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Multiple roles of β-catenin in controlling the neurogenic niche for midbrain dopamine neurons

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Stem cell-based replacement therapy has emerged as a potential strategy to alleviate specific features of movement disorder in Parkinson's disease. However, the current strategy to produce dopamine (DA) neurons from embryonic stem cells has many limitations, including the difficulty of generating DA neurons with high yields. Further insights into the mechanisms that control the neurogenesis of DA neurons will reduce or mitigate such limitations. It is well established that the ventral midbrain (vMB) contains the neurogenic niche that produces DA neurons. However, it is unclear how the microenvironment within this niche controls DA neurogenesis. Here, we show that β-catenin controls DA neurogenesis by maintaining the integrity of the neurogenic niche and the progression from progenitors to DA neurons. Using conditional gene targeting approaches, we show that regional deletion of β-catenin in the vMB by using *Shh-Cre* disrupts adherent junctions of progenitors and the integrity of radial glia in the vMB, which leads to a severe reduction in DA neurogenesis and perturbs the migration and segregation of DA neurons. By contrast, *Th-IRES-Cre* removes β-catenin in a subset of neural progenitor cells without perturbing the cellular and structural integrity of the vMB. Interestingly, loss of β-catenin in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants negatively regulates neurogenesis by interfering with the progression of committed progenitors to DA neurons. Taken together, these results provide new insights into the indispensable functions of β-catenin at multiple stages during DA neurogenesis. They also suggest that β-catenin-mediated signaling pathways can be targeted to promote and expand DA neurons in cell-based therapeutic strategies.

KEY WORDS: Dopamine neuron, Wnt signaling, β-catenin, Neurogenesis, Adherent junction, Radial glia, Mouse

INTRODUCTION

The molecular mechanism that governs the neurogenesis and differentiation of midbrain dopaminergic (DA) neurons has attracted intense investigations because of the prospect of using stem cells in restorative therapies for patients with Parkinson's disease. It is hoped that a thorough understanding of these mechanisms will lead to therapeutic strategies that improve the efficiency of DA neuron production, engraftment and function. Indeed, recent efforts have led to the identification of several intrinsic DA neuron determinants, including Nurr1, Pitx3, En1/2, Otx2, Foxa1/2, Ngn2 and Lmx1a, which enable stem and progenitor cells to establish proper cell identity, and control a cascade of transcriptional machinery that regulates the cell fate and differentiation of DA neurons at the early stages of neurogenesis (Andersson et al., 2006a; Andersson et al., 2006b; Ferri et al., 2007; Kele et al., 2006; Nunes et al., 2003; Omodei et al., 2008; Puelles et al., 2004; van den Munckhof et al., 2003) (for reviews, see Ang, 2006; Prakash and Wurst, 2006; Smidt and Burbach, 2007). DA neurons in mouse mutants lacking one of these transcription factors either fail to acquire proper cell identity or undergo accelerated degeneration due to aberrant cell death (Andersson et al., 2006a; Ferri et al., 2007; Kele et al., 2006; Kittappa et al., 2007; Nunes et al., 2003; Puelles et al., 2004; Saucedo-Cardenas et al., 1998; Sgado et al., 2006; Sonnier et al., 2007; van den Munckhof et al., 2003; Zetterstrom et al., 1997). Conversely, overexpression of Nurr1, Pitx3 and Lmx1a in embryonic stem cells promotes a robust generation of neurons with molecular profiles and functional properties that resemble ventral midbrain (vMB) DA neurons (Andersson et al., 2006b; Chung et al., 2005; Wagner et al., 1999). Despite these promising results, the efficiency of generating mature DA neurons from stem cells and with which these neurons engraft has been low, suggesting that additional mechanisms, such as signals from the microenvironment of DA progenitors, might be important for the neurogenesis of DA neurons.

Indeed, the development of DA neurons depends on a number of exogenous factors, such as sonic hedgehog (Shh) and fibroblast growth factor 8 (Fgf8), which are important for the patterning and expansion of these neurons (Hynes et al., 1995; Ye et al., 1998). Several lines of evidence indicate that members of the Wnt family might also regulate the development of DA neurons. For instance, addition of Wnt1 to vMB cultures from E14.5 mouse embryos promotes the expansion of DA progenitors, whereas addition of Wnt5a enhances the acquisition of DA neuron phenotype (Castelo-Branco et al., 2003; Schulte et al., 2005). Interestingly, Wnt1 is expressed both in the dorsal aspect of the midbrain-hindbrain junction and along the midline of the vMB (Omodei et al., 2008; Prakash et al., 2006; Puelles et al., 2004). Furthermore, lineage tracing using the inducible *Wnt1-CreER^T* allele shows robust recombination in the vMB, suggesting that Wnt1 can exert both long- and short-range effects on DA neuron development (Zervas et al., 2004). Consistent with these results, loss of Wnt1 results in a failure to induce DA neurons in the vMB, whereas overexpression of Wnt1 in the En1-expressing domain leads to an ectopic expansion of DA progenitors (Danielian and McMahon, 1996; Prakash et al., 2006; Prakash and Wurst, 2006). Although these results suggest that Wnt1 signaling mechanisms influence multiple aspects of DA neurogenesis, the detailed in vivo functions of Wnt1 on DA progenitors are not fully elucidated.

In this study, we investigated the roles of β-catenin, an essential component of the canonical Wnt signaling pathway and the cadherin-dependent cell adhesion complex (Logan and Nusse, 2004; Takeichi, 2007), in the development of DA neurons. Our results showed that β-catenin was abundant in two important locales within

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the neurogenic niche of DA neurons, namely the adherent junctions in the ventricular zone (VZ) and the radial glia in the vMB. To further investigate the functions of β-catenin, we generated two conditional mutants, one removed β-catenin from the neurogenic niche in the vMB and the other from the progenitor cells of DA neurons. The removal of β-catenin in the vMB using Cre recombinase that was inserted into the *Shh* locus (*Shh-Cre*) results in loss of cell polarity of DA progenitors and severe disruption of radial glia processes. As a consequence, neurogenesis of DA neurons and the migration and segregation of DA neurons to the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) were severely perturbed. By contrast, the removal of β-catenin in DA progenitors using *Th-IRES-Cre* resulted in a more modest phenotype due to the continuous role of β-catenin in cell cycle progression in DA progenitors. These results revealed previously unidentified functions of β-catenin in the neurogenesis of DA neurons, which can potentially be harnessed to improve the efficacy of stem cell replacement therapy for Parkinson's disease.

MATERIALS AND METHODS

Animals

To generate conditional knockout of β-catenin mice, β-catenin floxed (*β-Ctn^{fl}*) mice (Jackson Laboratories, Stock number 004152) were crossed with *Shh-Cre* (Jackson Laboratories, Stock number 005622) or *Th-IRES-Cre* mice (Harfe et al., 2004; Lindeberg et al., 2004). Reporter mouse line R26R was purchased from Jackson Laboratories (Stock number 003474). Animal care was approved by the Institutional of Animal Care and Use Committee and followed the NIH guidelines.

Histology and immunohistochemistry

Histology and immunohistochemistry (IHC) were the same as described previously (Zhang et al., 2007). Briefly, mouse embryos, from embryonic day 10.5 (E10.5) to E12.5, were fixed with 1% paraformaldehyde (PFA) in PBS (4% PFA for Nkx6.1 antibody). Mice at E18.5 and postnatal day 0 (P0) were perfused with 1% PFA, and the brain fixed in 1% PFA, cryoprotected in 15%-30% sucrose solution and sectioned in the coronal plane using a Leica cryostat. Mouse brains were sectioned at a thickness of 14 μm (for stereology counting, brains were cut at 40 μm), and mounted on Superfrost glass slides.

Sections were incubated with primary antibody overnight (details of primary antibodies used are available on request) and secondary antibodies to detect signals. For stereology counting, sections were incubated for 1 hour with biotinylated IgG and an avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA). Images were captured using a Nikon Eclipse E800 fluorescent microscope (Nikon, Japan), connected to a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI, USA), or a BX41 Olympus microscope equipped with an Olympus DP70 CCD camera (Olympus, Japan). Images were captured using Spot Advance or Olympus DP Controller software programs. To analyze the recombination pattern of *Th-IRES-Cre*, serial confocal images were captured, using a Leica confocal microscope (TCS SP, Leica Microsystems), in the entire vMB of *Th-IRES-Cre;R26R* embryos at E9.5, E10.5, E11.5 and E12.5 (n=3 for each stage). The extent of recombination was determined by the percentage of Sox2- or Ngn2-positive progenitors that co-expressed *lacZ*.

BrdU labeling

Pregnant mice were injected intraperitoneally with BrdU 50 μg/gm (BD Bioscience, San Jose, CA, USA) (Zhang et al., 2007). We carried out two injection schemes. In the first scheme, the pregnant mice were injected with BrdU at E10.5 and E12.5, respectively, and sacrificed 2 hours later. In the second scheme, the pregnant mice were injected with BrdU at E10.5 and E11.5, respectively, and sacrificed 24 or 48 hours later.

