

# Severe Defects in Dorsal Thalamic Development in Low-Density Lipoprotein Receptor-Related Protein-6 Mutants

Cheng-Ji Zhou,<sup>1</sup> Kathleen I. Pinson,<sup>2</sup> and Samuel J. Pleasure<sup>1</sup>

<sup>1</sup>Department of Neurology, Programs in Neuroscience and Developmental Biology, University of California, San Francisco, San Francisco, California 94143-0435, and <sup>2</sup>Department of Reproductive Medicine, University of California, San Diego, La Jolla, California, 92093-0674

Mice with mutations in the Wnt coreceptor low-density lipoprotein receptor-related protein-6 (LRP6) have a smaller and severely disorganized dorsal thalamus and lack thalamocortical projections. Using molecular markers, we showed that most dorsal thalamic and epithalamic neurons were missing, and most of the major dorsal thalamic nuclei were not identifiable. However, the ventral thalamus was essentially unaffected, although the dorsal thalamic defect leads to rostral displacement of portions of the ventral thalamus. Analysis of younger embryos showed that epithalamic and dorsal thalamic neurons were not produced at early stages of development, whereas ventral thalamic neurons were still produced. These defects were accompanied by improper formation of the boundary between dorsal and ventral thalamus, the zona limitans interthalamica (ZLI). Furthermore, the expression of an early marker of posterior forebrain development that marks the compartment from the midbrain–hindbrain junction to the ZLI (including the future dorsal thalamus, pretectum, and midbrain) was disrupted, supporting the idea that diencephalic development is abnormal from very early in embryogenesis. This study provides compelling *in vivo* evidence that thalamic development requires normal activity of the LRP6-mediated canonical Wnt signaling pathway.

**Key words:** diencephalon; differentiation; thalamus; Wnts; zona limitans; sonic hedgehog

## Introduction

During vertebrate development, Wnts are expressed in highly localized domains in the dorsal neural tube and dorsomedial cerebral cortex, and strong evidence links Wnts to regulation of dorsal precursor proliferation in the spinal cord and medial cortical derivatives, like the hippocampus (Ikeya et al., 1997; Grove et al., 1998; Lee et al., 2000; Zhou et al., 2004). It is somewhat controversial as to whether Wnts also regulate cell fate in the developing mammalian nervous system, although this is well established in lower vertebrates (Dorsky et al., 1998). However, recent studies reexamining the phenotype of Wnt1/3A double mutants and gain-of-function experiments in chicks (Muroyama et al., 2002; Caspary and Anderson, 2003; Helms and Johnson, 2003) and examining the role of Wnts in mouse neural crest cell fate using conditionally mutant mice (Lee et al., 2004) have established that Wnts do appear to regulate cell fate in mammalian

neural development. However, it is still unclear whether this is true in other regions of the mammalian CNS.

The thalamus is a crucial intermediate target for sensory input to the cortex and has a central role in regulating sensory processing. The prosomeric model of brain development divides the developing forebrain into neuromeric divisions called prosomeres arrayed in an anteroposterior (A-P) manner in the neuroaxis (Rubenstein et al., 1994). In this schema, the diencephalon (including the thalamus) arises from components of prosomeres p1 (the pretectum), p2 (the dorsal thalamus and epithalamus), p3 (the ventral thalamus), and hypothalamus. The boundary between dorsal and ventral thalamus is termed the zona limitans interthalamica (ZLI) and has been postulated to be a local signaling center (Larsen et al., 2001). The folding of the neural tube during gestation as the brain expands leads to the transition of these initial anteroposterior divisions into a dorsoventral orientation. Previous studies have shown that during submammalian vertebrate development, Wnt signaling is involved in the initial anteroposterior patterning of the neural tube and later in subdividing the brain into telencephalon, eye fields, and diencephalon (Heisenberg et al., 2001; Houart et al., 2002; Nordstrom et al., 2002). Also, an important functional role for Wnt signaling specifically in thalamic development has recently been suggested in chick, where Wnt signals were shown to be required at early stages to induce a posterior forebrain marker in the diencephalon and then again at later stages to induce dorsal thalamic markers within this posterior forebrain domain (Braun et al., 2003).

To study the function of the Wnt/ $\beta$ -catenin pathway in the developing thalamus, we examined the phenotype of low-density lipoprotein receptor-related protein-6 (LRP6) mutant

