Sonic Hedgehog Signaling Controls Thalamic Progenitor Identity and Nuclei Specification in Mice

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The mammalian thalamus is located in the diencephalon and is composed of dozens of morphologically and functionally distinct nuclei. The majority of these nuclei project axons to the neocortex in unique patterns and play critical roles in sensory, motor, and cognitive functions. It has been assumed that the adult thalamus is derived from neural progenitor cells located within the alar plate of the caudal diencephalon. Nevertheless, how a distinct array of postmitotic thalamic nuclei emerge from this single developmental unit has remained largely unknown. Our recent studies found that these thalamic nuclei are in fact derived from molecularly heterogeneous populations of progenitor cells distributed within at least two distinct progenitor domains in the caudal diencephalon. In this study, we investigated how such molecular heterogeneity is established and maintained during early development of the thalamus and how early signaling mechanisms influence the formation of postmitotic thalamic nuclei. By using mouse genetics and *in utero* electroporation, we provide evidence that Sonic hedgehog (Shh), which is normally expressed in ventral and rostral borders of the embryonic thalamus, plays a crucial role in patterning progenitor domains throughout the thalamus. We also show that increasing or decreasing Shh activity causes dramatic reorganization of postmitotic thalamic nuclei through altering the positional identity of progenitor cells.

Introduction

The thalamus is located in the vertebrate diencephalon and plays critical roles in controlling animal behavior. Each of the thalamic nuclei exhibits a unique pattern of gene expression and connectivity. In mammals, a vast majority of thalamic nuclei send axons to unique sets of distinct neocortical areas. For example, principal sensory nuclei (visual, somatosensory, and auditory) establish topographic and area-specific thalamocortical projections to primary sensory areas in the neocortex (Jones, 2007). Despite the functional importance of the thalamus, however, molecular mechanisms that control the specification of thalamic nuclei are not well understood. A particularly intriguing question is how a single developmental unit in the alar plate of caudal diencephalon [prosomere 2 in the study by Puelles and Rubenstein (2003)] can

generate such a diverse array of postmitotic nuclei at later stages of development.

Numerous studies have shown that ventral-to-dorsal gradient of Shh activity specifies different neuronal subtypes throughout the embryonic CNS (Fuccillo et al., 2006). In the diencephalon, Shh is not only expressed in the basal plate, which is ventral to the thalamus, but is also expressed in the zona limitans intrathalamica (ZLI), a dorsally extending group of cells located immediately rostral to the early embryonic thalamus (Puelles and Rubenstein, 1993; Shimamura et al., 1995). *In ovo* electroporation and grafting studies in chick showed that ectopic expression of Shh in the caudal diencephalon and mesencephalon induces the expression of *Gbx2* and reduces *Pax6* (Kiecker and Lumsden, 2004; Vieira et al., 2005). Conversely, inhibition of Shh signaling by a dominant-negative form of Shh receptor Ptc1 reduced *Nkx2.2*, *Ptc1*, and *Gbx2* expression (Kiecker and Lumsden, 2004). Together, these studies establish the importance of Shh signaling in the global regionalization of the diencephalon, particularly its role in specifying the identity of the thalamus as a whole. To date, however, whether Shh signaling also controls the identity of progenitor cells within the thalamus along the rostrocaudal axis and subsequently contributes to the specification of different thalamic nuclei has remained largely unknown.

We recently reported molecular heterogeneity of thalamic progenitor cells in the mouse and analyzed the postmitotic fate of each progenitor cell population using genetic lineage tracing methods (Vue et al., 2007). We proposed that the thalamic ventricular zone is marked by the expression of the basic-helix-loop-helix (bHLH) transcription factor Olig3, and is divided into two

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Figure 1. Thalamic progenitor cells are exposed to graded activity of Shh signaling. **A**, A schematic of the location of the thalamus (TH) in the caudal diencephalon of the mouse embryo. Embryo is facing left. The thalamus is bordered by the prethalamus rostrally, the habenula dorsally, the basal plate ventrally, and by the ZLI rostrally. The basal plate and ZLI are sources of Shh (blue). Red dashed line shows the curvature of the anterior–posterior (AP) axis of the diencephalon. The two vertical lines (1 and 2) represent the planes of frontal sections shown in **C–J**. **B**, Expression patterns of bHLH (Olig2, Olig3, Ngn1/2, Mash1) and homeodomain (Nkx2.2, Dbx1) transcription factors in the thalamic ventricular zone. Olig3 is expressed in the entire thalamic ventricular zone and the ZLI. Expression of Mash1, Nkx2.2, Ngn1, and Ngn2 divides the thalamus into two distinct progenitor domains, a small rostral domain (pTH-R) and a much larger caudal domain (pTH-C). Olig2 and Dbx1 are expressed in opposing gradients within pTH-C. **C–J**, Graded patterns of *Gli1* and *Ptc1* expression in thalamic progenitor cells. Frontal sections through the thalamus of E10.5 (**C, D, G, H**) and E12.5 (**E, F, I, J**) wild-type brains. Top panels (**C–F**) are sections at a more dorsal and rostral level (plane 1 in **A**) and the bottom panels (**G–J**) are for a more ventral and caudal level (plane 2 in **A**), showing similar results. **C, G**, Double immunofluorescence for Olig3 and Shh at E10.5 to show the location of the thalamus. Olig3 expression caudal to the ZLI marks the entire thalamic ventricular zone. **D, H**, *In situ* hybridization for *Gli1* on sections adjacent to **C** and **G**. *Gli1* expression is higher closer to the ZLI and decreases in a graded manner throughout the thalamic ventricular zone. Arrowheads indicate dorsal and caudal boundary of the thalamus delineated by Olig3 expression. **E, I**, *In situ* hybridization for *Gli1* at E12.5, showing a similar gradient to E10.5. **F, J**, *In situ* hybridization for *Ptc1* on adjacent sections to **E** and **I**, respectively, again showing similar gradients. Asterisk and dotted lines in **E** and **F** mark the thalamic ventricular zone, indicating that expression of *Gli1* and *Ptc1* is restricted to progenitor cells. 3V, Third ventricle; LV, lateral ventricle; PT, prethalamus; TH, thalamus; PTh, prethalamus. Scale bar, 500 μm.

distinct progenitor domains, pTH-R and pTH-C (Fig. 1A,B). The pTH-R domain is rostroventrally located within the thalamic primordium, expresses transcription factors Nkx2.2 and Mash1, and contributes largely to nuclei that do not project to the cortex. These nuclei were previously not considered to be part of the postmitotic thalamus. The other domain, pTH-C, expresses Ngn1 and Ngn2 and gives rise to all the classical thalamic nuclei projecting to the cortex. Within pTH-C, Olig2, a bHLH protein, is expressed in a high-rostroventral to low-caudodorsal gradient, while the homeodomain protein Dbx1 is expressed in the opposite gradient. Because Nkx2.2, Olig2, and Dbx1 are known to be

differentially regulated by Shh in the ventral spinal cord (Briscoe and Novitsch, 2008), our previous study predicted that differential Shh signaling may impart not only the specification of pTH-R and pTH-C, but also the molecular heterogeneity of progenitor cells within the pTH-C domain. To test this hypothesis, we used conditional gene activation or deletion as well as *in utero* electroporation in mice to increase or decrease Shh activity in thalamic progenitor cells in temporally and spatially restricted manners. We found that altering the level of Shh activity shifts the positional gene expression patterns in both pTH-R and pTH-C. In addition, this shift results in reorganization of postmitotic thalamic nuclei.

Materials and Methods

Mice. Care and experimentation on mice were done in accordance with the Institutional Animal Care and Use Committee of the University of Minnesota. Noon of the day on which the vaginal plug was found was counted as embryonic day 0.5 (E0.5). Stages of early embryos were confirmed by morphology (Kaufman, 1992). To generate *Nes^{Cre/+}*; *R26^{SmoM2/+}* compound heterozygous mice, we crossed heterozygous *Nestin-Cre* (*Nes^{Cre/+}*) transgenic mice (Tronche et al., 1999) with homozygous *ROSA26-stop-SmoM2-EYFP* (*R26^{SmoM2/SmoM2}*) mice (Jeong et al., 2004) and identified Cre-positive embryos by PCR. Cre-negative littermates were used as controls. *Nes^{Cre/+}*; *R26^{SmoM2/+}* mice died by the end of the first postnatal day (P0). For Shh conditional knock-out, we crossed *Nes^{Cre/+}*; *Shh^{cl/+}* mice with *Shh^{cl/c}* mice. *Shh^{cl}* is a conditional allele for *Shh* (Lewis et al., 2001). PCR was done to identify *Nes^{Cre}* allele as well as floxed and wild-type alleles of *Shh*. *Smo* conditional knock-out mice were generated by crossing *Nes^{Cre/+}*; *Smo^{cl/+}* mice with *Smo^{cl/c}* mice (Long et al., 2001). Postmitotic fates of Olig2-expressing progenitor cells were analyzed by crossing *Olig2^{Cre/+}* mice (Dessaud et al., 2007) with *R26-stop-EGFP* reporter mice. *Olig2* mutant embryos were obtained by crossing between *Olig2^{ires-EGFP/+}* mice (Mukoyama et al., 2006). For analysis of normal gene expression (Fig. 1) and *in utero* electroporation, timed pregnant CD1 mice (Charles River) were used. For all the other mice, colonies were maintained in C57BL/6J background.

