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Smoothened controls cyclinD2 expression and regulates the generation of intermediate progenitors in the developing cortex

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HIGHLIGHTS

- Impaired positioning of neurons in the superficial layer was observed in the Smo conditional knockout mice (Smo-CKO mice).
- The number of IPCs decreased in the neocortex of the Smo-CKO mice.
- Expression of cyclin D2 was suppressed in the Smo-CKO mice.
- The development of dopaminergic projections and the GFAP expression of astrocytes were affected in the Smo-CKO mice.

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ABSTRACT

Translocation of the Smoothened to the cell membrane is critical for sonic hedgehog activity. However, the biological importance of Smoothened itself has not been fully studied. To address this issue, we disabled Smoothened specifically in the dorsal telencephalon. Birth-date analysis and layer marker expression patterns revealed the slightly impaired development of the superficial layer neurons in the embryos of Emx1-Cre; Smoothened$^{fl/-}$ conditional knockout mice. Further analysis of the mutant embryos revealed a decrease in the number of intermediate progenitor cells. In the knockout mice, the expression of cyclin D2, but not cyclin D1 or cyclin E, was reduced in the dorsal telencephalon. In addition, the projections of dopaminergic neurons were affected during development, and the number of activated astrocytes was increased in the neocortex of the mutant mice. Our data suggest that Smoothened signaling, acting through cyclin D2, is critical for the proper development and maturation of the neocortex.

1. Introduction

Sonic hedgehog (Shh) is a secreted protein that acts as a morphogen and plays a pivotal role in the ventral patterning, proliferation, differentiation and survival of neuronal progenitors by controlling the expression of transcription factors in downstream signaling [1,3,4,23–25]. Indeed, when the Shh gene is knocked out, the ventral structures of the telencephalon are severely affected [3]. Shh signaling is also involved in the development of the dorsal part of the telencephalon: cortical thickness is reduced due to an increase in apoptosis and a decrease in the number of neurons in the dorsal telencephalon of the neural stem cell-specific Shh conditional knockout mice, whereas the neocortex becomes hyperplastic when Shh signaling is promoted [9,17,19,26,27]. These data indicate that Shh signaling regulates the growth and development of the cortex by controlling the proliferation, differentiation and survival of neural stem cells. More recently, the regulation of the development of the intermediate progenitor cells (IPCs) of the cortex via Shh activity has attracted attention because IPCs are important for brain evolution: IPCs give birth to the majority of layer II/III neurons, and the number of layer II/III neurons increases as the brain evolves [5,8,13,21].

The sonic hedgehog receptor Patched negatively regulates the 7-transmembrane receptor Smoothened (Smo) by inhibiting its translocation to the membrane in the resting state [25]. The binding of Shh to Patched relieves the inhibition of Smo, leading to Smo localization to the membrane, thereby stimulating Gli family of transcription factors [25]. Although the importance of Shh has been intensively investigated, the roles of the intervening molecules in

Abbreviations: Shh, Sonic hedgehog; Smo, Smoothened; IPCs, intermediate progenitor cells; GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase.

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the Shh signaling pathway, such as Smo, have not been fully studied in the brain [18].

In this study, we investigated the importance of Smo for cortical development using Emx1-Cre; $Smo^{fl/-}$ conditional knockout mice (Smo-CKO mice). Emx1, a transcriptional factor, is specifically expressed in the dorsal telencephalon from the embryonic stage to adulthood, and Smo expression was deleted from embryonic day (E) 10.5 in the dorsal telencephalon of the conditional knockout embryos [15].

2. Materials and methods

2.1. Generation of floxed Smo mice

$Emx1^{cre}$ mice were crossed with Smo heterozygous mutants to generate $Emx1^{cre}$; $Smo^{+/-}$ males. These mice were mated with $Smo^{f/f}$ (The Jackson Laboratory) [18] females to generate conditional knockout mice of the informative genotypes $Emx1^{cre}$; $Smo^{f/-}$ (Smo-CKO). The $Emx1^{cre}$ allele induces recombination in neural progenitor/stem cells and neurons of the dorsal telencephalon by E10 [15]. All experiments were conducted in accordance with the guidelines for animal experiments of the University of Fukui.