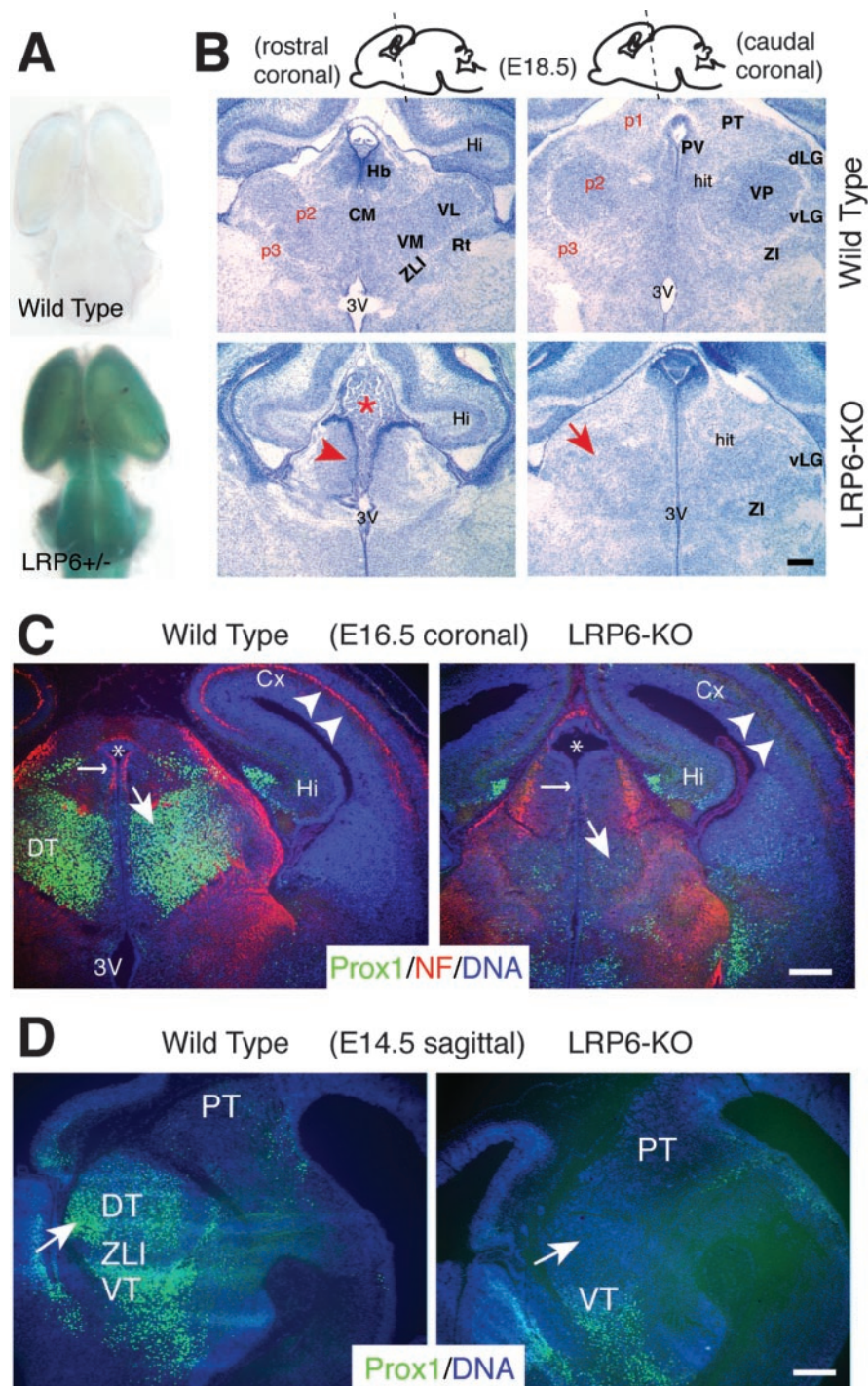
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Correspondence should be addressed to Dr. Samuel J. Pleasure, Department of Neurology, University of California, San Francisco, P.O. Box 0435, Room S-268, 513 Parnassus Avenue, San Francisco, CA 94143-0435. E-mail: samueljp@itsa.ucsf.edu.

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**Figure 1.** Disruption of the thalamus in LRP6 knock-out (KO) mice. *A*, X-gal staining (blue) in heterozygous (LRP6<sup>+/-</sup>) shows that LRP6 is ubiquitously expressed in entire developing brain at E12.5. *B*, Nissl staining in E18.5 coronal brain sections shows anatomic defects of thalamus in mutants. These defects are most severe at rostral levels where all lateral thalamic structures are missing, and only the medial thalamic regions remained with a disorganized ventricular zone (red arrowhead). Note the extra choroid plexus-like structure in the third ventricle (red star). At caudal levels, the major posterior dorsal thalamic nuclei are not identifiable (red arrow). *C*, Double immunofluorescence for Prox1 (green; a dorsal thalamic neuronal marker) and neurofilament (red) in coronal sections at E16.5 shows that most dorsal thalamic neurons (large white arrows) are missing and that there are few axonal projections to the cortical subplate (where thalamocortical axons project prominently at this age) (white arrowheads) in the mutants. In addition, the habenular recess is larger in the mutant (asterisk), and the fibers normally coursing adjacent to the habenular ventricular zone (small white arrows) are missing or misplaced in the mutant. *D*, Immunofluorescence of Prox1 (green) in sagittal brain sections of E14.5 mice shows a similar phenotype in the dorsal thalamus (white arrows). 3V, Third ventricle; Cx, cerebral cortex; CM, central medial thalamic nuclei; dLG, dorsal lateral geniculate nucleus; DT, dorsal thalamus; Hb, habenular nuclei; Hi, hippocampal formation; hit, habenulo-interpenduncular tract; p1, 2, 3, prosomeres 1, 2, 3; PT, pretectum; PV, paraventricular thalamic nuclei; Rt, reticular thalamic nucleus; VL, ventral lateral thalamic nuclei; vLG, ventral lateral geniculate nucleus; VM, ventral medial thalamic nuclei; VP, ventral posterior thalamic nuclei; VT, ventral thalamus; ZI, zona incerta. Scale bars, 50  $\mu$ m.

mice. LRP6 is a ubiquitously expressed Wnt receptor that acts along with members of the Frizzled family to transduce Wnt signals through the Wnt/ $\beta$ -catenin pathway (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Thus, LRP6 mutants represent a generalized hypomorphic (because of complementation by LRP5) loss of function for the Wnt/ $\beta$ -catenin pathway (Maretto et al., 2003; Kelly et al., 2004).