Stereology

The total number of Th$^+$ neurons in the SNpc and VTA was determined using the optical fractionator, an unbiased cell counting method not affected by the volume of the reference (i.e. SNpc or VTA) or the size of the counted elements (i.e. neurons) (Zhang et al., 2007). Neuronal counts were performed using a computer-assisted image analysis system consisting of an Olympus BX-51 microscope equipped with an XYZ computer-controlled motorized stage and the StereoInvestigator software (Microbrightfield, Villiston, VT, USA). Th$^+$ neurons were counted in the SNpc or VTA of every third section throughout the entire midbrain. Each section was viewed at lower power (4×) and outlined. At a random start, the numbers of Th-stained cells were counted at high power (60× oil, numerical aperture 1.4) using a 50×50 μm counting frame**.** For tissues from embryos and P0 mice, a 10 μm dissector was placed 1.5 μm below the surface of sections.

Statistical analyses

Data were analyzed by two-tailed Student's t-test. Values were expressed as mean±s.e.m. Changes were identified as significant if the P-value was less than 0.05.

RESULTS

Expression of β-catenin in adherent junctions and radial glia processes in the vMB

To understand the roles of β-catenin in neurogenesis of DA neurons, we examined the expression of β-catenin in the vMB during development. We found that β-catenin was abundant at the apical side of ventricular zone, where it colocalized with N-cadherin in adherent junctions (Fig. 1A-C′). Immunogold electron microscopy confirmed that β-catenin and N-cadherin were present in adherent junctions at the apical side of the ventricular zone (Fig. 1D-F). In addition, β-catenin and N-cadherin were also colocalized in numerous linear structures that extended from ventricular zone to marginal zone (Fig. 1A-C). These linear structures co-expressed radial glia markers, including RC-2 (Ifaprc2 – Mouse Genome Informatics), nestin, and Glast (Slc1a3 – Mouse Genome Informatics; Fig. 1G-I; data not shown).

To determine the expression of β-catenin in developing DA neurons, we used tyrosine hydroxylase (Th) as a marker and showed that newly born DA neurons expressed β-catenin in the cytoplasm (Fig. 1J-L, arrowheads). By E12.5, the cell bodies of DA neurons and Th-positive (Th$^+$) processes were in close proximity along the radial glia processes that were positive for Glast, RC-2 or nestin (Fig. 1M-O). Interestingly, different z-series of confocal images showed that a number of Th$^+$ processes also co-expressed the radial glia marker nestin (Shults et al., 1990) (Fig. 1P-R, arrowheads). These results were consistent with a recent study showing that radial glia in the vMB give rise to DA neurons (Bonilla et al., 2008), and further suggest that a subset of DA neurons might continue to express radial glia markers. The preponderance of β-catenin in adherent junctions and radial glia processes suggests that the primary function of β-catenin in DA neurogenesis might be in maintaining the integrity of these two important structures in vMB and the progression of DA progenitors to differentiated neurons (Fig. 1S).

Regional deletion of β-catenin in the vMB disrupts adherent junctions and radial glia processes

To characterize the roles of β-catenin during the development of DA neurons, we removed β-catenin from progenitor cells and differentiated DA neurons in the vMB. To this end, we generated conditional mutants that expressed Cre from the sonic hedgehog promoter (*Shh-Cre*) and were homozygous for a β-catenin floxed allele (*β-Ctn$^{fl/fl}$*; mutants were designated as *Shh-Cre;β-Ctn$^{fl/fl}$*) (Brault et al., 2001; Harfe et al., 2004; Kittappa et al., 2007). By using *R26R* as a reporter (Soriano, 1999), we found that *Shh-Cre* recombination occurred in the vMB from E10.5 to E12.5 in a discrete domain that included the floor plate and part of basal plate (Fig. 2A-C). At E10.5, Cre recombinase was expressed in Sox2-

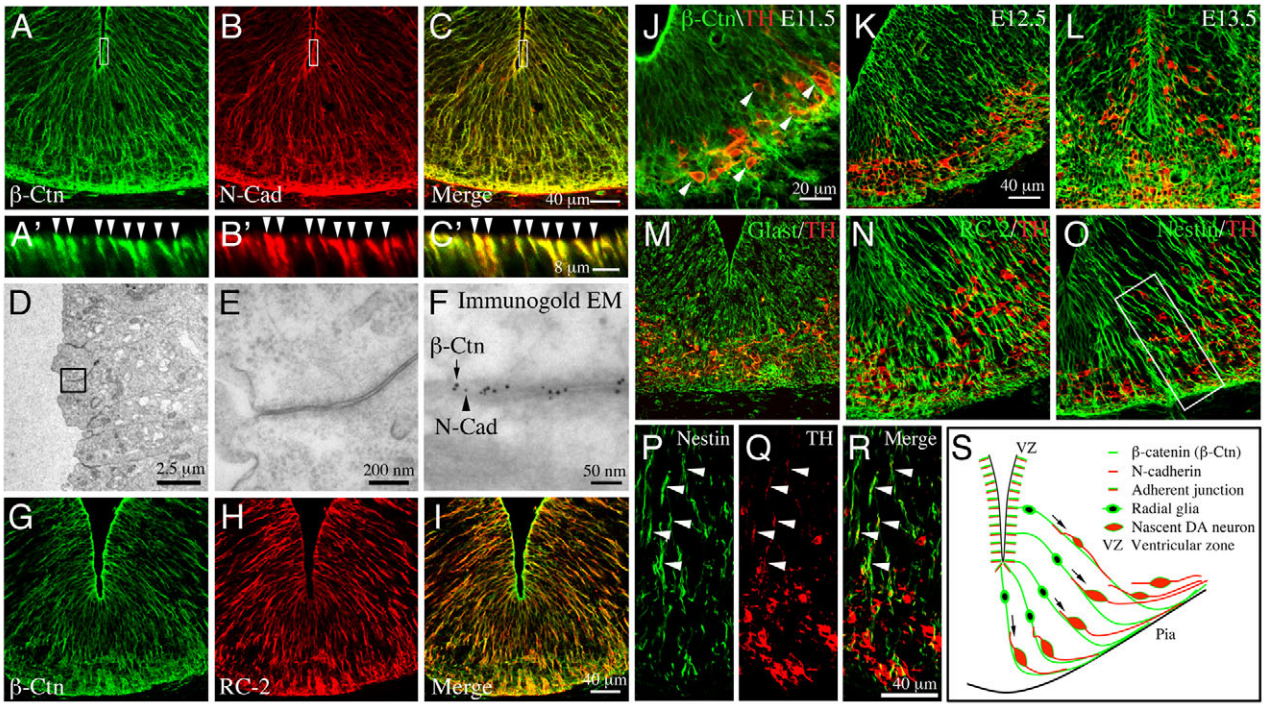

Fig. 1. Expression of β-catenin in adherent junctions, radial glia processes and DA neurons in ventral midbrain. (**A-C**) Confocal images of β-catenin and N-cadherin in adherent junctions and radial glia in the ventricular zone of the vMB. (**A′-C′**) Regions highlighted in panels A-C show colocalization of β-catenin and N-cadherin in adherent junctions (arrowheads). Scale bars: in C, 40 μm; in C′, 8 μm. (**D-F**) Immunogold electron microscopy images confirm colocalization of β-catenin (arrow, 15 nm particles) and N-cadherin (arrowhead, 10 nm particles) in adherent junctions. Scale bars: in D, 2.5 μm; in E, 200 nm; in F, 50 nm. (**G-I**) Confocal images showing coexpression of β-catenin with the radial glia marker RC-2. Scale bar in I: 40 μm. (**J-L**) Confocal images showing coexpression of β-catenin (green) in the cytoplasm of newly born DA neurons (TH, red) from E11.5 to E13.5. Arrowheads in J indicate β-catenin in Th^+ cells. (**M-O**) Confocal images show DA neurons are present in close proximity to radial glia processes highlighted by Glast (M), RC-2 (N) and nestin (O). Scale bars: in J, 20 μm; in K, 40 μm for K to O. (**P-R**) A small number of DA neuron processes (Q) coexpress the radial glia marker nestin (P), indicating that a subset of DA neurons might be radial glia. Panels P-R show higher magnification views of the region highlighted in O. Scale bar in R: 40 μm for P-R. (**S**) A diagram summarizing the presence of β-catenin in adherent junctions in the ventricular zone (VZ), radial glia processes, and cytoplasm of DA neurons.

positive ($Sox2^+$) progenitor cells (Fig. 2D-F) (Kittappa et al., 2007), and in newly formed DA neurons from E10.5 to E12.5 (Fig. 2G-I; arrowheads). Consistent with the Cre recombination pattern (Fig. 2A-C), immunohistochemistry showed loss of β-catenin in regions of the vMB that contained both progenitors and differentiated DA neurons (Fig. 2J,K; arrows indicate Th^+ DA neurons). The few linear structures that continued to express β-catenin were blood vessels that co-expressed the endothelial marker CD31 (Pecam1 – Mouse Genome Informatics; Fig. 2J,K, arrowheads; data not shown).

