Development/Plasticity/Repair

Interactions of Wnt/β-Catenin Signaling and Sonic Hedgehog Regulate the Neurogenesis of Ventral Midbrain Dopamine Neurons

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Signaling mechanisms involving Wnt/β-catenin and sonic hedgehog (Shh) are known to regulate the development of ventral midbrain (vMB) dopamine neurons. However, the interactions between these two mechanisms and how such interactions can be targeted to promote a maximal production of dopamine neurons are not fully understood. Here we show that conditional mouse mutants with region-specific activation of β-catenin signaling in vMB using the *Shh–Cre* mice show a marked expansion of Sox2-, Ngn2-, and Otx2-positive progenitors but perturbs their cell cycle exit and reduces the generation of dopamine neurons. Furthermore, activation of β-catenin in vMB also results in a progressive loss of Shh expression and Shh target genes. Such antagonistic effects between the activation of Wnt/β-catenin and Shh can be recapitulated in vMB progenitors and in mouse embryonic stem cell cultures. Notwithstanding these antagonistic interactions, cell-type-specific activation of β-catenin in the midline progenitors using the *tyrosine hydroxylase–internal ribosomal entry site–Cre* (*Th-IRES-Cre*) mice leads to increased dopaminergic neurogenesis. Together, these results indicate the presence of a delicate balance between Wnt/β-catenin and Shh signaling mechanisms in the progression from progenitors to dopamine neurons. Persistent activation of β-catenin in early progenitors perturbs their cell cycle progression and antagonizes Shh expression, whereas activation of β-catenin in midline progenitors promotes the generation of dopamine neurons.

Introduction

The developing ventral midbrain (vMB) in vertebrates contains a neurogenic niche that is enriched with progenitor cells for dopamine (DA) neurons (Bjorklund and Lindvall, 1984). Within this niche, progenitors for DA neurons undergo lineage specification, migration, and differentiation to become mature DA neurons (Ang, 2006; Prakash and Wurst, 2006; Smidt and Burbach, 2007; Arenas, 2008). Several lines of evidence indicate that two distinct genetic networks critically regulate the development of DA neurons. Sonic hedgehog (Shh) induces the expression of forkhead transcription factor Foxa2 in vMB through specific Gli (glioma-associated oncogene homolog) transcription factor binding elements in the enhancer sequence of *Foxa2* (Sasaki et al., 1997). Interestingly, the enhancer elements in *Shh* contain highly conserved binding sites for Foxa2 that regulate the expression of Shh in vMB (Jeong and Epstein, 2003), supporting the notion that Shh and Foxa2 constitute a feedback transcriptional mechanism for mutual expression. Consistent with this notion, mouse mutants with region-specific removal of Foxa2 in vMB show a severe loss of Shh (Lin et al., 2009). In addition to the Shh–Foxa2 regulatory loop, the canonical Wnt/β-catenin signaling mechanism controls a distinct set of transcription factors critical for the development of DA neurons. Specifically, genetic studies in several mouse mutants indicate that Wnt1 and Otx2 (Orthodenticle homeobox 2) form a feedback mechanism to regulate the expression for each gene (Puelles et al., 2004; Vernay et al., 2005; Prakash et al., 2006). Furthermore, in mouse embryonic stem cells (mESCs), Wnt1 and Lmx1a (LIM homeobox transcription factor 1, alpha) form a feedback regulatory mechanism similar to that in Shh–Foxa2 (Chung et al., 2009).

Several Wnts regulate the development of DA neurons in vMB. For instance, Wnt1 regulates proliferation, specification, neurogenesis in vMB DA progenitors, as well as the survival of

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DA neurons (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Danielian and McMahon, 1996; Prakash et al., 2006). Other components of the Wnt signaling pathway, including Wnt2, the Wnt receptors Fzd3 and Fzd6, and the Wnt coreceptor Lrp6, have been found to regulate the development of DA neurons (Castelo-Branco et al., 2010; Sousa et al., 2010; Stuebner et al., 2010). Similarly, β-catenin, a critical Wnt signaling component, is expressed in vMB DA progenitors and is required for the maintenance of adherent junctions, the integrity of radial glia processes, and cell cycle progression of DA progenitors (Joksimovic et al., 2009; Tang et al., 2009).

To further investigate the role of canonical Wnt signaling in DA neurogenesis, we generated conditional mouse mutants in which the glycogen synthase kinase 3β (GSK3β) phosphorylation sites in β-catenin (β-Ctn^{Ex3}) was removed from the neurogenic niche in vMB. Our results indicate that the activation of β-catenin in vMB promoted a marked expansion of DA progenitors but led to a reduced expression of Shh and Foxa2. Moreover, the antagonistic interaction between the Wnt and Shh pathways in the generation of DA neurons was also detected in the cultures of DA progenitors and mESCs. Conversely, cell-type-specific activation of β-catenin in midline progenitors promoted DA neurogenesis. These results provide strong evidence that Wnt/β-catenin and Shh signaling pathways control a delicate balance of target gene expression during DA neurogenesis.

Materials and Methods

Animals. To generate conditional activation of β-catenin in mice, β-$catenin^{Exon3}$ mice (β-Ctn^{Ex3}) were crossed with *Shh–Cre* (stock #005622; The Jackson Laboratory) or *tyrosine hydroxylase-internal ribosomal entry site-Cre* (*Th-IRES-Cre*) (Harada et al., 1999; Harfe et al., 2004; Lindeberg et al., 2004; Tang et al., 2009). Animal care was approved by the Institutional of Animal Care and Use Committee and followed National Institutes of Health guidelines.

Histology and immunohistochemistry. The protocols for histology and immunohistochemistry were the same as described previously (Zhang et al., 2007; Tang et al., 2009). 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, postnatal day 0 (P0), and P21 were perfused and fixed with 4% PFA, cryoprotected in 15–30% sucrose solution, and sectioned in the coronal plane using a Leica cryostat. Mouse brains were sectioned at 14 μm thickness (for stereology counting, brains were cut at 40 μm) and mounted on Superfrost glass slides.

Sections were incubated with primary antibody overnight and secondary antibodies for 1 h, followed by incubation in DAB solution to detect signals. The primary antibodies in this study included the following: anti-bromodeoxyuridine (BrdU) antibody (1:500; MAB3222; Millipore Bioscience Research Reagents), anti-Foxa2 [1:20; 4C7; Developmental Hybridoma Study Bank (DHSB)], anti-Ki67 (1:200; RM9106-S0; Thermo Fisher Scientific), anti-Lmx1a (1:1000; gift from Dr. Mike German, University of California, San Francisco, San Francisco, CA), anti-Ngn2 (Neurogenin 2) (1:10; gift from Dr. David Anderson, California Institute of Technology, Pasadena, CA), anti-Pitx3 (Pituitary homeobox 3) (1:300; gift from Dr. Marten Smidt, University Medical Center Utrecht, Utrecht, The Netherlands), anti-Nkx2.2 (NK2 transcription factor related, locus 2) (1:50; 74.5A5; DHSB), anti-Nkx6.1 (NK6 homeobox 1) (1:1000; gift from Dr. Mike German), anti-Nurr1 (Nuclear receptor related 1 protein) (1:500; sc-990; Santa Cruz Biotechnology), anti-Otx2 (1:200; ab21990; Abcam), anti-phospho-histone H3 (PH3) (1:200; 06-570; Millipore Corporation), anti-Shh (1:200; catalog #2207; Cell Signaling Technology), anti-TuJ1 class III β-tubulin (1:2000; MMS435P; Covance), anti-tyrosine hydroxylase (1:1000; AB157; Millipore Bioscience Research Reagents), anti-tyrosine hydroxylase (1:500; ab113; Abcam), and anti-β-catenin (1:200; catalog #9587; Cell Signaling Technology). For stereology counting, sections were incubated for 1 h with biotinylated IgG and avidin–biotin complex (Vector Laboratories). Images were captured using a Nikon Eclipse E800 fluorescent microscope connected to a SPOT RT camera (Diagnostic Instruments) or a BX41 Olympus microscope equipped with Olympus DP70 CCD camera. Images were captured using Spot Advance or Olympus DP Controller software programs or using an LSM 510 confocal microscope (Carl Zeiss 510 Microimaging).

BrdU labeling of dopaminergic progenitors. We performed two injection schemes. In the first scheme, the pregnant mice were injected with BrdU (50 μg/g) (BD Biosciences) at E10.5 and E12.5, respectively, and killed 2 h later. In the second scheme, the pregnant mice were injected with BrdU at E10.5 and E11.5, respectively, and killed 24 h later (Zhang et al., 2007; Tang et al., 2009).

