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# Effects of canonical Wnt signaling on dorso-ventral specification of the mouse telencephalon

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**Abstract**

Wnt signaling is involved in numerous processes during vertebrate CNS development. In this study, we used conditional Cre/loxP system in mouse to ablate or activate ß-catenin in the telencephalon in two time windows: before and after the onset of neurogenesis. We show that β-catenin mediated Wnt signals are required to maintain the molecular identity of the pallium. Inactivation of ß-catenin in the telencephalon before neurogenesis results in downregulated expression of dorsal markers *Emx1*, *Emx2* and *Ngn2*, and in ectopic up-regulation of ventral markers *Gsh2*, *Mash1* and *Dlx2* in the pallium. In contrast, ablation of ß-catenin after the onset of cortical neurogenesis (E11.5) does not result in a dorso-ventral fate shift. In addition, activation of canonical Wnt signaling in the subpallium leads to a repression of ventral telencephalic cell identities as shown by the down-regulation of subpallial markers *Dlx2*, *Nkx2.1*, *Gsh2*, *Olig2* and *Mash1*. This was accompanied with an expansion of dorsal identities ventrally as shown by the expanded expression domains of pallial markers *Pax6* and *Ngn2*. Thus, our data suggest that canonical Wnt signals are involved in maintaining the identity of the pallium by controlling expression of dorsal markers and by suppressing ventral programs from being activated in pallial progenitor cells.
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*Keywords:* β-catenin; Telencephalon; Patterning; Progenitor cell identity

## Introduction

The telencephalon is a complex protosegment that exhibits pattern singularities that are explained by its evolutionary roots and the complex and dynamic morphogenetic signaling in the area (Puelles and Rubenstein, 2003). Albeit in the telencephalon a clear confinement of subdivisions to dorsal or ventral is problematic, zones of gene expression offer a frame for analyzing the implications of cellular signaling on telencephalic fate decisions and the developmental plasticity of this process. In a simplified model, the two telencephalic subdivisions, pallium and subpallium, differ in their proliferative profile, cytoarchitecture, and in their spectrum of cell types. For instance, pallial domains produce predominantly glutamatergic neurons, whereas in the subpallium predominantly GABAergic, cholinergic neurons, and oligodendrocytes are produced (Rallu et al., 2002a; Wilson and Rubenstein, 2000). Morphogenetic signals from the anterior neural ridge (ANR) and from the dorsal and ventral midlines play important and dynamic roles in telencephalic patterning. They serve as sources of multiple secreted molecules, including Shh, Wnts, BMPs, and Fgf8, that impose alone

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or in concert fate and proliferative decisions to telencephalic progenitors (Grove and Fukuchi-Shimogori, 2003; Rubenstein et al., 1998).

Wnt signaling is central in telencephalic fate decisions. At early stages of CNS development, antagonism of posteriorizing Wnt signals is required for the establishment of the telencephalon (Houart et al., 2002; Mukhopadhyay et al., 2001; Yamaguchi, 2001). Subsequently, a number of *Wnt* genes are deployed in the anterior lateral neuroectoderm and later in the dorsal midline of the telencephalon, and the cortex (Lee et al., 2000; Parr et al., 1993), where they are important both for expansion and patterning of the pallium (Galceran et al., 2000; Lee et al., 2000). *Fgf8* is expressed in the ANR and later in the telencephalic midline and experiments in mouse and zebrafish models suggest that Fgf8, while being central in anterior signaling (Shimamura and Rubenstein, 1997), is also either directly or indirectly promoting ventral and dorsal fate decisions in the telencephalon (Crossley and Martin, 1995; Gunhaga et al., 2003; Kuschel et al., 2003; Shanmugalingam et al., 2000). *Shh* is expressed in the ventral midline of the anterior neuroepithelium and in the underlying prechordal plate and promotes ventral fates of telencephalic neural progenitors (Jessell, 2000; Rallu et al., 2002a). The inductive effects imposed by Shh, Wnts, BMPs, retinoic acid and Fgf8 signals depend on the specific time window, and any of these signals may have alternative effects at different developmental stages (Gunhaga et al., 2003; Machold et al., 2003; Nordstrom et al., 2002).

The information provided by morphogenetic signals defines the expression domains of transcription factors that are employed in a differential manner in the telencephalon. The pallium and subpallium differ in the expression of numerous transcription factors, including *Ngn1* and *Ngn2* that are expressed in the dorsal ventricular zone (VZ) and *Mash1* that has a complementary expression in the ventral part (Guillemot and Joyner, 1993; Sommer et al., 1996). Several of the pallial- and subpallial-specific transcription factors are instructive for region specific fate decisions. In *Ngn2* and *Ngn1/2* double mutants, progenitors of the pallium ectopically express *Mash1* and produce neuronal subtypes normally produced in the subpallium (Fode et al., 2000). The transcription factors *Pax6* and *Emx2* are expressed in gradients in the dorsal telencephalon where they are essential for cortical development (Grove and Fukuchi-Shimogori, 2003). In *Emx2/Pax6* double mutant mice, the dorsal telencephalon acquires ventral characters (Muzio et al., 2002). Homeobox transcription factors *Gsh2* and *Nkx2.1* are expressed in the ventral telencephalon where they are required for the maintenance of the ventral character of progenitor cells (Corbin et al., 2000; Sussel et al., 1999; Toresson et al., 2000).

At early stages of telencephalic development in the chicken, dorso–ventral fate specification by various morphogens is still flexible and recent data on chicken telencephalic explants provide evidence that Wnt3a, in concert with Fgf8 can induce the expression of dorsal markers *Pax6*, *Ngn2* and *Emx1*, while at the same time, it represses ventral marker *Nkx2.1* in neural progenitor cells (Gunhaga et al., 2003). However, it remains unclear whether Wnt signals are involved in dorso-ventral patterning of the mouse telencephalon. Wnt null mutants have not provided support to this idea (Jessell, 2000; Lee et al., 2000; Miller and Sassoon, 1998; Parr and McMahon, 1995; Parr et al., 2001; Shu et al., 2002). Moreover, the developmental flexibility of the later telencephalon has not been investigated in detail. In this study, we examine the role of the canonical Wnt signaling pathway in mouse telencephalic patterning by inactivating and constitutively activating the canonical Wnt pathway during the preneurogenic and neurogenic periods.

## Materials and methods

### *Mouse strains*

The ROSA26 reporter line (Soriano, 1999), was purchased from Jackson laboratory (stock #003309). The *Nes11Cre* (stock #003771) was purchased from Jackson laboratory (Tronche et al., 1999). The *Nes8Cre* line was kindly provided by Dr. W. Zhong (Petersen et al., 2002). The *β-catenin loxP exon2-6* mouse line (Brault et al., 2001), was kindly provided by Dr. R. Kemler and was kept in a 129/Sv mouse background. The *β-catenin loxP exon3* mouse line (Harada et al., 1999) was kindly provided by Dr. M. M. Taketo. To inactivate β-catenin in the telencephalon, the *Nes8Cre* (or *Nes11Cre*) line was crossed to *β-catenin loxP exon2-6*+/+ mice. Male *Nes8-Cre/β-catenin loxP exon2-6*+/− (or *Nes11Cre/β-catenin loxP exon2-6*+/−) offsprings, were subsequently crossed to *β-catenin loxP exon2-6*+/+ females to obtain *Nes8Cre/β-catenin loxP exon2-6*+/+ mice (or *Nes11Cre/β-catenin loxP exon2-6*+/+). To activate β-catenin in the forebrain *Nes8Cre* (or *Nes11Cre*) males were crossed to *β-catenin loxP exon3*+/+ females to get *Nes8Cre/β-catenin loxP exon3*+/−(or *Nes8Cre/β-catenin loxP exon3*+/−) animals.

### *Genotyping*

For genotyping by PCR the following primers were used. For genotyping of the *β-catenin loxP exon3* allele, ßcatF1ex2: 5′-GCTGCGTGGACAATGGCTAC-3′ and ßcatR1ex3: 5′-GCTTTTCTGTCCGGCTCCAT-3′ were used under the following PCR conditions: 94°C for 3 min; 30 cycles of 94°C 30 s, 56°C for 30 s, 72°C for 45 s; 72°C for 7 min, amplifying a 359-bp sequence from the wildtype allele and an approximately 550 bp sequence from the *β-catenin loxP exon3* allele. For genotyping of the *β-catenin loxP exon2–6* allele, RM41: 5′-AAGGTA-

GAGTGATGAAAGTTGTT-3′ and RM42: 5′-CACCATG-TCCTCTGTCTATTC-3′ were used, amplifying a 221 bp sequence from the wildtype allele and a 324-bp sequence from the *β-catenin loxP exon2–6* allele.