2.2. Antibodies

The following antibodies were used: anti-Cux1 (sc-13024; Santa Cruz), anti-Tbr1 (Millipore), anti-BrdU (chlorodeoxyuridine; CldU) (BU1/75; Oxford Biotechnology), anti-BrdU (iododeoxyuridine; IdU) (B44; Becton Dickinson), anti-Tbr2 (Abcam), anti-cyclin E (RB-012, Thermo), anti-cyclin D1 (RB-9041; Thermo), anti-cyclin D2 (sc-593, Santa Cruz) (MS-221; Thermo), anti-TH (Millipore), anti-GFAP (Dako), anti-S100 (B32.1; Abcam), anti-PCNA (sc-56; Santa Cruz), anti-β-tubulin HRP-DirecT and anti-GAPDH HRP-DirecT (MBL).

2.3. Immunohistochemistry and immunoblotting

Embryos were fixed for 3 h in periodate lysine paraformaldehyde at 4 °C. Certain sections (10 μm) were processed for hematoxylin-eosin staining. The immunohistochemistry was performed as described previously [17]. Three embryos from one dam were used in each immunostaining analysis.

For immunoblotting, cortices were dissected from E16.5 mice and homogenized with RIPA buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a conventional method. Immunoblotting was visualized using an Immobilon Western Chemiluminescent HRP Substrate (Millipore) on a LAS-3000 mini imaging system (Fujifilm).

2.4. CldU and IdU incorporation and birth-date analysis

For in vivo labeling of S-phase cells (thymidine analogue incorporation), a single intraperitoneal injection of CldU (105478; MP Biomedicals Inc.) was performed at 1 h prior to sacrificing at E13.5 or E16.5. For birth-date analysis, timed-mated pregnant females received an intraperitoneal injection of CldU at E13.5 and IdU (I7125; Sigma) at E16.5. Pups were allowed to develop to P14 and were processed for immunohistochemistry.

2.5. Cell cycle kinetics

To estimate the cell cycle length, we counted the number of IPCs, defined as Tbr2-positive cells, labeled by a 1 h pulse of CldU. The fraction of CldU/Tbr2 double-positive cells among all Tbr2-positive cells provided a rough estimate of the IPC cell cycle length. A smaller population of CldU-positive cells among the Tbr2-positive cells was indicative of a greater cell cycle length [2].

2.6. Quantification of cell numbers

Immunofluorescent staining in selected areas of the dorsal telencephalic region of the brain was quantified by manually counting the total number of cells (Hoechst 33342-stained) and the number of cells stained with each antibody in two anatomically matched sections from each embryo (three control and three mutant embryos) using Adobe Photoshop CS4 (Adobe). We performed the counting and quantitative procedures according to a previously reported method [17]. Identical areas in the dorsal telencephalic regions of the brain were selected from the control and mutant embryos, and the total number of pixels and the number of pixels showing immune-positive reactions were manually counted in 100 μm-wide (for E16.5 and E18.5) or in 200 μm-wide (for the adult) samples (examples are indicated by open boxes in Fig. 3A–C′). Two sections were chosen from each animal. Three control and three mutant embryos were used.

