dr Gord Fishell) and *Oct6*.

The primary antibodies for immunofluorescence labeling included anti-BrdU (mouse, Chemicon, 1:400; rat, Serotec, 1:200), calretinin (rabbit, Chemicon, 1:2000; mouse, Swant, 1:5000), Calbindin (rabbit, Swant, 1:5000), Dlx2 [rabbit (Porteus et al., 1994)], GABA (rabbit, Sigma, 1:5000), GFP (rabbit or chick, Molecular Probes, 1:2000), Gsh2 (rabbit, Kenneth Campbell, 1:5000), islet 1 (mouse, DSHB, 1:1000), NeuN (mouse, Chemicon, 1:1000), Nkx2.1 (rabbit, Biopat, 1:1000; mouse, Neomarkers, 1:100), Npy (rabbit, Immunostar, 1:2000), Olig2 (rabbit, 1:2000), somatostatin (rat, Chemicon, 1:400), parvalbumin (mouse, Chemicon, 1:5000), phospho-histone H3 (rabbit, Upstate Biotech 1:200), and Tbr1 [rabbit (Englund et al., 2005), 1:1000]. Fluorescent secondary antibodies were Alexa line (Molecular Probes, 1:500). Triple labeling of in vitro cell cultures was performed using a Cy5-conjugated secondary (Jackson ImmunoResearch) (Xu et al., 2004). The nuclear marker DAPI (300 nM) was applied with the secondary antibodies. Signal was detected by epifluorescence microscopy (Nikon E800), and images acquired (Coolsnap HQ, Roper; Metamorph software, Universal Imaging).

### Data collection and statistical analysis

Co-labeling of Nkx2.1 and BrdU was counted using a 60× oil immersion lens (n.a. 1.4) on a Nikon E600 microscope fitted with a motorized stage and Stereo Investigator software (MicroBrightField). Cell counts in a specified region were obtained using the Fractionator probe and systematic random sampling. For each embryo, data was pooled from both hemispheres of four non-consecutive sections.

For postnatal analyses, coronal cryosections were evaluated from the genu of the corpus callosum to the hippocampal commissure. The medial boundary of primary somatosensory cortex was identified in cresyl violet-stained sections, and the counting region extended about 2 mm lateral to that point, to the lateral boundary of the primary and secondary somatosensory cortex. The boundary between layers four and five was identified by Cresyl violet and DAPI staining, and the cortex was divided into two bins containing layers two to four, and layers five and six. For all counts, both hemispheres of at least four non-consecutive coronal sections for were examined. First, the relative areal densities (counts/mm<sup>2</sup>) of cell profiles labeled for DAPI (all cells) and Tbr1 (most or all projection neurons), and of NeuN-labeled cells (most or all neurons), were estimated using the Fractionator probe and systematic random sampling (MicroBrightfield StereoInvestigator Software). Other markers were quantified as total profile counts in the defined region (profiles/mm<sup>2</sup>), and are presented

as the ratio of these counts with all cells (DAPI), or with all NeuN+ profiles per unit area. Thus, these data provide relative counts for the comparison of cortical composition between mutant and control brains. Pilot studies revealed no consistent differences in profile size between any marker in mutant and wild-type sections. Statistical analysis (unpaired *t*-test) was performed with Excel software on data obtained from at least three mutants and three controls.

## Results

### Loss of Shh reduces the proportion of S-phase cells that express Nkx2.1 in the MGE

To examine the role of telencephalic Shh expression in the development of cortical interneurons, nestin (Ns) Cre:Shh<sup>F1/F1</sup> conditional mutants were generated as described by Machold et al. (Machold et al., 2003), except that in our studies recombination occurred on a flox/flox rather than a flox/Shh null background. Analysis of *Shh* mRNA expression revealed loss by E12.5 (data not shown; see also *Gli1* expression loss in Fig. 2). In addition, the postnatal phenotype of microcephaly (see Fig. S1 in the supplementary material) and death by P15 is very similar to that reported previously (Machold et al., 2003).

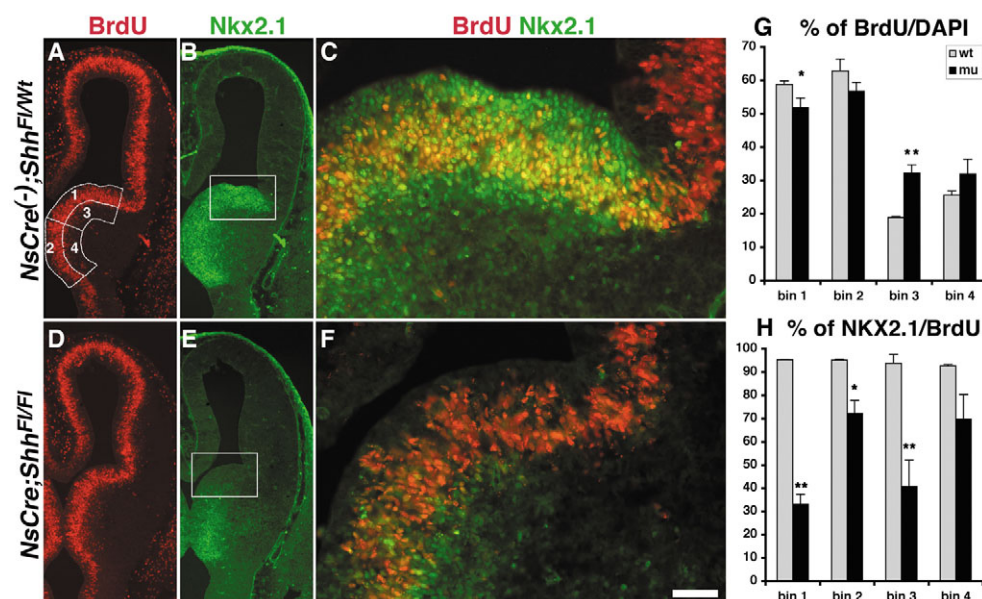
As noted previously, these NsCre:Shh<sup>F1/F1</sup> mutants have a marked reduction of Nkx2.1 expression, even though the density of S-phase cells is not grossly reduced and there is no increase in the numbers of apoptotic cells (Machold et al., 2003), leading to the possibility that loss of Shh signaling reduces the expansion of Nkx2.1-expressing progenitors. To examine this possibility, Nkx2.1 expression was examined in E12.5 embryos that had received the S-phase marker BrdU 1 hour prior to sacrifice. The BrdU-labeling index has subtle abnormalities in the mutant MGE at this age, with a trend (not significant after correction for multiple comparisons) towards reduced BrdU-labeling index in the ventricular zone (VZ) of both the dorsal and ventral halves of MGE (Fig. 1G). In the SVZ region of the MGE there is a significant increase of the BrdU-labeling index in the dorsal MGE ( $n=3$ ,  $P<0.01$ ). These results are difficult to interpret in absence of data on the total cell-cycle time, but could suggest that the loss of Shh results in

a decrease in symmetrical, progenitor-expanding divisions within the VZ and an increase in asymmetrical VZ divisions that then undergo terminal, 'transient-amplifying' divisions within the SVZ (Haubensak et al., 2004; Noctor et al., 2004).

