Hedgehog signaling is involved in development of the neocortex

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Sonic hedgehog (Shh) function is essential for patterning and cell fate specification, particularly in ventral regions of the central nervous system. It is also a crucial mitogen for cerebellar granule neuron precursors and is important in maintenance of the stem cell niche in the postnatal telencephalon. Although it has been reported that *Shh* is expressed in the developing dorsal telencephalon, functions of *Shh* in this region are unclear, and detailed characterization of *Shh* mRNA transcripts in situ has not been demonstrated. To clarify the roles of Shh signaling in dorsal pallium (neocortex primordium) development, we have knocked out the *Shh* and *Smo* genes specifically in the early developing dorsal telencephalon by using *Emx1[cre]* mice. The mutants showed a smaller dorsal telencephalon at E18.5, which was caused by cell cycle kinetics defects of the neural progenitor/stem cells. The cell cycle length of the progenitor/stem cells was prolonged, and the number of cycle-exiting cells and neurogenesis were decreased. Birth-date analysis revealed abnormal positioning of neurons in the mutants. The characteristics of the subventricular zone, ventricular zone and subplate cells were also affected. Weak immunoreactivity of Shh was detected in the dorsal telencephalon of wild types. Reduced Shh immunoreactivity in mutant dorsal telencephalons supports the above phenotypes. Our data indicate that Shh signaling plays an important role in development of the neocortex.

KEY WORDS: Neocortex, Hedgehog signaling, Cell cycle kinetics, Mouse

INTRODUCTION

The secreted protein sonic hedgehog (Shh) is involved in various aspects of embryogenesis. In the mammalian central nervous system, Shh plays an important role in ventral patterning along the entire neuraxis, including the telencephalon (Agarwala et al., 2001; Echelard et al., 1993; Ericson et al., 1995; Roelink et al., 1994; Ruiz i Altaba et al., 1995; Watanabe and Nakamura, 2000). GABAergic interneurons, which comprise 20-30% of cortical neurons, are important for both development and function of the cerebral neocortex (Anderson et al., 2002; Fuccillo et al., 2004; Gulacsi and Anderson, 2006; Xu et al., 2005). GABAergic interneurons have been shown to migrate tangentially into the developing dorsal pallium from medial ganglionic eminence (MGE) of the ventral regions (Anderson et al., 2002; Anderson et al., 2001; Lavdas et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001).

Previous studies have also illustrated that *Shh* is expressed in dorsal parts of three major brain structures, i.e. the neocortex of the telencephalon, tectum and cerebellum, at perinatal stages (Dahmane et al., 2001; Ruiz i Altaba et al., 2002). The common feature of these three structures is layer formation parallel to the pial surface. In the developing cerebellum, Shh produced by Purkinje neurons promotes proliferation of granule neuron progenitors and induces differentiation of Bergmann glia. Shh signaling coordinates development of cortical cell types in growth and patterning of the cerebellum (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999).

Similar molecular mechanisms might be applied to development of the cerebral neocortex. Indeed, it has also been reported that Shh is a mitogen for the neocortex and tectal progenitors, and that it modulates cell proliferation in vitro (Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004). In addition, Machold, Lai and Palma et al. have reported that Shh signaling controls stem cell behavior in adult brains (Machold et al., 2003; Lai et al., 2003; Palma et al., 2005). In *Shh*-null mutants, specification of ventral cell populations and general brain growth are all affected (Chiang et al., 1996). The telencephalon of *Shh* mutants forms as a single fused vesicle (holoprosencephaly) that is strongly dorsalized (Chiang et al., 1996; Rallu et al., 2002). Therefore, the roles of Shh in corticogenesis cannot be elucidated by using the conventional *Shh* knockout mice.

Recently, the roles of Shh signaling in initial patterning of the telencephalon have been analyzed by using *Smoothened* (*Smo*) conditional knockout mice (Fuccillo et al., 2004). *Smo* encodes a membrane protein that transduces Hh signaling into the cytoplasm. For example, Cre recombinase under control of *FoxG1* regulatory sequences (in telencephalic neural precursors) was used to inactivate *Smo* conditionally by embryonic day (E) 9.5 in the whole telencephalon and initial patterning of the ventral telencephalon failed. However, corticogenesis in the dorsal pallium appeared normal (Fuccillo et al., 2004). *Nestin-Cre*;*Smo* conditional knockouts lost Shh signaling by E12.5 (Machold et al., 2003) and showed grossly normal patterning in contrast to *FoxG1-Cre*;*Smo* (Fuccillo et al., 2004; Machold et al., 2003). These contrasting phenotypes imply that the time period between E9.5 and E12.5 is crucial for specification of ventral characters in the telencephalon, in keeping with observation that cellular response to Shh varies over time (Fuccillo et al., 2004; Machold et al., 2003). Furthermore, Xu et al. (Xu et al., 2005) reported that the cortical thickness was decreased in *Nestin-Cre; Shh* mice. Lien et al. (Lien et al., 2006) reported that the cortical hyperplasia was caused by abnormal activation of hedgehog signaling in *Nestin-Cre; αE-catenin* mice. Taken together, these findings suggest the possibility that Shh

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signaling controls the progenitor cell number in the developing dorsal pallium. However, one important caveat is that *Shh* expression in this region has not been well established. In the embryonic dorsal pallium, *Shh* mRNA expression level was reported to be too 'low' to be detected by in situ hybridization. *Shh* was detectable by only more-sensitive RT-PCR in this region from E14.5 to postnatal day 3 (Dahmane et al., 2001).

In this study, we clarify the roles of Shh signaling in dorsal pallium development by using *Emx1-Cre;Shh*^fl/– and *Emx1-Cre;Smo*^fl/– conditional knockout mice. *Emx1* is expressed in the dorsal pallium from embryonic stages to adulthood (Iwasato et al., 2004). The conditional knockout mice lost *Shh* and *Smo* expression very early by about E10.5 in the dorsal telencephalon. Analyses of the mutant embryos revealed decreased proliferation and neuronal differentiation and increased cell death at neurogenesis stages. The positioning of neurons in the neocortex was also affected. Our data suggest that Shh signaling controls cell cycle kinetics of cortical neural progenitor/stem cells and positioning of cortical neurons in the dorsal pallium during corticogenesis. We also report detailed localization of Shh protein in the developing dorsal pallium by immunohistochemistry. Shh was detected in neural progenitor/stem cells, Cajal-Retzius (CR) neurons, cortical plate (CP) neurons and GABAergic interneurons. The immunostaining data support the conditional knockout phenotypes.

MATERIALS AND METHODS

Generation of floxed *Shh* and *Smo* mice

Emx1^Cre mice (Iwasato et al., 2004) were crossed with *Shh* heterozygous (a gift from Dr McMahon) (St-Jacques et al., 1998) and *Smo* heterozygous (The Jackson Laboratory, Maine, USA) (Zhang et al., 2001) mutants to generate *Emx1*^Cre;*Shh*^+/– and *Emx1*^Cre;*Smo*^+/– males. These mice were mated with *Shh*^fl/fl (a gift from Dr McMahon) (Lewis et al., 2001) and *Smo*^fl/fl (The Jackson Laboratory, Maine, USA) (Long et al., 2001) females to generate conditional knockout mice of the informative genotypes *Emx1*^Cre;*Shh*^fl/– (*Shh*-CKO) or *Emx1*^Cre;*Smo*^fl/– (*Smo*-CKO). The *Emx1*^Cre allele induces recombination in neural progenitor/stem cells and neurons of the dorsal telencephalon by E10, consistent with the known properties of the dorsal telencephalon-specific enhancer of *Emx1* (Iwasato et al., 2004). Animals were maintained according to the guidelines from Animal Research Committee of Kyoto University.

Tissue preparation

For frozen sections, embryos were fixed for 30 minutes for Shh immunostaining or overnight for other staining in 4% paraformaldehyde at 4°C. These embryos were then washed in phosphate-buffered saline (PBS). Frozen samples were serially sectioned at 5 μm for in situ hybridization and immunohistochemistry. For paraffin sections, embryos were fixed for 3 hours in PLP fixative at 4°C. Tissues embedded in paraffin were sectioned at 5 μm for immunohistochemistry and histological analysis.