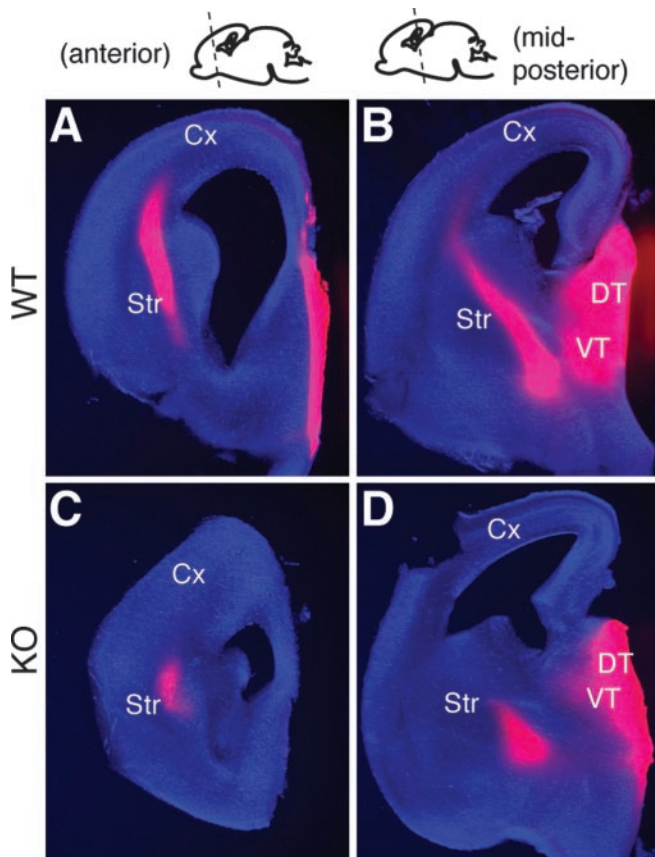
**Materials and Methods**

*Animals and tissue preparation.* All animals were treated according to protocols approved by the University of California, San Francisco. The LRP6 mutant mice were originally generated by gene trap insertion that joined the first 321 amino acids of the LRP6 protein in-frame with the  $\beta$ -geo reporter gene that is then trapped in the endoplasmic reticulum rendering it nonfunctional (Pinson et al., 2000). LRP6 mutant mouse embryos were obtained from timed matings. Females with semen plugs found the next morning were considered embryonic day 0.5 (E0.5). As described previously, the homozygous mutants showed severe anatomical defects in the limbs and tail and died at birth (Pinson et al., 2000). Embryos between E10.5 and E18.5 were genotyped by morphology and X-gal staining. Embryo heads or brains were fixed by immersion in 4% paraformaldehyde and sectioned on a cryostat at 7  $\mu$ m. Two to five normal or mutant embryos were used for each experiment at each time point.

*Antibodies and histology.* Rabbit antibodies to prospero-related homeobox-1 (Prox1) (1:5000), and Histone H3 (1:2000; Upstate Biotechnology, Lake Placid, NY), and mouse antibodies to neurofilament (RMO270; 1:3000; gift from Virginia Lee, University of Pennsylvania, Philadelphia, PA), Nkx2.2 (1:1000), and Pax6 (1:1000) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) were used according to widely published immunohistologic protocols and detected with secondary antibodies conjugated to Alexa fluorochromes (Molecular Probes, Eugene, OR) followed by nuclei counterstaining with Hoechst 33258 (Sigma, St. Louis, MO).

For bromodeoxyuridine (BrdU) labeling, pregnant mice were injected intraperitoneally with 100 mg/kg body weight and then the embryo brains were dissected out 1 hr later. The cryostat sections were pretreated with 2N HCl for 30 min at 37°C followed by general immunofluorescence with anti-BrdU antibody (1:1000; Roche Products, Hertfordshire, UK). For X-gal staining, embryo brains were dissected into 2% paraformaldehyde for 1 hr and stained with X-gal substrate solution.

Nonradioactive *in situ* hybridization histochemistry was performed using digoxigenin-labeled riboprobes for  $\beta$ 4, Dlx2, Gbx2, Id4, Nectrin, retinoic acid receptor-related orphan receptor (ROR $\alpha$ ), soluble Frizzled-related protein-2 (SFRp2), Shh, Tcf4, Irx3, Six3, Wnt3,



**Figure 2.** LRP6 mutants fail to produce thalamocortical projections. *A–D*, E18.5 wild-type (*A, B*) and mutant (*C, D*) brains after placement of DiI crystal in the thalamus. Wild-type mice, shown at two coronal levels, have fibers from the dorsal thalamus projecting through the internal capsule to the cortex dorsally. In mutants, the fibers that leave the thalamus get stalled and terminate in the striatum. Str, Striatum; Cx, cortex; DT, dorsal thalamus.

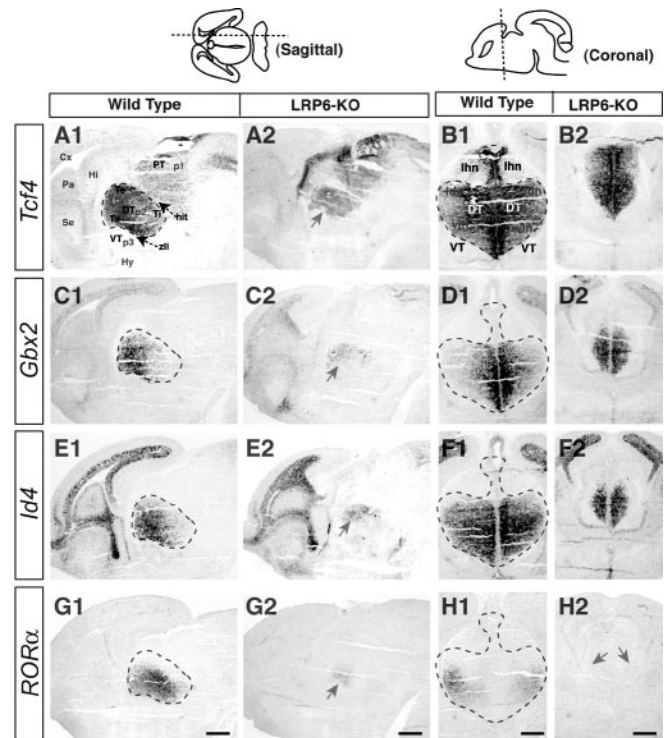
Wnt5a, and Wnt7b and detected with sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase and then color developed with the enzyme substrate solution as described previously (Kim et al., 2001).

Lipophilic dye labeling was performed in fixed brains. Brains were hemisected in the sagittal plane and large crystals of DiI placed in the thalamus using fine forceps. Brains were stored in 4% paraformaldehyde (changed weekly) at 37°C in the dark for 6–8 weeks. Brains were then sectioned and photographed.

