Development/Plasticity/Repair

The Role of Sonic Hedgehog of Neural Origin in Thalamic Differentiation in the Mouse

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The specification of the intricate neuronal assemblies that characterize the forebrain is not well understood. The ventral spinal cord is specified through a concentration gradient of Sonic hedgehog (Shh) protein secreted by the notochord. *Shh* is expressed also in the forebrain neuroepithelium (neural Shh) and the underlying notochord and prechordal plate. Neural Shh is essential for the development of the prethalamus (ventral thalamus), but its effects on the thalamus (dorsal thalamus) are still unclear. We hypothesized that neural Shh would act on a previously regionalized dorsal diencephalic region to promote the emergence of specific thalamic nuclear and histological traits. To find out, we generated a conditional mouse mutant line specifically lacking Shh expression in the diencephalic neuroepithelium. We show that the transcription factor Gbx2, required for thalamic development downstream *Shh*, is expressed in our mutant in a restricted thalamic region and is necessary and sufficient for the differentiation of the medial and intralaminar thalamic nuclei. In the rest of the thalamus, neural *Shh* is required to promote neuronal aggregation into nuclei as well as axonal extension. In this way, the individual thalamic nuclei show differential dependence on *Shh*, *Gbx2*, or both for their differentiation. Additionally, Gbx2 is required for the survival of thalamic neurons.

Key words: apoptosis; conditional mutant; *Foxb1*; *Gbx2*; *Shh*; thalamus

Introduction

Brain regions differentiate stepwise, receiving first general regional specification during gastrulation and progressing down a genetic regulatory hierarchy. This cascade ends in the effector genes that direct formation of neuronal nuclei and confer mature functional attributes to neurons. Transient embryonic structures called organizers and their secreted products are key in this process. The best known model is the specification of ventral spinal cord neurons through Sonic hedgehog (Shh) protein secreted by the notochord (Dessaud et al., 2008). *Shh* is expressed in the forebrain neuroepithelium (neural *Shh*) and the underlying notochord and prechordal plate. If and how the mechanisms found in the ventral spinal cord have become modified to specify the convoluted three-dimensional neuronal assemblies found in the forebrain is not well understood. The thalamus (dorsal thalamus) is a forebrain region constituted mostly by neurons projecting to cortex and striatum, and, in rodents, it can be subdivided into several nuclear groups harboring altogether >30 nuclei and subnuclei (Swanson, 1992). These differ from each other specifically in position, morphology, marker expression, membrane properties, and cortical targets (Jones, 2007a). Thalamic subdivisions

originate as five undifferentiated cell masses or pronuclei that gradually resolve into specific nuclear groups: central pronucleus (anterior, ventral, and intralaminar groups), dorsal pronucleus (lateral and posterior groups), medial pronucleus (medial group), lateral geniculate pronucleus, and medial geniculate pronucleus (Rose, 1942; Jones, 2007b).

Neural Shh [from the zona limitans interthalamica (ZLI) and diencephalic tegmentum] is essential for prethalamus (ventral thalamus) specification (Kiecker and Lumsden, 2004; Vieira et al., 2005; Zeltser, 2005; Scholpp et al., 2006; Vieira and Martinez, 2006), but its role in (dorsal) thalamic development is still unclear. *Shh* controls thalamic expression of transcription factor gene *Gbx2* (Hashimoto-Torii et al., 2003), required for thalamic development (Miyashita-Lin et al., 1999; Hevner et al., 2002). We hypothesized that the role of neural Shh would be to take over the last steps of the thalamic specification cascade, which lead to the emergence of specific thalamic histogenesis and nucleogenesis, and that this would be done in part through activation of *Gbx2*.

To address this question, we analyzed thalamic differentiation in the *Gbx2* mutant and in a conditional mutant lacking diencephalic *Shh* expression. We found that, in the neural *Shh*-deficient diencephalon, *Gbx2* is expressed by default in a restricted area and is necessary and sufficient for the development of the medial and intralaminar thalamic nuclei. The rest of the thalamus requires neural *Shh* to promote proper migration and neuronal aggregation into nuclei as well as axonal extension. Therefore, the individual thalamic nuclei form three groups according to their dependence on neural *Shh*, *Gbx2*, or both for their differentiation. Additionally, we show that Gbx2 is essential for neuronal survival in the prenatal thalamus.

Materials and Methods

Mouse lines

Experiments with animals were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and under authorization of Az 32.22/Vo ("Ordnungsamt der Stadt Göttingen"). To obtain embryos and fetuses for the analysis, timed-pregnant females of the appropriate crossings were killed by cervical dislocation.

Foxb1–Cre *mouse line* (Foxb1 $^{tm1(cre)Gabo}$). Homologous recombination was used to replace the *Foxb1* coding sequence by the Cre recombinase cDNA, and a mouse line was created (kept in the C57BL/6 background) (Zhao et al., 2007). This line expresses Cre under the control of the regulatory sequences of *Foxb1*. All mutant mice used for our study were heterozygous for *Foxb1^{Cre}*, and therefore they were heterozygous for *Foxb1* and showed normal phenotype as expected (Alvarez-Bolado et al., 2000; Kloetzli et al., 2001). No homozygotes were used in this study.

Floxed Shh *mouse line* (Shh ^{tm2Amc}). This line was generated by Dr. MacMahon (Harvard University, Cambridge, MA) (Dassule et al., 2000) and obtained through The Jackson Laboratory. In these mice, exon 2 of the Shh locus [which encodes approximately half of the active N-terminal Shh signal that is essential for Shh function (Fan et al., 1995; Hynes et al., 1995; López-Martínez et al., 1995; Martí et al., 1995; Roelink et al., 1995)] is flanked with loxP sites. The same floxed line has been used for the study of the developing limb (Lewis et al., 2001).

Foxb1 $^{tm1(cre)Gabo}$ × Shh ^{tm2Amc} (Shh-c). After crossing the floxed *Shh* line with a Cre line, a mutant mouse line is generated (*Shh-c*) carrying a recombined (i.e., inactive) *Shh* locus in Cre-expressing cells. Because the caudal diencephalon (including the thalamus) belongs entirely to the *Foxb1* lineage, i.e., expresses Cre in the *Foxb1–Cre* line (Zhao et al., 2008), this line lacks functional Shh in the diencephalic neuroepithelium (see Results). When *Shh* expression is activated, the recombined *Shh* locus produces a truncated mRNA lacking exon 2. *In situ* hybridization (ISH) probes can be designed against the 3′ untranslated region (UTR), labeling all *Shh* mRNA forms, either full length (functional) or truncated (nonfunctional). Probes against exon 2, however, label only the full-length form.

Gbx2 *mutant mouse line*. In this line, provided by Drs. Gail Martin and Alex Joyner, all coding sequences of exon 2, including the homeobox, have been deleted (Wassarman et al., 1997). A portion of the 3′ UTR of *Gbx2* remains unaltered in the targeted *Gbx2* locus (Wassarman et al., 1997), and *in situ* hybridization probes against this sequence can label the expression of *Gbx2* mRNA (although lacking the homeodomain and therefore nonfunctional) in the *Gbx2* mutant (see Results).

Reporter lines. To map the embryonic brain region in which Cre-mediated recombination takes place, we crossed our mice with the *ROSA26R* reporter mouse line (Soriano, 1999). In *ROSA26R* animals, the reporter gene β-galactosidase is inserted in the constitutively active ROSA locus downstream a floxed stop codon. During Cre-mediated recombination, the stop codon is deleted and β-galactosidase is constitutively produced. This reporter is a lineage marker because, in mice carrying both the *Foxb1^{Cre}* and the *ROSA26R* alleles, cells expressing *Foxb1* and any cell derived from them will permanently express β-galactosidase. To label the axons of neurons of the *Foxb1* lineage, we crossed our *Foxb1^{Cre}* mice with the Z/AP (Lobe et al., 1999) reporter mouse line (C57BL/6) (see below, Axonal labeling by alkaline phosphatase activity). In Z/AP animals, Cre-mediated recombination activates constitutive expression of human placental alkaline phosphatase (hPLAP). This reporter is also a lineage marker.

Axonal labeling by alkaline phosphatase activity

In *Foxb1^{Cre}* Z/AP animals, *Foxb1* lineage neurons will express hPLAP. Because hPLAP attaches to axonal membranes, it is a marker of axons of lineage-labeled neurons (Fields-Berry et al., 1992; Gustincich et al., 1997; Leighton et al., 2001).

Alkaline phosphatase activity detection. This protocol has been described previously (Lobe et al., 1999). Briefly, brains of the appropriate genotypes were collected, fixed (4% paraformaldehyde) on ice for 60 min, agarose embedded, cut into 150-μm-thick sections, fixed again (4% paraformaldehyde/0.2% glutaraldehyde) on ice for 60 min, incubated for 30 min at 72°C to inhibit endogenous phosphatase, rinsed in alkaline phosphatase buffer (in mM: 100 Tris-Cl, pH 9.5, 100 NaCl, and 10

MgCl$_2$), incubated with staining solution (250 μl of nitroblue-tetrazolium-chloride plus 187.5 μl of 5-bromo-4-chlor-indolyl-phosphate per 50 ml of alkaline phosphatase buffer) overnight (4°C), and fixed (4% paraformaldehyde) for 60 min (4°C).

In situ *hybridization in whole mount*

ISH was performed as described previously (Wilkinson, 1992). The probes were cloned by PCR (primers available on request). Briefly, the embryos were fixed in paraformaldehyde 4% in PBS overnight (4°C), washed in PBS plus 0.1% Tween 20 (PBT), and stored at −20°C in methanol. For ISH, the embryos were rehydrated, bleached (6% H$_2$O$_2$), digested [10 μg/ml proteinase K in PBT at room temperature (RT)], washed (2 mg/ml glycine/PBT), postfixed (4% paraformaldehyde/0.2% glutaraldehyde/PBT), prehybridized for 1–2 h (70°C), and hybridized overnight (70°C). They were then washed (50% formamide, 5× SSC, pH 4.5, and 1% SDS at 70°C), rinsed [100 mM maleic acid, 150 mM NaCl, 2 mM levamisole, and 0.1% Tween 20 (MAB)], and incubated in 10% sheep serum in MAB/2% blocking reagent (Roche Diagnostics) for 2–3 h (RT) and then in anti-digoxigenin alkaline phosphatase antibody (Roche Diagnostics) overnight (4°C). The embryos were rinsed and then left in MAB overnight (4°C). The embryos were then incubated in BM-Purple (Roche Diagnostics) with levamisole (RT) and, after color developed, washed in PBT, pH 4.5, fixed in 4% formaldehyde/0.1% glutaraldehyde overnight (4°C), and transferred into 80% glycerol/PBT.