For genotyping of *Nes8Cre and Nes11Cre* animals, Cre52: 5′-GTCCAATTTACTGACCGTACACC-3′; Cre32: 5′-GAAGCATGTTTAGCTGGCCC-3′ were used, amplifying a sequence of 293 bp.

### *Tissue preparation and histology*

Embryos were obtained by dissection in ice-cold PBS. The day of vaginal plug was considered as embryonic day 0.5 (E 0.5). All efforts were made to minimize the number of animals used and their suffering according to international ethical guidelines. For in situ hybridisation and immunohistochemistry, heads were fixed overnight in 4% paraformaldehyde in PBS and subsequently cryoprotected in 30% sucrose, embedded and frozen in OCT and sectioned at 10–12 μm.

### *β-Galactosidase assay*

The β-galactosidase assay was carried out as described by (Hogan et al., 1994). In short, embryos were fixed in 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$ and 5 mM EGTA, or in 0.2% PFA. Specimens were either washed in rinse buffer (0.1M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 20 mM Tris pH7.3, 0.01% sodium deoxycholate and 0.02% Nonidet P-40), and incubated as wholemounts overnight at 37°C in staining solution (rinse buffer supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH 7.3, and 1 mg/ml X-gal), or cryoprotected in 30% sucrose in PBS overnight at 4°C, embedded and frozen in OCT and sectioned at 10–14 μm, washed three times in rinse buffer and subsequently stained.

### *Immunohistochemistry and BrdU labelling*

For immunohistochemistry of β-catenin, heads were processed as described above. Immunohistochemistry β-catenin (SigmaC2206) was carried out as follows: sections were refixed in 4% PFA for 15 min, washed three times 5 min in PBS, permeabilized in 0.1% Triton X-100 in PBS for 15 min and saturated in blocking buffer (5% bovine serum albumin (BSA), 5% goat serum, 0.1% Triton-X100 in PBS). Subsequently, sections were incubated with a primary antibody diluted in 0.5% BSA, 0.5% goat serum, 0.1% Triton-X100 in PBS overnight at room temperature. After three washes in PBS, incubation followed with anti-rabbit peroxidase antibody (EnVision+ system kit HRP (DAB), DAKO) for 60 min at room temperature, followed by 3 washes in PBS. Peroxidase staining was done following the instructions of the manufacturer (EnVision+ system kit HRP (DAB), DAKO).

### *In situ hybridisation*

In situ hybridisation on cryosections was carried out as previously described (Machon et al., 2002) except that sections were incubated at 69°C overnight. Digoxigenin-labeled riboprobes were prepared by standard protocols. Probes were kindly provided by J. Rubenstein (*Dlx2*; *Dlx5*; *Emx1*; *Emx2*; *Gsh2*); F. Guillemot (*Ngn2*; *Mash1*); P. Gruss (*Pax6*); E. Lai (*Bf1*); A. McMahon (*Shh*); G. Martin (*Fgf8*); AL Joyner (*Nkx2.1*); S. Pleasure (*Wnt3a*); D. Rowitch (*Olig2*).

## Results

### *Canonical Wnt signaling in the telencephalon*

A number of Wnt genes are expressed in the anterior lateral neuroectoderm and later in the dorsal telencephalon (Lee et al., 2000; Parr et al., 1993). Wnt signals can be mediated through at least four intracellular branches: (i) the Wnt/$Ca^{2+}$-pathway; (ii) the planar cell polarity pathway; (iii) a pathway that regulates spindle orientation and asymmetric cell division; (iv) the canonical Wnt pathway (Huelsken and Behrens, 2002). Using a reporter line carrying a LacZ gene under the control of β-catenin/T cell factor responsive elements (BAT-gal), Maretto et al. (2003) showed that the canonical Wnt signaling pathway displays high activity in the pallium and no activity in the subpallium. In Fig. 1A, LacZ activity demonstrates activation of the BAT-gal transgene in the pallium of BAT-gal reporter mice between E11.5 and E16.5, and confirms that β-catenin dependent Wnt signals display a high-dorsomedial to low-lateral gradient in the pallium.

### *β-catenin is required for maintaining the molecular identity of preneurogenic pallial progenitor cells*

Recently, it has been shown that Wnt signaling is required for the initial specification of dorsal cell identities in the avian telencephalon (Gunhaga et al., 2003). However, it is not clear whether the canonical Wnt signaling pathway is involved in the induction or maintenance of dorsal character in the mouse telencephalon. To investigate whether the canonical Wnt signaling pathway plays a role in mouse telencephalic patterning, we used a nestin enhancer-driven Cre-recombinase (*Nes8Cre*) mouse line (Petersen et al., 2002) to inactivate β-catenin, the mediator of the canonical Wnt signaling pathway, in the telencephalon. To map the timing and area of *Nes8Cre* recombination, the *Nes8Cre* line was crossed to the reporter line ROSA26 (Soriano, 1999), which carries a *β-galactosidase* gene that is activated after Cre-mediated recombination. Consistent with Petersen et al. (2002), offspring from *Nes8Cre* × ROSA26 crosses showed extensive recombination in the somites and neuroectoderm at E8.5 (Fig. 1B,

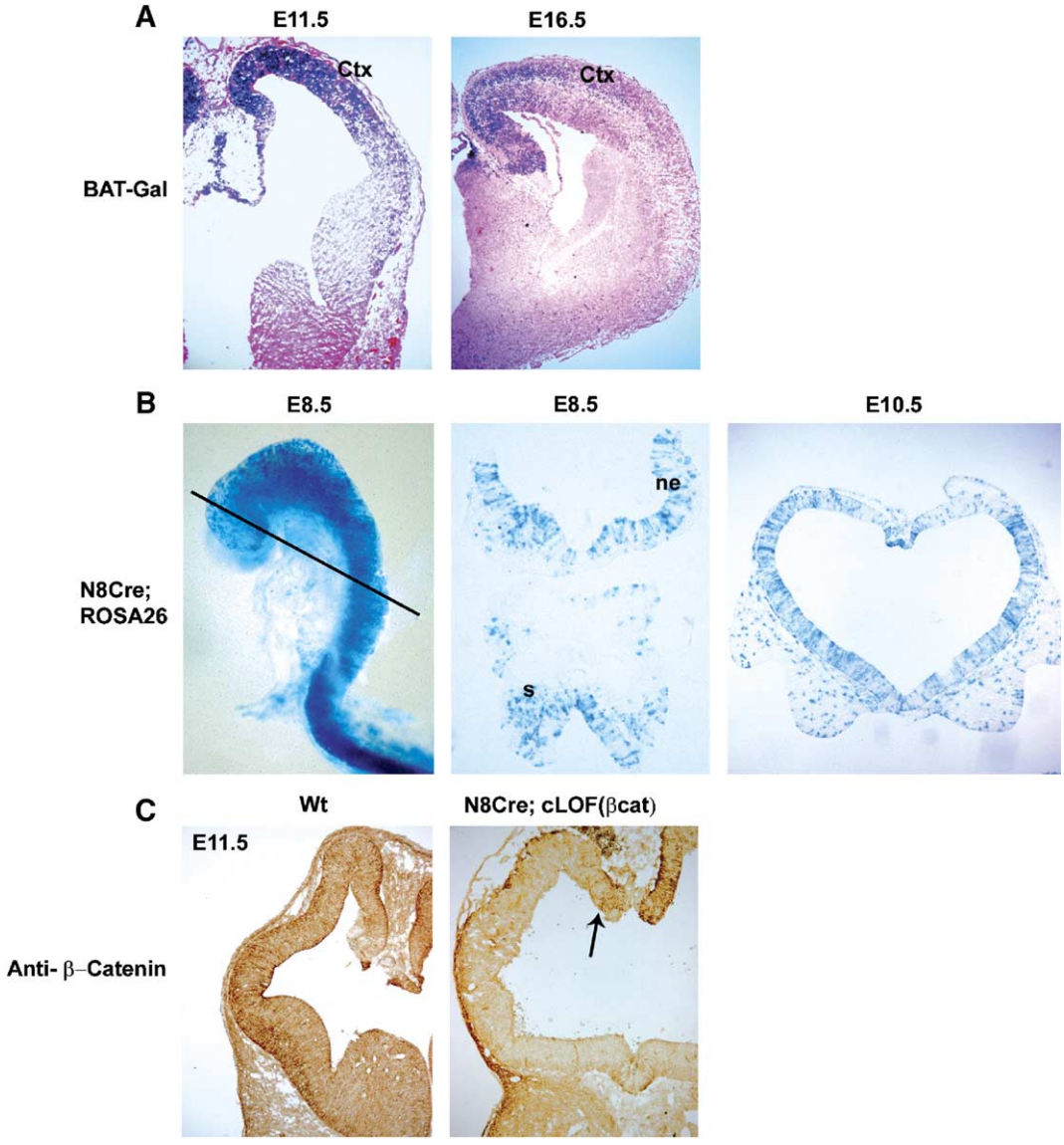


Fig. 1. (A) BAT-gal reporter line showing areas of canonical Wnt signaling activity in the telencephalon of E11.5 and E16.5 embryos. (B) Domains of *Nes8Cre* recombination indicated by ROSA26 LacZ reporter line staining. *Nes8Cre* activity was found throughout the neuroepithelium and in somites at E8.5. Coronal section of the telencephalon at E10.5 showing mosaic recombination in dorsal and ventral domains. (C) Anti-β-catenin staining on coronal sections at E11.5. Recombination is close to complete throughout the telencephalic neuroepithelium. A few cell in the dorsomedial wall (arrow) express detectable levels of β-catenin. Abbreviations: Ctx, cortex; ne, neuroepithelium; s, somite.