Analyses of *Shh-Cre;β-Ctn$^{fl/fl}$* embryos revealed a severe perturbation in the organization of the vMB, including the floor plate (Fig. 3). Compared with control *β-Ctn$^{fl/fl}$* littermates, *Shh-Cre;β-Ctn$^{fl/fl}$* embryos showed disorganized neural progenitors in the vMB at E12.5 (Fig. 3A,B). Many progenitors in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants were detached from the neural tube and became free-floating in the enlarged ventricle (Fig. 3B, arrowheads). To further characterize this phenotype, we used pericentrin as a marker for the dividing cells in the ventricular zone (Yingling et al., 2008). Whereas the dividing neural progenitors were aligned along the ventricular zone in E12.5 control embryos, those in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants either lost the distinct orientation of pericentrin or completely lacked the expression of this marker (arrowheads, Fig. 3C,D and insets). This phenotype resembled those in conditional mutants where N-cadherin was removed from the telencephalon (Kadowaki et al., 2007), and suggested that the removal of β-catenin might disrupt the organization of adherent junctions and polarity in neural progenitors. Indeed, electron microscopy showed that adherent junctions in the neuroepithelium in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants lacked the classical filamentous structures associated with cell membranes (Fig. 3E,F). These results indicated that loss of β-catenin perturbed other crucial components in adherent junctions, such as N-cadherin and α-E-catenin. Consistent with this notion, confocal microscopy showed a severe downregulation of N-cadherin and α-E-catenin in regions of the vMB in which loss of β-catenin was detected (Fig. 3G,H; see also Fig. S1 in the supplementary material). The residual N-cadherin and α-E-catenin proteins were diffusely distributed in the neuroepithelia of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (Fig. 3H, arrowheads; Fig. S1 in the supplementary material).

We analyzed the impact of removing β-catenin on DA neurogensis in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants. Schematic diagrams of sagittal and cross sections of a E12.5 mouse embryo to highlight the anteroposterior (AP) position of the vMB where analyses were performed are shown in Fig. 3I,J. In control embryos, DA neurons were detected in the vMB as early as E11.5, and showed a progressive increase in number from E12.5 to E18.5 (Fig. 3K-S). The newly born DA neurons formed compact aggregates in the marginal zone and projected processes to rostral targets. By contrast, far fewer DA neurons were detected in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E12.5 (Fig. 3L). Although DA neuron number in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants increased slightly after E12.5, many mutant DA neurons

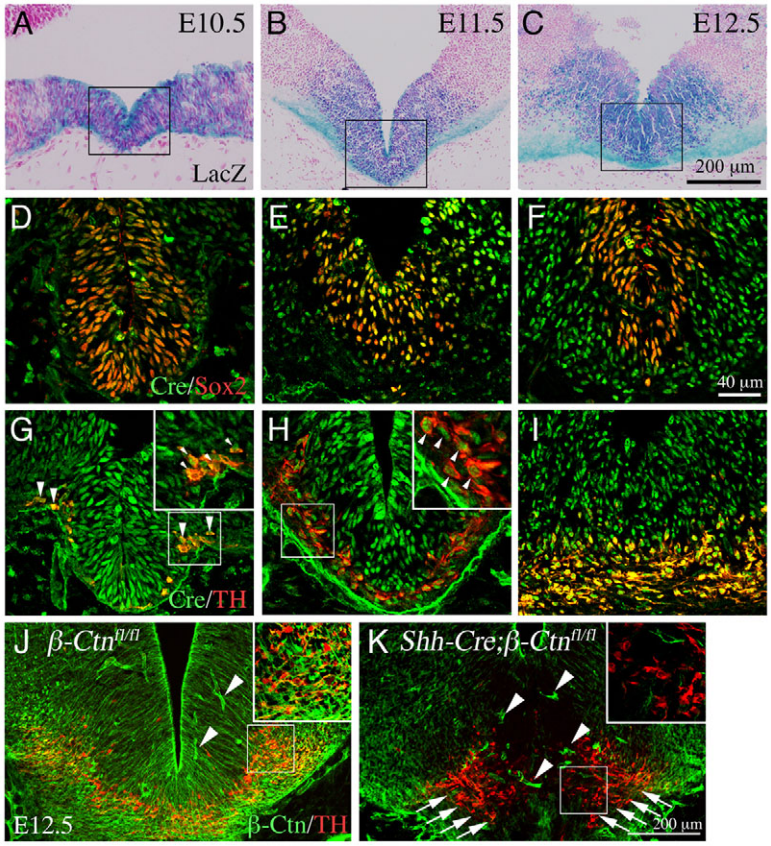

Fig. 2. Expression and recombination pattern of *Shh-Cre* in the neurogenic niche of DA neurons. (**A-C**) *Shh-Cre* drives the recombination of the *R26R lacZ* reporter gene in the vMB at E10.5 to E12.5. Scale bar: 200 μm. (**D-F**) Higher magnification of regions outlined in A-C shows the colocalization of Cre and the DA progenitor marker Sox2. (**G-I**) Expression of Cre in newly born DA neurons (arrowheads) from E10.5 (G), E11.5 (H), to E12.5 (I). Scale bar in F: 40 μm for D-I. Insets in G and H are higher magnification views of boxed regions to show the presence of Cre in Th$^+$ neurons. (**J**,**K**) Immunohistochemistry shows loss of β-catenin neural progenitors and differentiation of DA neurons (arrows). Insets are higher magnification views of boxed regions. Arrowheads indicate blood vessels that continue to express β-catenin. Scale bar in K: 200 μm for J,K.

were randomly scattered and showed disorganized processes (Fig. 3N,P). By E18.5, the subdivision of the SNpc and the VTA was poorly defined, although DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants continued to express other phenotypical markers, such as DAT (Scl6a3 – Mouse Genome Informatics), Pitx3 and Aldh1a (Fig. 3Q,R, arrows; data not shown). Overall, there was a >50% reduction of DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (Fig. 3S).

Loss of β-catenin severely reduces DA neurogenesis without affecting patterning of the vMB

In addition to the disruption in adherent junctions and cell polarity, loss of β-catenin in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants could increase cell death or alter cell fate. Indeed, there was a significant increase in activated caspase 3-positive cells in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E10.5 and E12.5 (see Fig. S2 in the supplementary material). However, none of the dying cells expressed Th, suggesting that cell death did not directly contribute to the loss of DA neurons (see Fig. S2 in the supplementary material). We then examined the expression of a number of lineage-specific neural progenitor markers in control and *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E10.5. We used Foxa2 and Lmx1a as region-specific markers for DA progenitors in the vMB, and Sox2, Ngn2 and Nurr1 as markers for DA progenitors (Andersson et al., 2006a; Andersson et al., 2006b; Ferri et al., 2007; Kele et al., 2006; Kittappa et al., 2007). In addition, we also used Nkx6.1 and Nkx2.2 as markers for more lateral domains of the vMB. Our results indicated that the expression of Foxa2 and Lmx1a in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants was similar to that in controls (Fig. 4A-D). Furthermore, domains in the vMB defined by progenitor markers Nkx6.1 and Nkx2.2 were not perturbed in *Shh-Cre;β-Ctn$^{fl/fl}$* embryos (Fig. 4E,F). These results indicated that patterning of the vMB was not perturbed in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants. Although the total number of Foxa2$^+$ and Lmx1a$^+$ cells was modestly reduced in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (Fig. 4G), this was most likely due in part to the increase in cell death of progenitors (see Fig. S2 in the supplementary material). Neural progenitors in the the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants continued to express the DA progenitor markers Sox2, Ngn2 and Nurr1. However, the organization of these progenitor cells was perturbed owing to loss of cell adhesion and polarity (Fig. 4H-M). The disruption in the organization of DA progenitors in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* embryos raised the possibility that loss of β-catenin might also affect radial glia in the vMB, which in turn might perturb the migration of DA neurons. Indeed, the radial glia processes in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* embryos, highlighted by nestin and RC-2, were severely interrupted (Fig. 4N-Q).