In situ *hybridization. In situ* hybridization were the same as described previously (Zhang and Huang, 2006). Briefly, RNA probes for *in situ* hybridization were prepared using plasmid cDNA clones for *Shh*, *cyclin D1*, and *Lmx1b* transcribed with T7 or T3 polymerase using digoxigenin (DIG)-labeling reagents and a DIG RNA labeling kit (Roche). Embryos were fixed overnight at room temperature in 4% PFA in DEPC-treated PBS, cryoprotected in 15 and 30% sucrose in DEPC PBS, and embedded in OCT. Sections were processed at 14 μm. During hybridization, sections were first postfixed with 4% PFA and then washed with acetylation solution and 1% Triton X-100. Then sections were prehybridized with hybridization buffer (Amresco) for 2–4 h before applying hybridization buffer containing DIG-labeled riboprobes (200–400 ng/ml) at 55°C overnight. The second day, slides were washed with 4× SSC, followed by RNase A (20 μg/ml) treatment at 37°C for 45 min and subsequent washes with 2× SSC, 1× SSC, and 0.5× SSC at room temperature. For visualizing the *in situ* hybridization results, we used DIG Nucleic Acid Detection kit (Roche). Finally, the slides were dried under room temperature and mounted with Crystal Mount (Biomeda).

Stereology. The total number of TH-positive (TH^+) neurons in substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) was determined using the optical fractionator, an unbiased cell counting method not affected by the volume of reference (i.e., SNpc or VTA) or the size of the counted elements (i.e., neurons) (Zhang et al., 2007; Tang et al., 2009). Neuronal counts were performed using a computer-assisted image analysis system consisting of an Olympus BX-51 microscope equipped with a *x–y–z* computer-controlled motorized stage and the StereoInvestigator software (MicroBrightField). TH^+ neurons were counted in 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.

Ventral midbrain DA progenitor cultures. Primary cultures for dopamine neurons were prepared from vMB using microisland methods according to published procedures (Takeshima et al., 1996). Briefly, mouse embryos were collected from time-pregnant CD-1 (for E10.5 and E13.5 wild-type cultures) or *Shh–Cre* (for E12.5 *Shh–Cre*;β-$Ctn^{Ex3/+}$ cultures) females. The ventral midbrain was dissected, dissociated after treatment with trypsin, and cultured on coverslips coated with poly-D-ornithine (Sigma) and laminin (Sigma) at the density of 1.2×10^6/ml. The dissociated cells were maintained in the DMEM/F-12 (1:1) medium containing 10% FBS overnight. Then, the differentiated neurons were changed to DMEM/F-12 (1:1) medium containing N2 supplements (Invitrogen), 20 ng/ml FGF2 (Millipore Corporation), 100 ng/ml FGF8 (Peprotech), and designated factors, including Shh (Peprotech), Wnt1 (Peprotech), Wnt5a (R & D Systems), and the GSK3β inhibitor CT99021 (Axon Medchem) before they were fixed with 4% PFA. The number of mature DA neurons in culture were determined by counting the total number of TH^+ neurons per 20× field (Parish et al., 2008).

Mouse embryonic stem cell cultures. Differentiation of R1 mESCs into DA neurons was performed using a slightly modified protocol (Barberi et al., 2003). Briefly, R1 mESCs were seeded at a density of 50 cells/cm^2 on mitomycin-treated stromal cell PA6 and cultured in ES-Serum Replacement Media, composed by KnockOut-DMEM (Invitrogen), 15% KnockOut serum replacement (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), 200 mM L-glutamine (Invitrogen), 1% nonessential amino acids (Biochrom AG), and 2000 U/ml penicillin/streptomycin (Invitrogen).

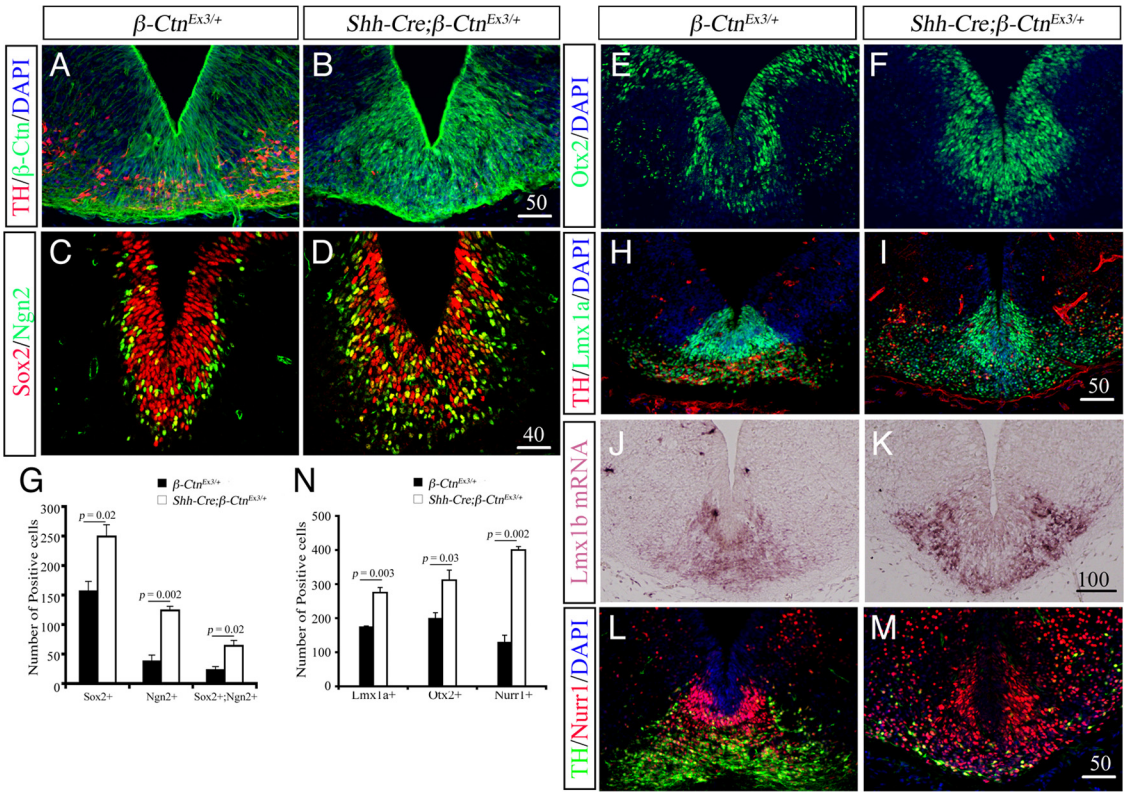

Figure 1. Stabilization of β-catenin in vMB leads to expansion of DA progenitors. ***A***, ***B***, β-Catenin staining in the vMB of *β-Ctn*$^{Ex3/+}$ and *Shh–Cre;β-Ctn*$^{Ex3/+}$ mice at E12.5. Scale bar (in ***B***), 50 μm. ***C***, ***D***, Immunofluorescent staining at E12.5 shows significant increases in the numbers of Sox2$^+$, Ngn2$^+$, and Sox2$^+$;Ngn2$^+$ progenitor at E12.5. ***E–N***, In addition, the number of Otx2$^+$ (***E***, ***F***), Lmx1a$^+$ (***H***, ***I***), Lmx1b$^+$ (***J***, ***K***), and Nurr1$^+$ (***L***, ***M***) progenitors are all increased in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. Scale bars: ***D***, 40 μm; ***I***, 50 μm; ***K***, 100 μm; ***M***, 50 μm. ***N***, Quantifications of Lmx1a$^+$, Otx2$^+$, and Nurr1$^+$ cells show a consistent increase of these progenitors in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. The data are represented as number of positive cells per section as described previously (Tang et al., 2009). DAPI, 4′,6′-Diamidino-2-phenylindole.

After 5 d, medium was changed and supplemented with 25 ng/ml FGF8 (R & D Systems) and different concentrations of Shh (R & D Systems) and the GSK3β inhibitor CT99021. From day 8 to day 11, cells were cultured in N2 medium consisting of F-12 and MEM mixture at 1:1 (Invitrogen), glucose, N2 supplement (Invitrogen), 15 mM HEPES (Invitrogen), 200 mM L-glutamine, and 3 mg/ml AlbuMax I (Invitrogen) supplemented with 50 ng/ml FGF8 and 10 ng/ml FGF2 (R & D Systems) and the same concentration of Shh and CT99021 as in days 5–8. From day 11 to day 14, the media was replaced with N2 medium supplemented with 30 ng/ml brain-derived neurotrophic factor (BDNF), 30 ng/ml glial derived neurotrophic factor (GDNF) (both from R & D Systems), and 200 μM ascorbic acid (Sigma).

After *in vitro* differentiation, cells were fixed in 4% PFA (10′, RT), serum blocked, and incubated in the appropriate primary and subsequently secondary antibodies as described previously (Parish et al., 2005). Nuclear counterstaining was performed using Hoechst. The following antibodies were used: mouse monoclonal anti-III-tubulin (TuJ1; 1:500; Promega), rabbit polyclonal anti-tyrosine hydroxylase (1:500; Pel-Freez Biologicals), mouse monoclonal anti-tyrosine hydroxylase (1:500; ImmunoStar), rabbit anti-Foxa2 (1:500; Cell Signaling Technology), rabbit anti-Pitx3 (1:100; Zymed Laboratories), rabbit anti-Nurr1 (1:500; Santa Cruz Biotechnology), and Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 donkey anti-rabbit (1:500; Invitrogen).

Statistical analyses. Data were analyzed by two-tailed Student's *t* test. Values were expressed as mean ± SEM. Changes were identified as significant if the *p* value was <0.05.