Petersen et al., 2002). The *Nes8Cre* driver line can be used to analyze effects of canonical Wnt signaling in the preneurogenic CNS. *Nes8Cre* mice were crossed to transgenic mice having exons 2 to 6 of the *β-catenin* gene flanked by *loxP* sites (*β-catenin loxP exon2-6+/+*) (Brault et al., 2001). *NesCre8/β-catenin loxP exon2-6+/−* males were subsequently crossed to *β-catenin loxP exon2-6+/+* females to generate a conditional loss-of-function (cLOF) mutation of *β-catenin* (referred to as *N8Cre/*cLOF). Recombination deletes exons 2 to 6 and results in an inactive β-catenin protein and defective canonical Wnt signaling (Brault et al., 2001). Immunostaining against β-catenin at E11.5 showed that in *N8Cre/*cLOF embryos, the level of the β-catenin protein is greatly reduced in the majority of telencephalic neuroepithelial cells (Fig. 1C). Mutant embryos at E9.5 appeared similar to wildtypes on gross inspection (data not shown). Expression of telencephalic marker *Bf1* (Li et al., 1996) and the pallial marker *Emx2* (Simeone et al., 1992) was similar in wildtypes (Figs. 2a, b) and *N8Cre/cLOF* mutants (Figs. 2a', b') at E9.5, indicating that the telencephalon is established and the pallium is specified in these mutants. At E10.5 and E11.5, the embryos homozygous for the β-catenin mutation were found in a ratio at 23% by genotyping, however, of those about half were found to be arrested in development. The other half of homozygous mutants continued development but they were smaller than wild type littermates and displayed failure of anterior neural tube closure and had deposits of

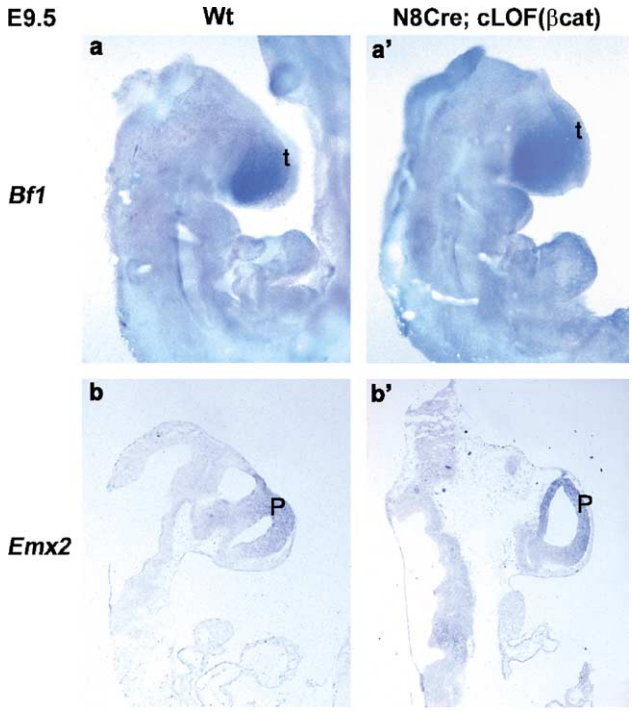


Fig. 2. Initial dorso-ventral specification of the telencephalon was established in *N8Cre*/β-catenin loss-of-function mutants (*N8Cre*/cLOF). Detection of *Bf1* (a, a') and *Emx2* (b, b') transcripts by RNA in situ hybridisation at E9.5 showing that the telencephalon and dorsal domains of the telencephalon was initially established in *N8Cre*/cLOF mutants. Abbreviations: P, Pallium; t, telencephalon.

blood cells in the brain (Figs. 3a, a'). The dorso-ventral subdivisions of the telencephalon were analyzed using RNA probes specific for discrete regions of the telencephalic progenitor zones. At E11.5, transcription factor *Bf1* is expressed throughout the dorsal and ventral domains of the telencephalic neuroepithelium of wild-types and mutants (Fig. 3b, b'). Notably, the neuroepithelium is disorganised, likely due to adherens junction defects (Figs. 3b'–g') as previously reported (Machon et al., 2003).

Homeobox transcription factors *Pax6*, *Emx1* and *Emx2* are expressed in the pallial neuroepithelium and are involved in maintaining the molecular identity of the dorsal telencephalon (Muzio et al., 2002; Stoykova et al., 2000). While pallial progenitor cells of *N8Cre/cLOF* mutants still expressed *Pax6* (Fig. 3c'), expression of *Emx1* and *Emx2*

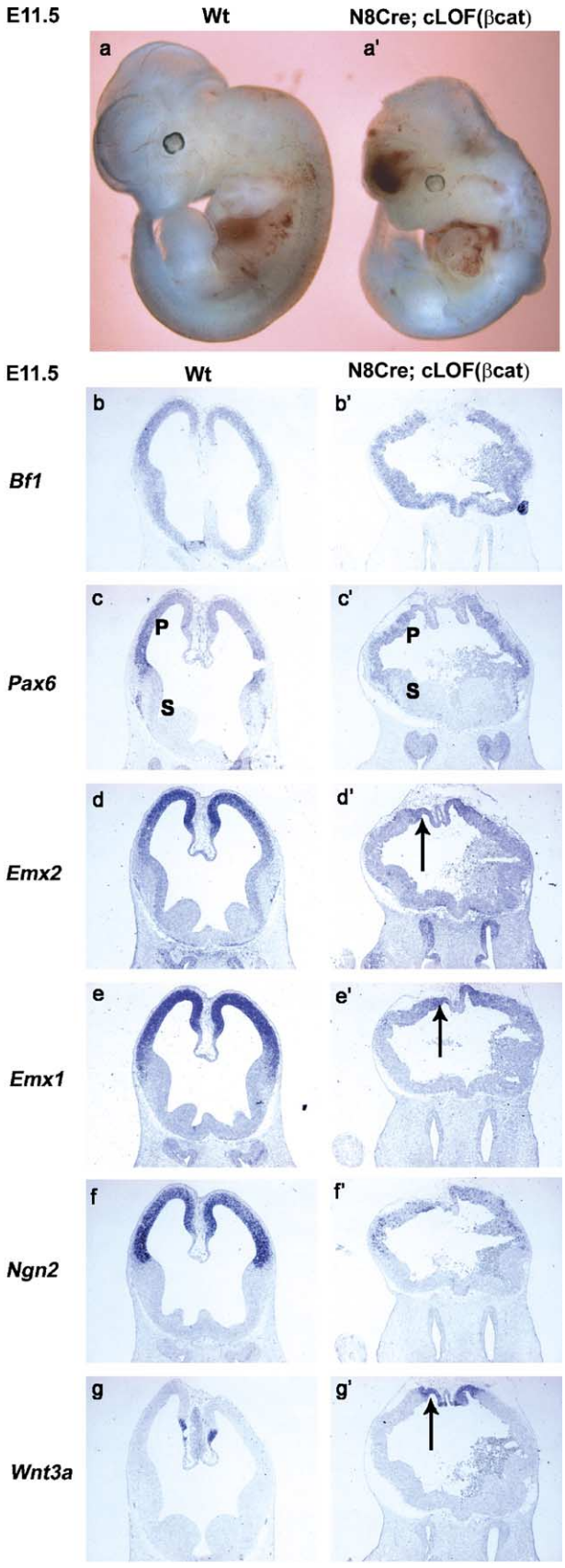


Fig. 3. Canonical Wnt signaling is essential for maintaining the expression of dorsal markers in the pallium. (a, a') *N8Cre*/β-catenin loss-of-function mutants (*N8Cre*/cLOF) were smaller than wild type littermates and displayed failure of anterior neural tube closure. (b–g) RNA in situ hybridisation on coronal sections of *N8Cre*/cLOF mutants and wildtype littermates at E11.5. *Bf1* (b, b') and *Pax6* (c, c') was retained to their endogenous expression domains, while expression of *Emx2* (d, d'), *Emx1* (e, e') and *Ngn2* (f, f') was reduced in the pallium of *N8Cre*/cLOF mutants. *Emx2* and *Emx1* expression remains (arrows in d', e') at low levels in the dorsomedial neuroepithelium in a domain coinciding with *Wnt3a* expression (g, g'). Abbreviations: P, Pallium; S, Subpallium.

was greatly reduced and was detectable only in a small region of the dorsal midline (Figs. 3d', e'), a domain that exhibits lower rates of recombination (Fig. 1C). This remaining *Emx1/2* positive domain was also shown to express *Wnt3a*, a marker of the dorsomedial neuroepithelium referred to as the hem (Figs. 3g, g'). In wildtype animals bHLH transcription factor *Ngn2* is expressed in neuronal progenitors of the pallium (Fig. 3f) and is involved in maintaining dorsal identity of pallial progenitor cells (Fode et al., 2000; Sommer et al., 1996). In contrast, the pallium of *N8Cre/cLOF* mutants contained only a few cells expressing *Ngn2* (Fig. 3f'). We conclude that in the preneurogenic phase, the canonical Wnt signaling pathway is involved in maintaining the dorsal molecular identity of pallial progenitor cells.