ISH on sections

Nonradioactive ISH was performed on cryosections (15 μm thick) that were fixed in 4% paraformaldehyde and acetylated after sectioning. Prehybridization, hybridization, and washing steps were performed with the help of an automatic liquid-handling unit (Genesis RSP 200; Tecan), and the digoxigenin-labeled probe was detected by a dual-amplification procedure (Yaylaoglu et al., 2005; Visel et al., 2006).

Bromodeoxyuridine labeling

Pregnant mice from appropriate crossings were intraperitoneally injected with bromodeoxyuridine (BrdU) (RPN201; GE Healthcare) (50 μg/g body weight). For short survival data, we injected at approximately embryonic day 12.75 (E12.75) (at 6:00 P.M. on day 12 postcoitum) and collected the embryos either 1.5 or 3 h later. According to thalamic birthdating data (Altman and Bayer, 1988; Clancy et al., 2001, 2007), at this stage, neurons are being produced in all areas of the thalamic neuroepithelium. For long-term survival, we injected BrdU on day E12.5 at 12:00 P.M., 3:00 P.M., and 6:00 P.M. (Takahashi et al., 1993) and collected the fetuses at E18.5.

We detected cell proliferation on cryosections (10 μm) by means of anti-BrdU antibody M0744 (1:100) (Dako), after epitope retrieval in 2 M HCl for 30 min at 37°C. We used the nuclear marker 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen) as a counterstain. In the short-survival animals (E12.75), we examined the sections under confocal microscopy and counted BrdU-labeled and unlabeled cells in 100-μm-wide bins encompassing the width of the neuroepithelium (apical to basal side) at three rostrocaudal levels and calculated the labeling index (BrdU-labeled cells as percentage of total cells) (Takahashi et al., 1993; Warren et al., 1999; Ishibashi and McMahon, 2002).

Apoptosis detection

We selected cryostat sections of E12.5 and E18.5 brains at three thalamic rostrocaudal levels in three individuals per genotype (wild type, *Shh-c*, $Gbx2^{-/-}$). We pretreated the sections with 4% paraformaldehyde (20 min) and proteinase K (1.5 μg/ml, 5 min) at room temperature and then labeled the apoptotic cells with the ApopTag TUNEL (terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling) kit (Millipore Bioscience Research Reagents) according to the instructions of the manufacturer. We used DAPI as counterstain and counted absolute number of apoptotic cells per histological section of the thalamus (both sides) under high magnification in three individuals per genotype.

Statistical analysis

ANOVA of the cell counting results (BrdU and apoptosis) was performed with Prism software (GraphPad Software).
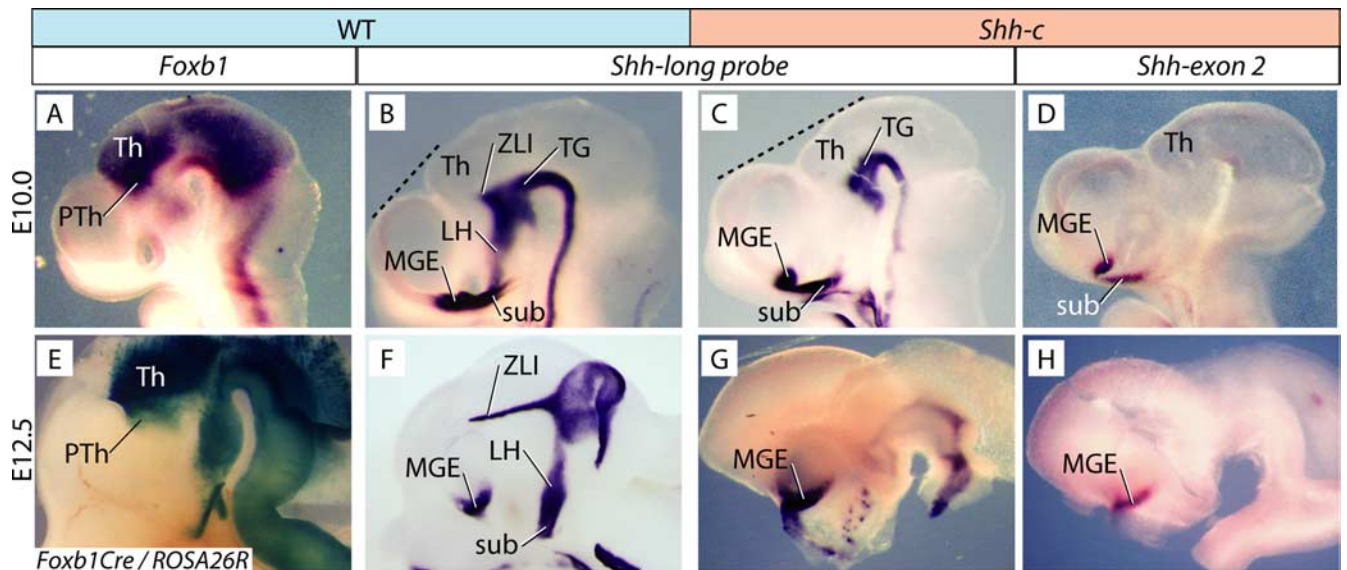
Figure 1. Abolition of functional *Shh* expression in the *Shh-c* caudal diencephalon. ***A–D, F–H,*** Whole-mount *in situ* hybridization for the genes and genotypes indicated. ***E,*** *Foxb1* lineage mapping by β-galactosidase detection in *Foxb1^{Cre}/ROSA26R* heterozygotes. ***A, B,*** At E10.0, *Foxb1* expression (***A***) overlaps with the incipient ZLI as labeled by *Shh* (***B***). ***C, D,*** In the *Shh-c* mutant, *Shh* transcription is not active in the ZLI but in the diencephalic tegmentum (***C***). An exon 2 probe shows that all diencephalic *Shh* mRNA at this age is nonfunctional (***D***). The dotted lines in ***B*** and ***C*** show that the mutant thalamus is smaller. ***E, F,*** At E12.5, *Foxb1* lineage labeling is present in most of the thalamic region (***E***), overlapping thalamus and ZLI as labeled by *Shh* (***F***). The *Shh-c* mutant shows almost no *Shh* transcriptional activation in the caudal diencephalon (***G***) and no functional *Shh* at all (***H***). For the abbreviations used in the figures, see Table 1.

Table 1. Abbreviations used for thalamic and other structures

Pronucleus	Abbreviation	Name
Central	ATN	Anterior thalamic nuclei
	VEN	Ventral group
	VM	Ventromedial
	VMb	Ventromedial basal
	VP	Ventral posterior
Medial	PV	Paraventricular
	PT	Paratenial
	MV	Medioventral
	MD	Mediodorsal
	CM	Centromedial
	CL	Centrolateral
Dorsal	LD	Laterodorsal
	LP	Lateral posterior
	Po	Posterior
Lateral geniculate	LGd	Lateral geniculate dorsal
Medial geniculate	MG	Medial geniculate
Other structures	Ctx	Cortex
	DG	Dentate gyrus (hippocampus)
	Em	External medullary lamina
	H	Habenula (epithalamus)
	Ic	Internal capsule
	LH	Lateral hypothalamus band
	MB	Midbrain
	MGE	Medial ganglionic eminence
	PTh	Prethalamus (ventral thalamus)
	Rf	Retroflex fascicle
	RT	Reticular nucleus
	sub	Suboptical *Shh* domain
	Te	Thalamic eminence
	TG	Diencephalic tegmentum
	Th	Thalamus (dorsal thalamus)
	ZLI	Zona limitans interthalamica

Table 2. Pronuclear markers in the wild-type thalamus

Pronuclei	Nuclei	*Calb2*	*Gbx2*	*Lhx2*	*Neurog2*	*Cdh6*
Central	ATN				+	+
	VP				+	+
	VM			+	+	+
	VMb	+	+	+		+
Medial	PV, PT, MV	+	+	+		
	MD		+			
	CM	+	+	+		+
	CL	+	+	+		
Dorsal	LD	+		+	+	
	LP	+	+	+		+
	Po					+
Lateral geniculate	LGd				+	+
Medial geniculate	MG	+	+	+		+

+ indicates expression in a recognizable structure (listed in the left column). Intensity of expression has not been recorded.