## Results

### LRP6 mutants have thalamic defects

LRP6 was ubiquitously expressed during development of the brain (Fig. 1*A*), as shown previously in other embryonic regions (Pinson et al., 2000; Kelly et al., 2004), and LRP6 mutant mice had distinctive thalamic abnormalities evident by midgestation (Fig. 1). In Nissl stained sections, the dorsal thalamic compartment was affected, and it was difficult to identify individual dorsal thalamic nuclei, particularly more rostrally (Fig. 1*B*). This was also noticeable when we stained sections with antiserum for the divergent homeobox gene *Prox1*, which is expressed strongly in most dorsal thalamic neurons (Bagri et al., 2002). In these sections, very few *Prox1*-immunolabeled dorsal thalamic neurons were detected compared with wild-type mice in both coronal and sagittal section planes (Fig. 1*C, D*). In addition, the third ventricle and habenular recess were enlarged, and there was excess choroid plexus in the third ventricle (Fig. 1*B, C*). In parasagittal sections, the anteroposterior boundaries between the pretegmentum–dorsal thalamus and dorsal–ventral thalamus were less distinct, and the



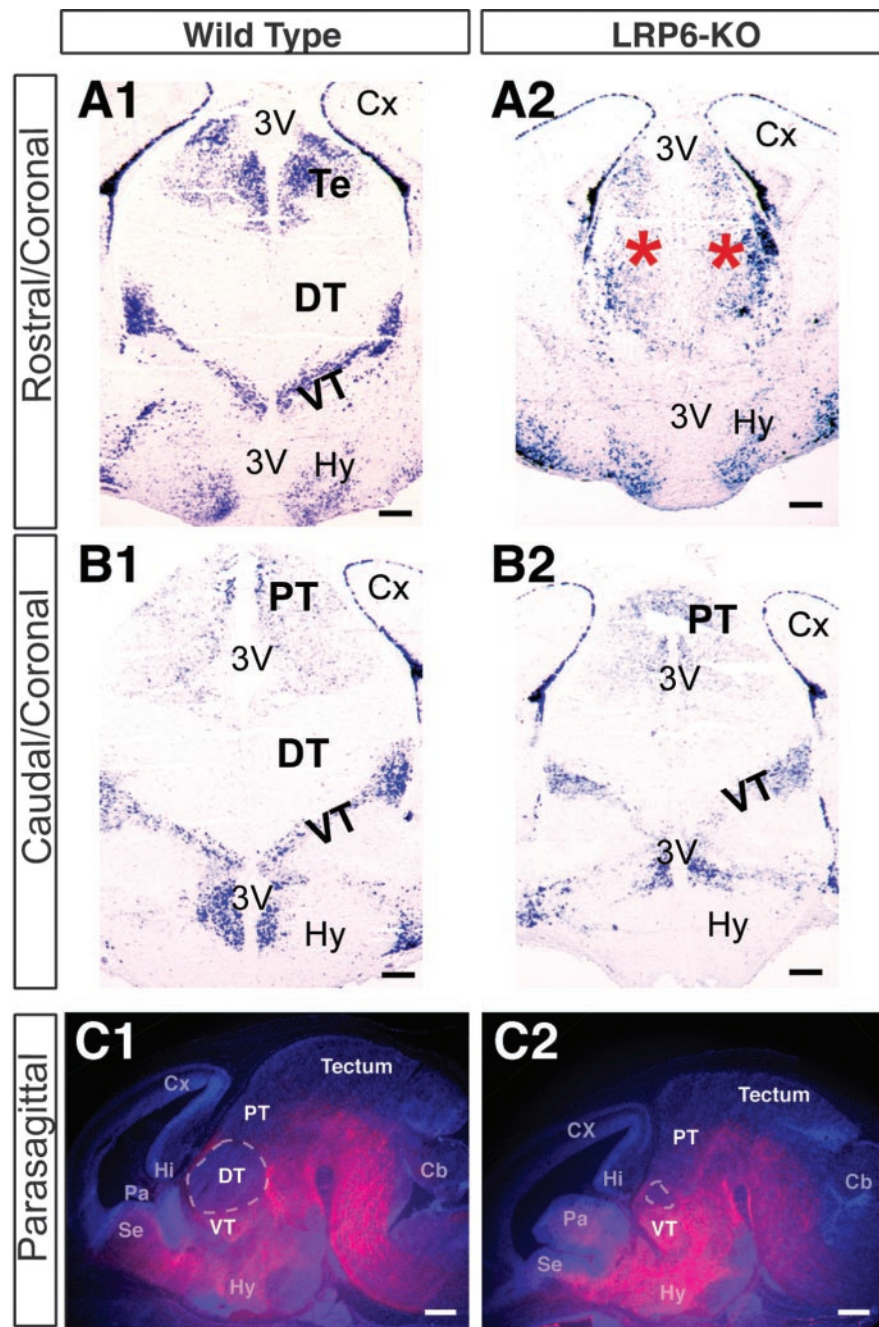
**Figure 3.** Molecular anatomy of the dorsal thalamus in LRP6 mutants. *A–H*, *In situ* hybridization of dorsal thalamic markers *Tcf4* (*A, B*), *Gbx2* (*C, D*), *Id4* (*E, F*), and *RORα* (*G, H*) are used to examine the details of the thalamic defects in sagittal and coronal sections at middle–late gestation. The black dashed line area (*A–H*) indicates the entire dorsal thalamic region as defined by the expression of *Tcf4* in adjacent sections. The arrows (*A2, C2, E2, G2, H2*) are used to show the location of residual staining for each marker in the small remaining dorsal thalamic structure. In LRP6 knock-out (KO) mice, the anterior, ventral, and lateral regions of the dorsal thalamus are most affected. *RORα* is a marker for thalamic sensory relay neurons (25) (*G, H*), and these neurons are especially severely affected in the mutant brains. Cx, Cerebral cortex; DT, dorsal thalamus; Hi, hippocampus; hit, habenulo–interpeduncular tract; Hy, hypothalamus; imt, intermediate thalamic neuroepithelium; ldn, laterodorsal nuclei; lhn, lateral habenular nucleus; p1, 2, 3, prosomere 1, 2, 3; Pa, pallidum ganglion; PT, pretegmentum; SE, septal ganglion; Ta, anterior thalamic region; Te, epithalamus; Ti, internal thalamic region; vln, ventral lateral nucleus; VT, ventral thalamus. Scale bars, 100  $\mu$ m.

reduced size and disorganization of the dorsal thalamus were quite apparent (Fig. 1*D*).

