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Otx1l, Otx2 and Irx1b establish and position the ZLI in the diencephalon

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The thalamic complex is the major sensory relay station in the vertebrate brain and comprises three developmental subregions: the prethalamus, the thalamus and an intervening boundary region – the zona limitans intrathalamica (ZLI). Shh signalling from the ZLI confers regional identity of the flanking subregions of the ZLI, making it an important local signalling centre for regional differentiation of the diencephalon. However, our understanding of the mechanisms responsible for positioning the ZLI along the neural axis is poor. Here we show that, before ZLI formation, both Otx1l and Otx2 (collectively referred to as Otx1l/2) are expressed in spatially restricted domains. Formation of both the ZLI and the Irx1b-positive thalamus require Otx1l/2; embryos impaired in Otx1l/2 function fail to form these areas, and, instead, the adjacent pretectum and, to a lesser extent, the prethalamus expand into the mis-specified area. Conditional expression of Otx2 in these morphant embryos cell-autonomously rescues the formation of the ZLI at its correct location. Furthermore, absence of thalamic Irx1b expression, in the presence of normal Otx1l/2 function, leads to a substantial caudal broadening of the ZLI by transformation of thalamic precursors. We therefore propose that the ZLI is induced within the competence area established by Otx1l/2, and is posteriorly restricted by Irx1b.

KEY WORDS: Forebrain patterning, Thalamus development, Zona limitans intrathalamica (ZLI), Hedgehog, Zebrafish

INTRODUCTION

The regionalisation and refinement of naïve neural tissue is a prerequisite for developing an elaborate structure such as the brain. During the development of this organ, highly differentiated, information-processing structures are formed – such as the cerebrum in the telencephalon, the tectum in the mesencephalon and the cerebellum in the rhombencephalon – which all have to be wired up with the information-generating sensory systems. The central relay station in this flow of information is the thalamic complex in the diencephalon. Furthermore, this complex does not only physically connect various brain areas, but it also modulates and eventually processes the stream of conscious sensations; for example, it is involved in filtering unwanted stimuli (Sherman and Guillery, 2001). The thalamic complex differentiates from the mid-diencephalic territory (MDT) and consists of three parts: the anteriorly located prethalamus, the posteriorly located thalamus and an intervening ventricular ridge – the zona limitans intrathalamica (ZLI). An essential step towards the establishment of a functional thalamic complex is the release of Sonic hedgehog (Shh) from the ZLI; this protein is necessary and sufficient to induce the characteristic set of pro-neural transcription factors within the MDT (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp et al., 2006). *shh* expression within the ZLI is induced in a characteristic ventral-to-dorsal progression from the basal plate into the alar plate, suggesting a dependency on the basal plate; however, studies in zebrafish have shown that neither the *shh*-positive basal plate nor the underlying axial mesoderm per se are required for ZLI formation (Scholpp et al., 2006). Positioning this signalling centre along the anteroposterior axis is less well understood. It is believed that, in mouse, the ZLI forms directly above the anterior tip of the notochord, and thus at the interface between the prechordal neuraxis and the epichordal neuraxis (Shimamura et al., 1995). Grafting experiments in chick have suggested that alar *Shh* expression similar to that observed in the ZLI is induced by an ectopic interface between prechordal and epichordal neuroepithelia (Vieira et al., 2005). Fate-mapping studies in chick have suggested that the pre-ZLI is a wedge-shaped region of neuroepithelium – characterised by the absence of lunatic fringe (*L-fng*) expression – that gives rise to the ZLI compartment by presumed morphological changes in the diencephalon (Larsen et al., 2001; Zeltser et al., 2001). A further model suggests that mutual repression between Six3 anteriorly and Irx3 posteriorly is involved in positioning the ZLI in chick (Kobayashi et al., 2002; Braun et al., 2003). So far, absence of *Six3* expression in the presumptive ZLI of zebrafish and chick, and the presence of a ZLI in *Six3* knock-out mouse embryos, has raised doubts about the direct involvement of a Six3-Irx3 interaction (reviewed in Wilson and Houart, 2004; Kiecker and Lumsden, 2005). Recently, it has been shown that mice deficient for both forebrain-embryonic zinc finger gene (*Fezf1*, formerly known as *Fez*) and *Fezf2* (formerly known as *Fezl*) gene functions, and fish deficient for *fezf2* only, lack the prethalamus and show a mis-specification of the ZLI, suggesting that either the ZLI precursors require Fezf1/Fezf2 cell-autonomously or that the ZLI is induced by interaction between the prethalamus and thalamus via an unknown posterior molecular component (Hirata et al., 2006; Jeong et al., 2007).

Members of the Otx family, which comprises orthologues of the *Drosophila orthodenticle* (also known as *ocelliless* – FlyBase) gene, play an important role in the regionalisation of the vertebrate rostral neural tube (Simeone et al., 2002). During neural plate specification, the Otx genes – in zebrafish, *otx1l* and *otx2* (collectively referred to as *otx1l/2*) – are expressed in a highly regulated spatiotemporal pattern (Mercier et al., 1995). During gastrulation, Otx responds to the Wnt gradient within the neural plate, and becomes downregulated posteriorly by a combination of high Wnt signalling (Wnt8 from the marginal zone) and activation of the Gbx genes in

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the prospective hindbrain (Kiecker and Niehrs, 2001; Rhinn et al., 2005). At the end of the neural plate stage, Otx gene expression marks the forebrain and midbrain primordia, forming a distinctive border at the midbrain-hindbrain boundary (reviewed in Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001), and is required to maintain midbrain identity (Acampora et al., 1997; Foucher et al., 2006). A previous study involving *Otx2*$^{+/-}$; *Otx1*$^{-/-}$ double-mutant mice suggested that the Otx genes might contribute to the specification of the diencephalon, because the mutant embryos lack expression of *Shh* and diencephalic markers by embryonic day 9.5 (Acampora et al., 1997). Recently, it has been shown that, when clones of *Otx2*$^{-/-}$; *Otx1*$^{-/-}$ cells develop in a thalamic location in mosaic mice, they show posterior diencephalic, most probably pretectal, characteristics, such as expression of GABAergic markers, suggesting that Otx genes drive glutamatergic thalamic fate (Puelles et al., 2006).

Another regionally expressed group of genes in the early neural plate is the Irx family of transcriptional control genes – homologues of the *Drosophila* Iroquois gene complex (Dambly-Chaudiere and Leyns, 1992; Gomez-Skarmeta et al., 1996). In zebrafish, expression of two members of this family, *irx1b* and *irx7*, mark the posterior diencephalon and the midbrain from the neural plate stage onwards (Wang et al., 2001; Itoh et al., 2002). In contrast to the Otx genes, expression of the Irx genes is upregulated by Wnt signalling (Itoh et al., 2002). Although it is known that both Otx gene-family expression and *irx1b* expression persist within the diencephalon, especially in the thalamus, their functions in this area have yet to be elucidated.