To further investigate the mechanism of reduced DA neurogenesis in *Shh-Cre;β-Ctn$^{fl/fl}$* embryos, we characterized proliferation and cell cycle progression in neural progenitors. Using phospho-histone 3 (PH-3) as a marker, we showed a consistent reduction in the number of progenitor cells in M-phase along the ventricular zone of *Shh-Cre;β-Ctn$^{fl/fl}$* embryos at E10.5 and E12.5 (Fig. 5A-E). The number of neural progenitor cells in S-phase, marked by 2-hour pulse labeling of BrdU, was also reduced in *Shh-Cre;β-Ctn$^{fl/fl}$* embryos at E10.5 and E12.5 (Fig. 5F-J). To provide a more dynamic analysis of DA neurogenesis, we performed neuronal birth dating experiments by labeling progenitor cells with a single injection of BrdU at E10.5 or E11.5, allowing the embryos to develop for 24 hours, and analyzing the number of BrdU and Th double-positive cells (BrdU$^+$;Th$^+$). Our results showed that the efficiency of generating BrdU$^+$;Th$^+$ cells, determined by dividing the number of BrdU$^+$;Th$^+$ cells by the total number of DAPI$^+$ cells, within a 24-hour interval,

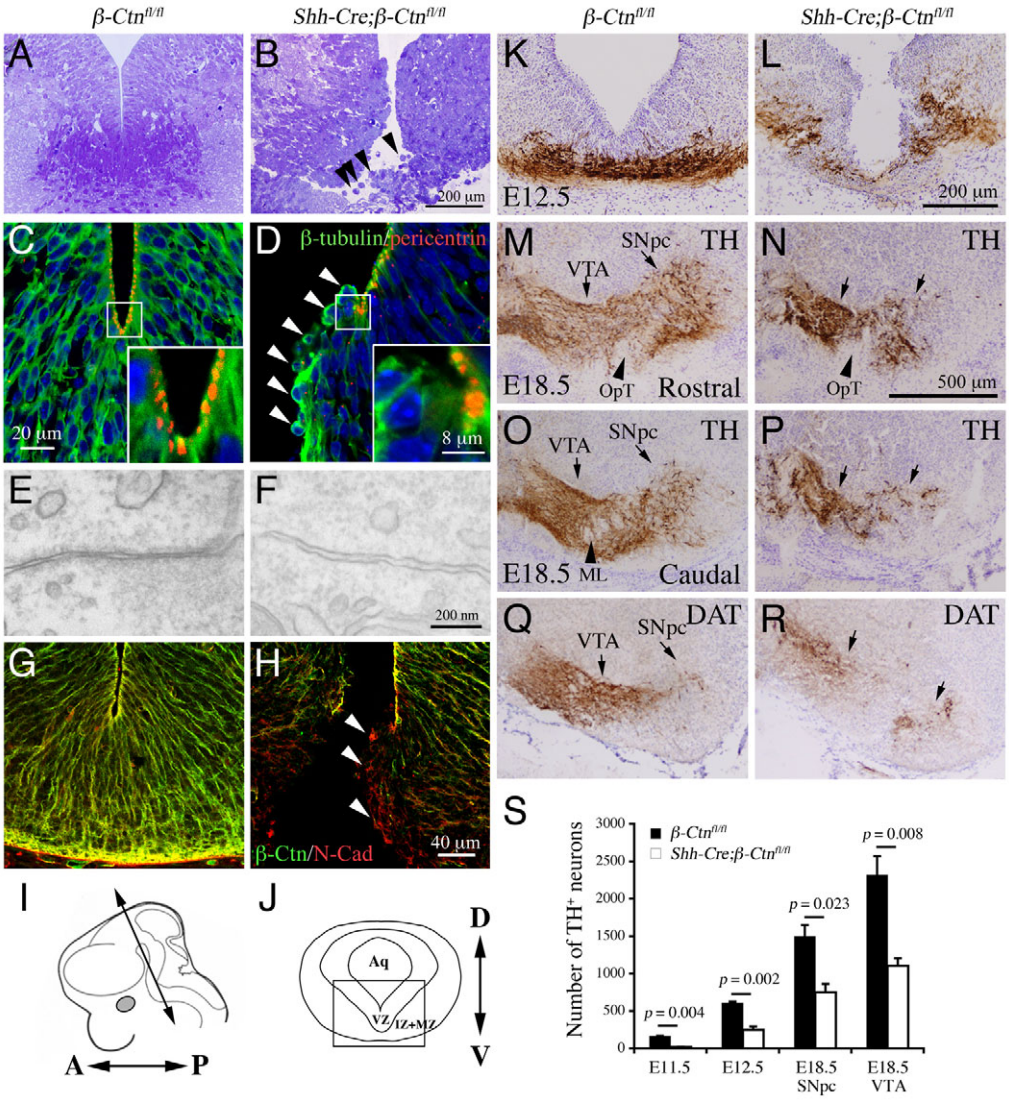

Fig. 3. Perturbation of cell polarity in DA progenitors and reduced DA neurogenesis in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants. (**A**,**B**) Toluidine Blue sections demonstrate loss of cell polarity in neural progenitors in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (arrowheads in B). Scale bar: 200 μm. (**C**,**D**) Loss of cell polarity in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (D) leads to perturbation in division planes in neural progenitors (arrowheads). Scale bar: 20 μm (8 μm in insets). (**E**,**F**) Electron microscopy reveals a complete absent of adherent junctions in neural progenitors of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (F). Scale bar: 200 nm. (**G**,**H**) Immunofluorescence images show an absence of β-catenin in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (arrowheads, H). Scale bar: 40 μm. (**I**,**J**) Schematic diagram of sagittal and cross sections of an E12.5 mouse embryo to highlight the AP position where analyses were performed. (**K**,**L**) In contrast to E12.5 control (K), DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants are reduced and poorly organized (L). Scale bar: 200 μm. (**M-P**) The segregation of DA neurons into the SNpc and VTA (arrows) is poorly developed in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E18.5 (N,P). Two landmarks (arrowheads), the optic tract (OpT) and the medial lemniscus (ML), indicate the rostral and caudal part of the vMB (M-P). (**Q**,**R**) Similar to control, DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants continue to express the DA marker DAT (arrows). Scale bar in N: 500 μm for M-R. (**S**) Stereological counting shows a severe reduction of DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E11.5 and E18.5 (Student's *t*-test, *n*=3).

was significantly reduced in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* embryos at E11.5 and E12.5 (Fig. 5K-M). Together, these results support the notion that the removal of β-catenin in DA progenitors significantly reduces the efficiency with which DA neurons are generated in the vMB.

Deletion of β-catenin using Th-IRES-Cre negatively regulates neurogenesis in progenitors of DA neurons

The reduced DA neurogenesis in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* embryos can be attributed to the loss of cell polarity, the disruption in radial glia processes and the reduced proliferation in DA progenitors. However, the persistent expression of β-catenin in radial glia and postmitotic DA neurons suggests that β-catenin might also regulate the differentiation in DA neurons in a cell-autonomous manner (Fig. 1). To test this hypothesis, we generated β-catenin conditional mutants using the *Th-IRES-Cre* mouse line (Lindeberg et al., 2004), in which the *IRES-Cre* cassette was inserted into the 3′UTR of *Th* locus and presumably should be expressed in differentiated DA neurons. Indeed, Cre was co-expressed with Th in more than 90% of DA neurons at E12.5, E15.5, P0 and P28 (Fig. 6A-E). Consistent with these results, we found that Th-IRES-Cre activated the *lacZ* reporter in similar numbers of DA neurons in *R26R* reporter mice during development and in postnatal life (Fig.

