nature neuroscience

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Hedgehog signaling from the ZLI regulates diencephalic regional identity

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The zona limitans intrathalamica (ZLI), a narrow compartment in the vertebrate forebrain that bisects the diencephalon transversely, expresses the secreted factor sonic hedgehog (Shh). Because genetic disruption of *Shh* in mouse causes severe early developmental defects, this strategy has not been useful in identifying a ZLI-specific role for this gene. To modulate Shh signaling in a spatiotemporally restricted manner, we carried out gain- and loss-of-function experiments in chick embryos using *in ovo* electroporation and found that Shh signaling is required for region-specific gene expression in thalamus and prethalamus, the major diencephalic brain areas flanking the ZLI. We further show that differential competence of thalamic and prethalamic primordia in responding to Shh signaling is regulated by the transcription factor Irx3. We show that, through the release of Shh, the ZLI functions as a local signaling center that regulates the acquisition of identity for these important diencephalic regions.

Development of the vertebrate nervous system involves compartition of the embryonic neuroepithelium and the activity of local signaling centers[1–4]. Various attempts have been made to describe the embryonic forebrain as a segmented structure[5], culminating in the 'prosomeric model,' which proposes a metameric groundplan for the forebrain based principally on morphology and marker expression[6,7]. Recent lineage-tracing experiments have revealed two cell-lineage-restriction boundaries in the early avian forebrain[8]. These flank a wedge-shaped region amounting to approximately one-third of the forebrain anlage, which is characterized by a gap in the expression of *Lfng*[9]. Subsequently this wedge undergoes a marked reduction in size relative to other parts of the brain such that it seems to 'collapse' into a narrow band of cells that separates the thalamus (previously referred to as the dorsal thalamus) from the prethalamus (previously ventral thalamus; see ref. 7 for terminology, but see ref. 10)[8,9]. This band of cells is known as the zona limitans intrathalamica (ZLI) and may mark the interface between regions of differential competence to respond to inductive signals (**Fig. 1**)[4,11,12]. It has been proposed that, in the early embryo, the site of prospective ZLI formation is marked by the abutting expression domains of two homeobox genes, *Six3* anteriorly and *Irx3* posteriorly, both of which have been suggested to mediate differential competence at pre-ZLI stages[12,13]. Activation of the Wnt signaling pathway induces *Irx3* and represses *Six3*, suggesting that the anteroposterior location of ZLI formation is a direct consequence of the earliest signals that pattern the neural plate[13].

Once the *Lfng*-free wedge has narrowed to form the definitive ZLI, it starts to express sonic hedgehog (Shh)[9], a member of the hedgehog family of secreted signaling molecules. Elsewhere, Shh is expressed first in the axial mesendoderm underlying the neural plate and later in the ventral midline along the entire neuraxis[14,15]. One of the best-characterized functions of Shh is its ventralizing effect on the neural tube: numerous studies have shown that a ventral-to-dorsal gradient of Shh activity is both necessary and sufficient to specify neuronal subtypes within ventral spinal cord and hindbrain[16–18]. Similarly, Shh regulates the development of ventral fore- and midbrain[19–21]. In addition to its roles in patterning, Shh promotes growth and proliferation[22,23], functions in axon guidance[24], maintains stem cell character[25] and may act as a survival factor[26]. Targeted disruption of the *Shh* gene in mouse produces embryos with microcephaly and cyclopia, underlining that the protein is required for ventral specification, midline formation and growth of the neural tube[27]. Similarly, mutations in the human *SHH* gene result in holoprosencephaly, a birth defect characterized by forebrain and craniofacial malformations[28].

For a considerable period during development, the ZLI is the only place in the neural tube where Shh expression expands dorsally and reaches into the alar plate, forming a conspicuous 'peak' within the posterior part of the forebrain, the diencephalon (**Fig. 1**). This observation prompted us to ask whether the ZLI might act as a local signaling center that regulates the development of the adjacent thalamic regions through Shh activity. Diencephalon and midbrain are significantly reduced in size in $Shh^{-/-}$ mice, indicating that Shh promotes growth of these tissues[29]. The knockout approach[27], however, does not permit the timing of different Shh activities to be dissected and, indeed, undergrowth of the entire brain is already detectable at embryonic day 9.5 (E9.5), before the definitive ZLI has formed[29]. Thus, we performed *in ovo* electroporation experiments in chick embryos, which allows spatially and temporally defined modulation of Shh signaling in the diencephalon at later stages[30]. We show that the identities of both thalamus (posterior to the ZLI) and prethalamus (anterior to the ZLI) depend on Shh signaling and that these two regions respond differently to Shh. We further show that ectopic misexpression of the transcription factor Irx3 is sufficient to provide the

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Published online 24 October 2004; doi:10.1038/nn1338

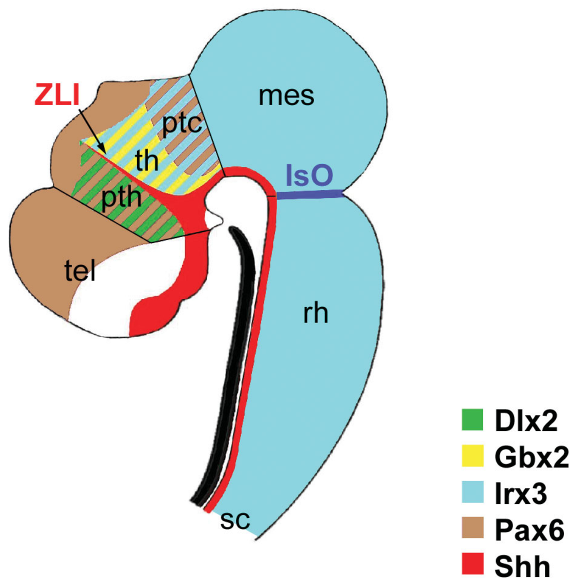

Figure 1 Schematic illustration of embryonic chick brain. Expression patterns of regionally expressed genes are color coded. The notochord, a mesodermal signaling center, is shown in black. A 'peak' of Shh expression is visible in the ZLI (red). IsO, isthmic organizer (midbrain-hindbrain junction); mes, mesencephalon (midbrain); ptc, pretectum; pth, prethalamus; rh, rhombencephalon (hindbrain); sc, spinal cord; tel, telencephalon; th, thalamus; ZLI, zona limitans intrathalamica.