In the present study, we mapped Otx gene-family expression during segmentation and pharyngula periods, confirming that this expression is maintained in the presumptive ZLI and thalamus during the segmentation period. We show that this persisting expression is a prerequisite for the formation of the ZLI, because lack of Otx1l/2 function led to the absence of ZLI markers. In addition, thalamic fate is not maintained in these Otx1l/2 hypomorphic (OtxH) (Foucher et al., 2006) embryos. The mis-specified area is transformed mainly into pretectum and, to a lesser extent, into prethalamus, indicating that Otx1l/2 are required for the formation of both the ZLI and thalamus. Rescue of the MDT in OtxH morphants by conditional spatiotemporal induction of Otx2 expression shows that the Otx proteins are necessary to specify ZLI identity as well as thalamic identity, depending upon the position along the anteroposterior axis. Furthermore, loss-of-function experiments demonstrate that expression of *irx1b* in the thalamus is necessary to restrict *shh* expression and thus to establish the posterior boundary of the ZLI. Therefore, we propose a novel model of ZLI formation: Otx1l/2 are required for *shh* expression in the ZLI and set the anterior boundary of the ZLI, whereas Irx1b, which is maintained by Otx1l/2 function, represses the expression of *shh* and therefore determines the posterior boundary of the ZLI. By discovering this novel role for Otx1l/2 during regionalisation of the neural tube, we have directly linked the early Wnt-dependent patterning events with the formation of the presumptive ZLI and thus with the positioning of the thalamus during early neural development.

MATERIALS AND METHODS

Maintenance of fish

Breeding zebrafish (*Danio rerio*) were maintained at 28°C on a 14 hour light/10 hour dark cycle (Brand et al., 2002). Embryos were staged according to Kimmel et al. (Kimmel et al., 1995). To prevent pigment formation, some embryos were raised in 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma). The data we present in this study were obtained from analysis of King's College wild-type (KWT) fish and the Shh-GFP transgenic line was generated by Carl Neumann (Neumann and Nuesslein-Volhard, 2000).

Injections

For transient knock-down of gene expression, morpholino-antisense oligomers (morpholinos; MO) were dissolved in 5 mM Hepes buffer with 0.2% phenol red (Nasevicius and Ekker, 2000). *otx1l*- and *otx2*-MOs (see Foucher et al., 2006) and *Irx1b*-MO [originally described as *iro1*-MO (Itoh et al., 2002)] were back-loaded into borosilicate capillaries prepared on a Sutter micropipette puller. The MO-antisense oligomers were injected at a concentration of 0.5 mM between the one- and eight-cell stage into the yolk cell close to the blastomeres, and the injected amount was estimated from the concentration and volume measured by injection of a sphere of MO solution into oil at the same pressure settings. As a control, a non-priming morpholino (Scholpp et al., 2006) was used, which showed no effect on embryos injected at 0.5 mM but caused unspecific effects at a concentration of 1.5 mM.

For mis-expression experiments, full-length *otx2* was cloned into a pCS2+ vector (Rupp et al., 1994) and, from this template, mRNA was synthesised in vitro (Message Machine kit, Amersham). Together with rhodamine dextran as the lineage tracer, 120 pg mRNA was injected into 1 out of 64 cells (Miniruby, Invitrogen).

The heat-shock (hs)-inducible *otx2-GFP* construct was generated by using the *Xba*I and *Bam*HI cloning sites in the hs-GFP-vector described in Lewis et al. (Lewis et al., 2004). The generated *otx2* gene lacks its 5′ UTR, preventing binding to the *otx2*-MO. The DNA construct (60 ng) was injected into the first blastomere. At tailbud stage, embryos were heat-shocked for 1 hour at 37°C. Injected embryos were fixed in 4% PFA overnight at 4°C before in situ hybridisation or antibody staining.

Whole-mount in situ hybridisation

Whole-mount mRNA in situ hybridisations (ISHs) were performed as previously described (Scholpp et al., 2003). Digoxygenin- and fluorescein-labelled probes were prepared from linearised templates using an RNA labelling kit (Roche). Blue staining was achieved using 1 mg/ml NBT/BCIP in NTMT (pH 9.5), whereas red staining was performed using Fast Red (Roche) dissolved in 0.1 M Tris-HCl (pH 8.0). Stained embryos were dissected and mounted in 70% (v/v) glycerol/PBS. Expression patterns have been described for *otx1l* (originally described as *otx3*) and *otx2* (Mercier et al., 1995), *shha* [originally described as *shh* (Krauss et al., 1993)], *shhb* [originally described as *twhh* (Ekker et al., 1995)], *dlx2a* (Akimenko et al., 1994), *dbx1a* [originally described as *hlx1* (Fjose et al., 1994)], *lhx5* [originally described as *lim5* (Toyama et al., 1995)], *ptc1* (Concordet et al., 1996), *dmbx1a* (Kawahara et al., 2002), *epha4a* [originally described as *rtk1* (Xu et al., 1994)], *fezf2* (originally described as *fezl*) (Hashimoto et al., 2000), *pax6a* (Macdonald et al., 1994), *gsh1* (Cheesman and Eisen, 2004), and *pax7* (Seo et al., 1998).

Embryos were photographed on a Zeiss Axiocam/Axioskop or were imaged using a Nikon C1 confocal microscope. The data sets were 'deconvolved' by AutoDeblur X Gold-Edition (AutoQuant) and further processed using Imaris 4.1.3 (Bitplane AG). Composites were assembled in Adobe Photoshop 7.0.

RESULTS

Persistent Otx expression in the ZLI and thalamus anlagen

During gastrulation, *otx2* marks the entire forebrain and midbrain primordia in all vertebrate embryos (reviewed in Rhinn and Brand, 2001). To follow changes in expression of the Otx genes during the subsequent period of forebrain subdivision in zebrafish, we examined the expression of *otx2* and of regionally expressed markers of the MDT (Krauss et al., 1993; Mercier et al., 1995) from early segmentation to the pharyngula period. During this time period, we found a very similar expression pattern for *otx1l* (formerly described as *otx3*) and *otx2*, as previously described (Mercier et al., 1995). We therefore only show the expression of *otx2*, described below (Fig. 1).

At the 10-somite stage, *otx2* was downregulated in the most anterior part of the neural tube and formed a defined anterior boundary, which was maintained through the pharyngula stages. The