BrdU incorporation and birth date analysis

For in vivo labeling of S-phase cells (BrdU incorporation), a single intraperitoneal injection of 50 mg/kg BrdU (Sigma B5002-1G) was made 1 hour prior to sacrificing. For birth-date analysis, the timed-mated pregnant females received a single intraperitoneal injection of CldU (MP Biomedicals 105478) and IdU (Sigma 17125-5G) at E13.5 and E15.5, respectively. Pups were allowed to develop to E18.5 and processed for CldU and IdU immunohistochemistry. The quantification of young neurons and their distribution within cortical layers was analyzed according to Molyneaux et al. (Molyneaux et al., 2005) on anatomically matched sections from each mouse (three wild-type and three mutant mice) (details can be provided on request).

Immunohistochemistry

Standard immunostaining procedures were used. To analyze Shh localization in the dorsal telencephalon, samples must be kept at 4°C during all procedures. Details of antibodies used in this study can be provided on request.

In situ hybridization

Section RNA in situ hybridization was performed as previously described (Ishii et al., 1997). cDNA probes used were *Smo* (Akiyama et al., 1997), *Wnt7b* (Parr et al., 1993) and *Tbr1* (Bulfone et al., 1995). Digoxigenin-labeled probes were synthesized using a digoxigenin RNA labeling kit (Roche 1362372).

In utero electroporation

For introduction of pCIG-Shh expression vector (Sun et al., 2008), pregnant mice were deeply anesthetized and a ventral midline incision was made to perform in utero manipulation. The expression vector (1 μg/μl) was injected through the uterine wall into the telencephalic vesicle of each embryo (E12.5). Electroporation was performed as described previously (Saito and Nakatsuji, 2001). The electroporated embryos were analyzed at E18.5.

RESULTS

Using RT-PCR, it has been reported that *Shh* mRNA is expressed in the developing dorsal pallium, the neocortex anlage, from E14.5. However, the expression level is too low to be detected by in situ hybridization (Dahmane et al., 2001). Therefore, functions of *Shh* in this region were unclear.

Size reduction of the dorsal telencephalon at E18.5 in *Shh*-CKO and *Smo*-CKO mice

To clarify directly the roles of Shh signaling in the development of the neocortex, we knocked out *Shh* and *Smo* genes specifically in the developing dorsal telencephalon using *Shh* and *Smo* conditional alleles (Lewis et al., 2001; Long et al., 2001) and *Emx1*^Cre knock-in mice. The *Emx1* promoter directs expression of Cre recombinase specifically in the developing dorsal telencephalon including the dorsal pallium (Iwasato et al., 2004) (see Fig. S1D in the supplementary material). We confirmed *Smo* mRNA expression. Although *Smo* mRNA was observed in the subventricular zone (SVZ)/the ventricular zone (VZ) of wild-type brains, it was not detectable specifically in the dorsal pallium of *Smo*-CKO embryos (Fig. 1C,C′). We also examined Shh immunoreactivity (see below).

In conventional *Shh*-null mutants, which show rhinocephaly (Chiang et al., 1996), most aspects of ventral and dorsal patterning in the telencephalon are abrogated, leading to difficulty in analysis of corticogenesis. By contrast, *Shh*-CKO and *Smo*-CKO embryos showed almost normal appearance (data not shown). When the brains were excised at E18.5, their morphology seemed to be grossly normal, although they were significantly smaller than wild-type brains (Fig. 1A-A″), based on measures of the rostral-caudal length, the width and the area (measured on the photos) of the telencephalon (Table 1). Furthermore, the coronal and parasagittal sections revealed the decreased size of the dorsal telencephalon, while the morphology of the ventral telencephalon was not significantly changed (Fig. 1B-B″). In addition, we performed immunostaining with anti-neuronal class III β-tubulin (Tuj1), a pan-neuronal marker, and quantified the total thickness of the dorsal pallium and the thickness of CP (Tuj1-positive layer). There was a significant reduction of the thickness of both of them in *Shh*- and *Smo*-CKO mutants at E15.5, and in *Smo*-CKO mutants at E18.5 (see Fig. S2 in the supplementary material). These results indicate that Shh signaling regulates neurogenesis in the dorsal telencephalon and that its absence results in a smaller dorsal pallium.

Shh signaling in the dorsal pallium maintains proliferation and survival of neural progenitor/stem cells

Possible mechanisms that might account for the size reduction of the dorsal telencephalon are decreased proliferation and increased cell death. To examine whether cell proliferation is decreased in the
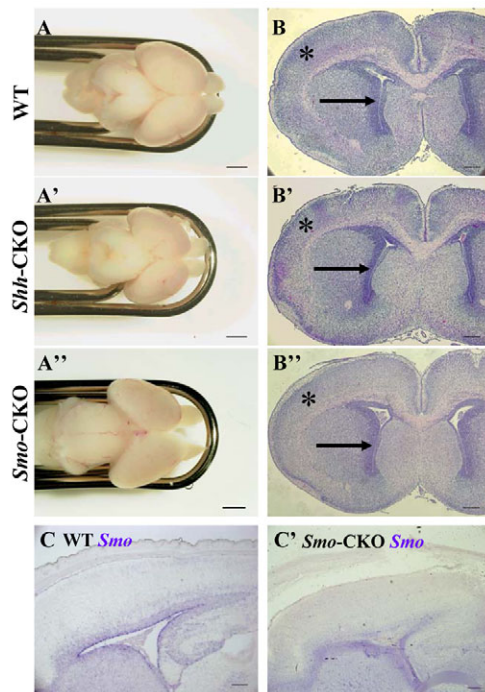
Fig. 1. The size of the dorsal telencephalon is reduced in *Shh*-CKO and *Smo*-CKO embryos. Dorsal views of wild-type (**A**), *Shh*-CKO mutant (**A'**) and *Smo*-CKO (**A''**) mutant brains at E18.5. The appearance of the mutant brains was grossly normal. Coronal sections of wild-type (**B**), *Shh*-CKO (**B'**) and *Smo*-CKO (**B''**) telencephalons at E18.5 were stained with HE. These panels illustrated that the size of the dorsal telencephalon was slightly, but significantly reduced in the mutants (see Table 1). Note that the thickness of the mutant cortices is slightly thinner than that of the wild-type cortex (asterisk). The angle of the lower extension of the lateral ventricle (arrow in B) is almost perpendicular in wild type, while that of the mutants is medially inclined (arrow in B',B''). By contrast, the ventral parts were not apparently affected. In situ hybridization of *Smo* on E18.5 wild-type (**C**) and *Smo*-CKO mutant (**C'**) embryo brains. The expression of *Smo* was undetected specifically in the dorsal telencephalon in the mutant brain. Scale bar: 1 mm in A-A''; 200 μm in B-B''; 100 μm in C,C'.

mutants, we performed an analysis of BrdU incorporation (details can be provided on request) (Fig. 2A-D). At E13.5, the BrdU-incorporation index was significantly decreased in the dorsal pallium of both *Shh*-CKO and *Smo*-CKO embryos (Fig. 2D). Interestingly, in *Shh*-CKO embryos at E15.5, there was no significant change in the BrdU-incorporation index (Fig. 2D), suggesting that proliferation defects were somehow rescued at this stage. However, the BrdU-incorporation index in *Smo*-CKO embryos was significantly reduced at E15.5 compared with wild type (Fig. 2D), consistent with inability of *Smo* mutant cells to respond to Shh.

To determine weather the size reduction of the mutant dorsal pallium resulted from increased cell death, we performed immunostaining for cleaved caspase 3 to identify apoptotic cells (Fig. 2E-G). In comparing with wild type, *Shh*-CKO and *Smo*-CKO embryos showed significantly increased cell death at E13.5 (Fig. 2H). These results indicate that the size reduction of the dorsal pallium was caused, at least partly, by proliferation defects and increased cell death in the absence of Shh signaling.