Regardless of subtle changes in BrdU-labeling index, the percentage of S-phase cells that co-expressed Nkx2.1 in the MGE drops from over 90% in NsCre(-):Shh<sup>F1/Wt</sup> controls to less than 50% in the NsCre:Shh<sup>F1/F1</sup> embryos (Fig. 1A-F). Phospho-histone H3 (PH3) staining that primarily labels cells in M-phase appears to be grossly normal, suggesting that cell-cycle progression is occurring in the mutant telencephalon (see Fig. S2 in the supplementary material). These findings suggest that progenitor cells in the VZ and SVZ of the MGE respond to a reduction in Shh signaling by dramatically reducing their expression of Nkx2.1.

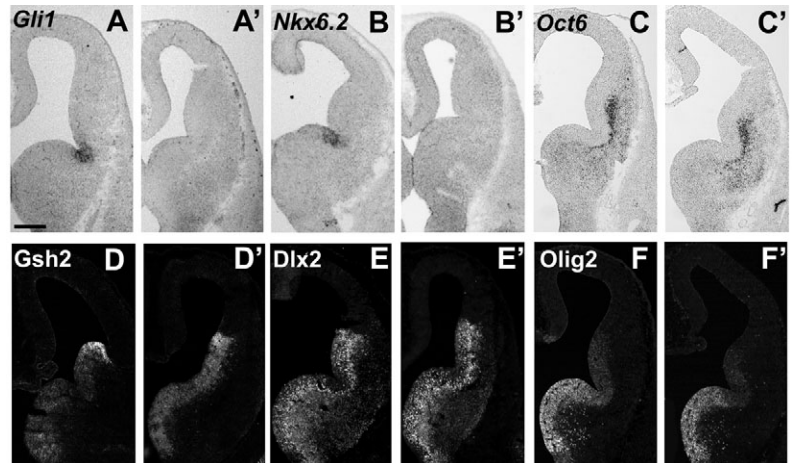
### Loss of Shh signaling in the nestin-Cre:Shh<sup>F1/F1</sup> telencephalon results in a subtle disruption of MGE patterning

Because a reduction in Shh signaling leads to a decrease in Nkx2.1 expression, we examined whether this reduction is indicative of a gross re-patterning of the MGE or is an effect specific to certain Shh-responsive genes. First the expression of *Gli1*, a marker of Shh signaling (Lee et al., 1997), was investigated (Fig. 2A). At E12.5, two of the three mutants showed no detectable *Gli1* expression, while the other showed a marked reduction of expression in cells located in the dorsal MGE when compared with wild types. By E14.5, all NsCre:Shh<sup>F1/F1</sup> mice lack *Gli1* expression (not shown), consistent with a loss of Shh signaling. We also examined the expression of *Nkx6.2*, a target of Shh signaling in the spinal cord, which is also expressed in the dorsal-most MGE (Vallstedt et al., 2001; Stenman et al., 2003b). At E12.5, *Nkx6.2* expression was not detected in any NsCre:Shh<sup>F1/F1</sup> mutant (Fig. 2B;  $n=3$ ). By contrast, more general markers of pallidal telencephalic patterning appear to be normal in the VZ, SVZ and mantle, as the expression of *Gsh2* (Fig. 2D), *Dlx2* (Fig. 2E) and islet 1 protein (see Fig. S3 in the supplementary material) is unchanged in the mutants (see also Machold et al., 2003).



**Fig. 1.** Telencephalic loss of Shh reduces Nkx2.1 expression in progenitors of the MGE. Shown is the S-phase marker BrdU (red) and Nkx2.1 (green) immunolabeling on coronal 12  $\mu$ m cryosections from E12.5 embryos. The outline in A indicates the bins of MGE used for quantification in G,H. Boxed areas in B and E are shown at higher magnification in C and F. (A-C) In NsCre(-):Shh<sup>F1/+</sup> controls, roughly 90% of BrdU-labeled nuclei co-label with Nkx2.1. Over the entire MGE, this proportion falls to roughly 50% in the NsCre:Shh<sup>F1/F1</sup> mutants, although effect was strongest in the dorsal MGE (*t*-test: \* $P<0.05$ , \*\* $P<0.01$ ). Note, the bin 1 result in G and the bin2 result in H are not significant after adjustment for multiple comparisons. Scale bar: 200  $\mu$ m in A,B,D,E; 50  $\mu$ m in C,F.

**Fig. 2.** NsCre:Shh<sup>F1/F1</sup> mutants lose the expression of Shh responsive genes at the MGE-LGE sulcus, but maintain other aspects of MGE patterning. (A-C') In situ hybridization of E12.5 forebrain for (A,A') *Gli1*, (B,B') *Nkx6.2* and (C,C') *Oct6* in wild-type (A,B,C) and NsCre:Shh<sup>F1/F1</sup> mice (A',B',C'). (D-F') Immunofluorescence of E12.5 forebrain for (D,D') Gsh2, (E,E') Dlx2 and (F,F') Olig2 in NsCre(-):Shh<sup>F1/wt</sup> control (D,E,F) and NsCre:Shh<sup>F1/F1</sup> mutant mice (D',E',F'). Despite the loss of expression of *Gli1* and *Nkx6.2* in the mutant MGE, other markers of MGE patterning in the VZ (Olig2) and SVZ (*Oct6*) appear to be unaltered. Scale bar: 200  $\mu$ m.



This loss of *Nkx6.2* expression raises the possibility that dorsal MGE patterning is shifted in favor of LGE-expressed genes, as occurs in *Nkx2.1* nulls (Sussel et al., 1999). However, markers of both the VZ (identified by the higher expression level of *Olig2* in the MGE than the LGE), and the SVZ/mantle (identified by the higher expression of *Oct6* in the LGE than the MGE) are preserved in the NsCre:Shh<sup>F1/F1</sup> mutants (Fig. 2C,C',F,F'). These results suggest that despite the loss of *Gli1* and *Nkx6.2*, and the reduction of *Nkx2.1*, other aspects of MGE progenitor identity are maintained.

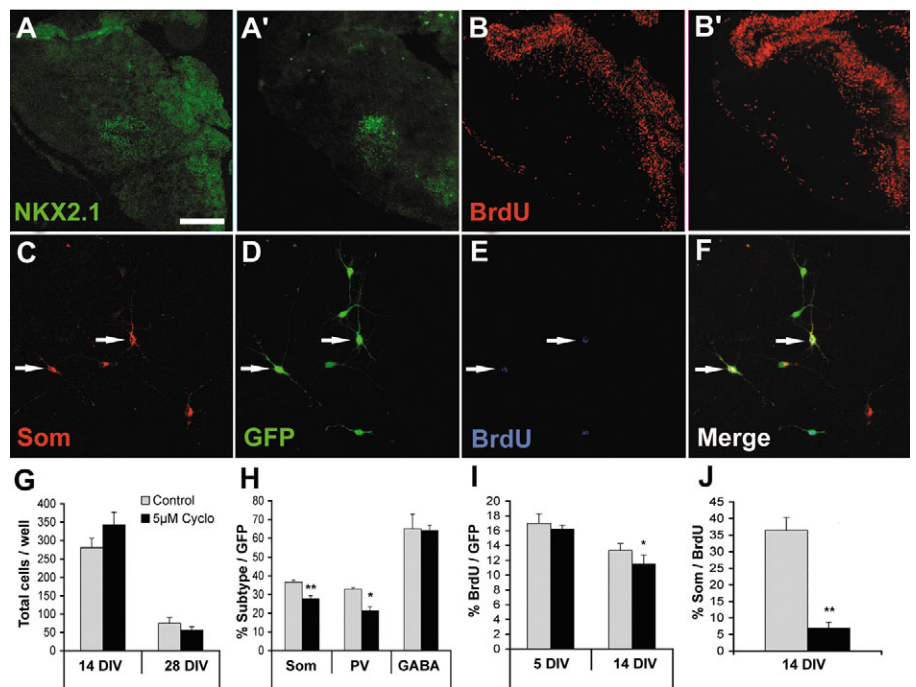
### Inhibition of Shh signaling in vitro reduces *Nkx2.1* expression and alters progenitor fate