Results

Activation of Wnt/β-catenin in vMB leads to expansion of DA progenitors but reduces DA neurogenesis

To determine whether activation of canonical Wnt/β-catenin signal in vMB affects the development of DA neurons, we generated conditional mutant mice in which the floxed exon 3 of *β-catenin* (*β-Ctn*^{EX3}) was removed using *Shh–Cre* (named *Shh–Cre;β-Ctn*$^{Ex3/+}$). Expression of one copy of *β-Ctn*^{EX3} allele using *Shh–Cre* leads to perinatal lethality as a result of a robust gain-of-function phenotype in multiple organs, including limbs (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Consistent with the anticipated recombination of *Shh–Cre* (Tang et al., 2009), *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants showed a much higher level of β-catenin protein in vMB at E12.5, with a significant accumulation of the mutant proteins in the nuclei of the neural progenitors (Fig. 1*A*,*B*) (data not shown). Compared with control (*β-Ctn*$^{Ex3/+}$) embryos, the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ embryos showed a marked expansion of Sox2-, Ngn2-, and Otx2-positive progenitors in the ventricular zone (VZ) (Fig. 1*C–G*). In addition, DA progenitors expressing Lmx1a, Lmx1b, and Nurr1 also showed significant increases in the intermediate zone and marginal zone (Fig. 1*H–N*).

We next examined whether the constitutive activation of Wnt/β-catenin in vMB could have altered cell cycle progression in DA progenitors, as described previously for Wnt1 in the neural tube (Megason and McMahon, 2002). To test this hypothesis, we performed a short-term (2 h) BrdU labeling to determine the number of progenitors in the S phase of cell cycle. Although E10.5 and E11.5 *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants showed no detectable difference in the number of BrdU$^+$ progenitors in the vMB VZ (Fig. 2*A–D*), a significant increase was detected at E12.5 (Fig. 2*E*,*F*,*M*). Furthermore, a longer BrdU labeling time interval (24 h) showed an even more drastic increase in the number of progenitors that incorporated BrdU (Fig. 2*G*,*H*,*N*). In contrast,

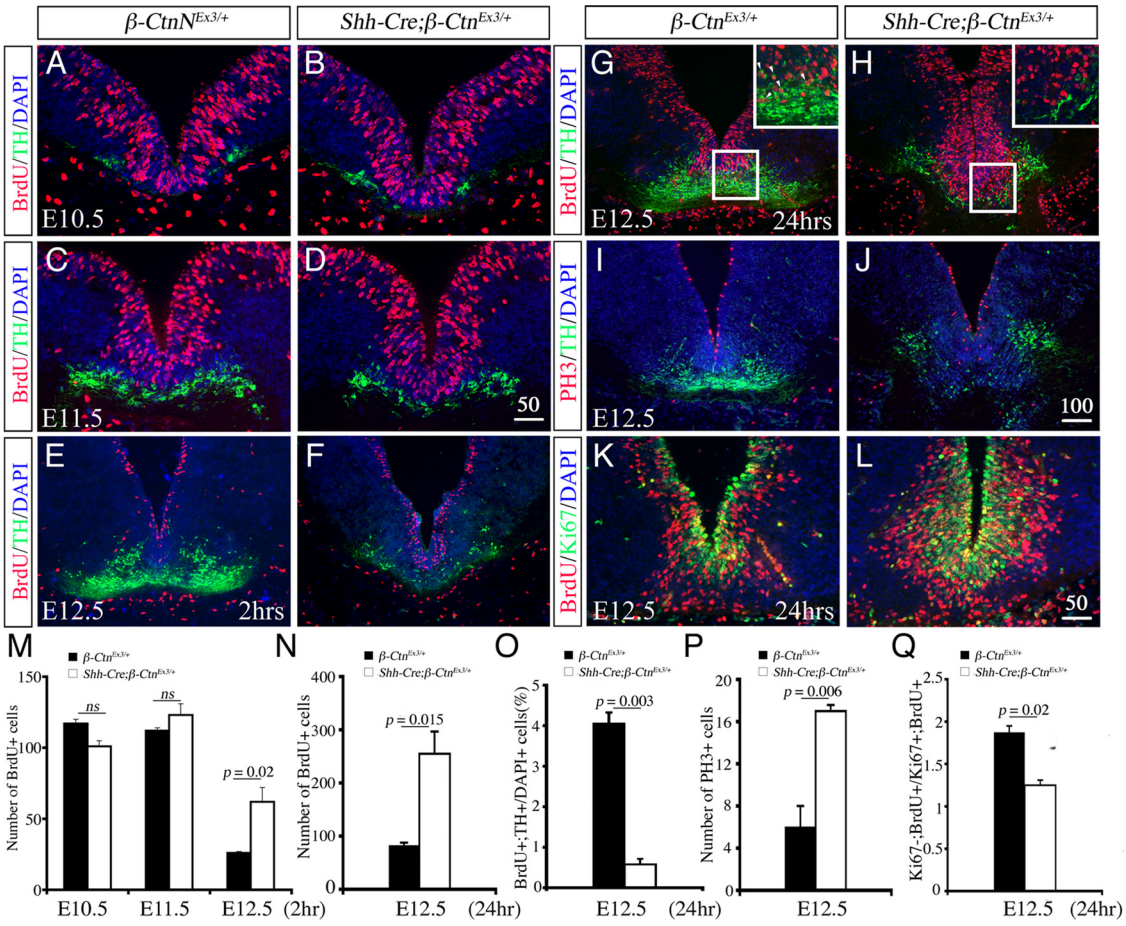

Figure 2. Cell cycle progression of the DA progenitors in vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. ***A–F***, Stabilization of β-catenin in vMB results in increased proliferation in early progenitors (2 h BrdU injection scheme) in the ventricular zone at E12.5 (***E***, ***F***) but not at E10.5 and E11.5 (***A–D***). Student's *t* test ($n = 3$); ns, Not significant. ***G***, ***H***, Neuronal birthdating experiments using a 24 h BrdU injection scheme also reveal a robust increase in BrdU$^+$ cells in vMB. However, there is a reduction in newly born DA neurons, highlighted by BrdU and TH double-positive (BrdU$^+$;TH$^+$) cells in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants (arrowheads in the insets). ***I***, ***J***, Similarly, the number of PH3$^+$ progenitors also increase in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. Scale bars: (in ***D***) ***A–D***, 50 μm; (in ***J***) ***E–J***, 100 μm. ***K***, ***L***, Cell cycle exit experiments show that fewer progenitor cells exit cell cycle in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. Scale bar (in ***L***): ***K***, ***L***, 50 μm. ***M–Q***, Quantification results confirm the increases in BrdU$^+$ progenitors (2 and 24 h injection schemes) (***M***, ***N***), the reduced production of BrdU$^+$;TH$^+$ neurons (***O***), the increase in PH3$^+$ progenitors (***P***), and the decrease in cell cycle exit (***Q***) after the activation of β-catenin in vMB.

much fewer BrdU and TH double-positive neurons were generated in the *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants within the same time interval (Fig. 2*G*,*H*, insets, *O*). Many of the apical progenitors in the VZ of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants continued to show positive PH3 staining, indicating that they were in the M phase of cell cycle (Fig. 2*I*,*J*,*P*). The increases of progenitors in S and M phases of cell cycle in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants at E12.5 suggested that the constitutive activation of Wnt/β-catenin signaling may affect the cell cycle progression in DA progenitors. To address this, we performed birthdating of DA neurons by pulse labeling the progenitors with BrdU for 24 h and then determined the number of progenitors that have exited cell cycle [BrdU-positive; Ki67-negative (BrdU$^+$; Ki67$^-$)] within this time interval. Consistent with our prediction, there were much fewer progenitors in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants that have exited the cell cycle during the 24 h time interval (Fig. 2*K*,*L*,*Q*). Together, these results supported the notion that constitutive activation of Wnt/β-catenin signal in vMB led to the expansion DA progenitors by reducing their exit from the cell cycle.

In analyzing the phenotype of the constitutive activation of Wnt/β-catenin signaling in DA progenitors, we noticed that the number of newly born DA neurons, marked by TH-positive staining, was reduced in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants at E12.5 (Fig. 2*E–H*). To provide a more quantitative analysis of DA neurons in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants, we used stereology to determine the total number of DA neurons in vMB from E12.5 to E18.5. Our results showed that, compared with control littermates, there were consistently fewer DA neurons in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants (Fig. 3*A–G*). Interestingly, a small ectopic cluster of DA neurons was identified the interpeduncular nucleus (Fig. 3*D*,*F*). At E18.5, the reduction in DA neurons was more prominent in the SNpc compared with the VTA (Fig. 3*E–G*).