Production of *Olig3^{Cre/+}* mice. We produced *Olig3^{Cre/+}* mice by homologous recombination in embryonic stem (ES) cells. In short, R1 ES cells were transfected with a targeting vector so that the entire coding region of the *Olig3* gene is replaced by a cassette encoding Cre recombinase followed by frt-neo-frt (FNF) sequence (see Fig. 3K). G418-resistant clones were selected and analyzed by Southern blot analysis for homologous recombinants. Two positive clones were microinjected into blastocysts obtained from the mating of C57BL/6Ncr1 female mice with (C57BL/6J × DBA/2J) F1 male mice (performed at Transgenic Animal Model Core of University of Michigan), and male chimeras were further bred with C57BL/6J females to produce heterozygous *Olig3^{Cre/+}* mice. *Olig3^{Cre/+}* mice were bred with *R26^{SmoM2/SmoM2}*

mice to obtain *Olig3*^{Cre/+}; *R26*^{SmoM2/+} mice. To produce conditional *Smo* knock-out mice, we crossed *Olig3*^{Cre/+}; *Smo*^{c/+} and *Smo*^{c/c} mice. To monitor the spatial and temporal patterns of Cre-mediated recombination, *Nes*^{Cre/+} or *Olig3*^{Cre/+} mice were bred with *R26-stop-EYFP* reporter mice (Srinivas et al., 2001), and embryos were analyzed for the expression of EYFP and various progenitor cell markers.

In utero electroporation. *In utero* electroporation was performed as described previously (Saito and Nakatsuji, 2001). Timed pregnant (E10.5 or E11.5) CD1 females were anesthetized by intraperitoneal injection of pentobarbital sodium (2.0 mg for 30 g dams). Embryos were visualized by illumination of the uterus with fiber optics, and a pulled micropipette (1B120F-6, World Precision Instruments) loaded with DNA was inserted into the third ventricle of each embryo. Approximately 0.5 μ l of the DNA solution was injected with Picospritzer III (Parker Instruments). Four pulses of square-wave current were applied (35 V, 30 ms on and 100 ms off), using CUY21EDIT electroporator (NEPAGENE) and paddle- or needle-type electrodes. We used 4 μ g/ μ l *pCAG-Olig2* (from Masato Nakafuku, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) plus 0.5 μ g/ μ l *pCAG-nuclear EGFP* and 2 μ g/ μ l *pCAG-SmoM2-EYFP* (from Andrew McMahon, Harvard University, Cambridge, MA). After the injection, the dam was sutured and allowed to recover until analysis.

In situ hybridization and immunohistochemistry. *In situ* hybridization and immunohistochemistry were done based on Vue et al. (2007). In this study, goat anti-Sox1 antibody from R&D Systems was used at 1:100. cDNAs for *BHLHB4*, *Gli1*, *Ptc1*, *Dlx2*, *Sox14*, and *Pdlm3* were obtained from Open Biosystems. *Irx2* and *Spry1/Spry2* cDNAs were obtained from Peter Gruss (Max Planck Society, Munich, Germany) and Gail Martin (University of California, San Francisco, San Francisco, CA), respectively. Sox1-positive cells in the intergeniculate leaflet (IGL) nucleus were counted and compared between *Olig3*^{Cre/+}; *Smo*^{c/c} and control embryos. Serial sections from each brain were collected onto 8 slides, and Sox1-positive cells within NPY-expressing IGL nucleus were counted with ImageJ software. Average cell counts per slide were compared between the two genotypes (4 thalami in each group), and Student's *t* test was performed to test the significance of difference. An SEM was used for presentation of data.

Analysis of cell proliferation in the thalamic ventricular zone of *SmoM2*-expressing or *Shh* knock-out brains. Brains of E11.5 *Olig3*^{Cre/+}; *R26*^{SmoM2/+} and control litter mates, or E12.5 *Nes*^{Cre/+}; *Shh*^{c/c} and control littermates were cryosectioned at 20 μ m thickness and then immunostained with anti-phospho histone H3 (anti-PH3) antibody (1:100, Millipore #06-570) to label cells in M-phase. PH3-positive cells situated caudal to the ZLI and within the *Olig3*-expressing domain in the diencephalon were manually counted, and the numbers were compared between *Olig3*^{Cre/+}; *R26*^{SmoM2/+} or *Nes*^{Cre/+}; *Shh*^{c/c} with their respective controls. Student's *t* test was performed to test the significance of difference, and an SEM was used for presentation of data.

Anterograde labeling of retinogeniculate axons and their targets in the thalamus. Approximately 2 μ l of cholera toxin B subunit [CTB (5 μ g/ μ l), List Biological Laboratories or Invitrogen] was injected into the right retina of neonatal *Olig3*^{Cre/+}; *R26*^{SmoM2/+} mice and their control littermates. After an overnight survival period, pups were perfused with 4% paraformaldehyde and processed for cryosectioning. Goat anti-CTB antibody (1:100, List Biological Laboratories) was used to detect the retinal ganglion cell axons as well as their targets in the contralateral diencephalon, the dorsal lateral geniculate (dLG) and ventral lateral geniculate (vLG) nuclei as well as IGL. We compared the areas of terminations by measuring the dLG between *Cre*+ pups and *Cre*− controls. The border between dLG and IGL was identified by DAPI staining and immunohistochemistry with anti-Sox2. Scanned images were analyzed by ImageJ software for area measurement. We measured the area of every section containing dLG (21–29 sections per brain, 20 μ m thickness) and calculated the estimated volume of dLG by summing the values of area $\times$ thickness of each section. Student's *t* test was performed to test the significance of difference, and an SEM was used for presentation of data.

Results

Shh signaling is graded within the thalamic progenitor zone

Within the diencephalon, *Shh* is expressed in the ZLI and the basal plate, which directly border the thalamus rostrally and ventrally, respectively (Fig. 1A). In this study we asked whether differential *Shh* signaling is responsible for setting up the expression patterns of the various transcription factors that we described previously (Fig. 1B) and later contributes to the specification of different thalamic nuclei. To determine whether the embryonic thalamus is indeed exposed to graded *Shh* activity, we analyzed the expression of *Gli1* and *Ptc1* in mouse embryos from E10.5 to E12.5. *Gli1* and *Ptc1* are direct target genes of the *Shh* transduction pathway, and their expression levels reflect the levels of *Shh* activity in the cell (Agren et al., 2004; Bai et al., 2004). We predicted that if thalamic progenitor cells are differentially patterned by *Shh* signaling, then both *Gli1* and *Ptc1* should be expressed in graded manners across pTH-R and pTH-C. Indeed, at both E10.5 and E12.5, *Gli1* and *Ptc1* were expressed in a high-rostral to low-caudodorsal gradient in wild-type brains (Fig. 1D–F,H–J; thalamus is defined by *Shh* and *Olig3* in C and G); both genes were expressed at the highest levels in pTH-R, and their expression gradually tapered off caudally and dorsally away from the ZLI and the basal plate. With this finding, we hypothesized that the highest level of *Shh* signaling defines pTH-R, and progressively lower levels define different positional identity of progenitor cells within pTH-C depending on the distance from the ZLI and the basal plate. It was also evident that expression of *Gli1* and *Ptc1* is found only in progenitor cells of the thalamic ventricular zone (Fig. 1E,F,I,J) and not in postmitotic cells in the mantle zone, demonstrating that *Shh* signaling, whose direct output is gene activation mediated by Gli transcription factors, is restricted to neural progenitor cells.

Ectopic enhancement of *Shh* signaling strongly induces *Gli1* and *Ptc1* in the entire thalamic progenitor domains

Based on the above findings, we examined whether increased *Shh* activity in thalamic progenitor cells can cause a rostroventral shift in their positional identity. More specifically, we predicted that if caudodorsal progenitors are exposed to a higher level of *Shh* activity than normal, these progenitor cells would express rostroventral molecular markers rather than caudodorsal ones. To test this possibility, we crossed *Nestin-Cre* (*Nes*^{Cre/+}) transgenic mice (Tronche et al., 1999) with *ROSA26-stop-SmoM2-EYFP* (*R26*^{SmoM2/SmoM2}) mice (Jeong et al., 2004) to express the fusion protein of *SmoM2-EYFP* broadly in neural progenitor cells, including those in the thalamus. *SmoM2* carries a missense mutation in the *Smoothed* (*Smo*) gene, which encodes a transmembrane *Shh* effector. *SmoM2*-expressing cells undergo cell-autonomous activation of *Shh* signaling independent of ligand binding (Xie et al., 1998). Thus, we expected that mis-expression of *SmoM2* would cause elevated *Shh* signaling in all thalamic progenitor cells. By comparing *Nes*^{Cre/+}; *R26*^{SmoM2/+} and *Cre*-negative, *R26*^{SmoM2/+} controls and wild-type embryos, we found that *Cre*-negative control and wild-type embryos show the same gene expression and morphology at all stages. In contrast, brains of *Nes*^{Cre/+}; *R26*^{SmoM2/+} mice were larger in size than *Cre*-negative littermates, especially in the dorsal telencephalon.