The reduction of *Nkx2.1*, a gene required for the specification of MGE-derived interneurons (Xu et al., 2004), as a

consequence of decreased Shh signaling suggests that the fate of MGE progenitors would be affected by a decrease in Shh signaling in vitro. Telencephalic slice cultures from E12.5 embryos were treated with cyclopamine (5  $\mu$ M) (Incardona et al., 1998) for 24-48 hours. This treatment dramatically reduces the expression of *Nkx2.1* in the proliferative zones within 24 hours (Fig. 3A,A'). Interestingly, cyclopamine has little apparent impact on *Nkx2.1* expression in the mantle region in these short-term cultures, possibly due to the lack of detectable message for the Shh signaling effector Smoothed (data not shown; see also [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gensat&cmd=retrieve&dopt=docsum&list\\_uids=14003](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gensat&cmd=retrieve&dopt=docsum&list_uids=14003) and Fig. S7 in the supplementary material).

To examine whether the loss of *Nkx2.1* expression is due to reduced proliferation within the MGE, BrdU (100 ng/ml)

**Fig. 3.** Cyclopamine reduces *Nkx2.1* expression and alters MGE progenitor fate in vitro. (A-B') Immunofluorescence labeling of re-sectioned (12  $\mu$ m) E12.5 telencephalic explants cultured for 24 hours. (A,B) *Nkx2.1* and BrdU labeling of control sections cultured in 0.05% EtOH. (A') Slices cultured in 5  $\mu$ M cyclopamine have reduced expression of *Nkx2.1* in the ventricular zone, whereas (B') BrdU incorporation appears unchanged. (C-F) Immunofluorescence labeling of somatostatin (Som, red), GFP (green) and BrdU (blue) in cell cultures grown for 14 DIV. The vast majority of transplanted cells from donors of this age have neuronal morphologies. Arrows indicate neurons triple-labeled for Som/GFP/BrdU. (G) Overall transplant cell number is unaffected by cyclopamine. (H) Cyclopamine reduces the percentage of Som- and Pv-expressing interneurons generated from MGE transplants. (I) The percentage of surviving, transplanted neurons (GFP+) that incorporated BrdU prior to transplant is unaffected by cyclopamine after 5 DIV, and slightly reduced after 14 DIV. (J) Cyclopamine dramatically reduces the number of BrdU+ transplanted cells co-labeling for somatostatin. *t*-test: \**P*<0.05, \*\**P*<0.005. Scale bar in A: 200  $\mu$ m.



was added to the cultures 6 hours prior to fixation (labeling cells in S-phase plus some that would have advanced through G2 and M). BrdU incorporation is grossly unchanged in both the control and cyclopamine-treated slices (Fig. 3B,B'). This result appears to reflect continued proliferation rather than S or G2-phase arrest, as cells expressing the M-phase marker PH3 are also plentiful in the MGEs of cyclopamine-treated slices (not shown). However, after 24 hours of treatment, cyclopamine treatment greatly reduced the proportion of S-phase cells in the MGE that strongly express Nkx2.1 (Fig. 3A,B).

Because cyclopamine treatment of slice cultures replicates the observed decrease in Nkx2.1 expression in the NsCre:Shh<sup>F1/F1</sup> mice, we examined whether inhibition of Shh signaling alters the fate of MGE progenitors and whether this alteration differentially affects cells that entered S-phase after the longest exposure to cyclopamine. To address this issue, we used a co-culture system in which the fates of progenitors from the embryonic MGE are assessed 2-4 weeks after culturing on a feeder layer made from neonatal cortex. In this system, MGE progenitors from wild-type embryos, but not from Nkx2.1<sup>-/-</sup> embryos, give rise to Pv and to Som-expressing interneurons (Xu et al., 2004). Slice cultures from E12.5 GFP-expressing donor mice were treated with cyclopamine and then BrdU as above, then the MGEs of these slices were dissociated and cells were plated over cortical feeder cells (Xu et al., 2004). After 2-4 weeks in vitro, the fates of all MGE cells (GFP+), and of those that were in S-phase within 6 hours of transplantation (BrdU+), were examined by co-labeling with neurochemical markers of differentiated interneurons (Fig. 3). Cyclopamine treatment of the slices has no effect on the total number of GFP-expressing cells with neuronal morphology that survive 14 or 28 days in vitro (DIV; Fig. 3J). By contrast, cyclopamine treatment results in a significant reduction in the percentage of these cells that express Som (at 14 DIV, 36.6±1.2% versus 27.8±1.7%; *n*=5; *P*<0.005) or Pv (at 28 DIV, 32.9±0.8% versus 21.2±2.3%, *n*=3; *P*<0.05).

To examine whether there is a differential effect of cyclopamine on the fate of cells that were in S-phase during the final 6 hours of the treatment period, triple immunolabeling of GFP, Som and BrdU was performed on cultures after 14

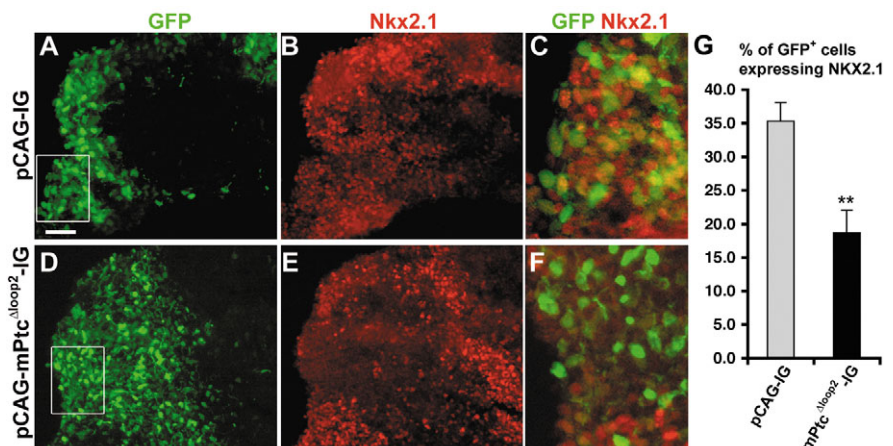
DIV. Cyclopamine pre-treatment has no influence on the numbers of BrdU-labeled cells after 5 DIV, and a modest but statistically significant effect after 14 DIV (13.4±0.9% versus 11.5±1.2%, *n*=3, *P*<0.05). However, cyclopamine pre-treatment results in a dramatic reduction in the percentage of BrdU-labeled, MGE progenitors with neuronal morphology that expressed Som (Fig. 3J, 36.4±3.9% versus 6.8±1.9%, *n*=3, *P*<0.005).