### *Canonical Wnt signaling is required for suppressing ventral cell identities in preneurogenic pallial progenitor cells*

To examine whether canonical Wnt signaling is required to repress ventral cell fates in pallial progenitor cells, we analyzed the distribution of progenitor cells with subpallial identities in N8Cre/cLOF mutants. Coronal sections of the telencephalon at E11.5 were analyzed by RNA in situ hybridisation using the subpallial markers Nkx2.1, Gsh2, Mash1 and Dlx-2.

The homeobox transcription factors *Nkx2.1* and *Gsh2* are expressed in the subpallium (Figs. 4a, b, c) where they are required for the maintenance of the ventral character of subpallial cells (Corbin et al., 2000; Sussel et al., 1999; Toresson et al., 2000). While *Nkx2.1* expression is confined to the MGE in both wildtypes and *N8Cre/cLOF* mutants (Figs. 4a, a'), *Gsh2* was ectopically expressed in the pallium of the mutants (Figs. 4b', c'). Transcription factors *Mash1* and *Dlx2* are expressed in neuronal progenitors of the subpallium (Figs. 4d–g) and play important roles in neurogenesis and neuronal differentiation (Anderson et al., 1997; Bulfone et al., 1993; Casarosa et al., 1999; Guillemot and Joyner, 1993; Yun et al., 2002). Similar to *Gsh2*, both *Mash1* and *Dlx2* were ectopically expressed in pallial progenitor cells in the absence of canonical Wnt signaling (Figs. 4d'–g'). Thus, in the absence of canonical Wnt signaling in the telencephalon, pallial progenitors ectopically express transcription factors known to promote differentiation of subpallial neuronal phenotypes (Fode et al., 2000; Stuhmer et al., 2002). Next, we examined whether pallial progenitor cells that ectopically express supallial progenitor markers continue to differentiate along a path

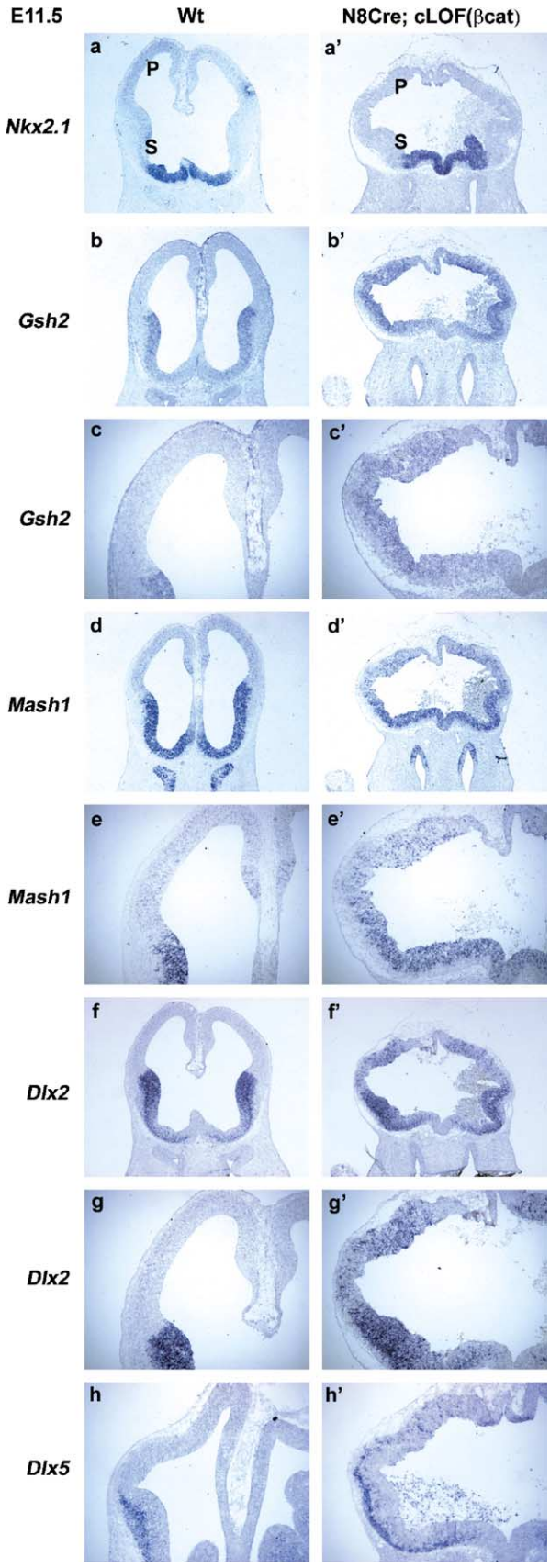


Fig. 4. Canonical Wnt signaling are required to repress expression of ventral markers in the pallium. RNA in situ hybridisation on coronal sections of *N8Cre*/β-catenin loss-of-function mutants (*N8Cre*/cLOF) and wildtype littermates at E11.5. *Nkx2.1* (a, a') was retained to its endogenous expression domain, while *Gsh2* (b, b', c, c'), *Mash1* (d, d', e, e'), *Dlx2* (f, f', g, g') and *Dlx5* (h, h') was expressed ectopically in the pallium. Abbreviations: P, Pallium; S, Subpallium.

typical for subpallial derived neurons. *Dlx5* is normally expressed in the subventricular zone (SVZ) and mantle zone (MZ) of the MGE and LGE, and has been suggested to be expressed in more differentiated cells compared to *Dlx2* and *Mash1* (Fig. 4h, Simeone et al., 1994; Yun et al., 2002). In addition, *Dlx5* has been shown to induce expression of glutamic acid decarboxylae 65 (GAD65), a marker for GABAergic neurons, when ectopically expressed in the cortex (Stuhmer et al., 2002). The ectopic upregulation of *Dlx5* in *N8Cre/cLOF* mutants suggests that in the absence of canonical Wnt signaling pallial progenitors are programmed to follow a differentiation path typical for subpallial cell derivates (Fig. 4h'). These data suggest that canonical Wnt signaling is required to restrict pallial progenitor cells from acquiring ventral characters.

### *β-catenin is not required for maintaining the molecular identity of pallial progenitor cells in the neurogenic period*

Our previous data suggest that canonical Wnt signaling is necessary for maintaining the identity of the preneurogenic pallium. To address if canonical Wnt signaling continues to be important for maintaining the dorsal identity during the neurogenic period, we generated a disruption of the *β-catenin* gene in the forebrain at the beginning of cortical neurogenesis (~E11)(Takahashi et al., 1995). To do so, we used a nestin enhancer based *Cre* recombinase driver line that induces recombination at E11 in the pallium, referred to as *Nes11Cre*. To analyze the timing and area of *Nes11Cre* recombination, the *Nes11Cre* line was crossed to the reporter line ROSA26 (Soriano, 1999). As seen in Fig. 5, *Nes11Cre* activity was first detected in a few cells of the anterior ventral telencephalon at embryonic day (E) 9.5 (Figs. 5a, d). At E11.5, *Nes11Cre* targets the entire subpallium and pallium except the hem and the choroid plexus (Figs. 5c, f).

