β-Catenin signaling specifies progenitor cell identity in parallel with Shh signaling in the developing mammalian thalamus

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SUMMARY
Neural progenitor cells within the developing thalamus are spatially organized into distinct populations. Their correct specification is critical for generating appropriate neuronal subtypes in specific locations during development. Secreted signaling molecules, such as sonic hedgehog (Shh) and Wnts, are required for the initial formation of the thalamic primordium. Once thalamic identity is established and neurogenesis is initiated, Shh regulates the positional identity of thalamic progenitor cells. Although Wnt/β-catenin signaling also has differential activity within the thalamus during this stage of development, its significance has not been directly addressed. In this study, we used conditional gene manipulations in mice and explored the roles of β-catenin signaling in the regional identity of thalamic progenitor cells. We found β-catenin is required during thalamic neurogenesis to maintain thalamic fate while suppressing prethalamic fate, demonstrating that regulation of regional fate continues to require extrinsic signals. These roles of β-catenin appeared to be mediated at least partly by regulating two basic helix-loop-helix (bHLH) transcription factors, Neurog1 and Neurog2. β-Catenin and Shh signaling function in parallel to specify two progenitor domains within the thalamus, where individual transcription factors expressed in each progenitor domain were regulated differently by the two signaling pathways. We conclude that β-catenin has multiple functions during thalamic neurogenesis and that both Shh and β-catenin pathways are important for specifying distinct types of thalamic progenitor cells, ensuring that the appropriate neuronal subtypes are generated in the correct locations.

KEY WORDS: β-Catenin, Wnt, Neurogenesis, Patterning, Thalamus

INTRODUCTION
The mammalian thalamus performs a diverse array of critical brain functions, each of which is subserved by specific neuronal groups or nuclei (Jones, 2007). Most thalamic nuclei are composed of glutamatergic excitatory neurons. These nuclei show differential patterns of gene expression and form connections with the neocortex in unique patterns (Nakagawa and O’Leary, 2001; Jones and Rubenstein, 2004). A second group of thalamic nuclei, including intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLG), is composed predominantly of GABAergic inhibitory neurons that do not project to the neocortex (Jones, 2007). Generation and positioning of different types of thalamic neurons depend on the proper patterning of the embryonic thalamus into distinct progenitor cell populations (Vue et al., 2009). However, its underlying molecular mechanisms are only beginning to be explored (Kiecker and Lumsden, 2004; Vieira et al., 2005; Kataoka and Shimogori, 2008; Scholpp and Lumsden, 2010; Jeong et al., 2011; Nakagawa and Shimogori, 2012).

During thalamic neurogenesis, many transcription factors are heterogeneously expressed by progenitor cells, and their expression patterns delineate two distinct progenitor domains, pTH-R and pTH-C (Vue et al., 2007). Each domain generates distinct subtypes of neurons; pTH-C generates nuclei that project to the cortex, whereas pTH-R generates GABAergic nuclei (Vue et al., 2007; Jeong et al., 2011).

The Wnt/β-catenin and Shh signaling pathways are crucial for various aspects of thalamic development. During early brain patterning, Wnts are expressed outside the nervous system (Nordstrom et al., 2002) and specify caudal forebrain fate, including the thalamus, and suppress rostral forebrain fate, including the prethalamus, through regulation of transcription factors of the Irf, Fez and Six families (Braun et al., 2003; Kobayashi et al., 2002; Shimizu and Hibi, 2009). The prethalamus is immediately rostral to the thalamus and gives rise mainly to GABAergic neurons that populate the vLG, zona incerta and reticular nucleus (Vue et al., 2007; Delaunay et al., 2009). In mice deficient in Lrp6, a Wnt co-receptor, the entire thalamic tissue expressed prethalamic genes and the zona limitans intrathalamicica (ZLI), the tissue that separates the thalamus from the prethalamus and produces signaling molecules such as Shh and various Wnts, did not form properly (Zhou et al., 2007). Thus, early Wnt signaling is crucial for establishing thalamic identity and forming the ZLI. Wnt ligands are induced within the caudal forebrain itself shortly before ZLI formation and they are still expressed within and near the thalamus during neurogenesis (Salinas and Nusse, 1992; Bulfone et al., 1993; Louvi et al., 2007; Quinlan et al., 2007).
al., 2009). Wnt ligands and their downstream transcription factors Lef1 and Tcf4 are expressed in unique patterns within the embryonic mouse thalamus and/or the ZLI and transcriptional activity of the target gene, *Axin2*, is differential within the thalamus (Bluske et al., 2009). These results raise the possibility that Wnt/β-catenin signaling continues to function during thalamic neurogenesis. However, no specific roles at this stage have been directly assessed either in chick or mouse.

During thalamic neurogenesis, Shh is produced by the ZLI and basal plate, a tissue located ventrally to the developing thalamus (Echelard et al., 1993). Graded Shh signaling, high rostroventrally and lower caudodorsally, regulates the position-specific fates of thalamic progenitor cells (Kiecker and Lumsden, 2004; Vieira et al., 2005; Vue et al., 2009; Jeong et al., 2011). Because β-catenin and Shh signaling, as evaluated by their transcriptional readouts, are active in partially complementary patterns across the thalamus (Bluske et al., 2009), it is possible the two pathways functionally oppose each other to control the identity of thalamic progenitor domains.