To characterize the reduced DA neuron phenotype in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants, we first determined whether there was an increase in cell death. Using activated caspase 3 as a marker, we found no detectable increase in cell death in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). We next examined whether the ability of DA progenitors to differentiate was impaired in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. To test this hypothesis, we cultured vMB progenitors from E12.5 control and *Shh–Cre;β-Ctn*$^{Ex3/+}$ embryos in conditions that have been shown previously to promote differentiation of DA neurons (Takeshima et al., 1996; Ye et al., 1998; Schulte et al., 2005). Consistent with the *in vivo* phenotype, progenitors from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mu-

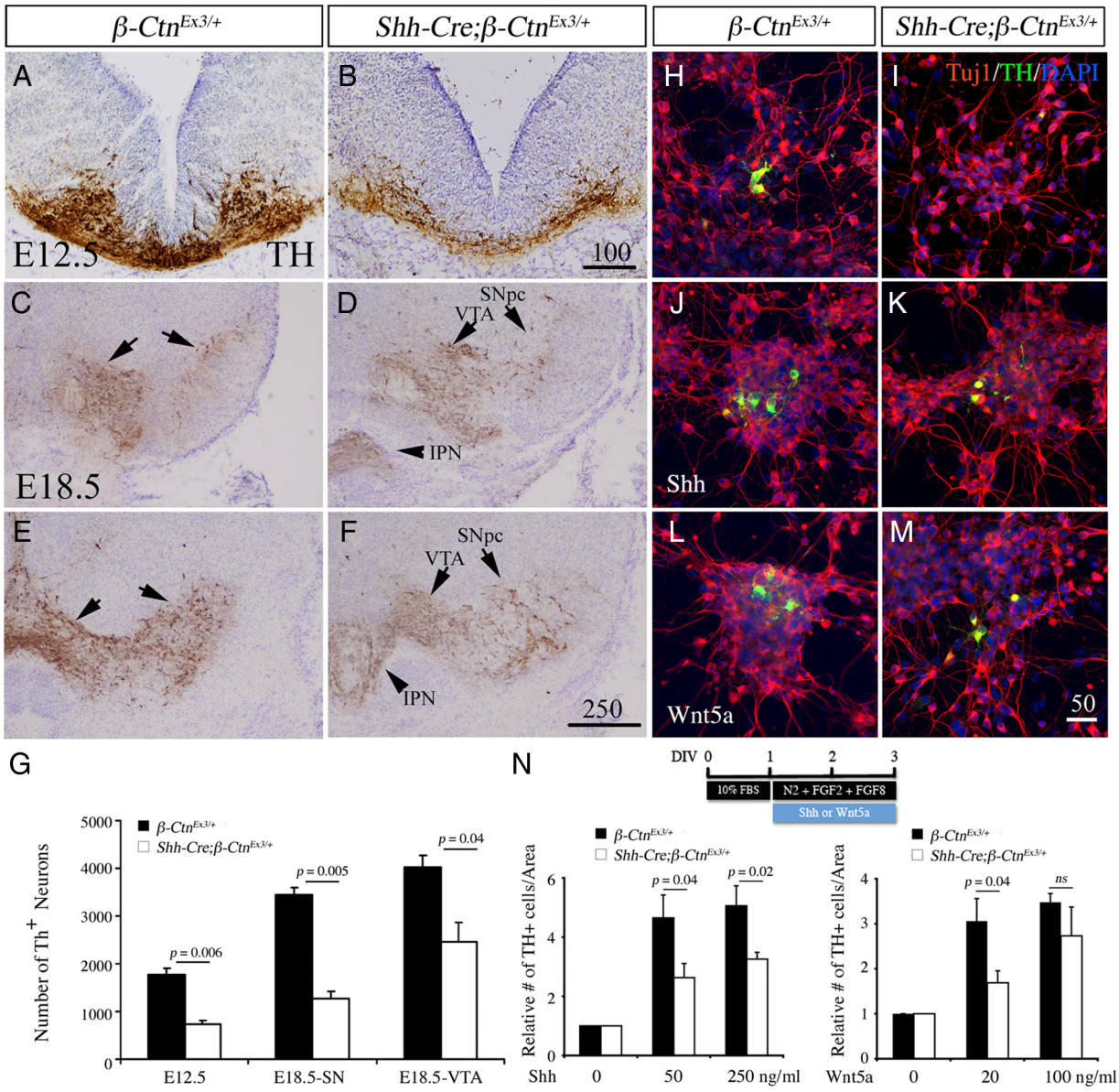

Figure 3. Ventral midbrain progenitors in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants show a significant reduction in DA neurons *in vivo* and *in vitro*. ***A–F***, Compared with control (*β-Ctn*$^{Ex3/+}$), vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants show much fewer DA neurons at E12.5 (***A***, ***B***) and E18.5 (***C–F***). Scale bars: (in ***B***), ***A***, ***B***, 100 μm; (in ***F***) ***C–F***, 250 μm. IPN, Interpeduncular nucleus. ***G***, Quantification by stereology confirms the reduced number of DA neurons at E12.5 and P0. Student's *t* test, $n = 3$. ***H–M***, Progenitors derived from the vMB of control (*β-Ctn*$^{Ex3/+}$) and *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants can be differentiated into DA neurons during the addition of Shh (***J***, ***K***) and Wnt5a (***L***, ***M***). Scale bar (in ***M***): ***H–M***, 50 μm. ***N***, However, the relative number of TH$^+$ cells per areas observed in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants are significantly less in the presence of Shh (left) but similar to control at higher concentration of Wnt5a (100 ng/ml) (right). Culture scheme is shown on the top of ***N***. The numbers of TH$^+$ neurons per area are normalized to control for each genotype. Student's *t* test ($n = 3$); ns, Not significant. DIV, days *in vitro*.

tants gave rise to fewer number of DA neurons under basal culture conditions (Fig. 3*H*,*I*). Interestingly, the addition of increasing doses of Shh only promoted a very modest increase in the number of DA neurons in progenitors from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants (Fig. 3*J*,*K*,*N*). However, when treated with Wnt5a, progenitors from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutant embryos showed an increase in DA neuron numbers in a manner similar to those from control (Fig. 3*L–N*).

Activation of Wnt/β-catenin antagonizes expression of Shh and Shh targets in vMB

The results from Figure 3 supported the notion that treatments with additional exogenous factors, such as Shh or Wnt5a, can indeed promote the generation of DA neurons from the progenitors of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. However, the fewer number of DA neurons from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants suggested that the regional activation of canonical Wnt/β-catenin signal may have altered the milieu in the neurogenic niche of DA neurons or the intrinsic properties of DA progenitors in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. To test these hypotheses, we examined Shh expression, an important exogenous factor that regulates the neurogenesis of DA neurons (Hynes et al., 1995). Our results showed that *Shh* mRNA was diffusely expressed in the floor plate at E10.5 (Fig. 4*A*). By E12.5, *Shh* mRNA became more restricted to the VZ of vMB, immediately adjacent to the neurogenic niche of DA progenitors (Fig. 4*C*). Despite the restricted expression pattern of *Shh* mRNA, Shh proteins were more widespread in the vMB, extending from VZ to the pia surface, suggesting that Shh proteins may be transported along the radial glia (Fig. 4*E*). This was confirmed by confocal imaging, which showed an extensive

