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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Neural stem cells are increased after loss of β-catenin, but neural progenitors undergo cell death

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Abstract

Neurons and glia in the central nervous system originate from neural stem and progenitor cells that reside in the ventricular zones. Here we examine the role of β-catenin in neural stem cell (NSC) regulation in mouse embryos lacking *β-catenin* specifically in the brain germinal zone. An *in vitro* clonal neurosphere assay was performed in order to ascertain the status of the NSC population. Intact neurospheres did not form from *β-catenin-null* cells due to a loss of cell adhesion and the number of expanded cells was reduced. Rescue of *β-catenin* expression restored adhesion and revealed that the number of NSCs increased in the knockout population. Using a clonal colony-forming assay, which confines precursor cells within a solid collagen matrix, we show that the number of NSCs in the hippocampus is unchanged although the *β-catenin* knockout striatum actually contains a larger proportion of NSCs. However, these colonies were smaller than those of control cells, due to increased apoptosis in the progenitor population. Furthermore, *β-catenin* knockout NSCs also retained multipotentiality as shown by their ability to clonally differentiate into neurons and glia. The effects on neural precursor cells were not due to loss of downstream T-cell factor signaling, as this pathway is not active *in vivo* in regions of the embryonic brain where NSCs and progenitor cells reside, nor is it active *in vitro* in NSC colonies. These data reveal that β-catenin is not required for the maintenance or differentiation of NSCs, but is required for the adhesion and survival of neural progenitor cells.

Introduction

During embryogenesis, neural stem cells (NSCs) are believed to be the precursor to all neurons and glia (Tropepe *et al.*, 1999; Seaberg & van der Kooy, 2003). Because a unique molecular marker for NSCs is currently unavailable, the 'neurosphere assay' is commonly used to determine the number of NSCs present within a population of cells. In this assay, single cells isolated from brain tissue are cultured *in vitro* to give rise to clonal free-floating neurosphere colonies (Reynolds & Weiss, 1992). The number of neurosphere colonies corresponds to the number of NSCs in the original population. The NSCs in each neurosphere can be dissociated and passaged clonally numerous times. However, the vast majority of cells in each neurosphere are neural progenitor cells, which have decreased passaging ability, limited self-renewal and readily produce terminally differentiated cells. Therefore, NSC-derived neurospheres exhibit the characteristics that define NSCs – self-renewal (as demonstrated by clonal passaging) and multipotentiality (as demonstrated by their ability to differentiate into numerous cell types).

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One pathway that can potentially regulate NSCs is the Wnt/β-catenin pathway. Wnt ligands activate a 'canonical' β-catenin pathway, as well as β-catenin-independent 'non-canonical' pathways. In the canonical Wnt/β-catenin pathway, Wnt receptor binding leads to the stabilization of cytoplasmic β-catenin. Some of this β-catenin translocates to the nucleus, where it can associate with the lymphocyte enhancer factor (LEF)1-T-cell factor (TCF) family of transcription factors and activate downstream genes (Clevers, 2006). β-catenin also has an important role in cell adhesion and tissue polarity as it links membrane-bound cadherins to the actin cytoskeleton (Lien *et al.*, 2006).

Several studies have examined the role of Wnt/β-catenin during development of the central nervous system. Neural precursors appear to be dependent on intact β-catenin signaling as conditional ablation of *β-catenin* leads to smaller brain structures (Machon *et al.*, 2003; Zechner *et al.*, 2003; Junghans *et al.*, 2005; Gulacsi & Anderson, 2008). Conversely, overexpression of a stabilized *β-catenin* allele resulted in abnormal expansion of the neuroepithelium (Chenn & Walsh, 2002; Zechner *et al.*, 2003). Therefore, β-catenin levels appear to be correlated with neuroepithelial growth. It is not clear, however, whether β-catenin regulates the NSC or neural progenitor populations. Furthermore, the expansion of neuroepithelial cells seen *in vivo* following overexpression of β-catenin is not consistent with

β-catenin's effects on neurosphere cells *in vitro*. In some cases, overexpression of Wnt or β-catenin in neurosphere cells lead to loss of NSCs (Hirabayashi *et al.*, 2004; Muroyama *et al.*, 2004; Kuwahara *et al.*, 2010) and enhanced neuronal differentiation (Hirabayashi *et al.*, 2004; Muroyama *et al.*, 2004; Lie *et al.*, 2005; Kuwabara *et al.*, 2009; Kuwahara *et al.*, 2010). Other studies suggest that Wnt-7a (Viti *et al.*, 2003) or β-catenin (Israsena *et al.*, 2004) increases the number of NSCs. However, it is not clear whether Wnt-7a acts through a non-canonical, TCF/β-catenin-independent pathway (Kengaku *et al.*, 1998; Le Grand *et al.*, 2009). In the light of these findings, we sought to determine whether β-catenin is required for regulating the balance between NSCs, neural progenitor cells and differentiated cells. Here we test the status of these specific cell populations in a conditional loss-of-function mutant specific to the brain germinal zone.

Materials and methods

Animals and genotyping

The *Nestin:Cre* mice (Tronche *et al.*, 1999) were purchased from the Jackson Laboratory and genotyped according to the company's protocol. These mice are of C57Bl6 background and express Cre recombinase in the developing neuroepithelium (stem and progenitor cells) under the control of the Nestin promoter. The generation and genotyping of *β-catenin*$^{lox/lox}$ mice (129 × C57Bl6 mixed background) has been described previously (Huelsken *et al.*, 2001). Homozygous conditional *β-catenin* knockout embryos were obtained by mating *Nestin:Cre*/β-catenin$^{+/lox}$ females with *β-catenin*$^{lox/lox}$ males. Control wildtype and heterozygous embryos were obtained from the same litters. The generation of *TCF-lacZ* mice was described by Mohamed *et al.* (2004). Matings were timed such that midday of the day that the vaginal plug was found was considered to be embryonic day (E)0.5. For each of the *Nestin:Cre* and *β-catenin*$^{lox/lox}$ strains, approximately 50 adult animals were used for this study. Approximately 25 adult animals of the TCF-lacZ strain were used for this study. All animal experiments were carried out according to the protocols approved by the University of Toronto Animal Care Committee.