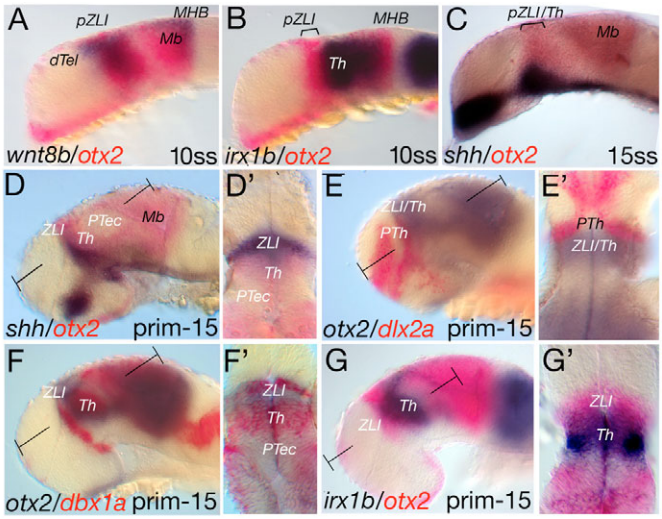

Fig. 1. Mapping of *otx2* expression relative to markers of the MDT. Whole-mount double in situ-hybridised wild-type embryos are mounted with anterior to the left and dorsal up. The markers and stages are indicated. (**A**) *wnt8b* marks the pZLI territory and shares the same anterior border as *otx2* at ten somites (14 hpf). (**B**) At the same stage, *otx2* is expressed in the territory from the pZLI to the midbrain, whereas *irx1b* and *otx2* are co-expressed in the presumptive thalamic area and anterior midbrain. (**C**,**D**) At 15 somites (16.5 hpf), *shha* expression is induced within the *otx2*-positive domain (C) and its anterior border coincides with the anterior expression border of *otx2* at prim-15 (30 hpf; D). (**D′**) A cross-section shows that the *otx2* expression domain marks the ZLI and the thalamus whereas the ZLI is only *shha* positive. In the pretectum, *otx2* is weakly expressed. (**E**,**E′**) *dlx2a* marks the territory of the prethalamus and is anteriorly adjacent to the *otx2* expression domain (E); the cross-section reveals the abutting expression domains (E′). (**F**,**F′**) Similarly to the *shha* expression domain, the expression domain of *dbx1a* overlaps with that of *otx2* at the anterior ZLI domain and at the thalamus. (**G**,**G′**) At 30 hours, the expression domain of *irx1b* persists in the thalamus, whereas the ZLI domain is only *otx2* positive. T-shaped brackets mark the plane of the section in the following picture. *dTel*, dorsal telencephalon; *Mb*, midbrain; *MHB*, midbrain-hindbrain boundary; prim-15, primordium stage 15; *PTec*, pretectum; *PTh*, prethalamus; *pZLI*, presumptive zona limitans intrathalamica; ss, somite stage; *Th*, thalamus; *ZLI*, zona limitans intrathalamica.

anterior *otx2* expression co-localised with most of the *wnt8b* expression domain, a marker of the presumptive ZLI (pZLI), as described in chick (Fig. 1A). At the same stage, *irx1b* is expressed in the posterior diencephalon, marking the thalamic anlage (Lecaudey et al., 2004), whereas the *otx2*-positive pZLI was negative for *irx1b* (Fig. 1B). At the 15-somite stage, *shha* expression is detectable in the basal root of the pZLI – precisely between the prethalamic and thalamic anlage (Fig. 1C) (Barth and Wilson, 1995; Scholpp et al., 2006). *otx2* expression was additionally downregulated in an area of the posterior-most forebrain, encompassing the pretectal anlage. Interestingly, the anterior boundary of the remaining *otx2* expression domain later coincided precisely with the anterior boundary of the *shha*-positive ZLI domain at pharyngula stage [prim-15; 30 hours post-fertilisation (hpf); Fig. 1D,D′]. Posterior to the MDT, *otx2* was very weakly expressed in the pretectum and strongly maintained in the midbrain (Fig. 1D,D′). To further examine the molecular profile of the MDT, we analysed the expression of well-characterised markers of the forebrain primordium at prim-15. We found that *otx2* expression abuts the prethalamic expression domain of *dlx2a* (Fig. 1E,E′) (Akimenko et al., 1994; Scholpp et al., 2006). Similarly to *shha*, the anterior border of *dbx1a* [formerly described as *hlx-1* (Fjose et al., 1994; Scholpp et al., 2006)] expression – marking the anterobasal part of the ZLI – coincided with the *otx2* expression domain (Fig. 1F,F′). Thus, *otx2* expression splits the MDT at the position of the presumptive prethalamus-ZLI border. In addition, *dbx1a* expression also marked the thalamus. This thalamic expression domain overlapped with that of *otx2* (Fig. 1F,F′). Interestingly, the posterior boundary of the thalamic domain of *dbx1a* expression did not fully resemble the *otx2* expression border, suggesting that *otx2* expression might not entirely cover the thalamic anlage (Fig. 1F′). At this stage, *irx1b* expression still marked the thalamic territory but not the ZLI anlage, which remained *otx2*-positive (Fig. 1G,G′) (Lecaudey et al., 2004). Therefore, we found the following gene expression code within the diencephalon from anterior to posterior: the prethalamus is *dlx2a*-positive, *otx2*-negative; the ZLI primordium is *otx2*- and *shh*-positive; the anterobasal part of the ZLI is additionally *dbx1a*-positive; the thalamus is *otx2*-, *irx1b*- and *dbx1a*-positive; the pretectum shows weak expression of *otx2*. In summary, these data show that *shha* expression in the ZLI is detected exclusively at the rostral part of the Otx-positive diencephalic domain.

Otx1l/2 function is required for the formation of the ZLI

To elucidate the function of Otx1l and Otx2 during forebrain regionalisation, we blocked their function by the injection of morpholino antisense oligonucleotides (MOs) against them (Foucher et al., 2006). Due to a strong maternal contribution, Otx1l and Otx2 protein can be detected by western blot analysis until mid-somitogenesis in embryos injected with Otx1l- and Otx2-MO; these are thus hypomorphs and are referred to as OtxH (Foucher et al., 2006). This approach provides a tool to address the late functions of the Otx gene family without interfering with those functions occurring earlier, which have been studied in mouse (reviewed in Simeone et al., 2002). To address whether the forebrain territory per se is initiated and formed correctly in OtxH embryos during the segmentation period, we studied the expression of well-described regional markers – *pax6a*, *epha4a* and *barhl2* (Macdonald et al., 1994; Xu et al., 1994; Colombo et al., 2006) – at the 12-somite and prim-15 stages. Although the hindbrain expression domains of *pax6a* and *epha4a* have already started to expand anteriorly at 12-somites (Foucher et al., 2006), we found no significant change in the diencephalic expression domains of these markers in OtxH embryos (Fig. 2A-F). At prim-15, the forebrain territory still expressed *pax6a* and *epha4a*, but their expression domains were shorter along the anteroposterior axis (Fig. 2G-J). Interestingly, the downregulation of these two markers within the ZLI territory at prim-15 that occurred in control embryos was not observed in OtxH embryos (Fig. 2H,J). Furthermore, we found a dramatic reduction in the expression of the mid-diencephalic marker *barhl2* (Fig. 2K,L) while its expression persisted unaltered in the telencephalon and in a small patch of cells in the posterior hypothalamus.

To further investigate the OtxH phenotype at the axial level of the ZLI, we analysed GFP expression in *shha-GFP* transgenic embryos (Fig. 3A-H) (Neumann and Nuesslein-Volhard, 2000). At the 15-somite stage, the phenotype of OtxH embryos was indistinguishable from control-injected transgenic siblings (Fig. 3A,B). From the 20-somite stage to prim-18, Shha-GFP expression in the ZLI progressed from ventral to dorsal in control siblings (Fig. 3C,E,G), whereas, in OtxH embryos, GFP expression was not initiated in the ZLI, although the basal plate expression domain