Cell cycle kinetics are altered in the mutants: prolonged cell cycle length and reduced cell cycle exit

To investigate the cause of proliferation defects, we examined cell cycle kinetics (cell cycle length and cell cycle exit) of neural progenitor/stem cells (details can be provided on request) (Chenn and Walsh, 2002). A smaller proportion of BrdU-labeled cells in Ki67-positive cells indicates a longer cell cycle length. In *Shh*-CKO embryos, length of cell cycle was significantly prolonged at E13.5, but not significantly changed at E15.5 (Fig. 3A-B',G) compared with wild type, consistent with the above results of proliferation analyses. However, *Smo*-CKO neural progenitor/stem cells divided significantly more slowly than wild-type cells at both E13.5 and E15.5 (Fig. 3A,A',C,C',G).

As it has been reported that Hh signaling also accelerates cell cycle exit in the amphibian and fish developing retina (Locker et al., 2006), we examined how cell cycle exit was affected in the mutants (methods can be provided on request) (Chenn and Walsh, 2002). The quantification of these experiments showed the significantly decreased cell cycle exit in *Shh*-CKO at E13.5 and in *Smo*-CKO at E13.5 and E15.5 (Fig. 3D-F',H).

To distinguish which cell population (neural stem cells versus basal progenitors) was affected, we performed double-immunostaining with anti-Pax6 and anti-Ki67 antibodies at E15.5 (methods can be provided on request). Pax6-positive cells represent neural stem cells in VZ (Englund et al., 2005) and Pax6-negative/Ki67-positive cells are basal progenitors in SVZ. There was no significant difference in the Pax6-positive cell number among wild-type, *Shh*-CKO and *Smo*-CKO mice (see Fig. S3A-D in the supplementary material). In addition, no significant difference was observed in the number of Pax6-negative/Ki67-positive basal progenitors, although there was a tendency towards a decrease in both mutants (see Fig. S3E in the supplementary material). Next, we used anti-Phospho histone H3 (PHH3) antibody to count the number of M-phase cells among Pax6-positive and Pax6-negative cells. The number of Pax6-positive/Phh3-positive cells was not significantly different among wild type, *Shh*-CKO and *Smo*-CKO mutants (see Fig. S3F-I in the supplementary material). By contrast, Phh3-positive basal progenitors (Pax6 negative) showed a tendency to decrease in number in *Shh*-CKO and were significantly decreased in number in *Smo*-CKO (see Fig. S3F-H, J in the supplementary material). Altogether, the basal progenitors in both mutants seemed to be more severely reduced in number.

To further examine how loss of Shh signaling affects the character of the basal progenitors, Tbr2 immunostaining was employed (methods can be provided on request). Tbr2, another T-domain transcription factor in the developing brain, is expressed at high levels in the basal progenitors in SVZ/VZ of the dorsal pallium (Bulfone et al., 1995; Englund et al., 2005). In *Shh*-CKO and *Smo*-CKO embryos, the number of Tbr2-positive cells was significantly decreased (see Fig. S4 in the supplementary material), suggesting that the basal progenitors were decreased in SVZ/VZ of the dorsal pallium.

Table 1. Size of the telencephalon

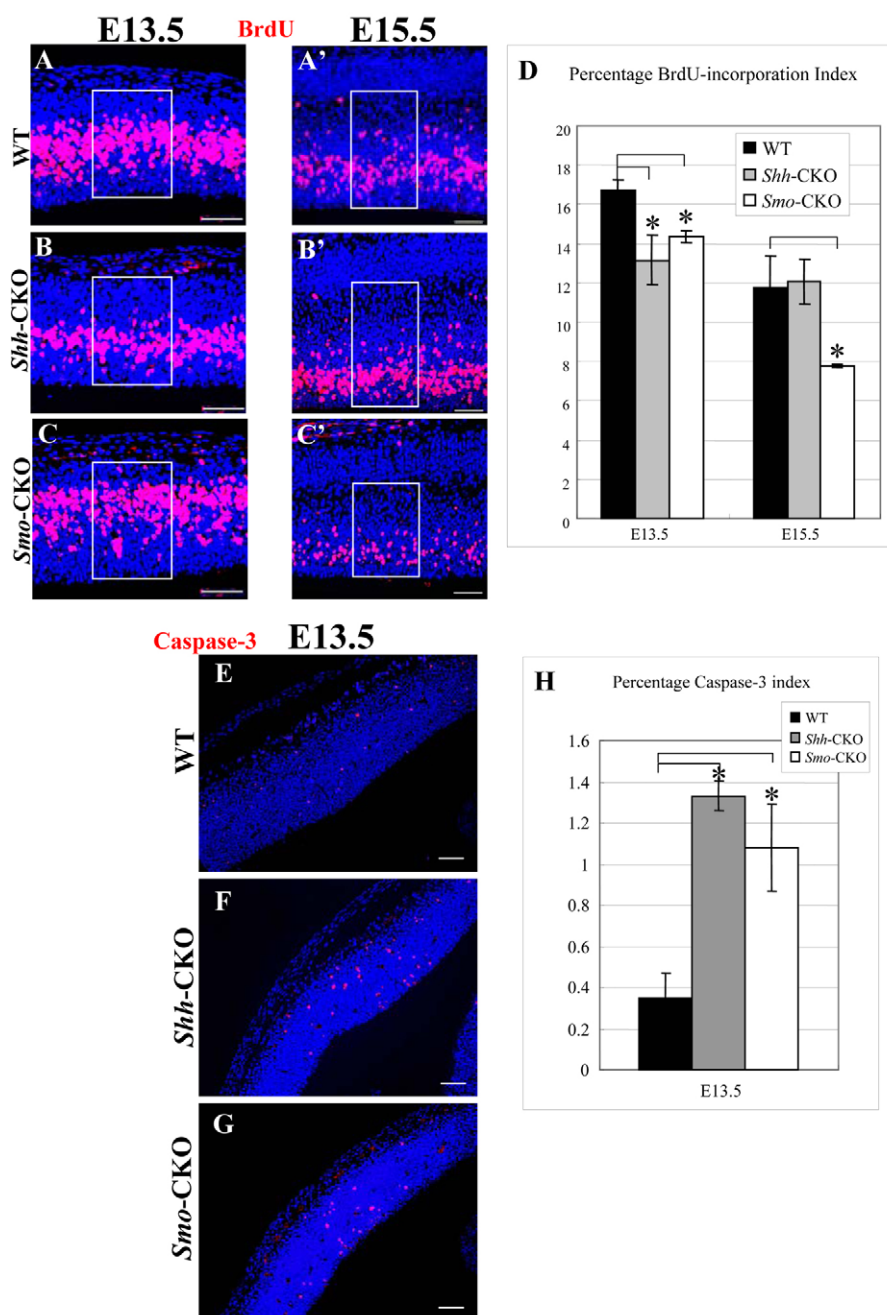| E18.5 telencephalon	Length	Width	Area
Wild type	100±1.1%	100±1.0%	100±3.6%
Shh-CKO	97.1±6.4%	92.8±1.9%*	75.9±8.5%*
Smo-CKO	89.4±1.7%*	92.8±2.1%*	80.6±13.2%*

*$P<0.01$.

Fig. 2. Proliferation of progenitor/stem cells in the dorsal pallium is reduced and apoptosis is increased in the absence of Shh signaling. BrdU-incorporation analysis at E13.5 (**A-C**) and E15.5 (**A'-C'**). Cells were counted in 100 μm-wide sampling boxes (white boxes). Examples of anti-BrdU labeling (red) of parasagittal sections of the dorsal pallium of wild-type (A,A'), *Shh*-CKO mutant (B,B') and *Smo*-CKO mutant (C,C') embryos. (**D**) Both *Shh*-CKO and *Smo*-CKO embryos showed significantly decreased BrdU incorporation index at E13.5 (13.2±1.26% and 14.4±0.29%, respectively; *n*=3) compared with wild type (16.8±0.47%, *n*=3). At E15.5, *Smo*-CKO showed significantly decreased index (7.78±0.10%; *n*=3), while *Shh*-CKO showed comparable index (12.04±1.15%, *n*=3) to wild-type embryos (11.75±1.60%, *n*=3). Parasagittal sections of E13.5 dorsal pallium were immunostained with anti-cleaved caspase 3 antibody (**E-G**) and cells were counted on the entire sections (**H**). There were a few stained cells (red) in wild-type brain (E,H, 0.35±0.118%; *n*=3). Apoptotic cells were increased in the dorsal pallium of *Shh*-CKO (F,H, 1.33±0.075%; *n*=3) and *Smo*-CKO embryos (G,H, 1.07±0.210%; *n*=3). **P*<0.01. Scale bar: 50 μm.