Analysis of *SmoM2-EYFP* expression as detected by anti-EGFP antibody showed that in *Nes*^{Cre/+}; *R26*^{SmoM2/+} embryos,

Figure 2. Ectopic expression of SmoM2 in neural progenitor cells elevates the level of Shh signaling throughout the entire thalamic ventricular zone. **A–Q**, Frontal sections of *Nes^{Cre/+}; R26^{SmoM2/+}* (**F–Q**) and *Cre*— control (**A–E**) embryos at E9.5 (**A–J**), E10.0 (**K–N**), and E10.5 (**O–Q**). Midline is to the left. **A–J**, Controls (**A–E**) are compared with *Nes^{Cre/+}; R26^{SmoM2/+}* littermates (**F–J**) at E9.5. **A, B, F, G**, Double immunofluorescence for Shh and Olig3 to show the locations of the ZLI and thalamus, which are similar between control and *Nes^{Cre/+}; R26^{SmoM2/+}* embryos. EYFP immunofluorescence shows that Cre-mediated misexpression of *SmoM2-EYFP* in the thalamus starts to occur at E9.5, but only in the vicinity of the ZLI (**C, H**, arrowheads). **C** and **H** are adjacent to **B** and **G**, respectively. *In situ* hybridization analysis for *Gli1* and *Ptc1* at E9.5 (**D, E, I, J**) shows that *Gli1* and *Ptc1* are not yet expressed in *Cre*— controls (**D, E**), but in *SmoM2*-expressing brain, both *Gli1* and *Ptc1* are prematurely induced in the thalamus (**I, J**, arrowheads) as well as in the prethalamus. **K–N**, In *Nes^{Cre/+}; R26^{SmoM2/+}* embryos at E10.0, recombination as indicated by *SmoM2-EYFP* expression spans the entire thalamus marked by Olig3 expression. *Gli1* expression follows this. Arrowhead indicates the ZLI, and arrow shows the caudodorsal border of the thalamus. **O–Q**, In *Nes^{Cre/+}; R26^{SmoM2/+}* embryos at E10.5, recombination has further progressed, and *SmoM2-EYFP* expression is found in most of the diencephalon. The arrow indicates the most caudal–dorsal part of the diencephalon that has not yet undergone recombination. Triple arrows show that the mantle zone of the thalamus does not express *Gli1*. Scale bar, 200 μm for **A–N**, 500 μm for **O–Q**.

Cre-mediated recombination starts to occur in the diencephalon by E9.5 (Fig. 2*A–C, F–H*). At this stage, Shh expression in the ZLI has just started (Fig. 2*A, F*), and *Gli1* and *Ptc1* were not clearly detectable in control embryos (Fig. 2*D, E*). However, in

Nes^{Cre/+}; R26^{SmoM2/+} embryos, *Gli1* and *Ptc1* were already expressed ectopically in thalamic progenitor cells near the ZLI (Fig. 2*I, J*). Subsequently, recombination progresses through the thalamus into the more caudodorsal region, and by E10.0, *SmoM2-EYFP* and *Gli1* expression covered the entire thalamus labeled by Olig3 (Fig. 2*K, L, M, N*). By E10.5, most embryos we analyzed showed broad expression of *SmoM2-EYFP* and *Gli1* in the entire diencephalon except the most dorsal portion (Fig. 2*P, Q*). Analysis of recombination using *R26-stop-EYFP* reporter mice showed a similar rostroventral to caudodorsal progression of EYFP expression (supplemental Fig. S1*A–D*, available at www.jneurosci.org as supplemental material). These results demonstrate that in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos, Shh signaling is elevated in a rostroventral to caudodorsal direction, and by E10.0, there is a uniform enhancement in signaling within the entire thalamic ventricular zone.

Increased Shh activity induces rostroventral markers in thalamic progenitor cells

We then asked whether the increased Shh signaling in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos alters the expression profile of transcription factors in the thalamus. In *Nes^{Cre/+}; R26^{SmoM2/+}* embryos, expression of Shh in the ZLI was not affected (Fig. 3*A, B, E, F*). Nonetheless, expression of *Nkx2.2* and *Mash1*, two markers for the rostral progenitor domain, pTH-R, was expanded (Fig. 3*C, D, G, H*). This expansion results in more than two-fold increase in the size of pTH-R and disrupts the normally distinct gene expression boundary between pTH-R and pTH-C. The once distinct boundary is now replaced by a “mixed zone (pTH-R/C)” in which some cells expressing only pTH-R markers (*Nkx2.2* and *Mash1*) are intermingled with cells expressing only pTH-C markers (*Ngn2* and *Olig2*) (Fig. 3*G, H*). Such a zone of mixed pTH-R and pTH-C identity was not seen in control embryos (Fig. 3*C, D*). Although expression of *SmoM2-EYFP* appeared ubiquitous and homogeneous throughout the thalamic ventricular zone of *Nes^{Cre/+}; R26^{SmoM2/+}* embryos at E11.5 (supplemental Fig. S1*E–H*, available at www.jneurosci.org as supplemental material), it is possible that only a small number of cells

in this zone expressed ectopic SmoM2 early enough to take on the pTH-R fate, whereas the remaining cells were irreversibly determined to become pTH-C progenitor cells.

SmoM2 acts cell autonomously to enhance Shh signaling (Xie

et al., 1998). Thus, the observed changes in marker expression are likely to be caused by intrinsic changes in thalamic patterning. Nonetheless, since most CNS progenitor cells are forced to express SmoM2 in $Nes^{Cre/+}; R26^{SmoM2/+}$ embryos, it is possible that some secondary, indirect effects from outside the thalamus may have contributed to the expansion of pTH-R and/or the formation of the “mixed zone.” To investigate this possibility, we took two additional approaches to further restrict the SmoM2 expression spatially and temporally within the thalamus.

First, we performed *in utero* electroporation of SmoM2 plasmid (*pCAG-SmoM2-EYFP*) at E11.5 and analyzed the embryos a day later. We found that similar to $Nes^{Cre/+}; R26^{SmoM2/+}$ embryos, both Nkx2.2 (Fig. 3I,J) and Mash1 (data not shown) are expanded and are ectopically expressed more caudally on the electroporated side compared with the control side. As a second approach, we generated *Olig3^{Cre}* mice (Fig. 3K), so that we can express Cre recombinase in pTH-R, pTH-C, and the ZLI, but not in surrounding regions of the forebrain such as the prethalamus, the habenula, the pretectum, the diencephalic basal plate or the telencephalon. Cre-mediated recombination as assessed with *R26-stop-EYFP* reporter mice (Fig. 3L–O) and *ROSA26-stop-SmoM2-EYFP* mice (supplemental Fig. S1I,J, available at www.jneurosci.org as supplemental material) faithfully followed the expression of the endogenous *Olig3* gene. Thus, *Olig3^{Cre}* mice are useful in thalamus-specific gene manipulation. We found that brains of *Olig3^{Cre/+}; R26^{SmoM2/+}* are morphologically indistinguishable from control littermates, and some of these pups survive postnatally. Neuronal differentiation as determined by the expression of TuJ1, a neuronal marker, was also not affected in these embryos (supplemental Fig. S2A,B, available at www.jneurosci.org as supplemental material). Additionally, analysis of cell proliferation in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos at E11.5 revealed that BrdU-incorporation and phospho-histone H3 (PH3), an M-phase marker, also appeared normal (supplemental Fig. S2A,B, available at www.jneurosci.org as supplemental material). Quantification of PH3-positive cells in *Olig3*-expressing thalamic progenitor cells showed that there was no significant difference in number between *Olig3^{Cre/+}; R26^{SmoM2/+}* and control embryos (4440 ± 206.0 , $n = 2$ for *Olig3^{Cre/+}; R26^{SmoM2/+}*, 4906 ± 449.5 , $n = 2$ for *Cre*– controls; $p = 0.45$).

Analysis of progenitor markers in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos showed similar changes in gene expression compared with $Nes^{Cre/+}; R26^{SmoM2/+}$ embryos; pTH-R was expanded, and a “mixed zone (pTH-R/C)” was formed between pTH-C and the

Figure 3. Ectopic SmoM2 expression causes caudodorsal expansion of pTH-R marker genes Nkx2.2 and Mash1 in thalamic progenitors. **A–H**, Frontal sections of $Nes^{Cre/+}; R26^{SmoM2/+}$ (**E–H**) and *Cre*– control (**A–D**) embryos at E12.5. Midline is to the left. Immunofluorescence for Shh shows the location of the ZLI, which is similar between $Nes^{Cre/+}; R26^{SmoM2/+}$ (**A**) and control brains (**E**). **B, F**, High-magnification views of the boxed areas in **A** and **E**, respectively. These are adjacent to **C/D** and **G/H**, respectively. Double immunofluorescence for Nkx2.2 and Olig2 (**C, G**) and double immunofluorescence for Mash1 and Ngn2 (**D, H**) show that in the control brain, Nkx2.2 and Mash1 are expressed in pTH-R and form a distinct boundary with pTH-C, which expresses Olig2 and Ngn2 (**C, D**). In $Nes^{Cre/+}; R26^{SmoM2/+}$ brains, both Nkx2.2 and Mash1 are expanded caudodorsally, increasing the size of pTH-R. Only in $Nes^{Cre/+}; R26^{SmoM2/+}$ embryos, there is an additional domain between pTH-R and pTH-C that contain both pTH-R markers Nkx2.2/Mash1 and pTH-C markers Olig2/Ngn2. These two sets of markers are not colocalized (arrows in **G, H**). Dotted lines outline the ZLI in each panel. Arrowheads indicate the domain with both pTH-R and pTH-C markers, which we refer to as pTH-R/C. **I, J**, Analysis of E12.5 embryos electroporated with *pCAG-SmoM2-EYFP* plasmid at E11.5 in the thalamus. Double immunofluorescence of Nkx2.2 and YFP on frontal sections. Right side was transfected. Left side is control. Nkx2.2 is ectopically induced in the thalamic progenitor cells only on the transfected side (double arrows in **I**). Transfection occurred only in the thalamus, not in the prethalamus, as shown by YFP expression (**J**). **K**, Strategy for making the *Olig3^{Cre}* mice. The exon encoding for Olig3 was replaced by the *Cre*-frr-neo-frr (*Cre*-FNF) cassette. **L–O**, Analysis of recombination in *Olig3^{Cre}* mice. **L–O**, *Olig3^{Cre/+}* mice were bred with *ROSA26-stop-EYFP* mice and embryos were analyzed at E11.5. Double immunofluorescence of YFP (**L**) and Olig3 (**M**) shows that YFP is expressed in the thalamus, where endogenous Olig3 is expressed. Low-magnification images of the same embryo indicate the specificity of *Cre*-mediated recombination in the forebrain (**N, O**). Neither other regions of the diencephalon or the neocortex have undergone recombination (arrows). **P–S**, Frontal sections of *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos at E12.5, showing that thalamus-specific, ectopic expression of SmoM2 causes the same marker changes in progenitor cells as seen in $Nes^{Cre/+}; R26^{SmoM2/+}$ embryos (**A–H**). pTH-R is expanded (bracket), and the mixed zone (pTH-R/C) emerges between pTH-R and pTH-C (arrowheads). Scale bar, 200 μm for **A–M** and **P–S**, 500 μm for **N** and **O**.

expanded pTH-R (Fig. 3P–S). The results of these two additional approaches, *in vivo* electroporation and the use of *Olig3^{Cre}* mice, further reinforce our conclusion that it is the intrinsic increase in Shh signaling that causes the caudodorsal shift of pTH-R markers in thalamic progenitor cells.