We were unable to determine a cell type that is increased in the cyclopamine-treated cultures. However, consistent with the unchanged expression of Gsh2 and Dlx2 in the MGE of NsCre:Shh<sup>F1/F1</sup> mutants (Fig. 2) (see also Machold et al., 2003), cyclopamine treatment does not alter the percentage of transplanted neurons that express detectable levels of GABA (Fig. 3H; *n*=3, 65±8% versus 64±3%). These results suggest that although the ability of the MGE progenitors to make Pv- or Som-expressing interneurons is altered in association with the loss of Nkx2.1-expressing MGE progenitors, a ventral (pallidal) telencephalic fate has been maintained.

### Cell-autonomous blockade of Shh signaling reduces Nkx2.1 expression in vitro

The results presented above suggest that Shh signaling maintains Nkx2.1 expression in the MGE and thereby promotes Pv/Som interneuron fate determination. To confirm that Shh signaling blockade results in a cell-autonomous downregulation of Nkx2.1, telencephalic slices were transfected with a dominant-negative version of the Shh receptor Patched, mPTC1<sup>Δloop2</sup> (a gift from Gary Struhl), that is insensitive to Shh, causing a blockade of Shh signal transduction (Briscoe et al., 2001). Electroporation of pCAGGS-mPTC1<sup>Δloop2</sup>-ires-GFP (mPTC1<sup>Δloop2</sup>) into the MGE of telencephalic slices (E12.5 + 1 DIV) produces a large decrease in the percentage of GFP+ cells that co-label with Nkx2.1 relative to pCAGGS-ires-GFP controls (Fig. 4). In summary, although we cannot rule out the possibility that Shh blockade results in the relative expansion of a non-Nkx2.1 expressing lineage, these results strongly support the idea that MGE progenitors downregulate Nkx2.1 but continue to cycle in response to a cell-autonomous reduction of Shh signaling.

**Fig. 4.** Cell-autonomous blockade of Shh signaling also reduces Nkx2.1 expression in the MGE of telencephalic slices. Shown are 12 μm sections made from slices cultured from E12.5 + 1 DIV. Slices had been transfected with either pCAGGS-ires-GFP control vector (A-C) or pCAGGS-mPTC1<sup>Δloop2</sup>-ires-GFP (D-F) by electroporation. Sections were immunolabeled with GFP (A,E) and Nkx2.1 (B,F). The boxed areas in A and D are enlarged, and the GFP signal intensity equally reduced to show co-labeling of GFP with Nkx2.1 in C and F, respectively. The area of pCAGGS-mPTC1<sup>Δloop2</sup>-ires-GFP expression in D has a marked decrease in Nkx2.1-expressing cells (E), and a corresponding loss of GFP-Nkx2.1 co-labeled cells (compare C with F). (G) Quantification of the percentage of GFP-expressing cells that co-labeled with Nkx2.1 in the MGE of these slices found a significant reduction in the mPTC1<sup>Δloop2</sup> transfected slices (*n*=6; \*\**P*<0.004). Scale bar: 50 μm.



### Exogenous Shh rescues the loss of Nkx2.1 expression and interneuron fate effects in slices from NsCre:Shh<sup>F1/F1</sup> mice

Because cyclopamine treatment reduces the number of Pv- or Som-expressing interneurons generated from cultured MGE progenitors, we next investigated whether a similar loss occurs in MGE transplants from NsCre:Shh<sup>F1/F1</sup> mutant mice. Telencephalic slices from NsCre:Shh<sup>F1/F1</sup> litters were cultured for 24 hours, with BrdU added for the last 6 hours. The proliferative region of the MGE was transplanted onto cortical feeders as described above. After 10 DIV, these cultures were fixed and processed for the immunodetection of BrdU and Som, the interneuron subgroup marker that is most affected in NsCre:Shh<sup>F1/F1</sup> mutants in vivo (Fig. 6). Whereas wild-type transplants showed an expected co-localization of BrdU with Som, the transplants made from NsCre:Shh<sup>F1/F1</sup> brains showed little co-labeling (Fig. 5E;  $17.5 \pm 4.5\%$  versus  $2.4 \pm 0.4\%$ ,  $P < 0.03$ ).

If Shh signaling maintains Nkx2.1 expression within MGE progenitors, the addition of exogenous Shh to slices from NsCre:Shh<sup>F1/F1</sup> mutants should restore Nkx2.1 expression in S-phase cells. Thus, slices from E13.5 NsCre:Shh<sup>F1/F1</sup> embryos were cultured for 24 hours in the presence or absence of exogenous Shh (10 nM). BrdU was again added six hours prior to fixation. The mutant slices were then re-sectioned at 12  $\mu\text{m}$ , and immunolabeled for BrdU and Nkx2.1. As shown in Fig. 5C, Shh-treatment of NsCre:Shh<sup>F1/F1</sup> slices does not alter the density of MGE cells in S-phase (BrdU/DAPI). By contrast, this treatment results in a nearly threefold increase in the percentage of BrdU<sup>+</sup> cells expressing detectable levels of Nkx2.1 (Fig. 5A-C;  $67.0\%$  versus  $24.6\%$ ;  $P < 0.008$ ).

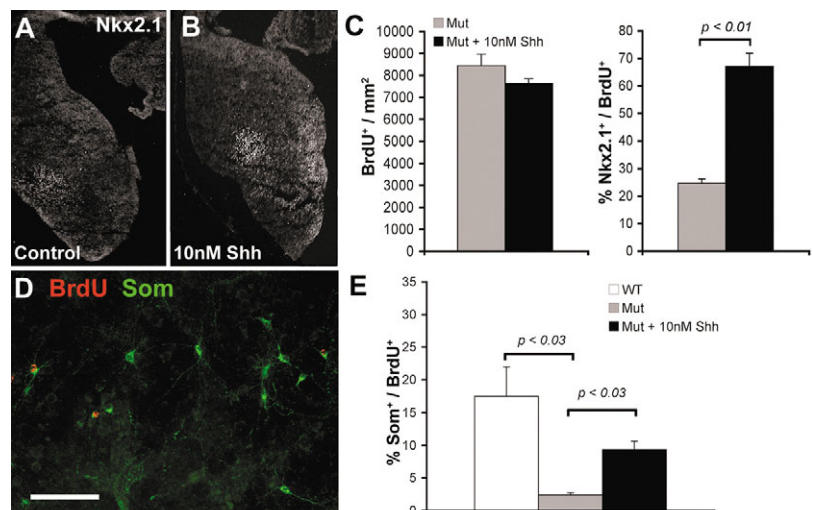
Because Shh-treatment of NsCre:Shh<sup>F1/F1</sup> slices rescues Nkx2.1 expression, this treatment might also rescue the reduction of interneurons generated from NsCre:Shh<sup>F1/F1</sup> MGE transplants. Slices were treated as above and then cultured on cortical feeder cells for 10 days. BrdU<sup>+</sup> cells from mutant slices treated with Shh showed a 4-fold increase in the percentage of double labeling for Som (Fig. 5;  $2.4 \pm 0.4\%$  versus  $9.6 \pm 1.3\%$ ,  $P < 0.03$ ,  $n = 3$ ).

