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ScienceDirect
Developmental Biology 311 (2007) 223–237
DEVELOPMENTAL BIOLOGY
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A dynamic gradient of Wnt signaling controls initiation of neurogenesis in the mammalian cortex and cellular specification in the hippocampus

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Received for publication 3 May 2007; revised 5 August 2007; accepted 21 August 2007
Available online 28 August 2007

Abstract

Neurogenesis in the developing neocortex is a strictly regulated process of cell division and differentiation. Here we report that a gradual retreat of canonical Wnt signaling in the cortex from lateral-to-medial and anterior-to-posterior is a prerequisite of neurogenesis. Ectopic expression of a β-catenin/LEF1 fusion protein maintains active canonical Wnt signaling in the developing cortex and delays the expression onset of the neurogenic factors Pax6, Ngn2 and Tbr2 and subsequent neurogenesis. Contrary to this, conditional ablation of β-catenin accelerates expression of the same neurogenic genes. Furthermore, we show that a sustained canonical Wnt activity in the lateral cortex gives rise to cells with hippocampal characteristics in the cortical plate at the expense of the cortical fate, and to cells with dentate gyrus characteristics in the hippocampus. This suggests that the dose of canonical Wnt signaling determines cellular fate in the developing cortex and hippocampus, and that recession of Wnt signaling acts as a morphogenetic gradient regulating neurogenesis in the cortex.

Keywords: Corticogenesis; Hippocampus; Neurogenesis; Wnt signaling

Introduction

The mammalian cerebral cortex is derived from the dorsal telencephalon. Telencephalic development requires a precise interplay of inductive signals that control cell proliferation and direct spatial and temporal patterning. During early steps of its development, the majority of neuroepithelial progenitor cells undergo symmetric cell divisions that result in an expansion of the neuroepithelial progenitor population (Gotz and Huttner, 2005). At the onset of cortical neurogenesis (around embryonic day 10 (E10) in mouse), neuroepithelial progenitors transform to radial glial cells (RGC) that divide asymmetrically in the ventricular zone (VZ) (Malatesta et al., 2000, Noctor et al., 2001, 2002; Gotz and Barde, 2005). The asymmetric (neurogenic) division mode gives rise to one daughter progenitor and one neuron, or another progenitor that translocates to the subventricular zone (SVZ). These non-surface (basal) progenitors in the SVZ divide symmetrically to generate another progenitor pair or two postmitotic neurons (terminal division) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). The cortical neurogenesis is initiated in anterior and lateral domains and it progresses towards posterior and medial domains (McSherry, 1984; McSherry and Smart, 1986; Takahashi et al., 1995, 1999). Signals that elicit the spatiotemporal control of cortical neurogenesis have not been clearly understood but several transcription factors are known to be involved in this control. For instance, Pax6 promotes generation of neurons during asymmetric division of RGC progenitors (Heins et al., 2002). Neurogenins (Ngn1/2) are proneural factors that are essential for the commitment to the neuronal lineage (Ma et al., 1996; Parras et al., 2002). Tbr2 is expressed in basal progenitors in the SVZ (Englund et al., 2005) that probably generate neurons for upper cortical layers (Tarabykin et al., 2001).

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doi:10.1016/j.ydbio.2007.08.038

Wnts are secreted molecules that regulate numerous developmental processes (http://www.stanford.edu/~rnusse/wntwindow.html) including proliferation and fate determination in the vertebrate CNS (Ciani and Salinas, 2005). Wnt signals are mediated through several intracellular pathways, including the β-catenin-mediated canonical Wnt signaling pathway. In the hippocampal primordium, the canonical Wnt signaling is present in a medio-lateral gradient (Grove et al., 1998) and it has been shown that the expansion of the progenitor pool is positively regulated by canonical Wnt signaling (Lee et al., 2000; Galceran et al., 2000; Machon et al., 2003). In the developing neocortex and spinal cord, activated β-catenin controls cell proliferation by regulating cell cycle exit in progenitors (Chenn and Walsh, 2002; 2003; Megason and McMahon, 2002; Zechner et al., 2003). Wnt signaling can trigger contradictory cellular processes depending on a developmental stage. For instance, at earlier stages (E8–E10), Wnts have mitogenic effect on acutely dissociated cortical progenitors while later, β-catenin-dependent signaling promotes differentiation (Hirabayashi et al., 2004; Hirabayashi and Gotoh, 2005; Hirsch et al., 2007). In addition, at early neurogenesis, Wnts play an essential role in establishing a dorso-ventral identity (Gunhaga et al., 2003; Backman et al., 2005). Later during corticogenesis, a gradient of Fgf8, Pax6 and Wnt-dependent Emx2 regulate area identity in the cortex. The anterior signaling centre expressing Fgf8 establishes the anterior–posterior axis in the cortex (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003) that is counteracted by the gradient of Emx2 expressed posteriorly (Mallamaci et al., 2000; Fukuchi-Shimogori and Grove, 2003, Hamasaki et al., 2004; Shimogori et al., 2004). It has been proposed that the gradient of Pax6 from the anterior and lateral pole together with the opposite gradient of Emx2 establish the major area map in the cortex (Bishop et al., 2000; Muzio et al., 2002; Rash and Grove, 2006). Wnt signaling is partly mediated by Wnt-dependent Emx2 (Theil et al., 2002) that is necessary for hippocampal development (Tole et al., 2000). However, the Wnt–Emx2 hierarchy is more complex since not all canonical Wnt signals are transmitted via Emx2. Furthermore, Emx2 also positively regulates the Wnt pathway (Muzio et al., 2005).

Published data document well an important role of canonical Wnt signaling in cell proliferation by regulation of cell cycle exit during development of CNS. Here we focus on genetic events preceding differentiation of progenitors into neurons. We show that the gradually weakening gradient of the canonical Wnt activity controls initiation of neurogenesis by regulation of known neurogenic genes. Further, the function of Wnts in cell fate determination during cortical neurogenesis is largely unknown. Our data suggest that the Wnt gradient determines cell identity along the latero-medial axis in the developing dorsal telencephalon.

Materials and methods

Animals

D6-CLEF transgenic mice were created at the Norwegian Transgenic Centre by pronuclear injection of a plasmid construct in which the activation domain of β-catenin was linked to LEF-1 (Hsu et al., 1998) and this fusion gene was coupled to a D6 promoter (Machon et al., 2002). Wnt reporter mice BAT-Gal (Maretto et al., 2003) were used for mapping Wnt activity in normal mice and for monitoring ectopic activity in D6-CLEF/BAT-Gal crosses. D6-Cre mice were previously created in our laboratory (Van den Bout et al., 2002). For conditional activation and inactivation of the canonical Wnt pathway, transgenic mice with loxP-flanked exon3 (Harada et al., 1999) or exons(2–6) (Brault et al., 2001) in β-catenin gene were crossed to D6-Cre. For conditional inactivation of Pax6, transgenic mice with loxP-flanked Pax6 (Ashery-Padan et al., 2000) were crossed to D6-Cre.

Immunohistochemistry

8-μm-thick frozen tissue sections were permeabilized in 0.1% Triton X-100 in PBS and saturated in 5% BSA plus 5% goat serum. Sections were incubated overnight in the primary antibody solution (0.5% BSA, 0.5% goat serum, 0.1% Triton X-100 in PBS). For some antibodies, brief boiling of sections in 0.01 M citrate buffer was required. Secondary antibody staining was performed for 30–60 min. Primary antibodies: rabbit anti-Pax6 (Covance, 1:250), rabbit anti-β-catenin (Sigma, 1:500), rabbit anti-Tbr1 (Chemicon, 1:500), rabbit anti-Tbr2 (R. Hevner, Chemicon, 1:1000), rabbit anti-Meis2 (A. Buchberg), rabbit anti-Prox1 (Chemicon, 1:1000), rabbit anti-Sox2 (J. Muhr). Secondary antibodies: anti-mouse or anti-rabbit ALEXA594 or 488 (Molecular Probes, 1:500). Nuclei were visualized by 4,6-diamidino-2-phenylindol (DAPI, Roche, 0.1 μg ml^{-1}). Fluorescence images were obtained on an Axioskop2 microscope (Zeiss) using Axiovision software. Electronic images were further processed using Adobe Photoshop and Illustrator software.

In situ hybridization

In situ hybridization on 8-μm-thick cryosections was carried out according to standard protocols and hybridization was incubated overnight at 68 °C. Plasmids for antisense probes were kindly provided: KA1 (J. Boulter), Ngn2 (J. Rubenstein), vGlut2 (M. Gotz) and Neuropilin-2 (NP2) was purchased as an IMAGE clone BE915536.

Acetylcholinesterase staining

Acetylcholinesterase staining was carried out as described in Lim et al. (2004).

