The Wnt/β-catenin pathway directs neuronal differentiation of cortical neural precursor cells

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Summary

Neural precursor cells (NPCs) have the ability to self-renew and to give rise to neuronal and glial lineages. The fate decision of NPCs between proliferation and differentiation determines the number of differentiated cells and the size of each region of the brain. However, the signals that regulate the timing of neuronal differentiation remain unclear. Here, we show that Wnt signaling inhibits the self-renewal capacity of mouse cortical NPCs, and instructively promotes their neuronal differentiation. Overexpression of Wnt7a or of a stabilized form of β-catenin in mouse cortical NPC cultures induced neuronal differentiation even in the presence of Fgf2, a self-renewal-promoting factor in this system. Moreover, blockade of Wnt signaling led to inhibition of neuronal differentiation of cortical NPCs in vitro and in the developing mouse neocortex. Furthermore, the β-catenin/TCF complex appears to directly regulate the promoter of neurogenin 1, a gene implicated in cortical neuronal differentiation. Importantly, stabilized β-catenin did not induce neuronal differentiation of cortical NPCs at earlier developmental stages, consistent with previous reports indicating self-renewal-promoting functions of Wnts in early NPCs. These findings may reveal broader and stage-specific physiological roles of Wnt signaling during neural development.

Key words: β-catenin, Wnt, Neurogenesis, Neocortex, Neural precursor cell, Mouse

Introduction

Neurons and glia in the central nervous system originate from common precursor cells (neural precursor cells, or NPCs) that proliferate in the ventricular zone (VZ) of the fetal brain and spinal cord (Temple, 2001). In the developing mouse brain, NPCs give rise to neurons mostly between embryonic day (E) 10 and E17, and to astroglia after birth (Qian et al., 2000). The balance between proliferation and differentiation of NPCs is essential in determining the size of each region within the brain. This fate decision is made by a complex interplay between extrinsic signals and intrinsic genetic mechanisms. Growth factors such as fibroblast growth factor (Fgf) 2 and epidermal growth factor (Egf), as well as activation of the transmembrane receptor Notch, inhibit neuronal differentiation and promote the self-renewal capacity of NPCs (Johe et al., 1996; Panchision and McKay, 2002; Temple and Qian, 1996). Neuronal differentiation involves the proneural basic helix-loop-helix (bHLH) transcription factors. For example, the bHLH proteins neurogenin (Ngn) 1 and Ngn2 are essential for neurogenesis in the neocortex (Schuurmans and Guillemot, 2002). The Notch signaling inhibits neuronal differentiation through a mechanism mediated by the bHLH proteins Hes1 and Hes5, which inhibit neurogenesis by antagonizing the proneural bHLH proteins (Ohtsuka et al., 2001). However, relatively little is known about the extrinsic cues that 'trigger' neuronal differentiation during cortical development. Previous studies have suggested that platelet-derived growth factor (Pdgf) cause an increase in neuronal cell number in cortical cultures, but this effect was shown to be the result of selective expansion of immature neurons (neuronal progenitors), rather than from instruction of neuronal fate (Erlandsson et al., 2001; Johe et al., 1996; Williams et al., 1997). Although erythropoietin appears to induce de novo neurogenesis in the adult brain in response to hypoxic insults (Shingo et al., 2001), its involvement in embryonic neurogenesis has not been demonstrated.

Wnt genes encode secreted factors that regulate various cell fate decisions depending on the cellular context. In mammals, 19 Wnt genes have been identified to date, and several of these (such as Wnt7a, Wnt7b, Wnt2b and Wnt8b) are expressed during cortical development in complex spatiotemporal patterns (Fougerousse et al., 2000; Grove et al., 1998; Kim et al., 2001a; Lee et al., 2000). Wnt proteins signal through a receptor complex composed of members of the Frizzled (Fz) and low-density lipoprotein receptor-related protein (Lrp) families, and activate a number of intracellular signaling pathways including the β-catenin/TCF pathway (known as the canonical Wnt pathway) (Brantjes et al., 2002; Wodarz and

Nusse, 1998) and non-canonical pathways, including those mediated by Jun N-terminal kinase (Jnk) or Ca^{2+} (Habas et al., 2003; Huelsken and Birchmeier, 2001; Kuhl et al., 2000). Recent reports have shown that the canonical Wnt pathway promotes the self-renewal capacity of some tissue stem cells such as hematopoietic stem cells (Reya et al., 2003; Willert et al., 2003). The Wnt signals have also been implicated in promoting self-renewal during neural development; ectopic expression of Wnt1 or stabilized β-catenin in the early stages of chick spinal cord or mouse forebrain development, respectively, has been shown to result in an increased number of NPCs and suppression of neuronal differentiation (Chenn and Walsh, 2002; Megason and McMahon, 2002). Infection of mouse forebrain explants with a retrovirus expressing HA epitope-tagged Wnt7a also promoted proliferation and suppressed neuronal differentiation of NPCs (Viti et al., 2003). However, activation of a TCF-dependent reporter gene construct was detected in differentiating neurons of mouse cortical development (Maretto et al., 2003), implying a potential role of the canonical Wnt pathway in the differentiation of cortical neurons. In addition, Wnt1 promotes neuronal differentiation of the embryonic carcinoma cell line P19 (Lyu et al., 2003; Smolich and Papkoff, 1994; Tang et al., 2002). It has remained unclear, however, whether Wnt is important for promoting neuronal differentiation in the developing neocortex.

In contrast to the recent reports mentioned above that indicate a role for Wnts in the expansion of NPCs, we now show that Wnt7a promotes neuronal differentiation of NPCs in the developing mouse neocortex, at the expense of NPCs. Inhibition of the canonical Wnt pathway suppressed neuronal differentiation in vitro and in the developing neocortex. This effect appears to be mediated at least in part by direct regulation of *Ngn1* promoter by the β-catenin-TCF complex. Importantly, Wnt signaling promotes neuronal differentiation only at late stages of cortical development. These results demonstrate that Wnt7a or related Wnt proteins may play an essential role in triggering neurogenesis during cortical development in a stage-specific manner and imply that the timing of differentiation can be determined not only by the expression of differentiation-inducing factors, but also by the responsiveness of the cells to these factors.

Materials and methods

Expression constructs and antibodies

The plasmid pEF-BOS-Wnt7a and pMX-IRES-EGFP were kindly provided by M. Nakafuku and T. Kitamura, respectively. Dkk1 and Fz5 cDNAs were amplified by PCR from a mouse embryonic brain cDNA library and their coding regions were verified by DNA sequencing. These cDNAs were subcloned into pcDNA3 (Invitrogen), pMX-IRES-EGFP [a replication-incompetent retroviral vector containing an internal ribosome entry site (IRES) sequence followed by the coding sequence for enhanced GFP] (Morita et al., 2000) or $pCS2^{+}$. pMX-Wnt7aHA-IRES-EGFP was constructed by introducing an HA epitope at the C terminus of Wnt7a, according to the sequence of Wnt7aHA used by Viti et al. (Viti et al., 2003) (pUSEamp Wnt-7a, Upstate Biotechnology). We confirmed the expression of Wnt7aHA protein by western blotting with an anti-HA antibody (Santa Cruz). The β-catenin mutant ΔN90 (Barth et al., 1997) was amplified by PCR from the β-catenin cDNA and verified by DNA sequencing. The MKK7-JNK fragment was constructed by ligation of the Flag-JNK1 cDNA into the pEF-Flag-MKK7 plasmid, and was subcloned into pMX-IRES-EGFP.