To resolve these unanswered questions, we used mouse genetics to conditionally increase or decrease β-catenin activity in thalamic progenitor cells during neurogenesis. In addition, we deleted Shh and β-catenin (*Ctnnb1*) in the same embryos to determine the relative contributions of these two pathways to the positional identity of thalamic progenitor cells. Our results indicate that thalamic identity continuously requires β-catenin signaling for its maintenance and that normal patterning of the thalamus requires the complementary activity of both Shh and β-catenin pathways. We also found that ablation of β-catenin signaling during neurogenesis results in aberrant organization of glutamatergic and GABAergic neuronal populations later in development.

### MATERIALS AND METHODS

#### Mice

The mutant mouse alleles used in this study have been reported previously: *Bat-gal* (Maretto et al., 2003), *Ctnnb1pro exon2-6* (β-catenin<sup>2-6Δ</sup> (Brault et al., 2001), Jackson Laboratory), *Ctnnb1pro exon3* (β-catenin<sup>ex3</sup>) (Harada et al., 1999), *Olig3Cre* (Vue et al., 2009), *Neurog<sup>C<sub>CreERT2</sub></sup>* (Kim et al., 2011a), *Shhc* (St-Jacques et al., 1998; Jackson Laboratory), *Shh<sup>Δ</sup> (Sht); Lewis et al., 2001; Jackson Laboratory), *Neurog1*, *Neurog2GFP* (Ma et al., 1998; Seibt et al., 2003; obtained from F. Guillemot), *Ascl1CreERT2* (Kim et al., 2011b), *Rosa26<sup>STOP</sup>-GFP* (Srinivas et al., 2001), and *CAG-loxP-stop-loxP-ZsGreen* (Ai6) (Madisen et al., 2010; Jackson Laboratory).

Tamoxifen was dissolved in corn oil (20 mg/ml), and one dose (0.2 ml) was administered via oral gavage to pregnant female mice containing *CreERT2* alleles. Noon of the day of the vaginal plug was considered embryonic (E) day 0.5; stages of embryos was confirmed by morphology (Kaufman, 1992). Care and experimentation was carried out in accordance with the Institutional Animal Care and Use Committee of the University of Minnesota.

#### Histology

Mouse embryos were fixed in 4% paraformaldehyde (PFA) and cryoprotected in 30% sucrose, embedded in OCT compound and cryosectioned at 20 μm. All sections were cut frontally; see Fig. 1 and published literature (Vue et al., 2007; Nakagawa and Shimogori, 2012) for description of section planes and nomenclature.

#### Immunofluorescence and in situ hybridization

Immunofluorescence was carried out based on Vue et al. (Vue et al., 2007). Primary antibodies unique to this study included: Dlx2 and Dlx5 (1:3000; guinea pig; Kawasaki et al., 2006), Gata2 and Gata3 (1:200; rabbit; Santa Cruz Biotechnology), Helt/Heslike (1:500; guinea pig; Miyoshi et al., 2004), Islet1 (Is1 – Mouse Genome Informatics) (39.4D5) and Pax6 (1:10; mouse, Developmental Studies Hybridoma Bank). Ki67 (Mki67 – Mouse Genome Informatics) (1:100; mouse; BD), Lhx2 (1:100; goat; Santa Cruz Biotechnology; note: based on the expression pattern, this antibody may also recognize Lhx9, so we refer to this as Lhx2/9) and β-catenin (1:200; mouse; BD) and Pdgfrα (1:100; rat; BD). Secondary antibodies were DyLight- or Cy2/3/5-conjugated from Jackson ImmunoResearch (West Grove, PA, USA). Most immunofluorescence images were taken with a confocal Olympus Fluoview 1000 microscope. Optical sections were collected at 2 μm intervals and ImageJ software (NIH) was used to merge channels. Immunofluorescence images were also captured with a fluorescent microscope and analyzed as previously described (Vue et al., 2007) and are indicated in the figure legend.

In situ hybridization was performed as described (Vue et al., 2007). lacZ mRNA shown in Fig. 1 was detected by Tyramide Signal Amplification (TSA) (Perkin Elmer) combined with peroxidase-conjugated antidigoxigenin antibody (Roche).

#### Electroporation and whole-embryo culture

E10.5 embryos were dissected in 1% PBS according to Takahashi et al. (Takahashi et al., 2008). We utilized the roller tube/bottle system for whole embryos culture using a rotating bottle holder mounted inside an incubator. Embryos were precultured for 1 hour at 37°C in 100% rat serum (Gemini, Woodland, CA) supplemented with 2 mg/ml glucose with 95% O2/5% CO2. After preculture, embryos were individually electroporated with pCAG-ΔNlefl1-2A-H2BEGFP vector (based on pCAG-TdTomato-2A-H2BEGFP (Trichas et al., 2008)) or co-electroporated with pCAG-Neurogenin<sup>2</sup> (Mizuguchi et al., 2001) into the third ventricle (Vue et al., 2009). After electroporation, embryos were transferred to the culture medium, re-gassed, and returned to the roller tube/bottle apparatus for another 24 hours.