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prethalamus with thalamic competence to respond to Shh. Our results show that the ZLI is a local signaling center that regulates development of the adjacent diencephalic primordia and that differences between these regions are defined by a prepattern conferred by the differential expression of transcription factors such as Irx3.

RESULTS

Hedgehog signaling on both sides of the ZLI

The definitive ZLI expresses Shh along its full dorsoventral extent from chick stage HH18 onwards[9] (see Methods). To test whether hedgehog signaling is active adjacent to the ZLI, we analyzed by *in situ* hybridization the expression of two *bona fide* target genes of the Shh pathway: *Nkx2-2*, encoding a homeodomain transcription factor[11,31,32], and *Ptc*, which encodes patched, a multipass transmembrane protein of the Shh receptor complex[15,33,34]. Both *Nkx2-2* and *Ptc* are expressed in thalamus and prethalamus in two domains straddling the ZLI from stage HH18 onwards (**Fig. 2a,b**). The induction is strongest close to, and gradually fades with increasing distance from, the ZLI. Hence, Shh target genes are expressed in thalamus and prethalamus, suggesting that Shh from the ZLI activates its signaling pathway in neighboring tissues.

We also examined the expression of dispatched (encoded by *Disp*), a modulator of hedgehog proteins that is conserved between fly and mouse and is required for long-range Shh signaling[35–37]. We searched the available mouse *Disp* sequences against the BBSRC ChickEST Database (http://www.chick.umist.ac.uk) for avian *Disp* orthologs using the BLAST algorithm and found an EST with 78% identity to mouse *Disp1* (ChEST 700n22; E value: 3E-31). *In situ* hybridization using a probe derived from this clone showed that, like mouse *Disp1*, the gene corresponding to this EST is expressed in the vicinity of Shh expression and is therefore likely to represent chick *Disp1* (not shown). *Disp1* is also expressed in the ZLI, where it may function to release Shh, enabling long-range signaling (**Fig. 2c**).

Zinc finger transcription factors of the Gli family transduce hedgehog signals intracellularly[23]. All three *Gli* genes are expressed broadly in the diencephalon, except for the ZLI itself, and may thus act in concert to transduce Shh signals (**Fig. 2d–f**)[38]. Taken together, these observations support the idea that Shh signaling is able to operate on both sides of the ZLI.

Asymmetric homeobox gene expression flanking the ZLI

The diencephalic areas flanking the ZLI, the thalamus and prethalamus, are marked by the differential expression of homeobox genes. Before stage HH18, *Gbx2* is expressed at low level throughout the undifferentiated neuroepithelium of the prospective thalamus, and it is likely that this expression depends on Wnt signaling (**Fig. 2j**)[13]. At later stages, as neurogenesis begins, a more distinctive *Gbx2* expression becomes detectable in the mantle zone of the thalamic primordium[8]. The weak expression throughout the neuroepithelium

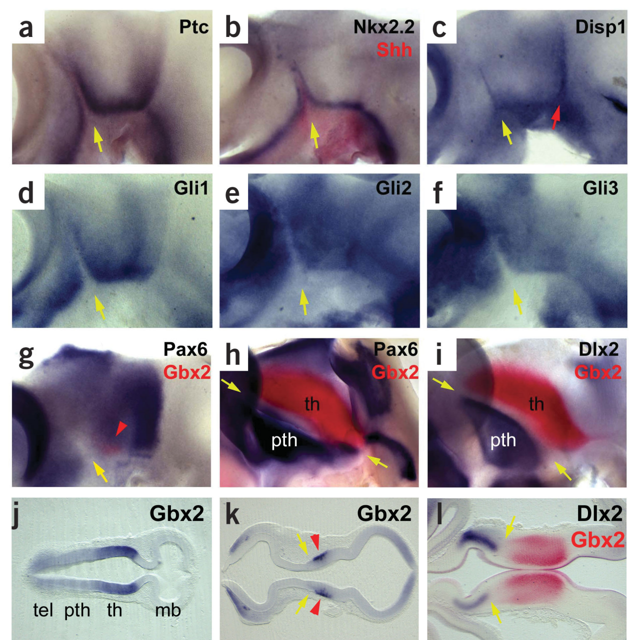

Figure 2 Gene expression in the diencephalon at definitive ZLI stages. (**a**–**k**) Gene expression was analyzed at HH20 (**a**–**g**), E5 (**h**,**i**,**l**), HH16 (**j**) and HH18 (**k**), respectively, by *in situ* hybridization for *Ptc* (**a**), *Shh* (red) and *Nkx2-2* (blue) (**b**), *Disp1* (**c**), *Gli1* (**d**), *Gli2* (**e**), *Gli3* (**f**), *Gbx2* (red) and *Pax6* (blue) (**g**,**h**), *Gbx2* (red) and *Dlx2* (blue) (**i**,**l**), and *Gbx2* (blue) (**j**,**k**). (**a**–**i**) Lateral views facing the ventricular surface of hemisected brains (mid-sagittal) are shown; anterior points to the left, dorsal to the top. The ZLI is highlighted with yellow arrows. Expression of *Disp1* in the forebrain-midbrain boundary (red arrow) and a ventral patch of *Gbx2* expression in **g** (red arrowhead) are visible. The strong *Gli2* and *Gli3* expression on the top left side of panels **e** and **f**, respectively, are in the dorsal telencephalon. (**j**–**l**) Coronal sections through diencephalon; anterior points to the left. *Gbx2* is expressed at low level throughout thalamic neuroepithelium (th) in **j** (embryo was deliberately overstained) and strongly induced in the mantle zone next to the forming ZLI in **k**. Expression of both *Dlx2* and *Gbx2* is absent from the ventricular layer in **l**. mes, mesencephalon; pth, prethalamus; tel, telencephalon; th, thalamus.