Results

Canonical Wnt signaling gradually recedes from the neocortical primordium

Wnt activity in the medial wall is well described because several Wnts are expressed in the medial cortical wall (Grove et al., 1998; Lee et al., 2000). To monitor a dynamic nature of canonical Wnt activity in the developing telencephalon we employed a transgenic BAT-Gal mouse reporter line (Maretto et al., 2003). In this mouse, multiple TCF binding sites are coupled to a heterologous minimal promoter that drives expression of a β-galactosidase (*β-gal*) reporter gene. Thus, the expression of *β-gal* reflects the activity of canonical Wnt signaling. However, as the β-gal protein is very stable and persists in a tissue long after its expression ceases, measurement of its enzymatic activity may not reflect canonical Wnt activity in real time (Supplementary Fig. 1). Therefore we performed *in situ* hybridization with a *β-gal* riboprobe on tissue sections from the telencephalon of BAT-Gal mice as well. Wnt signaling is strongly active in broad areas of the developing head at E8, before closure of the neural tube (Figs. 1A–C). While Wnt activity was detected in the dorsal telencephalon, including the

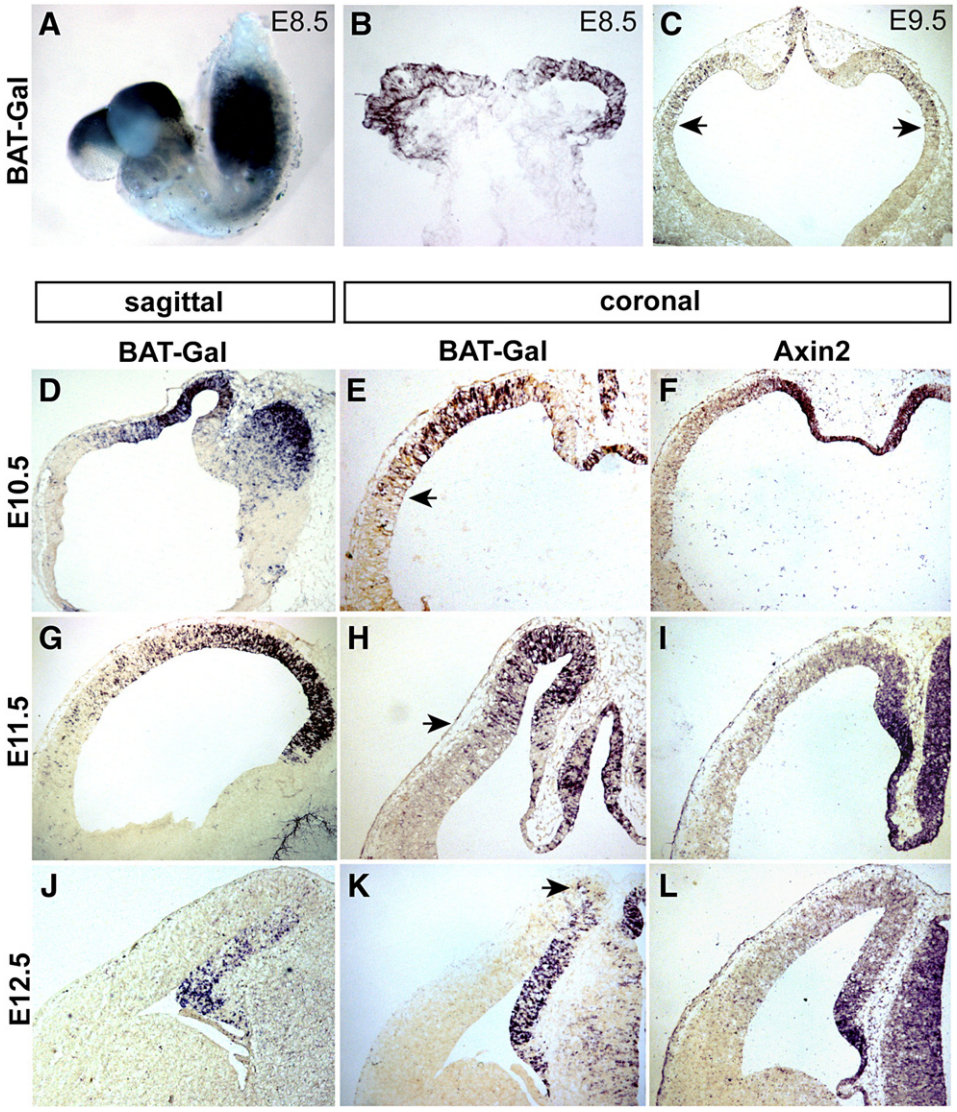

Fig. 1. Gradual movement of the canonical Wnt activity away from the lateral cortex. (A) Whole mount *in situ* hybridization with the *β-gal* probe of an embryo at E8.5 from the BAT-Gal mouse that serves as a Wnt reporter. (B) Transversal section of the embryo shown in panel A. (D, G, J) *In situ* hybridization on sagittal sections from the BAT-Gal mouse between E10.5 and E12.5. (C, E, H, K) BAT-Gal staining on coronal sections E9.5–E12.5. Note the gradual disappearance of the activity in anterior-to-posterior and lateral-to-medial direction; arrows show expression boundaries. (F, I, L) *In situ* hybridization with the *Axin2* probe on coronal sections E10.5–E12.5.

most lateral margins between E8.5 and E9.5 (arrows in Fig. 1C), the most anterior areas including the anterior neural ridge were always Wnt negative. From E10.5 onwards, we noticed that Wnt activity gradually moved away from the anterior (Figs. 1D, G, J) and also the lateral parts of the cortex (arrows in Figs. 1E, H, K) such that by E12.5, only the hippocampal primordium in the medial wall retained Wnt activity. A similar gradient was observed with a riboprobe for *Axin2*, a gene that is upregulated in areas of strong canonical Wnt signaling (Figs. 1F, I, L). As expected, *Axin2* was expressed only in the medial wall with the strongest canonical Wnt activity. A higher sensitivity of the BAT-Gal reporter may be explained by the presence of at least five TCF binding sites upstream of β-gal and multiple integrations of the BAT-Gal transgene compared to endogenous *Axin2* gene. Between E13.5 and newborn stage (P0) (Supplementary Fig. 1), Wnt activity gradually weakened and was retained only in the medial margin of the hippocampal primordium while the lateral and dorso-medial ventricular zone (VZ) showed very weak or no Wnt activity. At birth (P0), only the dentate gyrus migratory stream, the hilus and a few cells in the VZ showed detectable levels of the Wnt signaling. Thus, the activity of canonical Wnt signaling in the developing cortex is dynamic and gradually weakens in lateral and anterior areas until it disappears by birth.

Neurogenesis progression in the neocortex is complementary to the retreating gradient of the canonical Wnt signaling

Previous experiments in ferrets and mice have shown that differentiation of neural progenitors into neurons is a gradual process that begins at anterior and lateral margins of the developing neocortex (McSherry and Smart, 1986; Takahashi et al., 1999). Thus, the wave of neurogenesis advances from the areas with very weak or undetectable levels of the canonical

Wnt signaling. To study a possible time correlation of the Wnt gradient with the complementary gradient of neurogenesis in more detail, initiation and progression of neurogenesis was monitored in mouse embryos at critical time points between E10.5 and E12.5. Expression of several genes that are known to be involved in neurogenesis was examined. Immunohistochemistry of *Pax6* and *in situ* hybridization of *Ngn2* showed that their expression started at E10, at the boundary between the pallium (dorsal telencephalon) and subpallium (ventral) (Figs. 2A–B). Within 2 days, expression of these genes followed the receding Wnt gradient and expanded towards the medial cortical wall (arrows in Figs. 2F, G, K, L; compare with Fig. 1). Tbr2 expression started in the Cajal–Retzius cells at E10 (Englund et al., 2005) (arrow in Fig. 2C) but its expression in the SVZ spread from the lateral pole of the cortex at E11 towards the dorsomedial cortex (arrows in Figs. 2H, M). Interestingly, the expression of *Meis2*, a gene with hitherto unknown function in the forebrain, spread in the same manner but it was initiated in the subpallium (Figs. 2D, I, N), and it was mutually exclusive with the Wnt activity (arrows show expression boundaries). The