### LRP6 mutants lack thalamocortical projections

We noticed decreased numbers of neurofilament-stained axonal fibers in the subcortical white matter of mutant brains (Fig. 1*C*, arrowheads), implying that, in addition to its apparent anatomic disorganization, the dorsal thalamus may fail to appropriately produce thalamocortical projections. DiI tracing in E18.5 wild-type brains with dye crystals placed into the thalamus strongly labeled fibers leaving the thalamus and projecting laterally into the internal capsule before heading dorsally to penetrate the subcortical white matter (Fig. 2*A, B*). In mutant brains, some DiI-labeled fibers still projected out of the thalamus but failed to reach the cortex (Fig. 2*C, D*). These findings suggest that even those dorsal thalamic neurons that might remain in the mutants may be functionally abnormal and unable to produce axons that properly pathfind to the cortex.

We have shown previously that the telencephalon of LRP6 mutant mice is somewhat abnormal (Zhou et al., 2004); however, the most obvious defect (in the dentate gyrus) has no bearing on the ability of thalamocortical axons to pathfind to the cortex. We



**Figure 4.** Analysis of ventral thalamic markers in middle-late gestation. *A1–B2*, Reelin expression in coronal brain sections (E15.5) by *in situ* hybridization shows that the caudal ventral thalamus appears quite normal (*B1, B2*), whereas the rostral ventral thalamus may have been partially displaced by the absence of the dorsal thalamus rostrally (*A2*, red stars). Note that the reelin expression pattern in other regions, including pretegmentum and hypothalamus of the wild type and mutants, is also comparable. *C1, C2*, Immunofluorescence of GAD65 (red) in E17.5 sagittal brain sections (with a DNA staining in blue) shows that the GAD65-immunopositive ventral thalamus is largely intact, whereas the rostral portion of the ventral thalamus is shifted dorsally in the mutants. This is consistent with the reelin expression in the mutant rostral ventral thalamus (*A2*). Note that the anatomical structure of the tectum (dorsal midbrain) appears normal in the mutants. The white dashed line area indicates the normal and defective dorsal thalamus (*C1, C2*). 3V, Third ventricle; Cb, cerebellum; Cx, cerebral cortex; DT, dorsal thalamus; Hi, hippocampus; Hy, hypothalamus; Pa, pallidum; PT, pretegmentum; SE, septal ganglion; Te, epithalamus; VT, ventral thalamus. Scale bars, 100  $\mu$ m.

have recently noted additional neocortical abnormalities in LRP6 mutants, but these also are unlikely to be germane to this question because these defects are primarily attributable to defects in precursor proliferative behavior in the cortical wall (Zhou et al., 2004).

#### Loss of multiple dorsal thalamic molecular markers in mutant mice

The histologic abnormalities and loss of one marker (Prox1) in the dorsal thalamus of mutant mice suggested that there were alterations in the proper production of differentiated dorsal thalamic neurons. To evaluate this more fully, we examined the expression of several other more established dorsal thalamic neuronal markers, Tcf4, Gbx2, and Id4 (Miyashita-Lin et al., 1999; Rubenstein et al., 1999), in coronal and sagittal brain sections of late gestation embryos (Fig. 3*A–H*). Tcf4 was broadly expressed in the pretegmentum, epithalamus, and dorsal thalamus of wild-type mice, whereas in mutants, the dorsal thalamic component of the Tcf expression domain was reduced (Fig. 3*A, B*). Gbx2 and Id4 were both more limited to the dorsal thalamus in wild-type mice but were also dramatically reduced in their expression in the dorsal thalamus of mutant mice (Fig. 3*C–F*). Within the dorsal thalamus, the most affected subregions were the anterior and ventrolateral regions, the site of important primary sensory neurons. Because thalamic primary sensory neurons express retinoic acid receptor-related orphan receptor ROR $\alpha$  (Steinmayr et al., 1998; Nakagawa and O'Leary, 2002), we examined the distribution of this marker and found that LRP6 mutants almost entirely lacked expression of ROR $\alpha$  (Fig. 3*G, H*).

These results raise an interesting question. Why were Prox1 and ROR $\alpha$  almost entirely missing in the mutant brains, whereas the other thalamic markers were still expressed in a medial domain of the dorsal thalamus? Because Prox1 was expressed only relatively late in thalamic development (not easily detectable with our antibody until E13.5; data not shown) and ROR $\alpha$  is expressed in only a restricted subset of neurons, it seems likely that these two are expressed in a more differentiated state for dorsal thalamic neurons, whereas Id4, Gbx2, and Tcf4 are expressed considerably earlier. Thus, we conclude that LRP6 mutants had developmental disorganization of the dorsal thalamus, failure to properly project thalamocortical fibers to the cortex, and reduced expression of five separate dorsal thalamic molecular markers.

#### Formation of the ventral thalamus in LRP6 mutants

We also examined two ventral thalamic neuronal markers, Reelin and glutamate decarboxylase (GAD), to determine whether the diencephalic defects include the ventral thalamus. In contrast to

