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/\textsuperscript{loxP} system in mouse to ablate or activate $\beta$-catenin in the telencephalon in two time windows: before and after the onset of neurogenesis. We show that $\beta$-catenin mediated Wnt signals are required to maintain the molecular identity of the pallium. Inactivation of $\beta$-catenin in the telencephalon before neurogenesis results in downregulated expression of dorsal markers \textit{Emx1, Emx2} and \textit{Ngn2}, and in ectopic up-regulation of ventral markers \textit{Gsh2, Mash1} and \textit{Dlx2} in the pallium. In contrast, ablation of $\beta$-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 \textit{Dlx2, Nkx2.1, Gsh2, Olig2} and \textit{Mash1}. This was accompanied with an expansion of dorsal identities ventrally as shown by the expanded expression domains of pallial markers \textit{Pax6} and \textit{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.

Keywords: $\beta$-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
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+/- mice (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, βcatFl1ex2: 5'-GCTGGTGGCAATGGCTCAT-3' and βcatR1ex3: 5'-GCTTTTCTGTCCGGCTCAT-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-
GAGTGAAGGTGTGTT-3' and RM42: 5'-CACCATGTCCTCTGTCTATTCC-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'–GTCTAATTCTGACCGTACACCC-3' ; Cre32: 5'–GAAGCATGTGTTAGCGGC-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 MgCl2 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 MgCl2, 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 ferrocyanide, 5 mM potassium ferrocyanide, 20 mM Tris pH7.3, and 1 mg/ml X-gal), or cryoprotected in 30% sucrose, embedded and frozen in OCT and sectioned at 10–14 μm.

**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. Guillelmo (Ngn2; Mash1); P. Gruss (Pax6); E. Lai (Bfl1); A. McMahon (Shh); G. Martin (Fg8); 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/Ca2+-pathway; (ii) the planar cell polarity pathway; (iii) a pathway that regulates spindle orientation and asymmetric cell division; (iv) the canonical Wnt pathway (Huelsen 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,
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 NesCre/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 NesCre/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 NesCre/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. 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.
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...
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 Dlx2.

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 supapallial progenitor markers continue to differentiate along a path
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 derivatives (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.
Partial dorsalisation of the subpallium by constitutive active \(\beta\)-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 \(\beta\)-catenin in the telencephalon and analyzed whether activation of the Wnt/\(\beta\)-catenin signaling pathway in the subpallium could alter the identities of ventral progenitors. The \(Nes8\text{Cre}\) line was crossed to transgenic mice in which exon 3 of the \(\beta\)-catenin gene is flanked by \(loxP\) sites (\(\beta\)-catenin \(loxP\) exon3\(^{+/+}\)) to generate a conditional gain-of-function mutation of \(\beta\)-catenin (referred to as \(N8\text{Cre}/c\text{GOF}\)). Upon recombination, phosphorylation sites critical for the regulation of \(\beta\)-catenin degradation are deleted, resulting in a stabilisation of \(\beta\)-catenin, and a constitutive activation of downstream Wnt target genes (Harada et al., 1999).

The neuroepithelium of \(N8\text{Cre}/c\text{GOF}\) 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 \(N8\text{Cre}/c\text{GOF}\) mutants and wildtype littermates were analyzed by RNA in situ hybridisation using the telencephalic marker \(Bf1\) and the pallial markers \(Pax6\), \(Emx2\) and \(Ngn2\). \(Bf1\) 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 \(N8\text{Cre}/c\text{GOF}\) mutants (Figs. 7c, c’). Interestingly, \(Ngn2\) expression was induced in the subpallium of \(Nes\text{Cre8}/c\text{GOF}\) mutants (Fig. 7d’). Repression of subpallial markers by constitutive active \(\beta\)-catenin

To address the question whether canonical Wnt signaling can repress ventral characters in subpallial progenitor cells, the telencephalon of \(N8\text{Cre}/c\text{GOF}\) 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
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
found in the subpallium of cGOF mutants (Figs. 7d', i'), the repression of subpallial markers also occurred in domains where 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 suggest that canonical Wnt signals can suppress ventral cell fates in subpallial progenitor cells, and can do so independently of Emx1, Emx2 and Ngn2.

Discussion

\( \beta \)-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 \( \beta \)-catenin in the telencephalic neuroepithelium, we show that \( \beta \)-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 \( \beta \)-catenin loss-offunction (N8Cre/cLOF) mutants, suggesting that pallial progenitors continue to differentiate along a path typical to subpallial cell derivatives in the absence of \( \beta \)-catenin mediated Wnt signals.

In contrast to the effects of canonical Wnt signaling during the preneurogenic phase, we find that \( \beta \)-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 \( \beta \)-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 \( \beta \)-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.

\( \beta \)-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 \( \beta \)-catenin N8Cre/cLOF mutants and their gradual reduction in the subpallium of \( \beta \)-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 \( \beta \)-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 (Sommmer 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 \( \beta \)-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 X(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 X(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 Wnt5a, is reduced in X(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).

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 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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