Results

Abolition of functional *Shh* expression and of *Shh* signaling in the caudal diencephalon of *Shh-c* mutants

At E10.0, *Foxb1* is expressed in the prethalamus and thalamus (Fig. 1*A*), overlapping the domain of the incipient ZLI (Fig. 1*B*) (Zhao et al., 2008). In *Shh-c* mutants at this age, *Shh* expression has been abolished in the ZLI as well as in domains of the rostral diencephalon (Fig. 1*C*). As expected (Ishibashi and McMahon, 2002), the caudal diencephalon is smaller in the mutant (Fig. 1*B,C*, dotted line). An *in situ* probe specifically labeling full-length *Shh* mRNA (*Shh*–exon 2 probe; see Materials and Methods) failed to label the mutant diencephalon (Fig. 1*D*), demonstrating that the *Shh-c* neuroepithelium does not produce functional *Shh* mRNA. The telencephalic *Shh* expression domains were intact (Fig. 1*D*, MGE and sub), because they do not overlap with *Foxb1* expression (Fig. 1*A*). At E12.5, all major regions of the brain can be recognized by specific marker expression (Shimamura et al., 1995). At this age, *Foxb1* drives Cre recombination activity in the caudal diencephalon, dorsal and ventral (as reflected by lineage labeling in *ROSA26R* crossings; Fig. 1*E*) (Zhao et al., 2008). In wild-type embryos, *Shh* showed a very pronounced and characteristic expression pattern in the caudal diencephalon (Fig. 1*F*, ZLI and TG) as well as the rostral diencephalon (hypothalamus) (Fig. 1*F*, LH). In the *Shh-c* mu-
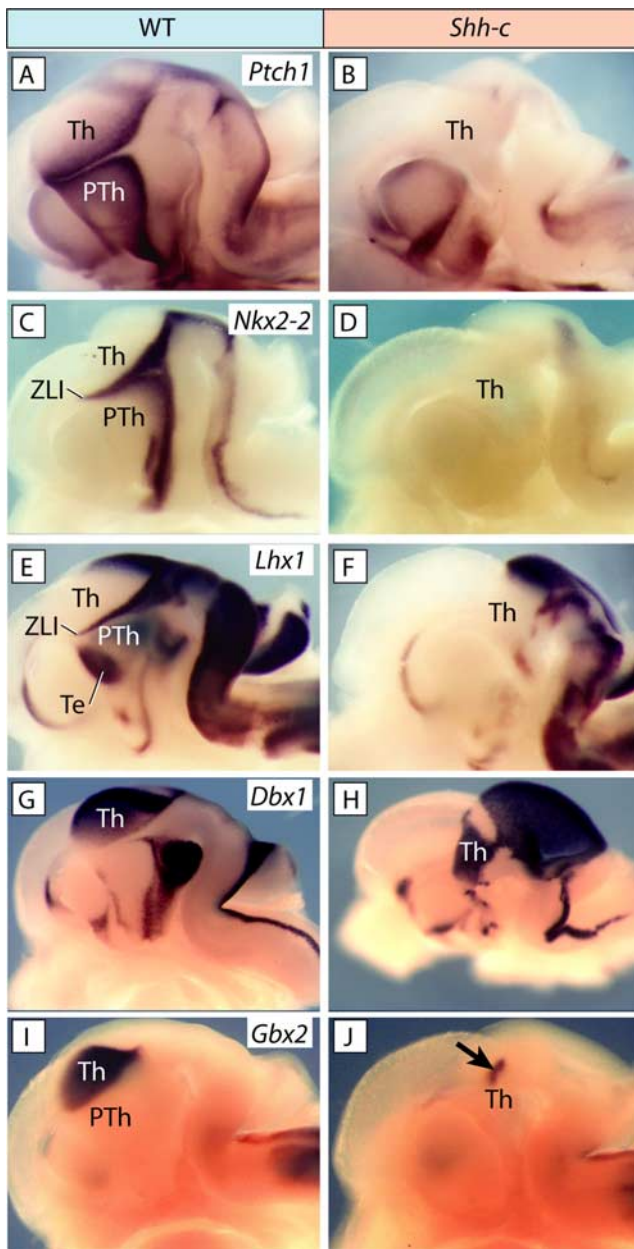
Figure 2. Thalamic regionalization in *Shh-c* mutants. Whole-mount *in situ* hybridization of hemisected E12.5 mouse brains, probes, and genotypes as indicated. *A*, *B*, Lack of *Ptch1* expression indicates that the *Shh* signaling pathway is abolished in the *Shh-c* mutant diencephalon. *C–F*, The ZLI has disappeared in the *Shh-c* mutant, as determined by expression of *Nkx2-2* and *Lhx1*. *G*, *H*, Thalamic expression of *Dbx1* is preserved in the mutant. *I*, *J*, *Gbx2* is expressed in a small restricted thalamic domain at E12.5 in the *Shh-c* mutant (arrow in *J*).

Table 3. Markers and nuclei in the *Shh-c* thalamus

Pronuclei	Nuclei	Calb2	Gbx2	Lhx2	Neurog2	Cdh6
Central	ATN				−	?
	VP				?	?
	VM			?	?	?
	VMb	?	+	?		?
Medial	PV, PT, MV	+	+	+		
	MD		+			
	CM		+	+		?
	CL		+	+		
Dorsal	LD	?		−	−	
	LP	+	+	−		?
	Po					?
Lateral geniculate	LGd				?	?
Medial geniculate	MG	+	+	−		?

? indicates "normal" marker expression in a morphologically altered part of the thalamus identified by its position along the anteroposterior and dorsoventral axes and comparison with wild type. − indicates abnormal lack of expression. + indicates normal expression in a readily recognizable structure.

sion of transcription factor gene *Lhx1*, a marker of the zona limitans, prethalamus, and thalamic eminence (Bachy et al., 2001), was also absent from the *Shh-c* ZLI (Fig. 2 *E,F*). To evaluate the thalamus of the *Shh-c* mutant, we used expression of specific markers *Dbx1* (Shoji et al., 1996; Ishibashi and McMahon, 2002) and *Gbx2* (Miyashita-Lin et al., 1999; Hevner et al., 2002). In the *Shh-c* thalamus, *Dbx1* was still expressed (Fig. 2 *G,H*), indicating that initial regional specification of the thalamus has taken place in the mutant. *Gbx2* was reduced to a very small domain (Fig. 2 *I,J*).

Specific markers in the wild-type thalamus

To explore thalamic differentiation, we chose five marker genes whose thalamic expression has been established: *Calb2–Calretinin* (Arai et al., 1991, 1994; Winsky et al., 1992; Frassoni et al., 1998), *Gbx2* (Jones and Rubenstein, 2004), *Lhx2* (Nakagawa and O'Leary, 2001), *Neurog2* (Nakagawa and O'Leary, 2001), and *Cdh6* (Jones and Rubenstein, 2004). Because the *Shh-c* mutants survive up to E18.5, we performed our analysis of the wild type also at this age. We first determined marker expression on transverse sections of E18.5 wild-type thalamus, at four rostrocaudal thalamic levels (Fig. 3, Tables 1, 2).

Central pronucleus (anterior and ventral nuclear groups)
The anterior group was labeled by *Lhx2* (weakly), *Neurog2*, and *Cdh6* (Fig. 3*C–E,H–J*, ATN). The ventral group was labeled by *Lhx2*, *Neurog2*, and *Cdh6* (Fig. 3*M–O*, VP and VM). The "tail" of the central pronucleus, represented by the ventromedial basal, was labeled by *Calb2*, *Gbx2*, *Lhx2*, and *Cdh6* (Fig. 3*P–R,T*, VMb).

Medial pronucleus (medial and intralaminar nuclear groups)
We include the intralaminar nuclei in the medial pronucleus (not in the central, as did Rose, 1942) because of marker and phenotype similarity (see below and Discussion). All derivatives of this pronucleus expressed *Gbx2* (Fig. 3*B,G*, L, CL, CM, MD, MV, PT, PV). Additionally, they (except the mediodorsal nucleus) expressed *Calb2* and *Lhx2* (Fig. 3*A–C, F–H, K–M*).

Dorsal pronucleus (lateral and posterior nuclear groups)
The laterodorsal nucleus expressed *Calb2*, *Lhx2*, and *Neurog2* (Fig. 3*F,H,I*, LD). The lateral posterior nucleus was labeled by *Calb2*, *Gbx2*, and *Cdh6* (Fig. 3*K,L,O*, LP). The posterior nucleus expressed *Cdh6* (Fig. 3*O*, Po).

tant, expression of *Shh* was absent from the diencephalon (Fig. 1 *G*), and this was confirmed by an exon 2 probe (Fig. 1 *H*). Expression of Shh receptor *Ptch1* is diagnostic of a functioning *Shh* signaling pathway (for review, see Lewis et al., 2001). *Ptch1* labeled the wild-type thalamus at this age (Fig. 2 *A*) but was absent in the *Shh-c* mutant caudal diencephalon (Fig. 2 *B*), indicating *Shh* pathway abolition in this region.

Abolition of the ZLI in the *Shh-c* mutant mouse

Other ZLI markers confirmed lack of this domain in the mutant. Expression of ZLI marker gene *Nkx2-2*, which is downstream *Shh* (Kitamura et al., 1997; Kiecker and Lumsden, 2004), was absent from the *Shh-c* mutant (Fig. 2*C,D*). Expres-
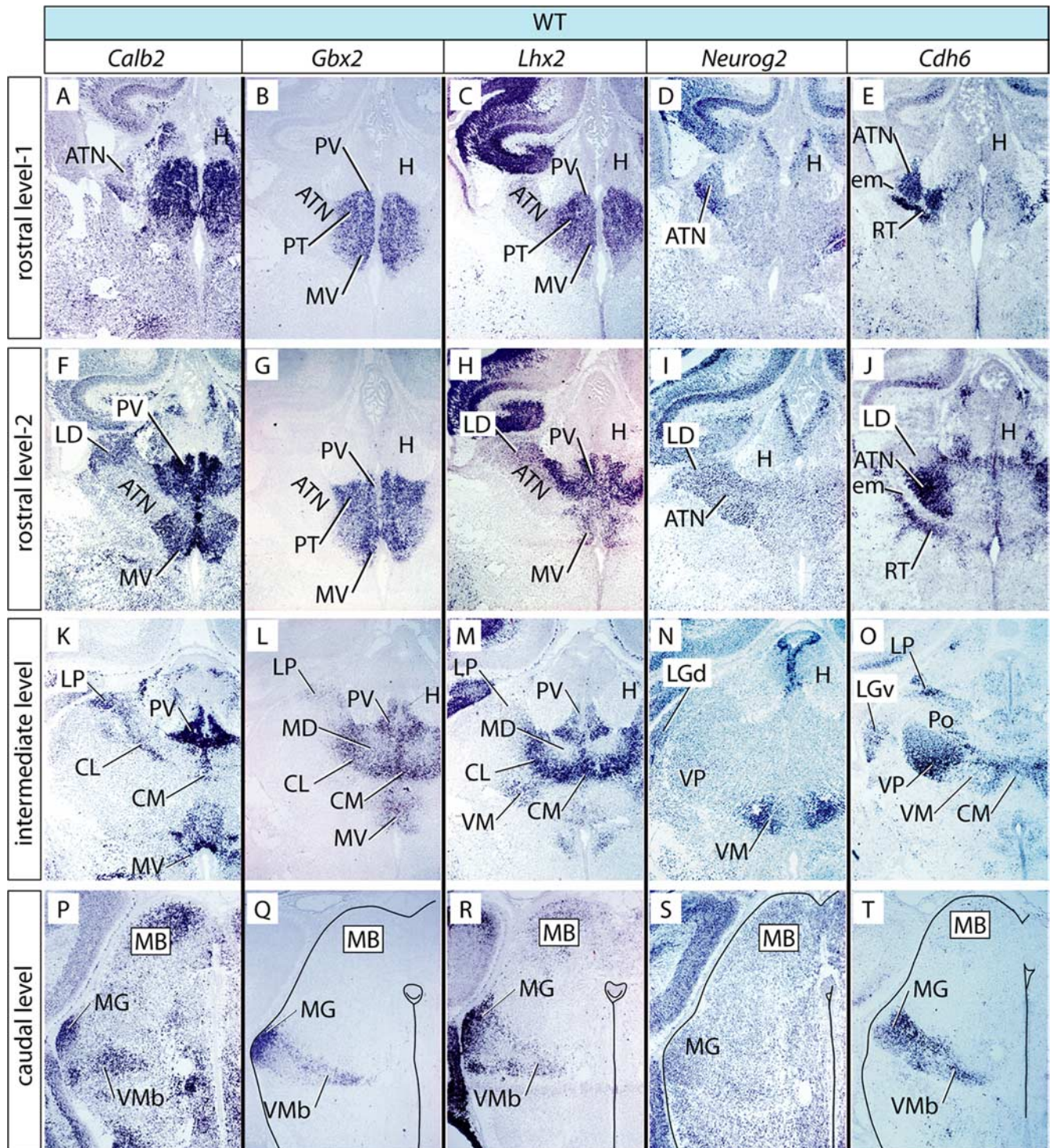
Figure 3. Marker expression in the E18.5 wild-type thalamus. *A–T*, *In situ* hybridization on E18.5 mouse brain sections for five marker genes as indicated on top of each column. Rows show sections at given rostrocaudal levels, as indicated on the left side, labeled for each of the five markers. Columns correspond to four rostrocaudal levels labeled with the same probe. Some structures have been outlined for clarity. For details, see Results.