#### RESULTS

### β-Catenin-controlled transcription occurs in multiple cell populations during thalamic neurogenesis

The embryonic mouse thalamus is partitioned into two progenitor domains, rostroventrally located pTH-R and the caudodorsally located pTH-C (Fig. 1) (Vue et al., 2007). Our previous work indicated that β-catenin-controlled transcription is active in a spatially and temporally dynamic pattern during thalamic neurogenesis, where it was highest in pTH-C and lower in pTH-R (Bluske et al., 2009). Our recent study (Wang et al., 2011) showed the presence of two distinct types of thalamic progenitor cells: (1) radial glial cells that divide apically at the surface of the third ventricle; and (2) basal progenitor cells that divide away from the surface of the ventricle and form the subventricular zone (SVZ) in addition to also residing in the ventricular zone (VZ). lacZ expression in *Bat-gal* transgenic mice reports Tcf/Lef1-dependent transcription that requires β-catenin activity (Maretto et al., 2003). At E12.5, lacZ mRNA was robustly expressed in pTH-C in the VZ (Fig. 1A-F), which contained apically dividing, phospho-histone-H3 (PH3)-positive cells (Fig. 1B’D’F’). lacZ expression further extended into the mantle zone (Fig. 1D,F). This indicates that within the pTH-C domain of the thalamus, β-catenin-controlled transcription is active in radial glial cells, basal progenitor cells and postmitotic cells in the thalamus.

**Neurog<sup>1</sup>C<sub>CreERT2</sub> and Olig3<sub>Cre</sub> alleles cause recombination in the thalamus during neurogenesis**

To investigate the roles of β-catenin signaling, we deleted the β-catenin gene (*Ctnnb1*) in a spatially and temporally regulated manner using two different Cre alleles, *Olig3<sub>Cre</sub>* (Vue et al., 2009) and *Neurog1<sup>C<sub>CreERT2</sub></sup>* (Kim et al., 2011a). Olig3 is a basic helix-loop-
helix (bHLH) transcription factor expressed from E10.5 to E13.5 in most thalamic or in the pretecutum (Fig. 1 for orientation) (Vue et al., 2007). Neurog1 is another bHLH factor expressed in thalamic pTH-C domain and the ZLI, but not within pTH-R or in the prethalamus (Vue et al., 2007). Our recent work showed that at E11.5 and E12.5, Neurog1 is expressed in VZ but showed minimal overlap with a radial glial marker, NICD (intracellular domain of Notch), indicating it is expressed in basal progenitor cells, whereas Olig3 is expressed in both radial glial cells and basal progenitor cells (Wang et al., 2011). Crossing Olig3Cre mice with the CAG-loxP-stop-loxP-ZsGreen reporter mouse line (Madsen et al., 2010) showed near complete recombination in E12.5 thalamus, including the entire VZ (Fig. 2A,B). The tamoxifen-inducible Neurog1CreERT2 allele causes recombination in diverse but discrete populations of neuron-committed progenitor cells in different brain regions, including the thalamus (Kim et al., 2011a). With tamoxifen administration at E10.5, we obtained less complete recombination in thalamic progenitor cells compared with Olig3Cre mice (Fig. 2C,D). Although a vast majority of progenitor cells expressing Pax6, a marker for radial glial cells, had undergone recombination with the Olig3Cre allele (Fig. 2A’ ,B’), a smaller proportion of Pax6-positive cells showed recombination with the Neurog1CreERT2 allele (Fig. 2C’ ,D’). Recombination was particularly limited in ventral sections (Fig. 2D,D’). Thus, within the pTH-C domain of the thalamus, the Neurog1CreERT2 allele causes less recombination in VZ radial glial cells than the Olig3Cre allele.

**Deletion of Ctnnb1 during thalamic neurogenesis causes ectopic induction of prethalamic markers**

We deleted Ctnnb1 in the thalamus by using these two Cre alleles. Expression of Axin2, a downstream target gene of β-catenin-controlled transcription, was reduced in the thalamus at E12.5 in both Olig3Cre ; Ctnnb11-2c/c [Olig3-Ctnnb1 conditional knockout (cko)] and Neurog1CreERT2 ; Ctnnb11-2c/c (tamoxifen administered at E10.5) (Neurog1-Ctnnb1 cko) mice (supplementary material Fig. S1). The decrease in β-catenin signaling resulted in ectopic induction of prethalamic progenitor marker Dlx2 (Fig. 3A-I) in the entire pTH-C domain. Dlx5 and Islet1, postmitotic prethalamic markers, were also present within the thalamus of both ckos (data not shown and supplementary material Fig. S2). The degree of prethalamic marker induction in pTH-C correlated with the efficiency of Cre-mediated recombination. In Neurog1-Ctnnb1 ckos, middle sections (Fig. 3H), which underwent more Cre-mediated recombination in the VZ than ventral sections (Fig. 2), contained more ectopic Dlx2-expressing cells than ventral sections (Fig. 3I). Olig3-Ctnnb1 cko showed robust ectopic prethalamic cells throughout the thalamus (Fig. 3D-F). The amount of prethalamic marker induction also varied with the timing of recombination in Neurog1-Ctnnb1 ckos. Administering tamoxifen at E9.5 and E10.5 resulted in robust induction of Islet1 (supplementary material Fig. S2A,B), whereas administering tamoxifen at E11.5 resulted in very few Islet1+ cells within the thalamus (supplementary material Fig. S2C), suggesting β-catenin may not play a role in regulating progenitor fate at later stages of