### Reductions of somatostatin- and parvalbumin-expressing interneurons in somatosensory cortex of NsCre:Shh<sup>F1/F1</sup> mice

Because inhibition of Shh signaling in vitro results in a decreased specification of cortical interneuron subtypes (Fig. 3), and transplants of S-phase cells from the MGE of NsCre:Shh<sup>F1/F1</sup> mice fail to generate Som-expressing interneurons (Fig. 5), we hypothesized that these mutants would have cortical interneuron deficits in vivo. NsCre:Shh<sup>F1/F1</sup> mice survive gestation and begin to fail during the second postnatal week (Machold et al., 2003). At this time they show a marked reduction in growth, pronounced extension of the hindlimbs in response to handling, and seizure-like behavior. Most of these mice die by P17. To examine their cortical development, NsCre:Shh<sup>F1/F1</sup> mutants and littermate controls were sacrificed at P12, prior to severe sickening. At this age, mutants show microcephaly and a 10% decrease in cortical thickness (see Fig. S1 in the supplementary material). This decrease may largely represent a loss of neuropil, as there is a roughly 10% increase in both total cell and neuronal density (Fig. 6).

To examine the differential effects on excitatory versus inhibitory neurons, the density of markers for projection neurons [Tbr1 (Hevner et al., 2001; Englund et al., 2005)] or interneurons (GABA) were determined in both superficial (layers 2-4) and deep (layers 5 and 6) cortical layers and expressed as a percentage of NeuN (Neuna60 – Mouse Genome Informatics)-expressing profiles. In both wild-type and NsCre:Shh<sup>F1/F1</sup> mice, about 70% of all cells are neurons at P12. As shown in Fig. 6, NsCre:Shh<sup>F1/F1</sup> mice show a significant increase in the percentage of Tbr1<sup>+</sup> profiles in the superficial cortex when compared with wild type ( $n = 3$ ;  $P < 0.009$ ). This increase is accompanied by a decrease in the percentage of GABA<sup>+</sup> profiles ( $n = 3$ ;  $P < 0.003$ ), although, overall, a lower than expected number of profiles labeled for GABA in both wild-type and mutant brains, probably due to the absence of glutaraldehyde in the fixative. In the deep layers of the cortex, a similar decrease in the percentage of GABA<sup>+</sup> profiles is observed ( $n = 3$ ;  $P < 0.009$ ), although the small increase in Tbr1

**Fig. 5.** Exogenous Shh rescues Nkx2.1 expression and somatostatin fate of NsCre:Shh<sup>F1/F1</sup> MGE progenitors in vitro. (A,B) Sections (12  $\mu\text{m}$ ) from slices cultured from E13.5 NsCre:Shh<sup>F1/F1</sup> + 1 DIV. BrdU was added to the culture medium 6 hours before fixation. Control sections (A) show a marked reduction of Nkx2.1 in the MGE, primarily in the proliferative region. When cultured with 10 nM Shh (B), Nkx2.1 expression is rescued in the proliferative region of the MGE. (C) Quantification of similarly treated sections co-labeled for Nkx2.1 and BrdU. Although the addition of Shh does not affect the overall percentage of BrdU<sup>+</sup> cells (left), the percentage of these cells that co-express Nkx2.1 is increased nearly 3-fold (right). To determine the fate potential of the BrdU-labeled cells, the proliferative region of the MGE was transplanted onto cortical feeders and immunolabeled for BrdU and somatostatin (D). (E) Quantification of the percentage of BrdU<sup>+</sup> cells that co-labeled with Som, showing a marked reduction in NsCre:Shh<sup>F1/F1</sup> compared with wild type, and a partial restoration of Som-BrdU co-labeling following the addition of Shh ( $n = 3$ ). Scale bar in D: 200  $\mu\text{m}$ .



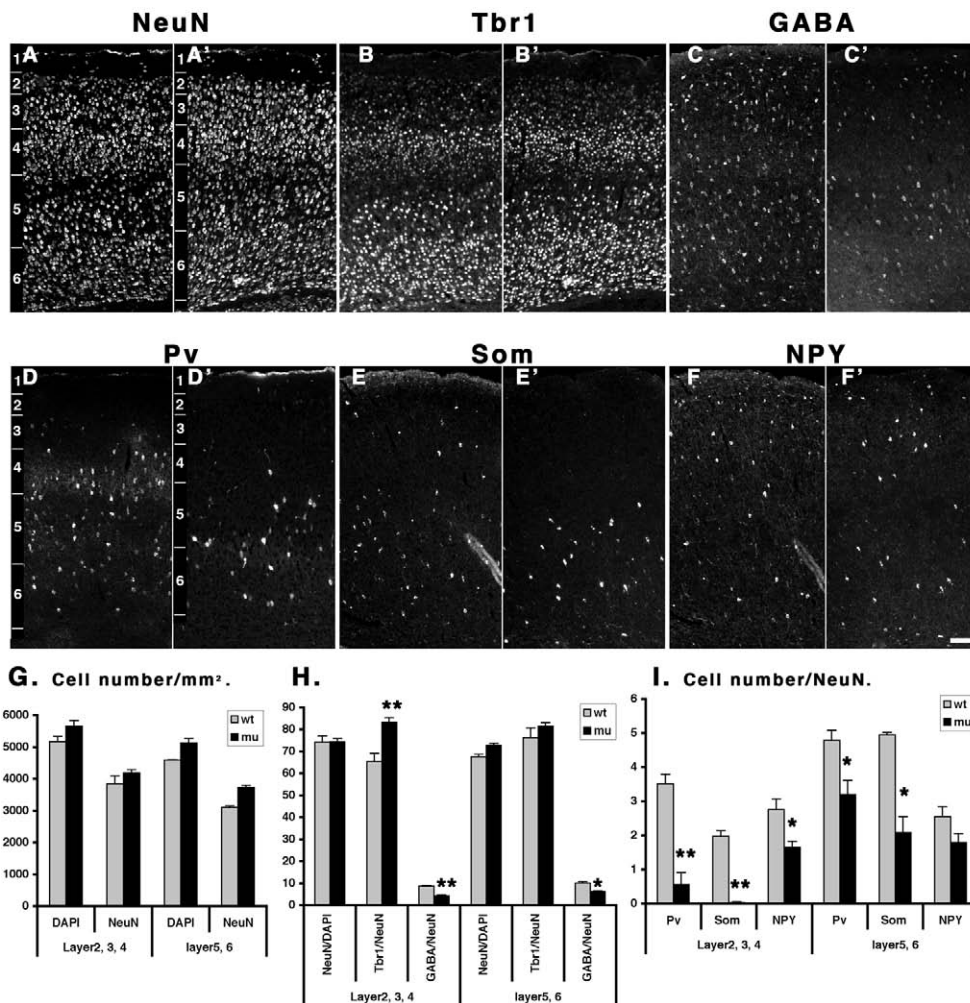
expression in the deep cortical layers is not statistically significant.