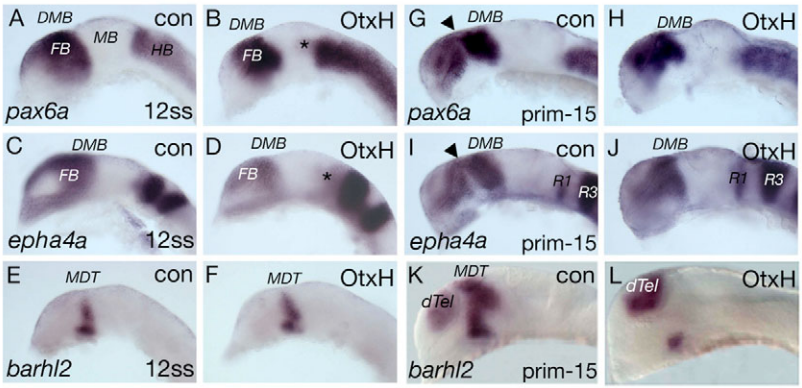

Fig. 2. Forebrain marker gene expression is unaltered in Otx1l/2 hypomorphic (OtxH) embryos at early stages. Gene expression profile of OtxH embryos at 12 somites (15 hpf; **A-F**) and prim-15 (30 hpf; **G-L**). (A,C) *pax6a* and *epha4a* mark the forebrain anlage and demarcate at the diencephalic-mesencephalic boundary. (B,D) Although the hindbrain expression domains start to shift anteriorly (asterisks), the forebrain expression domains of these genes are unaltered in OtxH embryos at this stage. (G-J) At prim-15, in control siblings, *pax6a* and *epha4a* become downregulated in the mid-diencephalon (marked by arrowheads); this structure is not detectable in OtxH embryos (*pax6a*: 26/32; *epha4a*: 36/41). Compared with control siblings, the expression domains of *pax6a* and *epha4a* shrink in the anteroposterior direction. (E,F) *barhl2* is expressed in the MDT at 12 ss (E) and its expression is indistinguishable from OtxH embryos at the same stage (F). (K) At prim-15, *barhl2* marks the dorsal telencephalon and the MDT, including the ZLI (12/30). (L) In OtxH embryos, the ZLI expression is absent, whereas the dorsal telencephalic domain persists. con, control; *dTel*, dorsal telencephalon; *DMB*, diencephalic-mesencephalic boundary; *FB*, forebrain; *HB*, hindbrain; *MB*, midbrain; *MDT*, mid-diencephalic territory; prim-15, primordium stage 15; *R1*, rhombomere 1; *R3*, rhombomere 3; ss, somite stage.

persisted (Fig. 3D,F,H). As described previously, the posterior commissure (PC) does not form in OtxH embryos (Foucher et al., 2006). However, we found that the nucleus of the medial longitudinal fascicle (nMLF) was detectable (Fig. 3G,H). To investigate this phenotype further, we performed an in situ hybridisation (ISH) analysis for markers of the ZLI: *shha*, the Shh-dependent target gene *ptc1*, and *wnt8b*. Expression of both *shha* and *ptc1* was absent from the pZLI in OtxH fish, whereas their expression was unchanged in their other expression domains, such as the hypothalamus, basal plate and floor plate, at prim-10 (Fig. 3I-L). In addition, diencephalic expression of *wnt8b*, a marker for the pZLI, was unchanged at the 18-somite stage in OtxH embryos [although the midbrain-hindbrain boundary (MHB) expression domain shifted anteriorly; Fig. 3M,N]. However, by prim-10, *wnt8b* ZLI expression was missing, while *wnt8b* expression was maintained in the dorsal telencephalon and posterior hypothalamus – presumably the equivalent to the retromamillary region in chick (Garda et al., 2002) – in these embryos (Fig. 3O,P).

Otx1l/2 function is required to establish the thalamus

Given the organising role of the ZLI in forebrain regionalisation, we assessed the development of the MDT (comprising the prethalamus, ZLI and thalamus) and the pretectum in OtxH embryos at prim-10 (after ZLI formation). We studied whether the prethalamic territory (Fig. 1D) was affected by the absence of the ZLI in OtxH embryos by analysing *fezf2*, *lhx5*, *lhx1* and *dlx2a* expression. In OtxH embryos, the expression domains of *fezf2*, *lhx5* and *lhx1* were maintained in the prethalamic primordium, and the ventral aspect of its expression domain – the preoptic area – was sometimes slightly broadened posteriorly compared with control siblings (Fig. 4A-F, arrows). Interestingly, in OtxH embryos, the expression domain of *lhx1* in the nucleus of the PC was missing, providing a molecular explanation for the lack of this commissure (Fig. 4F and Fig. 3H). The expression in the prethalamus seems reduced eventually due to a lack of maintenance of Shh signalling (Ericson et al., 1995). The persistence of these expression domains demonstrates the

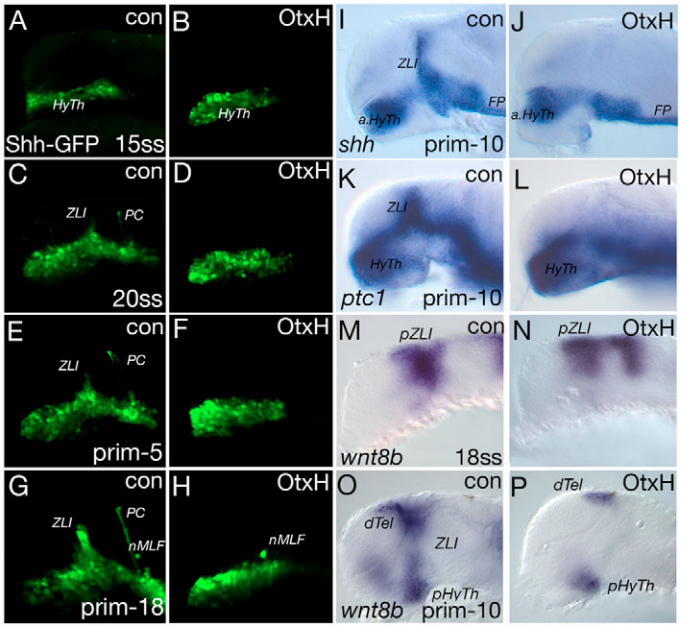

Fig. 3. Analysis of the ZLI in OtxH embryos. Confocal microscopy analysis of transgenic *shh-GFP* OtxH embryos and controls (con) (A-H) and gene expression analysis of OtxH embryos (I-P); the markers and stages are indicated. (**A**,**B**) Shh-GFP is detectable in the hypothalamic primordium (HyTh) in control embryos as well as in OtxH embryos at 17.5 hpf. (**C-H**) From 21 to 32 hpf, the ZLI develops from ventral to dorsal and the posterior commissure (PC) forms at the diencephalic-mesencephalic boundary in control embryos (C,E,G), whereas, in OtxH embryos, neither the ZLI nor the PC is detectable (D,F,H). Interestingly, the nucleus of the medio-longitudinal fascicle (nMLF) is visible in control embryos as well as in OtxH embryos (G,H). (**I**,**J**) At prim-10 (28 hpf), the Shh expression domains of the basal plate are unaltered in the anterior hypothalamus (aHyTh) and floor plate (FP) in OtxH embryos, whereas the ZLI, located in the alar plate, is missing compared with control embryos (42/51). (**K**,**L**) Similarly, the expression domain of *ptc1* is absent at the ZLI in OtxH embryos (31/48). (**M**,**N**) Expression of the marker of the presumptive ZLI (pZLI), *wnt8b*, is unaltered in the diencephalic expression domain at 18 somites (18 hpf), although the MHB expression shifts anteriorly. (**O**,**P**) At prim-10, *wnt8b* is absent in the ZLI (10/12) but persists in the dorsal telencephalon (dTel) and posterior hypothalamus (pHyTh). prim, primordium stage; *ZLI*, zona limitans intrathalamica.