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**Fig. 1. β-Catenin-controlled transcriptional activity in multiple cell populations during thalamic neurogenesis.** (Top left) Side view of the diencephalon. The axis refers to the orientation in the thalamus. Three frontal section planes (1-3) are shown (gray lines). (Top right) A frontal section from plane 2. Because frontal sections are between horizontal and coronal orientations, several diencephalic regions can be observed in the same section. The bottom of the section contains rostroventral regions of the diencephalon and the top contains caudodorsal regions. Comparing sections 1-3 also reveals the dorsoventral difference within the thalamus; section 1 contains dorsal regions of the thalamus; section 3 contains ventral regions. (A-F) In situ hybridization (ISH) for lacZ mRNA on frontal sections from E12.5 BAT-gal transgenic mice. A-B are from approximately section plane 1, C-D’ from plane 2, and E-F’ from plane 3. The dotted line in A and C indicates the midline. The arrows in D and F indicate lacZ expression in the mantle zone. B’, D’, F’ are higher magnification images of the boxed regions in B, D, F, respectively; midline is on the right. The arrows in D and F indicate apically dividing PH3+ cells surrounded by lacZ signal, and the arrowheads (B’, D’, F’) indicate basally dividing PH3+ cells surrounded by lacZ signal. Scale bars: 100 μm in A-F; 50 μm in B’, D’, F’.
neurogenesis. It is also possible that with the delayed Cre activation, deletion of Ctnnb1 occurred only in postmitotic cells and did not affect the progenitor cell fate. Olig3, a thalamic radial glial and basal progenitor marker, and Otx2, a thalamic postmitotic marker, were reduced in both ckos (supplementary material Fig. S3A-D and data not shown), indicating loss of thalamic identity in some thalamic progenitor cells. However, Otx2, which is required for the specification of glutamatergic identity in the thalamus (Puuelles et al., 2006), was unchanged in the Olig3-Ctnnb1cko embryos (supplementary material Fig. S3E,F), indicating that Otx2 may be either upstream or independent of the β-catenin signaling pathway. In summary, β-catenin is required during thalamic neurogenesis to maintain thalamic fate and prevent prethalamic fate.

Deletion of Ctnnb1 causes a pTH-C to pTH-R fate switch

Because β-catenin-controlled transcription is active in pTH-C and is much weaker in pTH-R (Bluske et al., 2009) (Fig. 1A,B), we next determined if β-catenin is required in the pTH-C domain to suppress not only prethalamic fate, but also pTH-R fate. For this purpose, we first identified transcription factors that are expressed in pTH-R but not in the prethalamus or pTH-C. We found that Helt (Nakatani et al., 2007; Miyoshi et al., 2004; Guimera et al., 2006) and Gata2 (Kala et al., 2009) were specifically expressed in pTH-R progenitor cells and Gata2 and Gata3 can be used as markers of postmitotic, pTH-R-derived cells (supplementary material Fig. S4).

In E12.5 Olig3-Ctnnb1 and Neurog1-Ctnnb1cko embryos, pTH-R markers Helt and Gata2 were ectopically induced within the entire pTH-C (Fig. 3A-R), whereas the pTH-C marker Neurog2 was downregulated in cells expressing ectopic Helt (Fig. 3J-R). Induction of pTH-R specific markers often occurred in the same cells that had ectopic prethalamic markers. For example, in Neurog1-Ctnnb1cko embryos, 20% of ectopic cells expressed only Dlx2 and 25% expressed only Gata2, whereas 55% of ectopic cells expressed both (Dlx2+/Gata2+) (Fig. 3G-I; a total of 700 cells were counted in four brains of each genotype). Dlx2 and Gata2 were normally co-expressed in a few pTH-R cells at E10.5 (supplementary material Fig. S5), but Dlx2+/Gata2- cells were no longer found at E12.5 (Fig. 3A-C), suggesting that there is a thalamic-specific mechanism that downregulates Dlx2 after E10.5, which is lost in Ctnnb1cko mice.

In addition to the pTH-C domain, pTH-R also undergoes recombination with the Olig3Cre allele (Vue et al., 2009), but deletion of Ctnnb1 using the Ascl1CreERT2 allele (Kim et al., 2011b), which causes recombination in pTH-R and the prethalamus and not in pTH-C, did not result in induction of the prethalamic marker Dlx2 (supplementary material Fig. S6). This indicates that the requirement for β-catenin signaling in preventing prethalamic fate is unique to the pTH-C domain.

Cellular basis for the induction of prethalamic and pTH-R markers in Ctnnb1cko mice

β-Catenin has a variety of important functions within the cell, including cell adhesion and activating transcription via Tcf/Lef family transcription factors (Grigoryan et al., 2008). In control embryos, β-catenin was ubiquitously expressed in the thalamus, including in the pTH-C domain (supplementary material Fig. S7). In Neurog1-Ctnnb1 and Olig3-Ctnnb1ckos, ectopic expression of Helt and Dlx2 was found in cells that lack strong β-catenin expression (supplementary material Fig. S7D-F, data not shown). Ascl1, which is expressed in both the prethalamus and pTH-R, was also expressed in cells that lack β-catenin (supplementary material Fig. S8G-I). Not all the cells that did not express β-catenin showed aberrant patterns of gene expression, which is probably due to the heterogeneity of these cells in terms of the duration of the β-catenin loss or in their differentiation state. In summary, these results suggest that induction of prethalamic and pTH-R markers is a cell-autonomous phenotype of β-catenin deletion.