Lack of Shh signaling leads to abnormal neuronal positioning and differentiation in corticogenesis

To examine possible defects associated with neuronal positioning and differentiation, neuronal birth-date analysis was performed (methods can be provided on request). In wild-type embryos, neurons born at E13.5 (CldU-positive) settled down mainly in the deeper layers (Bin3 and Bin4 in Fig. 4D) with the peak in Bin4. Neurons born at E15.5 (IdU-positive) migrated mostly to the superficial layer of the CP (Bin5 in Fig. 4G) at E18.5. Because migration of E15.5-born neurons was not completed at E18.5, a substantial number of labeled cells remained in Bin1-3 (Fig. 4G).

In *Shh*-CKO embryos, neurons born at E13.5 distributed mainly in Bin3 and Bin4, as in wild-type embryos (Fig. 4D,E). However, the labeled neurons in Bin4 and Bin5 (* and ** in Fig. 4D,E) were significantly decreased in number, implying differentiation and/or positioning defects of E13.5-born neurons. Distribution of E15.5-born neurons in *Shh*-CKO mice showed a similar pattern to that in wild type (Fig. 4G,H). Here again, like proliferation defects, the phenotype might be consistently rescued.

Smo-CKO embryos showed a severely impaired neuronal positioning pattern at both stages. As shown in Fig. 4F, E13.5-born neurons were scattered throughout the dorsal pallium. We observed an increased proportion of E13.5-born neurons in Bin5 (* in Fig. 4F when compared with D), suggesting reduced numbers of late-born neurons with relatively superficial position. The increased CldU index in Bin1 (**** in Fig. 4) might reflect slower cycling cells stuck in SVZ/VZ or E15.5-born neurons that failed to migrate to the superficial layer (Bin5, # in Fig. 4G,I) of CP through the early-born neurons and remained in SVZ/VZ
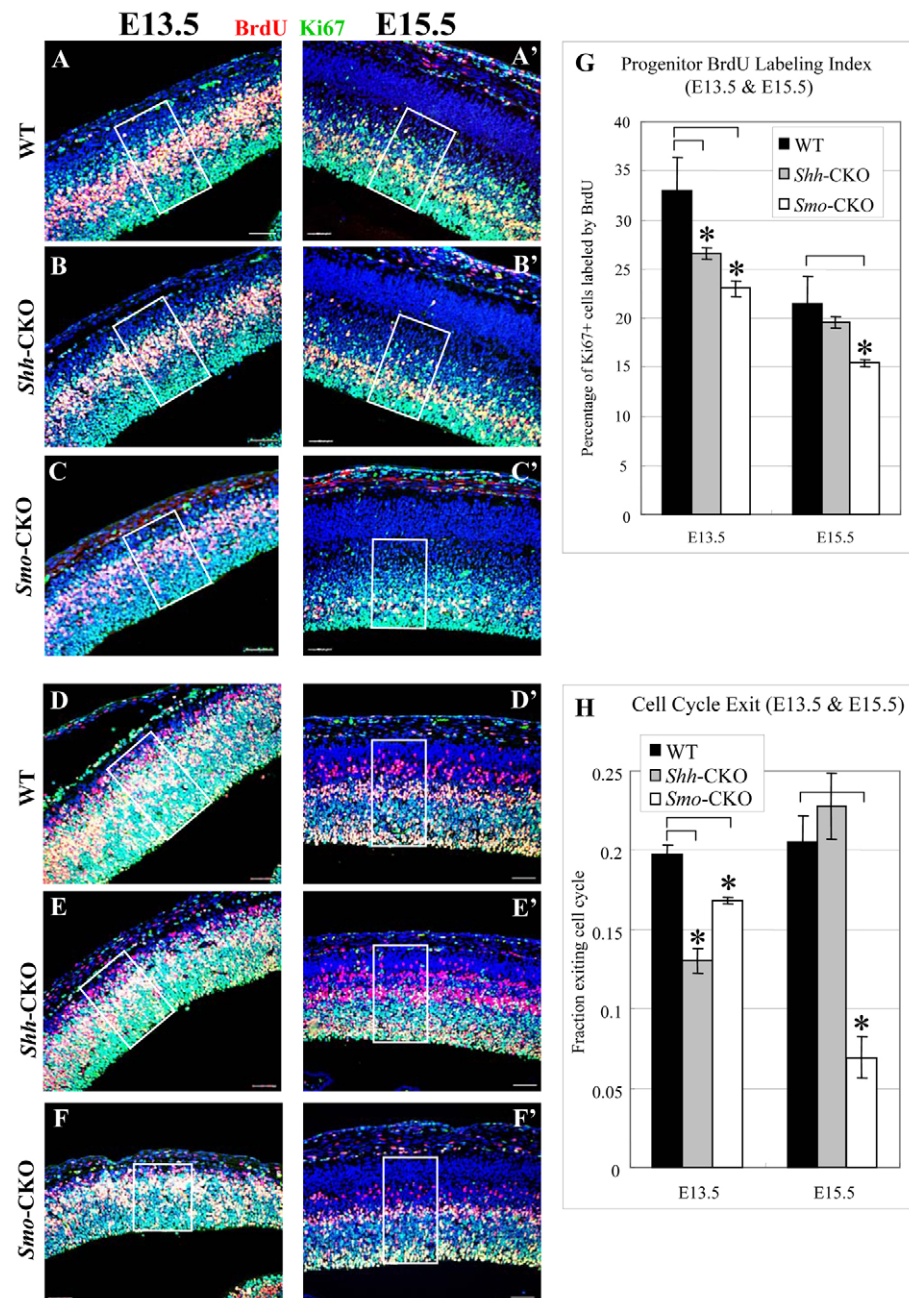
Fig. 3. Loss of Shh signaling leads to prolonged cell cycle length and reduced cell cycle exit of the neural progenitor/stem cells. (**A-C′**) After 1-hour pulse labeling of BrdU, immunostaining of parasagittal sections was performed with anti-Ki67 (green) and anti-BrdU (red) antibodies at E13.5 (A-C) and E15.5 (A′-C′). (**G**) Cell cycle length was estimated as percentage of Ki67 and BrdU double-positive cells in all Ki67-positive cells. Smaller percentage represents longer cell cycle. At E13.5, both *Shh*-CKO and *Smo*-CKO embryos showed significantly prolonged cycle length (26.5±0.005% and 23.0±0.004%, respectively, *n*=3) compared with wild-type embryos (33.0±0.034%, *n*=3) (A-C,G). At E15.5, cell cycle length of *Shh*-CKO was not significantly prolonged (19.6±0.005%, *n*=3) whereas that of *Smo*-CKO was significantly longer (15.4±0.003%, *n*=3) than that of wild type (21.5±0.027%, *n*=3) (A′-C′,G). (**D-F′**) To estimate cell cycle exit, sections were stained with anti-Ki67 and anti-BrdU antibodies 24 hours after BrdU pulse (E13.5, D-F; E15.5, D′-F′). (**H**) Cell cycle exit was determined as a ratio of the cells that exited the cell cycle (red, BrdU+/Ki67–, no longer dividing) to all cells labeled with BrdU (red+yellow) after 24 hours labeling. In *Shh*-CKO embryos, the ratio was significantly decreased at E13.5 (0.131±0.008; wild type, 0.198±0.005; *n*=3), but not different at E15.5 (0.230±0.021, *n*=3; wild type, 0.205±0.016, *n*=3). In *Smo*-CKO embryos, the ratio was significantly decreased compared with wild type at both E13.5 and E15.5 (0.168±0.002 and 0.069±0.013, respectively; *n*=3). *P<0.01. Scale bar: 50 μm.