3. Results

3.1. Neuronal positioning is impaired in the neocortex of the Smo CKO mice

Despite increasing evidence on the roles of Shh, the importance of Smo in the development of the dorsal telencephalon, especially during the postnatal period, has not been fully investigated. Here, we histologically examined the structure of the neocortex of postnatal 14-day-old (P14) Smo-CKO mice. Compared with the wild type littermates, the neocortex of the Smo-CKO mouse was thinner and the cells in layer II/III were less abundant (Fig. 1A and A′), which is consistent with our previous observation [17]. We also showed that cells born at approximately E15.5 are accumulated deep in the cortex of the Smo-CKO mice at E18.5, whereas the majority of cells born at E15.5 are located in the superficial layers of the cortex in the wild type. It is possible that such mal-positioning of later born neurons in the deep layers can be attributed to the poor development of the superficial layers. To assess this possibility and examine the roles of Smo in final cell positioning, we consecutively injected CldU at E13.5 and IdU at E16.5 and observed the distribution of the CldU- or IdU-labeled cells at P14. Unlike the positioning pattern at E18.5 [17], the IdU-labeled cells in the Smo-CKO mice were more superficially situated relative to the location in the wild type at P14 (Fig. 1 B-C). Therefore, it is likely that later born neurons were slow to start radial migration and/or migrated slowly, but they finally moved up to form the superficial layers. Subsequently, we examined whether the layer identity was altered in the Smo-CKO mice using Cux1 and Tbr1 immunostaining, which are markers for neurons of layer II/III and layer V/VI. Overall, no obvious difference was observed between the wild type and the Smo-CKO mice except for the accumulation of Tbr1-positive cells in the deepest bin (future VIb) of the Smo-CKO mice (Fig. 1D-E).

3.2. Smo regulates the number and the localization of intermediate progenitor cells in the dorsal telencephalon

Recently, it has been shown that some layer II/III cells are generated from the IPCs [20]. It is possible that fewer IPCs results in poor layer II/III organization. Indeed, it has been reported that the number of IPCs, which were identified based on Tbr2 expression [7], decreased at E18.5 in the Smo-CKO embryos. To further support this observation, we sought to determine whether the number of Tbr2-positive cells in the cortex changed in the earlier developmental stages. The number and proportion of Tbr2-positive cells decreased