In agreement with the differential frequency of recombination in radial glial cells between the Neurog1CreERT2 and Olig3Cre alleles, we observed different degrees of disruption of adherens junctions at the surface of the third ventricle. Olig3-Ctnnb1cko embryos showed severe disorganization of β-catenin distribution at E12.5, whereas β-catenin smoothly delineated the ventricular surface in control and Neurog1-Ctnnb1cko embryos (supplementary material Fig. S9). Despite the differences in ventricular architecture, prethalamic fate was induced in the thalamus of both ckos at this stage, suggesting that the role of β-catenin in maintaining progenitor cell identity is mediated by transcription, rather than cell adhesion.

To directly test if the conversion of pTH-C progenitors to pTH-R identity was mediated by β-catenin-controlled transcription, we overexpressed a dominant negative Lef1 (dnLef1) in wild-type embryos and found that the pTH-R marker Helt and the prethalamic marker Dlx2 are induced at varying levels in many of the transfected pTH-C cells (supplementary material Fig. S10A-F). This result supports the conclusion that induction of these markers is autonomous to reduced β-catenin-controlled transcription.
The presence of prethalamic progenitor cells within the thalamus was also not due to aberrant movement of prethalamic cells across the ZLI, a boundary tissue that separates the prethalamus and thalamus. We generated Olig3-Ctnnb1cko embryos that express YFP in a Cre-dependent manner. YFP-positive cells co-labeled with prethalamic marker Dlx2 in the Olig3-Ctnnb1cko (supplementary material Fig. S11).

The above results suggest that β-catenin maintains thalamic progenitor fate cell-autonomously by controlling transcription. Deletion of Ctnnb1 affects the types of postmitotic cells generated in the thalamus

We next determined if mis-specified progenitor cells produce postmitotic cells that are characteristic of prethalamic and/or pTH-R origins in Ctnnb1cko embryos. In E14.5 Neurog1-Ctnnb1cko embryos, Pax6, a postmitotic prethalamic marker, was detected in both the mantle zone and near the third ventricle within the thalamus (Fig. 4A-B’). Because these ectopic Pax6+ cells did not express the progenitor cell marker Ki67 (supplementary material Fig. S12), it is unlikely that they are aberrant progenitor cells. Moreover, ectopic Pax6+ cells had low levels of β-catenin (supplementary material Fig. S12G-G’). At E17.5, another postmitotic prethalamic marker, Islet1, was also ectopically induced (Fig. 4I,J). Thus, deletion of Ctnnb1 during neurogenesis causes aberrant generation of prethalamic neurons within the thalamus.

We also examined markers for pTH-R-derived postmitotic cells. A cluster of Gata3+ cells that appeared to be the enlarged vLG/IGL nuclei was found (Fig. 4D), and scattered, ectopic Gata3+ cells were observed in the medial region of the thalamus (Fig. 4D,D’). The SRY-box transcription factor Sox1 is normally expressed in pTH-R-derived postmitotic cells without overlapping with Sox2 (Vue et al., 2009). At E14.5 and E17.5, a number of ectopic, Sox1+/Sox2- cells were found in the thalamus (Fig. 4H,L-L’). Tight clusters of Sox1+ cells were not only found in lateral locations where pTH-R-derived vLG/IGL cells normally populate, but also in more medial locations (Fig. 4H,L’). These results showed that vLG/IGL nuclei, which contain the normal postmitotic derivatives of the pTH-R domain, were expanded in Ctnnb1cko embryos.

Within the thalamus, Sox2 is expressed in pTH-C-derived postmitotic cells in addition to progenitor cells (Vue et al., 2009). In Neurog1-Ctnnb1cko embryos, Sox2 expression was lost in cells that ectopically express Gata3 or Pax6 (data not shown), indicating that expression of postmitotic Sox2 depends on the pTH-C identity in progenitor cells. Similarly, Rora (RAR-related orphan receptor α), which is expressed in pTH-C-derived cells (Nakagawa and O’Leary, 2003), showed reduction in cko embryos (Fig. 4F). Although Wnt/β-catenin signaling inhibits the formation of oligodendrocyte precursor cells (Zhong et al., 2011; Ye et al., 2009; Langseth et al., 2010), we did not find an increase in cells expressing Pdgfrα, a marker for oligodendrocyte precursor cells (supplementary material Fig. S12E,F).

Fig. 3. Prethalamic and pTH-R markers are ectopically induced within pTH-C in Ctnnb1cko embryos. E12.5 control (from Olig3-Ctnnb1 litter), Olig3-Ctnnb1cko, and Neurog1-Ctnnb1cko (tamoxifen administered at 10.5) immunostained sections from three dorsoventral levels. The third ventricle is on the left. (A-C) Normal expression of the prethalamic marker Dlx2 and pTH-R marker Gata2. (D-F,G-I) Induction of both Dlx2 and Gata2 in pTH-C of Olig3-Ctnnb1 and Neurog1-Ctnnb1ckos (the arrows and arrowheads indicate Dlx2+/Gata2+ cells). (J-L) Normal expression of Helt and Neurog2 in the pTH-R and pTH-C domains, respectively. (M-O,P-R) Ectopic Helt+ cells scattered throughout pTH-C in the Olig3-Ctnnb1 and Neurog1-Ctnnb1cko embryos (arrows, arrowheads). Scale bar: 50 μm.
These data are consistent with the idea that correct specification of pTH-R and pTH-C progenitor identity by β-catenin signaling is critical for the generation and positioning of the neuronal subtypes generated from these domains. However, it is also possible that β-catenin plays a role in postmitotic thalamic cells as well (Fig. 1) and may contribute to the phenotypes we observed at late embryonic stages shown in Fig. 4.