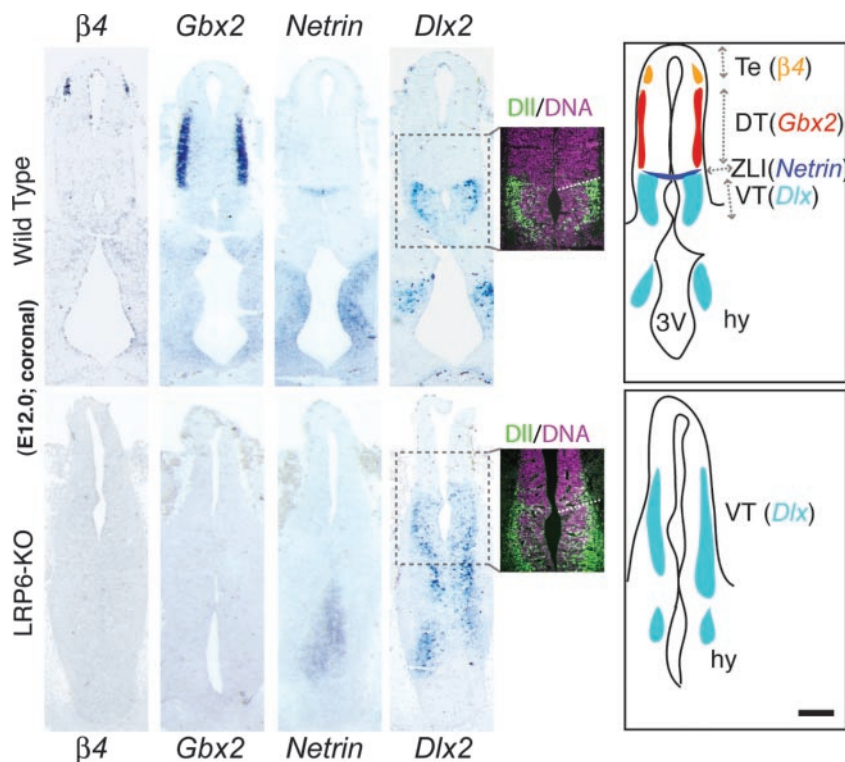
the situation in the dorsal thalamus, the ventral thalamus appeared largely intact in the mutant mice (Fig. 4A–C). Interestingly, in the rostralmost diencephalon, in the most abnormally formed region of the dorsal thalamus, there was displaced expression of Reelin (Fig. 4A). This may be attributable to failure to suppress ventral thalamic territory as suggested recently in chick (Braun et al., 2003). Alternatively, this could also be caused by simple displacement of the ventral thalamus rostrally or ectopic migration of ventral thalamic neurons.

#### Early neuronal defects in LRP6 mutants

To determine whether dorsal thalamic development is deranged in LRP6 mutants caused by early defects in neuron production rather than a secondary destructive lesion, we examined the expression of markers for the dorsal thalamus, epithalamus, ventral thalamus, and the compartmental boundary between them, the ZLI at earlier ages. In coronal brain sections of early embryos (E12), the epithalamic neuronal marker  $\beta 4$  (Bramblett et al., 2002) was normally expressed selectively in epithalamic neurons, whereas in mutants, these cells were missing (Fig. 5). In wild-type mice, *Gbx2* was abundantly expressed in paired stripes of neurons in the dorsal thalamus adjacent to the ventricular zone and were missing at this coronal level in the mutant diencephalon (Fig. 5). The axon guidance molecule *Netrin* and the transcription factor *Nkx2.2* were expressed at the ZLI in wild-type mice but not in the mutants (Fig. 5) (data not shown). *Dlx2* mRNA and protein expression, early markers of ventral thalamic neurons, occupied the area vacated by the dorsal thalamic markers in the mutant embryos (Fig. 5). We found no evidence of increased cell death at any age in the diencephalon of mutant mice (data not shown). Therefore, it seems that in LRP6 mutants, dorsal thalamic and epithalamic neurons were not properly generated and that the ZLI was also not properly formed. The expression of *Dlx2*+ cells more dorsally could be attributable to ectopic production of these cells or abnormal migration because of a breakdown of the normal boundary function of the ZLI (Larsen et al., 2001), perhaps even related to the loss of *Netrin* expression, which can act as a chemorepellant for migrating neurons (Bloch-Gallego et al., 1999).

#### Defects in thalamic neural precursor domains, Wnt ligands, and ZLI signaling molecule function

To determine whether the failure to produce certain types of thalamic neurons was associated with alterations in the distribution of regionally specified neuronal precursor zones, we examined markers that distinguish these precursor domains. The Wnt responsive transcription factor *Tcf4* and the Wnt ligand *Wnt3* were expressed in the ventricular zone of the epithalamus and dorsal thalamus in wild-type mice at this stage, but in mutants, the level of expression and size of the domain of expression were dramatically reduced (Fig. 6). In wild-type mice, the boundary