The pMX-IRES-EGFP vector was used for the production of recombinant retroviruses. Ecotropic virus-packaging (PLAT-E) cells were transfected with the desired plasmids with the use of FuGENE 6 (Roche) and then cultured for 3 days at 32°C. Retroviral particles were collected from the culture supernatant by centrifugation at 6000 ***g*** for 16 hours at 4°C and were resuspended in serum-free Dulbecco's modified Eagle's medium (DMEM)–F12 (1:1) (Invitrogen).

Primary antibodies used in this study included mouse monoclonal antibodies to βIII-tubulin (TuJ1, Babco), to GFAP (Chemicon), to Nestin (Becton Dickinson) and to HuC/D (Molecular Probes), as well as rabbit polyclonal antibodies to the 150 kDa neurofilament protein (Chemicon) and to GFP (MBL). Alexa-labeled secondary antibodies were from Molecular Probes.

Primary NPC culture and immunostaining

Primary NPCs were prepared from the dorsal cerebral cortex of ICR mouse embryos at E11.5 (E1 was defined as 12 hours after detection of the vaginal plug). Dissected cortices were transferred to artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 10 mM glucose) containing 0.05% trypsin (Sigma) and incubated for 10 minutes on ice to remove overlying epidermal ectoderm. The cortices were then transferred to aCSF containing 0.1% trypsin, DNase I (0.1 mg/ml) (Roche) and hyaluronidase (0.67 mg/ml) (Sigma), and incubated at 37°C for 10 minutes. After the addition of an equal volume of aCSF containing trypsin inhibitor (0.7 mg/ml) (Sigma), the neuroepithelium was transferred to DMEM-F12 (1:1) and mechanically dissociated into single cells. The dissociated cells were cultured in DMEM-F12 (1:1) supplemented with B27 (Invitrogen), Fgf2 (20 ng/ml) (Roche) and Egf (20 ng/ml) (Upstate Biotechnology). For retroviral infection, cells were mixed with recombinant viruses for 24 hours, washed with phosphate-buffered saline (PBS) and then incubated in the absence or presence of Fgf2. For immunostaining, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 for 30 minutes, incubated with primary antibodies for 2 hours and then with secondary antibodies for 30 minutes, and mounted in Mowiol (Calbiochem).

Clonal analysis

NPCs (7.9×10^2 cells/mm^2) were plated on dishes coated with poly-D-lysine and infected with retroviruses encoding either GFP alone (control) or both GFP and S33Y β-catenin at a low titer (0.21 infected cells/mm^2). To confirm each colony was a 'clone' generated from a single cell, we infected NPCs with a mixture of retroviruses encoding GFP and those encoding DsRed2 at the same titer. After incubation for 3 days, no colonies in the culture contained both GFP^{+} cells and $DsRed2^{+}$ cells, showing that each colony was derived from a single cell under this condition.

In utero electroporation

Introduction of plasmid DNA into the neuroepithelial cells of mouse embryos in utero was performed as described (Tabata and Nakajima, 2001). Plasmid DNA for histone H2B-GFP (pH2B-EGFP) (0.1 mg/ml) and the test plasmid (0.4 mg/ml) were injected into the lateral ventricle of each littermate at E13.5; in other experiments, the pMX-based vectors were injected at a concentration of 5 μg/μl. Electrodes were placed flanking the equivalent ventricular region of each embryo, covered with a drop of PBS and pulsed 8 times at 40 V for 50 ms separated by intervals of 950 ms with an electroporator (CUY21E; Tokiwa Science). The uterine horn was placed back into the abdominal cavity to allow the embryos to continue development. Two days after electroporation, the embryos were harvested, and the brains were removed and fixed with 4% paraformaldehyde in PBS at 4°C overnight. After equilibration with 30% (w/v) sucrose in PBS, the fixed tissue was embedded in OCT compound (Sakura) and frozen.

Coronal sections were prepared by cutting the frozen brain with a cryostat (CM1850, Leica) at a thickness of 6 or 10 μm and mounted on glass slides coated with MAS (Matsunami). Sections with a thickness of 6 μm were used for quantitative analysis.

Chromatin immunoprecipitation assay

Primary NPCs were cultured in suspension for 3 days, after which neurospheres were collected and dissociated. The dissociated cells were plated on dishes coated with poly-D-lysine and harvested after incubation for 2 days. The cells were suspended in lysis solution [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)] and sonicated to shear genomic chromatin into DNA fragments of ~0.5 to 1.0 kb. The lysate was incubated for 2 hours with protein A-conjugated beads, after which the beads were removed and the lysate was incubated overnight at 4°C with antibodies to β-catenin or control IgG (Santa Cruz Biotechnology). After the addition of protein A beads, the mixture was incubated with rotation for 1 hour. The beads were then isolated and washed consecutively with a low-salt solution [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], with a high-salt solution [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], with a LiCl solution [0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)] and twice with a Tris-EDTA solution [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. Immune complexes were then eluted from the beads with a solution containing 10 mM dithiothreitol, 1% SDS and 0.1 M $NaHCO_3$, after which NaCl was added to a final concentration of 0.2 M and the elute was incubated at 65°C overnight to induce the dissociation of proteins from DNA. The proteins were eliminated by digestion with proteinase K at 45°C for 1 hour and the DNA was purified with a QIAquick spin column (Qiagen). PCR was performed with the eluted DNA and primers specific for the TCF binding element within the promoter region of the mouse Ngn1 gene (forward, 5′-CTGCCCAAGA-GCTGCTACAGAGGG-3′; reverse, 5′-GCCGGACAGCAATAGAG-GCTCAGG-3′). The PCR products were labeled by incorporation of [α-^{32}P]dCTP and were detected by electrophoresis on a 5% polyacrylamide gel and autoradiography. The PCR reaction was performed within the range that the PCR products increased approximately twofold every cycle.

Luciferase assay

NPCs (8.0×10^5 cells/ml) were plated on dishes coated with poly-D-lysine and transfected with expression plasmids and the pRL-TK plasmid encoding *Renilla* luciferase (Promega) with the use of Lipofectamine 2000 (Invitrogen). Cell extracts were subsequently prepared and assayed for luciferase activity (Toyo Ink). Firefly luciferase activity was normalized relative to the activity of *Renilla* luciferase.

Quantitative analysis of immunohistochemistry

Immunohistochemistry of the brain sections was carried out as described (Shen et al., 2002). Quantitative analysis of marker expression was performed by Laser Scanning Cytometry (LSC, Olympus) as follows. The area of the nucleus of each electroporated cell was outlined automatically from the fluorescence of histone H2B-GFP with the use of a CCD camera and LSC software. Within each outlined area, the integral of the fluorescence intensity of the Alexa633-conjugated secondary antibodies was measured. This analysis was performed doubly blind with several sections, and a total of at least 200 electroporated cells were examined per sample. As LSC software can show the location of each electroporated cell in the neocortex and its fluorescence intensity, we determined the approximate expression level of the marker proteins at the border between the ventricular zone (VZ) and the intermediate zone (IMZ). We confirmed that the cells exhibiting lower and higher expression levels of the neuronal marker proteins relative to the expression level at this border were predominantly localized in the VZ or in the IMZ and cortical plate, respectively (data not shown). Other fluorescence images were obtained with a confocal laser microscope (LSM510, Zeiss).