**Elevated Wnt/β-catenin signaling induces pTH-C fate in the thalamus**

We next tested whether ectopically activated β-catenin signaling is sufficient to induce pTH-C fate at the expense of pTH-R fate. Deletion of exon 3 of Ctnnb1 causes truncation of the protein that renders it resistant to degradation and activates downstream genetic programs (Grigoryan et al., 2008). We found that E12.5 Olig3-Ctnnb1(ex3) embryos showed significant downregulation of pTH-R markers Helt (cell count: mean ± s.e.m.; control: 431.33 ± 70.46; exon3+: 217.67 ± 27.84; P = 0.048), Ascl1 (control: 491.33 ± 70.37; exon3+: 269.32 ± 32.13; P = 0.045) and Gata2 (control: 736 ± 92.32; exon3+: 449.33 ± 28.81; P = 0.041) (Fig. 5A-F; cells were counted in three brains for each genotype). Nkx2-2-positive cells were also reduced within each genotype. Nkx2-positive cells were also reduced within the thalamus of the cko. (C-D') Immunostaining for pTH-R-derived marker Gata3. Scattered Gata3+ cells were found in medial regions of the thalamus (arrow, D, D'); the arrowheads indicate the expanded domain of Gata3+ cells in the IGL/vLG in the cko. B', D' are high-magnification images of the indicated region in B and D, respectively. (E,F) ISH for pTH-C-derived postmitotic marker RORα; note reduction in the cko (F). (G,H) Immunostaining for Sox1 and Sox2. The arrowheads in H indicate clusters of ectopic Sox1+/Sox2+ cells in the thalamus. (I-L') E17.5 control and Neurog1-Ctnnb1 cko (tamoxifen at E10.5) sections. (J,J) Immunostaining for prethalamic postmitotic neuronal marker Islet1. The arrow in J indicates Islet1+ cells within the thalamus of the cko. (K-L') K', K', L', L' are high-magnification images of the boxed regions in K and L, respectively. Note expanded Sox1+ region in IGL/vLG (arrowhead in L') and medial clusters of Sox1+ cells in the cko embryos (arrow in L'). Scale bars: 100 µm in A-D,G-J,K'-L'; 200 µm in K,L; 50 µm in B', D'.

**Neurog1 and Neurog2 are required for proper specification of pTH-C Identity as thalamic progenitor cells differentiate**

Neurog1 and Neurog2 were downregulated in Ctnnb1 cko embryos (data not shown; Fig. 3J-R) and ectopically induced with elevated β-catenin signaling (Fig. 5F). Therefore, we hypothesized that Neurog1/2 may be downstream of β-catenin-controlled transcription and mediate its function in regulating the regional fate of thalamic progenitor cells.

In Neurog1/2 double knockout (dko) embryos, the prethalamic progenitor markers Dlx2 and Ascl1 were ectopically induced throughout the thalamus (Fig. 6A', C'), as shown previously (Fode et al., 2000). The postmitotic prethalamic marker Islet1 was also induced in the thalamic mantle zone (Fig. 6B'). Furthermore, pTH-R progenitor marker Helt and pTH-R-derived postmitotic marker Gata3 were expanded (Fig. 6D'). Olig3, which is expressed in radial glial and basal progenitor cells in pTH-C and pTH-R, was still expressed in the dko embryo (Fig. 6E'). Thus, Neurog1 and 2 are required to suppress both prethalamic and pTH-R fates. Pich1 (Agrén et al., 2004) and Axin2 expression was not changed in dko embryos (data not shown), indicating Shh and β-catenin-controlled transcription was not affected. In accordance with the changes in progenitor cell identity, we found that the pTH-C basal progenitor cell marker NeuroD1 (Wang et al., 2011) was absent (Fig. 6F'), as was a marker for postmitotic cells derived from pTH-C, Lmx2/9 and Sox2 (Fig. 6G' and data not shown). Thus, Neurog1/2 are required as pTH-C cells undergo differentiation to properly specify pTH-C fate and inhibit prethalamic and pTH-R fates.
Fig. 5. Stabilized Ctnnb1 in thalamic progenitor cells is sufficient to downregulate pTH-R markers and induce pTH-C markers. (A, C) Normal expression of pTH-R markers Helt and Gata2 (bracket). The third ventricle is on the left. (B, D) Reduced number of Helt+ and Gata2+ cells within pTH-R in Olig3-Ctnnb1<sup>1<sub>loxP</sub></sup> embryos. (E) Normal expression of Neurog2 in pTH-C and Ascl1 in pTH-R. (F) Decrease in the number of Ascl1<sup>+</sup> cells and ectopic appearance of Neurog2<sup>+</sup> cells within pTH-R of Olig3-Ctnnb1<sup>1<sub>loxP</sub></sup> embryos (arrows). Scale bar: 50 μm.