We next examined how the pTH-C domain was affected by the increase in Shh signaling. First, in $Nes^{Cre/+}; R26^{SmoM2/+}$ embryos, *Olig3* was expanded caudodorsally into the pretectal/habenular region at E12.5 (Fig. 4A, G). At this stage, *Gbx2* and *RORα*, markers of the postmitotic thalamus derived from the pTH-C domain (Vue et al., 2007), were also expanded accordingly (supplemental Fig. S3A–C, F–H, available at www.jneurosci.org as supplemental material).

Figure 4. Ectopic SmoM2 expression in neural progenitor cells causes the caudal diencephalon to acquire rostral thalamic identity. **A–L**, Frontal sections of *Nes^{Cre/+}; R26^{SmoM2/+}* (**G–L**) and *Cre*— control (**A–F**) embryos at E12.5. Midline is to the left. In *Nes^{Cre/+}; R26^{SmoM2/+}* thalamus, Olig3 expression maintains its rostral boundary between the ZLI and the prethalamus, but is expanded caudodorsally (**A**, **G**; arrows in **G** indicate the area of expansion). Similarly, Olig2 and *Pdlim3*, both of which are expressed in the rostroventral part of pTH-C in *Cre*— embryos (**B**, **C**), are significantly expanded in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (**H**, **I**; arrows show the extent of expansion). *Dbx1*, which is normally expressed in the caudal pTH-C and the rostral pretectum (**D**, arrow), is not significantly altered in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (**J**, arrows). *Pax7* is expressed in the pretectum and the habenula of *Cre*— controls (**E**, arrow), but is reduced in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (**K**, no expression between arrowheads). *Ngn2* is expressed in pTH-C and the rostral pretectum, whereas *Mash1* is expressed in the caudal pretectum (**F**, arrows to show *Mash1* expression). In *Nes^{Cre/+}; R26^{SmoM2/+}* brains, *Ngn2* is induced in the caudal pretectum and *Mash1* is repressed (**L**, no expression between the arrows). **M–R**, *Olig3^{Cre/+}; R26^{SmoM2/+}* (**P–R**) and *Cre*— control (**M–O**) embryos at E12.5. Midline is to the left. In *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos, Olig3 expression is not expanded, unlike in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (**M**, **P**, arrow showing the caudodorsal boundary of pTH-C), indicating that SmoM2 expression is indeed limited to the thalamus. Within the thalamus, however, *Ptc1* expression is increased and now occupies the entire pTH-R and pTH-C (**N**, **Q**). Olig2 is also induced more caudodorsally within pTH-C (**O**, **R**). Arrow in **M–R** indicates the caudal–dorsal limit of gene expression, showing that unlike in the *Cre*— control, *Ptc1* and Olig2 are now expressed in the entire pTH-C in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos. **S**, **T**, E12.5 embryo in which *pCAG-SmoM2-EYFP* was electroporated into two focal diencephalic regions at E11.5. The right side was electroporated. Olig2 is ectopically expressed in the caudal group of electroporated cells in an isolated manner (arrow). Scale bar, 200 μ m.

jneurosci.org as supplemental material). In contrast, *Pax7*, which is normally expressed in the pretectum and habenula, but not in thalamic progenitor cells, was not detected in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (Fig. 4*E*, *K*). Moreover, expression of *Mash1* in the caudal pretectum was replaced by *Ngn2*, which normally spans from pTH-C only into the rostral pretectum (Fig. 4*F*, *L*). *Irx2* and *BHLHB4*, early postmitotic markers of the pretectum, were also reduced at E12.5 (supplemental Fig. S3*D*, *E*, *I*, *J*, available at www.jneurosci.org as supplemental material). These results show that a high Shh signal

in the caudal diencephalon induces the thalamic progenitor domain, pTH-C, at the expense of the pretectum and habenula. Olig2, which is normally expressed in a rostral–ventral to caudal–dorsal gradient within pTH-C, was homogeneously expressed in this expanded pTH-C domain of *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (Fig. 4*B*, *H*). Another marker gene, *Pdlim3*, which is normally expressed in pTH-R as well as the rostroventral part of pTH-C (Fig. 4*C*) (Gray et al., 2004), also expanded (Fig. 4*C*, *I*). These results demonstrate that enhanced Shh signaling in the entire diencephalon transformed it into the rostral–ventral thalamus, which is composed of the pTH-R domain and the rostral–ventral part of the pTH-C domain (summarized in Fig. 9*A*, *B1*, *B2*, *C1*, *C2*).

Surprisingly, the expression of *Dbx1*, which is normally expressed in the caudodorsal part of pTH-C as well as in the pretectum and habenula, was not significantly reduced in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (Fig. 4*D*, *J*), suggesting that *Dbx1* is positively regulated by some unknown mechanisms operating in the caudodorsal diencephalon. Expression of *Dlx2*, a prethalamic marker, was not ectopically induced in the thalamic progenitor cells in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (data not shown), consistent with a previous study showing that the thalamus and the prethalamus are distinctly prepatterned and express different sets of genes in response to Shh signaling (Kiecker and Lumsden, 2004).

To ensure that the above changes were also not the product of indirect effects, we again analyzed embryos electroporated with the *pCAG-SmoM2-EYFP* plasmid, as well as *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos. In electroporated embryos where an isolated population of progenitor cells in the pretectum were transfected, Olig2 was ectopically induced (Fig. 4*S*, *T*). In *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos, although the pretectum and the habenula are spared and the extent of Olig3 expression was similar to controls (Fig. 4*M*, *P*), *Ptc1* and Olig2 were homogeneously elevated within the entire pTH-C domain (Fig.

4*N*, *O*, *Q*, *R*), demonstrating that thalamus-specific elevation of Shh signaling intrinsically induced the rostral pTH-C markers.

Expression of rostral postmitotic thalamic markers is expanded in SmoM2-expressing embryos

Does increased Shh signaling in thalamic progenitor cells lead to reorganization of postmitotic thalamus? To address this question, we examined the expression of postmitotic markers that are

differentially expressed within nuclei of the late embryonic thalamus (Fig. 5).

First, we analyzed nuclei that are derived from the pTH-R domain. We previously found that progenitor cells in pTH-R generate part of the vLG as well as the IGL, two nuclei that do not contain neurons projecting to the neocortex (Vue et al., 2007). Consistent with this fate analysis and the expanded pTH-R markers in *Nes^{Cre/+}; R26^{SmoM2/+}* as well as *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos, vLG/IGL was expanded caudodorsally along the lateral surface of the diencephalon (Fig. 5*A, G* for *Nes^{Cre/+}; R26^{SmoM2/+}*, and data not shown for *Olig3^{Cre/+}; R26^{SmoM2/+}*). These expanded nuclei are marked by expression of *Nkx2.2*, *Sox14* (Fig. 5*A–C, G–I*), and *Sox1* (data not shown). The expanded *Nkx2.2* and *Sox14* expression is consistent with previous studies using chick and mouse embryos (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005) and establishes that IGL and the lateral part of vLG are specified by high Shh activity in pTH-R. *Nkx2.2*-positive cells were also found in a more caudolateral region that is away from the large cluster of expanded vLG/IGL cells (Fig. 5*H*, arrowheads). These cells did not express *Sox2* but intermingled with *Sox2*-expressing cells (Fig. 5, compare *G, H, J*). This intermingling of two molecularly different cell types reflects our earlier observation that two completely separate progenitor cell populations, one with pTH-R identity and the other with pTH-C identity, are mixed in the SmoM2-expressing, thalamic ventricular zone (Fig. 3*G, H*).

Next, we tested whether markers of thalamic nuclei that are derived from the pTH-C domain are also shifted in SmoM2-expressing embryos. In control embryos, *Sox2* is expressed in the rostroventral part of the pTH-C-derived thalamic mantle zone (Fig. 5*D, M, O*) (Vue et al., 2007). In E16.5 *Nes^{Cre/+}; R26^{SmoM2/+}* brains, *Sox2* expression was expanded caudally and dorsally, occupying essentially the entire diencephalic mantle zone (Fig. 5*D, J*). Similarly, another rostral nucleus marker, *RORα*, which is expressed more specifically in ventral posterior nucleus (VP) and dLG (Nakagawa and O'Leary D, 2003), was expanded caudally and dorsally in *Nes^{Cre/+}; R26^{SmoM2/+}* brains compared with controls (Fig. 5*E, K*). To determine whether the expansion of these rostral nucleus markers is accompanied by the repression of caudal nucleus markers, we analyzed the expression of *Gbx2*. Although *Gbx2* is initially expressed in the entire postmitotic thalamus (supplemental Fig. S3*A, B, F, G*, available at www.jneurosci.org as supplemental material), its expression later becomes restricted to medial and caudodorsal nuclei and is nearly