RT-PCR

Total RNA was obtained from infected NPCs using TRIzol (Invitrogen) following the instructions of the manufacturer. Reverse transcription (RT) was performed with 10 μg of total RNA, oligo $d(T)_{12\text{-}18}$ (Invitrogen) primers and ReverTra Ace (TOYOBO). cDNA was amplified by PCR using ex Taq (TaKaRa). The sense and antisense primers used were as follows: neurogenin 1, sense 5′-ATGCCTGCCCCTTTGGAGAC-3′ and antisense 5′-TGCATGCGG-TTGCGCTCGC-3′; and Gapdh, sense 5′-CATTGACCTCAAC-TACATGG-3′ and antisense 5′-TTGCCCACAGCCTTGGCAGC-3′. PCR products were labeled by adding [α-^{32}P] dCTP, separated on a 5% polyacrylamide gel, and detected by autoradiography.

Results

The canonical Wnt pathway promotes neurogenesis in cortical NPC culture

To identify extrinsic cues that might specify neuronal development in the neocortex, we examined whether Wnt signaling is able to promote cortical neuronal differentiation. Given that Wnt7a and Wnt receptors Fz5 and Fz8 are expressed in the VZ of the developing neocortex (Borello et al., 1999; Fougerousse et al., 2000; Grove et al., 1998; Kim et al., 2001b; Lee et al., 2000), we first investigated the effect of ectopic expression of Wnt7a in cortical NPCs in vitro. Dissociated neuroepithelial cells derived from the neocortex of mouse embryos at E11.5 were cultured as a suspension for 3 days in the presence of Fgf2 and Egf to facilitate removal of differentiated cells and enrichment of NPCs. The resulting neurospheres were dissociated, plated onto coverslips coated with poly-D-lysine and infected with retrovirus harboring green fluorescence protein (GFP). Under this condition, the majority (>99%) of the infected (GFP^+) cells expressed the NPC marker nestin; very few cells were immunoreactive either with antibodies to βIII-tubulin (TuJ1), an early neuronal marker that is expressed by both neuronal progenitors and post-mitotic neurons (Qian et al., 1997), or antibodies to glial fibrillary acidic protein (Gfap), an astrocytic marker. Upon growth factor withdrawal, these NPCs differentiated into neurons, astrocytes and oligodendrocytes as described previously (Johe et al., 1996; Temple and Qian, 1996). Retrovirus-mediated expression of Wnt7a resulted in a marked increase in the population of $TuJ1^+$ cells after culture for 3 days in the absence of growth factors, compared with cells expressing only GFP (Fig. 1A,B), but it had no effect on the proportion of $GFAP^+$ cells (Fig. 1C).

This result appears contradictory to a recent report by Viti et al., which showed that expression of HA-tagged Wnt7a in mouse cortical explants resulted in the suppression of $TuJ1^+$ cell proportion (Viti et al., 2003). A major difference in their study was the use of an HA-tagged Wnt7a instead of non-tagged Wnt7a. Therefore, we compared the effects of HA-tagged and non-tagged Wnt7a under the same conditions. In order to detect the activation of the canonical Wnt pathway, we used a luciferase reporter gene construct under the control of TCF-responsive elements (TOP-FLASH) (Korinek et al., 1997). Expression of non-tagged Wnt7a increased the activity of TOP-FLASH, whereas that of HA-tagged Wnt7a had little effect, if any (see Fig. S1 at http://dev.biologists.org/supplemental/).

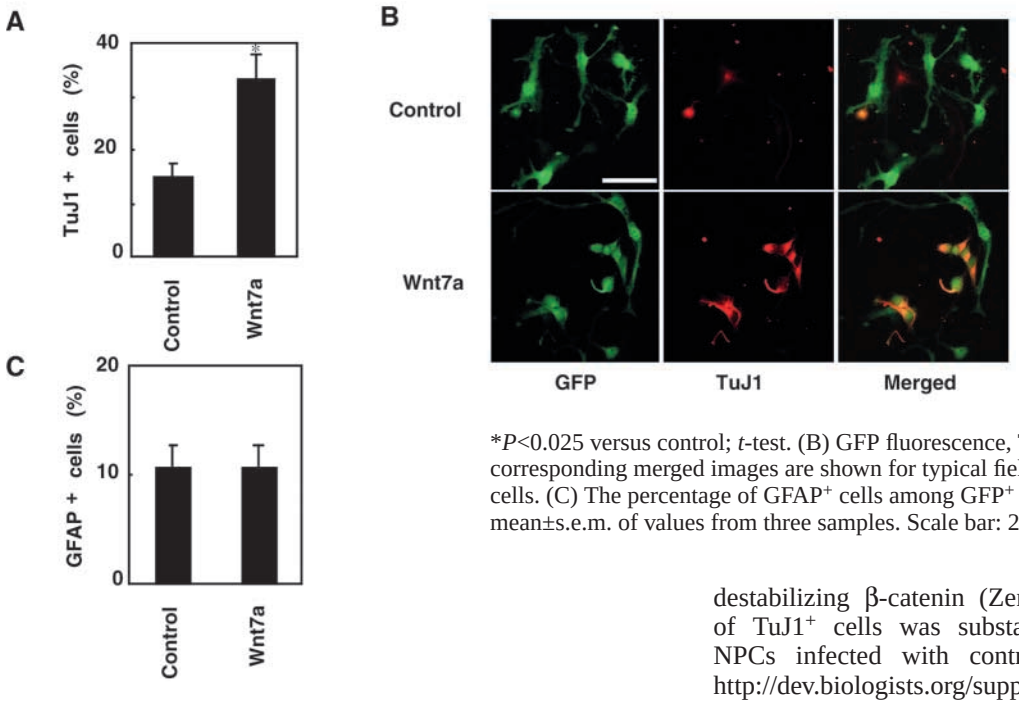

Fig. 1. Ectopic expression of Wnt7a promotes neuronal differentiation in cortical NPCs. Dissociated cortical NPCs were infected with a retrovirus encoding either GFP alone (control) or both GFP and Wnt7a. The cells were then incubated for 3 days in the absence of Fgf2. (A) The percentage of TuJ1$^+$ cells among GFP$^+$ cells was determined. Data are the mean±s.e.m. of values from three samples. Similar results were obtained in four independent experiments. *P<0.025 versus control; t-test. (B) GFP fluorescence, TuJ1 immunofluorescence and the corresponding merged images are shown for typical fields of control and Wnt7a-expressing cells. (C) The percentage of GFAP$^+$ cells among GFP$^+$ cells was determined. Data are the mean±s.e.m. of values from three samples. Scale bar: 25 μm.

Both Wnt7a constructs were expressed from the same vector. Importantly, expression of HA-tagged Wnt7a decreased the population of TuJ1$^+$ cells (see Fig. S2 at http://dev.biologists.org/supplemental/). This result suggests that modification with the HA epitope tag might interfere with the activity of Wnt7a, and we therefore used only non-tagged Wnt7a for our studies.