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becomes downregulated at this stage (**Fig. 2k,l**). The second wave of *Gbx2* induction starts at stage HH19 in a small ventral patch of the thalamus adjacent to where the ZLI merges with the floor plate (**Fig. 2g**), and subsequently expands dorsally and posteriorly until it covers the thalamic area completely from E5 (HH26) onwards (**Fig. 2h**)[8,39]. It is this second phase of *Gbx2* expression that we have analyzed in this study.

Pax6 is a homeobox gene expressed in various parts of the developing neural tube[40]. Early *Pax6* expression marks the entire forebrain (**Fig. 2g**). After stage HH18, it becomes downregulated in the thalamus in a dorsally and posteriorly expanding region, virtually mirroring the upregulation of *Gbx2*, until the entire thalamus is devoid of *Pax6* expression (**Fig. 2h**).

Anterior to the ZLI, *Dlx2* marks the prethalamus from stage HH19 onwards (**Fig. 2i**) and its expression is restricted to the mantle zone, as for *Gbx2* (**Fig. 2l**)[7]. Notably, *Pax6* does not become downregulated in the prethalamus (**Fig. 2h**). In summary, a marked asymmetry between thalamus and prethalamus becomes apparent by their differential expression of homeobox genes (**Fig. 1**).

Hedgehog promotes thalamic and prethalamic gene expression

To test whether Shh signaling promotes thalamic development at definitive ZLI stages, we overexpressed Shh by unilateral electroporation of an expression construct, pXeX-Shh[21], into the diencephalon of stage HH18 embryos. First, we analyzed the expression of *Shh*, *Nkx2-2* and *Ptc* in electroporated embryos at E3.5 (HH21-22) to ensure that the Shh pathway had been activated efficiently. Ectopic *Shh* mRNA was readily detectable in the electroporated area (n = 3; **Fig. 3a–c**), and *Ptc* (n = 4; **Fig. 3d–f**) and *Nkx2-2* (n = 3; not shown) were upregulated on the electroporated side both anterior and posterior to the ZLI. We are thus confident that we are able to force Shh signaling efficiently and in a spatially restricted manner in the early diencephalon.

To assay the effect of overactivating Shh signaling in the thalamus, we analyzed the expression of *Gbx2* at E5. Electroporation of pXeX-Shh resulted in a posterior expansion of *Gbx2* expression (n = 7; **Fig. 3g–i**). Next we asked whether forced Shh signaling also affects the prethalamus and found that *Dlx2* expression was expanded after overactivation of the Shh pathway (n = 5; **Fig. 3j–l**). Taken together, these data indicate that Shh signaling promotes an expansion of regionally specific marker gene expression in the areas flanking the ZLI.

Shh has been shown to negatively regulate *Pax6* expression in the spinal cord[31], prompting us to ask whether it is also able to downregulate *Pax6* in the thalamus. Electroporation of pXeX-Shh resulted in premature downregulation of *Pax6* expression in the thalamus, but not in the prethalamus, suggesting that the two regions have different competence to respond to Shh (n = 5; **Fig. 3m–o**).

Requirement for hedgehog in thalamus and prethalamus

To analyze whether Shh signaling not only promotes, but also is required for, prethalamic and thalamic development, we electroporated an expression construct encoding a mutated form of mouse Ptc, pmPtc1$^{\Delta loop2}$-IRES-GFPnls[41], at stage HH18. The Ptc$^{\Delta loop2}$ mutant does not bind Shh and renders receiving cells insensitive to it, as shown previously in *Drosophila* and in chick spinal cord[41]. First, we analyzed the expression of *Nkx2-2* and *Ptc* at E5 in electroporated embryos. Electroporation resulted in perturbed expression of both marker genes, with patches of cells that do not express *Nkx2-2* (n = 4; **Fig. 4a–c**) or *Ptc* (n = 3; **Fig. 4d–f**) both anterior and posterior to the ZLI. Notably, the patches that did not express *Nkx2-2* or *Ptc* did express green fluorescent protein (GFP) and, consequently, also Ptc$^{\Delta loop2}$ (insets in **Fig. 4c,f**). We conclude that