(Bin1, Bin2 in Fig. 4I). These data suggest that Shh signaling controls the timing of neuronal differentiation and the positioning of neurons during corticogenesis.

Characters of each zone are affected in CKO mutants

Because timing of differentiation and migration are closely linked to the layer identities during corticogenesis, the above results prompted us to investigate specification defects in the dorsal pallium. The marginal zone (MZ), CP, IZ and SVZ/VZ were histologically distinct in the E18.5 dorsal pallium (Fig. 5A). The mutants showed a comparable structure (Fig. 5B,C). To investigate specification defects, we analyzed the expression of *Wnt7b*, a marker of layer VI in the dorsomedial cortex (Rubenstein et al., 1999) (Fig. 5D). In wild-type embryos, *Wnt7b* was expressed weakly in SVZ/VZ and intensely in the deep layer of CP and SP at

E18.5, but not in IZ, making an unstained gap between CP and SVZ/VZ (Fig. 5D). In *Shh*-CKO embryos, this unstained gap was lost at E18.5 (Fig. 5E), suggesting that IZ cells have been changed in character and/or reduced to almost none. In *Smo*-CKO embryos, discrete expression in SP and weak expression in SVZ/VZ were reduced to the background level, while the expression in the deep layer of CP was comparable with wild type (Fig. 5D-F). The results imply that the character of the neural progenitor/stem cells in SVZ/VZ was affected in *Smo*-CKO mutants. In addition, the *Wnt7b*-negative upper layers of CP in both CKO brains seemed to be thinner than those in wild-type brains, consistent with the above birth-date analyses.

Tbr1 is a transcription factor gene of the T-box family that is expressed soon after cortical progenitors begin to differentiate. It is highly expressed in early-born postmitotic neurons of MZ, layer VI in CP and SP (Bulfone et al., 1995). SVZ/VZ and superficial layers
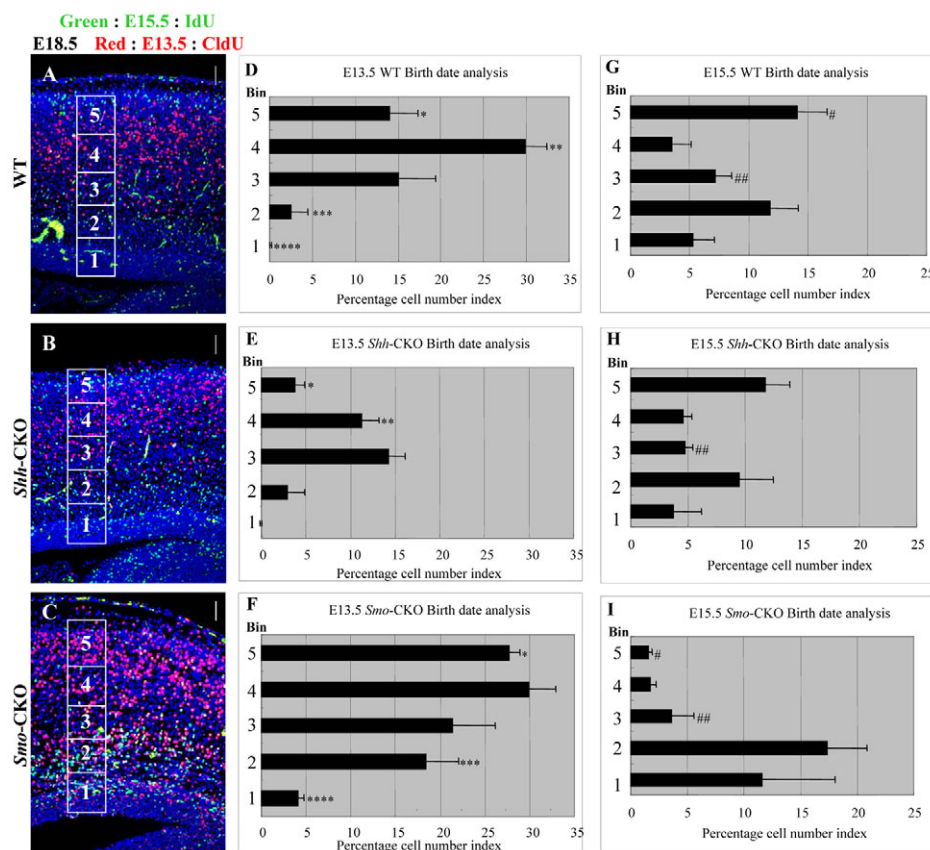
Fig. 4. Positioning of postmitotic neurons was affected in CKO mutants. (**A-C**) Birth-date analysis was carried out by CldU and IdU double staining of E18.5 the dorsal pallium sections after a single pulse of CldU at E13.5 and IdU at E15.5. (**D-I**) Quantification of CldU- and IdU-positive cell index in each Bin. In wild-type embryos, E13.5-born neurons (CldU+, red) mostly settled down in the deep cortical layers with a peak in Bin4 (**29.92±2.45%; *n*=3, in D) and E15.5-born neurons (IdU+, green) distributed mainly to the superficial layer, Bin5 (#14.12±2.51%; *n*=3, in G). In *Shh*-CKO embryos, E13.5-neurons positioned similarly to wild type though neurons in Bin4 were significantly decreased (**11.24±1.89% in E compared with D; *n*=3, *P*<0.01). E15.5 neurons in *Shh*-CKO were positioned in the almost same pattern as in wild type (compare G with H). In *Smo*-CKO embryos, E13.5 neurons were differently scattered throughout the dorsal pallium (Bin1****, 4.06±0.669%; Bin2***, 18.38±3.69%; Bin5*, 28.07±0.76%; *n*=3, *P*<0.01 in F) compared with wild type (Bin1****, 0.09±0.09%; Bin2***, 2.56±1.86%; Bin5*, 14.00±3.29%; *n*=3, in D). E15.5 cells mostly remained in SVZ/VZ (Bin1 and 2 in I) and significantly decreased in CP (Bin3##, 3.63±1.97%; *n*=3, *P*<0.05 and Bin5#, 1.51±0.38%; *n*=3, *P*<0.01 in I) compared with wild type (Bin3##, 7.37±1.02% and Bin5#, 14.12±2.51%; *n*=3, in G). Scale bar: 50 μm.

of CP were weakly stained as well. (Fig. 5G). In both *Shh*-CKO and *Smo*-CKO embryos, there was a tendency towards reduction of the *Tbr1*-expressing layer VI relative to wild type (Fig. 5G-I). In addition, *Tbr1* expression in SP (Fig. 5G,H) was not detected in *Smo*-CKO embryos (Fig. 5I). Weak expression in SVZ/VZ and superficial layers of CP was reduced to almost the background level, suggesting that characters of cells in these regions, especially neural progenitor/stem cells in SVZ/VZ were also affected.

To examine whether SP was correctly formed in the mutants, we performed the immunostaining using anti-Map2 antibody, which can distinguish SP neurons from CP neurons (Shinozaki et al., 2002) and anti-chondroitin sulfate (CS56), a SP marker (Gilmore et al., 1998). At E18.5, in *Smo*-CKO mutants, fewer Map2-positive neurons were observed in SP than in wild type (Fig. 5J,L), while *Shh*-CKO showed comparable Map2-positive neurons in SP with wild type (Fig. 5J,K). At E15.5, CS56 immunoreactivity of *Smo*-CKO was substantially decreased in SP, when compared with wild type and *Shh*-CKO mutants (Fig. 5M-O, arrow). These results indicate that SP was underdeveloped in the absence of Shh signaling.