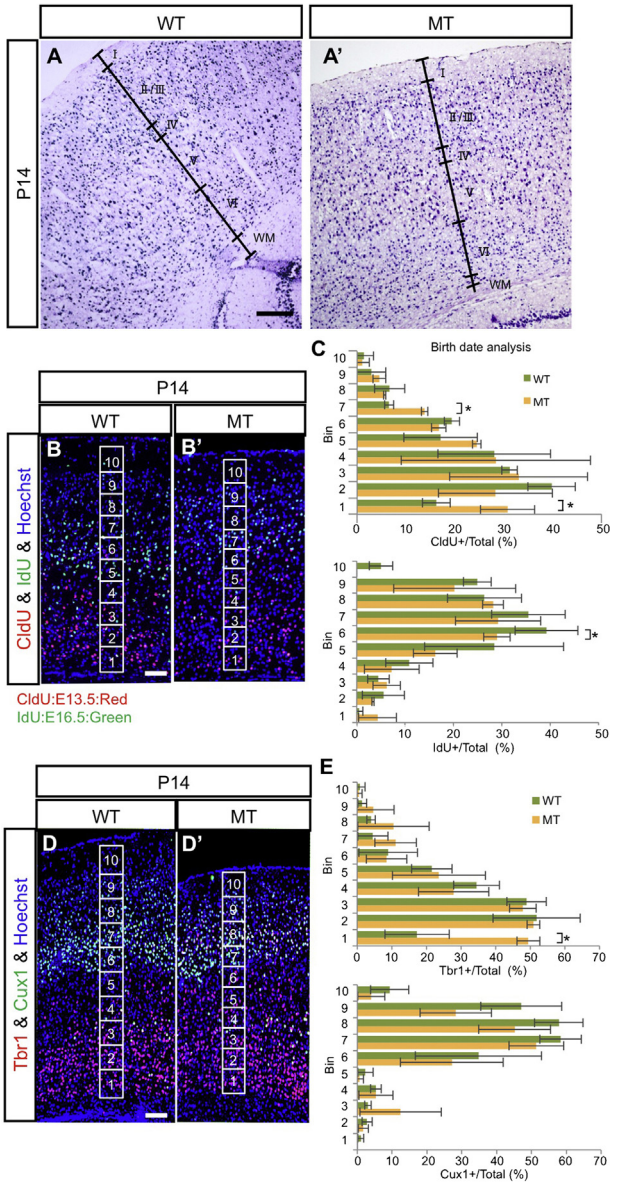

Fig. 1. Neuronal positioning in the neocortex was impaired in the Smo CKO mice. Coronal sections of wild-type littermate (WT) and the Smo CKO (MT) mouse neocortices at P14 were stained with hematoxylin-eosin (A and A′). Birth-date analysis was performed using CldU and IdU double-staining of coronal sections of P14 neocortex after a single pulse injection of CldU at E13.5 and IdU at E16.5 (B and B′). Cortices were divided into 10 bins, and the ratios of the number of CldU- and IdU-positive cells to the total number of cells, visualized by Hoechst-staining, in each bin were calculated (C). In the Smo CKO mice, E13.5-born neurons were increased in bin1 ($n=3$, $30.8\pm5.53\%$, $P=0.0154$) and decreased in bin7 ($n=3$, $13.9\pm0.67\%$, $P=0.0118$) compared with the WT mice ($n=3$, bin1: $16.2\pm2.85\%$, $n=3$, bin7: $6.56\pm0.92\%$). E16.5-born neurons were less abundant in bin6 ($n=3$, $29.0\pm2.70\%$, $P=0.0245$) compared with the WT mice (bin6 $n=3$, $39.2\pm6.52\%$). The analysis using layer specific markers Tbr1 for the deep layers (layers V and VI) and Cux1 for the upper layers (layers II and III) was performed on P14 neocortices (D and D′). Ratios of the number of Tbr1- or Cux1-positive cells to the total number of cells in the each bin were quantified (E). In the Smo CKO mice, Tbr1-positive neurons were significantly more abundant in bin1 ($n=3$, $49.4\pm3.33\%$, $P=0.004$) compared with the WT mice ($n=3$, $17.3\pm9.3\%$). WM, white matter. Scale bar, 100 μm. Values are means ± SD, $^*P<0.05$ (Student's *t*-test).