Lateral geniculate pronucleus
The lateral geniculate pronucleus was labeled by *Neurog2* and *Cdh6* (Fig. 3 N,O, LG).

Medial geniculate pronucleus
The medial geniculate pronucleus was labeled by *Calb2*, *Gbx2*, *Lhx2*, and *Cdh6* (Fig. 3P–R,T, MG).

General appearance of the *Shh-c* thalamus
In the *Shh-c* brain, the prethalamus is absent so that the thalamus "hangs" over the third ventricle, particularly on rostral sections (Fig. 4A–J). At caudal levels, the *Shh-c* thalamus was embedded into the midbrain (Fig. 4K–T). By counting the number of histological sections, we estimated that the *Shh-c* thalamus was smaller than the wild type, on the rostrocaudal
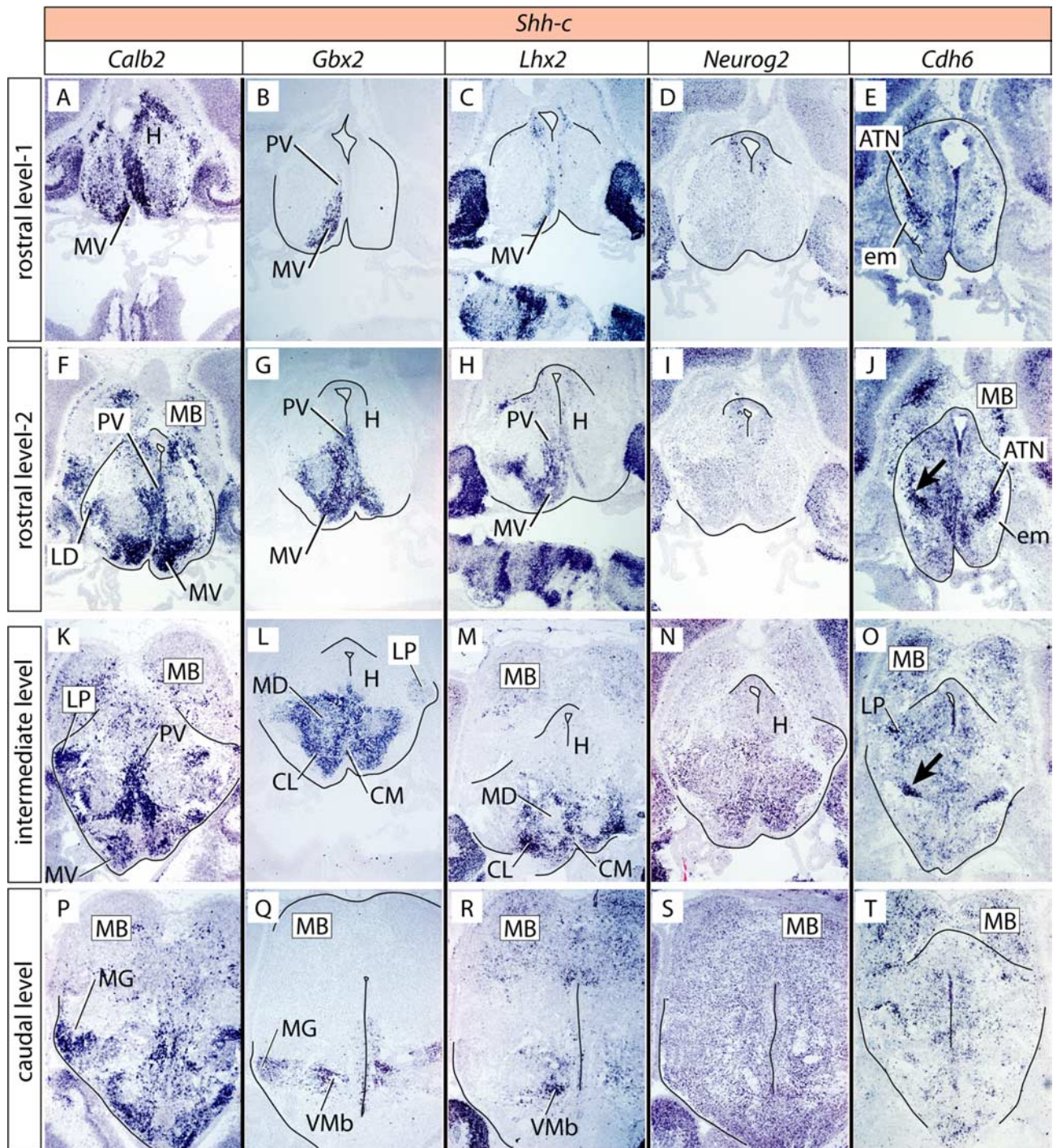
Figure 4. Marker expression in the E18.5 *Shh-c* thalamus. *A–T*, *In situ* hybridization on E18.5 mouse brain sections for five marker genes as indicated on top of each column. Rows show sections at a given rostrocaudal level, as indicated on the left side, labeled for each of the five markers. Columns correspond to four rostrocaudal levels labeled with the same probe. The arrows in *J* and *O* mark continuous rostrocaudal expression of *Cdh6* in the central pronucleus. Some structures have been outlined for clarity. For details, see Results.

axis, by ∼50%, as could be expected (Ishibashi and McMahon, 2002).

Despite gross morphological alteration, the general position of the nuclear groups in the rostrocaudal and dorsoventral axes was correct. Comparison of Tables 2 and 3 shows that, in the *Shh-c* thalamus, the medial pronucleus was well preserved, whereas the rest of them, particularly the central and dorsal pronuclei, were morphologically very altered but mostly preserved specific marker expression (only *Lhx2* and *Neurog2* were abolished in some nuclei).

The medial pronucleus is preserved in the *Shh-c* thalamus
Central pronucleus (anterior and ventral nuclear groups)
The mutant anterior thalamic nuclei could be recognized by their preserved *Cdh6* expression and their relation to the external medullary lamina. The anterior thalamic nuclei lost, however, *Lhx2* and *Neurog2* expression (Fig. 4C,D). The ventral group lost the expression of *Neurog2* (Fig. 4I) and preserved only the most reduced expression of *Cdh6* (Fig. 4J,O). The entire central pronucleus (anterior plus ventral groups) seemed reduced to a rostro-
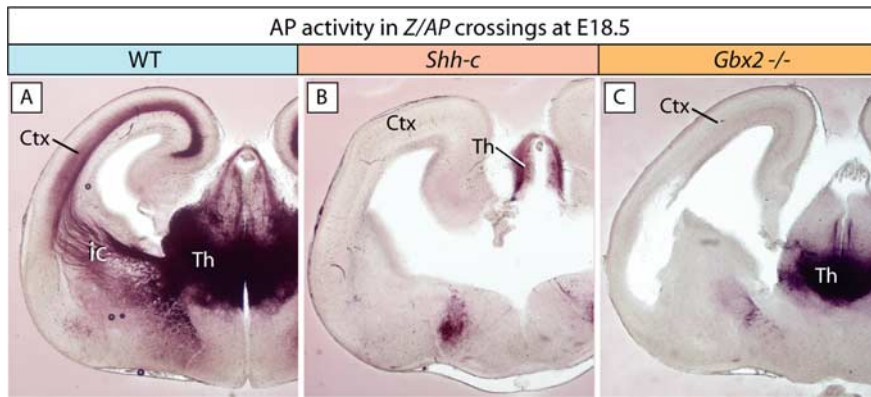
Figure 5. No thalamic axons reach the cortex in the *Shh-c* brain. Detection of alkaline phosphatase activity in transverse sections of wild-type (**A**), *Shh-c* (**B**), and *Gbx2*$^{-/-}$ (**C**) brains at E18.5. The wild-type cortex (**A**) shows abundant labeled thalamic axons in the cortex, whereas the *Shh-c* (**B**) and the *Gbx2*$^{-/-}$ (**C**) cortices are completely unlabeled.