Overexpression of *Shh* increases proliferative cells in SVZ/VZ of the dorsal pallium

The above results are consistent with the previous report about the developing retina (Locker et al., 2006), suggesting that Shh signaling promotes: (1) transition from slow cycling stem cells to fast cycling progenitors; as well as (2) cell cycle exit (neuronal differentiation) in the dorsal pallium. To confirm these roles of Shh signaling, we performed a gain-of-function experiment by in utero electroporation. The pCIG-Shh expressing vector, which expresses both Shh and EGFP, was electroporated into the dorsal pallium at E12.5. At E18.5, these transfected embryos were subjected to analyses. Both control (EGFP-expression vector only) and pCIG-Shh transfected embryos, most of EGFP-positive cells were present in CP at E18.5 (data not shown and Fig. 6A). In pCIG-Shh transfected regions, CP was expanded in a planar direction and the thickness of SVZ/VZ was extremely increased (Fig. 6B). The number of the proliferative cells with Ki67 immunoreactivity was increased on the pCIG-Shh transfected side (Fig. 6C,C′). To investigate whether cell characters are altered in each layer, we examined expression of *Wnt7b* and *Tbr1*. On the pCIG-Shh
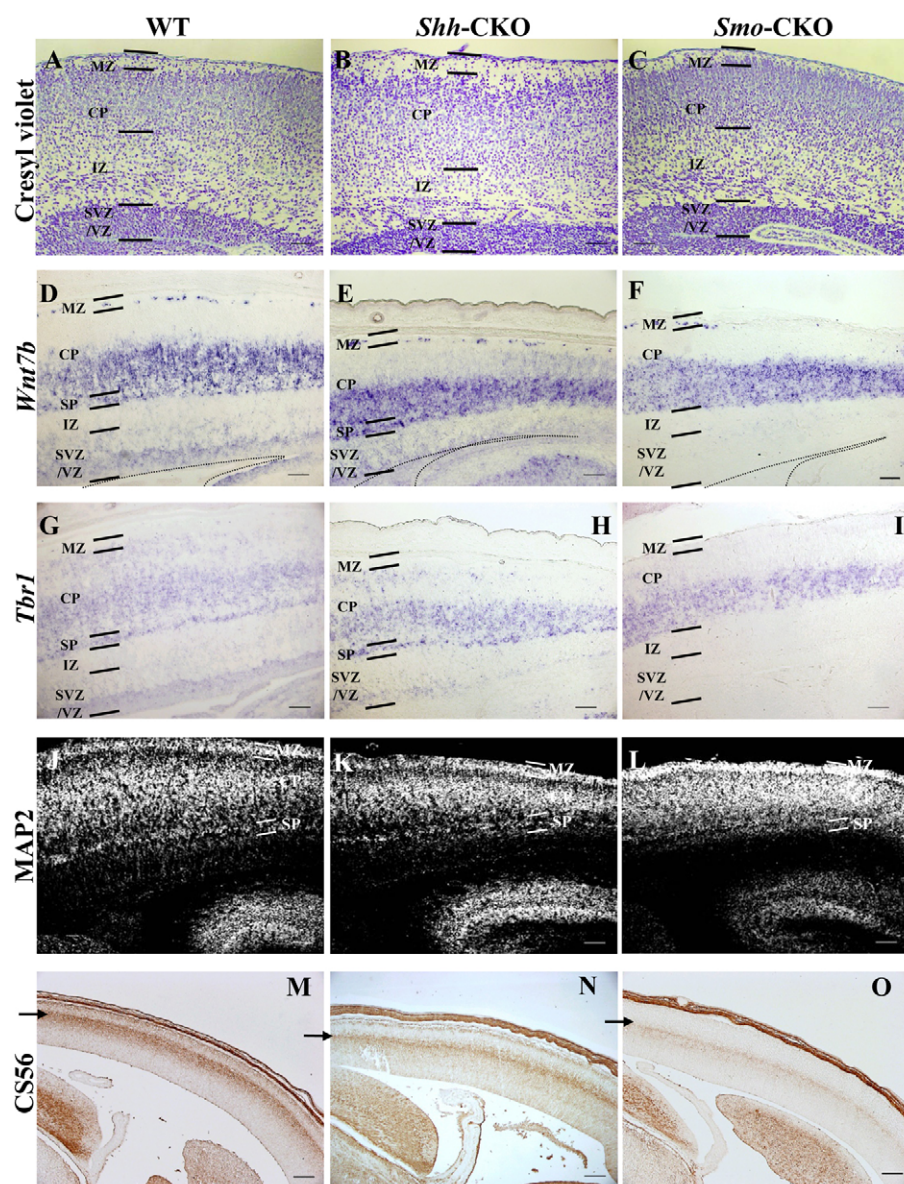
Fig. 5. Shh signaling was required for formation of the deep cortical layer and subplate. Cresyl violet staining of the E18.5 dorsal pallium (**A-C**). In situ hybridization on parasagittal sections of the E18.5 dorsal pallium with *Wnt7b* (**D-F**) and *Tbr1* probes (**G-I**). In wild type, *Wnt7b* and *Tbr1* were intensely expressed in the deep cortical layer and SP, and weakly expressed in SVZ/VZ, but not in IZ (D,G). In *Shh*-CKO, the unstained gap of *Wnt7b* expression was not observed (E). *Tbr1* expression was comparable with wild type (H). In *Smo*-CKO, *Wnt7b* expression in SP and SVZ/VZ was undetectable (F). *Tbr1* expression in SP and SVZ/VZ was not detected either (I). MAP2 and chondroitin sulfate were detected by immunohistochemistry. In wild type and *Shh*-CKO, MAP2 (**J-L**) and chondroitin sulfate (CS56) (**M-O**) expression in SP neurons was detectable at E18.5 and E15.5, respectively (J,K; arrow in M, N). In *Smo*-CKO, MAP2 and Chondroitin sulfate were weakly expressed in SP neurons at E18.5 (L) and E15.5 (arrow in O), respectively. Scale bar: 100 μm.

transfected side, SVZ/VZ where *Wnt7b* and *Tbr1* were not expressed, were greatly expanded (Fig. 6D-E′). TBR2-positive basal progenitors were increased and some of them were scattered distantly from the ventricular surface (Fig. 6F′, arrows). These results demonstrated that CP-derived Shh can accelerate proliferation of neural progenitor/stem cells in SVZ/VZ and neuronal differentiation.

Shh immunoreactivity is weakly detected in both the proliferative zones and postmitotic neurons in the dorsal pallium

The above phenotypes in *Shh*- and *Smo*-CKOs demonstrated that Shh signaling plays physiological roles in the developing dorsal telencephalon. To examine Shh localization in detail, immunohistochemistry was performed. At E13.5, Shh was detected in almost all layers of the dorsal pallium, including VZ, SVZ, CP and MZ (Fig. 7A). The nuclei were not stained with anti-Shh antibody; consistent with this, Shh was in the cytoplasm and extracellular space. Although Shh was still detected in all layers at

E15.5, the MZ and intermediate zone (IZ) were more abundantly stained than the other layers (Fig. 7B). To confirm that this abundantly stained region coincides with IZ, we performed double immunostaining with anti-Shh and anti-N-cadherin antibodies. N-cadherin is intensely expressed in IZ (Ajioka and Nakajima, 2005) (Fig. 7C). The Shh staining overlapped with N-cadherin in IZ at E15.5 (Fig. 7C-E). As a whole, the intensity of Shh immunoreactivity in the dorsal pallium was much lower than that in other regions such as the floor plate of the telencephalon at E13.5 and E10.5 (arrow in Fig. S1A,C in the supplementary material) The intense specific staining was also observed in the floor plate and notochord at E8.5 (data not shown).