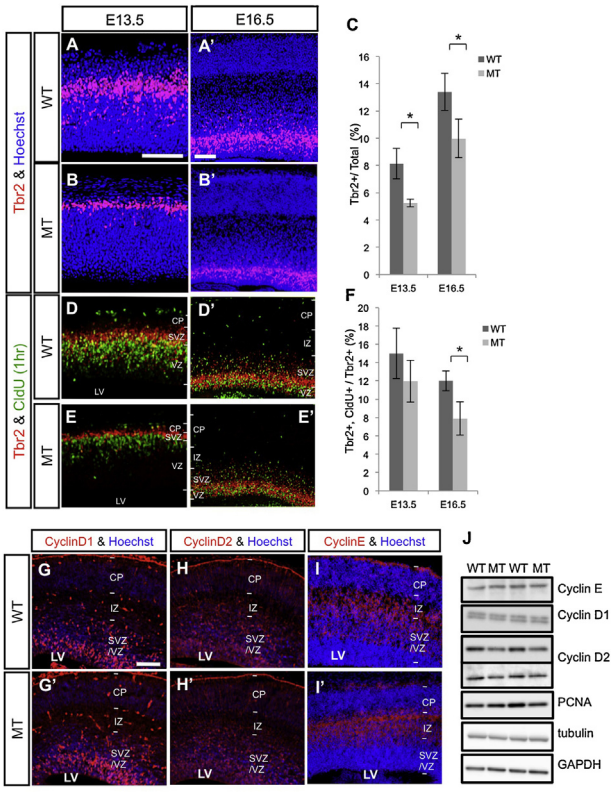

Fig. 2. The number of IPCs decreased and the expression of cyclin D2 was down-regulated in the cortex of the Smo CKO. Coronal sections of E13.5 and E16.5 cortices were immunostained with anti-Tbr2 antibody, a marker for IPCs (A-B′). (C) At E13.5 and E16.5, the number of IPCs was reduced in the Smo CKO mice (E13.5; $n=3$, $5.25\pm0.28\%$, $P=0.0012$, E16.5; $n=3$, $9.98\pm1.4\%$, $P=0.039$) compared with the WT mice (E13.5; $n=3$, $8.12\pm1.12\%$, E16.5; $n=3$, $13.4\pm1.36\%$). After 1 h of pulse labeling with CldU, immunostaining of parasagittal sections was performed with anti-Tbr2 (red) and anti-CldU (green) antibodies at E13.5 and E16.5 (D-F′). If all IPCs are not in the resting phase of cell cycle, the cell cycle length for IPCs can be estimated as the percentage of all Tbr2-positive cells that are double-labeled for Tbr2 and CldU. A smaller percentage of double-labeled cells indicates a longer cell cycle. In the Smo CKO embryos, the IPC cell cycle length was not affected at E13.5 ($n=3$, $12.0\pm2.23\%$, WT; $n=3$, $15.0\pm2.75\%$), whereas the IPC cell cycle length was significantly increased at E16.5 ($n=3$, $7.90\pm1.81\%$, $P=0.0281$) compared with WT embryos ($n=3$, $12.0\pm1.08\%$). Coronal sections of E16.5 cortices were immunostained with anti-cyclin D1, cyclin D2 or cyclin E antibodies (G–I′). Immunoblotting was used to quantify the expression of these cyclins in E16.5 cortices (J). The expression of cyclin D2 was down-regulated in the Smo CKO (MT). Upper row: visualized using mouse anti-cyclin D2 (MS-221; Thermo); lower row: visualized using rabbit anti-cyclin D2 (sc-593; Santa Cruz) antibodies. The expression of cyclin D1 and cyclin E was not different between the CKO and WT embryos. PCNA was used to identify the total fraction of proliferating cells. Tubulin and GAPDH were used as loading controls. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; LV, lateral ventricle. Scale bar, 100 μm. Values are means ± SD, $^*P<0.05$ (Student's *t*-test).

in the cortex of E13.5 and E16.5 Smo-CKO embryos (Fig. 2A–C). In addition, the Tbr2-positive cells were less widely distributed in the cortex of the Smo-CKO embryos compared with the wild type (Fig. 2A–B′). We then asked how the number of Tbr2-positive cells decreased in the absence of Shh signaling. We injected the CldU, and mice were sacrificed at 1 h post-injection. The proportion of cells that were positive for both Tbr2 and CldU, which are the IPCs that had exited S phase within 1 h, decreased at E16.5 in the Smo-CKO embryos (Fig. 2D–F).

3.3. *Cyclin D2 is down-regulated in the cortex of the Smo CKO embryo*

We subsequently explored the mechanisms underlying the decreased IPC generation. Cyclin D1, D2 and E comprise part of the Smo signaling pathway and are regulated by Gli transcription factors in many biological systems [25]; therefore, we studied the expression of cyclin D1, D2 and cyclin E in the developing neocortex of the mutant embryos. Surprisingly, the expression of cyclin

D2 was suppressed, whereas that of cyclin D1 and cyclin E was not noticeably suppressed (Fig. 2G–J).