Table 4. Markers and nuclei in the *Gbx2*$^{-/-}$ thalamus

Pronuclei	Nuclei	Calb2	Gbx2*	Lhx2	Neurog2	Cdh6
Central	ATN	!			−	?
	VEN			?	?	?
	VMb	+	+	−		+
Medial	PV, PT, MV	−	?	?		
	MD		?			
	CM		?	?		?
	CL		?	?		
Dorsal	LD	−		−		−
	LP	−	−	−		+
	Po					+
Lateral geniculate	LGd				+	+
Medial geniculate	MG	+	+	+		+

? indicates "normal" marker expression in a morphologically altered part of the thalamus identified by its position along the anteroposterior and dorsoventral axes and comparison with wild type. − indicates abnormal lack of expression. + indicates normal expression in a readily recognizable structure. ! indicates ectopic expression. * indicates ISH detection of the truncated, nonfunctional *Gbx2* mRNA in the *Gbx2* mutant.

caudal row of *Cdh6*-expressing cells (Fig. 4*J*,*O*, arrows). The tail of the central pronucleus (the ventromedial basal) was an exception, because it lost *Calb2* and *Cdh6* but still expressed *Gbx2* and *Lhx2* in the mutant (Fig. 4*Q*,*R*).

Medial pronucleus (medial and intralaminar nuclear groups)
This pronucleus was well preserved in morphology and marker expression, including *Calb2* (Fig. 4*A*,*F*,*K*), *Lhx2* (Fig. 4*C*,*H*,*M*), and *Gbx2* (Fig. 4*B*,*G*,*L*). Expression of *Gbx2* labeled the entire pronucleus and is an argument toward including the intralaminar nuclei in this medial pronucleus (not the central) (Rose, 1942).

Dorsal pronucleus (lateral and posterior nuclear groups)
The laterodorsal nucleus, expressing *Calb2*, was present in the mutant (Fig. 4*F*) but lacked expression of markers *Lhx2* and *Neurog2* (Fig. 4*H*,*I*). The lateral posterior nucleus preserved *Calb2*, *Gbx2*, and *Cdh6* expression (Fig. 4*K*,*L*,*O*) but lost *Lhx2* (Fig. 4*M*). The posterior nucleus appeared lost, to judge by *Cdh6* expression (Fig. 4*J*).

Lateral geniculate pronucleus
We could not identify a lateral geniculate nucleus in the *Shh-c* thalamus.

Medial geniculate pronucleus
The medial geniculate pronucleus preserved expression of *Calb2* and *Gbx2* (Fig. 4*P*,*Q*) but lost *Lhx2* and *Cdh6* in the mutant (Fig. 4*R*,*T*).

In summary, the medial pronucleus (in which we include the intralaminar group) was comparatively little affected by lack of neural Shh, whereas the other four pronuclei showed major alteration.

No thalamocortical axons in the *Shh-c* mutant
Extension of axons projecting to the cortex is an important differentiation trait of thalamic neurons (for review, see López-Bendito and Molnár, 2003). To explore this capability in the *Shh-c* mutant thalamus, we crossed our *Shh-c* line with the reporter mouse line Z/AP (Lobe et al., 1999), which results in labeling of the axons of *Foxb1* lineage cells (see Materials and Methods). Because the entire thalamus belongs to the *Foxb1* lineage (Fig. 1*E*) (Zhao et al., 2008), *Foxb1–Cre* Z/AP mice showed staining of thalamocortical axons as expected (Fig. 5*A*). The *Shh-c* mutant, however, showed a completely blank cortex (Fig. 5*B*). The second mutant used in this study, the *Gbx2*-deficient mouse (see below), also lacked thalamocortical axons (Fig. 5*C*) as reported previously (Miyashita-Lin et al., 1999; Hevner et al., 2002).

No regionalization defect in the *Gbx2* mutant
Gbx2 is a transcription factor specifically expressed in the entire mouse thalamic primordium starting at E11.5 (Bulfone et al., 1993a,b; Hashimoto-Torii et al., 2003) and is essential for thalamic development (Miyashita-Lin et al., 1999; Hevner et al., 2002). Because thalamic expression of *Gbx2* is under the control of *Shh* (Hashimoto-Torii et al., 2003), we were surprised to find a small domain of *Gbx2* expression in the *Shh-c* thalamus at E12.5 (Fig. 2*J*, arrow), as well as essentially normal thalamic expression at E18.5 (Fig. 4*B*,*G*,*L*,*Q*). We asked whether this late, default expression of *Gbx2* in the absence of neural Shh was perhaps "rescuing" some traits of the wild-type phenotype in the *Shh-c* mutant, making its phenotype milder. Therefore, we wanted to compare the *Gbx2*$^{-/-}$ thalamus with the *Shh-c* thalamus. We used a *Gbx2* mutant in which most of exon 2, containing the homeobox-coding sequence, has been deleted (Wassarman et al., 1997) (see Materials and Methods). First, we detected regional marker genes at E12.5 to assess regionalization in this mutant. Probes against *Ptch1*, *Gli1*, *Nkx2-2*, *Lhx1*, *Emx2*, *Pax6*, *Dlx2*, *Dbx1*, and *Irx3* detected no changes in the regionalization of the caudal diencephalon, except for the smaller size of the thalamus (data not shown). We then proceeded to analyze thalamic differentiation in the *Gbx2* mutant at E18.5.

Abolition of the medial pronucleus in the *Gbx2* mutant
As was the case for the *Shh-c* mutant, the *Gbx2*-deficient thalamus was smaller than normal but showed correct dorsoventral and anteroposterior axes, as judged from the presence of a morphologically recognizable habenula (epithalamus) dorsally and the medial geniculate nucleus caudally (see below) (Fig. 6, Table 4). Comparison of Tables 2–4 shows that, in this mutant, the medial pronucleus is specifically altered in morphology, although it maintains marker expression. The central and dorsal pronuclei were very affected (like in the *Shh-c*), but the lateral and medial geniculate pronuclei seemed relatively intact.
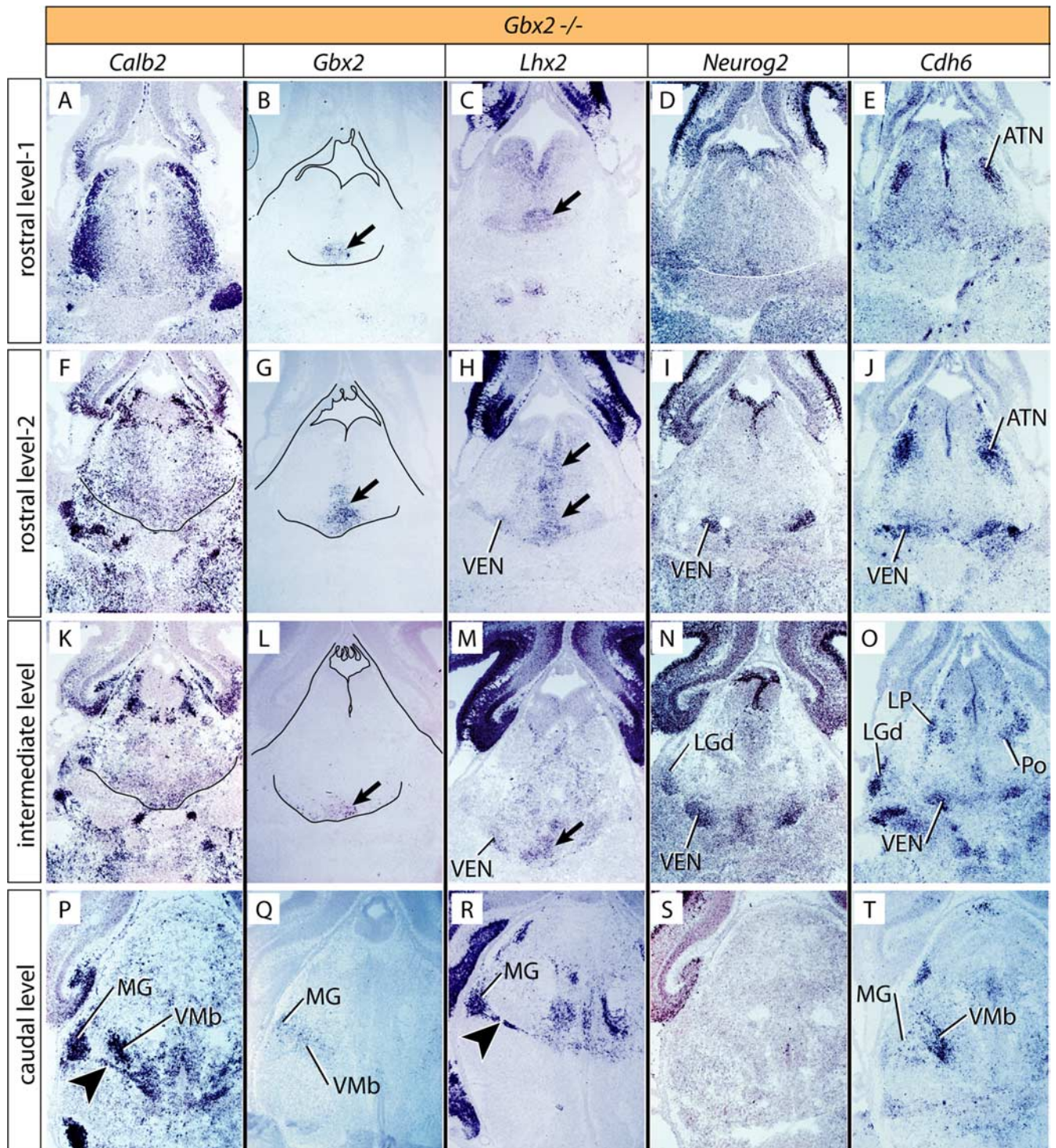
Figure 6. Marker expression in the E18.5 *Gbx2* thalamus. *A–T*, *In situ* hybridization on E18.5 mouse brain sections for five marker genes as indicated on top of each column. Rows show sections at a given rostrocaudal level, as indicated on the left side, labeled for each of the five markers. Columns correspond to four rostrocaudal levels labeled with the same probe. The arrows in *B*, *C*, *G*, *H*, *L*, and *M* indicate marker expression in morphologically abolished medial pronucleus. Some structures have been outlined for clarity. For details, see Results.