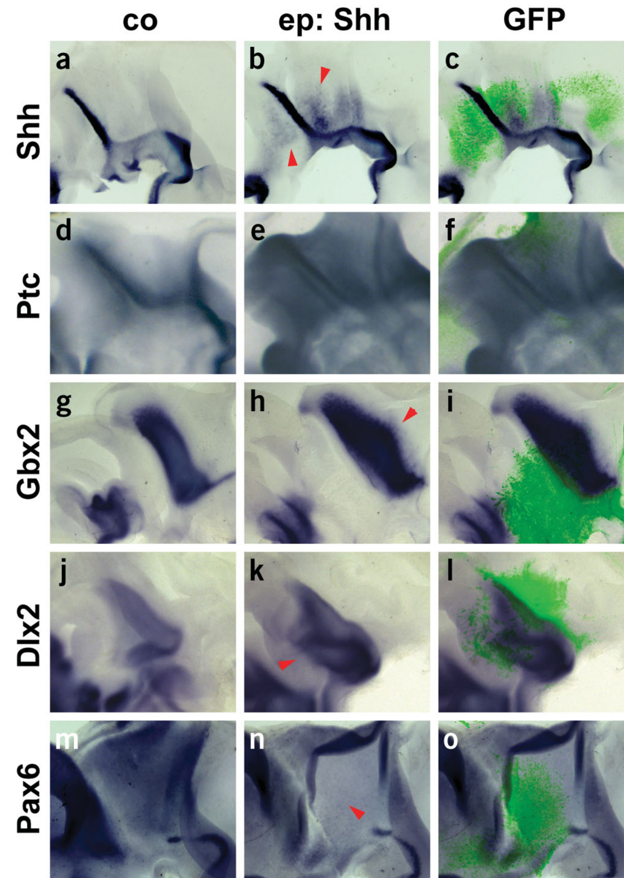

Figure 3 Overexpression of Shh results in expansion of thalamus and prethalamus. (**a**–**o**) Embryos were coelectroporated with pXeX-Shh and pCAβ-eGFP at stage HH18, cultured to E3.5 (**a**–**f**, **m**–**o**) or E5 (**g**–**l**) and analyzed by *in situ* hybridization for *Shh* (**a**–**c**), *Ptc* (**d**–**f**), *Gbx2* (**g**–**i**), *Dlx2* (**j**–**l**) and *Pax6* (**m**–**o**), followed by immunochemical detection of GFP. Lateral views facing the ventricular surface of hemisected brains (mid-sagittal) are shown; anterior points to the left, dorsal to the top. (**a**,**d**,**g**,**j**,**m**) Nonelectroporated control side (co); (**b**,**e**,**h**,**k**,**n**) electroporated (ep); (**c**,**f**,**i**,**l**,**o**) overlay with anti-GFP fluorescence in green. Expanded expression of *Shh*, *Gbx2* and *Dlx2* and thalamic downregulation of *Pax6* (red arrowheads) are visible.

hedgehog signaling was blocked in a mosaic pattern that corresponded to the pattern of electroporation.

To assay the effect of blocking hedgehog signaling in the thalamus, we analyzed the expression of *Gbx2*. Electroporation of Ptc$^{\Delta loop2}$ resulted in patches of thalamic cells that did not express *Gbx2* (n = 13; **Fig. 4g–i**). Thus, thalamic cells require functional hedgehog signaling at definitive ZLI stages in order to express *Gbx2*. Next we asked whether prethalamic gene expression also requires hedgehog. Similar to *Gbx2* in the thalamus, prethalamic cells expressing Ptc$^{\Delta loop2}$ did not express *Dlx2* (n = 6; **Fig. 4j–l**), indicating that hedgehog signaling is required at definitive ZLI stages for prethalamic regional identity.

We have shown above that Shh is able to downregulate *Pax6* expression in the thalamus (**Fig. 3m–o**). Conversely, Ptc$^{\Delta loop2}$-expressing thalamic cells did not downregulate *Pax6*, resulting in *Pax6*-positive patches in the otherwise *Pax6*-negative thalamus. To our surprise, *Pax6* expression was also affected in the prethalamus of the same embryos, where electroporated cells now did not express *Pax6* (n = 6;

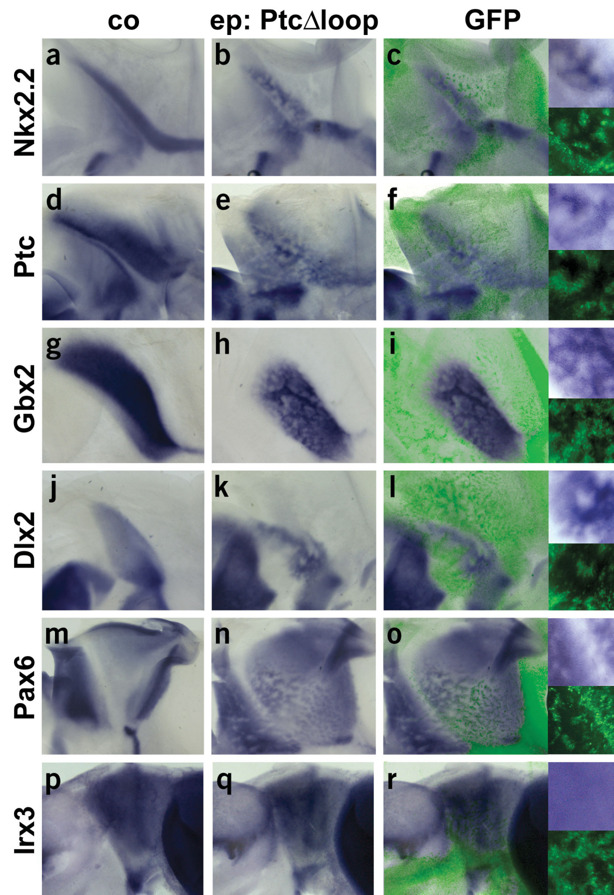

Figure 4 A requirement for Shh signaling in prethalamus and thalamus. Embryos were electroporated with pmPtc1$^{\Delta loop2}$-IRES-GFPnls at stage HH18, cultured to E5 and analyzed by *in situ* hybridization for (**a**–**c**) *Nkx2-2*, (**d**–**f**) *Ptc*, (**g**–**i**) *Gbx2*, (**j**–**l**) *Dlx2*, (**m**–**o**) *Pax6* and (**p**–**r**) *Irx3*, followed by immunochemical detection of GFP. Lateral views facing the ventricular surface of hemisected brains (mid-sagittal) are shown; anterior points to the left, dorsal to the top. (**a**,**d**,**g**,**j**,**m**,**p**) Nonelectroporated control side (co); (**b**,**e**,**h**,**k**,**n**,**q**) electroporated (ep); (**c**,**f**,**i**,**l**,**o**,**r**) overlay with anti-GFP fluorescence in green. Insets in panels **c**,**f**,**i**,**l**,**o**,**r** show the *in situ* signal (upper) and the corresponding anti-GFP fluorescence (lower) of a representative thalamic area at higher magnification.