The *Nes11Cre* line was crossed to the *β-catenin loxP exon2-6+/+* line (Brault et al., 2001). Subsequently, *Nes11Cre/β-catenin loxP exon2–6+/–* offsprings were crossed to *β-catenin loxP exon2-6+/+* mice to obtain homozygous loss-of-function mutant embryos (referred to as *N11Cre*/cLOF). Embryos were analyzed at E14.5 with the pallial markers *Pax6*, *Emx2*, *Emx1* and *Ngn2* and the subpallial markers *Mash1* and *Dlx2*. Similar to *N8Cre/*cLOF mutants, the expression of *Pax6* was maintained in the pallium of *N11Cre/*cLOF although the *Pax6* positive ventricular zone (VZ) is broader (Figs. 6a, a') likely due to morphological changes in the VZ as a consequence of defect cell-to-cell adherence as previously reported (Machon et al., 2003). In contrast to *N8Cre/*cLOF, the expression of *Emx1* and *Ngn2* was maintained in the pallium of *N11Cre/*cLOF mutants (Figs. 6c, c', d, d'), and no ectopic upregulation of *Mash1* and *Dlx2* expression in progenitor cells of the pallium was found at E14.5 (Figs. 6e, e', f, f'). However, a gradual downregulation of *Emx2* was noted in pallial progenitors of *N11Cre/*cLOF mutants (Figs. 6b, b'). This demonstrates that in the neurogenic phase, the overall expression profile of dorsal and ventral markers is maintained in the absence of canonical Wnt signaling. The reduced expression of *Emx2* in the cortical ventricular zone (VZ) is in line with data showing that *Emx2* expression is under control of canonical Wnt signals (Theil et al., 2002). Thus, canonical Wnt signaling is not required to maintain the overall molecular identity of pallial progenitors after E11.5.

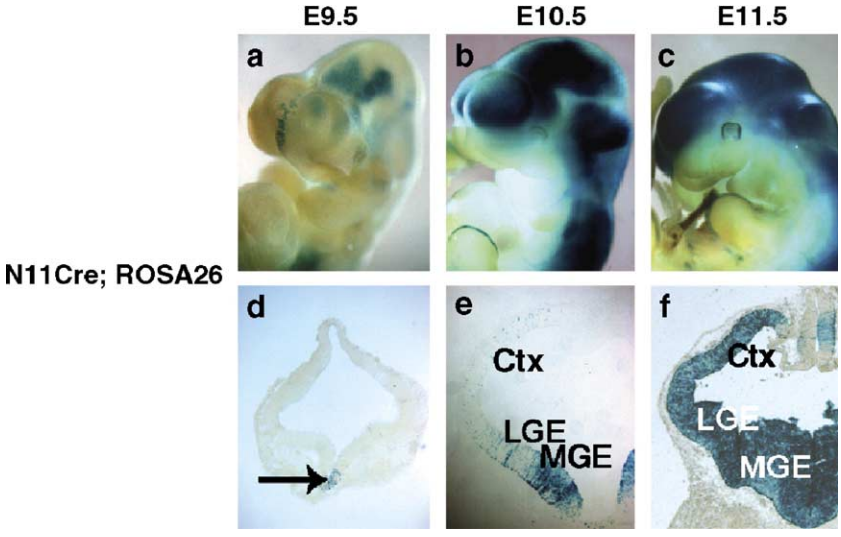


Fig. 5. Domains of *Nes11Cre* mediated recombination indicated by ROSA26 LacZ reporter line staining. (a–c) Whole mount stained *N11Cre*/ROSA26 embryos at E9.5 to E11.5. (d–f) Coronal sections of the telencephalon showing recombination by the *N11Cre* driver line between E9.5 to E11.5. (d) *N11Cre* activity in the anterior midline at E9.5 (arrow). (e) E10.5 strong LacZ staining in the MGE. (f) E11.5 recombination was high in both ventral and dorsal domains. Abbreviations: Ctx, cortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence.

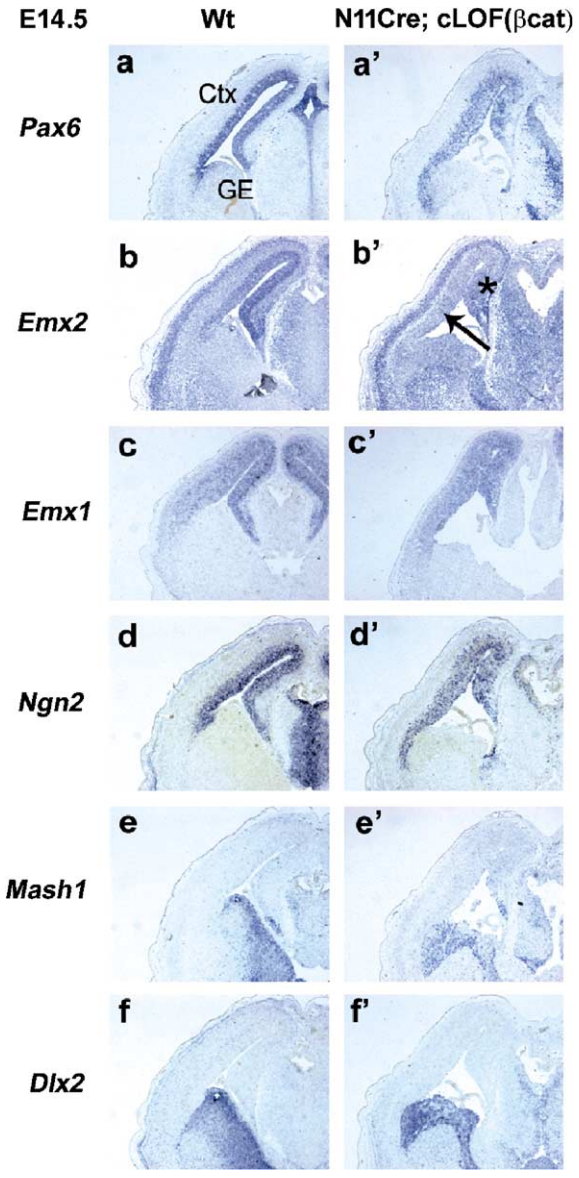


Fig. 6. Canonical Wnt signaling is not essential for maintaining the overall molecular integrity of the pallium during neurogenesis. RNA in situ hybridisation on coronal sections of *N11Cre*/β-catenin loss-of-function mutants (*N11Cre*/cLOF) and wildtype littermates at E14.5. Expression of pallial markers *Pax6* (a, a'), Emx1 (c, c') and *Ngn2* (d, d') remains present in the pallium of *N11Cre*/cLOF mutants, while *Emx2* (b, b') expression in the pallial VZ was downregulated (arrow in b'). Low expression of *Emx2* persists in the dorso-medial cortical wall (asterisk in b'). Expression of subpallial markers *Mash1* (e, e') and *Dlx2* (f, f') was not ectopically upregulated in the pallial progenitor zones of *N11Cre*/cLOF mutants. Abbreviations: Ctx, cortex; GE, ganglionic eminence.

### *Partial dorsalisation of the subpallium by constitutive active β-catenin*

We next examined whether canonical wnt signaling maintains the molecular integrity of the preneuronogenic pallium primarily by promoting dorsal characters or by suppressing ventral characters. To address this question, we expressed a dominant active form of β-catenin in the telencephalon and analyzed whether activation of the Wnt/β-catenin signaling pathway in the subpallium could alter the identities of ventral progenitors. The *Nes8Cre* line was crossed to transgenic mice in which exon 3 of the *β-catenin* gene is flanked by *loxP* sites (*β-catenin loxP exon3+/+*) to generate a conditional gain-of-function mutation of β-catenin (referred to as *N8Cre*/cGOF). Upon recombination, phosphorylation sites critical for the regulation of β-catenin degradation are deleted, resulting in a stabilisation of β-catenin, and a constitutive activation of downstream Wnt target genes (Harada et al., 1999).

The neuroepithelium of *N8Cre*/cGOF mutants was expanded throughout the CNS (Figs. 7a'–d' and 8a'–c'), an observation that is in line with previous reports (Chenn and Walsh, 2002; Galceran et al., 2000; Lee et al., 2000; Machon et al., 2003; Zechner et al., 2003). Sagittal sections of E11.5 *N8Cre*/cGOF mutants and wildtype littermates were analyzed by RNA *in situ* hybridisation using the telencephalic marker *Bf1* and the pallial markers *Pax6*, *Emx2* and *Ngn2*. *Bf1* was expressed in the dorsal and ventral telencephalic neuroepithelium of wildtypes. In *N8Cre*/cGOF mutants, *Bf1* expression persisted but was reduced in both dorsal and ventral domains (Figs. 7a, a'). Expression of *Pax6* was reduced but persisted in the mutant pallium (Figs. 7b, b'). Notably, the expression domain of *Pax6* expanded ventrally into the ganglionic eminence (Fig. 7b'), while *Emx2* expression remained restricted to the dorsal domain of *N8Cre*/cGOF mutants (Figs. 7c, c'). Interestingly, *Ngn2* expression was induced in the subpallium of *NesCre8*/cGOF mutants (Fig. 7d'). Similar affects were observed using the *Nes11Cre* driver line to delete exon 3 from β-catenin (referred to as *N11Cre*/cGOF). In situ hybridisation analysis of the telencephalon of *N11Cre*/cGOF mutants at E13 showed that *Bf1* expression persisted but was reduced where recombination was initiated (Figs. 7e, e'). *Emx1* and *Emx2* expression remained restricted to the dorsal domains (Figs. 7g, g', h, h'), while *Ngn2* was induced around the ectopic source of activated β-catenin in the subpallium (Figs. 7i, i'). *Pax6* on the other hand was not induced as such but its expression levels appeared to increase in the LGE of *N11Cre*/cGOF mutants (Figs. 7f, f'). Taken together, these data show that constitutive activation of the Wnt/β-catenin pathway in the subpallium can induce a partial dorsalisation of the subpallium as shown by the induction of *Ngn2* and the expanded expression domain of *Pax6*.