We next investigated which intracellular signaling pathway mediates the effect of Wnt7a on neurogenesis in cortical NPC cultures. Wnt7a activates either the canonical β-catenin-dependent pathway or non-canonical pathways, depending on the cellular context (Kengaku et al., 1998; Lucas and Salinas, 1997). Expression of either of two stabilized mutants of β-catenin (ΔN90 or S33Y) (Morin et al., 1997) markedly enhanced the effect of growth factor withdrawal on the increase of TuJ1$^+$ cell number (Fig. 2A). Most of the TuJ1$^-$ cells were Nestin$^+$ and when the cells were cultured 3 days longer, many of them became TuJ1$^+$ (see Fig. S3 at http://dev.biologists.org/supplemental/). Expression of S33Y β-catenin also increased the population of cells positive for neurofilament (NF), which is largely restricted to postmitotic neurons with elaborate neurites (Lee and Cleveland, 1996) (Fig. 2B,C). We also examined the role of the JNK pathway on neurogenesis of NPCs, by use of a constitutively active form of JNK, the fusion protein MKK7-JNK in which JNK1 is intramolecularly phosphorylated and activated by MKK7. Expression of this construct did not result in an increase in the percentage of neurons in NPC cultures (Fig. 2A), although it did cause an increase in the transcriptional activity of an AP1-dependent luciferase reporter gene, which serves as a monitor of JNK pathway activity (data not shown). These results thus suggest that activation of the canonical Wnt pathway causes an increase in the neuronal population in cultured cortical NPCs, whereas activation of JNK does not. Moreover, when the canonical Wnt pathway was blocked in NPCs by ectopic expression of Axin, which inhibits Wnt signaling by destabilizing β-catenin (Zeng et al., 1997), the proportion of TuJ1$^+$ cells was substantially reduced compared with NPCs infected with control retrovirus (see Fig. S2 at http://dev.biologists.org/supplemental/), suggesting that the endogenous activity of the canonical Wnt pathway plays an important role in neuronal differentiation in neocortical NPCs in culture.

Wnt signaling instructs NPCs to commit to the neuronal lineage

Fgf2 prevents NPC differentiation into neurons, thus Fgf2 and Wnt exert opposite effects on neuronal differentiation. We therefore compared Wnt and Fgf2 to see which effect prevails, and found that expression of S33Y β-catenin resulted in a marked increase in the neuronal population even in the presence of Fgf2 (Fig. 3B). More than 75% of the cells expressing S33Y β-catenin became TuJ1$^+$ after 4 days of culture, compared with only 15% of the control cells. In addition, the percentage of cells positive for the neuron-specific RNA-binding protein HuC/D (Sakakibara and Okano, 1997) was significantly increased by the expression of S33Y β-catenin (Fig. 3C). Wnt is thus a potent inducer of neurogenesis that can override the effect of Fgf2.

The increase in the size of the neuronal population induced by activation of the canonical Wnt pathway might have been attributable to (1) inhibition of glial differentiation, (2) selective survival of neuronal progenitors and neurons, (3) selective proliferation of neuronal progenitors, or (4) instructive differentiation of NPCs into neuronal progenitors and neurons (see Fig. 3A). Given that Fgf2 maintains NPCs in the undifferentiated state and that Wnt increases the neuronal population even in the presence of Fgf2, it is likely that the canonical Wnt pathway directly affects the fate of NPCs and instructs their neuronal differentiation, rather than affecting the fate of neuronal progenitors or neurons. We performed several experiments to distinguish further among these possibilities.

After culture of NPCs for 6 days in the presence of Fgf2, we detected very few (<1%) GFAP$^+$ glial cells or cells positive for the oligodendrocyte marker O4 among GFP$^+$ NPCs, and the expression of S33Y β-catenin did not affect the proportion of these glial cells. Next, we stained for dying cells by

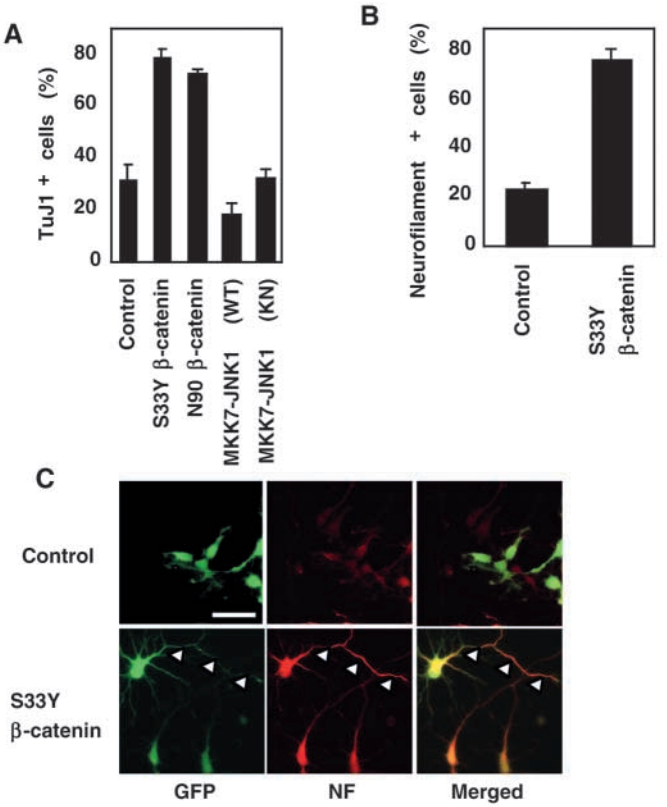

Fig. 2. Wnt7a promotes neuronal differentiation through the β-catenin/TCF signaling pathway. (A) NPCs were infected with a retrovirus encoding GFP alone (control) or GFP together with the β-catenin mutants S33Y or ΔN90 or with either wild-type (WT) or a kinase-negative mutant (KN) of MKK7-JNK1. The percentage of $TuJ1^+$ cells among GFP^+ cells was determined after culture for 2 days in the absence of Fgf2. Data are the mean±s.e.m. of values from eight samples, and similar results were obtained in three independent experiments. (B) NPCs were infected with a retrovirus encoding GFP alone (control) or GFP together with S33Y β-catenin. The percentage of neurofilament$^+$ cells among GFP^+ cells was determined after culture for 4 days in the absence of Fgf2. Data are the mean±s.e.m. of values from eight samples, and similar results were obtained in three independent experiments. (C) GFP fluorescence, neurofilament (NF) immunofluorescence and the corresponding merged images of control cells and cells expressing S33Y β-catenin are shown. Arrowheads indicate neurite-like protrusions induced by the expression of S33Y β-catenin. Scale bar: 25 μm. Data are the mean±s.e.m. of values from six samples, and similar results were obtained in three independent experiments.