Cell culture

Isolation of NSCs from E14 lateral ganglionic eminences (i.e. the future striatum) or the medial pallium (i.e. the future hippocampus) for neurosphere culture was performed as previously described (Tropepe *et al.*, 1999). Cells were plated at 10 cells/μL in serum-free medium (SFM) containing 10 ng/mL fibroblast growth factor (FGF)2 (Sigma, St Louis, MO, USA) and 1 μg/mL heparin (Millipore, Billerica, MA, USA) and grown for 1 week. Expanded cultures were mechanically dissociated using a fire-polished glass pipette. After the total cell counts were recorded, cells were passaged in bulk at a density of 10 cells/μL in 24-well plates. The neural colony-forming assay, in which NSCs were grown within a semi-solid collagen gel in 35 mm plates, was performed using NeuroCult NCFC Assay kit (mouse) from Stem Cell Technologies (Vancouver, BC, Canada) according to the manufacturer's instructions. FGF2 and heparin were added at the concentrations described above. Counting and passaging of single colonies were performed 2 weeks later. For passaging, single 500 μm diameter colonies were cut out of the collagen using dissection scissors and incubated in collagenase (Stem Cell Technologies) for 15 min at 37 °C. Note that these colonies were picked 1 week earlier than suggested in a published protocol (Louis *et al.*, 2008), and hence were smaller than the reported minimum size for passageable NSC colonies (2 mm diameter). When these colonies were allowed to grow for one more week, they typically reached 2 mm in diameter. However, we chose the 2-week time point for passaging because the colonies appeared to be in better condition compared with after 3 weeks *in vitro*. Gentle aspiration was used to break up the colonies and the resulting small clumps were cultured in 3 mL of SFM with FGF2 and heparin in six-well plates for 4–5 days. Cells were then pelleted and gently dissociated using the NeuroCult Chemical Dissociation kit (Stem Cell Technologies). One-tenth of the culture was plated into collagen gels in a 35 mm plate and the number of secondary colonies derived from a single collagen colony was assessed 10–14 days later.

Differentiation of colonies grown in collagen was induced by direct application onto the gel consisting of fetal calf serum to a final concentration of 1%. After 1 week of culture, colonies were fixed *in situ* and the degree of multipotentiality of individual colonies was assessed by immunostaining of multiple markers for differentiated cells. To assess the proportions of differentiated cell progeny, dissociated cells were plated at 20 cells/μL as a monolayer on Matrigel (BD Biosciences, Bedford, MA, USA) in SFM containing 1% fetal calf serum and fixed for immunostaining after 1 week in culture.

Retroviral infection of neurosphere cells

Mouse Wnt-3a (Kengaku *et al.*, 1998) and human wildtype β-catenin (Tetsu & McCormick, 1999) were subcloned into the pMXIE retroviral expression vector to generate pMXIE-mWnt-3a-IRES-gfp and pMXIE-h-b-cat-IRES-gfp. pMXIE-gfp (empty vector) was used as a control. Non-replicating viral particles were produced by transient transfection of plasmid together with the pVSVG plasmid (expressing the G protein of the vesicular stomatitis virus) into PlatE cells using Fugene6 (Roche, Basel, Switzerland), and culturing for 3 days. The supernatant was filtered and ultra-centrifuged at 52000 ***g*** to concentrate the virus.

The NSCs were obtained from the E14 embryonic striatum of TCF-lacZ mice for infection with Wnt-3a retrovirus. For rescue of *β-catenin* knockout cells, neurosphere cells underwent five passages *in vitro* followed by infection with wildtype β-catenin retrovirus. As a control, wildtype cells were cultured and infected with the same retrovirus. Cells were plated at a density of 100 cells/μL in 1.5 mL SFM/FGF/heparin in a 12-well plate, and infected by overnight incubation with 2×10^5 viral particles/mL. The following day, cells were mechanically dissociated and replated at 10 cells/μL in 24-well plates. The number of green fluorescent protein (gfp)-positive neurospheres (≥ 100 μm diameter) and/or degree of *lacZ* staining (Lobe *et al*., 1999) were assessed after 1 week of culture.

Immunocytochemistry

For *in vivo* staining of embryonic brains, tissue was dissected and fixed overnight at 4 °C in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS). Tissue was then transferred to 30% sucrose in PBS and stored at 4 °C. Prior to embedding, tissue was equilibrated with Cryomatrix (Thermo Scientific, Waltham, MA, USA) for 3 h at room temperature. Cryosections were prepared at −20 °C and at a thickness of 12 μm. Cells grown *in vitro* were fixed with freshly prepared 4% paraformaldehyde in PBS for 15 min at room temperature. Immunostaining was carried out as described previously (Tropepe *et al.*, 1999). For control staining, primary antibody was omitted from the staining solution. Specificity of the antibody procedure was confirmed by comparing the staining patterns

in wildtype tissue with those previously reported for a particular antibody. The primary antibodies employed in this study were as follows: mouse anti-β3-tubulin (#MAB1637, 1 : 500; Millipore), rabbit anti-β-catenin (#C-2206, 1 : 1000; Sigma), mouse anti-nestin (#MAB353, 1 : 400; Millipore), rabbit anti-Tbr1 (Hevner *et al.*, 2001, used at 1 : 2500), rabbit anti-Pax6 (#AB5409, 1 : 1000; Millipore), rabbit anti-Prox1 (#AB5475, 1 : 2000; Millipore), rabbit anti-activated Caspase-3 (#G748, 1 : 500; Promega, Madison, WI, USA), rabbit anti-lacZ (#ab616, 1 : 2000 dilution; Abcam, Cambridge, MA, USA), rabbit anti-glial fibrillary acidic protein (#ZO334, 1 : 1000; Wako, Richmond, VA, USA), mouse anti-microtubule-associated protein-2 (#MAB3418, 1 : 200; Millipore) and mouse anti-O4 (#MAB345, 1 : 500; Millipore). Anti-rabbit or anti-mouse Alexa Fluor 488 or 568 nm secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA) and used at a dilution of 1 : 400. Nuclei were visualized by incubating cells or slides for 20 min with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (Sigma) in PBS. To stain for filamentous actin, sections were incubated with 50 μg/mL rhodamine phalloidin (#P1951; Sigma) for 20 min followed by three 5 min washes with PBS. Cells undergoing apoptosis *in vivo* were revealed using the ApopTag Red *In Situ* Apoptosis Detection kit (#S7165; Millipore) according to the manufacturer's instructions. Staining was analyzed using an Olympus fluorescent microscope and OLYMPUS MICROSUITE image analysis software. Adjustments of brightness and contrast were applied to whole images and identical adjustments were made to images of both wildtype and knockout tissue. Confocal images of activated Caspase-3 staining in neural colonies grown in collagen were collected (within one confocal plane) using a Zeiss LSM-510 confocal microscope.

Quantitative polymerase chain reaction analysis

RNA was isolated from E14.5 brains using an RNeasy kit (Qiagen, Germantown, MD, USA). First-strand cDNA synthesis was carried out using a Superscript III kit (Invitrogen). Taqman qPCR primers and Master mix (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's recommendations. Polymerase chain reaction was performed using a 7900HT Real-Time polymerase chain reaction machine (Applied Biosystems). SDS-2.3 software was used to calculate relative differences in gene-expression levels with β-actin used for normalization.