3.4. The development of dopaminergic projections and the GFAP expression of astrocytes are affected in the Smo CKO mice

Finally, we attempted to determine whether the malformation observed in the cortex could alter other brain structures. It has been shown that during development, dopaminergic fibers originating in the brainstem tangentially enter the cortex in the middle of the cortical formation at the same time that neurons migrate radially from the ventricular zone and tangentially across the cortex to build up the neocortex [22]. Therefore, we assumed that the distribution of dopaminergic fibers is altered when the structure of the cortex is altered by impaired cell positioning. Dopaminergic fibers, visualized by tyrosine hydroxylase staining [16], were rare in the Smo-CKO mice compared with the control mice (Fig. 3A–B′ and D). However, the depletion of Smo may only affect the rate of maturation of these dopaminergic projections because the in later stages of development, no apparent difference in the distribution of tyrosine hydroxylase stained neurites was observed (Fig. 3C-D). Additionally, we investigated the influence of Smo on the astrocytes with our Smo-CKO mice based on the significance of astrocytes in the brain as well as the roles of Smo for the astrocytes demonstrated by glial fibrillary acidic protein (GFAP)-specific Smo conditional knockout mice [10]. We found an enhanced expression of GFAP in the cortex of the Smo-CKO mice, whereas no significant difference in the number or distribution of the astrocytes, revealed by S100, was observed, suggesting that astrocytes are activated in our Smo-CKO mice (Fig. 3E–G).

4. Discussion

Smo is a well-known downstream molecule of Shh signaling. However, based on our own observations and previously published data, there are differences in how Shh and Smo affect the development of the neocortex. Of particular note are differences in the positioning of postmitotic neurons in Shh and Smo conditional knockout mice [17]. It is possible that Shh secreted from the ventral area rescues the positioning of postmitotic neurons in the dorsal forebrain of the dorsal cortex-specific Shh conditional knockout mice.

The impaired development of the superficial layer neurons is consistent with our demonstration that the production of IPCs was impaired. The involvement of Shh signaling in the generation of IPCs has been intensively examined using Patched homolog 1 mutant mice [26]. Our observation supports the concept that Shh signaling cascades are critical for the development of IPCs. It has been shown that cyclin D1, D2 and E are major targets of Shh signaling through Gli transcription factors [25]. However, we observed that only the expression of cyclin D2 was suppressed in the absence of Smo in the developing cortex, suggesting that other cyclins are not similarly regulated through Smo in the cortex. Interestingly, IPC development is also impaired in cyclin D2 knockout mice but not cyclin D1 knockout mice [11]. The D2 knockout mice exhibited a thinner cortex, sparsely distributed neurons and a reduction in the number of Tbr2-positive IPCs, probably due to the impaired duration of the cell cycle. The phenotypes observed in the cortex of the D2 knockout mouse coincide well with those of our Smo-CKO mice. These observations strengthen our assertion that the impairment of IPCs in the Smo-CKO mice occurs due to insufficient cyclin D2 activity.

Recently, it has been demonstrated that Shh signaling is involved in the cortical microcircuit formation of corticofugal projection neurons [14]. We observed that dopaminergic projections