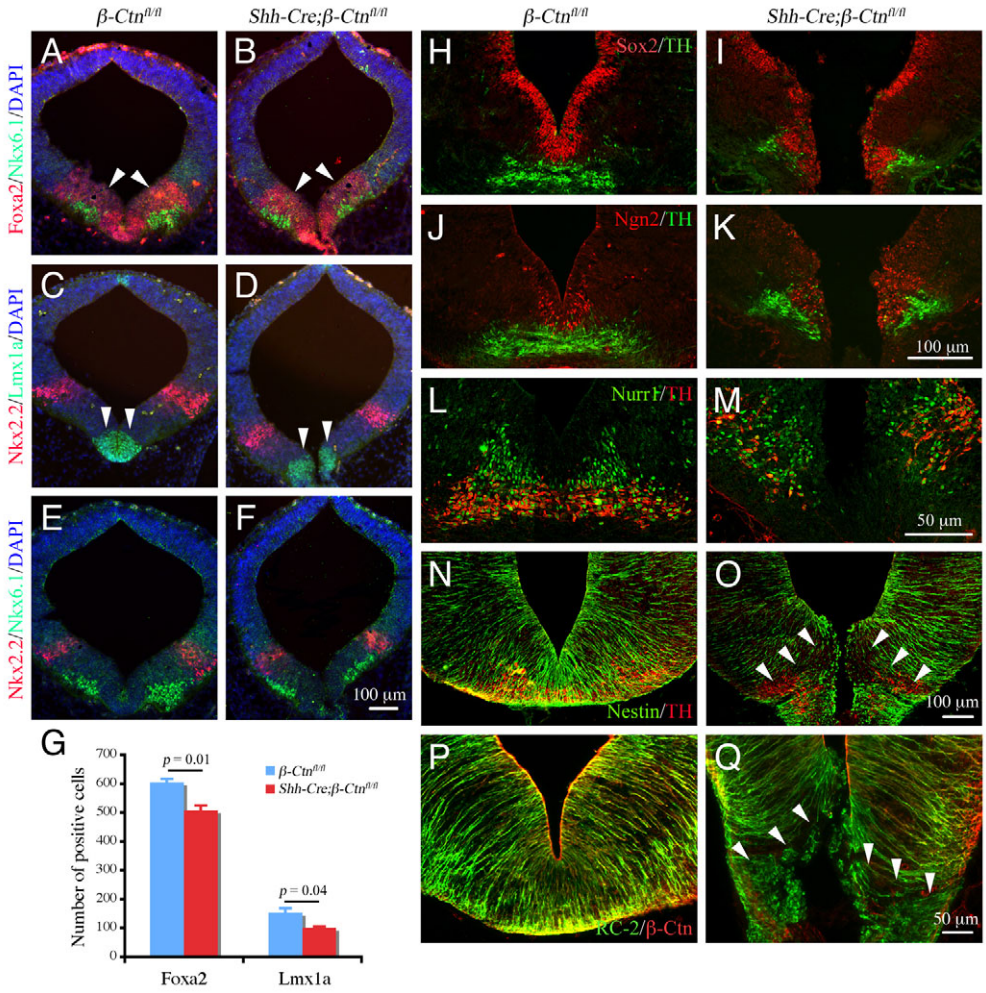

Fig. 4. Loss of β-catenin does not alter regional specification, but disrupts radial glia processes in the vMB. (**A-F**) Midbrain sections from E10.5 *Shh-Cre;β-Ctn*$^{fl/fl}$ mutants show no alteration in regional specification compared with controls (A,C,E). The neurogenic niche for DA neurons in the vMB of *Shh-Cre;β-Ctn*$^{fl/fl}$ mutants continues to express the transcription factors Foxa2 (A,B) and Lmx1a (C,D; arrowheads). Domains outside of the neurogenic niche for DA neurons continue to express regional markers, such as Nkx2.2 (C-F) and Nkx6.1 (E,F). Scale bar in F: 100 μm. (**G**) Quantification of Foxa2$^+$ and Lmx1a$^+$ cells show a consistent reduction of these progenitors in *Shh-Cre;β-Ctn*$^{fl/fl}$ mutants (Student's *t*-test, n=3). (**H-M**) Loss of β-catenin does not alter the expression of Sox2 (H,I), Ngn2 (J,K) or Nurr1 (L,M) in progenitors of DA neurons. Sections are prepared from control (*β-Ctn*$^{fl/fl}$) and *Shh-Cre;β-Ctn*$^{fl/fl}$ mutants at E12.5. Scale bars: in K, 100 μm for H-K; in M, 50 μm for L,M. (**N-Q**) Radial glia processes, which extend from the ventricular zone to the marginal zone in control (N,P), are severely disrupted in *Shh-Cre;β-Ctn*$^{fl/fl}$ mutants (O,Q). Arrowheads in O and Q mark the region where β-catenin is deleted. Radial glia processes are highlighted by nestin (N,O) and RC-2 (P,Q). Disorganization of DA neurons is highlighted by the Th antibody (N,O). Sections were obtained from E12.5 embryos. Scale bars: in O, 100 μm for N,O; in Q, 50 μm for P,Q.

6F-J). However, a small number of cells in the intermediate zone along the midline of the vMB showed positive *lacZ* expression at E12.5 (Fig. 6F, right box). Detailed analyses revealed that the β-galactosidase-positive (β-Gal$^+$) cells were not present at E9.5, but could be detected in ~10% of Sox2$^+$ or Ngn2$^+$ DA progenitors at E10.5 (Fig. 6K-M). By E12.5, ~20% of Sox2$^+$ and Ngn2$^+$ progenitors co-expressed β-Gal (Fig. 6M-O). These β-Gal$^+$ cells also express Nurr1 and the radial glia marker nestin (Fig. 6P-S). As no Cre was detected in β-Gal$^+$ cells (Fig. 6A-J), these results suggest that ectopic Cre recombination might have occurred transiently in a subset of DA progenitors from E10.5 to E12.5.

We generated *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ mutants and showed that β-catenin was efficiently removed in Sox2$^+$ progenitors and Th$^+$ neurons in the vMB (Fig. 6T-W). To determine the effect of β-catenin loss in *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ mutants, we first compared DA neurogenesis in control and conditional mutants from E11.5 to P0 (Fig. 7). Unlike the severe perturbation in adherent junctions and cell polarity in *Shh-Cre;β-Ctn*$^{fl/fl}$ embryos, the vMB of *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ embryos remained intact with no disruption in overall tissue organization or cell polarity (Fig. 7A,B). Furthermore, the regional organization of DA neurons in the SNpc or VTA in the vMB of postnatal brain was similar between control and *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ mutants (Fig. 7C,D). However, there was a 35-40% reduction in the number of DA neurons in *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ mutants, which began at E11.5 and persisted into postnatal life (Fig. 7E). Similar deficits were detected when Pitx3 was used as a marker for DA neurons (see Fig. S3 in the supplementary material). The reduced number of DA neurons in *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ mutants was not due to an increase in cell death. Rather, the number of activated caspase 3-positive DA neurons was reduced in *Th-IRES-Cre;β-Ctn*$^{fl/fl}$ mutants during development (see Fig. S4 in the supplementary material). We also determined the number of Ngn2$^+$

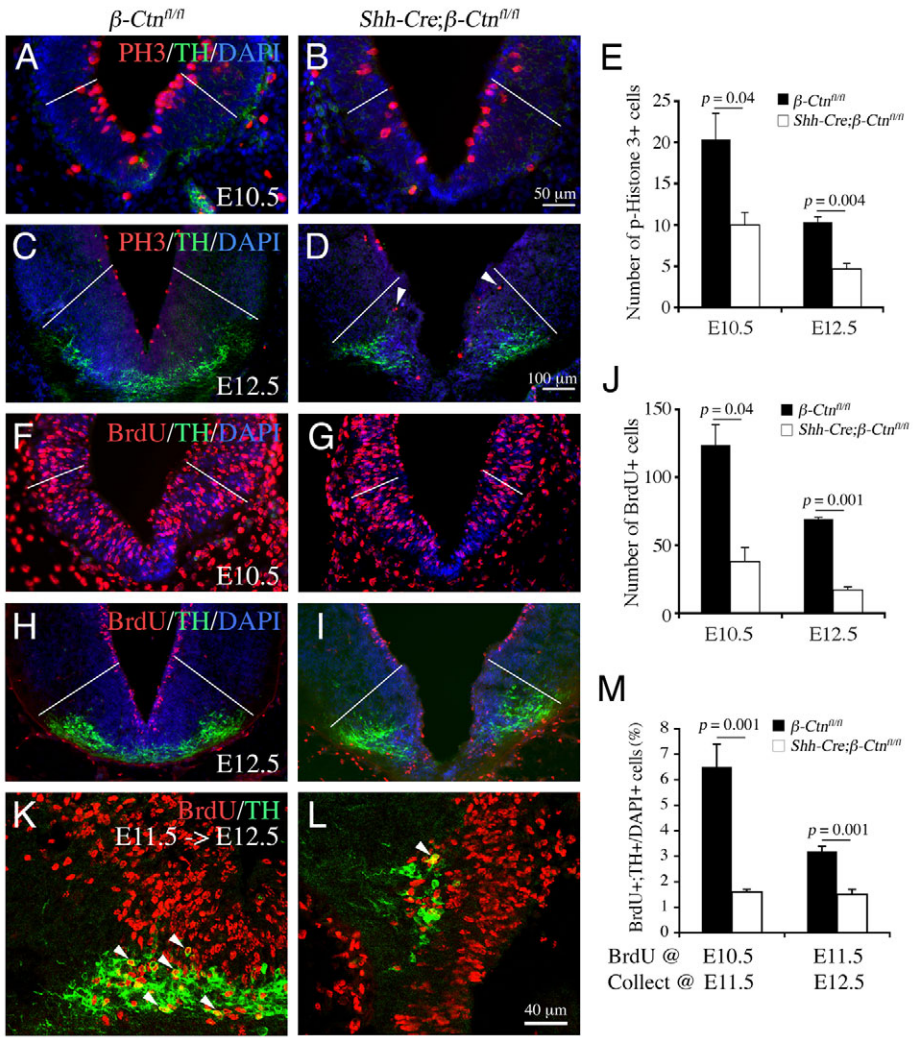

Fig. 5. Neurogenesis defects in the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants. (**A-D**) Loss of β-catenin results in reduced proliferation in neural progenitors at E10.5 and E12.5. The number of phospho-histone 3 (PH-3)-positive cells along the ventricular zone in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants is reduced compared with those in controls. Arrowheads in D show that many PH-3$^+$ progenitors are displaced from the ventricular zone in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants. Scale bars: in B, 50 μm for A,B; in D, 100 μm for C,D. (**E**) Quantification of PH-3$^+$ progenitors shows a >50% reduction in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E10.5 and E12.5 (Student's *t*-test, *n*=3). (**F-I**) Similarly, the number of BrdU$^+$ progenitors in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants is also reduced. White lines in A to I highlight the region in which β-catenin is removed by *Shh-Cre*. (**J**) Quantification shows a marked reduction of BrdU$^+$ cells in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants at E10.5 and E12.5. (**K**,**L**) Neuronal birth-dating experiments show a reduction in newly born DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants, highlighted by BrdU and Th double-positive cells (arrowheads). Scale bar in L: 40 μm. (**M**) Quantitative analyses show a marked reduction of newly born DA neurons at E11.5 and E12.5, normalized to the total number of DAPI$^+$ cells (Student's *t*-test, *n*=3).

progenitors in the ventricular zone of the vMB and found no difference between controls and conditional mutants (see Fig. S5 in the supplementary material). These results suggested that the reduced number of DA neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants was not due to a loss of progenitors in the ventricular zone.