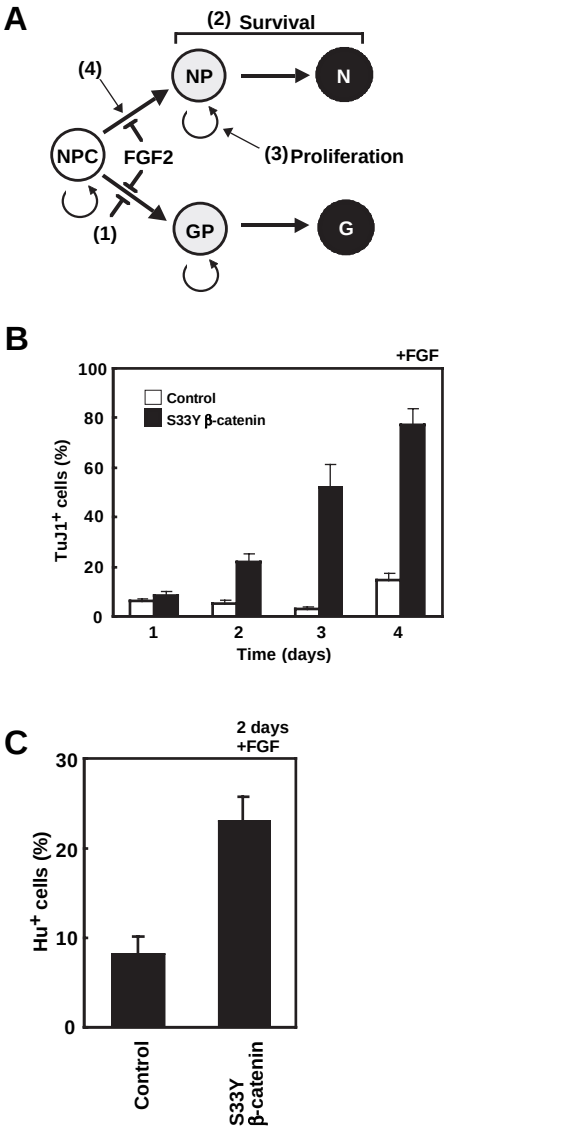

Fig. 3. Expression of a stabilized form of β-catenin promotes neuronal differentiation in the presence of Fgf2. (A) A model depicting the possible actions contributing to the increased neuronal population after activation of the canonical Wnt pathway. NP, neuronal progenitor; GP, glial progenitor; N, neuron; G, glia. (B) NPCs were infected with a retrovirus encoding either GFP alone (control) or both GFP and S33Y β-catenin, and the percentage of $TuJ1^+$ cells among GFP^+ cells was determined at the indicated times after culture in the presence of Fgf2. Data are the mean±s.e.m. of values from six samples, and similar results were obtained in two independent experiments. (C) The percentage of HuC/D^+ cells among GFP^+ cells after culture for 2 days in the presence of Fgf2 was determined. Data are the mean±s.e.m. of values from eight samples, and similar results were obtained in three independent experiments.

immunostaining with antibodies to cleaved caspase 3 (Srinivasan et al., 1998). We found that the percentage of cleaved caspase 3^+ cells was less than 1% among GFP^+ NPCs, regardless of the presence or absence of Fgf2, and regardless of whether S33Y-β-catenin was ectopically expressed (3 days) or not. Therefore, neither selective cell death nor suppression of glial differentiation appeared to account for the increase in the neuronal population induced by activation of the canonical Wnt pathway.

To determine whether Wnt signaling instructs the fate switch of NPCs, we carried out clonal analysis by using NPCs infected with control or S33Y β-catenin-encoding retroviruses at a low titer, allowing us to trace the fate of each infected cell. Expression of S33Y β-catenin significantly increased the proportion of neuron-only and neuron-containing clones and reduced that of non-neuronal clones (Fig. 4A), demonstrating that β-catenin can induce neuronal fate instructively. By contrast, expression of S33Y β-catenin did not substantially

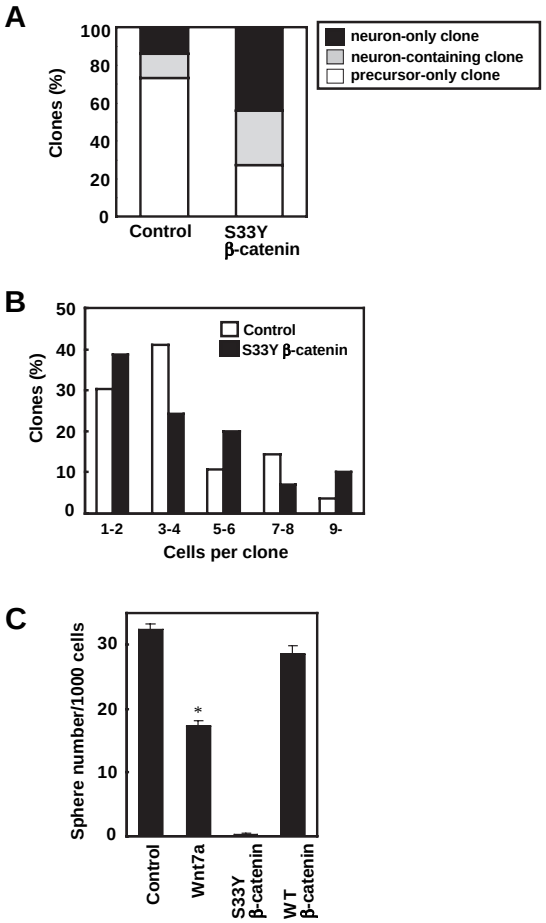

Fig. 4. Expression of a stabilized form of β-catenin instructively promotes neuronal differentiation. NPCs were infected with a retrovirus encoding either GFP alone (control) or both GFP and S33Y β-catenin at a low titer and subjected to clonal analysis. After incubation for 3 days in the presence of Fgf2, cells in each clone were stained with TuJ1 antibody and the clones were classified as containing either only TuJ1$^+$ cells (neuron-only clone), both TuJ1$^+$ and TuJ1$^-$ cells (neuron-containing clone), or only TuJ1$^-$ cells (precursor-only clone) (A). The cell number for each clone was determined (B). The results are representatives of three independent experiments. (C) NPCs were infected with a retrovirus encoding either GFP alone (control) or GFP together with Wnt7a, S33Y β-catenin or wild-type (WT) β-catenin. They were then plated at a density of 1000 cells/well in 96-well plates that had been coated with poly-HEME and incubated for 9 days in suspension culture in the presence of Fgf2, after which the number of GFP$^+$ primary neurospheres per 1000 founding cells/well was determined. Data are the mean±s.e.m. of values from 12 samples, and are representatives of three independent experiments. *$P<10^{-5}$ versus control.

increase the size of the clones (Fig. 4B). Expression of S33Y β-catenin also did not increase bromodeoxyuridine (BrdU) incorporation into cells measured after culture for 1 to 3 days in the presence of Fgf2, and in fact decreased it (data not shown), probably as a result of cell cycle arrest associated with neuronal differentiation.

Taken together, these results suggest that the canonical Wnt pathway may direct NPCs into the neuronal lineage instructively. If so, one would expect that the number of NPCs might be reduced in response to activation of the Wnt pathway. We tested this prediction by monitoring the abundance of NPCs using the neurosphere assay: when NPCs are cultured in suspension at a low density in the presence of Fgf2, they produce clonal cell aggregates, known as neurospheres, that contain progenitors to neurons, astrocytes and oligodendrocytes as well as secondary NPCs that are themselves able to produce neurospheres (Reynolds and Weiss, 1996). Expression of either Wnt7a or S33Y β-catenin in NPCs resulted in a marked decrease in the number of neurospheres (Fig. 4C). Activation of the canonical Wnt pathway thus appears to increase the number of neurons at the expense of neurosphere-forming NPCs.