Figure 5. Ectopic SmoM2 expression causes a rostroventral shift of postmitotic thalamus. ***A–L***, Frontal sections of *Nes^{Cre/+}; R26^{SmoM2/+}* (***G–L***) and *Cre–* control embryos (***A–F***) at E16.5. Midline is to the left. In control embryos, *Nkx2.2* and *Sox14* expression delineates the lateral vLG and IGL (***A–C***, arrowhead), which are derived from pTH-R. ***B***, High-magnification image of the boxed region in ***A***. In *Nes^{Cre/+}; R26^{SmoM2/+}* embryos, *Nkx2.2* and *Sox14* expression is expanded caudally and dorsally (***G–I***, arrowhead). ***H***, High-magnification image of the boxed areas in ***G***, showing that some cells ectopically expressing *Nkx2.2* (***H***, arrowheads) are intermingled with *Sox2*-expressing cells (***J***). In *Cre–* controls, *Sox2* is highly expressed in rostroventral part of the postmitotic thalamus, including VP and dLG, but not in more caudodorsal part (***D***, asterisk). In *Nes^{Cre/+}; R26^{SmoM2/+}* embryos, *Sox2* is robustly expanded caudodorsally (***J***, arrowheads). Similarly, *RORα* expression, which corresponds to rostral nuclei such as VP and dLG (***E***), is also expanded caudodorsally in *Nes^{Cre/+}; R26^{SmoM2/+}* embryos (***K***, arrowheads). Note that the medial part of the thalamus that is negative for *RORα* in control is also negative in SmoM2 embryos (***E, K***, asterisk). The caudodorsal expression border of *Gbx2* is elongated near the midline in SmoM2 embryos (***F, L***, arrow), but overall, *Gbx2* expression is reduced, especially from the medial dorsal part (***F, L***, asterisk). ***M, N***, E17.5 embryo in which pCAG-SmoM2-EYFP plasmid was electroporated at E11.5 into the thalamus. The right side was electroporated, and the left side is the control. On the electroporated side, *Sox2* is ectopically induced in a caudodorsal region (***M***, arrow), while this induction did not occur on the control side. Conversely, *Gbx2* is downregulated where *Sox2* is induced (***N***, arrow). ***O–T***, *Olig3^{Cre/+}; R26^{SmoM2/+}* and *Cre–* control embryos at E18.5. Midline is to the left. In *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos, *Sox2* expression is expanded and now occupies the entire thalamic mantle zone (***R***). The *Sox2*-negative, caudodorsal region (asterisk in ***O***) is undetectable in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos. The caudodorsal boundary of the thalamus is outlined by a dotted line. Similarly, expression of *RORα* is expanded (***P, S***). Unlike *Sox2*, *RORα* is not induced in the medial part of the thalamic mantle zone (***P, S***, asterisk). *Gbx2* expression is dramatically reduced in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos (***Q, T***). ***U–X***, Postnatal day 1 thalamus of *Olig3^{Cre/+}; R26^{SmoM2/+}* (***W, X***) and *Cre–* control (***U, V***) pups injected with CTB in the contralateral retina at P0. dLG nucleus is delineated as dashed lines by CTB immunohistochemistry (***V, X***) and DAPI staining (***U, W***). dLG is expanded by ~3.6 times (see Results). Scale bar, 200 μ m for ***B, H***; 500 μ m for other panels.

complementary to that of *Sox2* and *RORα* (Fig. 5*F, N, Q*). In *Nes^{Cre/+}; R26^{SmoM2/+}* brains, *Gbx2* expression was dramatically reduced, particularly in regions where both *Sox2* and *RORα* are ectopically induced (Fig. 5*L*).

To better demonstrate the local effects of enhanced Shh signal on postmitotic thalamic development, we again analyzed SmoM2-electroporated embryos and *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos. When caudodorsal thalamus was electroporated at E11.5

and analyzed at E17.5, Sox2 was highly induced in the cohort of cells on the transfected side (Fig. 5M). *Gbx2* was specifically repressed in these Sox2-expressing cells, but its expression on the control side was unaffected (Fig. 5M,N). In *Olig3^{Cre/+}; R26^{SmoM2/+}* brains at E18.5, the overall morphology of the thalamic mantle zone was normal. However, Sox2 was ubiquitously expressed in the entire thalamus, not just in rostroventral nuclei as seen in the control (Fig. 5O,R). *RORα* was also significantly expanded caudodorsally in the lateral part of the thalamus (Fig. 5P,S). Furthermore, *Gbx2* expression is almost completely absent in *Olig3^{Cre/+}; R26^{SmoM2/+}* brains (Fig. 5Q,T). These data demonstrate that in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos, the entire postmitotic thalamus is dominated by rostroventral identity.

Although the expansion of *RORα* implicates that rostroventrally located nuclei, VP, and dLG are increased in size in *Olig3^{Cre/+}; R26^{SmoM2/+}* embryos, we used criteria other than molecular markers that would definitively prove that individual nuclei are altered. For this purpose, we used anatomical criteria to define thalamic nuclei. Specifically, we injected anterograde axon tracer CTB to the retina of the neonatal *Olig3^{Cre/+}; R26^{SmoM2/+}* mice to label retinogeniculate axons in the dLG nucleus. Because *Olig3* is not expressed in the retina or along the retinogeniculate pathway to the thalamus, *Olig3^{Cre/+}; R26^{SmoM2/+}* mice provide a particularly useful model to evaluate the specific effects of enhanced Shh signal in thalamic progenitor cells on the size of postnatal thalamic nuclei. We found that the volume of the dLG nucleus as estimated by the CTB-labeled region in the thalamus, was increased by ~3.6 times in *Cre+* pups ($0.086 \pm 0.008 \text{ mm}^3$ in *Cre+* pups, $n = 3$; $0.024 \pm 0.003 \text{ mm}^3$ in *Cre-* pups, $n = 2$; $p = 0.0091$) (Fig. 5U–X).

In summary, we demonstrated that increasing Shh signaling in CNS progenitor cells results in expansion of rostral–ventral thalamic progenitor pools, pTH-R, and rostroventral pTH-C, at the expense of more caudal–dorsal thalamus as well as the pretectum and habenula (summarized in Fig. 9B1,B2,C1,C2). When the manipulation of the Shh signal was restricted to thalamic progenitor cells, pTH-R and rostroventral pTH-C domain still expanded and caudal–dorsal pTH-C was shrunken. These results strongly indicate that the high Shh signal biases thalamic progenitor cells toward more rostral–ventral fates in a cell-intrinsic manner. This conclusion is consistent with our observation that the number of PH3+ cells in the entire *Olig3*-expressing domain was similar between *Olig3^{Cre/+}; R26^{SmoM2/+}* and control embryos (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Compatible with changes in progenitor identity, postmitotic thalamic nuclei are expanded or shrunken based on their putative locations of origin (summarized in Fig. 9B3,C3).

Because *Gli1* and *Ptc1* are not ectopically expressed in the mantle zone of *SmoM2*-expressing brains, the altered organization of the postmitotic thalamus is unlikely to be due to the direct activation of Shh signaling in postmitotic cells. Instead, we conclude that shifted identity of thalamic progenitor cells resulted in corresponding alteration of the identity of postmitotic thalamic nuclei.

Overexpression of the bHLH transcription factor *Olig2* partially mimics the effect of *SmoM2* expression in the thalamus

If Shh signal directly controls the identity of thalamic progenitor cells and indirectly influences the specification of postmitotic thalamic nuclei, it is likely that transcription factors that are regulated by Shh signal in progenitor cells translate their positional information onto their postmitotic progeny. *Olig2* is normally

expressed in the rostroventral portion of pTH-C, and is induced in progenitor cells that ectopically express *SmoM2*. These *SmoM2*-expressing embryos also showed induction of Sox2 and *RORα* caudally and dorsally throughout the thalamic mantle zone. Thus, it is possible that *Olig2* functions to specify rostroventrally located thalamic nuclei that express Sox2 and *RORα*. To test this possibility, we first performed genetic lineage tracing of *Olig2*-expressing progenitor cells (supplemental Fig. S4A–C, available at www.jneurosci.org as supplemental material). We crossed *Olig2^{Cre/+}* mice (Dessaud et al., 2007) with *ROSA26-stop-EGFP* reporter mice, and analyzed E14.5 compound heterozygous embryos. At E11.5, GFP was expressed in the rostroventral part of pTH-C, as well as pTH-R (supplemental Fig. S4A, available at www.jneurosci.org as supplemental material). Expression of GFP in pTH-R suggests that there is transient expression of *Olig2* in this progenitor domain before E10.5, which is similar to the observation in ventral spinal cord (Dessaud et al., 2007). At E14.5, EGFP was expressed in the rostroventral portion of the postmitotic thalamus, which largely overlapped with Sox2 expression (supplemental Fig. S4B,C, available at www.jneurosci.org as supplemental material). VP and the ventral part of medial geniculate (MGv) nuclei were two nuclei that had particularly strong EGFP expression. In contrast, EGFP was not found in more caudodorsal nuclei. Thus, *Olig2*-expressing thalamic progenitor cells give rise to rostroventral part of the postmitotic thalamus, consistent with the possibility that *Olig2* plays a role in the specification of rostroventral nuclei such as VP and MGv.

We then tested whether *Olig2* is necessary and/or sufficient for the specification of these nuclei. When we ectopically expressed *Olig2* by *in utero* electroporation at E10.5 in the caudodorsal diencephalon, Sox2 was induced in the mantle zone only on the transfected side at E13.5 and E14.5 (supplemental Fig. S4E,F, available at www.jneurosci.org as supplemental material), which partially mimicked the effect of expressing *SmoM2*. Cell proliferation and neuronal differentiation did not seem to be affected in *Olig2*-transfected cells, and overexpressing EGFP alone did not induce Sox2 expression (data not shown). These results suggest that *Olig2* mediates the Shh signal and confers at least part of the rostroventral identity to the postmitotic thalamus. In contrast, *Olig2* null mice did not show altered expression of Sox2 or progenitor markers such as *Mash1*, *Ngn2*, and *Dbx1* (data not shown). Thus, *Olig2* is not necessary but is sufficient to induce at least some molecular features of the rostroventral thalamic nuclei.