Fig. 4m-o). These results are in line with our observations following Shh overexpression and reveal an asymmetry between thalamus and prethalamus with respect to their competence to respond to Shh: whereas in the thalamus Shh is required to downregulate *Pax6*, in the prethalamus it is necessary for maintenance of *Pax6*.

The homeobox gene *Irx3* is expressed posterior to the ZLI and has been proposed to mediate differential competence to respond to inductive signals at early stages[12]. Expression of *Irx3* is largely unaffected in Ptc$^{\Delta loop2}$-expressing thalamic cells ($n = 6$; **Fig. 4p–r**), indicating that blocking Shh signaling does not result in a global downregulation of regional gene expression and supporting the notion that our loss-of-function approach is specific.

A requirement for hedgehog signaling in the ZLI

In the floor plate, Shh is involved in a positive feedback loop promoting its own expression. To test whether a comparable mechanism is active in the ZLI, we analyzed ZLI marker genes in embryos expressing Ptc$^{\Delta loop2}$ and found that the expression of both *Shh* ($n = 5$; **Fig. 5a–c**) and *Foxa2* ($n = 3$; formerly *Hnf3β*, **Fig. 5d–f**) is downregulated in the ZLI on the electroporated side. This suggests that hedgehog signaling in the ZLI is required for the maintenance of ZLI-specific gene expression, including the expression of *Shh* itself. At first sight, this finding seems to contradict the apparent lack of expression of the Shh receptor Ptc within the ZLI (**Figs. 2a**, **3d** and **4d**). However, *in situ* hybridization does not reflect absolute levels of gene expression and our results suggest that *Ptc* may be expressed at low levels within the ZLI itself while it is strongly upregulated in its vicinity. In addition, Ptc protein may persist for some time in cells that have earlier downregulated *Ptc* transcription.

Contributions of ZLI and ventral midline

Although the relative topography of thalamus, ZLI and prethalamus, as well as the spatiotemporal dynamics of gene expression (with *Dlx2*, *Gbx2* and *Pax6* following *Shh* in a characteristic ventral-to-dorsal 'wave' of induction and downregulation), strongly suggest a specific role for Shh signaling from the ZLI, our results do not strictly distinguish between ZLI- and ventral midline-derived signals. However, closer inspection of the Ptc$^{\Delta loop2}$ overexpression results showed both autonomous and nonautonomous effects on marker gene expression. In particular, besides a patchy downregulation of *Gbx2*, the overall size of the expression domain of *Gbx2* was reduced in a majority of cases (8/13; **Fig. 4g–i**). Notably, the *Gbx2* domain does not appear to reach as far dorsally as on the unelectroporated side of the diencephalon. In combination with the earlier observation that Shh is required for its own maintenance within the ZLI (**Fig. 5a–c**), this is most simply explained by a downregulation of Shh in the ZLI that in turn results in a reduced induction of *Gbx2* in the thalamus. To specifically interfere with Shh signaling from the ZLI, we electroporated Ptc$^{\Delta loop2}$ dorsolaterally into the ZLI region, thereby avoiding local effects on ventral midline–derived signaling. Double *in situ* hybridization revealed a correlation between the dorsoventral extents of *Shh* expression in the ZLI and of *Gbx2* expression in the thalamus ($n = 5$; **Fig. 6a–c**). Although an absolute discrimination between ZLI- and ventral midline–derived Shh is probably not possible in our system, the interpretation that the dorsal extension of *Shh* expression in the ZLI is required for proper gene expression in thalamus and prethalamus seems inescapable in light of our observations.

Irx3 regulates thalamic competence

We have shown that region-specific gene expression in both thalamus and prethalamus depends on Shh signaling from the ZLI. This raises the question of how one signal can elicit different responses on either side of its source and implicates a prepattern of differential competence in the responding tissues. The homeobox gene *Irx3*, an ortholog of the iroquois family of prepattern genes in *Drosophila*[42], is expressed posterior to the ZLI (**Figs. 4p** and **7a**) and has been proposed to mediate differential competence to inductive signals at pre-ZLI stages[12]. The Irx3-related Irx2 has recently been shown to regulate cellular competence in the anterior hindbrain[43]. This prompted us to ask whether Irx3 might mediate differential competence in response to ZLI-derived Shh signaling. We electroporated an Irx3 expression construct, pCAGGS-cIrx3[12], into the prethalamic area of stage HH18 embryos and analyzed the expression of *Gbx2* and *Sox14* at E5. *Sox14* encodes a member of the HMG superfamily of transcription factors that has been shown to be regulated by Shh in the spinal cord[44]. Its expression domain in the thalamus is narrower

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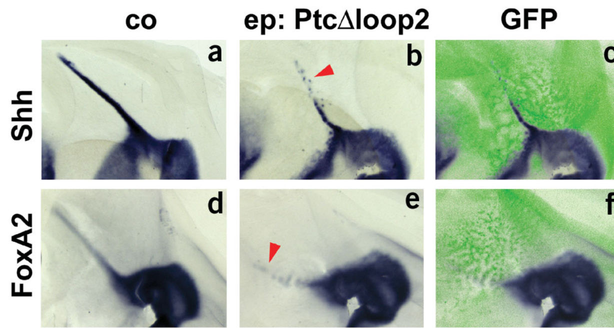

Figure 5 Shh signaling is required for ZLI-specific gene expression. Embryos were electroporated with pmPtc1$^{\Delta loop2}$-IRES-GFPnls at stage HH18, cultured to E5 and analyzed by *in situ* hybridization for *Shh* (**a**–**c**) and *FoxA2* (**d**–**f**), followed by immunochemical detection of GFP. Lateral views facing the ventricular surface of hemisected brains (mid-sagittal) are shown; anterior points to the left, dorsal to the top. (**a**,**d**) Nonelectroporated control side (co); (**b**,**e**) electroporated (ep); (**c**,**f**) overlay with anti-GFP fluorescence in green. Both ZLI markers are downregulated (red arrowheads).

than that of *Gbx2*, suggesting that its induction requires higher levels of Shh signaling[38]. Both *Gbx2* and *Sox14* were ectopically induced following expression of Irx3 in the prethalamus (n = 18 and 5, respectively; **Fig. 7b–d**). *Sox14* was consistently induced closer to the ZLI, resulting in a 'mirror image' duplication of thalamic marker gene expression. By contrast, *Dlx2* becomes downregulated in prethalamic cells ectopically expressing Irx3 (n = 4; **Fig. 8a**,**b**).