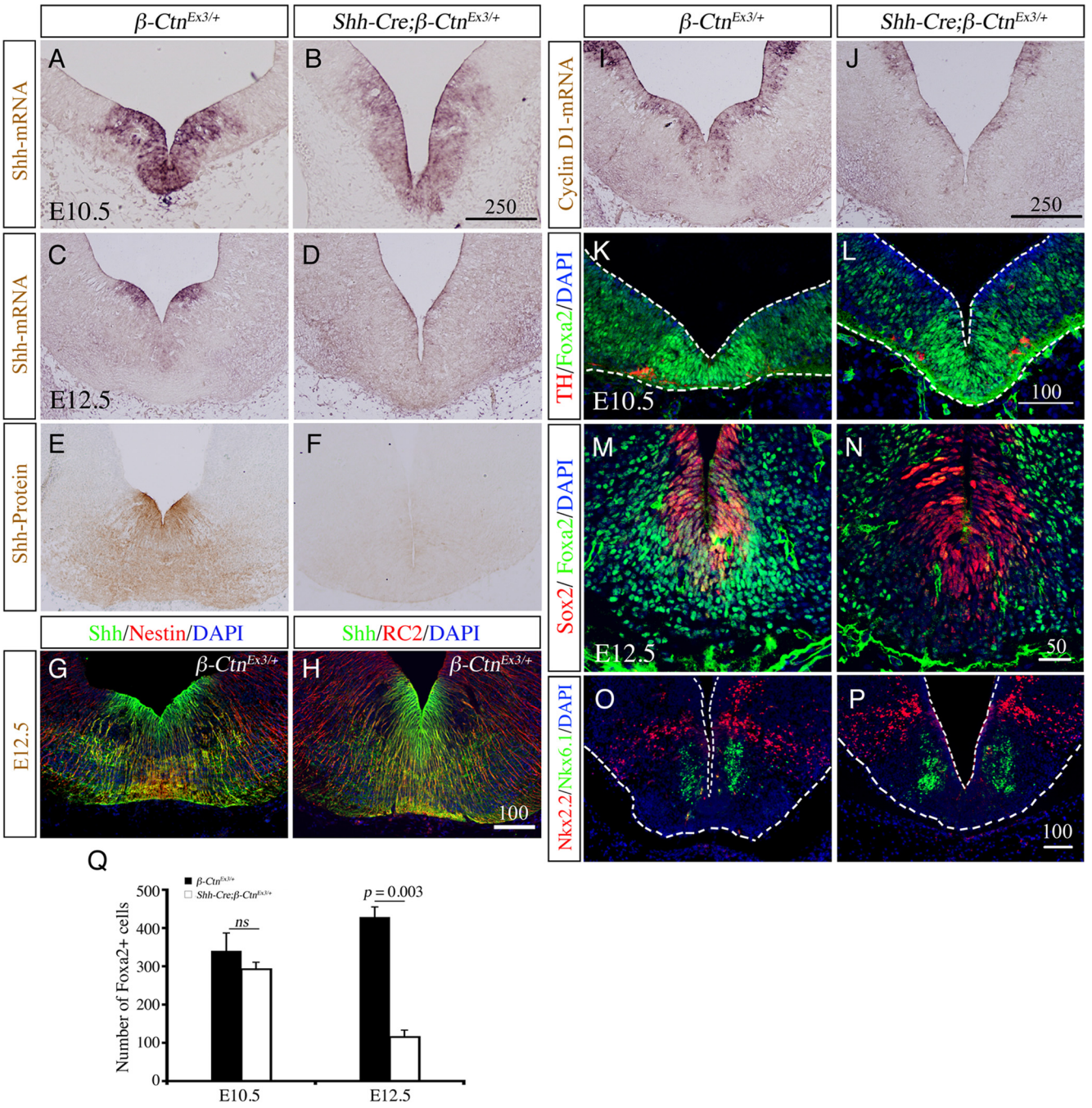

Figure 4. Activation of β-catenin in vMB antagonizes Shh expression and reduces Shh target genes. ***A***, ***B***, *Shh* mRNA expression is slightly reduced at E10.5 in vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. Scale bar (in ***B***): ***A***, ***B***, 250 μm. ***C–F***, By E12.5, *Shh* mRNA (***C***, ***D***) and protein (***E***, ***F***) expression are severely reduced in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. ***G***, ***H***, Two representative images show the extensive colocalization of Shh (green) and the radial glia marker Nestin (red) (***G***) and Shh and RC-2 (***H***). Scale bar: ***H***, 100 μm. ***I***, ***J***, Shh downstream target gene cyclin D1 show a similar reduction in the vMB of *Shh–Cre; β-Ctn*$^{Ex3/+}$ mutants. Scale bar (in ***J***): ***C–H***, 250 μm. ***K–N***, Compared with control (*β-Ctn*$^{Ex3/+}$), another Shh target Foxa2 shows no detectable reduction in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants at E10.5 (***K***, ***L***) but is significantly downregulated at E12.5 (***M***, ***N***). Scale bars: ***L***, 100 μm; ***N***, 50 μm. ***O***, ***P***, In contrast, expression of Nkx6.1 and Nkx2.2 in vMB shows no detectable changes in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. Scale bar: ***N***, 100 μm. ***Q***, Quantification of Foxa2^{+} progenitors at E10.5 and E12.5. Student's *t* test ($n = 3$). ns, Not significant.

colocalization of Shh proteins with radial glia markers, e.g., Nestin, RC-2, and Glast (glutamate–aspartate transporter) (Fig. 4*G*,*H* and data not shown). Unlike the wild-type embryos, constitutive activation of Wnt/β-catenin led to a modest decrease of *Shh* mRNA at E10.5 (Fig. 4*B*) but a near complete loss of Shh protein and mRNA in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants at E12.5 (Fig. 4*D*,*F*). Consistent with these results, the expression of Shh targets, such as cyclin D1 and Foxa2, was reduced in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants at E12.5 but not at E10.5 (Fig. 4*I–N*). In contrast, the expression of other regional vMB markers, such as Nkx2.2 and Nkx6.1, showed no detectable change (Fig. 4*O*,*P*). These results supported the hypothesis that persistent activation of Wnt/β-catenin could alter the neurogenic niche for DA neurons by antagonizing the expression of Shh and Shh target genes in the progenitors.

To further characterize the interactions between canonical Wnt/β-catenin and Shh in the generation of DA neurons, we cultured progenitors from the vMB of wild-type E10.5 embryos

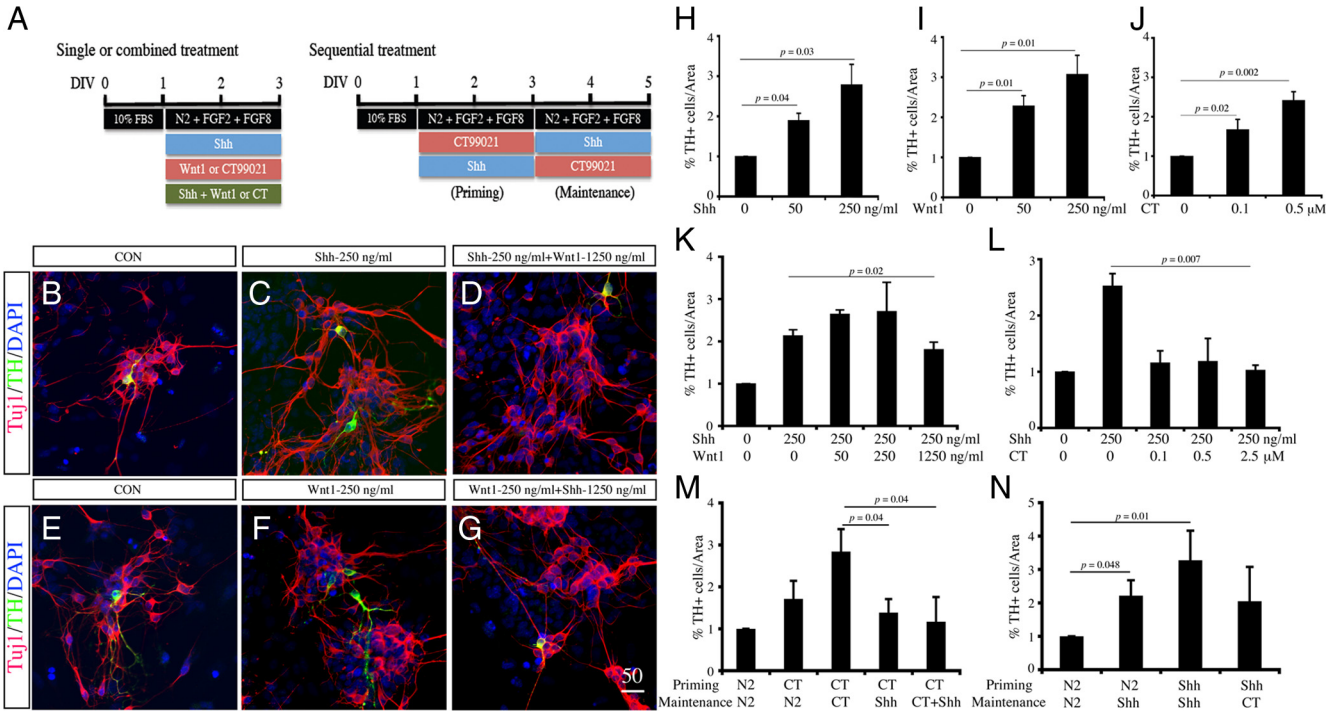

Figure 5. Ventral midbrain progenitor cultures show antagonistic effect between canonical Wnt/β-catenin signaling and Shh. ***A***, Schematic diagrams illustrating the two culture conditions for DA progenitors. In the "single or combined treatment" paradigm, vMB progenitors are treated with Shh, Wnt1, the GSK3β inhibitor CT99021 alone, or with a combination of Shh and Wnt1, or Shh and CT99021 (***B–L***). In the "sequential treatment" paradigm, the vMB progenitors are treated with Shh or CT99021 from day 1 to 3 and then switched to CT99021 or Shh from day 3 to 5 (***M***, ***N***). ***B–J***, Compared with control (***B***, ***E***), treatments with Shh or Wnt1 alone increase the number of DA neurons in a dose-dependent manner, with the maximal effect at 250 ng/ml (***C***, ***F***, ***H***, ***I***). Similarly, treatments with the GSK3β inhibitor CT99021 can also increase the number of DA neurons (***J***). ***K***, ***L***, To determine whether simultaneous treatments of Wnt1 and Shh can maximize the production of DA neurons, we treat the progenitor cultures with optimal concentration of Shh (250 ng/ml) and increasing amounts of Wnt1 or CT99021 (***D***, ***G***, ***K***, ***L***). Combined treatment of Shh and Wnt1 show a very modest increase in DA neuron number at 50 and 250 ng/ml Wnt1 but not at high dose (1250 ng/ml). On the contrary, combined treatments of Shh and CT99021 show an antagonistic effect ($n = 4$). Scale bar (in ***G***): ***B–G***, 50 μm. ***M***, ***N***, Compared with single treatment with CT99021 or Shh, sequential treatment of CT99021 and Shh decreases the DA neuron number ($n = 3$).