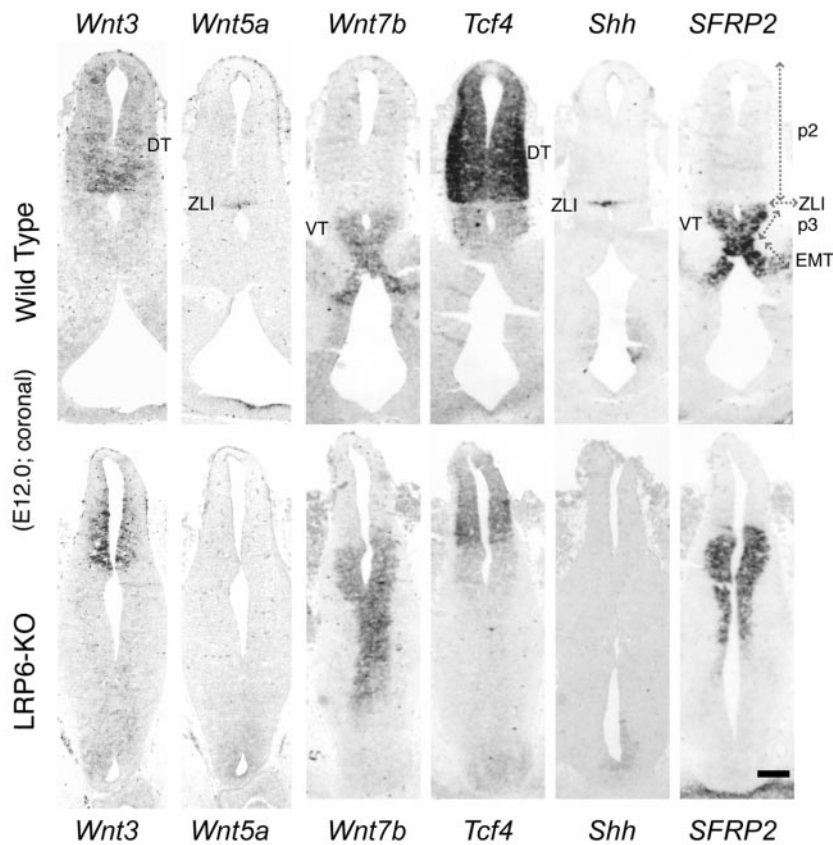


**Figure 5.** Analysis of regional thalamic neuronal cell fate early in thalamic development. The top panels are coronal sections of normal thalamus at E12 stained with  $\beta 4$ , *Gbx2*, *Netrin*, and *Dlx2* that mark differentiated neurons of the epithalamus (Te), dorsal thalamus (DT), ZLI, and ventral thalamus (VT) from dorsal to ventral, respectively. In the bottom panels, no  $\beta 4$  (in Te), *Gbx2* (in DT), or *Netrin* (in ZLI) are expressed in the LRP6-knock-out (KO) thalamus. However, the expression of ventral neuronal marker *Dlx2* (*Dlx2* by *in situ* hybridization and *Dll* by immunofluorescence) is slightly displaced into what should be the dorsal thalamic region (see schematic panel at bottom right). Scale bars, 50  $\mu$ m.

between the dorsal and ventral thalamus (the ZLI) expressed *Shh* and *Wnt5a*, but in LRP6 mutants, the expression of both was missing at E12 in the coronal plane (Fig. 6). In marked contrast, expression of the ventral thalamic ventricular zone markers *SFRP2* (a Wnt inhibitor) and *Wnt7b* (a Wnt ligand) was largely intact (Fig. 6). Because our analysis showed that the dorsal thalamic compartment and the segmental boundary between dorsal and ventral thalamus were abnormal, even at the point when precursor domains were established, it seemed likely that the phenotype begins even earlier in gestation at the time that diencephalic patterning is established. This was supported by our failure to find any obvious alteration in the distribution of dividing cells in the diencephalic ventricular zone between E10.5 and E13.5, indicating that the failure to produce an organized dorsal thalamus was not attributable to wholesale failure of precursor proliferation (data not shown).

#### Early defects in diencephalic development in LRP6 mutants

It was quite apparent from our analysis that the relationship of the ZLI and its expression of *Shh* to the loss of dorsal and epithalamic markers was likely to be important. Previous studies showed that the ZLI initially forms as a discrete compartment in the chick brain that subsequently is restricted to forming the thin boundary domain between prosomeres 2 and 3 (Larsen et al., 2001; Zeltser et al., 2001). We examined wild-type and LRP6 mutant embryos to determine whether ZLI defects were apparent at these earlier stages. Lunatic fringe was reported to be a marker for this domain at early stages of chick development (Zeltser et al., 2001), but we were unable to detect lunatic fringe in this region of the mouse brain (data not shown). By approximately E9.5, the



**Figure 6.** Progenitor zones and compartmentalization of the thalamus. *Tcf4* and *Wnt3* mark the ventricular zone of prosomere 2 (p2), including DT and Te, *Shh* and *Wnt5a* mark the p2/p3 boundary, and *SFRP2* and *Wnt7b* mark the ventricular zone of p3 (VT) and eminentia thalami (EMT). In LRP6 knock-out (KO) mutant brains, only weak expression of *Tcf4* and *Wnt3* is seen and no expression of *Shh* or *Wnt5a*. However, the ventral thalamic ventricular zone markers are intact. It is difficult to be sure whether the ventral thalamic ventricular zone is truly expanded or somewhat displaced rostrally because of the smaller size of the rostral dorsal thalamus. Scale bars, 50  $\mu$ m.

*Shh* expression spreads from the floor plate in a spur of expression to form the ZLI expression domain (Ishibashi and McMahon, 2002), and we were easily able to confirm this spur of expression in wild-type embryo brains by whole-mount *in situ* hybridization (Fig. 7A). Between E10.5 and E11.5, *Gbx2* expression also became well established in the forming dorsal thalamus (Fig. 7B,C). In contrast, in mutant embryos, *Shh* expression did not fully extend from the floor plate dorsally, and *Gbx2* was missing entirely at these ages (Fig. 7A–C).

There are no reliable specific markers for the future dorsal thalamus before the formation of the ZLI. However, recently in chick, *Ir3* was shown to be a marker for the forebrain posterior to the ZLI and *Six3* a marker anterior to the ZLI from early in gestation (Braun et al., 2003). We were able to confirm that *Ir3* was expressed in the future dorsal thalamus and pretectum (prosomeres P1 and P2) (Fig. 7C) but could not confirm the expression of *Six3* in the future ventral thalamus, rather, it was expressed in the hypothalamus and eye fields (data not shown). In LRP6 mutants, the *Ir3* expression domain was considerably decreased in distribution (Fig. 7C). This supports the idea that dorsal thalamic development was defective from the earliest developmental stages.

## Discussion

Our analysis with a variety of markers shows that the formation of the dorsal thalamus is disrupted in LRP6 mutant mice. This de-

fect is first apparent very early in the development of the diencephalon, with early defects in the expression of a posterior forebrain marker and a failure to properly form the boundary between dorsal and ventral thalamus.

### Defects in ZLI signaling molecule expression in LRP6 mutants

The failure of *Shh* expression in the ZLI in LRP6 mutant mice to properly extend dorsally and the abnormalities in two *Shh* pathway responsive genes (*Nkx2.2* and *Netrin*) provide strong evidence that *Shh* signaling is defective locally in the diencephalon. We also found that *Wnt5a*, a noncanonical Wnt ligand, expression is missing at the ZLI. Thus, normal Wnt signaling early in diencephalic development is required for the production of this secondary signaling structure. Whether the loss of dorsal thalamic neurons is a reflection of a cell autonomous loss of Wnt signaling in the dorsal thalamus or attributable to a secondary loss of *Shh* signaling is currently unclear. However, the even earlier alterations in *Ir3* expression and the previous studies in chick imply that there is a direct requirement for Wnt signaling (Braun et al., 2003).