Bromodeoxyuridine labeling and detection

Dissociated neurosphere cells were plated onto fibronectin-coated 24-well plates in SFM containing FGF and heparin at a density of 10 cells/μL and cultured for 4 days. Cells were exposed to a 1 h pulse of bromodeoxyuridine (BrdU) (0.6 μM) and then fixed with paraformaldehyde. To detect BrdU, cells were incubated in 4 N HCl for 30 min and immunostained using mouse anti-pan histone (#MAB3422, 1 : 500; Millipore) and rat anti-BrdU (#ab6326, 1 : 500; Abcam) antibodies. Secondary antibodies were used at a 1 : 200 dilution [Fluorescein isothiocyanate (FITC) donkey anti-rat IgG, #712-095-153 and Tetramethylrhodamine isothiocyanate (TRITC) goat anti-mouse IgG, #115-025-146; Jackson Immunochemicals, West Grove, PA, USA].

For *in vivo* BrdU labeling, pregnant mice were injected intraperitoneally at E14.5 with BrdU (65 mg/kg body weight). After 1 h, mice were killed by cervical dislocation and the brains of embryos were removed and fixed with paraformaldehyde. Cryosections were prepared and stained for BrdU as described.

Statistical analysis

For the quantification of cells positive for various markers, the numbers of antibody-stained cells were counted as a percentage of 4′,6-diamidino-2-phenylindole-positive or pan-histone-positive cells in at least eight random fields of view representing at least 400 cells. Data are expressed as means ± SEM. Statistical comparisons were performed using the SIGMASTAT 3.1 software package. All analyses used two-tailed tests. ANOVA or *t*-tests were used to analyze data as appropriate. The level of significance for all comparisons was $P < 0.05$.

Results

Tissue disorganization and hippocampal defects in β-catenin conditional knockout brains

Conventional *β-catenin null* embryos die at early gastrulation stages (Huelsken *et al.*, 2000). Therefore, in order to understand the role of Wnt/β-catenin signaling in neural stem and progenitor cell regulation, conditional *β-catenin* knockout mice were employed. We used *Nestin:Cre* mice (Tronche *et al.*, 1999) in order to restrict *Cre*-mediated excision of a floxed *β-catenin* allele (Huelsken *et al.*, 2001) to the neuroepithelium. Cre recombinase under the control of the rat nestin promoter and enhancer is specifically expressed in neural stem and progenitor cells (Mignone *et al.*, 2004) and can direct Cre-mediated excision as early as E9.5 (Haigh *et al.*, 2003). As determined by immunofluorescence staining, β-catenin protein expression was absent in the developing striatum (i.e. lateral ganglionic eminence) of E14.5 conditional knockout embryos (Fig. 1A and B).

How a tissue is organized can have a profound influence on cellular determinants that control the symmetry of division of stem cells and therefore the capacity for self-renewal (Lechler & Fuchs, 2005). β-catenin has a central role in linking cell surface cadherins to the actin cytoskeleton and in mediating tissue organization (Lien *et al.*, 2006). Filamentous actin and N-cadherin are normally both distributed on the ventricular surface of the striatum and to a lesser extent in the parenchyma (Supporting Information Fig. S1A and C). Deletion of *β-catenin* leads to a redistribution of both proteins to deposits located in deeper regions of the parenchyma (Supporting Information Fig. S1B and D). Furthermore, expression of markers for neural stem and progenitor cells, nestin and Mash1 was still apparent in the striatum following loss of *β-catenin* despite significant loss of tissue organization (Supporting Information Fig. S1E–H).

Changes in tissue organization were also observed within the cortex. When *β-catenin* is deleted in the cortex, markers that delineate the intermediate and ventricular zones appear to overlap (Supporting Information Fig. S2A–D). Hence, β-catenin is important in setting up tissue organization in both the striatum and cortex.

The hippocampus arises from the caudal dorso-medial region of the forebrain in response to Wnt signals from the cortical hem (Galceran *et al.*, 2000; Lee *et al.*, 2000). We observed that conditional loss of *β-catenin* caused a dramatic decline in the number of Prox1-positive presumptive hippocampal cells (Supporting Information Fig. S2E and F) and an overall shrinkage brain size (Supporting Information Fig. S2E and G). In summary, *β-catenin* is required in the forebrain for normal tissue organization and hippocampal development.

β-catenin is required for adhesion and maintenance of neurosphere cells

We next addressed the question of whether the smaller brain seen following deletion of *β-catenin* in the forebrain (Supporting Information Fig. S1E and G) was associated with an effect on the

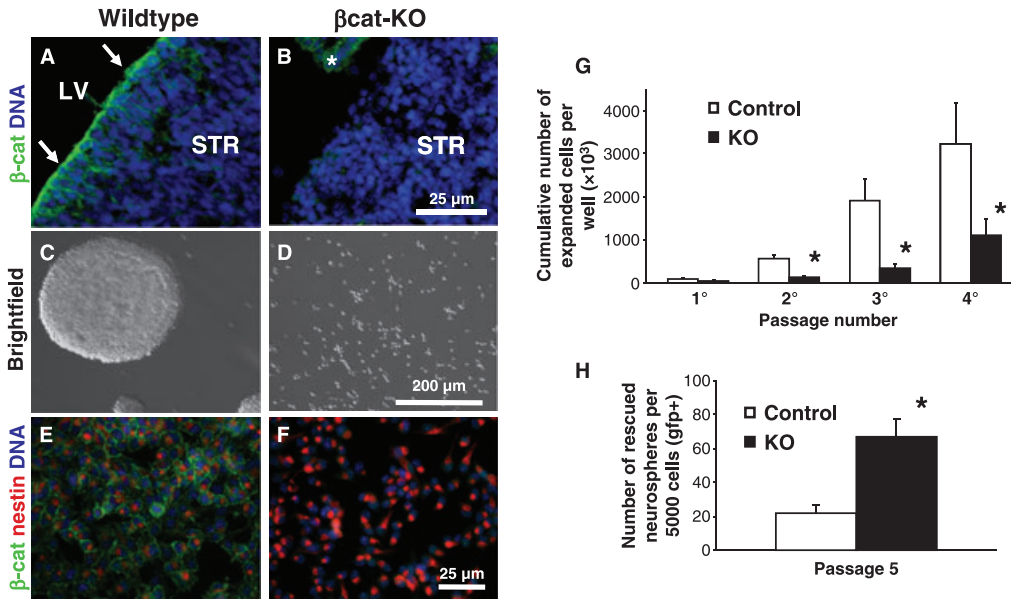

FIG. 1. β-catenin is required for adhesion and maintenance of neurosphere cells. (A) In the developing striatum (STR) of E14.5 wildtype embryos, β-catenin protein accumulation (arrows) is seen in cells lining the lateral ventricle (LV) and to a lesser extent in the parenchyma. (B) In β-catenin conditional knockout (KO) embryos, β-catenin expression is lost in the STR. Expression is retained in the non-neural choroid plexus (*). (C) When NSCs are isolated from the wildtype E14 STR and cultured at low density in SFM with FGF, they grow clonally to form large neurosphere colonies. (D) β-catenin conditional KO NSCs do not form intact neurospheres due to a lack of cell adhesion. Instead, individual cells are distributed throughout the bottom of the dish. (E) β-catenin antibody staining (green) in wildtype neurosphere cells shows distribution at the cell membrane. The neural progenitor marker, nestin (red), is also expressed. (F) In β-catenin conditional KO neurosphere cells, no β-catenin staining is detected but nestin (red) is robustly expressed. (G) Cumulative cell numbers after 1 week in culture are significantly lower in β-catenin conditional KO neurosphere cultures compared with wildtype for the secondary, tertiary and quaternary passages. (H) Rescue of β-catenin KO neurospheres. After five passages, control and β-catenin KO neurosphere cells were infected with a retrovirus expressing wildtype β-catenin. Significantly more gfp-positive intact neurospheres were found in the KO cultures compared with the controls, demonstrating the persistence and potential enhancement of NSCs in the KO population. All data areexpressed as mean ± SEM (*$P < 0.05$).