Attenuation of Shh signal in thalamic progenitor cells reduces the expression of rostroventral markers and increases caudodorsal markers

Previous studies on chick embryos showed that ectopic expression of *Ptc1Δloop2*, a dominant-negative form of the Shh receptor, *Ptc1*, reduced the expression of *Nkx2.2* and *Gbx2*, and increased the expression of *Pax6*, which is normally expressed highly in the caudal diencephalon (Kiecker and Lumsden, 2004). It was also reported that *Gli2/3* double knock-out mice showed reduced expression of *Gbx2*, whereas *Gli1* mutant mice showed reduced *Sox14* expression (Hashimoto-Torii et al., 2003). These studies demonstrated the requirement of Shh signaling in establishing the identity of the thalamus as a whole. However, it is still unknown whether Shh is required for the patterned expression of progenitor markers in pTH-R and pTH-C, or for the normal organization of postmitotic thalamic nuclei. Shh null mice show pronounced reduction in size of the diencephalon by E9.0, which reflects the early role of Shh in cell proliferation and survival

(Ishibashi and McMahon, 2002). Therefore, we analyzed conditional *Shh* knock-out mice by using the *Nes^{Cre}* allele to avoid such early effects. Conditional *Shh* mutant mice were smaller than control littermates, but they were postnatally viable and have relatively normal gross morphology of the brain (data not shown). The smaller brain size is reflected in part by a significant decrease in the number of PH3-positive progenitor cells in the thalamic ventricular zone of conditional *Shh* knock-out (*Nes^{Cre/+}; Shh^{c/c}*) embryos (3437 ± 146.0 , $n = 2$ for *Nes^{Cre/+}; Shh^{c/c}*, 6605 ± 472.0 , $n = 2$ for *Cre*– controls; $p = 0.024$) (supplemental Fig. S2C,D, available at www.jneurosci.org as supplemental material).

In *Nes^{Cre/+}; Shh^{c/c}* embryos, *Shh* expression in the forebrain was lost by E10.5 (data not shown; Machold et al., 2003). Consistent with the loss of *Shh* in the ZLI and the basal plate, *Ptc1* (data not shown) and *Gli1* mRNA was undetectable at E10.5 and E12.5 in *Nes^{Cre/+}; Shh^{c/c}* embryos (Fig. 6B,G and not shown). Furthermore, markers for the pTH-R domain were not induced. At E12.5, we observed a complete loss of *Nkx2.2* (Fig. 6C,H) and *Mash1* (Fig. 6D,I) within the thalamic ventricular zone, although their expression in the prethalamic progenitor domain remained at very low levels (Fig. 6H,I). Thus, *Nes^{Cre/+}* mice are useful in deleting the *Shh* gene in the brain when *Shh* signal is still critically required for the induction and/or maintenance of the expression of pTH-R markers. In addition to *Nkx2.2* and *Mash1*, expression of *Olig2*, a rostroventral pTH-C marker, was also lost in pTH-C of *Nes^{Cre/+}; Shh^{c/c}* embryos, although it was weakly detected in the prethalamus (Fig. 6C,H, arrowhead for the prethalamus). In contrast, markers that are expressed throughout the pTH-C domain, such as *Olig3* (Fig. 6A,F) and *Ngn2* (Fig. 6D,I) were still expressed in *Nes^{Cre/+}; Shh^{c/c}* embryos at E12.5 and earlier stages, although the size and intensity of the expression of *Olig3* seemed to be reduced. *Dbx1*, which is normally restricted to the caudodorsal pTH-C, was expanded rostroventrally in *Nes^{Cre/+}; Shh^{c/c}* thalamus (Fig. 6E,J). A pretectal marker, *Pax7*, was still expressed (data not shown). Together, these findings demonstrate that *Shh* activity is required for progenitor cell identity in pTH-R and rostroventral pTH-C and for suppressing the caudodorsal pTH-C identity (summarized in Fig. 9B1,B2,D1,D2).

Next, to more directly test the intrinsic requirement of *Shh* signaling in the thalamus, we conditionally knocked out the gene encoding *Smo*, a transmembrane effector of *Shh* signaling. Two *Cre* drivers, *Nes^{Cre/+}* and *Olig3^{Cre/+}*, were used. Both *Nes^{Cre/+}; Smo^{c/c}* and *Olig3^{Cre/+}; Smo^{c/c}* mice showed normal gross morphology of the brain and survived postnatally. We found that *Nes^{Cre/+}; Smo^{c/c}* embryos show lack of *Ptc1* expression in the thalamus at E12.5 (Fig. 7B,G) despite the normal expression of *Shh* in the ZLI (Fig. 7A,F). In these embryos, we also found that *Olig3* expression was reduced in the thalamic ventricular zone (Fig. 7A,F), and *Mash1* and *Olig2* were undetectable in pTH-R and pTH-C, respectively (Fig. 7C,D,H,I). Furthermore, *Dbx1* expression was expanded rostroventrally (Fig. 7E,J). However, un-

Figure 6. *Shh* is required for the normal organization of thalamic progenitor domains. Frontal sections of *Nes^{Cre/+}; Shh^{c/c}* forebrain (F–J) and *Nes^{Cre/+}; Shh^{+/+}* controls at E12.5 (A–E). Midline is to the left. *Shh* is expressed in the ZLI of control embryos (A, arrow) but not in the *Nes^{Cre/+}; Shh^{c/c}* mice (F, arrow). *Olig3* expression is maintained in *Nes^{Cre/+}; Shh^{c/c}* embryos, but the domain is smaller and density of *Olig3*-positive cells in this domain also seems reduced (F). *Gli1*, a target gene of *Shh* signaling, is lost in the thalamic progenitor cells of *Nes^{Cre/+}; Shh^{c/c}* embryos (G), while it is expressed in a graded manner in the control (B). Expression of *Nkx2.2* and *Olig2* is found in controls (C, arrow for *Nkx2.2*, double arrows for *Olig2*), but is completely lost in *Nes^{Cre/+}; Shh^{c/c}* embryos (H). A reduced number of *Olig2*-expressing cells are still found in the prethalamus (C, H, arrowhead). *Mash1* expression in the pTH-R domain is detected in the control but is lost in *Nes^{Cre/+}; Shh^{c/c}* embryos (D, I, arrowhead). *Mash1* expression in the posterior pretectum is maintained (D, I, arrow). Expression of *Ngn2* is intact and now occupies the entire thalamus, including the most rostroventral region (I, arrowhead). *Dbx1* expression is limited to the rostral pretectum and caudodorsal part of pTH-C in control embryos (E). The expression domain of *Dbx1* is expanded rostroventrally in *Nes^{Cre/+}; Shh^{c/c}* embryos (E, J, arrow). Scale bar, 200 μ m.

like in *Nes^{Cre/+}; Shh^{c/c}* embryos, *Nkx2.2* was still detectable though not as robustly as in the control (Fig. 7C,H).

Olig3^{Cre/+}; Smo^{c/c} embryos at E12.5 showed normal expression of *Shh* in the ZLI (Fig. 7K,P) and reduction of *Ptc1* expression in the thalamus but not in the prethalamus (Fig. 7L,Q), compatible with the restricted deletion of *Smo* and reduced *Shh* signal specific to the thalamus. The reduction of *Ptc1* expression in the thalamus was less significant than in *Nes^{Cre/+}; Smo^{c/c}* embryos, where *Ptc1* was undetectable in the thalamus and the prethalamus (Fig. 7G). Nevertheless, *Olig3^{Cre/+}; Smo^{c/c}* embryos showed reduction of *Mash1*-expressing cells in the thalamus (Fig. 7M,N,R,S). In addition, the decreased number of *Mash1*-expressing cells were intermingled with *Ngn2*-expressing cells, giving rise to the mixed zone (pTH-R/C) instead of pure pTH-R as we found in the control (Fig. 7N,S). Although *Nkx2.2* and *Olig2* were not consistently reduced in expression, *Dbx1* expression was grossly expanded rostroventrally (Fig. 7O,T), as in the *Nes^{Cre/+}; Shh^{c/c}* and *Nes^{Cre/+}; Smo^{c/c}* embryos. Together, these results indicate that the *Shh* signal is intrinsically required for the induction and/or maintenance of molecular characteristics of the pTH-R domain and for the suppression of caudodorsal part of the pTH-C domain.