The observed decrease in GABA-expressing profiles in the postnatal cortex of NsCre:Shh<sup>F1/F1</sup> mice raises the question of whether specific interneuron subgroups are preferentially affected by reduced Shh signaling. The MGE has previously been shown to generate at least two neurochemically distinct subgroups of cortical interneurons – those expressing Som and those expressing Pv (Wichterle et al., 2001; Anderson et al., 2002; Valcanis and Tan, 2003). Nkx2.1 expression within the MGE is required for the specification of these subgroups (Xu et al., 2004). To determine whether these subgroups are reduced in the cortex of NsCre:Shh<sup>F1/F1</sup> mice, we counted profiles of Pv- and Som-expressing cells in somatosensory cortex at P12. Indeed, there is a dramatic reduction of Som-expressing profiles, particularly in the superficial cortex (Fig. 6E,E',I). Pv-expressing profiles are also greatly reduced (Fig. 6D,D',I), although detection of this effect in superficial cortex is probably compromised by the protracted differentiation of this subgroup into the third postnatal week (Alcantara et al., 1996). Npy, also expressed in a Nkx2.1-dependent subgroup of cortical interneurons (Anderson et al., 2002; Xu et al., 2004), is also reduced in the superficial cortical layers (Fig. 6F,F',I). Similar

interneuron deficits are present in the somatosensory cortex of NsCre:Smoothed<sup>F1/F1</sup> mutants (see Fig. S4 in the supplementary material). Consistent with evidence that GABAergic interneurons of the cortex and striatum share common progenitors (Marin et al., 2000; Reid and Walsh, 2002), Som- and Npy-expressing interneurons are also reduced in the striatum of NsCre:Shh<sup>F1/F1</sup> mice (see Fig. S5 in the supplementary material).

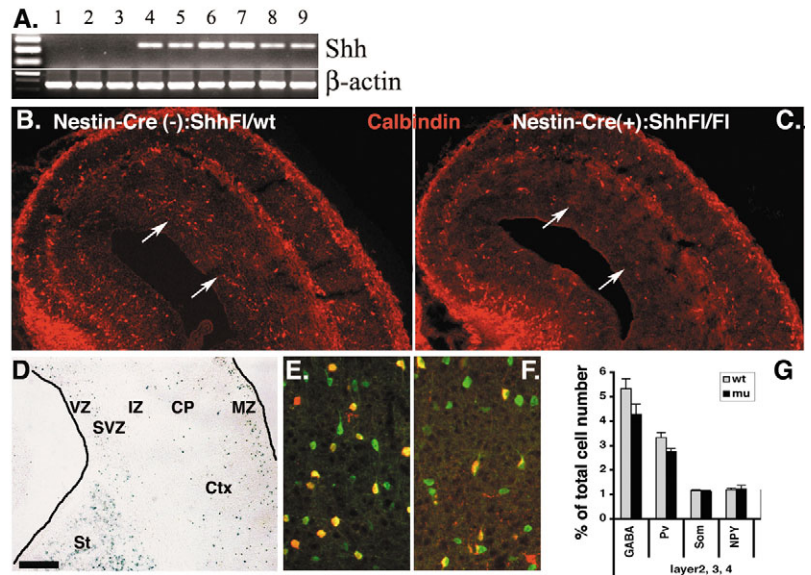
### Somatostatin- and Npy-expressing interneurons are not dependent on Shh signaling for migration, differentiation or survival

The link between loss of Nkx2.1-expressing progenitors in the MGE, and the loss of Pv- and Som-expressing interneurons in postnatal cortex, is suggestive of a novel, Shh-dependent regulation of cortical interneuron number. However, particularly in the light of evidence that, late in gestation, Shh becomes expressed within the cortex itself (Traiffort et al., 1998; Dahmane et al., 2001), and that this expression is also eliminated in the NsCre:Shh<sup>F1/F1</sup> mutants (Fig. 7A), direct or secondary effects of Shh loss on cortical interneuron migration, differentiation or survival must also be considered. To examine whether defects in cortical interneuron development can be identified prenatally in these mutants, the expression of



**Fig. 6.** Reductions in somatostatin and parvalbumin-expressing interneuron subgroups in NsCre:Shh<sup>F1/F1</sup> cortex. Shown are examples of immunofluorescence labeling with antibodies to the neuronal marker NeuN (A,A'), a marker of projection neurons, Tbr1 (B,B'), the interneuron marker GABA (C,C'), and three markers of MGE-derived, Nkx2.1-dependent interneuron subgroups, parvalbumin (Pv; D,D'), somatostatin (Som; E,E') and neuropeptide Y (Npy; F,F'). (A-F) Wild type; (A'-F') mutant. Data in G-I was collected from cryosections through the somatosensory cortex of three mutants and three controls at P12. Despite the trend towards a slightly increased density in both NeuN-labeled cells and in all cells in the mutants (G,H), there is a significant reduction in the percentage of neurons that express GABA (H). In the superficial cortex, this reduction is offset by an increased percentage of neurons that express Tbr1. Consistent with the reduction in GABA, reductions in cells expressing Pv, Som and Npy were observed, although after adjustment for multiple comparisons this effect was significant for only Pv and Som in layers 2-4. \* $P=0.05$ ; \*\* $P<0.01$ . Scale bar in F: 100  $\mu$ m for A-F.

**Fig. 7.** Interneuron losses in *NsCre:Shh<sup>F1/F1</sup>* mutants do not appear to result from defects in postmitotic development. (A) RT-PCR experiments on samples from P0 cortex collected from genotypes *NsCre:Shh<sup>F1/F1</sup>* (lanes 1, 2 and 3), *NsCre:Shh<sup>F1/Wt</sup>* (lanes 4 and 5), *NsCre(-):Shh<sup>F1/Wt</sup>* (lanes 6 and 7), and *NsCre(-):Shh<sup>F1/F1</sup>* (lanes 8 and 9). (B,C) Anti-calbindin immunolabeling of 12  $\mu\text{m}$  cryosections from E16.5 embryos shows a reduction of calbindin-expressing cells in the cortical subventricular zone (SVZ; arrows) of the *NsCre:Shh<sup>F1/F1</sup>* section (C). (D) *Dlx5/6Cre*:floxed  $\beta$ -gal reporter expression in a 12  $\mu\text{m}$  section at E16.5. Little reporter recombination is seen at E14.5 (not shown), but by E16.5 many stained cells are evident in the striatum (St) and cortex. (E,F) Immunofluorescence co-labeling of *Dlx5/6Cre*:floxed-GFP reporter and Pv (E) or Som (F) in layers III-V of somatosensory cortex at P25. Over 90% of cortical interneurons expressing either of these interneuron subgroup markers co-label with GFP. (G) Counts of interneuron subgroups in the postnatal (P25) somatosensory cortex of *Dlx5/6Cre:Sm<sup>F1/F1</sup>* mutants. CP, cortical plate; Ctx, neocortex; IZ, intermediate zone; MZ, marginal zone; VZ, ventricular zone. Scale bar: 50  $\mu\text{m}$ .