To characterize the mechanism of reduced DA neuron phenotype in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants, we examined the progression from progenitors, to committed DA progenitors (Nurr1$^+$;Th$^-$), to mature DA neurons (Nurr1$^+$;Th$^+$) using the scheme previously proposed (Kele et al., 2006). Our results indicated that the number of Nurr1$^+$;Th$^-$ DA progenitors in the intermediate zone of the vMB in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* embryos was reduced at E11.5 to E12.5 (Fig. 7F-J). Consistent with the results in Fig. 7E, Nurr1$^+$;Th$^+$ DA neurons in the marginal zone were also reduced in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at the same stages (Fig. 7K). Together, these results suggested that loss of β-catenin in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants led to a reduced generation of immature DA progenitors and mature DA neurons.

To test this hypothesis, we performed a series of birth-dating experiments to determine the efficiency with which DA neurons and progenitors are generated in control and *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants (Fig. 8A). Consistent with the results with Ngn2$^+$ progenitors (see Fig. S5 in the supplementary material), a short, 2-hour pulse label of BrdU revealed no difference in the number of progenitors in S-phase in the ventricular zone of control and *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at E10.5 and E12.5 (see Fig. S6A-F in the supplementary material). However, when the progenitors were labeled with a single dose of BrdU at E10.5 or E11.5 and allowed to develop for a further 24 or 48 hours in utero, we found no difference in the total number of BrdU$^+$ cells in the vMB (see Fig. S6G in the supplementary material). By contrast, BrdU$^+$;Th$^+$ cells were reduced by 33% and 55% in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at E11.5 and E12.5, respectively (Fig. 8B-F). Similar reductions of BrdU$^+$;Th$^+$ cells were also observed in embryos after a 48-hour development in utero (data not shown). When normalized to the number of BrdU$^+$ cells at E11.5 and E12.5, the number of newly born DA neurons was reduced by ~40% at E12.5 (Fig. 8F). Using the same approaches, we showed that the number of committed DA progenitors, namely Nurr1$^+$;BrdU$^+$ and Nurr1$^+$;BrdU$^-$ cells, was also reduced in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at the same stages (Fig. 8G-I; arrows in G and H indicate BrdU$^+$;Th$^+$ progenitors).

Another possible cause for the lower number of DA neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants was a change in cell fate due to loss of β-catenin in progenitors that transiently expressed Cre during neurogenesis (Fig. 6F-S). To address this issue, we used the R26R allele as a fate-mapping tool and generated *Th-IRES-Cre;β-Ctn$^{fl/+}$;R26R* and *Th-IRES-Cre;β-Ctn$^{fl/fl}$;R26R* mice. We reasoned that, if the reduced DA neuron number in *Th-IRES-Cre;β-Ctn$^{fl/fl}$*

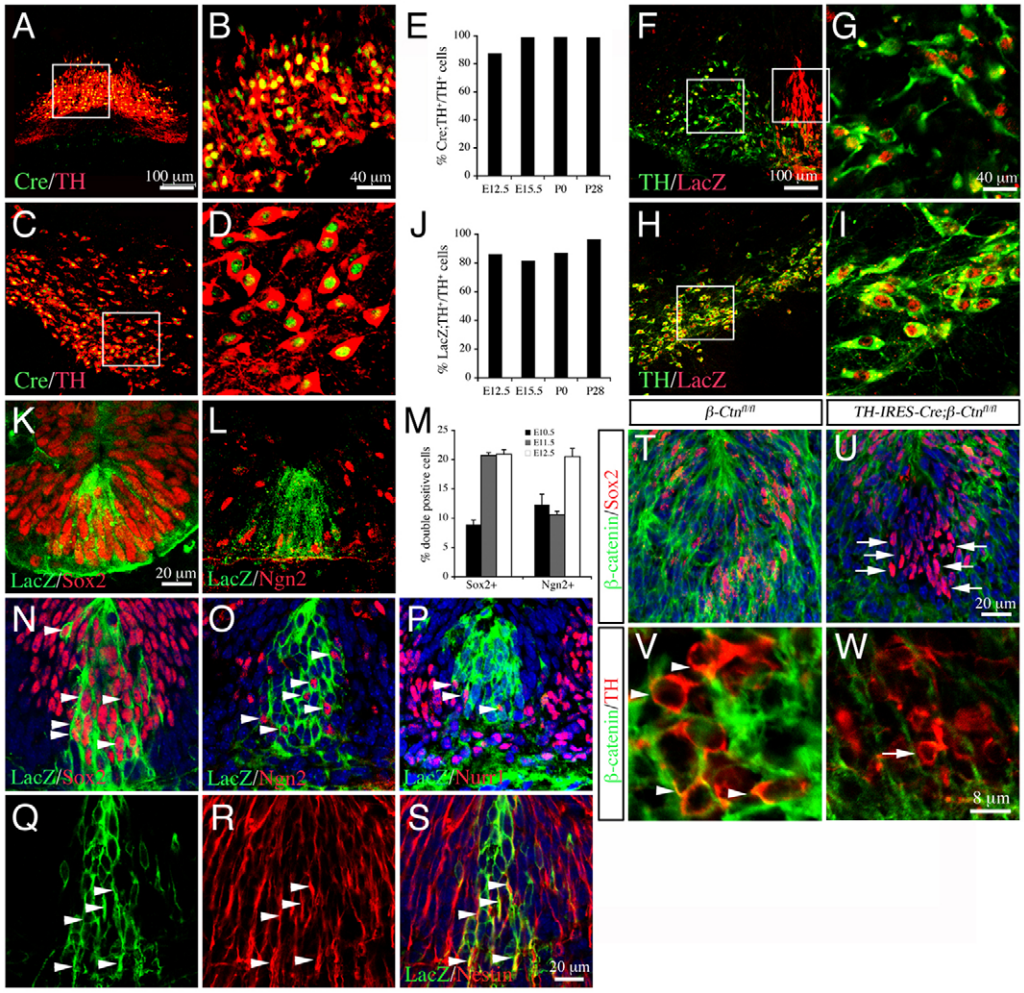

Fig. 6. Th-IRES-Cre targets recombination in progenitors and differentiated DA neurons. (**A-D**) Confocal images of Cre expression in DA neurons in *Th-IRES-Cre* mice at E12.5 (A,B) and P28 (C,D). B and D are higher magnification views of the boxed areas in A and C, respectively. (**E**) Quantitative analyses show that Cre can be detected in more than 85% of DA neurons in *Th-IRES-Cre* mice at E12.5. By E15.5, all DA neurons express Cre. (**F-I**) *Th-IRES-Cre;R26R* mice show recombination in the majority of DA neurons at E12.5 (F,G), which persists into P28 (H,I). Scale bars: in A,F, 100 μm; in B,G 40 μm. (**J**) Quantitative analyses of Cre recombination in *Th-IRES-Cre;R26R* mice from E12.5 to P28 show that >80% of DA neurons contain *lacZ*. (**K-S**) Confocal images of the boxed region to the right of panel F showing β-Gal expression in $Sox2^+$ (K,N) and $Ngn2^+$ (L,O) progenitors from E10.5 to E12.5. (M) Quantitative analyses show that 10-20% of DA progenitors express β-Gal (n=3). These $β\text{-}Gal^+$ cells also express Nurr1 (P) and the radial glia marker nestin (Q-S). Scale bars in K and S: 20 μm. (**T-W**) Confocal images showing that β-catenin protein is removed in $Sox2^+$ progenitors (arrows, U), and in Th^+ neurons (arrow, W) in E12.5 *Th-IRES-Cre;β-Ctn$^{fl/fl}$* embryos. Arrowheads in V indicate colocalization of β-catenin and Th in DA neurons. Scale bars: in U, 20 μm; in W, 8 μm.

mutants was due to a change in cell fate, there would be a significant increase in the number of $β\text{-}Gal^+$ and Th^- cells in *Th-IRES-Cre;β-Ctn$^{fl/fl}$;R26R* mice. Using confocal microscopy, we examined DA and non-DA neurons in the SNpc and VTA in *Th-IRES-Cre;β-Ctn$^{fl/fl}$;R26R* mice at P0, and found no detectable increase in $β\text{-}Gal^+$ and Th^- cells in *Th-IRES-Cre;β-Ctn$^{fl/fl}$;R26R* mice (see Fig. S7 in the supplementary material). These results indicated that the removal of β-catenin using Th-IRES-Cre reduced the number of progenitor cells and affected their ability to generate DA neurons without causing detectable changes in cell fate.

DISCUSSION

By analyzing *Shh-Cre;β-Ctn$^{fl/fl}$* mutants, we show that β-catenin is required to maintain adherent junctions and cell polarity of progenitor cells within the neurogenic niche of DA neurons (Fig. 3). Furthermore, β-catenin is also required to maintain the integrity of radial glia, which give rise to DA neurons and provide scaffolds for newly generated DA neurons to migrate on towards their final destinations (Fig. 4). Although these observations support the roles of β-catenin and N-cadherin in maintaining the integrity of adherent junctions and radial glia processes, it is possible that β-catenin in the canonical Wnt signaling pathway might control cell cycle progression during DA neurogenesis. Indeed, the removal of β-catenin in DA progenitors using *Shh-Cre* or *Th-IRES-Cre* reduces the progression from committed DA progenitors to DA neurons (Figs 5-8). Together, these results support the model that β-catenin regulates the neurogenesis of DA neurons at multiple crucial steps during development (see Fig. S8 in the supplementary material).