Central pronucleus (anterior and ventral nuclear groups)
The anterior group, although very much altered morphologically, showed ectopic *Calb2* expression (Fig. 6*A*), lost *Lhx2* (Fig. 6*C,H*) and *Neurog2* (Fig. 6*D,I*), and preserved *Cdh6* expression (Fig. 6*E,J*). The ventral group seemed reduced to a single rostrocaudal region preserving, however, all its markers (*Lhx2*, *Neurog2*, and *Cdh6*) (Fig. 6*H–J,M–O*, VEN). The ventromedial basal preserved three of its four markers (*Calb2*,

Gbx2, and *Cdh6*) (Fig. 6*P,Q,T*) and lost *Lhx2* (Fig. 6*R*). The smaller size of this nucleus in the *Gbx2* mutant brought to the fore a structure labeled by *Calb2* and *Lhx2* and perhaps corresponding to the subparafascicular nucleus (Fig. 6*P,R*, arrowheads).

Medial pronucleus (medial and intralaminar nuclear groups)
Truncated, nonfunctional *Gbx2* mRNA can still be detected in the *Gbx2* mutant (see Materials and Methods), allowing for rec-
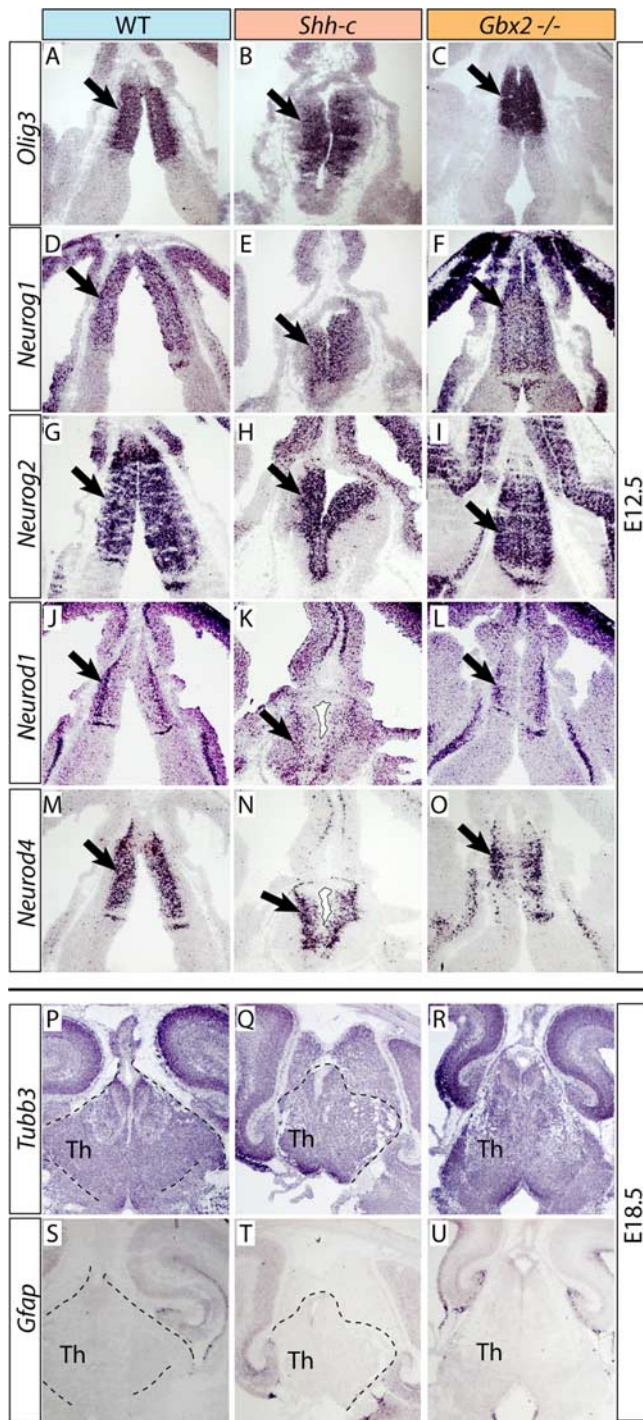
Figure 7. Differentiation markers in the thalamus. *In situ* detection of mRNA on transverse sections of E12.5 (*A–O*) or E18.5 (*P–U*) mouse brains for the genes and genotypes indicated. At E12.5, expression of *Olig3* (*A–C*), *Neurog1* (*D–F*), *Neurog2* (*G–I*), *Neurod1* (*J–L*), and *Neurod4* (*M–O*) is maintained in the mutants. At E18.5, expression of pan-neuronal marker gene *Tubb3* (*P–R*) and astroglial marker gene *Gfap* (*S–U*) does not show changes in the mutant brains. In *P*, *Q*, *S*, and *T*, the thalamus has been outlined for clarity.

Figure 8. BrdU labeling in the mutant thalamus. *A–C*, BrdU-labeled sections of E12.75 thalamus of the three genotypes as indicated. White arrows indicate the labeled thalamic neuroepithelium. *D*, *E*, Results from counting absolute numbers of labeled cells (*D*) and labeling index (*E*) in the E12.75 thalamic neuroepithelium (±SD) after 3 h. survival. No statistically significant differences were detected. *F–I*, BrdU-labeled cells (injection at E12.5, collection at E18.5) in the wild-type (*F*, *H*) and *Shh-c* (*G*, *I*) thalamus. The thalamus has been outlined in white. Rostrally (*F*, *G*), the wild type showed labeled cells at every mediolateral level, whereas in the *Shh-c* (*G*), they were confined to a band surrounding the ventricle (dotted line), and the mantle layer (arrow) showed sparse labeling. More caudally (*H*, *I*), most labeled cells in the *Shh-c* (*I*) did not progress beyond intermediate mediolateral levels (dotted line).

ognition of the medial pronucleus. This marker as well as *Lhx2* showed complete morphological abolition of the medial and intralaminar groups in the *Gbx2* mutant (Fig. 6*B,C,G,H,L,M*, arrows).

Dorsal pronucleus (lateral and posterior nuclear groups)
The laterodorsal nucleus had disappeared, whereas the lateral posterior preserved *Cdh6* expression (Fig. 6*O*) but lost *Gbx2*,
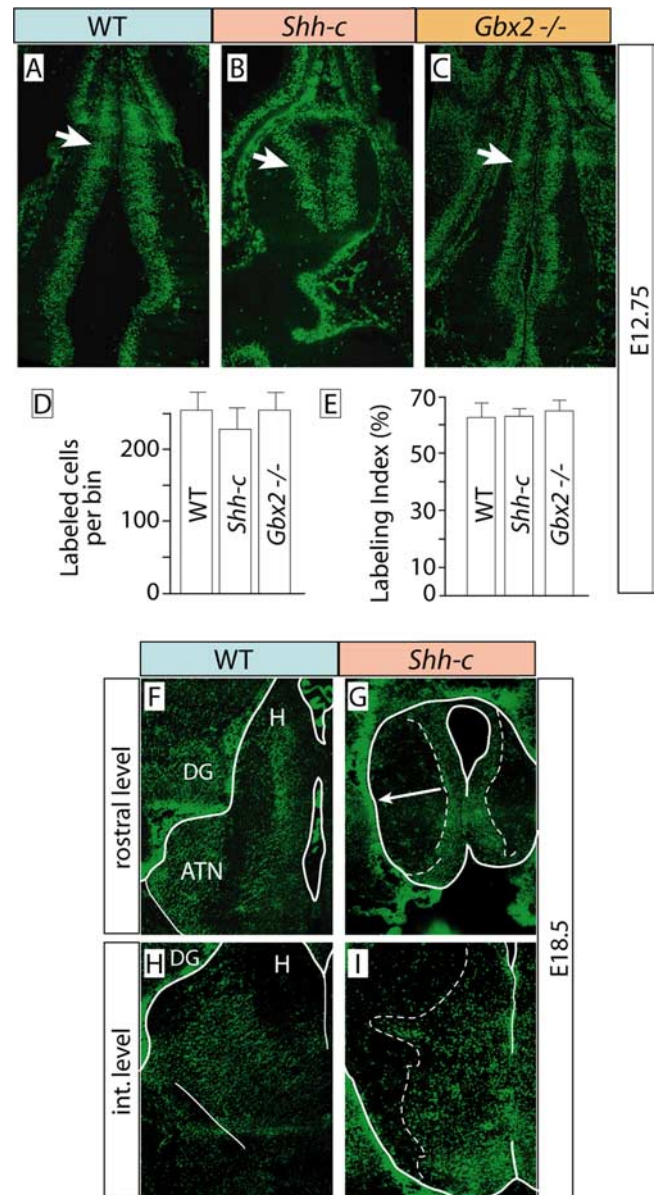
Calb2, and *Lhx2* expression. The posterior nucleus preserved *Cdh6* (Fig. 6*O*).

Lateral geniculate pronucleus
The lateral geniculate pronucleus preserved its two markers, *Neurog2* and *Cdh6* (Fig. 6*N*,*O*).

Medial geniculate pronucleus
The medial geniculate pronucleus preserved all its markers, *Calb2*, *Gbx2*, *Lhx2*, and *Cdh6* (Fig. 6*P–R,T*).

Basic helix–loop– helix gene expression in the mutant thalamic neuroepithelium

The phenotypes observed could be the result of transdifferentiation or lack of differentiation attributable to incorrect activation of proneural genes, a family of basic helix–loop– helix (bHLH) transcription factors governing fate acquisition (Guillemot, 2007a,b). *Neurog1*, *Neurog2*, and *Olig3* are bHLH transcription factors with major roles in telencephalic fate acquisition (Fode et al., 2000) and also expressed in the thalamic neuroepithelium (González et al., 2002; Vue et al., 2007). Mining the expression pattern database GenePaint (www.genepaint.org) (Alvarez-Bolado and Eichele, 2006) for bHLH genes with developmental thalamic expression yielded two additional ones (*Neurod1* and *Neurod4*). *In situ* hybridization labeling of the thalamic neuroepithelium at E12.5 did not show loss of expression of any of these five genes in the *Shh-c* and *Gbx2* mutants compared with the wild type (Fig. 7).