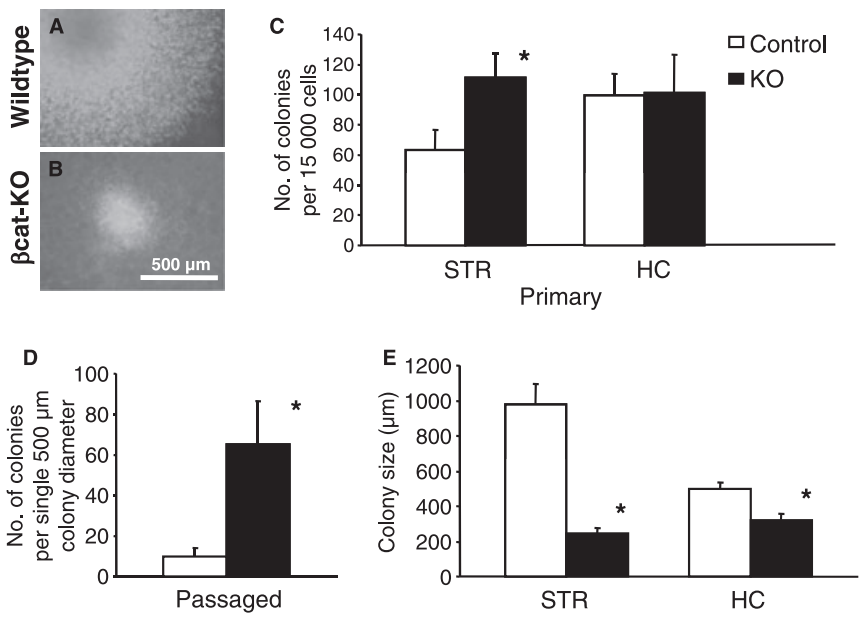

FIG. 2. The growth of neural progenitors in β-catenin knockout (KO) brains is impaired but NSCs are enhanced. When E14 striatal cells are plated at low density within a semi-solid collagen matrix, their progeny grow as single colonies. The number of clonal colonies in a dish indicates the number of NSCs. The size of the colonies indicates the number of neural progenitor cells. (A) Portion of a single wildtype colony that was derived from a single wildtype NSC. The center of the colony is in the top left corner of the photograph. (B) A β-catenin conditional KO colony that was grown from a single KO NSC. Note that the collagen matrix causes the images in A and B to be less distinct. (C) Loss of β-catenin leads to an increase in the number of primary striatal colonies, indicating that the KO striatum (STR) contains a greater proportion of NSCs (*$P < 0.05$). There was no significant difference in the number of primary NSC colonies obtained from the hippocampus (HC). (D) When single 500 μm diameter primary striatal colonies were passaged, a greater number of KO secondary collagen colonies were generated compared with controls. (E) Collagen colonies that lack β-catenin are significantly smaller than control colonies, indicating a decline in progenitor cell proliferation or decreased cell survival (*$P < 0.05$). All data are expressed as mean ± SEM.

neural stem and/or progenitor cell populations. Currently, no marker specific for these two populations is available; therefore, we performed an *in vitro* neurosphere assay using cells isolated from the striatum to assess their status. We confirmed deletion of the *β-catenin* allele in neurosphere cultures by genotyping (Supporting Information Fig. S3) as well as loss of β-catenin protein by immunofluorescent staining (Fig. 1E and F). Rather than proliferating to form intact neurospheres, *β-catenin* conditional knockout cells were distributed throughout the culture dish and did not adhere strongly to each other (Fig. 1C and D). For secondary, tertiary and quaternary passages, the total expanded cell number for the knockout cultures was significantly lower compared with that of dissociated control cultures (Fig. 1G; significant main effects were seen for genotype, $F_{322,1} = 6.396$, $P < 0.05$; passage, $F_{322,3} = 18.778$, $P < 0.05$; and interaction, $F_{322,3} = 13.142$, $P < 0.05$). Multiple comparison procedures revealed significant differences between control and knockout cell numbers for the secondary ($t_{124} = 2.883$, $P < 0.05$), tertiary ($t_{47} = 6.34$, $P < 0.05$) and quaternary passages ($t_{23} = 2.283$, $P < 0.05$). *β-catenin* therefore appears to be required for either cell growth or survival of neural stem and/or progenitor cells, and is also essential for proper cell adhesion.

Neural stem cells are enhanced in the brains of β-catenin knockout embryos but neural progenitors are impaired

In order to evaluate the ability of *β-catenin* knockout cells to form NSC colonies, it was necessary to overcome the problem of inadequate cell adhesion. To this end we used two approaches. First, we rescued β-catenin expression using a recombinant retrovirus. Second, we cultured NSCs in a semi-solid collagen gel that prevented cell progeny from floating away from each other. Upon reintroduction of wildtype *β-catenin* with a recombinant retrovirus it was possible to recover intact neurospheres from passage 5 *β-catenin* knockout cultures. Indeed, there were three times more rescued gfp-positive neurospheres in the knockout cultures compared with the number of gfp-positive neurospheres in the infected control cultures (Fig. 1H; $t_{56} = 3.49$, $P < 0.5$). Therefore, despite the inability to form cohesive neurospheres, NSCs were still present in the *β-catenin* knockout cell population even after prolonged passaging.