To reveal which neuron cell types are positive for Shh in MZ and IZ, we performed double immunostaining using anti-Shh antibody and cell-type specific markers. One of the main populations of MZ is CR neurons, the first-born neurons that invade tangentially into MZ from the extra-cortical origins (Garcia-Moreno et al., 2007). GABAergic interneurons have been shown to migrate tangentially from MGE into the presumptive neocortex (Wonders and Anderson,
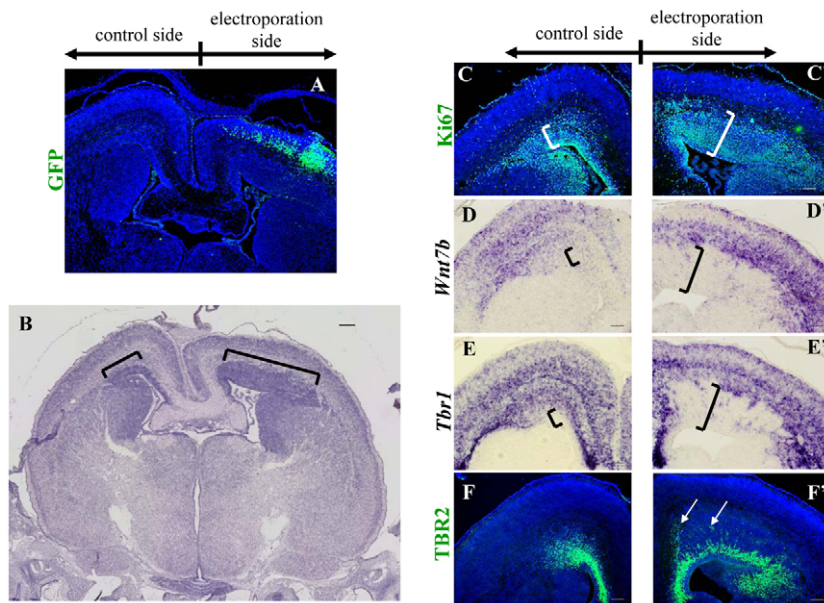
Fig. 6. Overexpression of Shh in utero leads to expansion of CP and SVZ/VZ. pCIG-Shh expression vector was electroporated into the dorsal pallium in utero at E12.5. At E18.5, EGFP was detected mostly in CP on the electroporated side (**A**). Note that the dorsal pallium was expanded in a planar direction on the electroporated side (brackets in **B**). Ki67-positive proliferating cells in SVZ/VZ were greatly increased so that SVZ/VZ was almost twice the thickness as on the control side (bracket in **C,C′**). In situ hybridization on coronal sections of E18.5 dorsal pallium with *Wnt7b* (**D,D′**) and *Tbr1* probes (**E,E′**). On the electroporated side, the non-expression regions in SVZ/VZ were expanded compared with the control side (bracket in D-E′). TBR2-positive progenitors were greatly increased in the SVZ on the electroporated side (**F,F′**) and their distribution was disturbed (arrows in F′). Scale bar: 100 μm in B; 50 μm in C-F′.

2006). The distribution of Shh-positive cells suggested that these cells include CR neurons and GABAergic interneurons. CR neurons express reelin, a large secreted protein that is associated with extracellular matrix (Ogawa et al., 1995; Schiffmann et al., 1997), and calretinin, a Ca^{2+}-binding protein (Alcantara et al., 1998; del Rio et al., 1995). At E13.5, we performed double-immunostaining with anti-Shh and anti-reelin or anti-calretinin antibodies on parasagittal sections of the embryonic brains. Several Shh-immunoreactive cells in MZ were positive for reelin (Fig. 7F, see Fig. S5A-A″ in the supplementary material) and calretinin (Fig. 7G, see Fig. S5B-B″ in the supplementary material), suggesting that some of the Shh-positive cells in MZ are CR neurons.

GABAergic interneurons are observed in the dorsal pallium at E12-E13 in the primitive plexiform layer. During the following embryonic stages (E14-E19), most GABAergic interneurons are present in MZ, subplate (SP), IZ and SVZ (Del Rio et al., 1992). At E15.5, some of the GABA-positive cells were immunopositive for Shh in MZ of the dorsal pallium (Fig. 7H; see Fig. S5C-C″ in the supplementary material). These results indicate that Shh is produced by GABAergic interneurons. The rest of the Shh-positive cells were probably projection neurons in CP and neural progenitor/stem cells in SVZ/VZ.

In *Shh*-CKO, Shh was removed from most of the cells, but not completely abolished in the dorsal pallium at E13.5 (Fig. 7A′; see Fig. 1SB,B′ in the supplementary material). In the ventral regions of the telencephalon including the floor plate, Shh immunoreactivity was not affected in the mutants (arrow, Fig. 1SB in the supplementary material). Therefore, conditional deletion of *Shh* was specific to the dorsal telencephalon. At E15.5, Shh was decreased in CP and SVZ/VZ, while abundantly observed in IZ of *Shh*-CKO embryos (Fig. 7B′). These Shh-positive cells in IZ are most probably interneurons derived from MGE because Shh immunoreactivity was not decreased in the ventral telencephalon of *Shh*-CKO embryos (see Fig. S1B in the supplementary material). Importantly, GABAergic interneurons, which were derived from MGE, were *lacZ* negative when the $Emx1^{Cre}$ mice were crossed with ROSA26 reporter mice (Iwasato et al., 2004).

DISCUSSION
Shh signaling regulates cell cycle kinetics of neural progenitor/stem cells
The involvement of Shh signaling for progenitor/stem cell proliferation has been demonstrated by in vitro assays (Palma et al., 2005; Palma and Ruiz i Altaba, 2004) and by analyses of the postnatal forebrain (Machold et al., 2003). Although Hh signaling has been proposed to be crucial for development of the neocortex (Ruiz i Altaba et al., 2002), this hypothesis has not been tested in vivo yet.

Our data suggest that Shh signaling regulates the cell cycle length and exit in the embryonic dorsal pallium (Fig. 3). Shh affects various checkpoints of the cell cycle by controlling activities of cyclins, cyclin-dependent kinases, etc. (Ruiz i Altaba et al., 2002). Because neuronal differentiation is closely linked to the cell cycle exit, the timing of differentiation also seemed to be regulated by Shh. In the amphibian and fish developing retina, Hh signaling speeds up the cell cycle by reducing the length of G1 and G2 phases. This leads to transition from slow cycling stem cells to fast cycling progenitors that are closer to cell cycle exit and differentiation. The cell cycle exit itself is also promoted by Hh signaling (Agathocleous et al., 2007; Locker et al., 2006). These previous reports are consistent with our results that proliferation of neural stem cells/basal progenitors and neurogenesis are decreased in our mutants (Figs 2, 3; see Figs S3, S4 in the supplementary material). In addition, the number of TBR2-positive basal progenitors was reduced in both mutants (Fig. S4 in the supplementary material), which is consistent with the fact that Shh signaling induces the transition from neural stem cells to basal progenitors. However, we cannot exclude the possibility that decreased basal progenitors and reduced neurogenesis are secondary effects of decreased proliferation and/or increased cell death.

It has also been reported that de-regulated Hh signaling in the dorsal pallium disturbed proliferation, differentiation and survival (Giros et al., 2007; Lien et al., 2006). The cortical thickness was decreased in *Nestin-Cre; Shh* mice (Xu et al., 2005). These reports are consistent with our results.
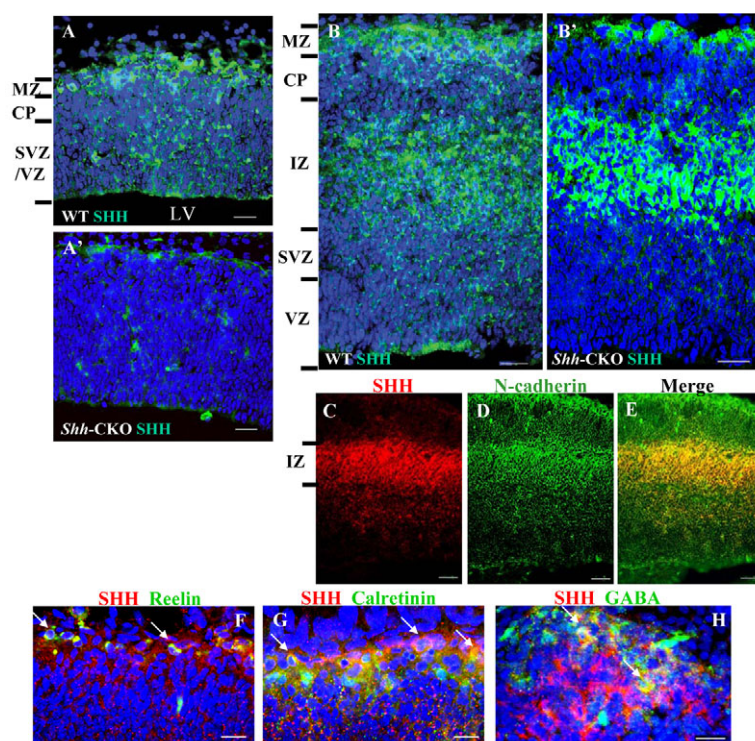
Fig. 7. Localization of Shh protein in the developing dorsal pallium. Shh protein was localized in MZ and SVZ/VZ of the wild-type dorsal pallium at E13.5 (**A**); there are few Shh-positive cells in *Shh*-CKO mutants (**A′**). At E15.5, Shh immunoreactivity was also found additionally in IZ and CP (**B**). Note that MZ and IZ were more intensely stained than other zones at E15.5. In *Shh*-CKO mutants, the Shh-immunoreactivity was sustained in IZ and MZ (**B′**). N-cadherin was intensely expressed in IZ (**D**), and Shh protein (**C**) was colocalized with N-cadherin in IZ (**E**). Reelin and calretinin, CR cell markers, were detected in some of Shh-positive cells in MZ at E13.5 (**F**,**G**, arrows). Shh protein was co-expressed in GABAergic interneurons in MZ at E15.5 (**H**, arrows). Sections were counterstained with DAPI (dark blue nuclei). The antibodies and colors used in each panel are as indicated. Scale bar: 100 µm in A-E; 50 µm in F,G.