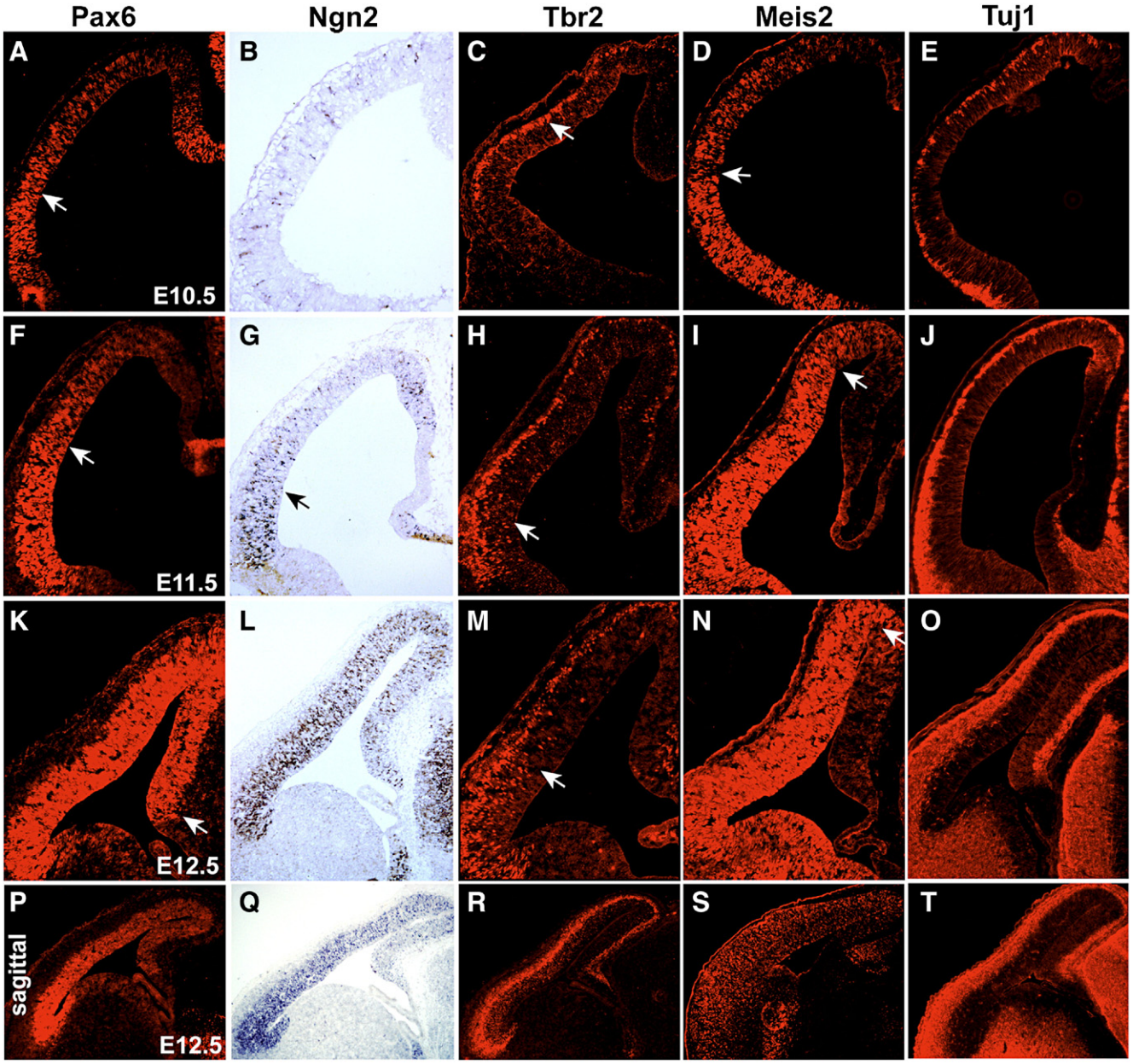

Fig. 2. Neurogenesis progresses in the opposite direction compared to the Wnt activity. (A–O) Coronal sections of the mouse cortex with visualized expression of neurogenic genes. (A, F, K) Pax6 immunofluorescence illustrating the latero-medial shift of the expression boundaries (arrows) between E10.5 and E12.5. (B, G, L) *Ngn2 in situ* hybridization shows the onset of expression at E11.5 and gradual expansion towards the medial wall. (C, H, M) Tbr2 immunostaining was seen in Cajal–Retzius cells at E10.5 (C) but the expression in the SVZ starts at E11.5 at the dorso-ventral boundary and moves dorsally over time (arrows in panels H, M). (D, I, N) Gradient of Meis2 immunofluorescence expands from the ventral telencephalon dorsally (arrows depict expression boundaries). (E, J, O) Tuj1 immunofluorescence between E10.5 and E12.5 with the latero-medial progression of neurogenesis. (P–T) Sagittal sections at E12.5 with Pax6, *Ngn2*, Tbr2, Meis2 and Tuj1 staining, respectively. Note the anterior–posterior gradient, anterior is on the left.

dynamics of the latero-medial gradient of neurogenic genes was also reflected in the wave of neurogenesis. The postmitotic layer labelled with the pan neuronal marker Tuj1 (Figs. 2E, J, O) and NeuN (not shown) spread in the same lateral to medial fashion. A similar gradient of expression of neurogenic genes as well as pan neuronal markers was seen along the anterior–posterior axis (Figs. 2P–T). In summary, the wave of neurogenesis progresses from the opposite pole of the neocortex with regard to the Wnt activity and its timing correlates with the recession of canonical Wnt signaling.

Ectopic activation of the canonical Wnt signaling impairs the onset of neurogenic genes

Is initiation of neurogenesis directly dependent on declining Wnt activity? To answer this question we ectopically activated canonical Wnt signaling in the lateral wall at the time point when it would normally have disappeared from this part of the cortex. We crossed D6-Cre mouse driver line (Van den Bout et al., 2002) to β-catenin$^{flox(exon3)}$ mice (Harada et al., 1999). D6-Cre-mediated recombination of exon3 in β-catenin leads to its stabilization and to permanent activation of the canonical Wnt pathway in the cortex and hippocampus from E11 onwards. D6-Cre activity was visualized with the β-gal enzymatic activity in R26R reporter mice (Soriano, 1999) at the critical stage E12.5 (Figs. 3A, D). Stabilization of β-catenin led to strong activation of the canonical Wnt pathway as measured by endogenous expression of *Axin2* or by β *-gal* mRNA in the BAT-Gal background (Figs. 3B, C, E, F). A dramatic downregulation of the neurogenic genes *Pax6*, *Ngn2*, *Tbr2* and *Meis2* was observed in the D6-Cre/β-catenin$^{flox(exon3)}$ cortex (Figs. 3G–N). Normal levels of expression were detected only at the

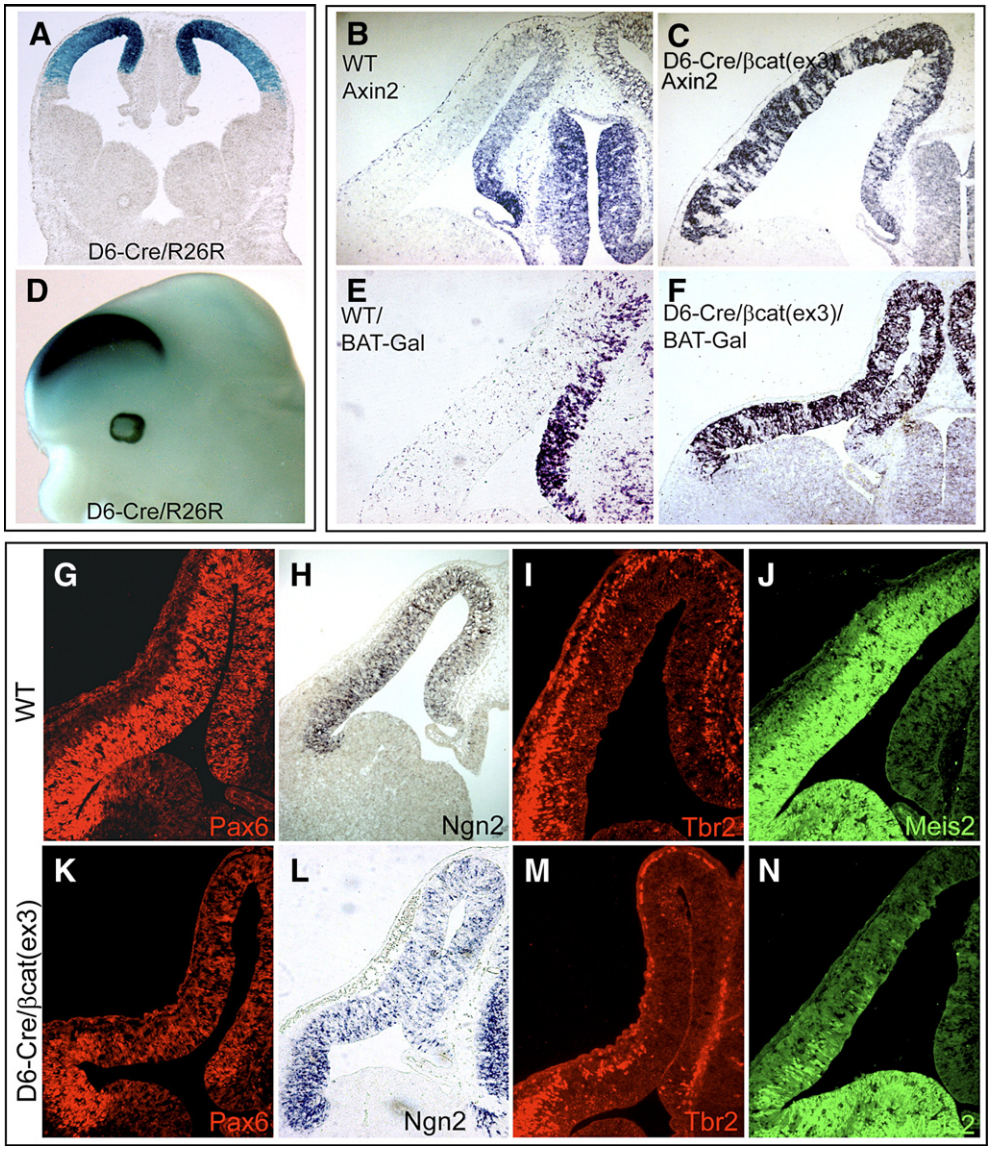

Fig. 3. Permanent activation of the canonical Wnt pathway in D6-Cre/β-catenin floxed (ex3) mice inhibits expression of neurogenic genes. (A, D) Activity of D6-Cre visualized in R26R reporter line (dark blue): (A) coronal section at E12.5; (D) side view. (B, C) *Axin2* mRNA in controls and mutants, coronal sections at E12.5. (E, F) Activation of *β-gal* mRNA in BAT-Gal background in D6-Cre/β-cat (ex3) mutants. (G–N) Downregulation of neurogenic genes in mutants at E12.5, in the area of the D6 activity, (G, K) anti-Pax6 immunofluorescence, (H, L) *Ngn2* mRNA, (I, M) anti-Tbr2 immunofluorescence, and (J, N) anti-Meis2 immunofluorescence.