We also used a three-dimensional semi-solid collagen matrix with the aim of confining single NSCs and their progeny within a single colony (Louis *et al.*, 2008). This assay does not rescue adhesion as such but merely prevents the NSC progeny from floating away from the cell that originated the colony. As in the conventional neurosphere assay, the colony number is an indication of the number of NSCs in the original population, whereas the colony size indicates the status of the more limited neural progenitor cells. Within the collagen, single wildtype cells grew to form separated large colonies of cells (Fig. 2A). After 2 weeks in culture, *β-catenin* conditional knockout cells from the E14 embryonic striatum formed significantly more colonies (Fig. 2B and C; $t_{26} = 2.287$, $P < 0.05$), but they were substantially smaller than the control colonies (Fig. 2E; $t_{193} = 8.120$, $P < 0.05$). For cells obtained from the developing hippocampus, there was no significant difference in the number of colonies that grew (Fig. 2C; $t_{24} = 0.0458$, $P = 0.964$). However, as seen with the striatum-derived colonies, hippocampal knockout colonies were significantly smaller compared with controls (Fig. 2E; $t_{182} = 3.452$, $P < 0.05$). The decreased size of *β-catenin* mutant colonies (Fig. 2E) reveals a decline in the number of neural progenitor cells due either to decreased cell proliferation or decreased cell survival (see below). When single collagen-embedded colonies were dissociated into single cells and replated, *β-catenin* knockout colonies generated more new clonal colonies than control cells (Fig. 2D). The increased numbers of colonies obtained from *β-catenin* mutant brains (Fig. 2C) and following single colony passaging (Fig. 2D) may indicate that the NSC population is undergoing enhanced stem cell self-renewal following loss of *β-catenin*. However, it is more likely that the passaged colonies comprised more stem cells simply due to losses in the progenitor population (see below).

β-catenin is required for survival of neural progenitors

Neural progenitor cells make up the majority of cells in clonal NSC colonies. The reduced total number of expanded cell numbers in suspension cultures (Fig. 1G) and the smaller size of *β-catenin* knockout colonies grown in collagen (Fig. 2D) indicated a massive loss of the neural progenitor population in *β-catenin* mutant cells. The loss of neural progenitor cells could be due to programmed cell death or to a decrease in cell proliferation. To determine whether the loss of neural progenitor cells is due to a decrease in cell survival, we immunostained neural colonies grown in collagen for activated Caspase-3 (Fig. 3A and B). *β-catenin* knockout colonies had considerably more activated Caspase-3-positive cells compared with wildtype ($t_{19} = 4.72$, $P < 0.05$). To monitor the number of cells that were proliferating, we exposed cells grown in suspension in SFM to a 1 h pulse of BrdU, dissociated the cells and counted the number of BrdU-positive cells. We did not find a significant difference between control and *β-catenin* knockout cells (control 37.3 ± 2.8%, knockout 34.0 ± 1.3%; $t_{14} = 1.09$, $P > 0.05$). Moreover, as assayed by immunostaining for β3-tubulin, we did not detect premature neuronal differentiation in proliferating knockout cultures compared with controls (data not shown). A similar scenario with respect to cell death and proliferation was evident in the embryonic striatum *in vivo*. We found more terminal deoxynucleotidyl transferase dUTP nick end labeling staining in *β-catenin* knockout striatum at E14 (Fig. 3C and C′; control, 5.0 ± 1.5 cells/section; knockout, 9.4 ± 1.1 cells/section, $t_8 = 2.365$, $P < 0.05$) and no change in the proportion of BrdU-positive cells (Fig. 3D and D′; control, 76.3 ± 2.4%; knockout, 71.7 ± 2.4%, $t_{12} = 1.361$, $P > 0.05$). These results indicate that the decline in neural progenitors in *β-catenin* knockout striatum is due to increased cell death and not due to decreased cell proliferation.

β-catenin is not required for the normal differentiation of neural stem cells

We next assayed the proportions of cells that differentiated into the three neural cell types: neurons, astrocytes and oligodendrocytes. We found no significant difference in neural cell marker expression that could be generated from *β-catenin* knockout NSCs compared with wildtype (Fig. 4A–C; $t_{16} = 0.053$, $P > 0.05$ for microtubule-associated protein-2; $t_{16} = 1.615$, $P > 0.05$ for glial fibrillary acidic protein; $t_8 = 0.510$, $P > 0.05$ for O4).

Multipotentiality is another important property of stem cells as the entire array of neural cell types are expected to arise from a single NSC colony. To determine if *β-catenin* knockout NSCs retained multipotentiality, we imposed differentiation conditions on clonal neural colonies grown in collagen and immunostained them *in situ* for markers of neurons, astrocytes (Fig. 4A and A′) and oligodendrocytes (Fig. 4B and B′). Like wildtype colonies, all of the *β-catenin* knockout colonies were able to generate astrocytes (glial fibrillary acidic protein-positive; wildtype 11/11, 100%; *β-catenin* knockout 35/35, 100%) and double staining for a neuronal marker revealed that nearly all of these colonies also contained neurons

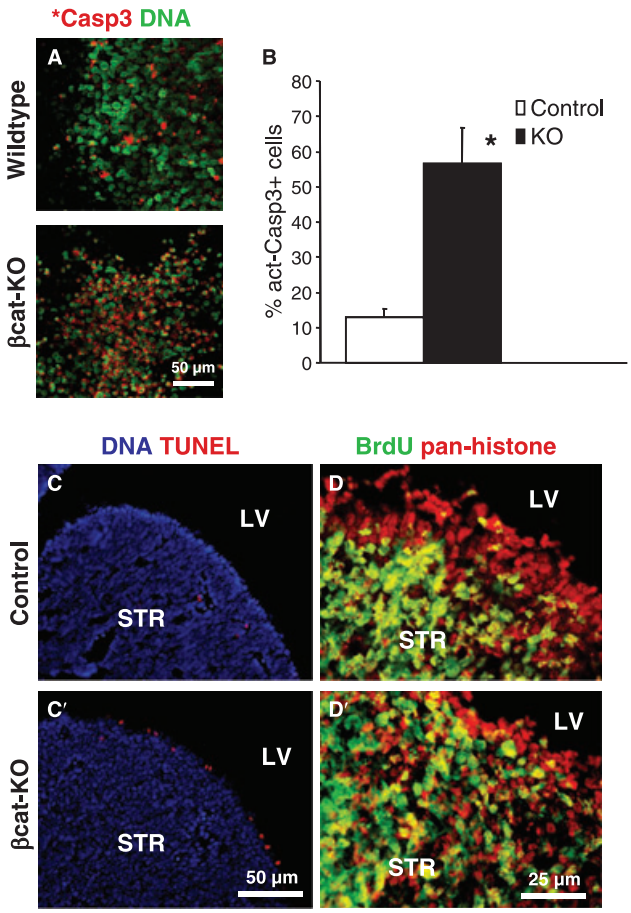

FIG. 3. The decline in neural progenitors is due to increased apoptosis and not to decreased cell proliferation. (A) Nestin-mediated β-catenin knockout (KO) leads to more cell death in collagen colonies as revealed by staining for activated Caspase-3 (red) and confocal microscopy. Anti-pan-histone staining (green) reveals nuclei. (B) The number of cells expressing activated Caspase-3 is dramatically increased in conditional β-catenin KO collagen colonies (*$P < 0.05$). Data are expressed as mean ± SEM. Apoptosis is increased in the brains of β-catenin conditional KO embryos *in vivo*. At E14, the germinal zone of the striatum (STR) of β-catenin KO mice (C′) contains more Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)--positive cells than wildtype (C) ($P < 0.05$). (D and D′) E14.5 embryos were treated with a 1 h pulse of BrdU, then fixed and sectioned to reveal labeling in the STR. No significant difference in the rate of cell proliferation was seen between control (D) and β-catenin KO (D′) brains ($P > 0.5$). Green, BrdU; red, pan-histone; LV, lateral ventricle.