Although *Pax6* expression is downregulated by Shh in the thalamus, it seems to depend on Shh signaling in the prethalamus (**Fig. 4m–o**). Is this differential response also regulated by Irx3? Ectopic expression of Irx3 in the prethalamic anlage resulted in patches of cells not expressing *Pax6* (n = 3; **Fig. 8c**,**d**). In conclusion, Irx3 expression in the prethalamus results in expression of thalamic marker genes and in repression of prethalamic marker genes.

The above results can be interpreted in two ways: either Irx3 regulates cellular competence to respond to Shh signaling or it is able to induce thalamic gene expression on its own. In the first case, it would act as a prepattern gene, which modulates the response to an inductive signal (Shh), and blocking this signal would prevent expression of downstream genes such as *Gbx2*, even if Irx3 is present. In the second case, a diffusible signal would be dispensable for downstream gene expression. To distinguish these possibilities, we coexpressed Irx3 with the blocking receptor Ptc$^{\Delta loop2}$ in the prethalamus and noted widespread expression of GFP and *Irx3* but only few *Gbx2*-positive cells (n = 6; **Fig. 8g**,**h**), whereas electroporation with Irx3 alone resulted in robust *Gbx2* induction (**Fig. 8e**,**f**). Thus, blocking the Shh pathway in prethalamic cells rescues the effect of overexpressing Irx3. These results suggest that Irx3 indeed acts as a competence factor modulating the cellular response to Shh rather than as an instructive upstream regulator of thalamic development.

DISCUSSION

The ZLI acts as a local signaling center

The establishment of cell lineage restriction and the consequential formation of compartments are crucial features of insect development. One important function of lineage restriction is to stabilize local signaling centers at compartment boundaries. Development of the vertebrate nervous system also involves compartition and largely depends on the simultaneous and sequential activity of various local signaling centers[1–4]. However, evidence for signaling centers that form along lineage restriction boundaries is essentially lacking to date. So far, the best case has been made for the 'isthmic organizer' (IsO; **Fig. 1**) that regulates development of the adjacent midbrain and hindbrain regions through the action of fibroblast growth factors[45–47]. Here we have provided evidence that the ZLI, a lineage-restricted compartment in the diencephalon[8–10], acts as a local signaling center that regulates development of its flanking regions by expressing Shh. We have shown that (i) local overexpression of Shh results in expansion of thalamic and prethalamic marker gene expression, (ii) blocking Shh signaling by means of a constitutively active form of Ptc inhibits the robust induction of these markers in postmitotic cells of the thalamic primordia and (iii) the differential response of thalamus and prethalamus to Shh is mediated, at least in part, by Irx3 (see **Supplementary Fig. 1** online). An absolute distinction between ZLI- and ventral midline–derived Shh may not be possible at this stage. However, the expression dynamics of the marker genes analyzed here strongly suggest a requirement for Shh expression in the ZLI, for example for *Gbx2* induction in the thalamus. This notion is strengthened by the observation of nonautonomous effects following Ptc$^{\Delta loop2}$ overexpression that are most simply explained by a reduction of Shh expression in the ZLI.

Sonic hedgehog in diencephalic development

The disproportionate undergrowth of the diencephalic primordium compared to other parts of the brain in *Shh* knockout mice suggests a specific requirement for Shh signaling in diencephalic development[27]. Expression of *Ccnd1* (encoding cyclin D1) in diencephalon and midbrain depends on Shh, indicating a role in promoting proliferation[29]. However, later requirements for Shh signaling in the diencephalon may be concealed by the severe early defects of knockout mice, which become evident long before the ZLI has formed. Through *in ovo* electroporation in chick embryos, we have been able to specifically characterize later effects of Shh signaling at definitive ZLI stages, which extend beyond growth promotion into the establishment of regional identity. On the basis of the observation in the *Shh* knockout that *Ccnd1* expression and growth are affected in both

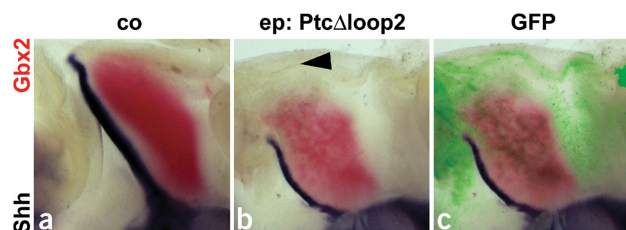

Figure 6 A 'dorsal truncation' of the ZLI results in a reduced *Gbx2* expression domain. Embryos were electroporated with pmPtc1$^{\Delta loop2}$-IRES-GFPnls at stage HH18, cultured to E5 and analyzed by *in situ* hybridization for *Shh* (blue) and *Gbx2* (red), followed by immunochemical detection of GFP. Lateral views facing the ventricular surface of hemisected brains (mid-sagittal) are shown, anterior points to the left, dorsal to the top. (**a**) Nonelectroporated control side (co). (**b**) Electroporated (ep). (**c**) Overlay with anti-GFP fluorescence in green. Reduced expression of *Shh* in the ZLI corresponds to a smaller *Gbx2* expression domain that does not extend as far dorsally as on the control side (black arrowhead).