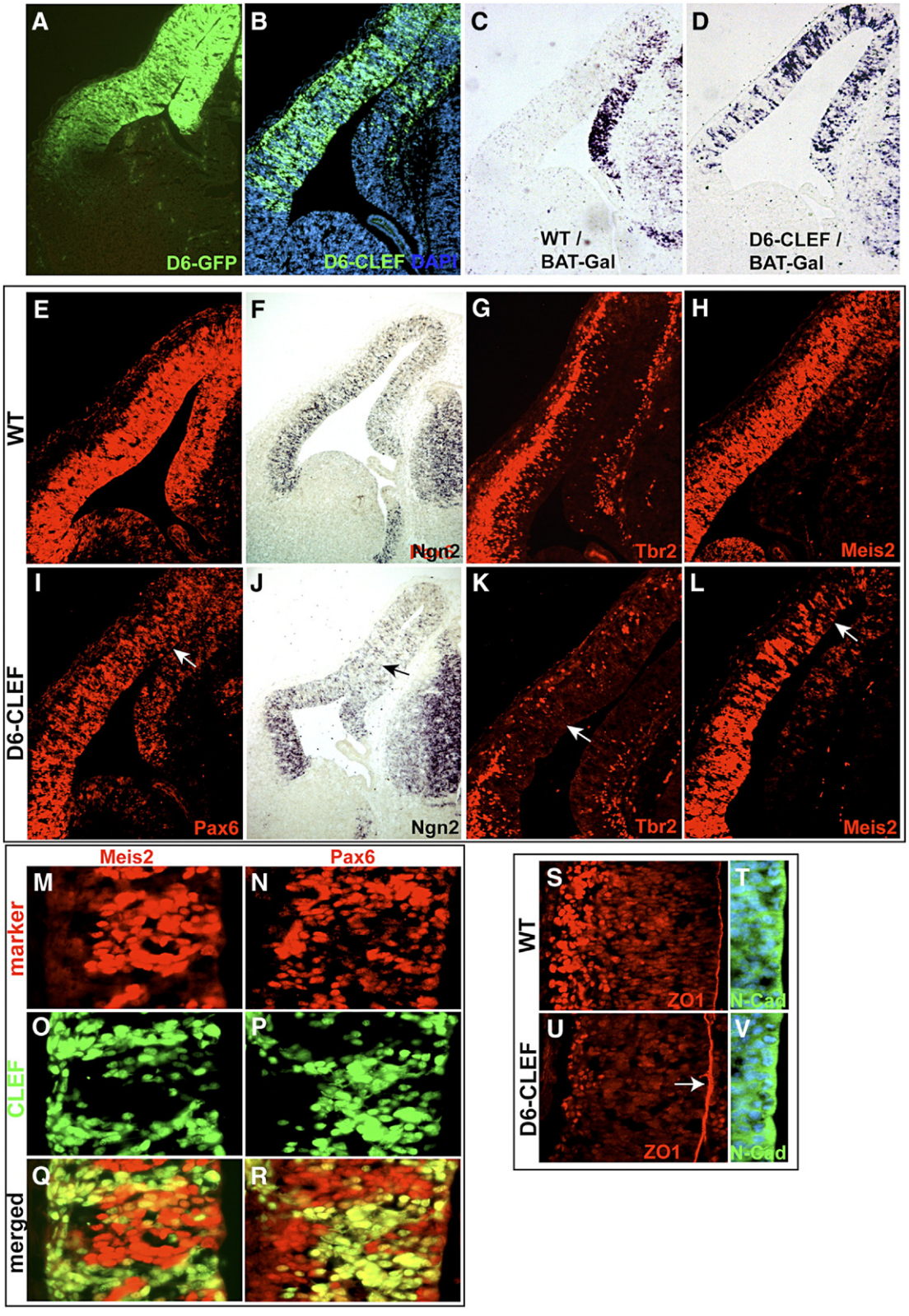

Fig. 4. Expression of neurogenic genes is inhibited by sustained Wnt activity in D6-CLEF mice. Coronal sections of the cortex at E12.5. (A) Activity of the D6 promoter/enhancer is shown in a D6-GFP mouse (green). (B) D6-CLEF transgenic mouse (β-catenin/LEF1 fusion gene driven by D6), CLEF immunofluorescence using HA-tag (green) counterstained with DAPI (blue). Note stripes of cells with CLEF expression. (C–D) D6-CLEF activates *β-gal* mRNA in a BAT-Gal (Wnt reporter) mouse in a striped pattern. (E, I) Pax6, immunohistochemistry in D6-CLEF and controls. (F, J) *Ngn2* mRNA. (G, K) Tbr2 immunohistochemistry. (H, L) Meis2 immunohistochemistry. Arrows in panels I–L show a lower expression of all the genes in the lateral wall. Note that the downregulation is not affected in the ventral margin of the cortex where D6-CLEF in not active (compare with the panel A). (M–R) Detail of D6-CLEF-HA-tag staining (green) together with Meis2 (M, O, Q) or Pax6 (N, P, R) immunofluorescence (red). Strong CLEF staining excludes the Meis2 or Pax6 expression. (S, U) ZO-1 and anti-N-cadherin (T, V) staining of intact adherens junctions.

pallial–subpallial boundary where the D6-Cre driver showed the weakest activity (compare with Fig. 3A). This strongly suggests that the gradual initiation of expression of known neurogenic genes is critically dependent on the weakening canonical Wnt activity in this area.

At later stages, however, activation of Wnt signaling in D6-Cre/β-catenin$^{flox(exon3)}$ mice influenced both the canonical Wnt pathway and cell adhesion. Cytoplasmic β-catenin is a part of the cytoskeleton that is crucial for proper cellular communication and migration and it is present in adherens junctions in the apical membrane of the neuroepithelium that maintain neuroepithelial integrity of the VZ. Interestingly, adherens junctions stained with anti-N-cadherin antibody or anti-ZO-1 were severely impaired at the apical side of the VZ from E13.5 onwards (Supplementary Figs. 2A, B, D, E). Loss of the epithelial character caused massive disorganization of the laminar structure of the cortex. Rosette-like structures were observed in the VZ at E14 and later (Supplementary Figs. 2C, F) which resembled phenotypic changes in N-cadherin mutants (Lele et al., 2002; Kadowaki et al., 2007). In late corticogenesis, many dividing progenitors were found at the outer margin of the cortex. In these ectopically located progenitors, the canonical Wnt pathway was strongly upregulated as it was assayed in the BAT-Gal background (Supplementary Figs. 2G, K). Further, in the same area with ectopic progenitors, overexpression of β-catenin, high cell proliferation (measured by BrdU incorporation) and absence of neuronal marker β-tubulin were observed (Supplementary Figs. 2H–N). Thus, the Cre-based approach influenced the function of β-catenin both in the cytoskeleton and in the canonical Wnt pathway. Further, dominant activation of β-catenin in all descendant cells of the D6 lineage produced a very severe phenotype in the cortex that complicated the interpretation of the results.

Because our Cre-based approach affected the cytoskeletal as well as nuclear function of β-catenin, we engineered a transgenic mouse D6-CLEF in which an activation domain of β-catenin was coupled to LEF1. The resulting fusion protein mimics physiological interaction of the two proteins leading to dominant activation of target genes of canonical Wnt signaling without affecting cell adhesion, as the stabilized form of β-catenin may do (Hsu et al., 1998). The *CLEF* gene was cloned under the control of the D6 promoter/enhancer that drives gene expression in the cortex from E10.5 onwards (Machon et al., 2002). Similarly to D6-Cre/β-catenin$^{flox(exon3)}$ mice, expression of D6-CLEF resulted in strong activation of the canonical Wnt pathway as monitored by BAT-Gal activity in D6-CLEF/BAT-Gal crossed mice (Figs. 4C–D). Activation of canonical Wnt signaling in D6-CLEF mice also induced expression of known Wnt targets such as *Axin2* and *Emx2* (data not shown). In contrast to the D6-Cre/β-catenin$^{flox(exon3)}$ mutants, the β-catenin/LEF fusion protein was expressed in cell nuclei (Fig. 4B) and adherens junctions stained with ZO-1 or N-Cadherin were intact in the D6-CLEF mutants (Figs. 4S, T, U, V).