calbindin, a protein expressed in many interneurons as they migrate from the ganglionic eminences into the cerebral cortex (Anderson et al., 1997), was examined at E16.5 (Fig. 7B,C). At this age a clear deficit in calbindin-expressing profiles is visible in the *NsCre:Shh<sup>F1/F1</sup>* mutant cortex, particularly in the stream that courses between the subventricular and lower intermediate zones. In slices from normal mice, this stream becomes robust after E14 (Anderson et al., 2001), consistent with the greater effect of the *NsCre:Shh<sup>F1/F1</sup>* mutation on interneurons destined for the superficial cortical layers (Fig. 6). These results suggest that the primary affect of this mutation on interneuron development is established prenatally. In addition, no increase in Caspase 3-expressing, presumably apoptotic, cells is apparent in the telencephalon at E12.5 (Machold et al., 2003), or at E14.5 and E16.5 (data not shown). Postnatally (P0 and P12) markers of cell death are evident in the striatal SVZ but not in the cortex (Machold et al., 2003) (data not shown).

To examine a potential cell-autonomous role of Shh signaling on cortical interneuron migration or differentiation, we next examined conditional loss-of-function mutations of the Shh receptor smoothed (Zhang et al., 2001; Gritli-Linde et al., 2002) mediated by Cre expression driven by the *Dlx5/Dlx6* enhancer (Zerucha et al., 2000; Stenman et al., 2003a). *Dlx5/Dlx6*-Cre-mediated recombination of a *lacZ* reporter appears to occur as or after the cells start migrating towards the cortex (Fig. 7D), when they are known to be postmitotic (Polleux et al., 2002; Xu et al., 2003). In addition, *Dlx5/Dlx6*-Cre-mediated recombination of a GFP reporter shows no co-labeling with the S-phase marker BrdU after a two-hour pulse (see Fig. S6 in the supplementary material). Consistent with evidence that this enhancer drives expression in essentially all interneurons of the murine cerebral cortex (Stuhmer et al., 2002b), fate mapping with the GFP reporter at P25 reveals that the *Dlx5/Dlx6*-Cre enhancer mediates recombination in roughly 90% of cortical Pv- and Som-expressing interneurons (Fig. 7E,F).

To determine whether Shh signaling has a cell-autonomous effect on postmitotic interneuron development, *Dlx5/6Cre:Sm<sup>F1/F1</sup>* mice were generated. In marked contrast to the *NsCre:Shh<sup>F1/F1</sup>* mutants, and to the *NsCre:Sm<sup>F1/-</sup>* mutants (Machold et al., 2003) (see also Fig. S4 in the supplementary material), *Dlx5/6Cre:Sm<sup>F1/F1</sup>* mice are viable with no gross phenotype. Cortical interneuron profiles labeled with GABA, Som, Pv or Npy showed no significant change when compared with controls in layers 2-4 (Fig. 7G), although there was a trend towards a decrease of GABA and Pv ( $n=3$ ,  $P<0.14$  for either marker, not corrected for multiple comparisons). Fewer differences between mutants and controls in the percentage of profiles expressing GABA or Pv were found in the deeper cortical layers (not shown;  $n=3$ ,  $P<0.18$  for either marker, not corrected for multiple comparisons). These results suggest that cell-autonomous Shh signaling is not crucial for the migration, differentiation or survival of most MGE-derived cortical interneurons. Interestingly, grossly normal expression of *Nkx2.1* in the striatum of *Dlx5/6Cre:Sm<sup>F1/F1</sup>* mutants at E16.5 and P25 suggest that factors other than Shh mediate *Nkx2.1* expression in postmitotic interneurons (see Fig. S7 in the supplementary material).

## Discussion

We have previously shown that *Nkx2.1* expression in the MGE is required for the specification of Pv, Som and Npy-expressing interneurons (Xu et al., 2004). In this paper, we provide evidence that Shh expression within the telencephalon maintains *Nkx2.1* expression by MGE precursors during the period of neurogenesis. Thus, Shh regulates the production of cortical interneurons by both expanding the numbers of neuronal progenitors (Machold et al., 2003) and by maintaining their identity. These results suggest that relatively subtle defects in Shh signaling could result in forebrain disease in the absence of gross morphological abnormalities, by altering the generation of cortical interneurons.



### Shh signaling maintains *Nkx2.1* expression in interneuron progenitors of the MGE

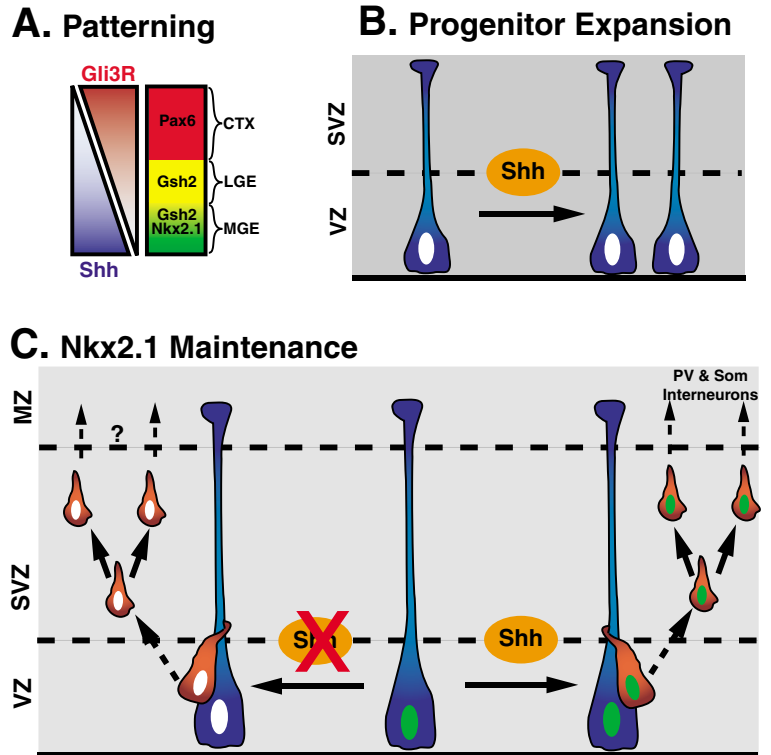
Loss of Shh signaling in the telencephalon of *NsCre:Shh<sup>F1/F1</sup>* mutants occurs by E12.5 (Fig. 2) (Machold et al., 2003), after initial patterning of the telencephalon has been established (Fuccillo et al., 2004). Indeed, in these mutants, general aspects of ventral telencephalon identity are preserved (Fig. 2) (see also Machold et al., 2003), including the LGE-MGE boundary (based on the expression of *Olig2* in the VZ, and *Oct6* in the SVZ and mantle zone; Fig. 2). Conversely, the *NsCre:Shh<sup>F1/F1</sup>* embryos also show reduced *Nkx2.1* expression that is most pronounced in the dorsal MGE, where there is also a loss of *Nkx6.2* expression (Figs 1, 2).