We then used *in situ* hybridization to label mRNA for the genes *Tubb3* (β tubulin 3; a pan-neuronal marker) and *Gfap* (glial fibrillary acidic protein; an astroglial marker) to assess possible changes in cell type in the E18.5 mutant thalamus. Our results (Fig. 7P–U) showed no change in the neuron versus glia composition of the mutant thalamus.

Cell proliferation in the mutant thalamic neuroepithelium

Next we asked whether alterations in proliferation could be involved in the phenotypes observed. It is known that Shh deficiency results in decreased proliferation at approximately E9.0, i.e., during the period of expansion of neural precursors (Ishibashi and McMahon, 2002), as witnessed by the very small size of the early thalamic primordium in our *Shh-c* mutants. We wanted to investigate the proliferative capabilities of the *Shh-c* neuroepithelium during the period of neuron production (i.e., after E11.0) (Altman and Bayer, 1988; Clancy et al., 2007). To this purpose, we labeled the thalamic neuroepithelium at E12.75 with BrdU (Fig. 8A–C). At this age, all rostrocaudal levels of the thalamic region should be actively proliferating (for details, see Material and Methods). We were not able to find statistically significant differences in absolute numbers of labeled cells or in labeling index between the three genotypes investigated, after either 1.5 h (data not shown) or 3 h survival (Fig. 8D,E).

Distribution of BrdU labeling in the E18.5 *Shh-c* thalamus

Migration alterations could contribute to the mutant thalamic phenotypes. To test this possibility, we wanted to examine the overall distribution in the thalamus of neurons born on a specific day. We labeled brains of the three genotypes with BrdU at E12.5 and collected them at E18.5, when most thalamic neurons have already been produced (Fig. 8F–I). In the wild-type thalamus (Fig. 8F,H) as well as the *Gbx2* mutant thalamus (data not
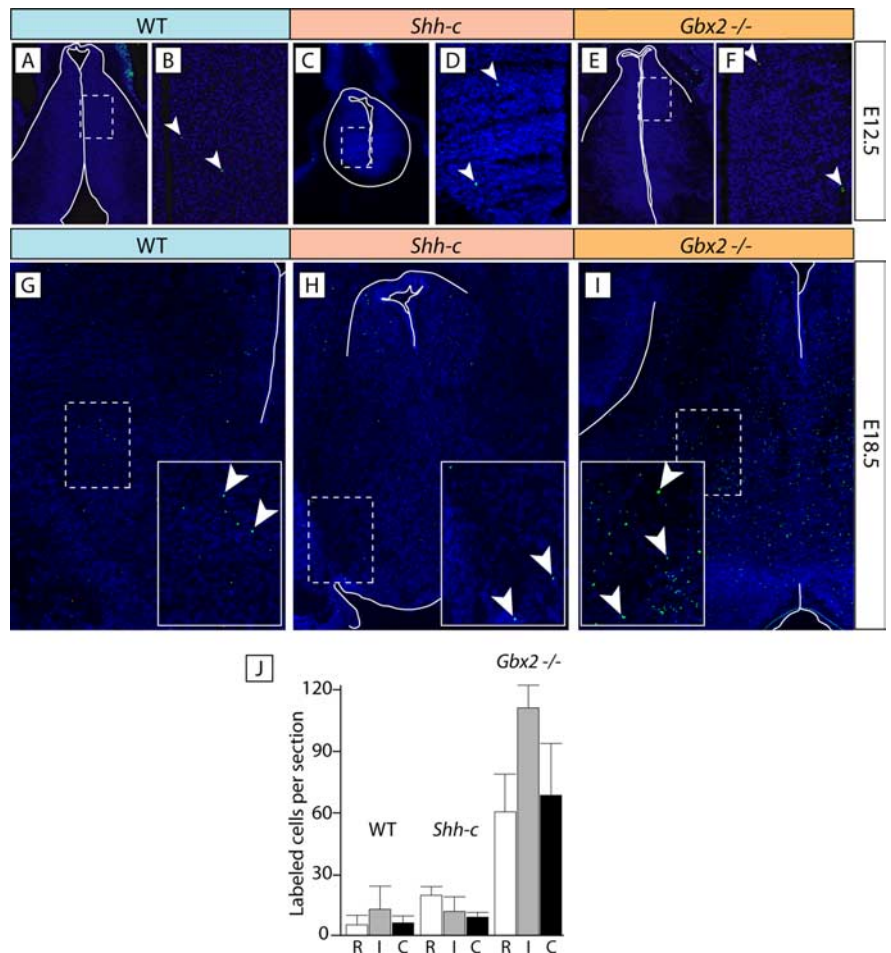

Figure 9. Apoptosis in the thalamus of the *Gbx2* mutant. *A–F*, TUNEL of apoptotic cells in the E12.5 thalamus of the three genotypes as indicated. *B*, *D*, and *F* show magnified views of the frames in *A*, *C*, and *E*. Arrowheads indicate apoptotic cells. *G–I*, Sections through intermediate rostrocaudal levels of the E18.5 thalamus of the three genotypes showing apoptosis detection. The frame in dotted line is shown magnified as inset in each picture. Some apoptotic cells are indicated by arrowheads. *J*, Mean ± SD of apoptotic cells per histological section of the E18.5 thalamus at rostral (R), intermediate (I), and caudal (C) levels in the three genotypes. The increase at every level in the *Gbx2* mutant is significant with respect to the other two genotypes ($p < 0.005$).

shown), we could find labeled neurons at every mediolateral level of the thalamus, reaching the periphery of this region. However, in the *Shh-c* thalamus at the rostral level almost all labeled cells were confined to a band around the ventricle (Fig. 8G). This phenotype was less striking at more caudal levels, in which most labeled cells had reached intermediate mediolateral levels (Fig. 8I, dotted line), although only few were located in the periphery of the primordium.

Increase in neuronal apoptosis in the *Gbx2* mutant thalamus

Increase in neuroepithelial (or "proliferative") apoptosis or in neuronal apoptosis could be at the root of the mutant phenotypes observed. Deficiency in Shh increases apoptosis in the diencephalic neuroepithelium during the phase of precursor expansion (Ishibashi and McMahon, 2002). To know whether Shh is necessary to prevent neuroepithelial cell death also during the phase of neuronal production, we labeled E12.5 thalamic sections by means of the TUNEL reaction. We found only very few apoptotic cells in the neuroepithelium of the three genotypes investigated and no evidence of an increase in apoptosis at this age (Fig. 9A–F).

Next we asked whether neuronal cell death would be increased in our mutants. TUNEL sections of E18.5 thalamus at three ros-
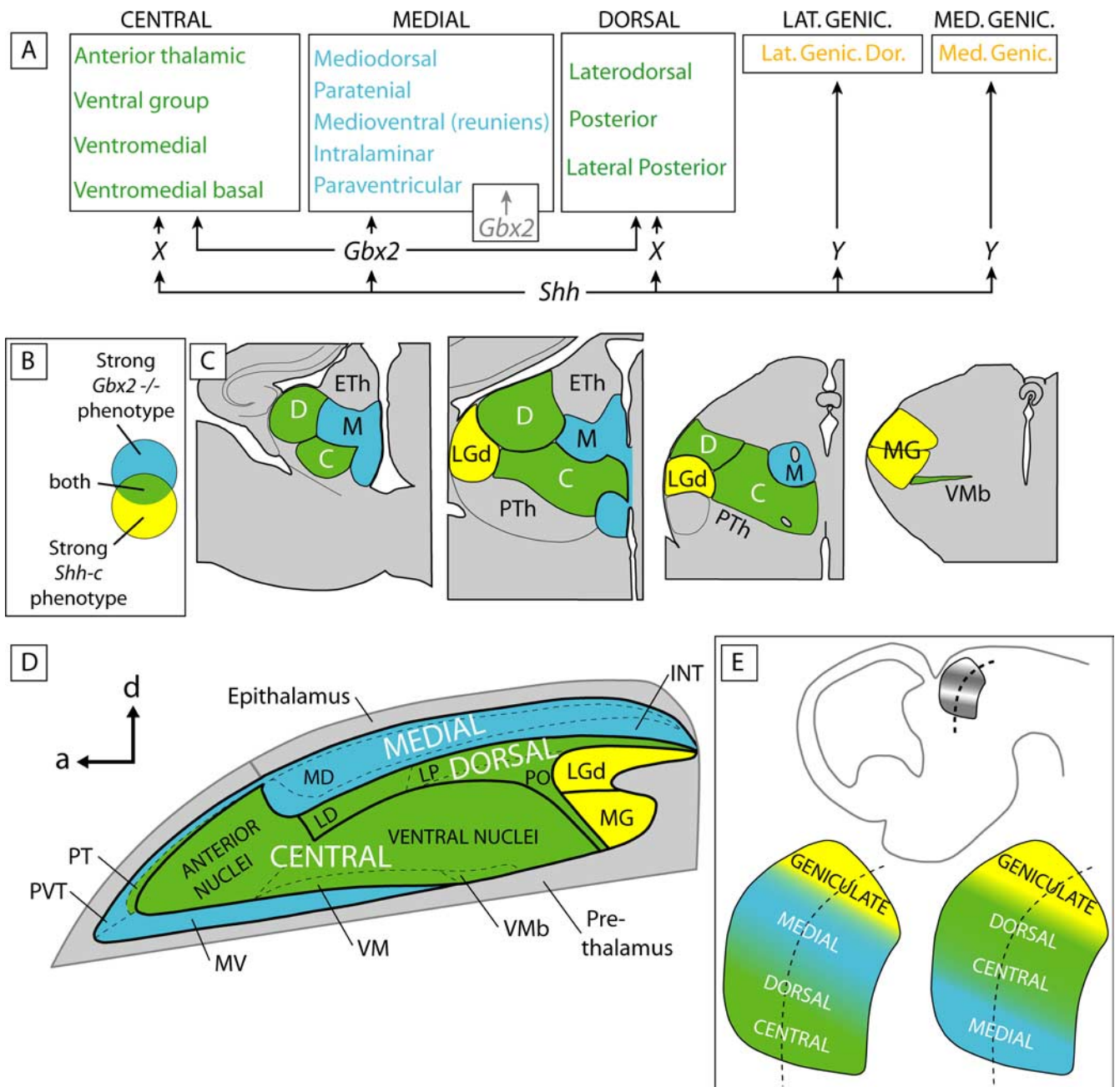
Figure 10. Summary of results. ***A***, Involvement of *Shh*, *Gbx2* and unknown factors (*X, Y*) in the differentiation of thalamic nuclear groups inferred from the mutant phenotypes. The *Gbx2* label in a closed box symbolizes restricted default expression of *Gbx2*. We include the intralaminar nuclei in the medial pronucleus (see Results). Color code as in ***B***. ***B***, Color code for this figure. ***C***, Diagram showing the thalamic pronuclei in four rostrocaudal levels labeled according to the severity of their phenotype in the *Shh-c* and *Gbx2* mutants. ***D***, Thalamic nuclear groups mapped on a flat map (Swanson, 1992) and labeled according to dependence on *Shh* and/or *Gbx2*. Each of the three groups encompasses contiguous nuclei. ***E***, Localization of the thalamic neuroepithelium (top) and possible arrangements of the three presumptive regions that will give rise to the different nuclear groups (bottom). Dotted line, Anteroposterior axis of thalamic primordium. C, Central; D, dorsal; M, medial; ETh, epithalamus.

trocaudal levels in the three genotypes (Fig. 9*G–I*) showed no change in neuronal cell death in *Shh-c* versus wild type (Fig. 9*J*). However, the *Gbx2* mutant thalamus showed a very significant increase ($p < 0.005$) in neuronal cell death (Fig. 9*J*).