Essential role of β-catenin in the integrity of the neurogenic niche for DA neurons

The notion that the vMB contains the neurogenic niche for midbrain DA neurons has been supported by neuroanatomical and fate-mapping approaches, which provide structural bases to and initial

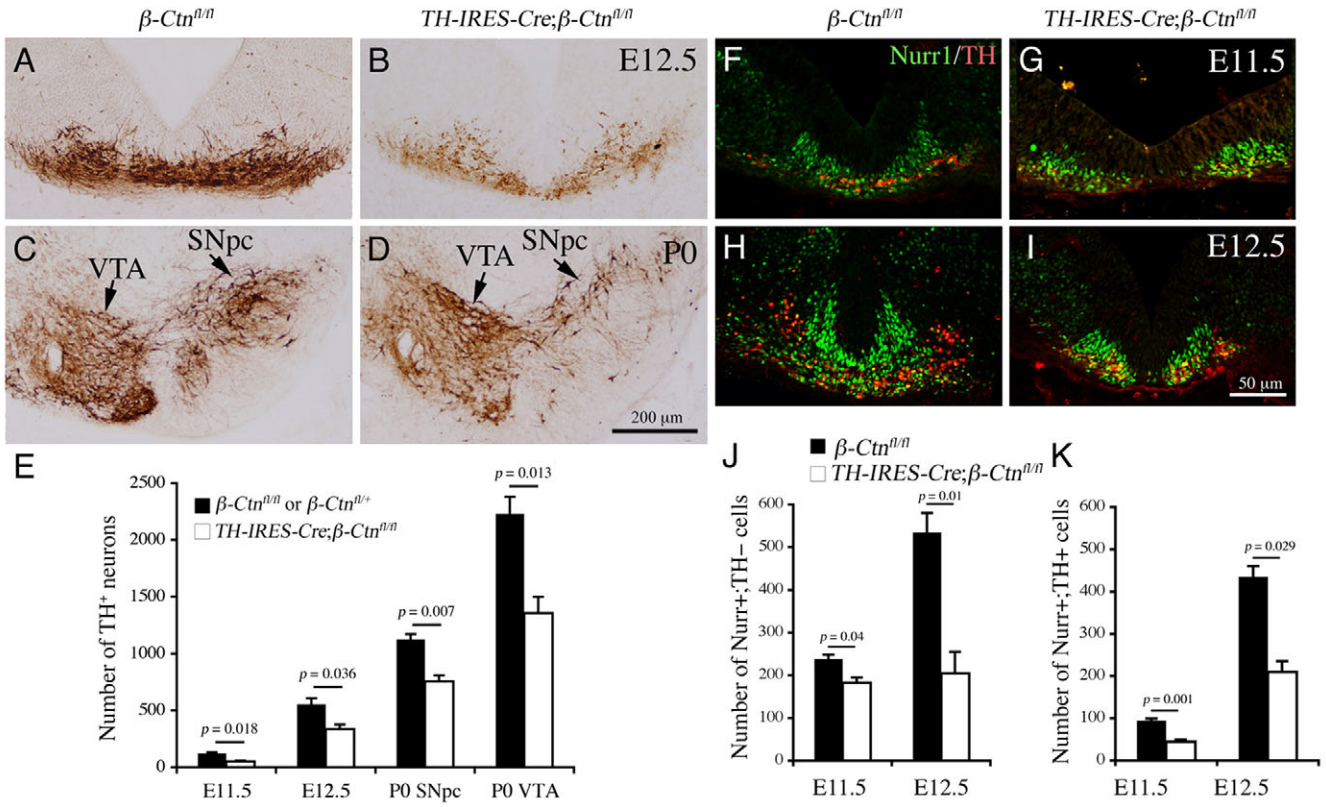

Fig. 7. Reduced DA progenitors and differentiated neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants. (**A-D**) Compared with in controls (*β-Ctn$^{fl/fl}$*), the number of DA neurons is reduced in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mice at E12.5 (A,B) and P0 (C,D). Unlike *Shh-Cre;β-Ctn$^{fl/fl}$* mutants (Fig. 3P), the segregation of DA neurons into the SNpc and VTA is not affected in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mice. Scale bar: 200 μm. (**E**) Quantification confirms the reduced number of DA neurons at E11.5, E12.5 and P0 (Student's *t*-test, *n*=3). (**F-I**) *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants show a persistent reduction in the number of DA progenitors (Nurr1$^+$;Th$^-$) and Nurr1$^+$;Th$^+$ neurons at E11.5 and E12.5. Scale bar: 50 μm. (**J**,**K**) Quantitative analyses for the number of Nurr1$^+$;Th$^+$ neurons and Nurr1$^+$;Th$^-$ progenitors at E11.5 and E12.5. A more dramatic reduction in Nurr1$^+$;Th$^-$ progenitors is noted at E12.5 (Student's *t*-test, *n*=3).

insights into the generation of DA neurons in the vMB (Bonilla et al., 2008; Kawano et al., 1995; Kittappa et al., 2007; Ono et al., 2007; Rothman et al., 1980; Shults et al., 1990; Specht et al., 1981). The identification of intrinsic determinants for DA progenitors, such as Nurr1, Pitx3, En1/2, Foxa1/2, Otx2, Ngn2 and Lmx1a, further defines the molecular hierarchies that control the process of DA neurogenesis (Ang, 2006; Prakash and Wurst, 2006; Smidt and Burbach, 2007). Interestingly, several recent studies have shown that a genetic network mediated by Wnt1-Otx2 regulates the proliferation and differentiation of DA neurons (Omodei et al., 2008; Prakash et al., 2006; Prakash and Wurst, 2007; Puelles et al., 2004). Consistent with these studies, results from this study provide further insights into the multiple crucial functions of β-catenin within the neurogenic niche of DA neurons.

At the stage of active neurogenesis of DA neurons, β-catenin and N-cadherin are present in high abundance in adherent junctions at the apical side of the ventricular zone in the vMB (Fig. 1). Because of the essential function of β-catenin in maintaining the cadherin complex at adherent junctions, removal of β-catenin in the vMB using *Shh-Cre* leads to a significant reduction in N-cadherin and α-E-catenin. As a consequence, the vMB of *Shh-Cre;β-Ctn$^{fl/fl}$* mutants exhibits perturbation in cell adhesion and a complete loss of cell polarity in DA progenitors (Fig. 3). The severe disruption in the organization of DA progenitors resembles that reported in mutants in which N-cadherin or β-catenin is removed from the developing telencephalon (Junghans et al., 2005; Kadowaki et al., 2007; Machon et al., 2003), and underscores a highly conserved function of β-catenin in maintaining cell polarity and adhesion in neural progenitors. Perturbation of this function disrupts the division planes of neural progenitors and leads to ectopic cell death in progenitors (Kadowaki et al., 2007).

Another prominent phenotype in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants is the reduced proliferation of progenitors (Fig. 5). This might be caused by the loss of cell polarity in progenitors, similar to the phenotype in N-cadherin conditional mutants (Kadowaki et al., 2007). Alternatively, it is possible that canonical Wnt signaling pathway of β-catenin might regulate cell proliferation and cell cycle progression in DA progenitors (Logan and Nusse, 2004). Consistent with this notion, several recent reports show that *Wnt1* mRNA signal can be detected in the ventral midbrain, in the vicinity of newly formed DA neurons, and that ectopic expression of Wnt1 in the En1 domain leads to an expansion of DA progenitors (Omodei et al., 2008; Prakash et al., 2006).