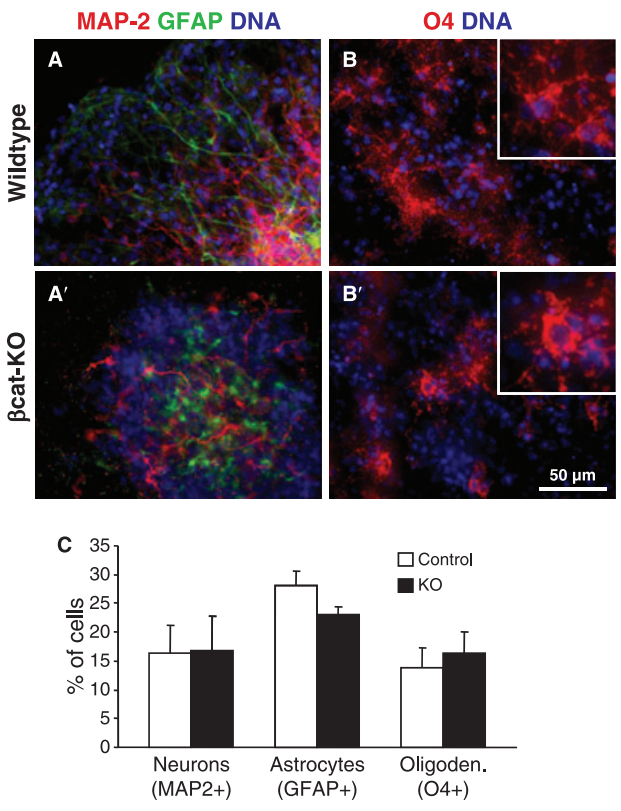

FIG. 4. β-catenin knockout (KO) NSCs are multipotential and exhibit normal neural differentiation. When single wildtype (A and B) or β-catenin KO (A′ and B′) NSCs are grown within a collagen matrix and differentiated *in situ*, they differentiate into all of the major neural cell types: neurons [microtubule-associated protein (MAP)-4-positive, red], astrocytes [glial fibrillary acidic protein (GFAP)-positive, green] and oligodendrocytes (O4-positive, red). Multipotentiality was retained after repeated passaging. Blue stain, Hoechst DNA. Insets in B and B′ show higher magnification view of O4 staining. (C) The proportions of neurons, astrocytes and oligodendrocytes relative to 4′,6-diamidino-2-phenylindole cells. β-catenin KO NSC cultures were not significantly different from controls, demonstrating that neural differentiation was normal ($P > 0.05$ for all comparisons). All data are expressed as mean ± SEM.

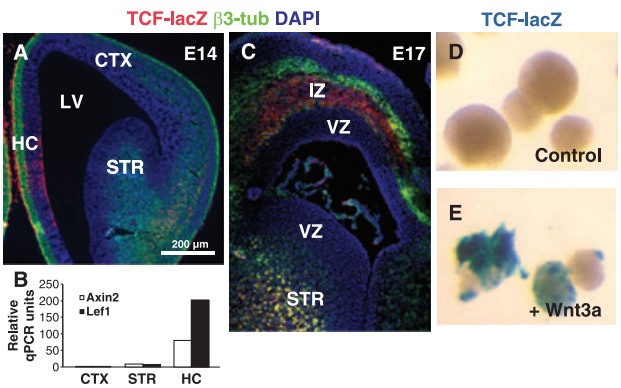

FIG. 5. Expression of TCF-lacZ in the embryonic brain and in neurospheres indicates zones that have active TCF signaling. (A) At E14, TCF-lacZ reporter activation (red) is seen in the future germinal zone and to a lesser extent in the cortical hem of the hippocampus (HC) but not in the striatum (STR) or in the dorsal cortex (CTX). DNA, blue. (B) Quantitative polymerase chain reaction of tissue dissected from the E14.5 dorso-lateral CTX, STR and presumptive HC showing highest levels of expression for Wnt-responsive genes axin-2 and lef-1. Levels are expressed using the delta–delta cycle threshold (CT) method with β-actin used as the housekeeping gene for normalization. (C) At E17, TCF-lacZ (red) is expressed in the intermediate zone (IZ) of the CTX and in the deeper regions of the STR. These are regions undergoing neuronal differentiation and are positive for the neuronal marker, β3-tubulin (green). TCF activation is not detected in the ventricular zone (VZ), where neural stem and progenitor cells reside. Yellow regions represent overlap of β3-tubulin and TCF-lacZ. (D) Clonal neurospheres that were obtained from the E14 STR of TCF-lacZ embryos (wildtype background) and that are comprised primarily of neural stem and progenitor cells do not express TCF-lacZ. (E) TCF activation (blue) is seen only when spheres are infected with a Wnt-3a retrovirus. DAPI, 4′,6-diamidino-2-phenylindole; LV, lateral ventricle; qPCR, quantitative polymerase chain reaction.

(microtubule-associated protein-2-positive; wildtype 10/11, 91%; *β-catenin* knockout 32/35, 91%). Oligodendrocytes also developed from nearly all wildtype and *β-catenin* knockout colonies (O4-positive; wildtype 6/7, 86%; *β-catenin* knockout 22/23, 96%). Multipotentiality was retained in *β-catenin* knockout NSCs despite the fact that these colonies contained far fewer cells than control colonies, even after they were passaged three times (data not shown). Therefore, loss of *β-catenin* does not change the relative types of differentiated cells, nor does it affect the ability of single NSCs to display multipotentiality.

Wnt/T-cell factor signaling is not activated in neural stem or progenitor cells

To evaluate Wnt/β-catenin-mediated transcriptional signaling, we analyzed the expression of a TCF-lacZ reporter in the forebrains of transgenic mice (Mohamed *et al.*, 2004). At E13 and E14, we found reporter expression in the dorso-medial portion of each cerebral cortical hemisphere (Fig. 5A) lying adjacent to the 'cortical hem', a source of Wnt proteins (Lee *et al.*, 2000). This is expected as development of the hippocampus is dependent on canonical Wnt/LEF1 signaling from the hem (Galceran *et al.*, 2000; Lee *et al.*, 2000). Interestingly, no LacZ expression was seen in other regions of the cortex or in the developing striatum. Further analysis of Wnt-TCF-responsive genes was assayed by quantitative polymerase chain reaction. Tissue taken from the E14 hippocampus showed very high expression of axin-2 and LEF1, whereas the dorsal cortex and germinal zone of the striatum expressed far less (Fig. 5B). At E17, TCF-lacZ expression was only seen in differentiating cells of the intermediate zone of the cerebral cortex and in the deeper layers of the striatum (Fig. 5C). The ventricular zones of both the cortex and striatum, where neural stem and progenitor cells are located (Tropepe *et al.*, 1999), were free of TCF-lacZ expression. We also examined the expression of this reporter in clonal neurospheres derived from E14 lateral and medial ganglionic eminences (from which the striatum and septum develop). Reporter expression could not be detected in normal neurosphere cells (Fig. 5D), indicating a lack of TCF/LEF activation in neural stem and progenitor cells. To eliminate the possibility that the TCF reporter is constitutively silent in these cells, we added a retrovirus expressing Wnt-3a to primary neurosphere cells in order to activate the reporter. Wnt-3a readily induced lacZ expression (Fig. 5E), demonstrating that the TCF-lacZ reporter is functional in neurosphere cells. Notably, Wnt-3a also caused a decrease in neurosphere number and greater adhesion of neurospheres to the culture dish (data not shown). These results, obtained both *in vivo* and *in vitro*, suggest that the downstream TCF/LEF signaling is not normally active in neural stem or progenitor cells.