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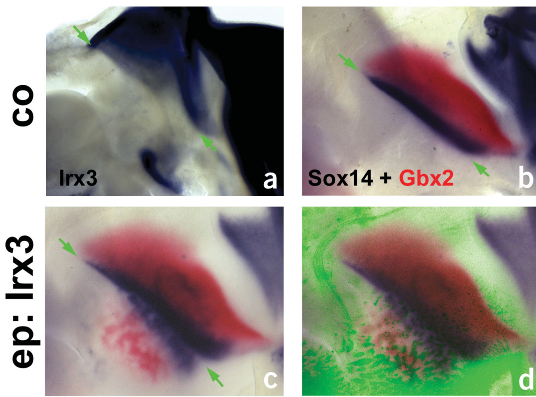

Figure 7 Ectopic Irx3 induces thalamic gene expression in the prethalamus. (**a**–**d**) E5 embryos stained for expression of *Irx3* (**a**) and of *Sox14* (blue) and *Gbx2* (red) (**b**–**d**). *Irx3* is expressed in thalamus and midbrain in **a**. The anterior limit of *Irx3* expression abuts the ZLI. (**b**) Nonelectroporated control side (co). (**c**) Electroporated with pCAGGS-cIrx3 at stage HH18 (ep). (**d**) Overlay with anti-GFP fluorescence in green. 'Mirrored' duplication of thalamic marker gene expression is visible in **c**,**d**. The ZLI is highlighted with green arrows.

ventral and dorsal diencephalon, it has been proposed that a signaling relay transduces the Shh signal to dorsal parts of the brain[29]. Although this may be the case at earlier stages, later *Shh* expression in the ZLI reaches far into, and Shh target genes become activated in, the dorsal diencephalon. Therefore, it is likely that the effects of Shh on the dorsal diencephalon are a combination of both relayed and direct signals.

The genes used here to log thalamic specification have characterized roles in the development of thalamic nuclei. *Gbx2* and *Sox14* mark different neuronal subtypes in the thalamus, and graded Shh signaling is required for their expression in diencephalic explants[38]. *Gbx2* is involved in thalamic differentiation and *Gbx2* knockout mice show a lack of thalamocortical innervation[48]. The specific effects of a lack of prethalamic *Dlx2* expression remain to be established. Subtle forebrain defects have been described in *Dlx2* knockout mice and late steps in neuronal differentiation are impaired in *Dlx1*/*Dlx2* double knockouts, suggesting a late role in cell type specification for this gene[49].

Pax6 has multiple roles in neural development, including patterning and cellular differentiation but also maintenance of cells in a proliferative (neurogenic) state[50]. Shh signaling seems to exert largely opposing effects on *Pax6* expression on either side of the ZLI: it is required for *Pax6* downregulation in the thalamus but for *Pax6* maintenance in the prethalamus. Based on the evidence presented above, we propose that Shh induces the conversion of thalamic progenitors from a '*Pax6*-positive/low *Gbx2*' into a '*Pax6*-negative/high *Gbx2*' state.

Secreted signaling factors of the Wnt family, notably *Wnt3*, *Wnt3a* and *Wnt8b*, are expressed in the posterior diencephalon before stage HH18 and have been implicated in diencephalic regionalization[10,13]. Specifically, a requirement for Wnt signaling for thalamic *Gbx2* expression has been demonstrated[13]. Is it possible that the effects Shh exerts on thalamic gene expression are indirectly mediated by Wnt proteins? In a preliminary study of the relationship between Shh and Wnt signaling during thalamic development (data not shown), we did not observe upregulation of diencephalic Wnt proteins after overexpression of Shh, even when *Gbx2* expression was strongly expanded in the same embryos. Furthermore, both *Wnt3* and *Wnt3a* expression was largely unaffected by electroporation of the blocking Ptc mutant (data not shown; *Wnt8b* becomes downregulated in the thalamus after stage HH18; ref. 10). This suggests that Shh does not simply act through upregulation of diencephalic *Wnt* expression. We have also carried out a series of electroporation experiments at stage HH18 using constructs activating (Wnt1, β-catenin) or blocking (dominant-negative disheveled, dominant-negative Lef1) the Wnt–β-catenin pathway but did not observe consistent autonomous effects on prethalamic or thalamic marker gene expression that were comparable with the effects resulting from activation or inhibition of the hedgehog pathway (data not shown). Thus, we propose that thalamic specification, similar to regional specification of the spinal cord, is a multistep process depending on sequential signals: first,