Interestingly, a recent study showed that loss of *Shh* signaling in *Shh* null mice also leads to diencephalic defects. These mice lack *Shh* protein in the floorplate from early development and in the ZLI later and also have reduced *Tcf4* expression in the thalamus (Ishibashi and McMahon, 2002). Also, a recent analysis of

the role of *Shh* signaling in thalamic development in chicks showed that *Shh* directly induces dorsal thalamic markers in tissue explants (Hashimoto-Torii et al., 2003). These studies, together with the work described here and the recent analysis of the direct role of Wnt ligands in diencephalic development (Braun et al., 2003), imply that integrity of both pathways is crucial. Whether the Wnt and *Shh* pathways directly collaborate molecularly or work separately to induce dorsal thalamic development remains to be examined.

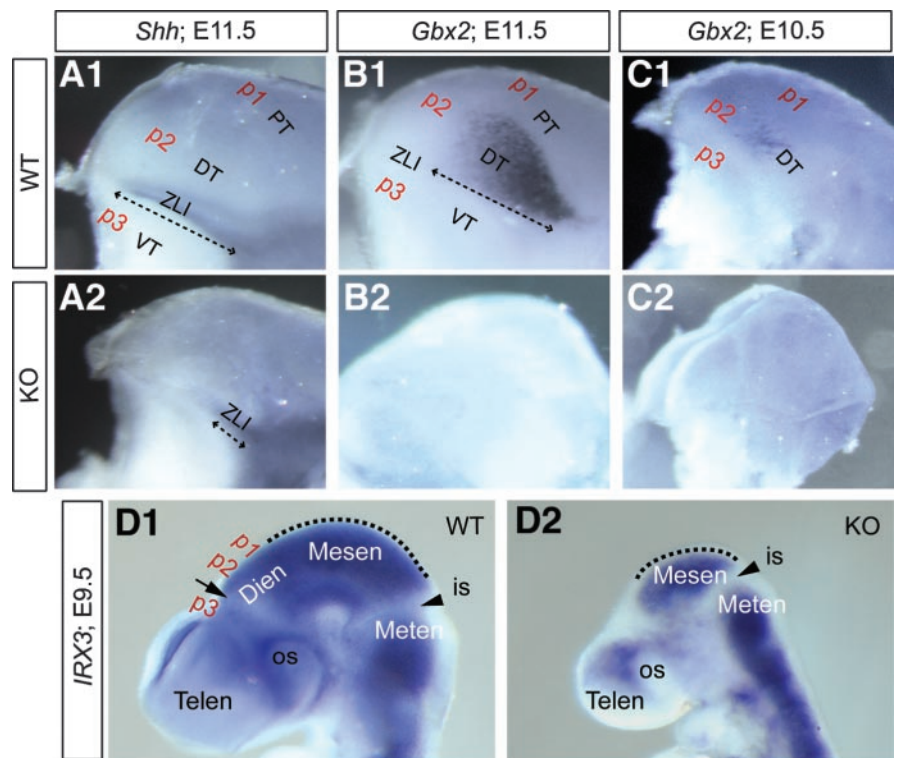
### Is there a reiterative role of Wnts in anteroposterior patterning in the brain?

Recent studies in zebrafish showed that a gradient of Wnt signaling regulates the anteroposterior partitioning of the brain with high Wnt tone inducing diencephalic fate at the expense of more anterior structures, whereas inhibition of high Wnt tone is required to induce the formation of the telencephalon and eyes (Masai et al., 1997; Heisenberg et al., 2001; Houart et al., 2002). Other studies showed regulation of anteroposterior neural fate in the neural tube as a whole in chick by Wnts (Nordstrom et al., 2002; Gunhaga et al., 2003). Taken with this earlier work, the recent study showing that Wnts are also required to properly induce posterior patterning of the diencephalon in chick supports a repeated role for Wnt signaling in regulating A-P development of several forebrain compartments during development.

Our analysis is quite consistent with these studies in that we found loss of dorsal–posterior thalamus and the boundary between anterior and posterior thalamus in the diencephalon in the setting of decreased Wnt signaling tone. The importance of the LRP6 mutants is that they extend this phenomenon to mammals and establish that Wnt signaling is required. It is likely that LRP6 mutants are so useful only because of the redundancy with LRP5; however, it remains to be seen whether these receptors are functionally identical in signaling activity but differ only subtly in expression levels and timing. A recent study examining this issue also showed a role for LRP6 and LRP5 in A-P patterning in the early embryo and showed that compound mutants of these two receptors are far more severely affected and at earlier stages (Kelly et al., 2004). Thus, it seems likely that Wnt signaling regulates anteroposterior development of the nervous system in a reiterated manner at multiple stages of embryonic development to allow proper axis formation within each successively more restricted brain compartment. This would make the role of Wnt signaling in brain development functionally quite similar to neural crest development, where recent work showed reiterative roles for Wnt signaling over several developmental stages (Lewis et al., 2004).

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**Figure 7.** Early diencephalic defects in LRP6 mutants. *A1–C2*, Whole-mount *in situ* hybridization for *Shh* and *Gbx2* in E10.5 and E11.5 brains. In E11.5 wild-type brains, *Shh* extends from the ventral floorplate in two spurs that project to the roofplate, whereas in mutant brains, this domain of expression stops less than midway (dashed arrow line). *Gbx2* expression is visible weakly at E10.5 and much more strongly by E11.5 in wild-type mice but is essentially absent in mutant brains at these time points. Note that the telencephalon was dissected free to allow ease of visualization of the diencephalon. *D1, D2*, Whole-mount *in situ* hybridization of *Irx3* in E9.5 embryos shows a defect in the caudal dorsal diencephalic domain in the mutants even before thalamic neuronal differentiation (see E10.5 in *C1* for the earliest onset of *Gbx2* expression). The arrow indicates the future ZLI, the boundary between dorsal and ventral thalamus. The arrowhead indicates the isthmus, the midbrain–hindbrain junction (which appears intact). The *Irx3* expression territory between the ZLI and isthmus in the mutants is much smaller than in wild type. Dien, Diencephalon; DT, dorsal thalamus; is, isthmus; Mesen, mesencephalon; Meten, metencephalon; os, optic stalk; p1, 2, 3, prosomere 1, 2, 3; PT, pretectum; Telen, telencephalon; VT, ventral thalamus.

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