Wnt signaling contributes to cortical neurogenesis in vivo

In addition to the in vitro culture experiments, we examined the role of Wnt signaling in the developing mouse neocortex by gene transfer in utero. Expression plasmids were injected into the lateral ventricles of mouse embryos at E13.5, and were introduced into NPCs in the VZ by electroporation with the electric pulses applied from outside the uterus. Two days after electroporation of a plasmid harboring GFP, a large fraction (30-50%) of the GFP$^+$ cells remained in the VZ and most of these cells were negative for TuJ1. By contrast, when a plasmid encoding S33Y β-catenin and GFP was introduced by electroporation, the population of GFP$^+$ cells that had migrated out of the VZ into the outer layers of the neocortex after 2 days was markedly increased (Fig. 5A). Similar results were obtained by electroporation of plasmids encoding Wnt7a and Fz5 (data not shown). In some cases, the VZ became thinner at the location where these proteins were ectopically expressed, probably because of premature neurogenesis of the NPCs (Fig. 5A and data not shown).

Next, we quantified the expression level of the neuronal marker HuC/D in individual electroporated (transfected) cells in situ, by employing a system called Laser Scanning Cytometry (LSC). In this system, the fluorescence intensity was quantified within each GFP$^+$ area (see Materials and methods for details). As it is difficult to distinguish neighboring cells when labeled with GFP, because of the presence of long and complex neurites, we used a fusion protein of GFP and histone H2B (histone H2B-GFP) to label just the cell nucleus, which enabled us to distinguish transfected cells more easily. The intensity of immunostaining for HuC/D, which localizes predominantly to the cell body of immature and mature neurons (Sakakibara and Okano, 1997), was determined within the histone H2B-GFP$^+$ area. Cells transfected with plasmids encoding S33Y β-catenin (Fig. 5B) or Wnt7a and Fz5 (data not shown) exhibited higher levels of expression of HuC/D than did control cells transfected with the corresponding empty vectors, again suggesting that activation of the canonical Wnt pathway promotes neurogenesis in vivo.

To test the hypothesis that the canonical Wnt pathway is necessary for neuronal differentiation in the developing neocortex in vivo, we blocked this pathway in cortical NPCs by in utero electroporation with a plasmid for either Axin or Dkk1 at E13.5. Ectopic expression of Axin increased the population of cells remaining in the VZ 2 days later (Fig. 5C) and reduced the levels of HuC/D expression (Fig. 5D). Expression of Dkk1 induced similar effects (Fig. 5E).

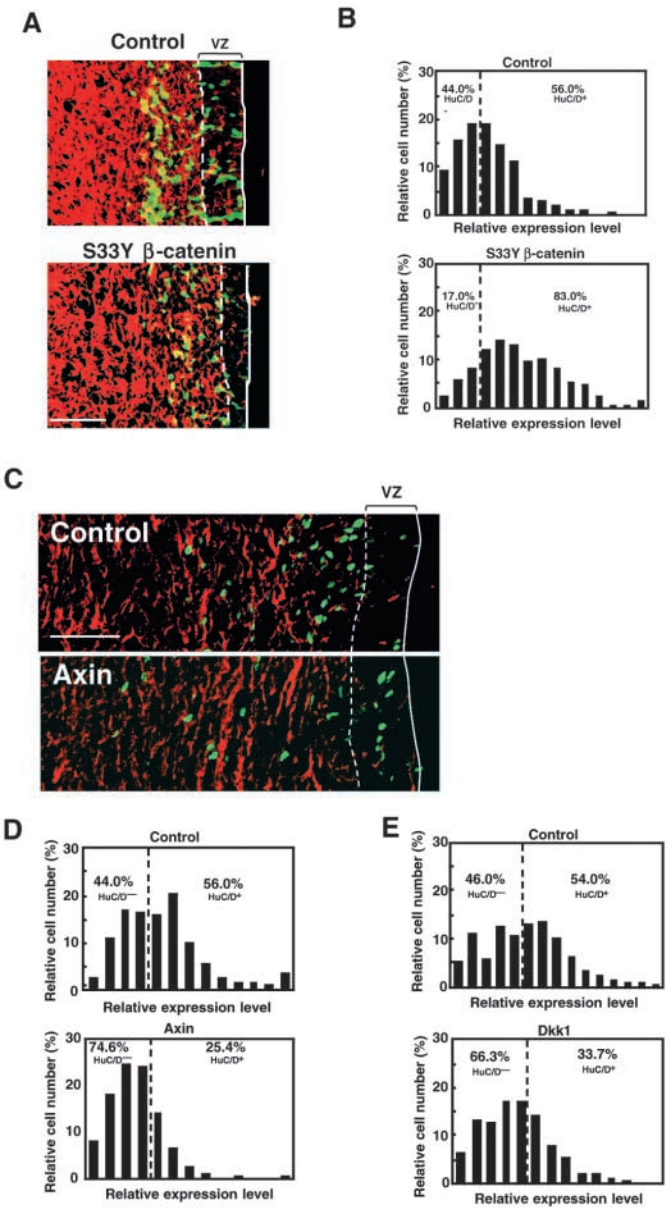

Fig. 5. The canonical Wnt pathway promotes NPC differentiation into neurons in the developing mouse neocortex. Mouse neuroepithelial cells were subjected to in utero electroporation at E13.5 with various constructs. Embryos were fixed 2 days after electroporation and subjected to immunostaining.
(A) Immunostaining with TuJ1 (red) and antibodies to GFP (green) in cells of the same region of the dorsolateral telencephalon electroporated with a vector for GFP alone (control) or for GFP and S33Y β-catenin. Broken lines indicate the boundary of $TuJ1^+$ and $TuJ1^-$ areas, which coincides with the basal edge of the ventricular zone (VZ) judged by the shape of the cells. Scale bar: 100 μm. Most of the cells expressing S33Y β-catenin migrated out of the VZ and only a small fraction of these cells (10.4%) remained in the VZ, whereas many control cells remained in the VZ (33.3%). The VZ of the cortex electroporated with S33Y β-catenin was thinner than that of control. Similar results were obtained from three independent experiments. (B) Quantitative analysis of HuC/D expression in individual electroporated cells. Embryos were electroporated with a vector expressing either histone H2B-GFP alone or together with a vector for S33Y β-catenin. The relative expression level (intensity/area) of the neuronal marker HuC/D within individual histone $H2B\text{-}GFP^+$ cells was quantified by immunostaining and LSC. The percentages of cells within each range of expression are shown in the histograms. The broken lines represent the approximate expression level apparent at the border between the VZ and the intermediate zone, and the percentages of cells with expression levels less than or greater than this value are indicated (see Materials and methods). Littermates were analyzed for each experiment.
(C) Immunostaining with TuJ1 (red) and antibodies to GFP (green) in cells of the same region of the dorsolateral telencephalon electroporated with vectors for histone H2B-GFP alone (control) or for histone H2B-GFP and Axin. A large number of the cells expressing Axin remained in the VZ (42.8%), compared with the number of control cells remaining (28.3%). Scale bar: 50 μm.
(D,E) Quantitative analysis of HuC/D expression in individual cells electroporated with a vector for either Axin (D) or for Dkk1 (E). All data are representative of results obtained from three independent experiments.