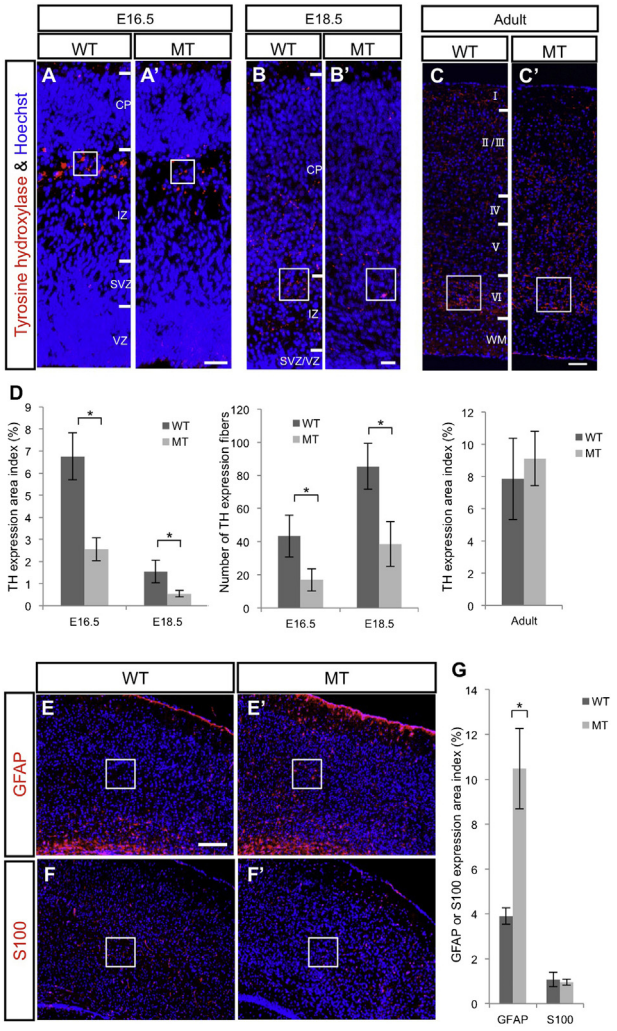

Fig. 3. The development of dopaminergic projections and the GFAP expression of astrocytes were affected in the Smo CKO mice. Coronal sections of E16.5, E18.5 and adult (P100) cortices were immunostained with anti-TH. The majority of TH-positive structures were located immediately below the cortical plate (A–C′). (D) The ratios of the areas of TH-positive structures to their total areas are shown as the TH expression area index. At both E16.5 and E18.5, TH-positive projections were decreased in the Smo CKO (E16.5: $n=3$, $2.55\pm0.5\%$, $P=0.0108$, E18.5: $n=3$, $0.54\pm0.14\%$, $P=0.0311$) compared with the WT embryos (E16.5: $n=3$, $6.76\pm1.56\%$, E18.5: $n=3$, $1.55\pm0.51\%$). In the adult cortex, no significant difference in the TH-positive fibers was observed. Coronal sections from P14 cerebral cortices were immunostained with anti-GFAP or anti-S100 antibody (E–F′). GFAP-positive cells decreased in the Smo CKO ($3.90\pm0.36\%$) compared with WT embryos ($10.47\pm1.78\%$) (E, E, and G), whereas the distribution of S100-positive cells in the cortex was not different between the CKO and the WT mice (F-G). Scale bars: 100 μm (C and C′), 200 μm (A–B′, E–F′). Values are means ± SD, $^*P<0.05$ (Student's *t*-test).

are developmentally compromised, which provides an additional new example of the involvement of Smo in circuit formation. Interestingly, dopaminergic projections apparently become normal in the adult, suggesting that dopaminergic projections are highly plastic after birth.

Astrocytes are an important cellular component in the brain and are sensitive to disturbances of the brain [6]. In damaging conditions, for example, astrocytes are activated and begin to express GFAP [6]. The fact that this astrocyte activation was inhibited by Smo suggests that Smo plays an important role in maintaining stable conditions in the brain. Highly activated astrocytes have also been observed in the GFAP-specific Smo knockout mice [10]. Therefore, it is probable that Shh signaling including Smo is essential for the regulation of the astrocyte activation state. Because

Emx1-positive progenitors differentiate into neurons as well as glia in the later stages of development [12], Smo is also ablated in the astrocytes in our Smo-CKO mice.

In conclusion, we reveal that neuronal positioning is impaired in the cerebral cortex of the Smo CKO mice, and that Smo regulates the number and the localization of IPCs. The Smo-CKO mice show down regulation of cyclin D2 expression, aberrations of dopaminergic projections, and the increasing expression of GFAP in the neocortex. Our data suggest that Smoothened signaling, acting through cyclin D2, is critical for the proper development and maturation of the neocortex.

Authors' contribution

M.K. and M.S. initiated this study. M.K., T.I. carried out most of the experiments. T.T. carried out experiments on TH immunostaining. M.S. wrote the manuscript together with M.K. and T.I. All authors reviewed the manuscript.

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