In addition to its role in adherent junctions, β-catenin can also regulate cell fate during neural development (Backman et al., 2005; Hari et al., 2002; Lee et al., 2004). For instance, removal of β-catenin using *Nestin-Cre* leads to ectopic expression of ventral markers in the dorsal telencephalon (Backman et al., 2005). Furthermore, the loss of β-catenin in migrating neural crest cells affects the development of distinct populations of sensory neurons in the first wave of neurogenesis (Hari et al., 2002). Conversely, the stabilization of β-catenin promotes sensory neuron fate in neural

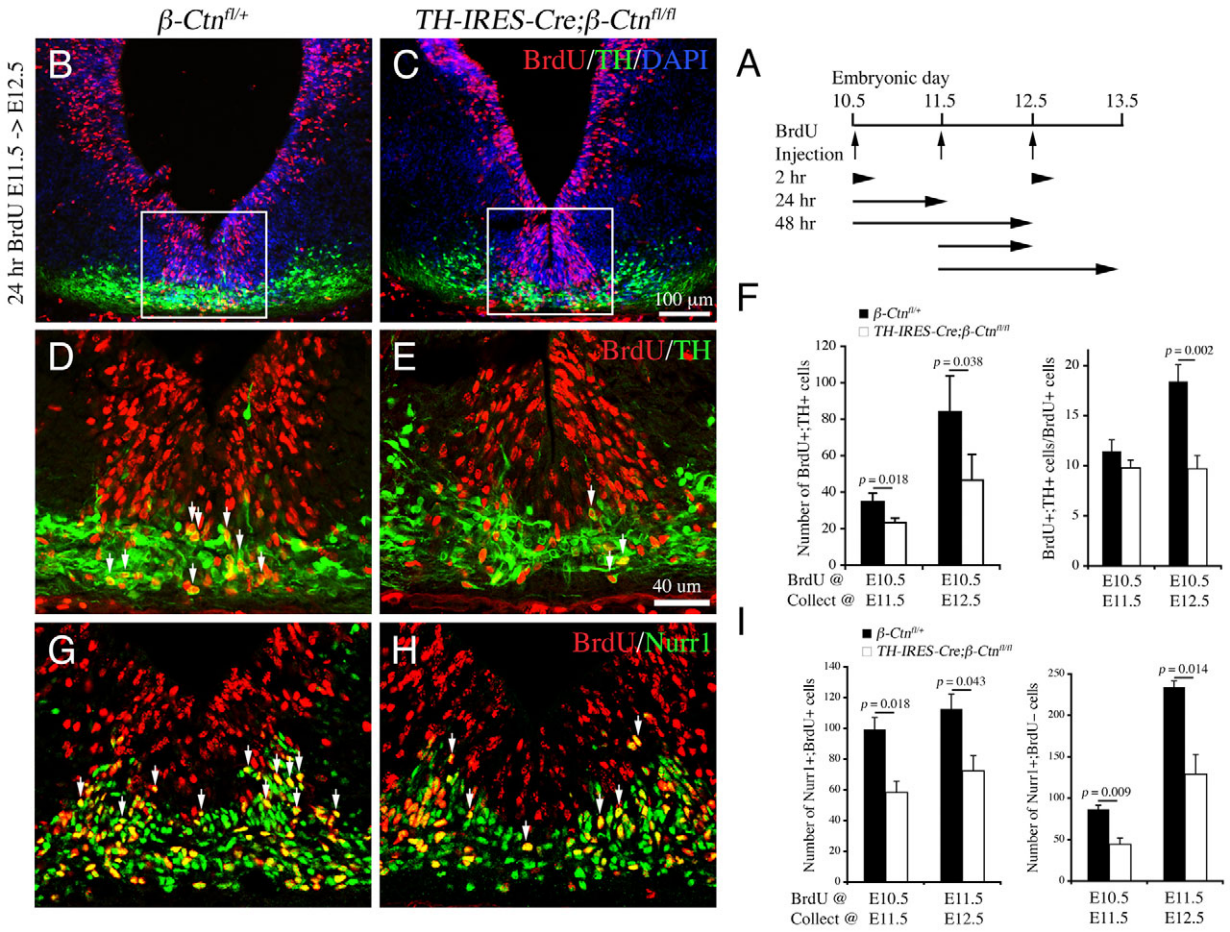

Fig. 8. Neuronal birth-dating experiments reveal reduced neurogenesis of DA neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants. (**A**) Illustration of the schedule for BrdU injection and embryo collection to analyze DA neurogenesis. Neural progenitors are labeled in S phase of cell cycle and allowed to develop for 2, 24 or 48 hours. (**B-E**) Representative images of DA progenitors and neurons in control (*β-Ctn$^{fl/+}$*) and *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants. In controls, there are many DA neurons positive for BrdU (BrdU$^+$;Th$^+$), indicating that these neurons are newly born from E11.5 to E12.5 (B,D). By contrast, the number of BrdU$^+$;Th$^+$ neurons is reduced in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at the same stage (arrows in D and E). Panels D and E are higher magnification views of boxed regions in B and C, respectively. (**F**) Quantification confirms the reduced number of newly born BrdU$^+$;Th$^+$ neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at E11.5 and E12.5. After adjusting for the total number of DA neurons, the percentage of newly born DA neurons is still reduced at E12.5 in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants (Student's *t*-test, *n*=3). (**G-I**) Using similar approaches, we analyzed the number of Nurr1$^+$ progenitors that are generated within 24 hours from E10.5 to E11.5, or E11.5 to E12.5. The results indicate a reduction in the number of newly born Nurr1$^+$;BrdU$^+$ or Nurr1$^+$;BrdU$^-$ cells in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at E11.5 (G) and E12.5 (H). (I) Quantification confirms the reduction of newly born DA progenitors in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants at E11.5 and E12.5 (Student's *t*-test, *n*=3). Scale bars: in C, 100 μm for B,C; in E, 40 μm for D,E,G,H.

crest cells (Lee et al., 2004). By contrast, removal of β-catenin in the vMB and DA progenitors appears to have no significant adverse effect on the patterning of neural tube or the expression of DA progenitor markers (Fig. 4). Furthermore, DA neurons in *Shh-Cre;β-Ctn$^{fl/fl}$* and *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants continue to express markers for mature DA neurons (Figs 3 and 7).

β-catenin in radial glia and its role within the neurogenic niche of DA neurons

Loss of β-catenin in dorsal telencephalon leads to the perturbation of radial glia processes (Machon et al., 2003). However, the underlying mechanism for such defects remains unclear. Our results show that the vMB is enriched with radial glia processes, which connect the ventricular zone and the marginal zone (Fig. 1). Interestingly, the majority of newly generated DA neurons are aligned directly onto the radial glia processes, suggesting that these processes might provide the structural support for DA neurons to migrate to their final destinations. These observations are intriguing in light of a recent study indicating that a significant number of DA neurons are derived from radial glia (Bonilla et al., 2008). Taken together, these two independent studies indicate that, similar to in the developing telencephalon (Gotz and Huttner, 2005; Kriegstein et al., 2006), radial glia in the vMB might serve dual roles during neurogenesis of DA neurons, one as transient amplifying cells that undergo symmetric cell division to give rise to DA neurons and the other to provide important scaffolds for the migration of newly born DA neurons. The fact that β-catenin is present in the processes of radial glia suggests that β-catenin might be involved in maintaining the integrity of these structures (Fig. 1). Indeed, the radial glia phenotype in *Shh-Cre;β-Ctn$^{fl/fl}$* mutants resembles that reported in *D6-Cre;Ncad$^{fl/fl}$* mutants (Kadowaki et al., 2007). Together, these results support the notion that interaction between N-cadherin and β-catenin might also regulate the migration and proper segregation of DA neurons to the SNpc and VTA (Figs 3, 4).

A continuous role of β-catenin in the expansion of DA neurons in vivo

It is well accepted that Th is expressed in cells in the vMB with distinct neuronal characteristics, such as an extension of neurites and the inability to incorporate ^{3}H-thymidine or BrdU (Rothman et al., 1980; Specht et al., 1981; Zhang et al., 2007). However, our results indicate that Th-IRES-Cre targets recombination in ~10-20% of DA progenitors from E10.5 to E12.5 (Fig. 6). These results, together with the reduction of DA neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants, suggest that β-catenin might continue to regulate the generation of DA neurons during the process of differentiation. Indeed, the reduction of DA neurons in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants occurs as early as E11.5 and is not due to an increase in apoptosis (Fig. 7; see also Fig. S4 in the supplementary material). Rather, neuronal birth-dating using BrdU pulse-chase labeling shows a significant reduction in newly born DA neurons from E10.5 to E11.5 and from E11.5 to E12.5 (Fig. 8F). In addition, the number of newly generated Nurr1^{+};Th^{-} and Nurr1^{+};BrdU^{+} progenitors is also reduced at similar stages (Figs 7, 8). The most parsimonious interpretation for the neurogenesis defects in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants is that loss of β-catenin reduces cell cycle progression in DA progenitors. This is supported by recent fate-mapping results showing that radial glia cells in the vMB are the progenitor cells for DA neurons (Bonilla et al., 2008). Furthermore, our results indicate that many Th^{+} neurons continue to express radial glial markers (Fig. 1). Interestingly, radial glia in developing telencephalon have the properties of transient amplifying cells and can undergo symmetric cell division to expand the neuronal population in cerebral cortex (Gotz and Huttner, 2005; Kriegstein et al., 2006). It is likely that the proliferative potential of radial glia in the vMB might also be regulated by β-catenin, through its function in the canonical Wnt signaling pathway (Castelo-Branco et al., 2003; Logan and Nusse, 2004).

In conclusion, this study provides several novel insights into the roles of β-catenin during the development of DA neurons. First, β-catenin is required to maintain the N-cadherin protein complex in adherent junctions and the cell polarity of progenitors in the neurogenic niche of DA neurons. Second, β-catenin crucially regulates the integrity of radial glia in the vMB. Loss of β-catenin might affect the conversion of radial glia into DA neurons and the migration of DA neurons. Finally, the defects of neurogenesis in *Th-IRES-Cre;β-Ctn$^{fl/fl}$* mutants suggest that β-catenin-mediated signaling contributes to the expansion of DA neurons from progenitors. Together, these results indicate that β-catenin regulates multiple steps during the generation of DA neurons.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/12/2027/DC1

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