and treated these progenitors with single, combined, or sequential treatment of Shh, Wnt1, or the GSK3β inhibitor CT99021 (Fig. 5*A*). Our results showed that treatment of these progenitors with increasing amount of recombinant Wnt1 or Shh led to a dose-dependent increase in DA neuron numbers, with the optimal concentration at 250 ng/ml (Fig. 5*B*,*C*,*E*,*F*,*H*,*I*). Consistent with these results, the selective GSK3β inhibitor CT99021 also promoted the generation of DA neurons (Fig. 5*J*). Surprisingly, combined treatments of Wnt1 and Shh did not show an additive or synergistic effect on the generation of DA neurons. Rather, higher doses of Wnt1 (1250 ng/ml) appeared to reduce DA neuron generation from the progenitors at the optimal condition for Shh (250 ng/ml) (Fig. 5*K*). Similarly, the GSK3β inhibitor CT99021 also showed inhibitory effects on the generation of DA neurons in the optimal conditions for Shh (250 ng/ml) (Fig. 5*L*). Such antagonistic effects between Wnt1 and Shh in the generation of DA neurons were also detected in cultures obtained from the vMB of E13.5 embryos (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

The lack of additive or synergistic effect between Wnt1 and Shh raised the possibility that a sequential activation of canonical Wnt/β-catenin and Shh signaling pathways may be able to better recapitulate the *in vivo* conditions of DA neurogenesis and maximize the yield of DA neuron generation in cultures. To test this hypothesis, we cultured progenitors from E10.5 embryos and first treated them with optimal concentration of the GSK3β inhibitor CT99021 or Shh (the "priming" stage), followed by switching culture conditions to optimal concentration of Shh or CT99021 (the "maintenance" stage) (Fig. 5*A*). Contrary to our expectations, sequential treatments with CT99021 followed by Shh, or Shh followed by CT99021, reduced the number of DA neurons compared with cultures treated with CT99021 or Shh alone (Fig. 5*M*,*N*).

The antagonistic interaction between Shh and Wnt1 in the generation of DA neurons from stem/progenitor cells was also examined in a previously established culture condition to generate DA neurons from mESCs (Barberi et al., 2003). This culture protocol consisted of a four-step protocol of treating mESCs cocultured with mitomycin-treated stromal cells PA6 in serum replacement media (SRM) (days 0–5), SRM plus FGF8 (days 5–8), N2 media plus FGF8 and FGF2 (days 8–11), and finally N2 media plus ascorbic acid, BDNF, and GDNF (days 11–14) (Fig. 6*A*). Under this condition, the majority of the TH^+ neurons expressed additional dopaminergic markers, including Foxa2, Nurr1, and Pitx3a (supplemental Fig. S4, available at www.jneurosci.org as supplemental material) (Rodríguez-Gómez et al., 2007; Hedlund et al., 2008). These results supported the notion that most TH^+ neurons derived from mESCs using this protocol exhibited a phenotype consistent with that of vMB DA neurons. Our results also showed that the addition of Shh (200 ng/ml) from days 5 to 11 further promoted the generation of TH^+ neurons from mESCs (Fig. 6*B*). Unlike the primary cultures, however, addition of the GSK3β inhibitor CT99021 had no effect on DA neurons (Fig. 6*B*). Here it is important to note that the baseline generation of DA neurons (and possibly the level of Wnt signaling) in ESC cultures is higher than in progenitors from

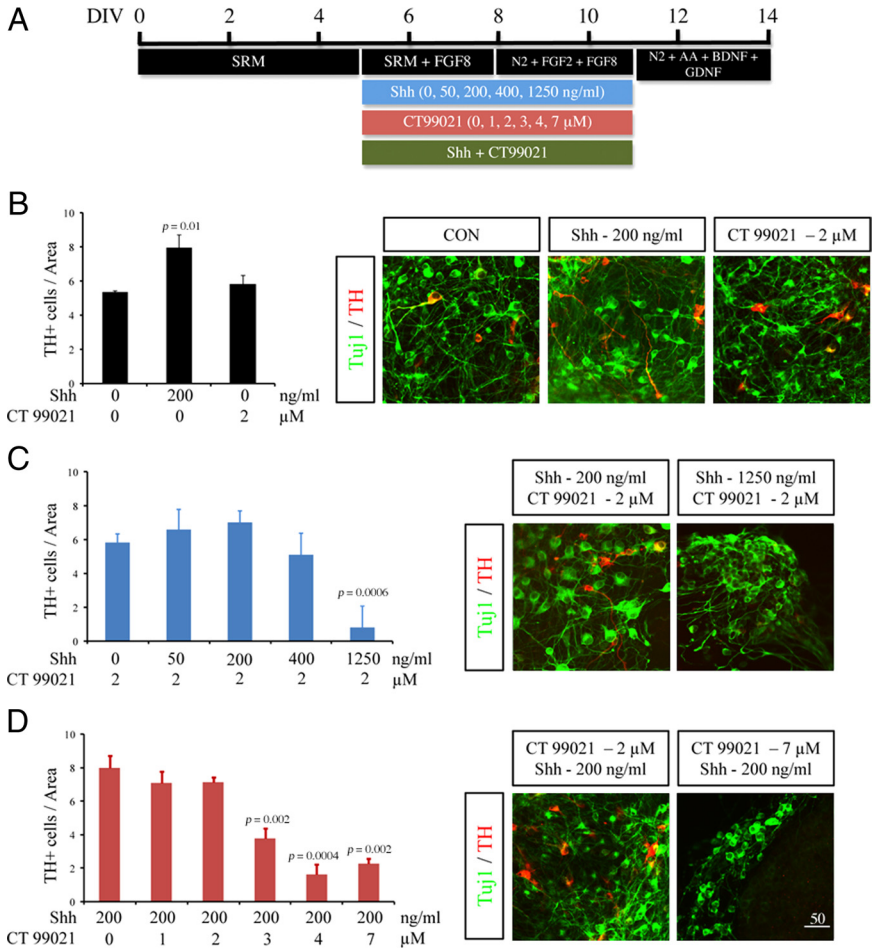

Figure 6. Antagonistic effects between Wnt/β-catenin and Shh in generating DA neurons from mESCs. ***A***, Schematic diagrams illustrating the culture conditions to generate DA neurons from mESCs. Mouse ESCs are cultured in the presence of SRM, followed by basal conditions, including SRM plus FGF8 (for 3 d), SRM plus FGF2 and FGF8 (for 3 d), and finally N2 supplement plus ascorbic acid (AA), GDNF, and BDNF (for 3 d). Modified treatment conditions include the addition of Shh, CT99021, or Shh and CT99021 from day 5 to 11. ***B***, Treatment with Shh (200 ng/ml) or CT99021 (2 μM) alone increases the DA neuron numbers. ***C***, Combined treatments with Shh and CT99021 do not show additive or synergistic effects in generating more DA neurons from mESCs. In contrast, higher concentration of Shh (1250 ng/ml) antagonizes the effects of CT99021 on DA neuron yield. ***D***, Similarly, the GSK3β inhibitor CT99021 also antagonizes on the effect of Shh in the generation of DA neuron in a range of concentrations ($n = 3$). DIV, Days *in vitro*; CON, control.

E10.5 embryos. Despite this difference, and similar to the observation in progenitor cultures from E10.5 embryos, combined treatments of Shh and CT99021 did not show additive or synergistic effects (Fig. 6*C*,*D*). Rather, higher doses of Shh suppressed DA neurogenesis from mESCs (Fig. 6*C*), and high doses of CT99021 inhibited the ability of Shh to generate DA from mESCs (Fig. 6*D*). Moreover, we also found that high doses of CT99021 inhibited overall neurogenesis in most of the colonies, as assessed by a reduction in the number of Tuj1$^+$ cells. Interestingly, Tuj1-positive neurons were mainly detected outside the colonies (Fig. 6*D*, right).

Activation of Wnt/β-catenin in midline progenitors promotes DA neurogenesis *in vivo*

The results in Shh–Cre;β-Ctn$^{Ex3/+}$ mutants indicated that the constitutive activation of the canonical Wnt/β-catenin signaling in the vMB led to the expansion of DA progenitors but reduced the neurogenesis of DA neurons (Figs. 1, 2). Based on these data, we reasoned that cell-type-specific activation of the Wnt/β-catenin signaling in midline progenitors may avoid the defect in DA neurogenesis seen in *Shh–Cre*;β-*Ctn*$^{Ex3/+}$ mutants. To test this hypothesis, we generated *Th–IRES–Cre*;β-*Ctn*$^{Ex3/+}$ mutants. We have shown previously that *Th–IRES–Cre* mediates recombination in essentially all postmitotic DA neurons and a subpopulation of midline progenitors at E10.5 (Tang et al., 2009). Unlike the phenotype in *Th–IRES–Cre*;β-*Ctn*$^{fl/fl}$ mutants, the number of DA neurons in *Th–IRES–Cre*;β-*Ctn*$^{Ex3/+}$ mutants showed a significant increase at E11.5 and E12.5 (Fig. 7*A–D*,*I*). By P0 and P21, *Th–IRES–Cre*;β-*Ctn*$^{Ex3/+}$ mutants showed an ~20% increase in DA neuron numbers compared with controls (Fig. 7*E–I*). In addition to the increase in DA neurons, *Th–IRES–Cre*;β-*Ctn*$^{Ex3/+}$ mutants also showed a persistent increase in the number of committed progenitors (Nurr1$^+$;TH$^-$ cells) in vMB at E11.5 and E12.5 (Fig. 7*J–N*). Furthermore, we performed 24 h neuronal birthdating experiments by labeling the progenitors with BrdU at E10.5 or E11.5 and allowed them to become TH$^+$ postmitotic DA neurons until E11.5 and E12.5, respectively. Our results showed that the number of newly born TH$^+$ neurons was significantly increased in *Th–IRES–Cre*;β-*Ctn*$^{Ex3/+}$ mutants (Fig. 7*O–S*).