The dramatic changes in the progenitor cell identity with conditional deletion of the *Shh* or *Smo* gene prompted us to examine which populations of postmitotic thalamus are affected in the absence of *Shh* or *Smo*. In E17.5 *Nes^{Cre/+}; Shh^{c/c}* embryos, as expected from the loss of pTH-R markers at E12.5 (Fig. 6C,D,H,I), *Nkx2.2* (Fig. 8A,C,F,H) and *Sox14* (data not shown) in vLG/IGL were both dramatically reduced, confirming the re-

Figure 7. *Smo* is required for the normal organization of thalamic progenitor domains. Frontal sections of *Nes^{Cre/+}; Smo^{c/c}* and *Nes^{Cre/+}; Smo^{c/c}* control embryos (**A–J**) and *Olig3^{Cre/+}; Smo^{c/c}* and *Olig3^{Cre/+}; Smo^{c/c}* control embryos (**K–T**) at E12.5. Midline is to the left. **A–J**, *Nes^{Cre/+}; Smo^{c/c}* embryos show normal expression of *Olig3* and *Shh* (**A, F**), but *Ptc1* (**B, G**, arrow) and *Olig2* (**C, H**, arrow) are lost in thalamic progenitor cells. *Nkx2.2* is still detectable in a reduced number (**C, H**, double arrows). *Mash1* expression in pTH-R is undetectable in *Nes^{Cre/+}; Smo^{c/c}* embryo (**D, I**, arrow), whereas it is still detectable in the prethalamus (**D, I**, double arrows). *Dbx1* expression is expanded in *Nes^{Cre/+}; Smo^{c/c}* embryos (**E, J**). Arrows in **E** and **J** indicate the boundaries of the expression domain of *Dbx1*. **K–T**, *Olig3^{Cre/+}; Smo^{c/c}* embryos show normal expression of *Olig3* and *Shh* (**K, P**). *Ptc1* expression is reduced in the thalamus (**L, Q**, arrow) but not in the prethalamus (**L, Q**, double arrows). *Mash1* expression in the thalamus is reduced in *Olig3^{Cre/+}; Smo^{c/c}* embryos (**M, R**, double arrow). In high-magnification views of **M** and **R**, *Ngn2*, a pTH-C marker, is now expressed more rostroventrally. *Ngn2*-expressing cells are intermingled with *Mash1*-expressing cells, forming a mixed zone of pTH-R/C at the expense of pTH-R (**N, S**, bracket). Expression of *Dbx1* is expanded in *Olig3^{Cre/+}; Smo^{c/c}* embryos, reaching near the rostroventral border of the thalamus (**O, T**, arrowhead). *Olig2* expression is still detected in the pTH-C domain of *Olig3^{Cre/+}; Smo^{c/c}* embryos (**O, T**). Scale bar, 100 μ m for **N, S**; 200 μ m for other panels.

requirement of Shh signaling for the vLG/IGL lineage. Similar changes were found in *Nes^{Cre/+}; Smo^{c/c}* embryos (Fig. 8K–N). We next analyzed the pTH-C derivative. Expression of *Sox2* (Fig. 8B,G) and *ROR α* (Fig. 8D,I) was profoundly reduced in *Nes^{Cre/+}; Shh^{c/c}* embryos. In contrast, *Gbx2* expanded and occupied almost the entire thalamic mantle zone (Fig. 8E,J). Again, similar changes were observed in *Nes^{Cre/+}; Smo^{c/c}* embryos (Fig. 8K,M and data not shown). In *Olig3^{Cre/+}; Smo^{c/c}* mice, IGL, as marked by *Sox1* and neuropeptide Y (NPY) expression, was dramatically reduced in size and cell number. *Sox1*-positive cells in this nucleus was reduced by $\sim 70\%$ (*Olig3^{Cre/+}; Smo^{c/c}*: 337.5 ± 13.80 cells/slide, *Olig3^{Cre/+}; Smo^{c/c}*: 103 ± 24.93 cells/slide, $n = 4$; $p = 0.0002$). This reduction likely reflects the reduction of *Mash1*-positive cells in the pTH-R at E12.5 (Fig. 7M,N,R,S).

In summary, decreasing Shh signaling resulted in reduction of

rostral–ventral progenitor pools, pTH-R, and the rostroventral part of the pTH-C domain, and expansion of caudodorsal progenitor pools (summarized in Fig. 9B1,B2,D1,D2). This change in thalamic progenitor cell identity corresponds to alterations in postmitotic populations (Fig. 9B3,D3). Although conditional deletion of *Shh* or *Smo* with two different Cre lines caused different degrees of reduction in Shh signal, we observed consistent changes in the progenitor cell populations as well as their postmitotic derivatives. These observations confirm the cell-intrinsic requirement of Shh signal in the specification of rostral–ventral progenitor cells in the thalamus.

Relationship between Shh and Fgf signaling pathways in thalamic patterning

A recent study by Kataoka and Shimogori (2008) reported that *Fgf8* is expressed in the dorsal midline of the caudal diencephalon and that this expression domain continues to a region immediately rostral to the dorsal tip of the ZLI, suggesting that Fgf signaling may have a role in thalamic patterning. Focal *in vivo* electroporation of *Fgf8* near the ZLI increased the size of pTH-R and its derivative, “Rim domain,” although overexpression of *Fgf8* did not change the expression pattern of *Shh* or *Ptc1* (Kataoka and Shimogori, 2008). These results suggest that Fgfs may play a patterning role independent of Shh signaling in the thalamus by regulating gene expression in pTH-R progenitor cells. Alternatively, *Fgf8* may be downstream of the Shh pathway and mediates part of the roles that Shh plays. In fact, Shh induces the expression of *Fgf4* in early limb development (Laufer et al., 1994), and complete *Shh* null embryos show reduced expression of *Fgf15* in mouse diencephalon at E8.5 (Ishibashi and McMahon, 2002). To assess the role of Shh in *Fgf8* expression and Fgf signal in the thalamus, we analyzed the expression of

Fgf8 and its downstream targets, *Spry1* and *Spry2* (Liu et al., 2003) in *Nes^{Cre/+}; R26^{SmoM2/+}* and *Nes^{Cre/+}; Shh^{c/c}* embryos (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). We found that in both control and *Nes^{Cre/+}; R26^{SmoM2/+}* embryos, *Fgf8* is expressed in the dorsal midline as well as in the region immediately rostral to the dorsal part of the ZLI (supplemental Fig. S4B,F, available at www.jneurosci.org as supplemental material). Expression of *Spry1* and *Spry2* was also similar in the control and *SmoM2*-expressing embryos (supplemental Fig. S4C,D,G,H, available at www.jneurosci.org as supplemental material). In addition, *Nes^{Cre/+}; Shh^{c/c}* embryos at E12.5 showed no significant reduction of expression of *Fgf8*, *Spry1*, or *Spry2* (supplemental Fig. S4J–L, available at www.jneurosci.org as supplemental material) near the ZLI. These results indicate that the changes to the pTH-R domain observed in

Nes^{Cre/+}; R26^{SmoM2/+} and *Nes^{Cre/+}; Shh^{c/c}* embryos are not due to alterations in *Fgf8* expression or *Fgf* signal.

Discussion

In this study, we used two lines of mice expressing Cre recombinase to conditionally increase or decrease Shh signaling in the thalamus, and performed *in vivo* electroporation to elevate the signal. When Shh signaling was globally increased using the *Nes^{Cre}* allele, the entire caudal alar diencephalon, but not the surrounding brain regions including the midbrain, took on the molecular properties of the thalamus, which is consistent with previous studies using chick embryos (Vieira et al., 2005). Our current study further demonstrates that the differential Shh signal plays a crucial role not only in the specification of the most rostroventral progenitor domain of the thalamus (pTH-R) and its postmitotic derivatives, but also in the patterning within the caudodorsal thalamic progenitor domain (pTH-C) and the formation of the cortex-projecting thalamic nuclei (summarized in Fig. 9).

A high Shh activity positively regulates the identity of the pTH-R domain and its postmitotic derivatives in the thalamus

Previous studies revealed that a high Shh activity positively regulates the expression of *Nkx2.2* and *Sox14* in chick and mouse thalamus (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005). We find that in the mouse, both *Nkx2.2* and *Sox14* are expressed in the lineage of the rostral progenitor domain, pTH-R. In our present study, ectopic expression of *SmoM2* resulted in increased Shh activity in thalamic progenitor cells as shown by the expression of the target genes *Gli1* and *Ptc1*, and expansion of pTH-R markers. Postmitotic populations derived from pTH-R progenitor cells also increased. Conversely, conditionally deleting *Shh* using the *Nes^{Cre}* allele completely abolished the expression of *Nkx2.2* and *Mash1* as well as the postmitotic markers of the pTH-R derivative. Deleting the *Smo* gene with either *Nes^{Cre}* or *Olig3^{Cre}* allele also reduced expression of pTH-R markers in progenitor cells, and resulted in corresponding decrease in its postmitotic derivative, further confirming the cell-autonomous requirement of Shh signaling in this cell lineage. Interestingly, even when *SmoM2* is ectopically expressed in the entire thalamic progenitor domains either with *Nes^{Cre}* or *Olig3^{Cre}* allele, only part of the thalamus became pTH-R; the caudodorsal part still expressed pTH-C markers (Fig. 3*G,H,R,S*). Because the Cre-mediated recombination seems to progress in a rostroventral to caudodorsal direction in these Cre mice, it is possible that the rostroventral part starts to express *SmoM2* earlier than caudodorsal part. If *SmoM2* can induce ectopic expression of pTH-R markers only before a certain stage of development, the differential onset of *SmoM2* expression across the

Figure 8. Shh signaling is required for the normal organization of postmitotic thalamus. **A–P**, Frontal sections of *Nes^{Cre/+}; Shh^{c/c}* and *Nes^{Cre/+}; Shh^{c/c}* control embryos at E17.5 (**A–J**), *Nes^{Cre/+}; Smo^{c/c}* and *Nes^{Cre/+}; Smo^{c/c}* control embryos at E17.5 (**K–N**), and *Olig3^{Cre/+}; Smo^{c/c}* and *Olig3^{Cre/+}; Smo^{c/c}* control embryos at E18.5 (**O, P**). Midline is to the left. **A–J**, Immunohistochemistry for *Nkx2.2* (**A, F**), *Sox2* (**B, G**), and *in situ* hybridization of *RORα* (**D, I**) and *Gbx2* (**E, J**). **C, H**, High-magnification images of **A/B** and **F/G**, respectively, showing the double immunofluorescence of *Nkx2.2* and *Sox2*. *Nkx2.2* is expressed in vLG/IGL in the control but is undetectable in *Nes^{Cre/+}; Shh^{c/c}* brains (**A, C, F, H**; see arrowhead in **C, H**). *Sox2* is expressed in the rostroventral part of the postmitotic thalamus, including VP and dLG in control embryos (**B**). In *Nes^{Cre/+}; Shh^{c/c}* thalamus, *Sox2* expression is severely reduced to a small, lateral region (**G**). Expression of *RORα* shows an even more dramatic reduction in *Nes^{Cre/+}; Shh^{c/c}* mice (**D, I**). *Gbx2* is expressed in many thalamic nuclei, but is excluded from the rostroventral nuclei such as VP and dLG in control embryos (**E**). In *Nes^{Cre/+}; Shh^{c/c}* brain, *Gbx2* is expanded, reaching the most rostroventral part of the thalamus in these mice (**J**, arrowheads). **K–N**, Immunohistochemistry for *Nkx2.2* and *Sox2* shows that *Sox2* expression is dramatically reduced in *Nes^{Cre/+}; Smo^{c/c}* embryos at E17.5. High-magnification images of the boxed areas in **K** and **M** clearly show the loss of *Nkx2.2* in the vLG/IGL region of *Nes^{Cre/+}; Smo^{c/c}* embryos (**L, N**). **O, P**, Immunohistochemistry for the two IGL markers, *Sox1* and *NPY*, shows that both markers are severely reduced in *Olig3^{Cre/+}; Smo^{c/c}* embryos at E18.5. Po, Posterior nucleus; LP, lateral posterior nucleus. Scale bar, 500 μ m for **C, H**; 500 μ m for other panels.

thalamic ventricular zone might explain the partial transformation of pTH-C into pTH-R. In addition, caudodorsally located thalamic progenitor cells may be less sensitive to enhanced Shh signaling than rostroventral cells. This may explain why *in vivo* electroporation of *SmoM2* at E11.5 caused the ectopic expression of pTH-R markers only in the most rostroventral part of pTH-C (Fig. 3*I, J*).