Discussion

The results presented here show that deletion of *β-catenin* in the mouse embryonic brain leads to death in the neural progenitor population, whereas the NSC population is enhanced. An *in vitro* collagen-based neural colony-forming assay revealed that *β-catenin* deletion leads to an increased number of striatal NSC colonies, whereas the numbers of hippocampal NSCs were unaffected. These colonies were small compared with controls but this did not affect their ability to differentiate into multiple neural cell types even after repeated passaging. The fact that these single *β-catenin* knockout stem cell-derived colonies still showed both self-renewal and neural multipotentiality suggests that loss of *β-catenin* and defects in adhesion do not limit the numbers of NSCs.

Three possible mechanisms could explain the increase in the striatal NSC colonies following ablation of *β-catenin*. First, as neural progenitors die due to apoptosis, the relative proportion of stem cells increases. Second, NSCs divide more symmetrically in an attempt to compensate for the death of neural progenitors. Third, the neural progenitor cells may have undergone dedifferentiation into NSCs upon loss of *β-catenin*.

With regard to the first mechanism, we carefully selected only 500 μm diameter colonies for single colony passaging. This size represented the smaller control colonies, but the largest knockout colonies. As knockout progenitors were being lost due to apoptosis, this allowed for a larger proportion of surviving NSCs to comprise the knockout colony. Hence a greater number of secondary colonies should be established by these surviving NSCs.

An explanation in terms of the second mechanism (i.e. more symmetric NSC divisions in the knockout) may be consistent with the significant tissue disorganization in the *β-catenin* knockout forebrain due to changes in cell adhesion. As polarized cells are more prone to undergo asymmetric cell divisions, the changes in tissue organization following ablation of *β-catenin* could support more symmetric cell divisions. Normally, single colony passaging would be sufficient to resolve whether this is the case. However, our single colony passaging experiments are confounded by apoptosis in the progenitor cell population. Under these circumstances, it would be necessary to follow single NSCs and their progeny. Because we cannot directly observe the NSCs (and distinguish them from the more abundant neural progenitor cells), this experiment awaits development of a unique live marker for NSCs.

The third possible mechanism for the increase in NSCs in the *β-catenin* knockout brain is the dedifferentiation of neural progenitor cells into NSCs. When we used a recombinant retrovirus expressing wildtype β-catenin to rescue neurospheres from knockout cultures, intact neurospheres were recovered. Nevertheless, because we did not see all of the infected knockout cells generating neurospheres, we can conclude that they were mainly progenitors and had not dedifferentiated into NSCs.

We believe that the first mechanism (i.e. that the proportion of NSCs is increased in knockout brains owing to the loss of progenitors due to apoptosis) is the more likely explanation for the increase in NSC colonies obtained from the brain as well as following repeated passaging. These increases reveal that the NSC population is not affected by loss of β-catenin in the same way that progenitors are, i.e. NSCs apparently survive and are able to generate new stem cell colonies.

In wildtype cells, *β-catenin* does not appear to exert its effects through TCF/LEF1 activation, as a TCF-lacZ reporter is not activated in the anterior ventricular zones of the striatum and lateral cortex where neural stem and progenitor cells reside (Zechner *et al.*, 2003). This is in agreement with previous studies of other TCF reporter strains that also show expression in deep striatal and cortical areas but absence in the ventricular zones (Machon *et al.*, 2005, 2007; Gulacsi & Anderson, 2008; Mutch *et al.*, 2009). In contrast, at E14.5, we found TCF-lacZ expression in both the ventricular zone and cortical plate only in the caudo-medial regions of the forebrain, corresponding to the future hippocampal primordium (Fig. 5A). Reverse transcription-polymerase chain reaction analysis of Wnt response genes supports the observation that the hippocampus has high TCF activity compared with the ventricular zone of the striatum (Fig. 5B). The areas of positive TCF-lacZ activation within this region may represent a regional patterning event, separate from neural stem and progenitor

cell regulation. TCF/LEF1 activation (Galceran *et al.*, 2000) and β-catenin expression (Backman *et al.*, 2005) are necessary for hippocampal development, and the TCF reporter activation described here and in previous studies (Maretto *et al.*, 2003; Machon *et al.*, 2005, 2007; Lien *et al.*, 2008) may reflect early specification of the hippocampal anlagen. Importantly, we did not find TCF/LEF1 activation *in vitro* in striatal neurospheres cultured in proliferative growth conditions. Therefore, the effects of *β-catenin* ablation on neural stem and progenitor cells are more likely to involve changes in β-catenin/cadherin-based cell adhesion rather than TCF/LEF1 signaling.

Our results are at variance with those obtained by *in utero* electroporation of the TOPFLASH reporter into cortices of E13.5 embryos for which reporter activation was seen in the ventricular zone (Woodhead *et al.*, 2006). This observed activation may have been related to regional specification of the hippocampus in the dorsomedial cortex. Expression in other regions of the forebrain containing neural stem and progenitor cells (such as the ventral ganglionic eminence) was not assessed in that study. Furthermore, the same study did not directly examine the status of the NSC population and focused rather on neuronal progenitor cells. The only available assays for NSCs at embryonic stages are the neurosphere assay and the collagen-based colony-forming assay described here.

Gain-of-function studies have shown that β-catenin overexpression can enhance the differentiation of neurons (Hirabayashi *et al.*, 2004; Israsena *et al.*, 2004), similar to the effect of canonical Wnt ligands (Hirabayashi *et al.*, 2004; Muroyama *et al.*, 2004; Lie *et al.*, 2005; Kuwabara *et al.*, 2009; Kuwahara *et al.*, 2010). It is interesting that the converse is not upheld. We found that loss of *β-catenin* did not change the relative ratios of differentiated neurons and glia that were generated. Furthermore, NSCs remained multipotential even after repeated passaging. This suggests that pathways in addition to Wnt/β-catenin act to regulate neural differentiation and multipotentiality in NSCs.