Despite the uniform and coherent activity of the D6 enhancer in the cortex (Fig. 4A), D6-CLEF expression was observed in stripes with small zones of adjacent cells where CLEF was not present (Fig. 4B) and this resulted in a mosaic activation of Wnt targets such as BAT-Gal (Figs. 4C–D). The mosaic expression pattern allowed direct analysis of induction of neurogenic transcription factors in the presence or absence of the canonical Wnt signaling. As expected, a high Wnt activity in the lateral cortex of D6-CLEF mice inhibited expression of the neurogenic genes *Pax6*, *Ngn2*, *Tbr2* and *Meis2* (Figs. 4E–L) and the glutamate transporter vGLUT2 (not shown). We observed patchy loss of immunoreactivity mainly in medial and dorsal regions with a weak effect in the most lateral cortical wall where the D6 driver is least active. Areas with the highest Wnt activity led to complete inhibition of Pax6 or Meis2 whereas cells with a moderate CLEF expression partially overlapped with Pax6 or Meis2 immunoreactivity (Figs. 4M–R). These results are consistent with the D6-Cre/β-catenin$^{flox(exon3)}$ mutants and suggest that the dose of the canonical Wnt signaling can modulate progression of neurogenesis in the dorsal telencephalon.

A gradient of Wnt activity determines the cellular specificity in the hippocampus

Wnt activity is gradually weaker in the lateral pallium but is maintained at relatively high levels in the caudomedial pallium during mid-corticogenesis. There is substantial evidence that proliferation of hippocampal progenitors in the medial pallium is ultimately dependent on Wnt signaling. Wnt3a-deficient mice, dominant-negative LEF1 mice, or mice with a conditional ablation of β-catenin show strong reduction or complete absence of the hippocampus and the dentate gyrus (DG), as well as decreased cell proliferation in respective progenitor domains (Galceran et al., 2000; Lee et al., 2000, Machon et al., 2003). In addition, cell proliferation in the adult DG is also Wnt-dependent (Lie et al., 2005). It remains unclear, however, whether the canonical Wnt signaling also affects a regional identity in the hippocampus and the DG, areas that maintain high levels of the canonical Wnt signaling from embryonic to adult stages. We therefore investigated the cellular character in the cortex of D6-CLEF mice with sustained Wnt activity in the lateral and medial cortical primordium. Coronal sections from mutant newborns were stained with markers that are specifically expressed in hippocampal layers and the DG but they are absent in the wild type cortex. In D6-CLEF mice, clusters of cells expressing *neuropilin-2* (*NP-2*) and *KA1* were detected in the lateral and dorsomedial cortex (arrows in Figs. 5B, D). In contrast, wild type littermates expressed *NP2* in the CA1–CA3 fields of the hippocampus and in the DG and *KA1* marked only CA2–CA3 fields (Figs. 5A, C) (Wisden and Seeburg, 1993; Chen et al., 1997; Tole et al., 1997). The presence of hippocampus-specific markers in the cortex of neonates in D6-CLEF suggests that hippocampal cell fate is specified by canonical Wnt signaling. If so, ectopic Wnt-induced cells expressing hippocampal markers should appear in the cortex at the expense of cortical cells. Indeed, ectopic presence of cells with hippocampal markers was accompanied with partial disappearance of cortical cellular character in D6-CLEF cortex. Tbr1 is normally expressed in deeper cortical layers but not in the hippocampus. In the mutants, the number of Tbr1-positive

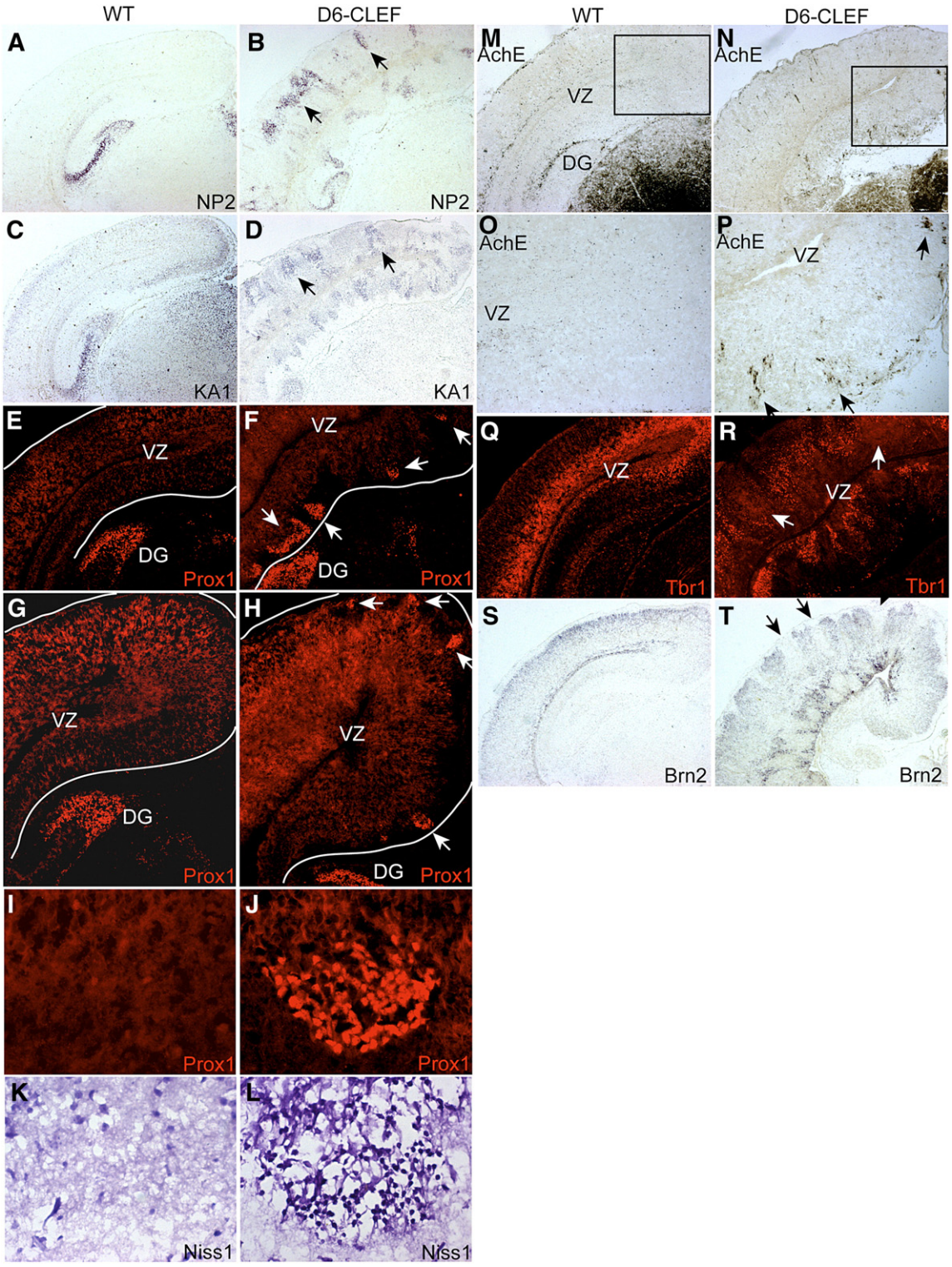

Fig. 5. Sustained Wnt activity induces hippocampal cellular character in the cortex. Coronal sections from newborn D6-CLEF mice and controls. (A, B) *NP2* mRNA is normally seen in hippocampal layers CA1–3 and in the dentate gyrus but $NP2^+$ cells are found in the lateral cortex in D6-CLEF mutants (arrows in panel B). (C, D) *KA1* mRNA marks hippocampal layers CA2–3 in a wild-type brain but clusters of $KA1^+$ cells are detected in the cortical plate from D6-CLEF (arrows in panel D). (E–H) Prox1 immunofluorescence reveals clusters of cells with the character of dentate granule cells in the medial and dorsomedial cortical wall in mutants (arrows in panels F, H) while normally Prox1 labels the granular layer in the dentate gyrus (DG). (G, H) more anterior sections from mutants and controls stained with anti-Prox1. White lines demarcate the edge of the cortical wall. (I, J) A high power image of ectopic $Prox1^+$ cells and Niss1 (cresyl violet) staining of a similar cluster on a parallel section (K, L). (M, N) Acetylcholinesterase (AchE) staining normally present in a part of the DG with abnormal patches in the hippocampus of D6-CLEF. (O, P) Details of depicted areas in panels M and N, arrows show ectopic AchE activity. (Q, R) Reduced number of $Tbr1^+$ cells in deeper cortical layers in the D6-CLEF mutants. (S, T) *Brn2* mRNA in specific areas in the upper cortical layers is downregulated in the mutants. DG, dentate gyrus; VZ, ventricular zone.

cells was lower than in wild type cortex and these cells were generally misplaced (Figs. 5Q–R). Specific zones in the cortex also lacked expression of Brn2, a marker of upper cortical layers, whereas Brn2-positive SVZ was severely disarranged (Figs. 5S–T). Such mosaic induction of the hippocampal cell fate was probably due to non-uniform activation of the Wnt pathway as shown in Figs. 4B–D.