To further investigate the mechanisms of the increased Nurr1$^+$;TH$^-$ progenitors in *Th–IRES–Cre*;β-*Ctn*$^{Ex3/+}$ mutants, we performed birthdating experiments in this population by labeling the progenitors with BrdU at E10.5 or E11.5 and allowed them to develop for 24 h. Our results showed an increase in the number of newly born Nurr1$^+$ precursors within the 24 h time intervals from E10.5 to E11.5 and from E11.5 to E12.5 (Fig. 8*A–E*). Together, these results indicated that the activation of Wnt/β-catenin signaling in a subpopulation of midline progenitors using the *Th–IRES–Cre* led to a significant increase in neurogenesis and DA neurons.

Discussion

The results from this study reveal an intricate, albeit primarily antagonistic, interaction between Wnt/β-catenin and Shh during DA neurogenesis in vMB progenitors as well as in mESCs (Fig. 8*F*). Activation of Wnt/β-catenin can promote the expansion of DA progenitors and the generation of DA neurons. However, these effects appear to be cell-context dependent such that constitutive activation of Wnt/β-catenin in vMB using *Shh–Cre* expands early progenitors but perturbs cell cycle progression in these progenitors and antagonizes the expression of Shh and Foxa2 in vMB. These phenotypes contribute to the reduced number of DA neurons. In contrast, a cell-type-specific activation of Wnt/β-catenin in the midline progenitors using *Th–IRES–Cre* circumvents these adverse effects and leads to a significant increase in DA neuron numbers.

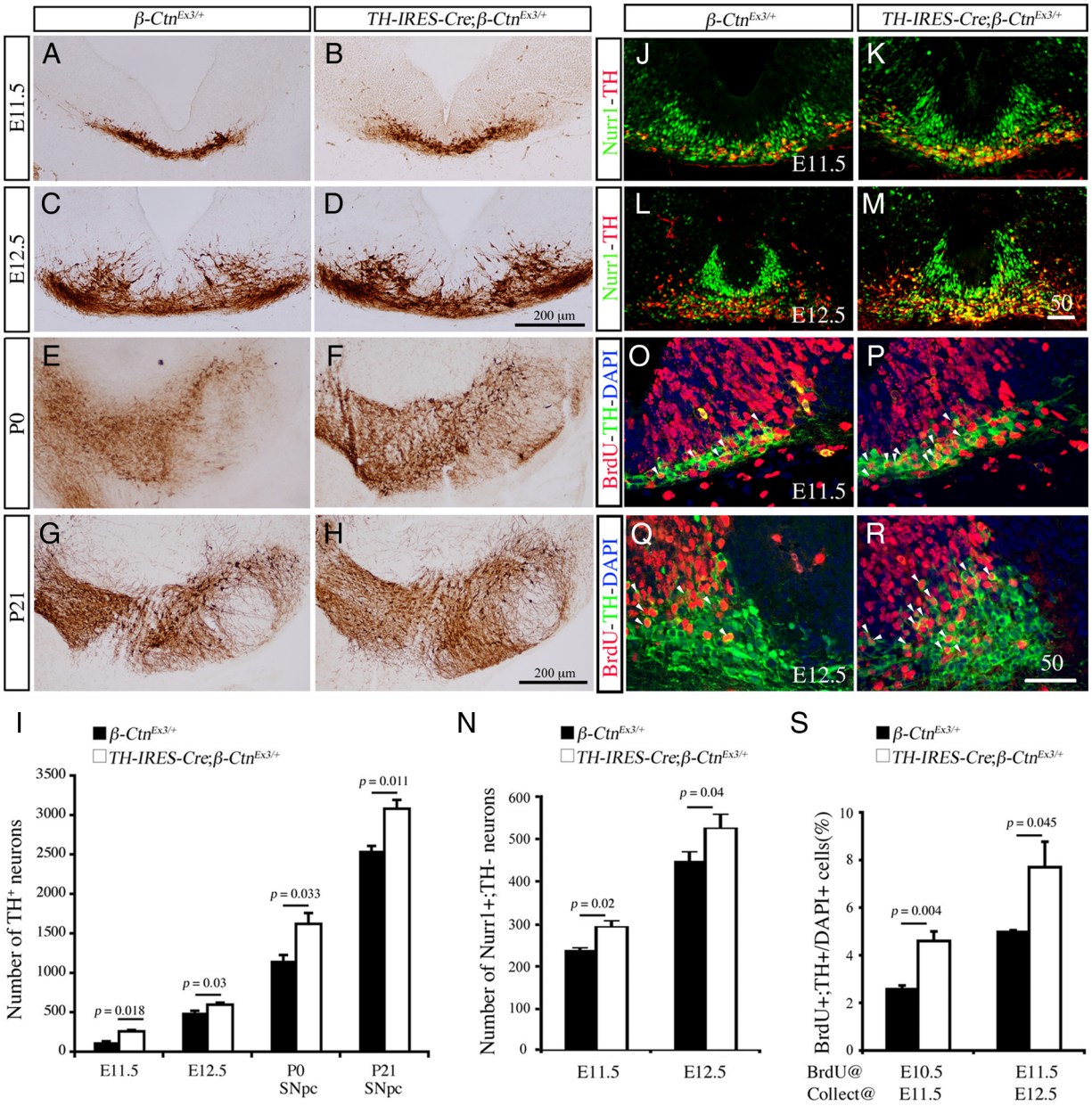

Figure 7. Increased DA neurons in *Th–IRES–Cre;β-Ctn*$^{Ex3/+}$ mutants. ***A–H***, Compared with control (*β-Ctn*$^{Ex3/+}$), the number of DA neurons is increased in *Th–IRES–Cre;β-Ctn*$^{Ex3/+}$ mice at E11.5 (***A***, ***B***), E12.5 (***C***, ***D***), P0 (***E***, ***F***), and P21 (***G***, ***H***). Scale bars: ***D***, ***H***, 200 μm. ***I***, Quantification using stereology confirms the increased number of DA neurons at E11.5, E12.5, P0, and P21. Student's *t* test, $n = 3$. ***J–M***, *Th–IRES–Cre;β-Ctn*$^{Ex3/+}$ mice show a persistent increase in the number of postmitotic DA precursors (Nurr1$^+$;TH$^-$) at E11.5 and E12.5. Scale bar: ***J–M***, 50 μm. ***N***, Quantitative analyses for the number postmitotic DA precursors (Nurr1$^+$;TH$^-$) at E11.5 and E12.5. ***O–R***, Neuronal birthdating experiments reveal increases of newly generated DA neurons (BrdU$^+$;TH$^+$) from E10.5 to E11.5 and from E11.5 to E12.5. The arrowheads indicate there are more BrdU$^+$;TH$^+$ cells in the mutants. ***S***, Quantification confirms the increased number of BrdU$^+$;TH$^+$ cells at E11.5 and E12.5.

Wnt/β-catenin signaling and the development of DA neurons

Several members of the Wnt family have been shown to regulate distinct aspects of the development of midbrain DA neurons. For instance, the canonical Wnt signaling mechanisms, mediated by Wnt1, Wnt2, and Wnt3a, control the patterning of midbrain–hindbrain junction and the initial generation of DA progenitors in vMB, whereas Wnt5a regulates the differentiation of DA neurons (Danielian and McMahon, 1996; Castelo-Branco et al., 2003; Castelo-Branco and Arenas, 2006; Andersson et al., 2008; Sousa et al., 2010). Consistent with these findings, analyses of *Wnt1*$^{-/-}$ and *En1*$^{Wnt1/+}$ mutant mice reveal a genetic network controlled by Wnt1 to regulate the establishment of DA progenitor domain and the full differentiation of DA neurons (Prakash et al., 2006; Omodei et al., 2008). Moreover, targeted deletion of β-catenin using either region-specific *Shh–Cre* in vMB or cell-type-specific *Th–IRES–Cre* in midline progenitors further demonstrate the essential role of Wnt/β-catenin signaling in the control of gene expression and in cell cycle progression during DA neurogenesis (Joksimovic et al., 2009; Tang et al., 2009). Remarkably, the effects of Wnt/β-catenin signaling appear to be highly conserved in mESCs in which β-catenin and Lmx1a cooperatively controls the differentiation of DA neurons through an autoregulatory feedback mechanism (Chung et al., 2009). Furthermore, similar roles for β-catenin have also been demonstrated in the regulation of cell cycle progression in neural progenitors of the ventral telencephalon (Gulacsi and Anderson, 2008).