### *Repression of subpallial markers by constitutive active β-catenin*

To address the question whether canonical Wnt signaling can repress ventral characters in subpallial progenitor cells, the telencephalon of *N8Cre*/cGOF mutants were analyzed by RNA in situ hybridisation using the subpallial markers *Nkx2.1*, *Mash1*, *Dlx-2* (Bulfone et al., 1993; Casarosa et al., 1999; Guillemot and Joyner, 1993; Sussel et al., 1999). At

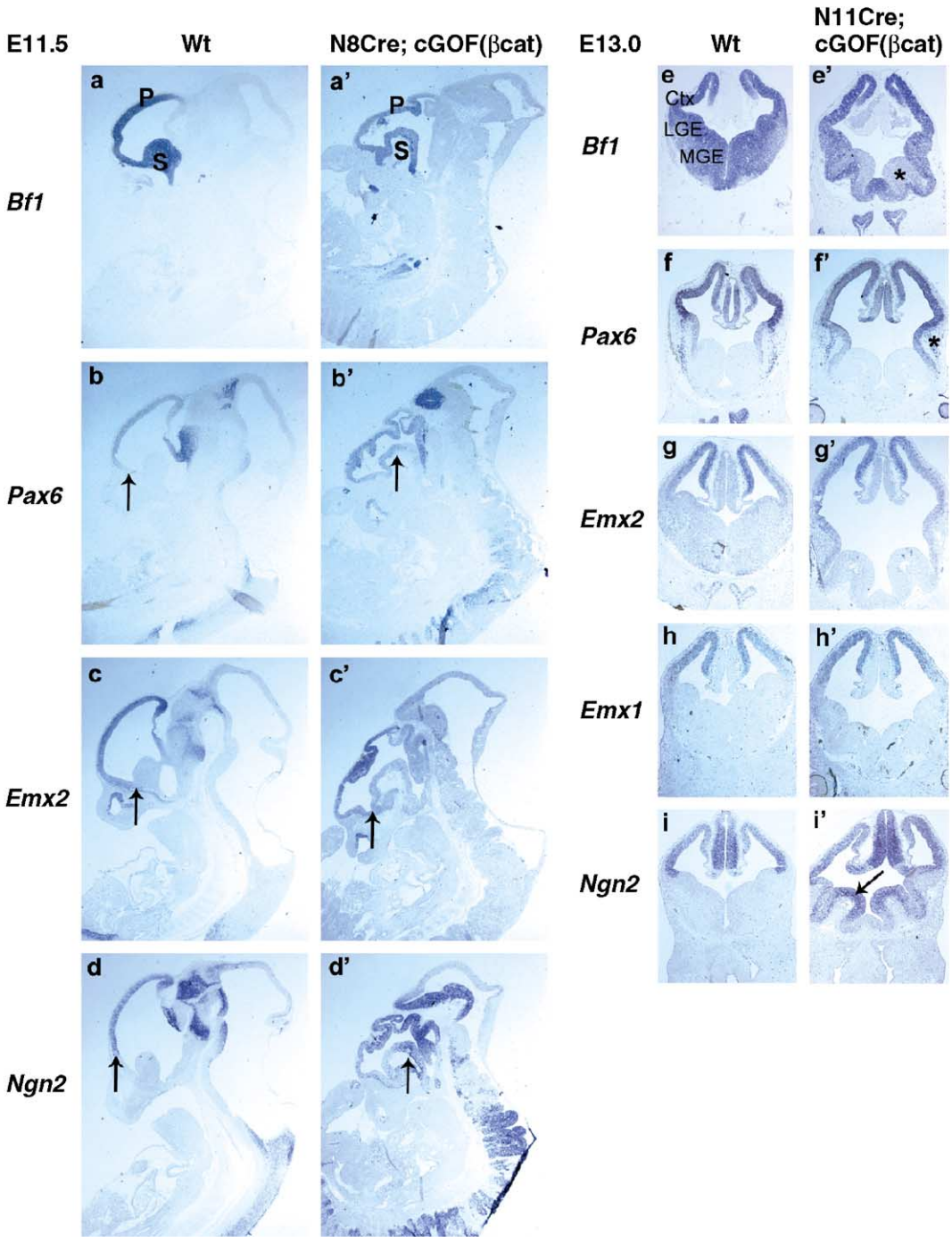


Fig. 7. Ectopic canonical Wnt signaling induces ectopic expression of *Ngn2*, but not *Pax6*, *Emx1*, or *Emx2* in progenitor cells of the subpallium of β-catenin gain-of-function (cGOF) mutants. (a–d) RNA in situ hybridisation on sagittal sections of *N8Cre*/cGOF mutants and wildtype littermates at E11.5. *Bf1* (a, a') expression was reduced in ventral and dorsal domains but persists. The domain of Pax6 expression (b, b') expands ventrally while Emx2 (c, c') was confined to its endogenous expression domains. Ngn2 expression was ectopically induced in the subpallium (d, d') (arrows in b–d denote ventral border of gene expression). (e–i) RNA in situ hybridisation on coronal sections of *N11Cre*/cGOF mutants and wildtype littermates at E13.0. Expression of *Bf1* (e, e') was reduced ventrally (asterisk in e'). *Pax6* (f, f') expression was slightly upregulated in the LGE of *N11Cre*/cGOF mutants (asterisk in f'). *Emx2* (g, g') and *Emx1* (h, h') expression was confined to their endogenous expression domains. *Ngn2* (i, i') was induced ectopically in the subpallium (arrow in i'). Abbreviations: Ctx, cortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; P, Pallium; S, Subpallium.

E11.5, *Nkx2.1* is expressed in the medial ganglionic eminence (MGE) of wildtype animals (Fig. 8a, Sussel et al., 1999). In *N8Cre*/cGOF mutants, the expression of *Nkx2.1* in the MGE was strongly reduced (Fig. 8a'). Similarly, expression of subpallial markers *Mash1* and *Dlx2* was greatly reduced and only detectable in the most caudal part of the subpallium of *N8Cre*/cGOF (Figs. 8b, b', c, c'). Although cells ectopically expressing *Ngn2* were