While the hippocampus develops in the medial cortical wall along the Wnt gradient, the developing DG emerges in the area of the highest Wnt activity, in the hem. Both the hem and granule cells of the DG specifically express Prox1 (Figs. 5E, G) (Oliver et al., 1993). Immunostaining of D6-CLEF cortical sections with anti-Prox1 antibody revealed ectopic clusters of $Prox1^+$ cells in the dorsomedial cortex (arrows in Figs. 5F, H), but very rarely in the lateral cortex where cells with the hippocampal character were seen in the mutants. This indicates that induction of the DG cell fate requires high levels of the canonical Wnt signaling while hippocampal CA layers may be specified in the presence of lower levels of the pathway. A higher magnification of $Prox1^+$ ectopic cells in the medial wall revealed strong presence of Niss1 nuclear substance in these cells which is typical of DG granule cells (Figs. 5J, L). As further evidence, cholinergic activity normally present in the outer blade of the DG in neonates was examined by acetylcholinesterase (AchE) staining. Again, irregular zones of cells expressing acetylcholinesterase were found in the mutant hippocampal wall, in a pattern resembling $Prox1^+$ patches (Figs. 5M–P). In summary, our data suggest that the gradient of the canonical Wnt signaling in the medial wall regulates graded specification of the area identity in the hippocampus and the DG.

Wnt activity regulates neurogenesis

Next we asked whether the delay in expression of the neurogenic genes, *Pax6*, *Ngn2*, *Tbr2* and *Meis2*, in mice with the ectopic canonical Wnt activity also affected neuronal differentiation and organization of the cortical plate. As expected, the layer of postmitotic neurons at E13 in the outer margin of the lateral cortical wall (preplate) of D6-CLEF mutants was thinner and irregular as visualized by NeuN, Tuj1 and Tbr1 immunofluorescence in D6-CLEF mice (Figs. 6A–C, E–G, I, L). The discontinuity of the preplate was probably caused by a mosaic expression of D6-CLEF in the postmitotic zone (Fig. 6K) and was also reflected in the presence of ectopic Hes5-positive progenitor cells in the postmitotic layer that are normally located in the VZ and SVZ (Figs. 6D, H). ZO-1, a marker of neuronal subtypes, was strongly inhibited in the postmitotic zone of D6-CLEF (Figs. 6J, M). In contrast to the D6-Cre/β-catenin$^{flox(exon3)}$ cortex, ZO-1 staining in apical adherens junctions was not changed. In summary, sustained canonical Wnt activity in the lateral cortical wall delays the wave of neurogenesis and supports the model of correlation between the progression of neurogenesis and the retreat of Wnts.

To further confirm the importance of the gradually retreating gradient of canonical Wnt signaling during corticogenesis, we inactivated β-catenin by crossing D6-Cre and β-catenin$^{flox(exons2–6)}$ (Brault et al., 2001). In the D6-Cre/β-catenin$^{flox(exons2–6\ -/-)}$ mutants, β-catenin is effectively deleted from E11 onwards (Machon et al., 2003) and therefore the gradient of canonical Wnt signaling was prematurely abolished in the cortex. As expected, by E13, expression of neurogenic genes *Tbr2*, *Ngn2* and *Meis2* expanded prematurely to the medial cortical wall that is normally positive for canonical Wnt signaling in wild type littermates (Figs. 7A–C, F–H). Their expression did not expand to the hem (arrow in the panel H) because the D6 driver is not active in the hem. As a consequence, the outer layer with differentiated $Tuj1^+$ or $NeuN^+$ neurons was thicker in the β-catenin loss-of-function mutants (Figs. 7D–E, I–J). In summary, several independent approaches demonstrate a clear functional link between canonical Wnt signaling and the inhibition of neurogenic transcription factors. It can be concluded that the gradual disappearance of Wnt activity is a prerequisite for initiation of neurogenesis in the cortex. Thus the gradual disappearance of Wnts from the lateral to the medial cortical wall may explain the complementary wave of neurogenesis.

Laminar organization of the neocortex is impaired upon ectopic canonical Wnt signaling

Previous studies used β-catenin gain-of-function or loss-of-function experiments for manipulation of canonical Wnt signaling in the developing central nervous system. However, as we have shown above, both experimental designs can affect organization of cortical layers by disruption of adherens junctions. Therefore, we examined cortical layering in D6-CLEF mutants that had no apparent defects in adherens junctions and the cytoplasmic level β-catenin was not affected. The slower differentiation rate in the mutants led to a hyperplasia of the cortex (Figs. 8A, E) which is in line with previously reported findings that investigated the ectopic Wnt activity in the cortex and spinal cord (Chenn and Walsh, 2002; 2003; Zechner et al., 2003). In addition, we observed that the boundary between the SVZ and the intermediate zone (IZ) was disrupted during later corticogenesis (E18) as shown by Tuj1/Sox2 (Figs. 8B, F) and Tbr2 immunofluorescence (Figs. 8D, H arrows). Furthermore, radial glial cells stained with the anti-GLAST antibody showed morphological abnormalities in the mutants (Figs. 8C, G). Since the cytoskeletal β-catenin was not directly affected in the mutants, all abnormalities in the organization of cortical layers were probably attributed to altered gene expression caused by the ectopic canonical Wnt signaling.

Hierarchy of neurogenic genes

As the onset of expression of *Pax6*, *Ngn2*, *Tbr2* and *Meis2* correlated with the receding gradient of canonical Wnt signaling, we further concentrated on details of this cascade. It is known that transcription factor Pax6 directly binds to a forebrain-specific enhancer of Ngn2 and regulates its expression (Scardigli et al., 2003) and thus Ngn2 is downstream of Pax6. To examine the relationship between Pax6 and Tbr2, we used *small eye* (*Sey*) mutant mice that are Pax6-deficient. I