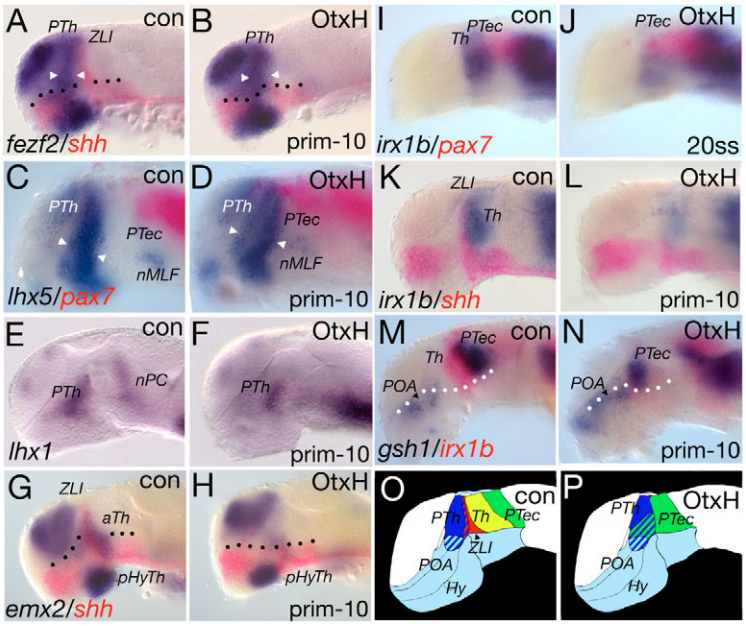

Fig. 4. Analysis of the MDT in OtxH embryos. (**A-H**) Single and double in situ hybridisation of OtxH embryos; the markers and stages are indicated. Shh expression is absent in the ZLI territory in OtxH embryos (B,H,L). Compared to control (con) siblings, markers for the prethalamic anlage – *fezl*, *lhx5* and *lhx1* – are still expressed in OtxH embryos and show a slight broadening in the ventral part of the anlage (*lhx5*: 11/15; *fezl* (*fezf2*): 12/20; A-F, marked by arrowheads). In OtxH embryos, the nuclei of the medio-longitudinal fascile is detectable by *lhx5* (C,D), whereas the *lhx1*-positive nuclei of the PC are absent (E,F). *emx2* expression in the anterior thalamus (aTh) is absent in OtxH embryos (23/30). (**I-N**) Prior to ZLI formation, at 20 somites (19 hpf), the *irx1b* expression domain is absent in OtxH embryos (I,J) and is still absent at prim-10 (28 hours; 24/60; K-N). (A-H) Dotted line marks the border between alar and basal plate. (C,D,M,N) *pax7* is expressed in the dorso-lateral part of the pretectum (C,D), whereas *gsh1* marks the ventro-medial domain of the pretectum (M,N). (C,D) In OtxH embryos, the *pax7*-positive part of the pretectum shifts anteriorly and a new border between the *lhx5*-positive prethalamus and the *pax7*-positive pretectum is observed (11/15). (M,N) Comparison of the *gsh1*-positive part of the pretectum in OtxH embryos and their wild-type siblings shows that the pretectum area abuts the *gsh1*-positive preoptic area (8/14). (**O**,**P**) Summary of the observations in OtxH embryos. In wild-type embryos, the MDT is organised in the following way: the lateral halves of the prethalamus (blue), the more medial ZLI (red), the thalamus (yellow) and the pretectum (green). In OtxH embryos, we still find the lateral halves of the prethalamus (blue) and the pretectum (green). Striped areas show the lateral to medial organisation of the area and not an overlap of expression domains. *aTh*, anterior thalamus; *Hy*, hypothalamus; *nMLF*, nucleus of the medio-longitudinal fascile; *nPC*, nucleus of the posterior commissure; *pHyTh*, posterior hypothalamus; *POA*, post-optic area; prim-10, primordium stage 10; *PTec*, pretectum; *PTh*, prethalamus; ss, somite stage; *Th*, thalamus; *ZLI*, zona limitans intrathalamica.

maintenance of a prethalamic territory. However, we found that *dlx2a* was absent from the prethalamus, as predicted by the known requirement for Shh signalling in the expression of this gene (see Fig. 5E,G) (Scholpp et al., 2006). Interestingly, the preoptic expression domain of *dlx2a* was expanded similarly to the prethalamic markers described above (Fig. 5G, arrow).

The thalamus maintains expression of the Otx genes during development, raising the possibility for a direct Otx requirement for thalamic identity. One of the earliest markers of the thalamic anlage is *irx1b* (Lecaudey et al., 2005). The *irx1b* expression domain abuts the ZLI rostrally (Fig. 4K), and the pretectal domain caudally marked by *gsh1* (Fig. 4M). We found that *irx1b* expression was absent in OtxH embryos during the early segmentation stages, before ZLI formation, suggesting that Otx function is independently required to maintain *irx1b* expression in the thalamus (Fig. 4I,J). At prim-10, we found that this Shh-independent *irx1b* expression domain was still downregulated in OtxH embryos (Fig. 4L,N). Moreover, expression of the Shh-dependent thalamic marker genes *emx2* and *dbx1a* was absent (Fig. 4G,H and data not shown) due to the lack of the ZLI (Scholpp et al., 2006) or due to an independent requirement for Otx function in the thalamus.

The loss of ZLI and thalamus markers in OtxH fish, with only the minor loss of neural tissue, raised the issue of fate identity of this tissue in OtxH morphant embryos. With the exception of the basal area, we showed that these cells are mostly not prethalamic, and therefore considered whether the mis-specified thalamus might possess posterior properties by examining the expression of two pretectal markers – *pax7* and *gsh1* (Seo et al., 1998; Cheesman and Eisen, 2004). Interestingly, *pax7* expression was already expanded anteriorly at the 20-somite stage (Fig. 4I,J). Similarly, we observed a significant rostral shift in the expression domains of these genes, suggesting a transformation of the ZLI/thalamus anlage into pretectum, at the prim-10 stage (Fig. 4C,D,M,N). This anterior shift of the pretectum was also detectable in the basal plate: the primordium of the nMLF, marked by *lhx5* expression, was also shifted rostrally (Fig. 4C,D). Our previous analysis (Foucher et al., 2006) excludes the possibility of restricted cell death in the diencephalon of OtxH embryos. We thus conclude that Otx1l/2 function is required to specify the fate of the ZLI and the thalamus: absence of these proteins led to transformation of the ZLI/thalamus into pretectum and, to a lesser extent, into basal prethalamus (Fig. 4O,P). Finally, loss of expression of the early Shh-independent thalamic marker *irx1b* in OtxH embryos indicates a direct Otx requirement for proper specification of the thalamus, possibly via the maintenance of Irx function.

Ectopic Otx2 can cell-autonomously rescue *shha* expression in the ZLI in OtxH morphant embryos

To find whether Otx1l/2 function is sufficient to induce *shha* expression in the diencephalic alar plate, we generated a heat shock-inducible construct driving expression of a GFP-tagged Otx2 protein (HS-Otx2-GFP), which could not be repressed by the *otx2* MO (see Materials and methods). After injection of this construct and the Otx1l- and Otx2-MO mix, we heat-shocked the embryos at the early neural plate stage (tail bud) and performed our analysis at prim-10 (Fig. 1A). To reliably detect co-localisation between cell clones positive for Shha and the Otx2-GFP fusion protein, we performed a single ISH for *shha* in combination with α-GFP-antibody staining (Fig. 5B-C″). Furthermore, to evaluate the position of the ectopically induced *shha* expression, a set of treated embryos was assessed for *shha* and for endogenous *otx2* transcripts (Fig. 5D,F,H). Finally, we tested whether expression of *dlx2a*, a Shh-dependent prethalamic marker gene, was rescued non-cell-autonomously by the diencephalic cells expressing *otx2-GFP* that were also positive for *shha* expression (Fig. 5C,G,I).