The timing of differentiation, characters of neural progenitor/stem cells and patterning of CP are regulated by Shh signaling in the dorsal pallium

It has been reported that the timing of differentiation is closely related to layer identities. However, the heterochronic transplantation experiments demonstrated that early-born neurons (i.e. layers IV) could change their identity to that of late-born neurons (i.e. layers II/III) when they were transplanted into an older host (Desai and McConnell, 2000).

In *Smo*-CKO embryos, the distribution of cortical neurons was impaired in the dorsal pallium at E18.5 (Fig. 4), implying differentiation defects. Although E13.5-born neurons resided in the more superficial layer in *Smo*-CKO CP, they did not seem to express the deep layer markers (Fig. 5). This result implies that E13.5-born neurons in the superficial layer changed their identity, which is consistent with the previous reports mentioned above.

Abnormal positioning of neurons in the mutants also implies migration defects. Loss of Shh signaling might lead to abnormal migrating behavior of young projection neurons in the mutants. Alternatively, Shh signaling may regulate the property of processes of radial glias. In *Smo*-CKO embryos, radially migrating neurons might not be able to hold the processes of neural progenitor/stem cells as appropriate scaffolds. Another possibility is that timing of migration might be retarded. In addition, selective cell death cannot be excluded as cell death was significantly increased in the CKOs (Fig. 2E-H). In either case, cell behaviors should be examined by time lapse video-microscopy and/or other methods in future studies.

Characters of neural progenitor/stem cells and patterning of CP are maintained by Shh signaling in the dorsal pallium

In this study, we showed that *Wnt7b* was undetectable in neural progenitor/stem cells of *Smo*-CKO in SVZ/VZ at E18.5 (Fig. 5). This result indicates that characters of neural progenitor/stem cells may be changed in SVZ/VZ of the dorsal pallium in this mutant. It

is possible that characters of neural progenitor/stem cells were changed by disturbance of cell cycle length and timing of cell cycle exit.

Tbr1 and *Wnt7b* were expressed in SP of wild-type and *Shh*-CKO embryos (Fig. 5D,E,G,H). However, in *Smo*-CKO embryos, they were undetectable in SP (Fig. 5F,I). MAP2 and chondroitin sulfate expression was decreased in SP neurons (Fig. 5L,O). The SP, MZ and CP are formed by splitting of the preplate at E13.5 (Allendoerfer and Shatz, 1994). Our data indicate that SP split from the preplate but was underdeveloped, and that SP neurons did not express *Tbr1* and *Wnt7b* in the absence of Shh signaling. Perlecan is a proteoglycan that is localized in the basal lamina of the neuroepithelium during development, and is required to spread and concentrate Shh in the developing brain (Giros et al., 2007). In perlecan-null mutants, SP did not segregate from CP, suggesting roles of Shh signaling in SP formation. Therefore, our results suggest that Shh signaling is required to form the mature SP and induce *Tbr1* expression in the early-born neurons of SP.

Shh immunoreactivity shows spatially and temporally distinct patterns in the developing dorsal pallium

Recently, *Shh* mRNA expression in the dorsal pallium was demonstrated by RT-PCR and the effects of Shh on neural stem/progenitor cells from the dorsal pallium were analyzed using an in vitro assay (Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004). Accumulating data suggest that endogenous Shh signaling plays important roles in the neocortical development (Giros et al., 2007; Lien et al., 2006; Xu et al., 2005). However, precise localization of Shh in the dorsal pallium has not yet been revealed in vivo.

In this study, we clarified the Shh protein-positive region in the dorsal pallium by immunohistochemistry (Fig. 7). Shh was mainly localized in MZ, IZ and SVZ/VZ of the dorsal pallium from E13.5 (Fig. 7A). Our data revealed that the Shh-positive cells were neural

progenitor/stem cells and CR neurons at early neurogenesis stages. At later neurogenesis stages, Shh was also detected in postmitotic neurons in CP (projection neurons) and in GABAergic interneurons in IZ (Fig. 7F-H; see Fig. S5 in the supplementary material). In the developing cerebellum, proliferation of granule cell progenitors is maintained by transient autocrine and paracrine Shh and then by Shh from Purkinje neurons. Purkinje neurons secrete Shh protein and play a central role in development of granule neurons (Dahmane and Ruiz i Altaba, 1999). Our data imply that similar molecular mechanisms exist in the developing cerebral neocortex. Most probably neural progenitor/stem cells and CR neurons in the dorsal pallium secrete Shh protein to maintain the stem cell niche by autocrine and/or paracrine mechanisms during early neurogenesis. During later neurogenesis, in addition to neural progenitor/stem and CR neurons, postmitotic neurons (including projection neurons and GABAergic interneurons) secrete Shh protein, which may also regulate cell cycle kinetics.

As there were few interneurons that produce Shh at E13.5 (Fig. 7A,A′), *Shh*-CKO showed the defects of proliferation and positioning at E13.5. By contrast, at E15.5, these phenotypes were rescued in *Shh*-CKO most probably by Shh secreted from many interneurons (Fig. 7B′), which were originated from MGE. This is consistent with the work of Xu et al. (Xu et al., 2005).

Interestingly, the expression level of Shh in the dorsal pallium is extremely low as mentioned above. There was in vitro evidence that 'high level' Shh instead suppresses cell proliferation, when compared with relatively 'low level' Shh. Palma and Ruiz i Altaba (Palma and Ruiz i Altaba, 2004) have reported that proliferation of neocortical precursors showed a Shh concentration-dependent increase between 1 and 5 nM in neurosphere assay. Very intriguingly, the proliferation was significantly decreased with higher concentration of Shh (25 nM), supporting our observation.

In addition, Shh is obviously required for cell survival because cell death was significantly increased in the absence of Shh signaling (Fig. 2). The hypomorphic phenotype of the dorsal telencephalon is partly caused by increased cell death.

Concluding remarks

The previous studies have reported that *Shh* mutations are found in human telencephalic anomalies, including holoprocencephaly (HPE) (Wallis and Muenke, 2000). This is consistent with the HPE phenotype of *Shh* KO mice (Chiang et al., 1996). It is also possible that abnormal Shh signaling in the dorsal telencephalon leads to macrencephaly and microcephaly (Lien et al., 2006; Xu et al., 2005).

As neuronal differentiation is closely linked to the cell cycle exit during neurogenesis, the regulation of cell cycle kinetics is very important for proper corticogenesis. Taken together, Shh signaling might be important for the maintenance of stem cell niches in the embryonic dorsal telencephalon and allow an appropriate probability of neuronal differentiation to form the precise layered structure. In conclusion, our findings suggest that 'low level' Shh signaling plays an important role in fine-tuning of cell cycle kinetics to form the proper neocortex.

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