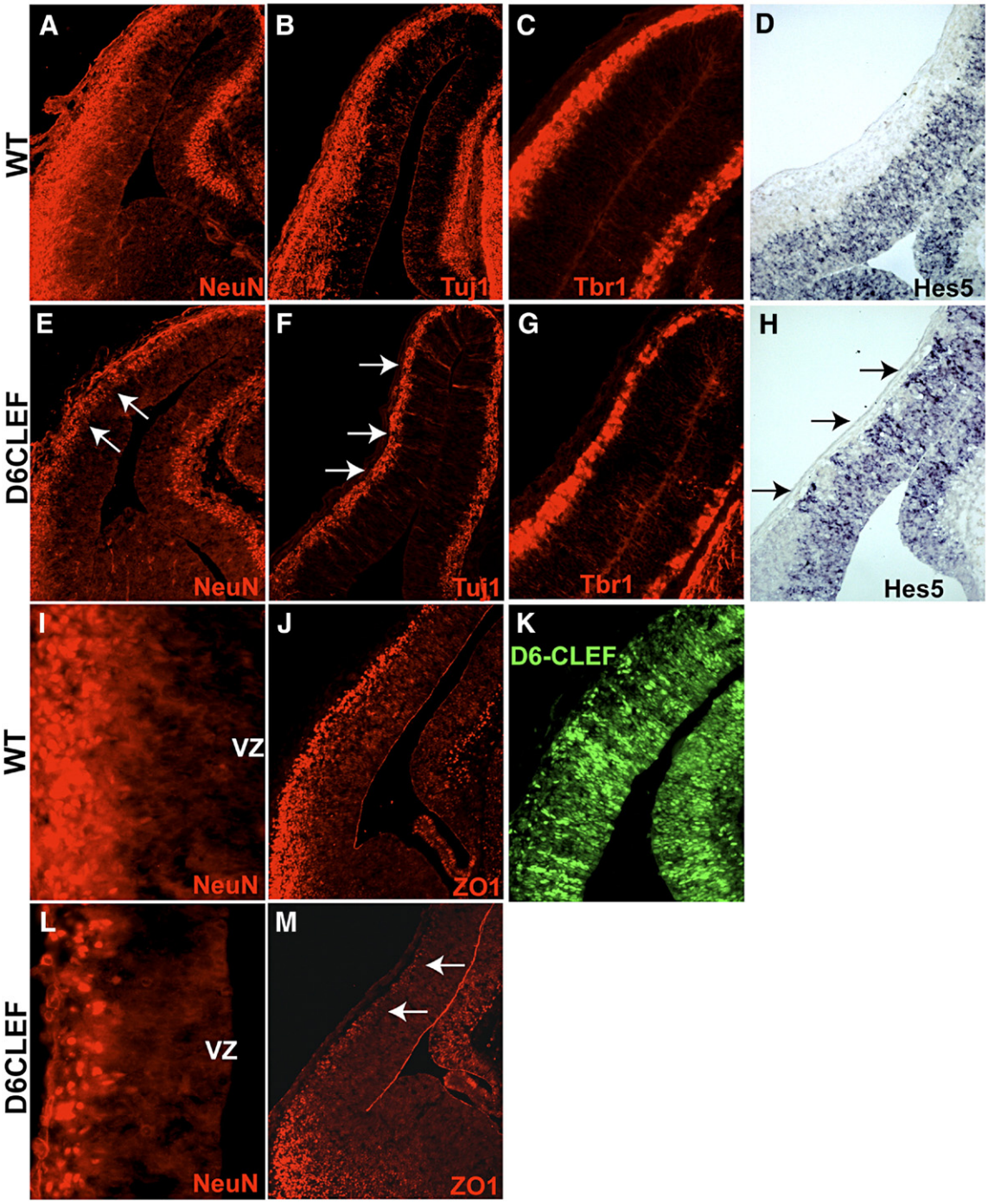

Fig. 6. The onset of neurogenesis in the cortex is delayed by ectopic Wnt activity. The layer of postmitotic neurons is thinner in D6-CLEF cortex at E13 as visualized with NeuN antibody (A, E), Tuj1 antibody (B, F) and anti-Tbr1 (C, G). (D, H) *Hes5* mRNA-positive cells are found in ectopic positions within the postmitotic layer in D6-CLEF (arrows in panel H) but normally Hes5 labels only the VZ/SVZ. (I, L) Detail of the lateral cortical wall showing less NeuN-positive postmitotic neurons stained with in D6-CLEF mice. (J, M) Downregulated expression of ZO-1 in the postmitotic layer (arrows in panel M) but adherens junctions are intact in the apical VZ. (K) anti-HA staining of the HA-tagged CLEF shows a mosaic expression in the postmitotic outer layer.

parallel, we conditionally deleted Pax6 by D6-Cre in D6-Cre/Pax6$^{flox(-/-)}$ hybrid mice. In both mutants, Tbr2 immunoreactivity in the SVZ was greatly reduced in the cortex at E13 showing that Tbr2 is at least in part downstream of Pax6 (Supplementary Figs. 3A–D). Pax6, however, is expressed in the VZ while Tbr2 in the adjacent SVZ and an overlapping expression was found only in a few cells (Englund et al., 2005) which is in line with the generally accepted scenario that SVZ progenitors arise from VZ progenitors (Noctor et al., 2004). In addition, two very recent reports indicated Tbr2 dependence on Pax6 (Holm et al., 2007; Quinn et al., 2007).

In contrast to the documented role of Meis2 in the control of Pax6 in the developing eye and pancreas (Zhang et al., 2002, 2006), the function of Meis2 in the forebrain is currently

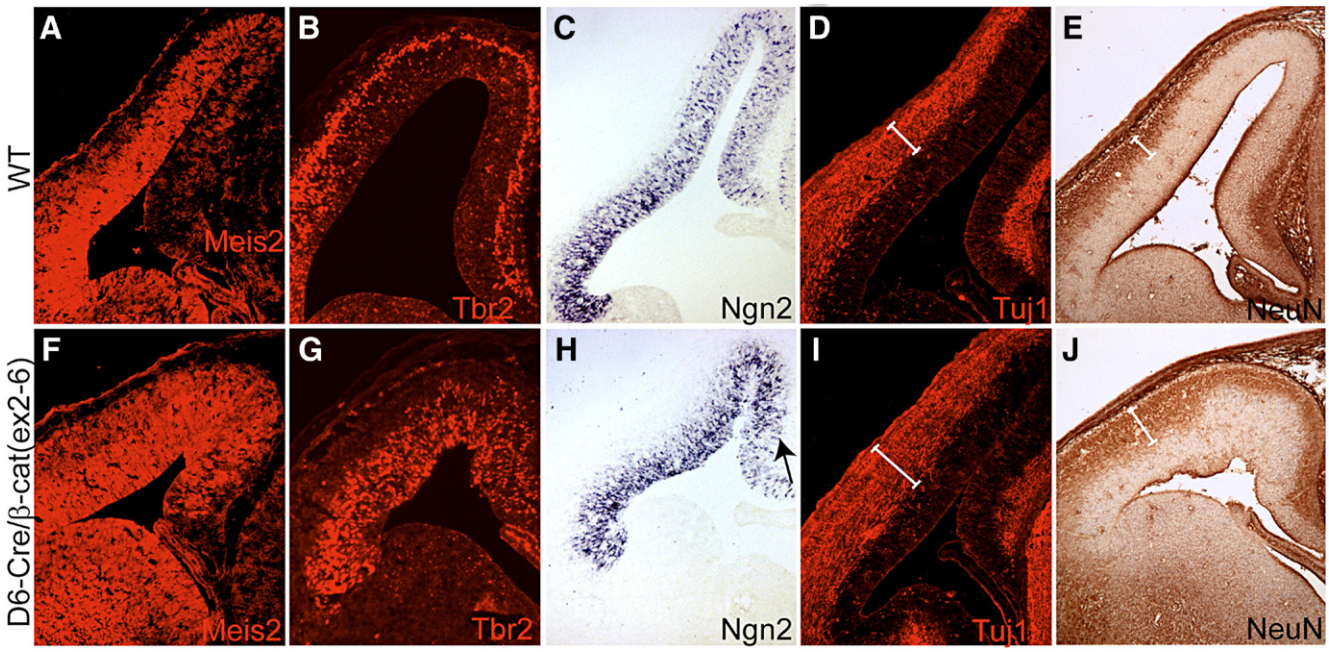

Fig. 7. Neurogenesis in the cortex is enhanced in the absence of β-catenin. Coronal sections from E13 brains in conditional D6-Cre/β-catenin$^{flox(exons2-6\ -/-)}$ mutants (lower panels) and control littermates (upper panels). Upon deletion of β-catenin expression of Meis2 (A, F) and Tbr2 (B, G) expands to the Wnt-positive medial cortical wall. (C, H) Increased expression of *Ngn2* mRNA in the medial wall (arrow in panel H). (D, I) The postmitotic layer visualized with the panneuronal marker Tuj1 (red) and NeuN (brown) (E, J) is thicker in the β-catenin loss-of-function mutants. White lines across the postmitotic layer depict its thickness.

unknown. In eyes and pancreas, the transcription factors Meis1/2 bind to respective enhancers in the *Pax6* gene which is required for their activities. We therefore examined the possibility that Meis2 also regulates the expression of Pax6 in the forebrain. Sequence analysis revealed a Meis binding site in the P1 promoter of the *Pax6* gene that has been reported to be responsible for Pax6 expression in the forebrain (Kammandel et al., 1999; Xu et al., 1999; Anderson et al., 2002) (Supplementary Figs. 4A–B). This Meis2 binding site is conserved among vertebrates and a Meis2 protein bound to its recognition site in the P1 promoter of Pax6 as shown by a chromatin immunoprecipitation assay (Supplementary Fig. 4C. Further, expression of Meis2 was normal in *Sey* mutants indicating that Pax6 is not upstream of Meis2 (not shown). These data indicate that Meis2 may contribute to regulation of the Pax6 expression in the forebrain.

Discussion

Wnts and the neurogenic cascade

The gradient of the Wnt activity in the telencephalon from an anterior to a posterior pole has been shown in the transgenic BAT-Gal mice (Maretto et al., 2003) in which the TCF-responsive promoter drives a β-gal reporter. However, its dynamics may have been overlooked in standard β-gal enzymatic assays because of the high protein stability of β-gal. In our experiments, *in situ* hybridization revealed the precise activity of canonical Wnts over time, specifically in the critical period of initiation of neurogenesis. At the onset of neurogenesis, neuroepithelial progenitors dividing symmetrically transform to radial glial cells that have an asymmetrical division mode. First newborn neurons migrate to the outer margin termed preplate. Thus the thickness of the preplate indicates the progression of neurogenesis. It is a gradual process starting at the anterior pole of the telencephalon that moves towards posterior and medial areas. Here we show for the first time that the gradient of canonical Wnt activity moves through the cortex in the fashion and timing that precisely correlates with the wave of neurogenesis. In analogy to *Drosophila* development, the movement of the gradient can be described as a morphogenetic wave. What directly causes the gradual retreat of canonical Wnt signaling and thus the morphogenetic movement remains to be analyzed. However, the molecular consequences can be precisely monitored by the induction of genes that have been shown to be directly or indirectly involved in generation of neurons, such as *Pax6*, *Ngn2* and *Tbr2*.

We suggest that the initiation of neurogenesis is dependent on gradual weakening of canonical Wnt signaling because sustained canonical Wnt signaling in D6-CLEF mice impairs the onset of neurogenesis after E12. Suppression of neurogenesis appeared to be dose-dependent because a lower expression of D6-CLEF was not able to completely downregulate the Pax6 or Meis2 expression. This is reasonable because Pax6 and subsequent neurogenesis normally appears in the medial cortical wall after E12 in the presence of physiological levels of the Wnt activity. At later corticogenesis, however, elevated Wnt signaling can induce differentiation of neuronal progenitors at the expense of proliferation (Hirabayashi et al., 2004). Our data suggest that cortical progenitors at stages E11–E12 respond to the canonical Wnt signaling by increased proliferation and the switch to increased differentiation occurs later.