Direct regulation of the *Ngn1* promoter by the β-catenin/TCF complex

We next investigated the mechanism by which Wnt signaling regulates neurogenesis. As our results implicated the β-catenin/TCF complex in neuronal differentiation, we searched for a proneural gene that might be under the control of these transcription factors. One such candidate is the bHLH transcription factor Ngn1, because this gene is expressed during early neurogenesis in the neocortex, and its expression, together with that of the *Ngn2* gene, is essential for development of the neocortex (Schuurmans and Guillemot, 2002). We found a consensus sequence for TCF binding (van de Wetering et al., 1997) located at nucleotide (nt) positions –1167 to –1160 relative to the transcription start site of the mouse *Ngn1* gene. This region within the promoter has been shown to be responsible for expression of the gene in the dorsal neocortex during neurogenesis (Murray et al., 2000). To determine whether this TCF binding element is functional, we compared the activities of the *Ngn1* gene promoter (nt –2670 to +74) containing either an intact or mutated version of this DNA sequence (Fig. 6A). Cultured NPCs were transfected with a luciferase reporter construct under the control of the wild-type or mutant *Ngn1* gene promoter. We found that the transcriptional activity of the mutant promoter was markedly reduced compared with that of the wild-type (Fig. 6B).

Next, we used ChIP assay to examine whether endogenous β-catenin was associated with the *Ngn1* gene promoter in cultured NPCs. Lysates of cultured NPCs were subjected to shearing of genomic chromatin followed by immunoprecipitation with antibodies to β-catenin. Polymerase chain reaction (PCR) analysis with primers targeted to the TCF binding element of the *Ngn1* gene promoter revealed the presence of this element in the immunoprecipitates (Fig. 6C). We then determined the level of Ngn1 mRNA by reverse transcription (RT)-PCR in cultured NPCs. Expression of S33Y β-catenin markedly increased the level of Ngn1 mRNA but not that of the control glyceraldehydes-3-phosphate dehydrogenase (Gapdh) mRNA, suggesting that transcription of the *Ngn1* gene is indeed under the control of the canonical Wnt pathway (Fig. 6D). Together, these results suggest that the β-catenin/TCF complex directly regulates transcription of the *Ngn1* gene during neuronal differentiation of cortical NPCs.

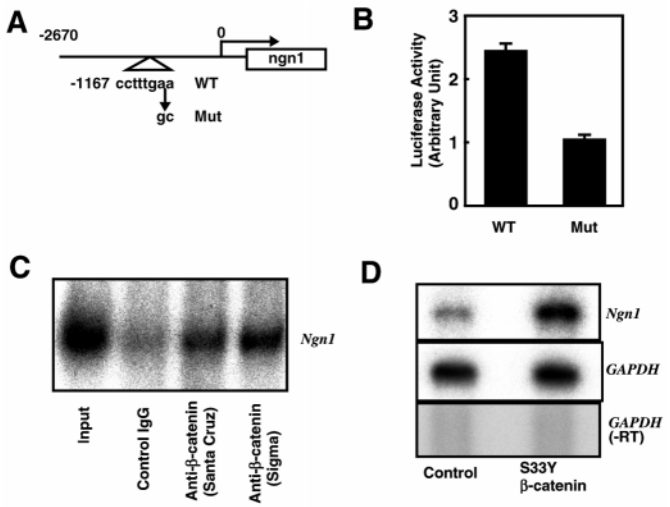

Fig. 6. β-catenin/TCF complex directly regulates the *Ngn1* promoter. (A) A schematic representation of the mouse neurogenin 1 (*Ngn1*) promoter (WT), and its mutant within the putative TCF binding site (–1167 to –1160) located in the *Ngn1* promoter (Mut). NPCs were transfected with a vector containing the *Ngn1* promoter (2.7 kb: wild type or mutant) driving luciferase expression. (B) Relative luciferase activity was measured after 13 hours of incubation. Mutation of the TCF binding site in the *Ngn1* promoter reduced endogenous transcriptional activity. (C) Chromatin complex was immunoprecipitated with anti-β-catenin (Santa Cruz Biotechnology and Sigma) or control IgG, and was subjected to PCR analysis to amplify *Ngn1* genomic sequence. (D) RT-PCR analysis of *Ngn1* expression. NPCs were infected with a retrovirus encoding either GFP alone (control) or both GFP and S33Y β-catenin, and the expression level of *Ngn1* mRNA was analyzed. *Gapdh* was used for standardization of the samples. No genomic amplification was observed from the RNA treated without reverse transcriptase (–RT).

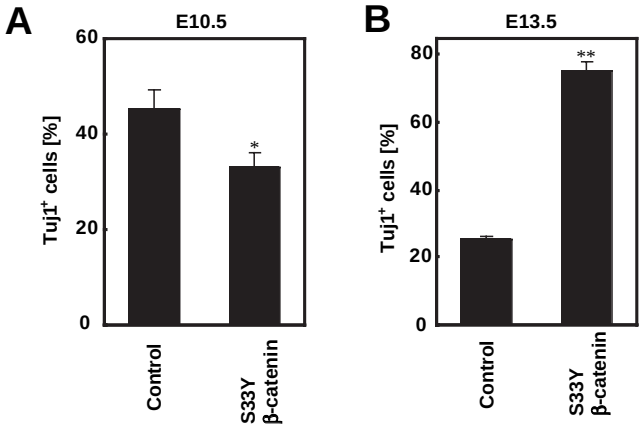

Fig. 7. Expression of a stabilized form of β-catenin does not promote neuronal differentiation in the cortical NPCs from E10.5. (A,B) NPCs isolated from E10.5 (A) or E13.5 (B) were acutely plated on poly-D-lysine coated coverslips and infected with a retrovirus encoding either GFP alone (control) or both GFP and S33Y β-catenin. The cells were incubated for 2 days with Fgf2, and the percentage of TuJ1$^+$ cells among GFP$^+$ cells was determined. *P< 0.02 and **P<10^{-9} versus control.

Stage-specific effects of Wnt signaling

Our results clearly indicate that stabilized β-catenin instructs neuronal differentiation of cortical NPCs prepared from mouse E11.5 neocortex and cultured for 3 days. However, Chenn and Walsh (Chenn and Walsh, 2002) have shown that ectopic expression of stabilized β-catenin by the nestin enhancer results in the expansion of NPC cell number and suppression of cell cycle exit. This difference might be due to the timing at which stabilized β-catenin was expressed, as the nestin enhancer is known to become active at around E8.5. To test this idea, we compared the effects of β-catenin on NPCs prepared from different stages of mouse neocortex development. Expression of stabilized β-catenin increased the population of TuJ1$^+$ cells in NPCs prepared from E13.5 neocortex, but reduced somewhat the population of TuJ1$^+$ cells among neuroepithelial cells acutely prepared from E10.5 neocortex (Fig. 7). This suggests that the response of NPCs to the canonical Wnt pathway depends on the stage of neural development.

Discussion

We have shown in this study that activation of the canonical Wnt pathway promoted, and inhibition of this pathway blocked, neuronal differentiation both in cortical NPC cultures and in the developing neocortex. We emphasize two aspects of these findings: (1) Wnts appear to function as an extracellular cue that instructively triggers neuronal differentiation; and (2) this effect of Wnts is dependent on the stage of development. In this Discussion, we address these aspects and their possible underlying mechanisms.