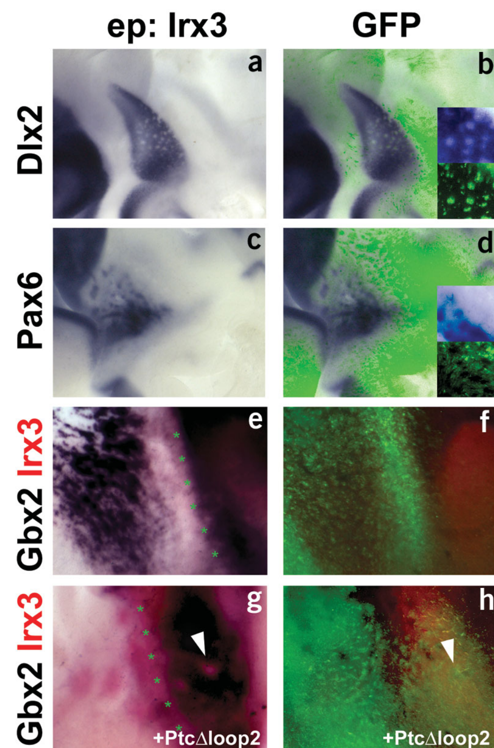

Figure 8 Ectopic Irx3 induces thalamic gene expression in the prethalamus autonomously and in a Shh-dependent manner. (**a**–**h**) Embryos were electroporated with pCAGGS-cIrx3 + pCAβ-eGFP (**a**–**f**) or pCASGGS-cIrx3 + pmPtc1$^{\Delta loop2}$-IRES-GFPnls (**g**,**h**) at stage HH18, cultured to E5 and analyzed by *in situ* hybridization for *Dlx2* (**a**,**b**), *Pax6* (**c**,**d**) or *Gbx2* (blue) and *Irx3* (red) (**e**–**h**), followed by immunochemical detection of GFP. Lateral views facing the ventricular surface of hemisected brains (mid-sagittal) are shown; anterior points to the left, dorsal to the top. Prethalamic genes are downregulated following ectopic expression (ep) of Irx3 in **a**–**d**. Insets in panels **b**,**d** show higher magnifications of the *in situ* signal of a representative prethalamic area and the corresponding anti-GFP fluorescence. (**e**–**h**) Higher magnification of diencephalic area in coelectroporated embryos; the prethalamus is on the left, the thalamus on the right side; the ZLI is indicated by green asterisks in **e**,**g**; **f**,**h** show anti-GFP fluorescence (no overlay). Strong ectopic induction of *Gbx2* is visible in the prethalamus after coelectroporation of Irx3 + GFP (**e**,**f**), but not Irx3 + Ptc$^{\Delta loop2}$ (**g**,**h**), although prethalamic cells express *Irx3* (red). Endogenous *Gbx2* expression is downregulated in the thalamus in Ptc$^{\Delta loop2}$-expressing cells, as described in **Figure 4** (white arrowheads in **g**,**h**).

Wnt proteins regulate anteroposterior regionalization of the diencephalon and define prospective thalamic and prethalamic areas; subsequently, Shh from the ZLI is required for robust expression of thalamic and prethalamic marker genes in these prepatterned regions. This model is consistent with the spatiotemporal expression dynamics of the different *Wnt* genes, *Shh* and the thalamic and prethalamic markers examined and is further supported by the observation that isolated diencephalic explants only express *Dlx2* or *Gbx2* robustly if ZLI tissue is included (data not shown). The establishment of a local signaling center between predefined regions, and the subsequent stabilization of their regional identities by a signal from this interface, are highly reminiscent of the development of the midbrain-hindbrain region[45–47].

Irx3 as a thalamic competence factor

The asymmetric effects in response to Shh signaling anterior and posterior to the ZLI suggest the presence of a prepattern. The homeobox transcription factor Irx2 has recently been shown to regulate differential competence at the IsO by mediating the response of hindbrain cells to FGF8, a diffusible signal released from the IsO[43]. *Irx3*, a close relative of *Irx2*, is induced by early Wnt signals that posteriorize the neural plate[13] and has been suggested to act as a competence factor for inductive signals from the IsO and the ventral midline of the central nervous system[12]. At later stages, *Irx3* is exclusively expressed posterior to the ZLI and is therefore a good candidate to mediate thalamic competence. Indeed, we found that ectopic expression of *Irx3* leads to a conversion of prethalamic into thalamic cells in a Shh-dependent manner, indicating that it acts as thalamic competence factor. This reflects the situation in the spinal cord, where *Irx3* expression defines a region in which different populations of interneurons are formed in response to graded Shh signaling[17].

In summary, we have identified the ZLI as a signaling center in the vertebrate forebrain that regulates the establishment of the regional identity of two major diencephalic regions, thalamus and prethalamus, through the action of Shh. Thalamus and prethalamus react differently to Shh, and this differential competence is—at least in part—mediated by expression of Irx3 posterior to the ZLI (**Supplementary Fig. 1**). Diencephalic development in vertebrates thus shows several characteristic features also found in insect embryos: (i) boundary formation and compartition, delineating (ii) a local signaling center that is stabilized by these boundaries and (iii) differential responsiveness of the adjacent tissues to the signal regulated by prepatterned differential competence.

METHODS

Embryos and *in situ* hybridization. Hen's eggs (Winter farm) were incubated in a humidified chamber at 38 °C. Embryos were staged according to Hamburger and Hamilton. *In situ* hybridization was performed as described[8].

***In ovo* electroporation.** Electroporation of stage HH18 embryos was done as described[9]. The expression plasmids for Shh (pXeX-Shh[21]) and Irx3 (pCAGGS-cIrx3[12]) were coelectroporated with a plasmid encoding a modified GFP (pCAβ-eGFP, J. Gilthorpe, MRC Centre for Developmental Neurobiology, King's College, London) to allow retrospective localization of the electroporated region. The plasmid encoding constitutively active Ptc (pmPtc1$^{\Delta loop2}$-IRES-GFPnls[41]) drives expression of GFP in electroporated cells via an internal ribosome entry site.

Immunostaining. GFP expression was detected by immunostaining using rabbit anti-GFP serum (Molecular Probes, Eugene) at 1:1,000 followed by a fluorophore-conjugated anti-rabbit secondary antibody (Alexa Fluor 488, Molecular Probes) and standard immuno-whole-mount protocols.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank J. Briscoe, J. Dodd, J. Gilthorpe, M. Goulding, M. Hargrave, A. Leutz, T. Ogura, C. Ragsdale, J. Rubenstein, A. Ruiz i Altaba, F. Schubert and C. Tabin for generously providing reagents and J. Gilthorpe, R. Wingate and other members of our laboratory for constructive criticism on the manuscript. This work was supported by the UK Medical Research Council and the Wellcome Trust.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests

Received 28 July; accepted 30 August 2004
Published online at http://www.nature.com/natureneuroscience/

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