Discussion
Assessing thalamic differentiation with marker genes
Our identification of thalamic nuclear markers (Table 2) agrees with the literature (Arai et al., 1991, 1992, 1994; Frassoni et al., 1991; Winsky et al., 1992; Nakagawa and O'Leary, 2001; Jones and Rubenstein, 2004) except for minor discrepancies probably attributable to species (mouse vs rat) or age

[E18.5 vs postnatal day 0 (P0), P2, or adult] differences. We have not found *Gbx2* expression in the anterior thalamus, but Jones and Rubenstein (2004) described it in the anterodorsal nucleus at P0. These authors do not mention lateral posterior nucleus expression of *Cdh6*, as we do. *Lhx2* expression in the anterior, ventral, and lateral groups, as well as *Neurog2* in the laterodorsal nucleus (Table 2), are not mentioned by Nakagawa and O'Leary (2001), working on P2. Finally, we do not find *Calb2* in the mediodorsal nucleus, contrary to Arai et al. (1994), working on adult rat.

These discrepancies do not bear on the reliability of our con-

clusions because, in every case, we have other markers plus clear ISH evidence obtained through a particularly sensitive dual-amplification protocol.

Neural Shh in the cascade of thalamic differentiation

The development of a brain region proceeds by steps regulated by a hierarchical cascade of gene activation. Because the *Shh-c* mutant expresses thalamic marker *Dbx1* (Fig. 2*G,H*), which is missing in the full *Shh* mutant (Ishibashi and McMahon, 2002), we can assume that the first steps of the thalamic cascade (regional specification) are not regulated by neural Shh. In any case, Shh is one among several factors in thalamic regionalization, because transcription factor gene *Pax6*, required for thalamic development (Warren and Price, 1997; Pratt et al., 2000, 2002), is expressed in the full Shh mutant (Ishibashi and McMahon, 2002). It is only after gastrulation (and general regional specification) that neural Shh signaling gets underway, activating *Gbx2*, *Nkx2-2*, and *Sox14* and repressing *Pax6* (Ishibashi and McMahon, 2002; Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004) in a thalamus already specified regionally.

We hypothesized that neural Shh would promote the last steps of the thalamic specification cascade (partially through *Gbx2* activation), leading to specific thalamic histogenesis and nucleogenesis. Our analysis of the mutant phenotypes confirms this hypothesis by showing that neural Shh has at least two roles: (1) promotion of specific neuronal aggregation, leading to the formation of recognizable thalamic nuclei; (2) promotion of axonal extension, leading [through a tightly regulated process (López-Bendito and Molnár, 2003; Price et al., 2006)] to appropriate thalamocortical connectivity.

Neural Shh and neuronal fate acquisition in the thalamus

However, earlier events such as incorrect neuronal fate acquisition could contribute to the mutant phenotypes reported here. The acquisition of general neuronal fate as well as some neuronal subtype traits are regulated by proneural genes, encoding bHLH transcription factors whose role in telencephalic development is well known (Guillemot, 2007a,b). Although there is evidence of a link between Shh and proneural and other bHLH genes (Blader et al., 1997; Lu et al., 2000; Ota and Ito, 2003; Andersson et al., 2006), the thalamic bHLH genes examined and the cell-type markers *Tubb3* and *Gfap* were not changed in the mutant. This agrees with our hypothesis that neural Shh influences later stages of differentiation.

Neurogenesis and proliferative cell death are not affected by neural Shh

Shh promotes precursor proliferation in the thalamus, hypothalamus, and cortex (Dahmane et al., 2001; Ishibashi and McMahon, 2002; Manning et al., 2006). The very small size of the early *Shh-c* thalamic primordium confirms this role. A role for Shh in neuron generation has not yet been documented (except for an indirect function in cortical interneuron generation) (Gulacsi and Lillien, 2003). In agreement, we show that deficiency in neuroepithelial Shh does not impair proliferation during the phase of neurogenesis.

Normally occurring cell death is a key developmental process that can affect the proliferative neuroepithelium (so-called proliferative cell death) (Thomaidou et al., 1997) as well as postmitotic neurons (see below). Although Shh is a neuroepithelial survival factor during precursor proliferation (Ishibashi and McMahon, 2002), we show that later deficiency in Shh does not increase neuroepithelial cell death. This suggests that Shh (of any

origin) acts specifically on the neuronal progenitors during the phase of expansion.

Migration in the Shh-c mutant thalamus

In the cortex, it is possible to follow neuronal migration to different layers by using precisely timed BrdU injections (Edgar and Price, 2001). Thalamic neuronal migration does not result in layer formation, but the principle of labeling a certain population with BrdU and recording its position at a later stage holds also for the thalamus. In this way, we have detected alteration in neuronal distribution in the E18.5 *Shh-c* thalamus, compatible with slow or failed migration. Inability of neurons to reach their settling places would alter specific neuronal aggregation (i.e., nucleogenesis and histogenesis) and would help explain the *Shh-c* mutant phenotype. Hedgehog signaling can influence the migration of glia and neurons in the developing fruit fly (Rangarajan et al., 2001). Shh has an indirect influence on the migration of newly born neurons in the adult mouse (Balordi and Fishell, 2007). Even if a comprehensive analysis of the role of Shh in migration is not intended here, our results indicate a possible function of neural Shh in the radial migration of newly born thalamic neurons.

Gbx2 is necessary for neuronal survival

Normally occurring developmental neuronal death (Oppenheim, 1991) appears in the rodent thalamus during the first postnatal week (Ashwell and Waite, 1991; Spreafico et al., 1995; Alcántara et al., 1997; Lotto et al., 2001; Luczyńska et al., 2003). Accordingly, we found few apoptotic figures in the wild-type and the *Shh-c* thalamus at E18.5. Thalamic neuronal survival during development depends on endogenous and target-derived factors (Lotto et al., 1997, 2001), a mechanism not altered by neural Shh deficiency.

We show, however, very increased neuronal cell death in the *Gbx2* mutant thalamus, indicating that *Gbx2* has a hitherto unknown role in the survival of specific neuronal populations. Obviously, a major cause of the *Gbx2* thalamic phenotype is massive cell death.

Gbx2 is necessary and sufficient to specify the medial pronucleus

Gbx2 is initially expressed at E11.5 in the entire thalamic primordium downstream *Shh* (Hashimoto-Torii et al., 2003). However, we show delayed and restricted expression of *Gbx2* independent of Shh. Because in the *Shh-c* mutant the medial pronucleus preserved general shape and gene expression whereas in the *Gbx2* mutant it showed complete and specific morphological abolition, it follows that *Gbx2* is necessary and sufficient to specify cell fate and position of the medial and intralaminar groups (in a thalamic primordium already specified as a region).

The lateral and medial geniculate pronuclei, however, depend on *Shh*, not *Gbx2*, for differentiation. Finally, the central and dorsal pronuclei depend on both neural *Shh* and *Gbx2*. The resultant *Shh–Gbx2*-thalamic differentiation cascade misses (at least) two components (Fig. 10*A*, *X* and *Y*) that would be responsible for the specific effects of *Shh*. Component *X* could simply be the early expression (E11.5) of *Gbx2* in the entire primordium.

Prepatterning the thalamic neuroepithelium

The existence of an early, Shh-independent pattern ("prepattern") in the spinal cord has been proposed to explain why the phenotype of *Gli* compound mutants is less severe than expected

(Ruiz i Altaba et al., 2003). Thalamic prepatterning depends on Wnt activity upstream *Irx3* (Braun et al., 2003), which makes the thalamic neuroepithelium competent to respond to Shh (Kiecker and Lumsden, 2004). We show an additional thalamic prepatterning phenomenon resulting in a late phase of neural Shh-independent *Gbx2* expression. The pronuclei, labeled according to their differential *Shh* or *Gbx2* dependence (Fig. 10 *A*, *B*), have a complex tridimensional relation to each other (Fig. 10 *C*). However, when they are depicted on a "flat map" (Fig. 10 *D*) (after Swanson, 1992), it becomes evident that each of the three groups encompasses adjacent nuclei that could originate in contiguous neuroepithelial areas. The thalamic neuroepithelium would then be patterned into at least three areas whose spatial arrangement is not yet clear (Fig. 10 *E*, bottom).

Finally, our results bear on a current question about the basic developmental mechanisms patterning the thalamus. Does neural Shh specify thalamic neurons through a concentration gradient? This mechanism is used by the notochord to specify the ventral spinal cord (Jessell, 2000), and it would be an elegant way to specify the intricate thalamic structure. However, the existence of a (neural) Shh-independent area in the thalamic neuroepithelium implies that a concentration gradient would have to work around a nonresponsive patch of tissue (Fig. 10 *E*). This suggests that any concentration gradient mechanism in the thalamus would have to adopt a more complex form than in the ventral spinal cord.

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