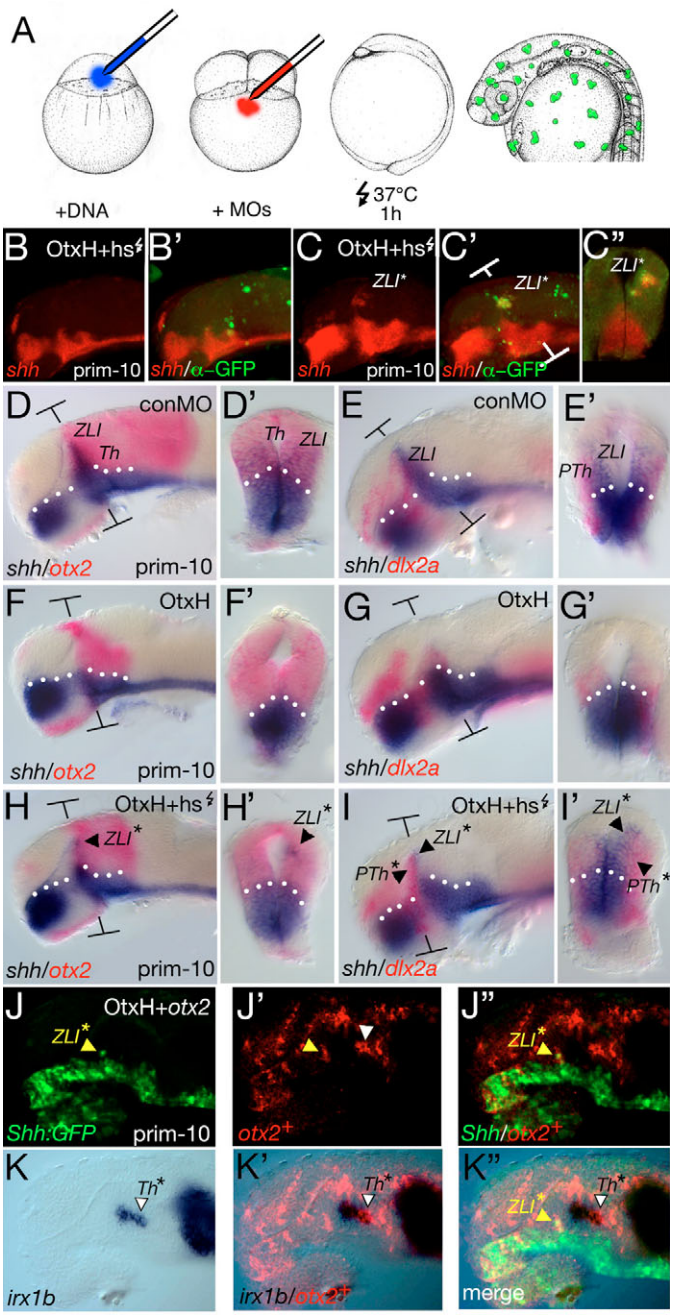

Fig. 5. Otx2 rescues the ZLI in OtxH embryos. Gene expression analysis of embryos at prim-10 (28 hpf); the markers are indicated on each panel. (**A**) Embryos were injected with 60 pg of the heat-shock-inducible *otx2-GFP* DNA construct into the one-cell-stage blastomere. Embryos were kept at 28°C until reaching the four-cell stage. Then, the double morpholino (MO) mix was injected to block transcription of endogenous *otx1l* and *otx2*. At the one-somite stage, the embryos were heat-shocked for 1 hour at 37°C and were fixed at the prim-12 stage. To reliably localise the Otx2-GFP-positive clone, an FITC-coupled antibody staining against GFP and a Fast Red single in situ hybridisation for *shh* were performed. Injection of *otx2-GFP* DNA and heat-shock activation led to a random induction of GFP-positive clones in the OtxH embryos. Confocal microscopy analysis of the embryos showed that ectopic induction of *shh* expression is generally not observed in *otx2-GFP* clones (**B**,**B'**). However, when these clones were located within the MDT, *shh* expression was induced in these cells (**C**,**C'** and cross-section in **C"**, 13/16-4/6 detected via α-GFP staining and 9/10 via *GFP*-ISH). (**D**,**D'**) To mark the prethalamic-thalamic boundary, a double in situ hybridisation of *otx2* and *shh* was performed. (**E**,**E'**) To mark the prethalamus, a double staining with *dlx2a* and *shh* was performed. (**F-G'**) In control (con) embryos, *shh* expression is absent at the ZLI. In addition, the *otx2* expression domain is smaller (F,F') and the *dlx2a* domain is absent (G,G'). By contrast, in embryos in which *otx2-GFP* is heat-activated, *shh* expression can be rescued in the presumptive MDT (**H**,**H'**), and a cross-section reveals that the induction is independent of the basal *shh* expression domain (H'). The rescue of *shh* in small clones is sufficient to induce the expression of *dlx2a* (5/10; **I**); for example, its expression was induced uni-laterally in the neural tube (**I'**). T-shaped brackets mark the plane of the section in the following picture. The dotted line marks the border between the alar and basal plate. (**J-K"**) *otx2* mRNA (150 pg; + red-lineage tracer) was injected into one of 64 cells of OtxH *shh-GFP* embryos. At 32 hpf, embryos were fixed and stained for GFP (green). The provision of an ectopic *otx2* message is able to rescue Shh-GFP expression within the ZLI in OtxH embryos (J-J", yellow arrowhead), as shown by a co-localisation of the red-lineage tracer of the Otx2$^+$ cells with the green GFP expression. The *irx1b* expression profile was assessed in the same embryo (K-K"). When Otx2$^+$ cells are located within the presumptive thalamic territory, they express *irx1b* (white arrowhead), shown by co-localisation of the red lineage tracer with *irx1b* detection (blue). (K") Merged picture of Shh-GFP expression, the red lineage tracer and *irx1b* expression. prim-10, primordium stage 10; *PTh*, prethalamus; *PTh**, rescued prethalamus; *Th*, thalamus; *ZLI*, zona limitans intrathalamica; *ZLI**, rescued zona limitans intrathalamica.

In OtxH embryos, *shha* expression was absent from the ZLI territory and the *otx2*-positive midbrain territory was lacking (Fig. 5B,F). After heat-shock, *otx2*-positive clones, marked by GFP, were randomly induced throughout the embryo (Fig. 5B,C) because of the random distribution of the injected DNA (Gilmour et al., 2002). In cases in which embryos were not treated by heat-shock, or when Otx2-GFP-positive clones were not located within the MDT primordium, we observed the phenotype described for OtxH embryos (Fig. 5B,G): the *shha*-positive ZLI was missing, the posterior diencephalon had shrunk and *dlx2a* expression was not induced in the prethalamic anlage due to the lack of ZLI signalling. Whenever Otx2-GFP-positive cell clones were located within the pZLI area, *shha* expression was induced in these clones cell-autonomously (Fig. 5C-C"). When the heat-shock was performed during mid-somitogenesis (15-somite stage), we found no rescue of *shha* expression within the ZLI territory (data not shown). To locate the position of the induced clones in the diencephalic territory, we performed another set of experiments using *otx2* expression itself as a marker to visualise the prethalamic-thalamic boundary (Fig. 5D,F,H). In control MO-injected embryos, the ZLI formed within the *otx2*-positive territory abutting the prethalamic domain (Fig. 1D and Fig. 5D). In OtxH embryos, the ZLI was missing, whereas the endogenous *otx2* transcript could still be detected and used as a landmark for the prethalamus-thalamus border (Fig. 5F). In OtxH/HS-Otx2GFP embryos, *shha* expression could only be detected at the anterior Otx2 expression border (Fig. 5H). A cross-section at the level of the ZLI anlage of the same embryo showed that the cell patch rescued for *shha* expression was not connected to the *shha* expression of the basal plate (Fig. 5H'). We never found ectopic induction of *shha* expression in the prethalamus or in the presumptive posterior thalamus or pretectum.