The reduction of these ‘class II’ effectors of Shh signaling, despite other evidence of preserved patterning, raises the question of whether Shh functions to maintain proliferation of the *Nkx2.1* lineage, or whether it functions to maintain *Nkx2.1* expression in previously specified MGE progenitors. Several lines of evidence support the latter possibility. Although there are subtle changes in the density of S-phase cells within the MGE of *NsCre:Shh<sup>F1/F1</sup>* embryos, there is a very dramatic reduction in the percentage of these cells that co-express *Nkx2.1* (Fig. 1). In addition, wild-type telencephalic slices treated with cyclopamine for just 24 hours show an even more robust loss of *Nkx2.1* expression, despite the relatively normal density of S-phase cells (Fig. 3). Finally, addition of exogenous Shh to slices from *NsCre:Shh<sup>F1/F1</sup>* embryos increased the percentage of BrdU and *Nkx2.1* co-expressing cells (Fig. 5), again without significantly altering the overall BrdU-labeling density. These results suggest that *Nkx2.1* expression is essentially ‘plastic’ within MGE progenitors, depending on their stimulation by Shh signaling. In the spinal cord, Shh also functions to maintain class II gene expression (*Nkx2.2*, *Nkx6.1*) at stage 10, after its role in initial patterning has been completed (Briscoe et al., 2000).

Also, similar to findings in the spinal cord (Ericson et al., 1996), our results suggest that Shh signaling during the final cell cycle determines both the expression of *Nkx2.1* and the interneuron fate of MGE progenitors. There was a striking loss of Som-expression in cultured cells that entered S-phase during the last 6 hours of treatment with cyclopamine (Fig. 3). However, the fate of MGE progenitors that no longer express *Nkx2.1* remains to be determined. Consistent with the continued expression of ventral markers in these mutants (Fig. 2) (Machold et al., 2003), cyclopamine-treated, BrdU-labeled cells continue to express GABA (Fig. 3). In the light of evidence that the LGE-MGE boundary is intact (Fig. 2), these cells may be partially-specified interneurons that fail to express the neurochemical subgroup marker but that retain other characteristics of these cells.

### Three actions of Shh on embryonic telencephalon development

The above results suggest the presence of three distinct actions of Shh signaling on embryonic development of the



**Fig. 8.** Three actions of sonic hedgehog on embryonic telencephalon development. (A) Early in development (~E9.0-E12.5), Shh forms a reciprocal gradient with the repressor form of *Gli3*. The relative expression levels of these two signals then work to establish dorsoventral patterning of the telencephalon. (B) Through prenatal development and possibly throughout life, Shh supports the expansion of radial glial stem cells (blue), promoting overall growth of the developing brain. (C) During neurogenesis in the MGE, Shh maintains the expression of *Nkx2.1* (green nuclei) in progenitor cells (blue) that asymmetrically divide into an additional radial glial cell and a transient-amplifying progenitor (red). In the presence of Shh signaling (right pathway), these progenitors migrate into the SVZ where they continue to divide to generate striatal and cortical interneurons. In the absence of Shh (left pathway), the progenitors continue to divide (albeit with reduction of progenitor self-renewal), but *Nkx2.1* expression is lost, as is their fate to become Pv- or Som-expressing interneurons.

telencephalon, with the bulk of evidence suggesting that the main role is played by Shh (Fig. 8). First, between E9 and E12.5, Shh, acting mainly by inhibiting the formation of the *Gli3* repressor (Rallu et al., 2002), contributes to the establishment of dorsoventral patterning (Chiang et al., 1996; Fuccillo et al., 2004). Second, Shh signaling also supports the expansion of progenitors of both the dorsal and ventral telencephalon (Dahmane et al., 2001; Britto et al., 2002; Machold et al., 2003). Third, Shh signaling maintains the expression of *Nkx2.1* and possibly also *Nkx6.2*. Based on the rescue of *Nkx2.1*/BrdU co-labeled cells in slices from *NsCre:Shh<sup>F1/F1</sup>* mutants cultured from E13.5 + 1 DIV (Fig. 5), this period extends at least until E14, well into the period of neurogenesis.

### Alterations in Shh-responsive gene expression correlate with alterations in neuronal fate in vivo

We have previously shown that expression of *Nkx2.1* in the MGE is required for the specification of several neurochemically defined subgroups of cortical interneurons

that originate there (Xu et al., 2004). Thus, the reduction in Nkx2.1-expressing progenitors in the MGE would be expected to result in reductions of Pv, Som and Npy-expressing interneurons in the postnatal cortex of the NsCre:Shh<sup>F1/F1</sup> mutants. Indeed, losses in each of these subgroups have been found, with the strongest effect on the superficial cortical layers (Fig. 6). Interneurons destined for the superficial cortical layers are later born, and thus are most likely to be affected in these mutants (Miller, 1985; Fairén et al., 1986; Cavanagh and Parnavelas, 1988). The correlation between the reduction in the proportion of all neurons (NeuN expressing) that express GABA, and the increased proportion of neurons that co-label for the projection neuron marker Tbr1, further supports the idea that NsCre:Shh<sup>F1/F1</sup> mutants have an actual decrease in cortical interneurons, as predicted by the loss of Nkx2.1-expressing progenitors in the MGE, rather than a reduction in the expression of the interneuron markers.

To what extent is this decrease attributable to the loss of Nkx2.1 expression in the embryonic MGE? As shown in Fig. 7, Shh expression in the cortex at P0 is also lost in the NsCre:Shh<sup>F1/F1</sup> mutants, raising the possibility that in addition to the effects of Shh loss on interneuron specification in the MGE, the NsCre:Shh<sup>F1/F1</sup> cortical interneuron phenotype may reflect later effects on interneuron migration, survival and differentiation. However, several lines of evidence do not support this interpretation. First, by E16.5, calbindin immunolabeling already reveals a profound reduction of migrating interneurons in the SVZ/lower intermediate zone stream of NsCre:Shh<sup>F1/F1</sup> mutants (Fig. 7), suggesting that the interneuron phenotype in these mutants is established during the age range of neurogenesis (E12.5-E16.5). Second, postnatal analysis of mice in which the Shh receptor smoothed has been inactivated in postmitotic interneurons by Dlx5/6Cre reveals normal densities of Som- and Npy-expressing interneuron profiles, suggesting that cell autonomous Shh signaling is not required for the migration, differentiation or survival of these subgroups.

In summary, during embryonic neuronogenesis, Shh signaling appears to maintain Nkx2.1 expression, and thereby interneuron fate determination, in MGE progenitors. These findings suggest that prenatal alterations in Shh signaling could disrupt the generation of cortical interneurons without causing a more gross disruption of telencephalic patterning. Such disruptions could result in the subtle alterations in interneurons that are implicated in a variety of cortical illnesses. Indeed, although loss-of-function mutations in SHH in humans can result in severe holoprosencephalies, more subtle mutations or variable penetrance can result in a spectrum of disorders that includes abnormalities of learning and attention, and seizures (Muenke and Beachy, 2000; Heussler et al., 2002; Lazaro et al., 2004; Nieuwenhuis and Hui, 2005).

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## Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/22/4987/DC1>

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