However, it is also possible that establishment of pTH-R identity requires not only high Shh signaling, but also activity of other signaling molecules that are expressed in or near the ZLI or the basal plate. An intriguing possibility is *Fgf8*, which was recently shown to play an independent role in specifying pTH-R and its derivatives (Kataoka and Shimogori, 2008). In this current study, we found that increasing Shh activity does not enhance the expression of *Fgf8* or its downstream targets, *Spry1* and *Spry2*, and that sustained expression of Shh is not required for the expression of *Fgf8*, *Spry1*, or *Spry2* in the diencephalon. Thus, if activation of both Shh and *Fgf8* pathways is required for the expression of pTH-R markers, the lack of increased *Fgf8* signaling in *SmoM2*-

Figure 9. Summary of the current study. **A**, Side view of the embryonic thalamus. The curved anterior–posterior (A–P) axis of the brain is shown in a red dotted line. The black vertical line indicates the orientation of frontal sections used throughout this study. In a given section, the top part is more dorsal and caudal, while the bottom part is more ventral and rostral. **B1, C1, D1**, Schematic frontal sections showing the progenitor domains at early embryonic stages in wild type (WT), *Nes^{Cre/+}; R26^{SmoM2/+}* (*SmoM2*), and *Nes^{Cre/+}; Shh^{fl/c}* (*Shh* *cko*) mice. Color gradients within pTH-C indicate the graded differential patterns of gene expression within this domain, which are altered when Shh signal is increased (**C1**) or decreased (**D1**). **B2, C2, D2**, Differential gene expression patterns in each genotype. **B3, C3, D3**, Schematic frontal sections indicating the postmitotic thalamus at late embryonic stages. The graded color of pTH-C-derived thalamus corresponds to the overall lineage relationship with the progenitor domains shown in **B1, C1**, and **D1**. Alterations in *SmoM2*-expressing mice and *Shh* *cko* mice indicate that the changes in the mantle zone are consistent with those observed in progenitor cells.

expressing thalamus may account for the limited expansion of pTH-R domain. However, since *Fgf8* is expressed only near the dorsal tip of the ZLI, not in the entire ZLI, it is unclear whether endogenous *Fgf8* signaling is required for all the progenitor cells in the pTH-R domain. Further studies are needed to identify the collaboration of signaling pathways responsible for the pTH-R identity.

How does Shh activity control the patterning of the pTH-C domain and the cortex-projecting thalamic nuclei?

Compared with the contribution of Shh signaling to the pTH-R lineage, understanding the role of Shh activity in the

caudodorsal progenitor domain, pTH-C, is more challenging. The pTH-C domain contains highly heterogeneous progenitor populations characterized by graded expression of transcription factors such as *Olig2* or *Dbx1*. In addition, the many dozens of distinct, cortex-projecting thalamic nuclei that are generated from this domain are not always identifiable by marker gene expression alone, and the use of cytoarchitectural features to discriminate individual nuclei is also limited. Because of these difficulties, how these nuclei are patterned and specified during embryogenesis is not well understood. We approached this question by first identifying and characterizing the molecular differences within pTH-C, which we found contributes to all the cortex-projecting thalamic nuclei. This analysis indicated that heterogeneity within this progenitor domain may contribute to the specification of multiple thalamic nuclei (Vue et al., 2007). Our current study now shows for the first time that the level of Shh signaling controls the identity of pTH-C progenitor cells, and that this in turn regulates the sizes and identity of postmitotic thalamic nuclei.

We showed that high Shh signaling positively regulates the expression of *Olig2* and *Pdlim3* in the rostral part of pTH-C. Conversely, conditional deletion of *Shh* or *Smo* with the *Nes^{Cre/+}* allele diminishes the expression of *Olig2* in pTH-C, while it expands the expression of *Dbx1*, a marker for caudodorsal pTH-C. In addition, increasing or decreasing Shh signaling altered regional patterns of gene expression in the postmitotic thalamus in a way that was consistent with the changes in progenitor cells. Among the postmitotic markers we analyzed, *RORα* and *Sox2* are expressed more strongly in the nuclei that are derived from the rostroventral part of the pTH-C domain. In *SmoM2*-expressing embryos, *Sox2* and *RORα* are expanded to occupy the entire rostrocaudal extent of the postmitotic thalamus, consistent with the expanded expression of *Olig2* and *Pdlim3*. *Shh* and *Smo* knock-out

brains show the opposite overall changes. These results clearly demonstrate that differential Shh activity controls not only the identity of pTH-C progenitor cells but also the spatial organization of the postmitotic thalamic nuclei. When *SmoM2* was conditionally expressed using the *Olig3^{Cre}* allele, the dLG nucleus, which is derived from the rostroventral part of pTH-C, was indeed expanded based on anterograde axon tracing from the retina. This confirms an intrinsic role of Shh signaling in the formation of a specific thalamic nucleus that projects to the cortex.

A recent study by Szabó et al. (2009) used *Foxb1-Cre* mice to broadly delete *Shh* in the developing CNS and found grossly abnormal thalamus and aberrant expression of postmitotic markers at E18.5. They also found that markers broadly expressed in thalamic progenitor cells such as *Olig3* and *Ngn2* were still expressed in the mutant mice. Based on these observations, they argued that Shh is mainly required in postmitotic cells in the thalamus to promote migration and aggregation into nuclei as well as axonal extension to the cortex rather than neuronal fate acquisition. In contrast, our current study strongly indicates that Shh acts primarily on thalamic progenitor cells. First, markers that are normally expressed in graded manners within the pTH-C domain such as *Olig2*, *Pdlim3*, and *Dbx1* were significantly affected when Shh activity was altered. The observed alteration of the postmitotic thalamus reflected the changes in the expression of these progenitor cell markers. Second, direct target genes of Shh signaling such as *Gli1* and *Ptc1* were expressed only in thalamic progenitors, even though *SmoM2* was mis-expressed in both progenitor and postmitotic cells after Cre-mediated recombination. *Gli1* and *Ptc1* expression was diminished in progenitor cells when the Shh signal was perturbed. Based on these observations, we conclude that Shh acts primarily on thalamic progenitor cells, and the graded Shh signal is essential to confer thalamic progenitors with position-dependent molecular heterogeneity, which forms a basis for generating a diverse set of postmitotic thalamic nuclei. Although the study by Szabó et al. (2009) and our current study used different Cre lines to delete the *Shh* gene, both observed the complete absence of the pTH-R marker *Nkx2.2*. Given the early and broad deletion of *Shh* in their conditional mutant mice, it is also likely that expression of graded pTH-C markers were altered as well. Thus, we think the different conclusions of the two studies have resulted from different interpretations of potentially similar data. Although we cannot completely rule out the possibility that Shh plays some role in postmitotic differentiation of thalamic neurons such as migration or axonal projection, demonstration of such roles would require different sets of experiments where Shh signal is altered in postmitotic cell-specific, conditional gene targeting in mice, or by *in vitro* assays that exclude the involvement of progenitor cells.

If Shh signaling regulates the expression of transcription factors in thalamic progenitor cells, then do these transcription factors play a functional role in specifying the formation of the various nuclei along the rostral–caudal and dorsal–ventral axes? We found by overexpression experiments that *Olig2* can induce rostroventral thalamic nucleus marker *Sox2* in the dorsal–caudal thalamus. However, *Olig2* mutant mice show normal expression of *Sox2*, indicating that other factors are also involved. Further studies are needed to identify such genes and to test the individual roles of each of the other transcription factors.

In conclusion, we believe that this study provides a unique perspective in neural development in several respects. First, we showed how Shh patterns a single developmental unit in the embryonic diencephalon (pTH-C) to generate multiple neuronal groups that are grossly arranged in similar rostrocaudal and dorsoventral locations to those of their precursor cells. This feature makes the developing thalamus a good model system to study how early patterning events contribute to the specification of multiple neuronal fates that share some basic properties such as the projection to the neocortex and use of glutamate as neurotransmitter. Second, connectivity and functions of thalamocortical projections are relatively well understood, especially for those

from the principal sensory thalamic nuclei. In this study, by using *Olig3^{Cre}* mice, we were able to generate postnatally viable mice in which Shh signaling was specifically enhanced or reduced in the thalamus without primarily affecting the development of the cortex or brain regions along the thalamocortical pathway. These mice will allow us to examine at the systems level the neuroanatomical and functional consequences of altering the early patterning of the thalamus.

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