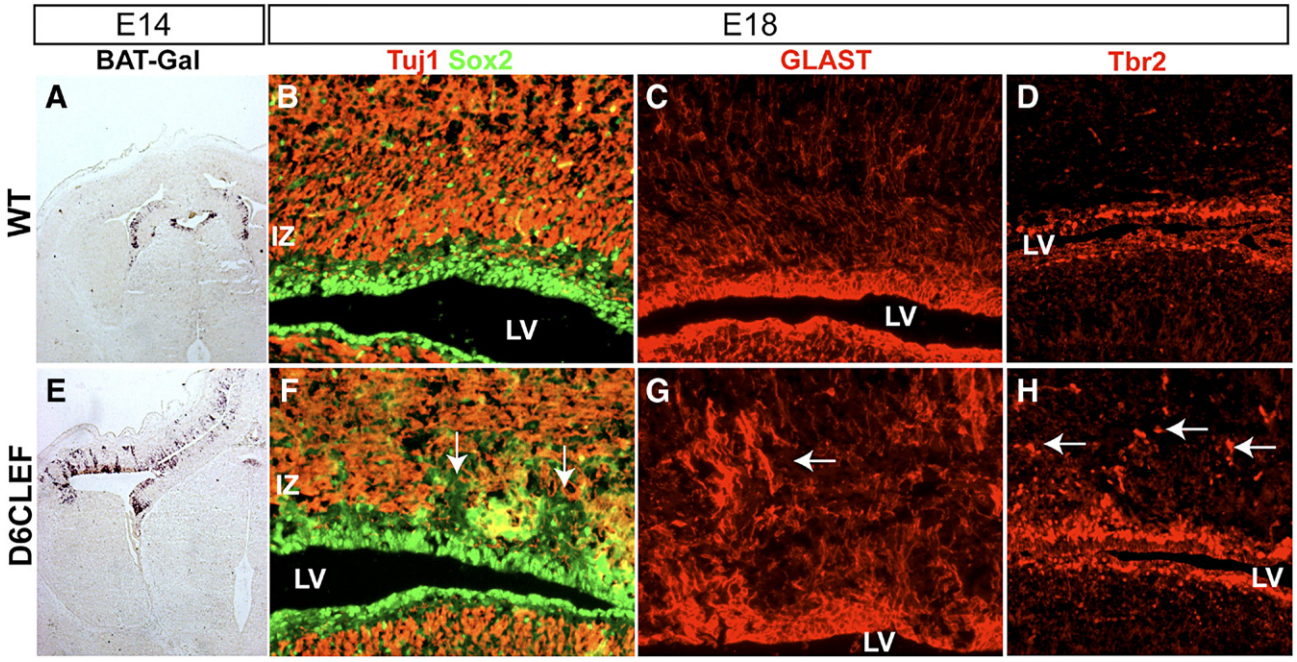

Fig. 8. Impaired pattern of cortical layers in D6-CLEF mice. (A, E) Cortical walls are larger in mutants; activation of the canonical Wnt pathway is shown by *β-gal* mRNA staining in D6-CLEF/BAT-Gal crosses at E14. (B, F) The intermediate zone (IZ) at E18, stained with Tuj1 (red), is not properly separated from the SVZ and VZ stained with Sox2 (green) (arrows in panel F). (C, G) Impaired boundary between the IZ and SVZ at E18 stained with anti-GLAST. (D, H) anti-Tbr2 immunofluorescence in the SVZ, arrows: ectopic $Tbr2^+$ cells in the cortical plate. IZ, intermediate zone; LV, lateral ventricle.

Further work is needed to elucidate what mechanism of gradual disappearance of the Wnt activity during corticogenesis is. We favour the idea that the expression of Wnt proteins gradually weakens in the growing tissue. However, it may also be an active suppressive process, for instance, via Fgf8 and Emx2 interplay that control the anterior–posterior fate map. In this context, it is noteworthy that Fgf8 function might be mediated by Pax6 (Sansom et al., 2005) but we did not notice any changes in the expression of *Axin2* in *Sey−/−* mice indicating that Pax6 is not actively involved in the retreat of the Wnt activity (unpublished data).

The Wnt gradient specifies cellular identity in the developing cortex and hippocampus

Wnt molecules are well-documented mitogens in developing neural tissue. During hippocampal development, the hem at the tip of the caudomedial cortical wall serves as a signaling centre which expresses Wnt3a. Inactivation of Wnt3a leads to down-regulation of proliferation in the medial wall and absence of the whole hippocampus. The Wnt3a signal is transmitted through the canonical pathway as dominant negative LEF1 yields a similar phenotype (Galceran et al., 2000). In the embryonic spinal cord, experiments using ectopic activation or conditional inactivation of β-catenin documented that canonical Wnts are of critical importance to cell proliferation (Megason and McMahon, 2002; Zechner et al., 2003). In the cerebral cortex, dominant active β-catenin leads to over-proliferation of cortical progenitors that do not exit the cell cycle (Chenn and Walsh, 2002). We provide evidence, for the first time, that canonical Wnt signaling also control cellular identity in the developing mammalian hippocampus. In a normal developing forebrain, the dentate gyrus emerges in the area of the strongest Wnt activity while hippocampal CA layers develop along the Wnt gradient. In D6-CLEF mice, sustained canonical Wnt activity in the dorsomedial and lateral cortex induces hippocampal cell identity in the same areas. It is interesting that ectopic $Prox1^+$ cells were found only in the medial cortex, with less in the dorsomedial, and no such cells in the lateral cortex. In contrast, $NP2^+$ or $KA1^+$ ectopic cells were clearly detected in the lateral cortex. This goes well with the hypothesis that strong Wnt activity specifies DG granule cells while a moderate Wnt gradient specifies CA layers. It has been shown that β-catenin-mediated Wnt signaling specifies cell fate of sensory neural cells that are derived from neural crest stem cells (Lee et al., 2004). Our findings are consistent with the idea that Wnts control cell fate in the neural tissue. The level of Wnt activity may be directly involved in cell specification along the anterior to posterior and the lateral to medial axis during forebrain development.

Cortical layer organization

When D6-Cre/β-$catenin^{flox(exon3)}$ was employed for ectopic activation of Wnt signaling, a delayed onset of neurogenesis was observed, however, at later corticogenesis, severe effects in the cortex were found. This may be due to several reasons. Firstly, the Cre-based approach affects the whole D6 lineage that includes nearly all cells in the dorsal telencephalon after E11, while D6-CLEF activity at later stages was more restricted to the germinal zone including the VZ and SVZ. Indeed, many dividing cells with a strong Wnt activity were found in the cortical plate in D6-Cre/β-$catenin^{flox(exon3)}$ mutants. Secondly,

dominant activation of β-catenin in D6-Cre/β-catenin$^{flox(exon3)}$ may influence the levels of β-catenin in the cytoplasm as the protein translocates to the nucleus which may result in temporary depletion of β-catenin in the cytoskeleton. Thus, cell adhesion was affected as it was seen in disrupted adherens junctions that are crucial for normal organization of the cortex (Cappello et al., 2006; Kadowaki et al., 2007). In this context, both dominant activation and inactivation of β-catenin (Machon et al., 2003) lead to impaired adherens junctions. Therefore, activation of canonical Wnts in D6-CLEF by a nuclear β-catenin/LEF1 fusion protein without affecting neuroepithelial integrity allowed us to draw clearer conclusions. It is interesting that canonical Wnts are involved in cellular migration of newborn neurons to the cortical plate. Such a possibility has been proposed (Chenn and Walsh, 2003) but the disorganization of the SVZ by genetic stabilization of the cytoplasmic β-catenin may also be caused by impaired cell adhesion. Recent report by Woodhead et al. (2006) provides evidence that the canonical Wnt signaling in the cortical VZ regulates cell cycle exit and subsequent progression of progenitors towards the SVZ and upper layers. Our results are in agreement with this finding. In D6-CLEF mice, the SVZ, the IZ and deeper cortical layers are disorganized. As the function of β-catenin in the cytoplasm seems to be minimally affected, altered expression of target genes by canonical Wnts is a plausible explanation. One such candidate is *Ngn2* that determines dendritic morphology and migration of neurons via inhibition of RhoA GTPase and this function is independent of its proneural activity (Hand et al., 2005; Britz et al., 2006). Another candidate downstream of Wnts is Pax6 that is involved in specification of later-born neurons in superficial cortical layers (Schuurmans et al., 2004).

Acknowledgments

We thank R. Grosschedl for the β-catenin/LEF1 fusion plasmid, R. Kemler for the β-catenin$^{flox(exons2–6\ -/-)}$ mice, M. Taketo β-catenin$^{flox(exon3)}$ mice, S. Piccolo for the BAT-Gal mice, P. Gruss for the Pax6$^{flox(-/-)}$ mice, M. Gotz the vGlut2 probe, J. Rubenstein for the Ngn2 probe, R. Hevner for the anti-Tbr2 antibody, A. Buchberg for the KA1 probe, J. Muhr for the anti-Sox2 antibody. We are also very grateful to James Booth for critical reading of the manuscript and helpful suggestions. The work was supported by the Research Council of Norway, by The Centre for Applied Genomics (grant 1M6837805002) and The Institutional Grant AVOZ50520514.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.08.038.

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