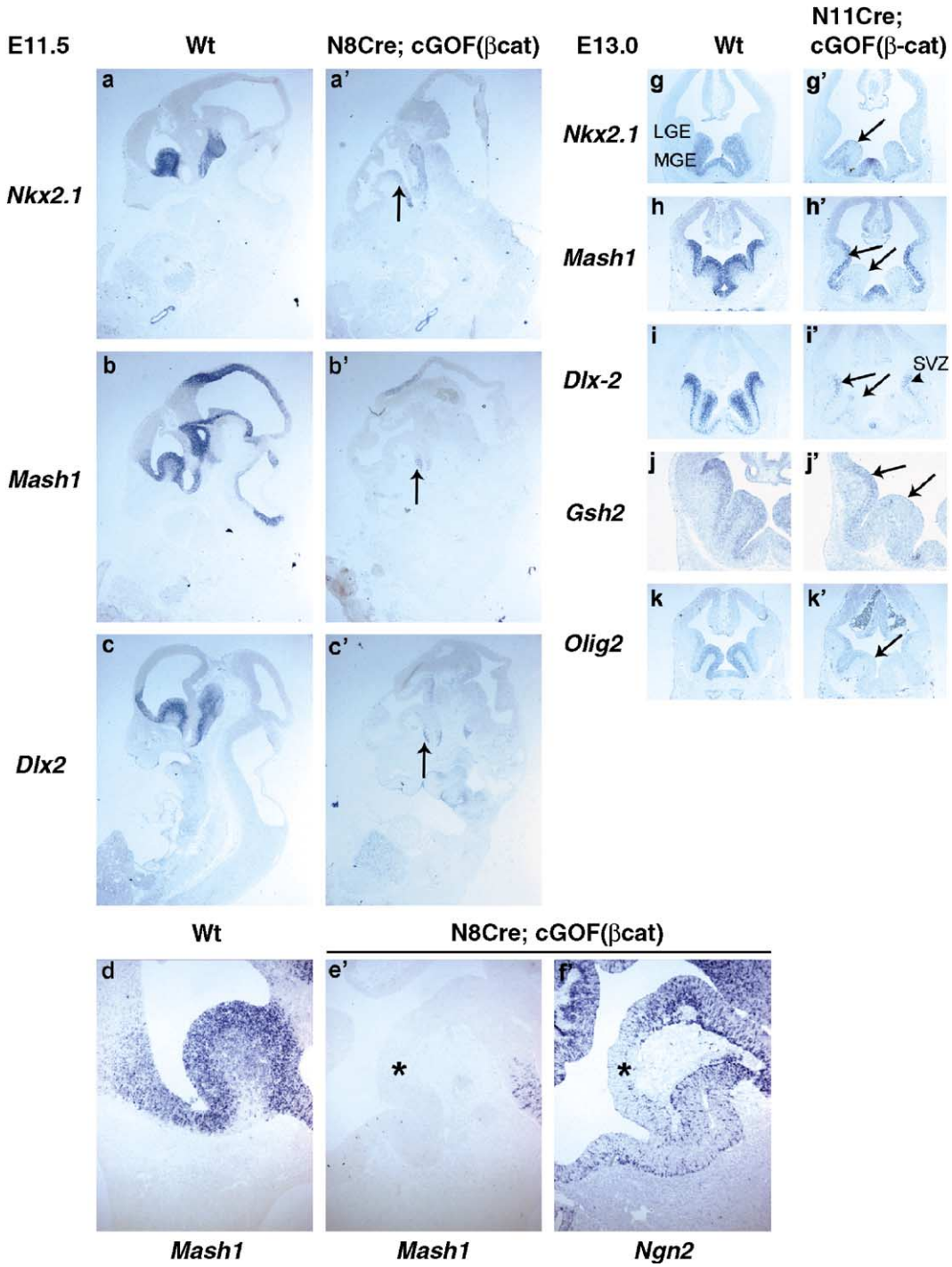


Fig. 8. Ectopic canonical Wnt signaling represses ventral cell identities in progenitor cells of the telencephalon in β-catenin gain-of-function (cGOF) mutants. (a–f) RNA in situ hybridisation on sagittal sections of *N8Cre*/cGOF mutants and wildtype littermates at E11.5. Expression of *Nkx2.1* (a, a'), *Mash1* (b, b') and *Dlx-2* (c, c') was greatly reduced in the subpallium of *N8Cre*/cGOF mutants (arrows denote areas of remaining expression). (d, e', f') Domains of *Mash1* repression (e') and ectopic *Ngn2* (f') expression was not entirely overlapping in *N8Cre*/cGOF mutants. (g–k) RNA in situ hybridisation on coronal sections of *N11Cre*/cGOF mutants and wildtype littermates at E13.0. Expression of *Nkx2.1* (g, g'), *Mash1* (h, h'), *Dlx-2* (i, i'), *Gsh2* (j, j'), and *Olig2* (k, k') was greatly reduced in the VZ of the MGE. Expression of *Mash1*, *Dlx2*, and *Gsh2* was reduced also in the VZ of the LGE (arrows in g'–k'). Expression of *Dlx2* in the SVZ of the LGE remained at levels similar as in wildtype animals (arrowhead in i'). Arrows denote sites of reduced expression. Abbreviations: MGE, medial ganglionic eminence; LGE, lateral ganglionic; SVZ, subventricular zone; VZ, ventricular zone.

found in the subpallium of cGOF mutants (Figs. 7d', i'), the repression of subpallial markers also occurred in domains were *Ngn2* was not ectopically activated (Figs. 8d, e', f'). Similar observations were made when the *Nes11Cre* driver line was used to delete exon 3 from β-catenin (*N11Cre*/cGOF), and thereby activating the canonical Wnt pathway

in the telencephalic neuroepithelium. In situ hybridization analysis of the telencephalon of *N11Cre*/cGOF mutants showed that the expression of *Nkx2.1* was reduced in the progenitor zone of the ventral MGE but remained expressed in the ventral midline (Figs. 8g, g'). The expression of pan ventral gene *Gsh2* (Corbin et al., 2000; Toresson et al., 2000) was reduced both in the MGE and the lateral ganglionic eminence (LGE) upon canonical Wnt signaling (Figs. 8j, j'). Moreover, *Mash1* and *Dlx-2* expression was not detectable in the VZ of the MGE but remained at reduced levels in the VZ of the LGE in *N11Cre*/cGOF mutants (Figs. 8h, h', i, i'). Expression of bHLH transcription factor *Olig2*, required for oligodendrocyte and motor neuron differentiation in the spinal cord (Lu et al., 2000, 2002; Zhou and Anderson, 2002), was also reduced in the subpallium of *N11Cre*/cGOF mutants (Figs. 8k, k'). Control experiments showed that *Pax6*, *Emx1* and *Ngn2* expression is maintained in the pallium of *N11Cre*/cGOF mutants at E16.5, suggesting that cell fate changes in *N11Cre*/cGOF mutants are specific to the subpallium (data not shown). Collectively, these observations suggests that canonical Wnt signals can suppress ventral cell fates in subpallial progenitor cells, and can do so independently of *Emx1*, *Emx2* and *Ngn2*.

## Discussion

### *β-catenin is required for maintaining the dorsal molecular identity of preneurogenic pallial progenitor cells*

Wnt signals are involved in patterning of the anterior neuroepithelium. At early stages of CNS development, antagonism of posteriorizing Wnt signals are required for the establishment the telencephalon (Houart et al., 2002; Mukhopadhyay et al., 2001; Yamaguchi, 2001). Furthermore, Wnt and Fgf8 signals are essential for the initial dorsal–ventral fate specification in the chicken telencephalon (Gunhaga et al., 2003). In this report, we demonstrate that canonical Wnt signaling is necessary for maintaining the molecular integrity of the pallium during the preneurogenic time period. By conditionally inactivating β-catenin in the telencephalic neuroepithelium, we show that β-catenin-mediated Wnt signals are required in the preneurogenic pallium to maintain the expression of dorsal markers *Emx1*, *Emx2* and *Ngn2*. Furthermore, these data demonstrate that canonical Wnt signaling is involved in maintaining the identity of the pallium by suppressing ventral genes (*Gsh2*, *Mash1*, *Dlx2*, *Dlx5*) in pallial progenitor cells. Interestingly, in addition to the upregulation of subpallial progenitor markers, we observed ectopic upregulation of *Dlx5* in the pallium of β-catenin loss-of-function (*N8Cre*/cLOF) mutants, suggesting that pallial progenitors continue to differentiate along a path typical to subpallial cell derivates in the absence of β-catenin mediated Wnt signals.

In contrast to the effects of canonical Wnt signaling during the preneurogenic phase, we find that β-catenin-dependent signals are dispensable for dorso-ventral cell fate specification after cortical neurogenesis is initiated (Takahashi et al., 1995). With the exception of *Emx2* that was downregulated in mice with deleted β-catenin (*N11Cre*/cLOF), expression of pallial markers *Emx1*, *Ngn2* and *Pax6*, and subpallial markers *Mash1* and *Dlx2* are maintained and restricted to their endogenous expression domains when β-catenin is deleted after E11. Thus, it seems that during neurogenesis, canonical Wnt signaling in the pallium is involved in regulating other aspects of cortical development such as differentiation (Hirabayashi et al., 2004; Viti et al., 2003) rather than specification of dorso-ventral cell identities.

### *β-catenin mediated Wnt signals repress ventral progenitor cell identities in the telencephalon*

The ectopic upregulation of subpallial markers *Gsh2*, *Mash1* and *Dlx-2* in the pallium of β-catenin *N8Cre/cLOF* mutants and their gradual reduction in the subpallium of β-catenin *N8Cre* and *N11Cre* GOF mutants, demonstrate that canonical Wnt signals can suppress ventral cell fates in telencephalic progenitor cells. One possible explanation would be that β-catenin-mediated signals repress expression of subpallial markers *Mash1*, *Gsh2* and *Dlx-2* directly or indirectly by regulating downstream effector(s). Since neither *Emx1* nor *Emx2* are induced in the subpallium of *N8Cre*/cGOF mutants and *Emx1/Emx2* double mutant mice do not display any dorsal to ventral fate shifts (Shinozaki et al., 2004), it is unlikely that these genes mediate the repressive actions of Wnt signals. On the other hand, the pallial-specific gene *Ngn2* (Sommer et al., 1996) is upregulated in the subpallium of *N8Cre* and *N11Cre*/cGOF mutants, and could in principle be a factor that downregulates supallial identities. The upregulation of *Dlx-2* and *Mash1* expression in the pallium of β-catenin *N8Cre*/cLOF mutants could be explained by the downregulation of *Ngn2*. In support for this interpretation, *Ngn2* knockout mice also display upregulation of *Dlx-2* and *Mash1* expression in the pallium (Fode et al., 2000). However, replacement of *Mash1* by *Ngn2*, using a knock in strategy, has been demonstrated that *Ngn2* alone cannot repress expression of subpallial markers (Parras et al., 2002). Furthermore, although ectopic *Ngn2* cells were found in the subpallium of *N8Cre*/cGOF mutants, we observed repression of subpallial markers in areas were *Ngn2* were not ectopically activated. Thus, it seems that canonical Wnt signals can repress ventral characters independent of *Emx1*, *Emx2* and *Ngn2*.