We first studied whether the rescued *shha* expression in the ZLI was able to activate transcription factors in the territory around the ZLI. The prethalamic marker *dlx2a* is expressed rostrally directly adjacent to the ZLI (Fig. 5E) (Scholpp et al., 2006) and, in a cross-section, the lateral position of the prethalamus could be visualised in relation to the ZLI (Fig. 5E′). In OtxH embryos, the expression of *dlx2a* was missing in the prethalamus (Fig. 5G,G′) but was sometimes present posterior to the prethalamus, close to the *shha*-positive basal plate (Fig. 5G,G′, arrows), similarly to the caudo-ventral expansion described for other prethalamic markers (Fig. 4). Most importantly, the alar *shha* expression induced by Otx2-GFP was sufficient to rescue the expression of *dlx2a* in the prethalamic primordium (Fig. 5I). In a cross-section, the rescue could be seen unilaterally, whereas the control side still lacked proper *dlx2a* expression (Fig. 5I′).

In a further set of experiments, we addressed the role of Otx2 in thalamic development. We injected the Otx1l/2-MO mix into transgenic *shha:GFP* embryos, followed by an injection of *otx2* mRNA with a red lineage tracer into 1 of 64 cells. By confocal microscopy analysis of prim-18-stage embryos, we found that *otx2* mRNA overexpression was able to rescue *shha* expression within the ZLI in OtxH embryos, confirming our heat-shock data (Fig. 4I-I″). Interestingly, when *otx2*-positive cells were located posterior to the pZLI territory, *irx1b* expression (and not Hh) was induced. This dual response to *otx* expression can be observed in the same embryo (Fig. 4K-K″). This shows that Otx2 promotes the expression of different sets of diencephalic markers, according to its anteroposterior location within the MDT.

Thus, we conclude that Otx1l/2 function is necessary cell-autonomously for both the formation of a functional ZLI organiser and the maturation of thalamic identity. Both the pre-ZLI and the prospective thalamus therefore independently require Otx activity for their development. In addition, the highly restricted spatial competence of Otx to induce alar *shha* expression suggests a mechanism by which *shha* expression is actively repressed posterior to the pZLI possibly via maintenance of *irx1b* expression.

Irx1b sets the boundary between the ZLI and the thalamus

From our analysis so far, we can conclude that Otx1l/2 are required for the induction of the ZLI and the thalamic anlage. However, although Otx1l and Otx2 are expressed in an area comprising both the pZLI and thalamus, our mosaic analysis showed that they are not able to induce ZLI identity ectopically in the presumptive thalamus. We therefore set out to address the issue of how ZLI formation is restricted posteriorly. Like the *Irx1* gene expression domain in mice, the expression domain of the zebrafish orthologue, *irx1b*, abuts on the ZLI territory (Bosse et al., 1997; Cohen et al., 2000; Lecaudey et al., 2005). We analysed the phenotype of embryos with reduced *irx1b* function by using the antisense morpholino approach (Itoh et al., 2002). To visualise the sequence of events during ZLI formation in *irx1b* morphants, we injected the MO into *shha-GFP* transgenic embryos. Until the 20-somite stage (19 hpf), *shha* expression was comparable to control siblings (compare to Fig. 6A with Fig. 3C). At prim-5 (26 hpf), we observed some cells posterior to the pZLI domain switching on GFP expression (Fig. 6B, arrowheads). At prim-18 (32 hpf), we observed a broadening of the Shha-GFP domain (Fig. 6C). At prim-10, we found that the ZLI territory marked by *shha* expression was massively expanded caudally, whereas the pretectal domain marked by *gsh1* was unaltered (Fig. 6D,E, arrows). Similarly, the expression of *shhb* (formerly known as *twhh*) was also broader, expanding posteriorly, whereas the pretectal

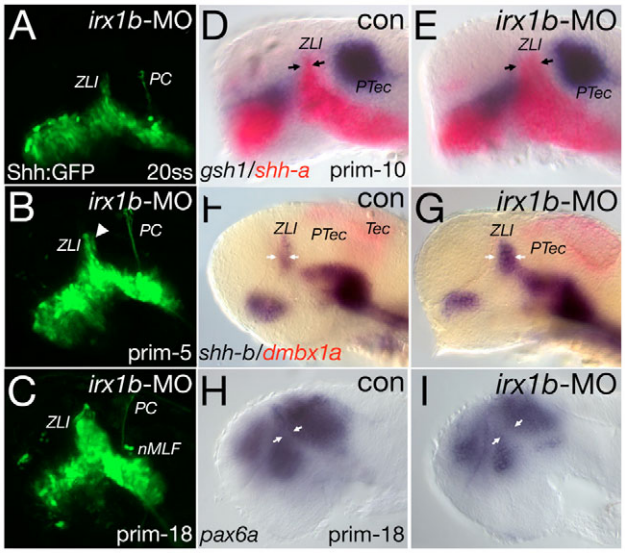

Fig. 6. Irx1b sets the posterior boundary of the ZLI. Confocal microscopy analysis of transgenic *shh-GFP; irx1b*-morpholino (MO) embryos (**A-C**) and in situ-hybridisation analysis of *irx1b*-MO embryos (**D-I**); markers and stages are indicated. (A) *irx1b*-MO-injected embryos resemble control (con) embryos at the same stage (compare with Fig. 3). (B) At 26 hpf, cells located posterior to the ZLI start to express Shh-GFP ectopically (arrowhead), whereas the posterior commissure (PC) and the nucleus of the medio-longitudinal fascicle (nMLF) form normally. (C) At 32 hpf, the Shh-GFP expression at the ZLI is broadened compared with control-injected embryos (see Fig. 3). (D,E) At prim-10, the Shh expression domain at the ZLI is broadened at the expense of the thalamus (arrows). (F,G) Similarly, the expression domain of *shhb* expands posteriorly in *irx1b* morphants (arrows). (D-G) Interestingly, the expression of markers of the pretectum, such as *gsh1* and *dmbx1a*, is unaltered. (H,I) The broadening of the ZLI is also visible by an in situ hybridisation for *pax6a*, revealing a much wider gap between the prethalamus and the remaining thalamic-pretectal tissue at prim-18 (32 hpf; arrows). *nMLF*, nucleus of the medio-longitudinal fascicle; *PC*, posterior commissure; prim, primordium stage; *PTec*, pretectum; *Tec*, tectum; *ZLI*, zona limitans intrathalamica.

dmbx1a expression domain was not visibly affected (Fig. 6F,G, arrows). Consequently, the *pax6a* expression domain within the thalamic and pretectal area shrinks upon expansion of the ZLI domain at prim-18 (Fig. 6H,I, arrows). These data show that, when Irx1b function is perturbed, the ZLI expands into the thalamic area. In addition, the observation of a normal pretectum and unaltered formation of the PC in *irx1b* morphant embryos, implies normal development of the diencephalic-mesencephalic boundary, in contrast to OtxH embryos (Scholpp et al., 2003; Foucher et al., 2006). We conclude that, in embryos lacking Irx1b function, the ZLI is normally initiated at the prethalamic-thalamic junction but subsequently expands posteriorly. Irx1b is therefore required to repress Shh expression and thus sets the posterior boundary of the ZLI in the diencephalic Otx-expressing domain.