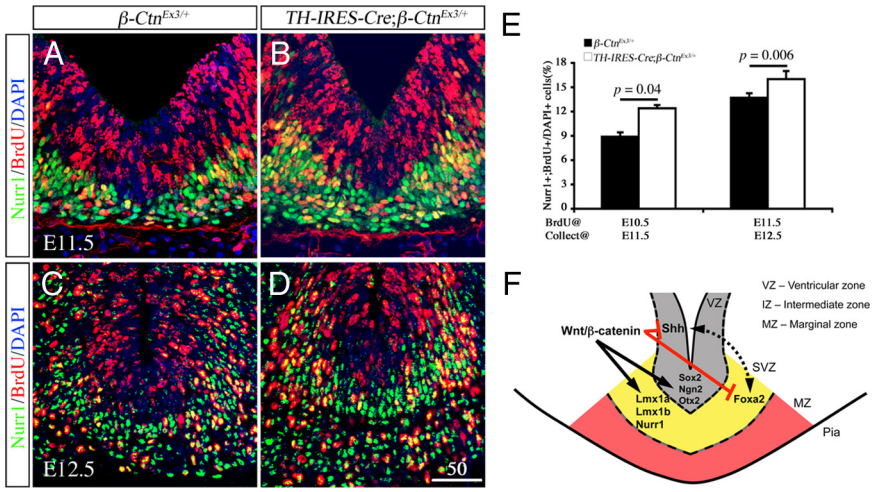

Figure 8. Increases in newly born postmitotic DA precursors in *Th–IRES–Cre;β-Ctn*$^{Ex3/+}$ mutants. ***A–D***, Neuronal birthdating experiments reveal increase newly generated postmitotic DA cells (BrdU$^+$;Nurr1$^+$) from E10.5 to E11.5 and from E11.5 to E12.5. Scale bar: ***A–D***, 50 μm. ***E***, Quantification confirms the increased number of BrdU$^+$;Nurr1$^+$ cells at E11.5 and E12.5. ***F***, A proposed model to indicate that Wnt/β-catenin and Shh control the expression of distinct sets of transcription factors in the progenitors in vMB. Constitutive activation of Wnt/β-catenin suppresses the expression of Shh and its downstream targets, including cyclin D1 and Foxa2.

Our current study provides additional *in vivo* evidence that activation of Wnt/β-catenin signaling leads to a marked expansion of early DA progenitors that express Sox2, Ngn2, and Otx2, as well as an increase in the progenitors that express Lmx1a, Lmx1b, and Nurr1 (Figs. 1, 8*F*). Despite the expansion of these progenitors, however, activation of Wnt/β-catenin perturbs cell cycle progression and reduces the production of TH$^+$ DA neurons in vMB (Figs. 2, 3). Interestingly, when cultured in the presence of Wnt5a, the progenitors from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants differentiate into DA neurons in a manner similar to those from control (Fig. 3*N*). These results provide important insights into the recently published results in which forced expression of Lmx1a in mESCs alone induces robust expression of Nurr1 and Pitx3, but only a limited number of these cells show properties of differentiated DA neurons (Chung et al., 2009). Furthermore, our results provide additional support that, when given the optimal growth conditions, such as excess Wnt5a, the progenitors expanded by the Wnt/β-catenin signaling mechanisms have the potential to differentiate into mature DA neurons.

Activation of Wnt/β-catenin antagonizes Shh and Foxa2 expression in the neurogenesis of DA neurons

Several explanations can account for the failure for constitutive activation of Wnt/β-catenin signaling to promote the differentiation of vMB progenitors into mature DA neurons in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. First, as indicated above, analyses of the proliferation and cell cycle progression in the DA progenitors in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants show much more progenitors in the S or M phase of the cell cycle. However, these mutant progenitors show reduced cell cycle exit (Fig. 2). Although the underlying cause(s) for the dysregulation of cell cycle progression in the DA progenitors of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants is not entirely clear, it is possible that the reduced expression of cyclin D1 and perhaps other cell cycle genes in the vMB of these mutants may have contributed to this phenotype. Second, the expanded progenitors may be exposed to a different environment that may prevent or delay their differentiation into committed progenitors or postmitotic neurons. Consistent with this notion, progenitors from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants can differentiate into DA neurons in the presence of Wnt5a just like those progenitors from control embryos (Fig. 3*N*).

The third explanation for the reduced production of DA neurons in *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants is the significant downregulation of Shh and forkhead transcription factor Foxa2 expression in the vMB (Fig. 4). The downregulation of Shh begins as early as E10.5, and, by E12.5, no detectable Shh is present in vMB in these mutants. In contrast, no detectable downregulation of Foxa2 is present until E12.5. The downregulation of Foxa2 may be attributable to the loss of Shh. Alternatively, activation of Wnt/β-catenin may directly or indirectly suppress the expression of Foxa2. Consistent with these results, expanded progenitors from *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants show limited potential to differentiate into DA neurons even when cultured in the presence of excess Shh, probably because of the severe reduction in Foxa2 expression (Figs. 3*N*, 4*L*). Similar antagonistic effects of Wnt/β-catenin activation on the expression of Shh in the developing hindbrain have been reported in a recent study (Joksimovic et al., 2009). Remarkably, the antagonistic effects between Wnt/β-catenin and Shh can be demonstrated in the differentiation of DA neurons using *in vitro* cultures of vMB progenitors and mESCs (Figs. 5, 6). These results support the model that Wnt/β-catenin and Shh each control distinct downstream target genes that work cooperatively to control the development of DA neurons (Fig. 8*F*) (Chung et al., 2009). Constitutive activation of one signaling mechanism may perturb a delicate balance between Wnt/β-catenin and Shh signaling mechanisms in the process of DA neurogenesis (Fig. 8*F*). Curiously, previous studies have shown that loss of Shh in the vMB of *Nestin–Cre;Shh*$^{flox/flox}$ or *En1*$^{KICre/+}$;*Shh*$^{flox/flox}$ mutants has no detectable effects on the expression of Lmx1a, Lmx1b, Foxa1, or Foxa2 (Ferri et al., 2007; Lin et al., 2009). These studies raise the possibility that loss of Shh alone may not be sufficient to cause the phenotypes in the progenitors of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants. It is possible that loss of Shh and Foxa2 in the *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants cooperatively block the differentiation of DA neurons. Alternatively, activation of Wnt/β-catenin in the vMB of *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants may suppress additional target genes that influence the generation of DA neurons.

The phenotype that *Shh–Cre;β-Ctn*$^{Ex3/+}$ mutants show a significant reduction in Foxa2 expression in vMB is reminiscent of those in *Nestin–Cre;Foxa2*$^{flox/flox}$ mutants, which show an expansion of Nurr1$^+$;TH$^-$ cells and a significant reduction in Nurr1$^+$;TH$^+$ DA neurons from E12.5 to E18.5 (Ferri et al., 2007). Although *Foxa1* null mutants also show a similar phenotype at E12.5, this deficit appears to be transient at E12.5 and is not detected at later developmental stages. It is unclear whether the effect of β-catenin activation to suppress the expression of Foxa2 is mediated through direct binding of lymphoid enhancer factor/T-cell factor to the enhancer sequence of *Foxa2*. Alternatively, it is possible that downstream targets activated by β-catenin may negatively regulate the expression of *Foxa2*. Regardless of the mechanism, it is most likely that downregulation of Foxa2 may in

part contribute to the reduced generation of DA neurons in these mutants.

Implications of Wnt/β-catenin and Shh in the generation of DA neurons from stem cells

The marked expansion of DA progenitors in the vMB of *Shh–Cre*; *β-Ctn*$^{Ex3/+}$ mutants raises the possibility that Wnt/β-catenin signaling may be a feasible target to promote the generation of DA neurons from neural stem cells. In support of this, both Wnt1 and the GSK3β inhibitor CT99021 promote the production of DA neurons in vMB progenitor cell and mESC cultures (Figs. 5, 6). Despite these encouraging results, simultaneous treatment with optimal doses of Wnt1 or CT99021 and Shh in vMB progenitors or mESCs shows no additive or synergistic effects, whereas treatments with higher doses of CT99021 and Shh actually suppress the generation of DA neurons (Figs. 5, 6). Furthermore, sequential treatments of Shh and CT99021 also do not show additional benefits (Fig. 5). These results suggest that a more detailed knowledge of the interaction between Wnt/β-catenin and Shh signaling in different vMB cells may further aid in the development of improved protocols for the generation of DA neurons in embryonic stem cell cultures. As a proof of principle, we report that cell-type-specific activation of Wnt/β-catenin in midline DA progenitors, using *Th–IRES–Cre*, leads to increases in Nurr1$^+$ precursors as well as in mature DA neurons both in prenatal and postnatal brains (Figs. 7, 8). Together, these encouraging results support the notion that, although a broad activation of Wnt/β-catenin remains an effective means to promoting the expansion of DA progenitors, a restricted activation in midline progenitors provides beneficial effects in promoting the generation of DA neurons. We suggest that Wnt/β-catenin activation in specific cell types may become a valuable strategy to improve the DA differentiation of embryonic stem cells.

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