A recent study (Kalani *et al.*, 2008) proposed that Wnt-3a protein could improve the colony-forming efficiency of embryonic forebrain cells that express the Wnt reporter gene axin2-lacZ but not of those that do not. This draws attention to the notion that there may be Wnt-responsive as well as Wnt-non-responsive NSC populations within the brain. Whether the self-renewal capacity is enhanced by Wnt-3a was not entirely clear from this study. Passaging of neurospheres was performed in bulk and the smaller size of Wnt-3a-treated neurospheres confounds a correct assessment of stem cell self-renewal. Another group has shown that β-catenin (Hirabayashi *et al.*, 2004) as well as Wnt-3a (Hirabayashi & Gotoh, 2005; Kuwahara *et al.*, 2010) overexpression increase proliferation of cortical neural progenitor cells at E10.5-11.5 but cause neuronal differentiation at E13.5. At earlier stages, the dorsal cortex may have included the hippocampal progenitor cells of the dorsal medial cortex that have activated TCF signaling and are Wnt-responsive (Machon *et al.*, 2005). At E13.5, it is likely that the dorsal cortex does not include these TCF-positive presumptive hippocampal cells (see Fig. 5).

In accordance with previous studies that reported an increase in cell death *in vivo* following decreased *β-catenin* expression (Zechner *et al.*, 2003; Junghans *et al.*, 2005; Tang *et al.*, 2009) or loss of *N-cadherin* (Kadowaki *et al.*, 2007), we saw an increase in cell death specifically in neural progenitor cells. *β-catenin* knockout NSCs were presumably not impacted as demonstrated by the ability of mutant colonies to passage more efficiently than controls. Cell–cell contact mediated by β-catenin and cadherins may be important for providing trophic support to neural progenitor cells, but exactly how cell adhesion mediates cell survival is not well understood (Grossmann, 2002). It may be that adhesion brings cells closer together so that more survival signals reach each neural progenitor.

Although increased Wnt/β-catenin signaling has been correlated with cyclin-D upregulation and increased cell proliferation (Clevers, 2006), we did not see a change in cell proliferation in the striatal germinal zone following loss of *β-catenin* either *in vivo* or *in vitro*. Two other studies (Machon *et al.*, 2003; Junghans *et al.*, 2005) have also reported unaltered mitotic activity in the dorsal-lateral telencephalon. Furthermore, it is well known that Wnt elicits changes in cell proliferation primarily via TCF/LEF1 activation (Clevers, 2006). As this pathway is not active in neural stem and progenitor cells, alternative pathways must be acting to control cell proliferation in the ventricular zone of the striatum.

Loss of *β-catenin* in the skin blocks differentiation into epidermis, but its specific effects on the epidermal stem cell population have not been studied (Huelsken *et al.*, 2001). In the intestine, conditional ablation of *β-catenin* leads to terminal differentiation of intestinal stem/progenitor cells (Fevr *et al.*, 2007). Interestingly, activation of canonical Wnt signaling with R-spondin is necessary for efficient *in vitro* growth and passaging of intestinal stem cell colonies (Sato *et al.*, 2009). In the hematopoietic system, *β-catenin* does appear to be required for long-term maintenance of the stem cell population (Zhao *et al.*, 2007; but also see Jeannet *et al.*, 2008 and Koch *et al.*, 2008). In contrast, canonical Wnt signaling facilitates differentiation of muscle satellite stem cells (Brack *et al.*, 2008; Le Grand *et al.*, 2009). Therefore, intestinal and hematopoeitic stem cells appear to be more dependent on canonical Wnt signaling/*β-catenin* than NSCs or muscle satellite stem cells.

In summary, our results show that β-catenin is not required for the maintenance of NSCs, nor is it required for the differentiation of multiple neural cell types from individual NSC clones. In contrast, β-catenin is critical for progenitor cell survival. We propose that, in neural stem and progenitor cells, the primary role of β-catenin may be to control cell adhesion rather than in regulating TCF/LEF1 activation.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Tissue disorganization and loss of the hippocampus in the brains of β-catenin conditional knockout (KO) embryos. (A) Filamentous actin (F-actin), as revealed by staining with rhodamine-phalloidin, is normally distributed in cells lining the lateral ventricle (LV) (arrows) at E14.5. Blue, 4′,6-diamidino-2-phenylindole (DAPI) staining of DNA. (B) In conditional KO embryos, F-actin accumulation is prevalent in deeper regions of the striatum (STR) (arrows). (C) Normally, N-cadherin (Ncad) occupies the lining of the LV (arrows). (D) In conditional KO brains, N-cadherin protein is more highly concentrated in apical regions (arrows). (E) Nestin is expressed in the periventricular region of control STR. (F) In conditional KO STR, nestin is still expressed in the same region despite significant tissue disorganization. Mash1 is similarly expressed in the periventricular region of both the control (G) and KO (H) STR. CTX, cortex.
Fig. S2. (A) In E17 wildtype cortex (CTX), Tbr1 staining (red) delineates the cortical plate (CP) and intermediate zone (IZ), whereas Pax6 staining (green) is restricted to the ventricular zone (VZ). Note that some of the Pax6 staining in the IZ may be attributed to background binding of the primary antibody to mouse IgG. (B) A more specific rabbit polyclonal antibody to Pax6 demarcates the VZ of the wildtype CTX. (C) In Nestin-mediated β-catenin knockout (KO)

embryos, Tbr1 expression extends ventrally toward the ventricle resulting in overlap of markers of the intermediate and ventricular cortical zone cells. (D) Extension of Pax6-positive VZ cells into more dorsal regions of KO CTX. (E) Prox1 (red) is expressed in the hippocampal primordium of E17 wildtype embryos. HC, hippocampus. (F) Higher magnification view of box in E showing Prox1-positive nuclei. (G) The neuroepithelium of mutant cortices is thinner. Also, much of the HC is lost in mutant mice. (H) Higher magnification view of box in G showing reduced number of Prox1-positive nuclei.
Fig. S3. Genotyping of neurosphere cultures to detect loxed (lox), wildtype (wt) and deleted (del) β-catenin alleles. Lane 1, Knockout (ko) culture that is *Nestin:Cre* positive and therefore does not possess a loxed or a wildtype β-catenin allele. Lane 2, Wildtype culture that does not carry the *Nestin:Cre* allele and that has one loxed β-catenin allele and one wildtype allele. Lane 3, Knockout culture that has a deleted β-catenin allele. Lane 4, Wildtype culture that does not have a deleted β-catenin allele. PCR, polymerase chain reaction.
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Abbreviations

BrdU, 5-bromo-2-deoxyuridine; Ct, Cycle threshold; E, embryonic day; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; gfp, green fluorescent protein; LEF, lymphocyte enhancer factor; NSC, neural stem cell; PBS, phosphate-buffered saline; SFM, serum-free medium; TCF, T-cell factor; TUNEL, Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; TRITC, tetramethyl rhodamine iso-thiocyanate.

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