Although there were some differences between Neurog1/2 dko and Ctnnb1 cko mice (e.g. ectopic expression of Dlx2 was found in the mantle zone in Neurog1/2 dko mice, but in the VZ/SVZ in Ctnnb1 cko, and smaller numbers of cells expressed ectopic markers in Ctnnb1 cko mice), these two mutants both showed similar changes in progenitor cell identity. In addition, induction of pTH-R and prethalamic markers in pTH-C cells electroporated with dnLef1 was suppressed when the cells were electroporated with both dnLef1 and Neurog2 (supplementary material Fig. S10G-P). The co-electroporated cells showed significantly reduced ectopic induction of prethalamic marker Dlx2 (ratio Dlx2<sup>+</sup> cells: total electroporated cells: mean ± s.e.m.; dnLef: 0.43±0.04; dnLef+Neurog2: 0.11±0.03; P=0.004) and pTH-R marker Helt (dnLef: 0.64±0.03; dnLef+Neurog2: 0.35±0.05; P=0.006) within pTH-C compared with electroporation of dnLef1 alone. Therefore, we propose that β-catenin-controlled transcription regulates the regional identity of thalamic progenitor cells at least partially through regulating neurogenins.

**Interactions of Shh and β-catenin pathways in specifying positional progenitor cell fate in the thalamus**

We have shown that β-catenin is required for maintaining pTH-C and preventing pTH-R and prethalamic fates. Previous studies have shown that high Shh signaling is required for pTH-R and rostral pTH-C identity (Vue et al., 2009; Jeong et al., 2011). Thus, induction of pTH-R markers in Ctnnb1 cko mice could be due to increased Shh signaling in the pTH-C domain. To test this possibility, we examined the expression of Shh and patched 1 (Ptc1), a direct target of Shh-controlled transcription via Gli family proteins (Agren et al., 2004). In both Neurog1-Ctnnb1 and Olig3-Ctnnb1 cko embryos, expression of Shh and Ptc1 was not significantly changed (supplementary material Fig. S13). These data indicate that mechanisms independent of Shh-mediated transcription are responsible for the induction of pTH-R markers with reduced β-catenin signaling.

To determine how Shh and β-catenin pathways interact to regulate positional progenitor cell fate in the thalamus, we analyzed embryos in which both signals were reduced. By taking advantage of the fact that Olig3 is not only expressed in thalamic progenitor cells, but also in the ZLI, we used the Olig3<sup>Cre<sub>+</sub></sup> allele to delete both Ctnnb1 and Shh. In single Shh cko embryos (Olig3<sup>Cre<sub>+</sub></sup>; Shh<sup>tdc<sub>+</sub></sup>), Shh is deleted in the ZLI but not in the basal plate (supplementary material Fig. S14D,J). The pTH-R markers Helt, Gata2, Ascl1 and Nkx2-2 were all absent in the thalamus except near the basal plate, whereas Neurog1 and Neurog2 expanded into pTH-R (Fig. 7D,J and supplementary material Fig. S14E,F,K,L). This result demonstrates the requirement of the ZLI as a continuous source of...
Shh in specifying pTH-R identity (summarized in Fig. 8I,J). Interestingly, conditional deletion of Shh only in the diencephalic basal plate resulted in a similar fate switch within pTH-R (Jeong et al., 2011). These, along with other studies (Vieira and Martinez, 2006; Kiecker and Lumsden, 2004), collectively demonstrate that both ZLI and basal plate Shh sources are required for appropriate patterning of thalamic progenitor cells.

In Ctnnb1; Shh double cko embryos (Olig3Cre<sup>+</sup>; Ctnnb1<sup>flox/flox</sup>; Shh<sup>fl/fl</sup>), robust induction of pTH-R markers Helt and Ascl1 was observed within the thalamus (Fig. 7C,G), similar to the single Ctnnb1 cko embryos (Fig. 7B,F). The prethalamic marker Dlx2 was also ectopically induced within the thalamus of the dko (Fig. 7G). However, another pTH-R gene, Nkx2-2, was not increased by changes in β-catenin signaling (Fig. 7F,G). Ptc1 expression was reduced in Ctnnb1; Shh double cko embryos, similar to single Shh cko embryos (Fig. 7K,L). Therefore, in the absence of active β-catenin signaling, Helt and Ascl1 are ectopically induced in the pTH-C domain even without high Shh signaling.

**DISCUSSION**

The transcriptional activity controlled by β-catenin signaling is regionally differential during thalamic neurogenesis (Bluske et al., 2009). To our knowledge, this current study is the first demonstration that β-catenin maintains regional thalamic fate and specifies the rostrocaudal positional identity of progenitor cells during neurogenesis. Such roles depend on β-catenin-mediated transcription and not on the role of β-catenin in maintaining the structural integrity of the radial glial scaffold. In the mouse, Wnt3, Wnt3a and Wnt7b are differentially expressed in the thalamus at E10.5 and E11.5, but none of their expression patterns completely recapitulate the pattern of Axin2 expression (Bluske et al., 2009). Therefore, it is likely that combined activity of these Wnt ligands play a role in activating the β-catenin-Lef1/Tcf signaling during thalamic neurogenesis. Although it is also possible that β-catenin signaling has a role in dorsoventral patterning of the thalamus, we did not detect ectopic expression of the basal plate marker Shh in the alar plate of Ctnnb1 cko embryos, indicating that alar-basal identity of the caudal diencephalon is not altered. Regulation of thalamic progenitor cell identity by β-catenin signaling was also shown to be important for the generation of neuronal subtypes in the correct location.