Another possible explanation for the observed alteration of gene expression in the pallium could be that the canonical Wnt signaling pathway act on a wider scale to suppress ventralizing programs from being activated in the pallium. Notably, transcription factor Gli3 and morphogen Shh have antagonizing functions in dorsal–ventral cell fate specifica-

tion in the telencephalon. While Gli3 promotes dorsal fates by restricting Shh ventralizing activities from being propagated in the pallium, Shh antagonizes Gli3 dorsalizing activities in the subpallium (Kuschel et al., 2003; Rallu et al., 2002b). Extra-toes mutants Xt(J), which carry a deletion in the Gli3 gene (Hui and Joyner, 1993), display a dorsal to ventral fate shift as shown by ectopic expression of subpallial markers *Mash1* and *Dlx-2* in the pallium (Kuschel et al., 2003; Rallu et al., 2002b; Tole et al., 2000). This alteration is most pronounced anteriorly (Kuschel et al., 2003; Tole et al., 2000), similarly to *N8Cre*/cLOF mutants. Moreover, expression of *Ngn2* is reduced anteriorly while *Emx1* and *Emx2* are reduced throughout the cortex of Xt(J) mutants (Kuschel et al., 2003; Theil et al., 1999; Tole et al., 2000). The expression of a number of *Wnt* genes in the hem, such as *Wnt2b*, *Wnt3a*, *Wnt7b* and *Wnt 5a*, is reduced in Xt(J) mutants suggesting that Gli3 function is required for the expression of *Wnt* genes in the hem (Grove et al., 1998; Theil et al., 2002). Based on these data and our own observations, it is tempting to speculate that Wnt signals from the hem, mediated through β-catenin, act downstream of *Gli3* to repress Shh ventralizing activities in the pallium (Fig. 9). Notably, members of the Wnt family have been shown to be targets of Gli2/3 and also mediators of Gli induced posterior mesodermal development in frogs (Mullor et al., 2001). Since Wnt signals display a high caudal to low rostral expression in the pallium (Maretto et al., 2003), it may be expected that the caudal part of the telencephalon would be more severely affected in LOF mutants in terms of dorsal–ventral cell fate specification. The fact that this does not occur could be explained if posterior telencephalic progenitors were more sensitive to Shh signals than caudal progenitors. This explanation is supported by experiments by Rallu et al. (2002a,b), showing that the anterior pallium respond to ectopic Shh signals (Activated Smoothened) by up-regulating pan ventral marker *Gsh2* while posterior domains do not (Rallu et al., 2002b).

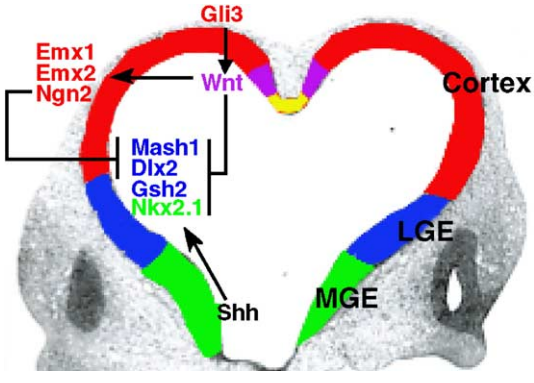


Fig. 9. β-catenin mediated Wnt signals are required to maintain the molecular identity of the pallium in the preneurogenic period. Canonical Wnt signals maintain the expression of dorsal markers *Emx1*, *Emx2* and *Ngn2* and repress ventral markers *Gsh2*, *Mash1* and *Dlx2* from being activated in the pallium. Gain-of-function experiments suggest that canonical Wnt signals can repress ventral telencephalic cell identities (*Nkx2.1*, *Gsh2*, *Mash1*, *Dlx2*) independent of *Ngn2*.

### *Constitutive active β-catenin maintain expression of dorsal markers Emx1, Emx2 and Ngn2*

Analysis of β-catenin *N8Cre*/cLOF mutants demonstrates that canonical Wnt signaling is required to maintain expression of *Emx1*, *Emx2* and *Ngn2* during the preneurogenic phase. Previous studies have shown that pallial markers including *Ngn2*, persists in *Emx1/Emx2* double mutant mice (Shinozaki et al., 2004), and *Pax6*, *Ngn2* and *Emx1* expression persist in *Emx2* knockout mice (Muzio et al., 2002; Yoshida et al., 1997). Moreover, *Pax6*, *Emx1* and *Emx2* expression is retained in *Ngn2* knockout mice (Fode et al., 2000). Collectively, these reports have shown that the expression of *Emx1/2* and *Ngn2* are not dependent on the expression of each other, suggesting that Wnt signals regulate the expression of these dorsal factors in a non-linear fashion. In support for this interpretation, expression of *Emx1* and *Emx2* was reduced throughout the cortex, while the reduction of *Ngn2* was most pronounced anteriorly. Furthermore, it has been demonstrated that pallial *Emx2* expression is regulated by TCF binding sites in the promoter (Theil et al., 2002). Previous studies have shown that *Pax6* directly regulates the expression of *Ngn2* in regions of the pallium with high expression of *Pax6* (Scardigli et al., 2003). However, *Pax6* is not necessary for the induction of *Ngn2* in a subset of cells in the pallial VZ, suggesting that other factors induce the expression of *Ngn2* in this subset of cells (Stoykova et al., 2000). Notably, *Pax6* expression remains expressed in *N8Cre*/cLOF mutants indicating that pallial *Ngn2* expression is also dependent on canonical Wnts signals. Moreover, *N8Cre* and *N11Cre* β-catenin gain-of-function mutants express *Ngn2* ectopically in the subpallium suggesting that *Ngn2* is directly regulated by canonical Wnt signals. In line with these data, a recent report has indicated that *Ngn2* is a target gene of β-catenin mediated signals (Israsena et al., 2004).

Analysis of *N8Cre* and *N11Cre* β-catenin gain-of-function mutants suggests that β-catenin-mediated signals alone cannot induce full dorsal identity in subpallial progenitors of the mouse telencephalon at the analyzed stages. While *Ngn2* is induced around the ectopic source of activated β-catenin in the supallium of *N8Cre* and *N11Cre*/cGOF mutants, neither *Emx1*, *Emx2* nor *Pax6* were induced in a similar fashion. Although *Pax6*-positive domains extended further into ventral domains in these mutants, this expansion could be explained by to the loss of *Gsh2* expression in the LGE, which has been shown to be required to antagonize *Pax6* expression in the LGE (Corbin et al., 2000; Toresson et al., 2000). One possible explanation is that other factors acting in concert with, or independently of canonical Wnt signaling, are necessary to induce full dorsal identity in the subpallium. Notably, Wnt signals can induce full dorsal telencephalic identity in avian dorsal telencephalic explants only in the presence of Fgf8 (Gunhaga et al., 2003). Another possible explanation would be that the

dorsalizing activities of Wnt signals are mainly attributed to its function in repressing ventralizing signals.

An alternative explanation of the gain-of-function data would be that β-catenin rather than having an instructive role in dorso-ventral patterning, inhibits the development of later sets of progenitors expressing markers (*Mash1*, *Dlx2*, *Ngn2*) (Chenn and Walsh, 2002; Zechner et al., 2003). Nevertheless, we favor the idea that these changes reflect the role of the canonical Wnt signaling in dorso-ventral patterning. First, cortical progenitors maintain expression of early pallial markers *Emx1* and *Pax6* at reduced levels at E16.5 (not shown), while the expression of the early ventral marker *Nkx2.1* is downregulated at E12.0 in *N11Cre*/cGOF mutants. This indicates that the down-regulation of early progenitor markers is specific to the subpallium. Second, the late pallial marker *Ngn2* remains expressed in the pallium at E16.5 suggesting that the development of late progenitors are not inhibited in the pallium of *N11Cre*/cGOF mutants. Pallial expression of *Ngn2* is induced at the start of cortical neurogenesis (~E10.5), suggesting that *Ngn2* is a hallmark of a relatively late set of progenitor cells (Sommer et al., 1996). Third, *Ngn2* expression is induced ectopically in ventral progenitors that suggest that late progenitor cells develop in the subpallium although their identity is shifted towards a dorsal identity.

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