In general, two models can explain how the fate of an uncommitted precursor cell is influenced by extrinsic cues. In one model, extrinsic cues instruct multipotent precursor cells to commit to a particular lineage. In the other model, multipotent precursor cells choose their fate stochastically, and the proliferation and/or survival of specific lineage-restricted cells is then supported by extrinsic cues. For example, Pdgf treatment increases the size of the neuronal population in cortical neuroepithelial cultures by acting as a mitogen in the early phase of NPC differentiation to expand the pool of neuronal progenitors (Erlandsson et al., 2001). Our results support a model in which the canonical Wnt pathway acts on cortical NPCs to instruct their neuronal differentiation, rather than to expand neuronal progenitors selectively, based on the following evidence. First, expression of a stabilized form of β-catenin did not induce overt cell proliferation in NPC cultures under the conditions examined, as assessed by BrdU incorporation. Second, the frequency of cell death was negligible in NPCs expressing S33Y β-catenin. Activation of the Wnt pathway thus does not appear to increase neuronal population by selective proliferation or survival of cells committed to the neuronal fate. Third, the frequency of glial differentiation in the presence of Fgf2 was low, and expression of S33Y β-catenin did not inhibit glial differentiation induced by Fgf2 withdrawal. It is therefore unlikely that the Wnt pathway increases the neuronal population indirectly by suppression of glial differentiation. Fourth, expression of either Wnt7a or S33Y β-catenin reduced the population of uncommitted precursors, as shown in the neurosphere assay, consistent with fate determination at the expense of NPCs. And, most importantly, clonal analysis revealed that expression

of S33Y β-catenin increased the ratio of neuronal clones to non-neuronal clones. In addition, ectopic expression of S33Y β-catenin overrode the inhibitory effect of Fgf2 on neurogenesis. As Fgf2 has been shown to maintain NPCs, it is likely that Wnt signaling directly regulates the fate of NPCs.

At present, it is not clear whether neuronal differentiation is actively 'triggered' by extracellular cues, or is induced passively when inhibitory signals are downregulated. Our results showing that Wnt signaling overcomes the inhibitory effect of Fgf2 clearly demonstrate that active triggering of neuronal differentiation can indeed take place during neuronal differentiation.

How does the Wnt pathway induce neuronal differentiation? In this study, we found that the canonical Wnt pathway regulates the *Ngn1* promoter. Ngn1 is a proneural bHLH transcription factor expressed in newly committed neuronal progenitors and immature neurons, and plays an essential role in neurogenesis and regional specification in the neocortex, together with Ngn2 (Schuurmans and Guillemot, 2002). We found that ectopic expression of stabilized β-catenin increased the level of Ngn1 mRNA. Importantly, a TCF binding element located at nucleotide positions –1167 to –1160 was found to be necessary for maximal transcriptional activity of the *Ngn1* gene promoter, and direct binding of β-catenin to the promoter was detected in NPCs. These results imply that a β-catenin/TCF complex directly regulates the *Ngn1* promoter, and that the canonical Wnt pathway induces neuronal differentiation through regulation of Ngn1. A recent report showing reduced expression of Ngn1 in β-catenin-deficient neural crest cells further supports a model of β-catenin-mediated regulation of Ngn1 (Hari et al., 2002). It is possible that other mechanisms than Ngn1 induction also contribute to Wnt induction of neurogenesis; for example, Wnt signaling may turn off the intracellular signaling events that inhibits neuronal differentiation. Notch signaling is known to inhibit neurogenesis, and an antagonistic interaction between the Notch and the Wnt pathways has been demonstrated. For example, mouse Wnt3a appears to be necessary for oscillating Notch signaling activity during somitogenesis (Aulehla et al., 2003). It would thus be of interest to determine whether Wnt signaling inhibits the Notch pathway in NPCs. As expression of S33Y β-catenin blocked the inhibitory effect of Fgf2 on neuronal differentiation, it is also conceivable that β-catenin somehow inhibits intracellular signaling downstream of the Fgf2 receptor. It is currently difficult to examine this possibility, as the mechanism by which Fgf2 blocks neurogenesis is unclear.

Recent experiments have suggested that Wnt signaling has the capacity to promote self-renewal in various tissue stem cells including neural stem cells and hematopoietic stem cells (Alonso and Fuchs, 2003; Reya et al., 2003; Willert et al., 2003). In the central nervous system, cells located in the midbrain or hippocampus are deleted in mice deficient in Wnt1 or Wnt3a, respectively (Lee et al., 2000; McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Mice lacking both Wnt1 and Wnt3a also manifest a reduction in the size of the caudal midbrain, rostral hindbrain, cranial and spinal ganglia, and dorsal neural tube (Ikeya et al., 1997; Megason and McMahon, 2002). Furthermore, ectopic expression of Wnt1 or stabilized β-catenin was shown to lead to a net increase in the size of the precursor pool in the chick spinal cord, in part through transcriptional regulation of cyclinD (Megason and McMahon, 2002), and infection of cortical explants with Wnt7aHA-expressing retrovirus induced expansion of neuronal precursors which accompanied expression of the Egf receptor (Viti et al., 2003). Consistently, transgenic mice expressing stabilized β-catenin in NPCs under the control of the nestin enhancer or Brn4 promoter also exhibited overgrowth of the brain and spinal cord, reflecting an expansion of the precursor population without alteration of the primary patterning of cell identities (Chenn and Walsh, 2002; Zechner et al., 2003). By contrast, in the present study, activation of the canonical Wnt pathway reduced the size of the precursor pool and promoted neuronal differentiation in the developing neocortex. We speculate that this difference might be attributable to differences in the developmental stage of the NPCs. Indeed, activation of the canonical Wnt pathway promoted neuronal differentiation of NPCs derived from E13.5 embryos, but not those acutely dissected from E10.5 embryos. It is possible that the chromatin region encompassing regulatory elements of genes crucial for neuronal differentiation (such as *Ngn1*) undergoes a change during development from a closed to an open state, as observed for the STAT-responsive element within the GFAP promoter, which becomes accessible to STAT3 only at later stages of neural development, owing to demethylation of the element (Takizawa et al., 2001). Therefore, chromatin state of the *Ngn1* promoter should be examined in future studies. In any case, our results have revealed that Wnt signals function in a stage-specific manner within the same region of the brain, analogous to the stage-specific functions of *Drosophila* Wg in wing disc development: Wg promotes cell proliferation and survival at early stages but determines the specification of sensory bristles of wing margin at later stages (Giraldez and Cohen, 2003; Johnston and Sanders, 2003; Phillips and Whittle, 1993).

One of the most fundamental questions about stem cells is what determines the timing of the fate switch from self-renewal to differentiation? Tissue stem cells appear to know how many times they should divide before undergoing differentiation, as this determines the size of each tissue. One might think that the expression of a differentiation-triggering cue is induced at the timing of the fate switch. However, our results suggest that what changes at the timing of cell fate switch might not be the expression of the extracellular cues, but the responsiveness of the stem cells to the cues, at least in the case of cortical development. These findings may shed lights on the nature of the stem cells.

Hundreds of distinct neuronal cell types are generated during development of the mammalian neocortex, establishing a diversity that is essential for the formation of complex neuronal circuits. Several extracellular factors, including PDGF (Erlandsson et al., 2001; Johe et al., 1996; Williams et al., 1997), insulin-like growth factor 1 (Arsenijevic and Weiss, 1998; Arsenijevic et al., 2001), brain-derived neurotrophic factor (Ahmed et al., 1995), bone morphogenetic protein 2 (Li et al., 1998) and erythropoietin (Shingo et al., 2001) have been implicated in the regulation of cortical neurogenesis, and interactions among these factors may be necessary to generate neuronal diversity in the neocortex (Song et al., 2000). It will be interesting to determine which types of neurons are generated by activation of the Wnt pathway and to investigate the interactions of this pathway with other extrinsic and intrinsic factors that participate in neurogenesis.

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