**Continuous requirement of β-catenin signaling in the regional fate specification of thalamic progenitor cells**

Previous studies using chicks (Braun et al., 2003) and mice (Zhou et al., 2004) showed that Wnt/β-catenin signaling is critical for the initial distinction between the thalamus and the prethalamus, as well as for ZLI formation (Zhou et al., 2004). The Irx, Six and Fez families of transcription factors are regulated by Wnt/β-catenin signaling and intrinsically control thalamic fate during early forebrain patterning (Shimizu and Hibi, 2009). However, it was unclear whether global regional fates of the thalamus and prethalamus continue to require extrinsic signals for their maintenance after they are established long before the onset of neurogenesis or whether intrinsic roles of transcription factors are enough to maintain the regional fates. Our study addressed this unresolved question and provided evidence for the requirement of β-catenin-controlled transcription in the maintenance of thalamic fate after the onset of neurogenesis. This is in a striking contrast to the telencephalon, where deletion of Ctnnb1 during neurogenesis did not affect the dorsal versus ventral fate (supplementary material Fig. S15) (Backman et al., 2005).

**Downstream mediators of β-catenin-controlled transcription in regional fate regulation in the thalamus**

This study demonstrated that expression of Neurog1/2 is dramatically reduced in Ctnnb1 cko embryos and is ectopically induced in mice with constitutively active β-catenin. Thus,
neurogenins are functionally downstream of β-catenin-controlled transcription (see supplementary material Fig. S16 for our working hypothesis). Neurog1/2 dko mice showed robust induction of prethalamic and pTH-R markers in both the thalamus, similar to Ctnnb1 cko mice. The induction of prethalamic and pTH-R markers within pTH-C cells electroporated with dominant negative Lef1 could be partially rescued by co-electroporating with Neurog2. Together, these results suggest that neurogenins mediate some of the roles of β-catenin signaling in controlling the positional identity of thalamic progenitor cells.

There are, however, some differences in the phenotypes between Neurog1/2 dko mice and Ctnnb1 cko mice. The smaller number of cells expressing ectopic markers in Ctnnb1 cko mice is likely to be due to the delayed and incomplete downregulation of neurogenins in these mice compared with Neurog1/2 dko mice, in which both genes are completely deleted in the germline. The incomplete deletion of Neurog1 and Neurog2 could also lead to the compensation by the remaining neurogenin for the loss of the other. The fact that ectopic, Dlx2-expressing cells were in the mantle zone of Neurog1/2 dko mice but in VZ/SVZ in Ctnnb1 cko mice suggests that β-catenin has functions other than regulating neurogenin expression, such as controlling cell adhesion and migration. The scattered nature of the ectopic cells in Ctnnb1 cko mice may also have contributed to their aberrant positioning. Reduction of Olig3 expression in Ctnnb1 cko but not in Neurog1/2 dko mice indicates the different roles of β-catenin and neurogenins in regulating Olig3 expression.

Previous in vitro studies demonstrated direct binding of Tcf/Lef1 factors to the proximal region of the Neurog1 gene in neocortical progenitor cells (Hirabayashi et al., 2004; Israsena et al., 2004). However, its in vivo significance has not been established. In fact, we did not detect a significant decrease in the expression of Neurog1 or Neurog2 in the neocortex of Ctnnb1 cko mice (supplementary material Fig. S15 and data not shown), implicating distinct regulatory mechanisms of β-catenin-controlled transcription between the thalamus and the neocortex. Further studies are needed to determine whether Tcf/Lef1 signaling directly regulates Neurog1 and/or Neurog2 transcription in the thalamus or in the neocortex in vivo.

**Interactions between β-catenin signaling and Shh signaling in thalamic patterning**

Our previous work showed that a high level of Shh signaling is required to specify the pTH-R domain (Vue et al., 2009). This function of Shh opposes that of β-catenin signaling, which we have shown in the current study to be necessary and sufficient to induce pTH-C fate and suppress pTH-R fate (summarized in Fig. 8).

Many different mechanisms have been proposed for the interactions between Shh and Wnt/β-catenin signaling during neural patterning (Alvarez-Medina et al., 2008; Yu et al., 2008; Tang et al., 2010; Joksimovic et al., 2009) and, depending on the temporal and spatial relationships between the two signaling pathways, they are likely to interact differently in different brain regions. By conditionally reducing both β-catenin and Shh signals in thalamic progenitor cells, we found that the expression of the pTH-R transcription factors Helt and Ascl1 in Ctnnb1;Shh cko mice is likely to be necessary and sufficient to induce β-catenin signaling and is independent of direct transcriptional regulation by Shh signaling. However, expression of another pTH-R transcription factor, Nkx2-2, relies solely on high Shh signaling. Therefore, different transcription factors expressed in the same pTH-R domain have different requirements for β-catenin and Shh signaling. Interactions between transcription factors have been demonstrated in many regions of the central nervous system (Gowan et al., 2001; Nakatani et al., 2007; Kala et al., 2009), and are likely to play critical roles in defining progenitor domains in the thalamus (Jeong et al., 2011; this study). Based on the results of this study as well as others, we propose a working hypothesis on the interactions of β-catenin and Shh pathways and transcription factors in specifying the regional identity of thalamic progenitor cells (supplementary material Fig. S16). This model proposes that the regional identity of neural progenitor cells is formed by a combination of different transcription factors, and yet individual transcription factors are regulated by different mechanisms downstream of signaling molecules, such as Shh, β-catenin, or other transcription factors. Such sophisticated mechanisms define the identity of progenitor cells and ensure that each progenitor cell population gives rise to the appropriate neuronal subtypes in the correct location.

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β-Catenin signaling patterns the thalamus