DISCUSSION

Establishment and positioning of a small group of cells – so-called local organisers or signalling centres – that secrete molecules to pattern and to further subdivide and differentiate the surrounding tissue, is a fundamental requirement for the regionalisation of the brain (reviewed in Puelles and Rubenstein, 2003; Wilson and Houart, 2004; Kiecker and Lumsden, 2005). To date, the

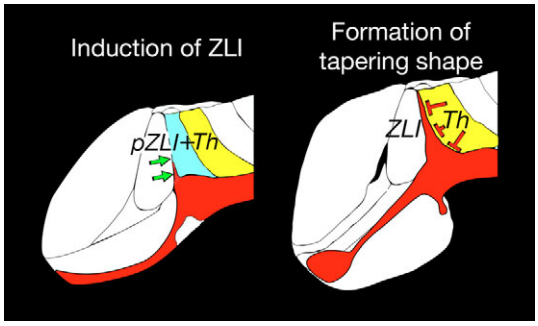

Fig. 7. Summary. Regionalisation of the diencephalon. At mid-somitogenesis stages, Otx1l/2 mark the presumptive ZLI (p-ZLI, light blue) and the thalamic anlage (Th, yellow), whereas *irx1b* is expressed only in the thalamic anlage. Shh marks the floor plate and basal plate (red) and starts to be induced in the Otx1l/2-positive pZLI (green arrows). At pharyngula stages, *shh* is expressed in the mature ZLI (red) and the territory of the Shh-positive ZLI is restricted by the thalamic expression of *irx1b* (red T-brackets).

mechanisms by which one of these local organisers, the ZLI, is precisely restricted spatially and temporally, have yet to be unravelled. Here, we describe a novel mechanism by which the ZLI is induced and positioned in the presumptive diencephalon. As early as ten somites, the anterior limit of *otx1l/2* expression marked the boundary between the prethalamus and the thalamus – the precise anteroposterior position of the future ZLI. We show that lack of *otx1l/2* function leads to the absence of the ZLI and subsequently of ZLI-dependent target genes in the MDT. Moreover, with a lack of Otx function, the thalamus was mis-specified prior to, and independently from, ZLI formation. The adjacent territories of the prethalamus and pretectum expanded into the mis-specified territory and formed a new interface. Conditional mosaic induction of Otx2 expression during somitogenesis, in Otx1l/2-deficient embryos, was able to rescue *shha* expression in the pZLI area as well as *irx1b* expression in the thalamic anlage. Therefore, Otx1l/2 is necessary for the specification of both the ZLI and thalamus. Finally, the fate distinction between the Otx-positive ZLI anlage and the Otx-positive presumptive thalamus is mediated by Irx1b, as shown by the broadening of the ZLI at the expense of the thalamus when Irx1b function is decreased.

Complex relationship between Shh and Otx in the neural tube

The *Otx2*$^{+/-}$ *Otx1*$^{-/-}$ mutant mouse is comparable to the OtxH zebrafish embryo; both animals have a transformation of the presumptive midbrain territory into an extended rhombomere 1, resulting in an anterior shift of the MHB and formation of an enlarged cerebellum (Acampora et al., 1997; Foucher et al., 2006). In the diencephalon, we found that ZLI formation, including *shha* induction, is dependent on proper Otx1l/2 function. Mice embryos with reduced Otx1/2 transcripts show a similar lack of *Shh* ZLI expression. In these mice, the direct or indirect nature of such loss could not be addressed and the lack of ZLI was proposed to be a consequence of the proximity of the MHB organiser and its repressive influence on the diencephalon (Acampora et al., 1997; Li and Joyner, 2001; Scholpp et al., 2003). In this report, we used a mosaic analysis and temporally controlled mis-expression in zebrafish, and were able to show that the formation of the ZLI is a novel specific function of Otx1l/2 proteins. By contrast, a recent study suggests that Otx2 protein is also able to restrict *Shh* expression in the floor plate in a dose-dependent manner during mouse midbrain development (Puelles et al., 2003). The authors propose a model in which Otx1/2 and Groucho, a family of transcriptional co-repressors, are needed to mediate a repressive interaction that limits *Shh* expression to the basal plate. Although Groucho activity has been described for the formation of the MHB organiser (Wurst and Bally-Cuif, 2001), there are no Groucho genes known to be involved in ZLI induction, neither in zebrafish nor in other species. This opens the possibility of a dual regulation of *shh* expression by Otx proteins: inhibitory in the presence of Groucho and activating in its absence.

Formation of the ZLI

There is an ongoing debate about the early regionalisation of the neural tube and the positioning of the ZLI. It is widely believed that, in mouse and chick, the ZLI forms directly above the anterior tip of the notochord, and thus at the interface between the prechordal neuraxis, induced by prechordal plate mesoderm, and the epichordal neuraxis, induced by the chordamesoderm (Shimamura et al., 1995). Because Fgf8-coated beads are able to induce *Foxg1* in the prechordal neural plate and *En1/2* in the epichordal neural plate in mice, it has been suggested that these two tissues have different competence mediated by the Iroquois complex (Shimamura and Rubenstein, 1997; Kobayashi et al., 2002). However, direct evidence of mesodermal inductive influence on ZLI positioning is lacking, and in other model organisms, such as fish, it has been shown that the influence of the mesoderm on induction and anteroposterior pattern of the neural tube, and therefore positioning and induction of the ZLI, is dispensable (Schier et al., 1997; Scholpp et al., 2006). It has been suggested that, in chick, the pre-ZLI territory is a Wnt8b-positive and L-fng-free wedge-shaped area that collapses during development and forms the definitive ZLI compartment with lineage restriction at both its rostral and caudal boundaries (Zeltser et al., 2001). Furthermore, grafting experiments in chick have shown that an ectopic border between neural tissue from a prechordal and epichordal origin is sufficient to induce an ectopic ZLI (Vieira et al., 2005).

The molecular players involved in ZLI induction have yet to be identified. Gain-of-function experiments in chick have suggested a model in which the interaction between *Six3* and *Irx3* induces the ZLI (Kobayashi et al., 2002; Braun et al., 2003). However, temporal analysis of the expression patterns for these two genes show a gap between the two domains at the time of ZLI formation, precluding direct interaction between the two territories at this stage (reviewed in Kiecker and Lumsden, 2005). Moreover, the ZLI forms in *Six3*-deficient mice, excluding an inductive role for Six3 (Lagutin et al., 2003). Finally, our data show that *irx1b* function is also dispensable for ZLI induction.

Recently, a new border of gene expression has been suggested to be involved in positioning the ZLI organiser, involving an interaction between the prechordally expressed *Fezf1* and *Fezf2* genes with an unknown epichordal factor (Hirata et al., 2006; Jeong et al., 2007). *Fezf1/Fezf2*-deficient mice lack Shh expression in the ZLI, due to mis-specification of the prethalamus. Interestingly, mis-expression of *Fezf2* shifts caudally and reduces the Shh-positive ZLI in fish and mice. We believe that our data allow us now to integrate the various morphological and genetic data and suggest a new model for the induction and positioning of the ZLI along the anteroposterior axis of the neural tube. The spatially restricted stripe-like expression domain of Otx1l/2 patterns the neural tube prior to ZLI formation, setting the anterior border of the pZLI. We show that the ZLI is formed by the rostral half of this Otx-expressing domain, which co-

localises with the stripe of *wnt8b* expression. Thus, this Otx1l/2/Wnt8b domain would appear to correspond directly with the L-fng-negative/Wnt8b-positive compartment that was previously described as the pZLI in chick (Larsen et al., 2001; Zeltser et al., 2001; Garcia-Lopez et al., 2004). This expression domain abuts the *Irx*-expressing territory in fish, chick and mouse. Finally, in both chick and zebrafish, it collapses into a narrow stripe of Hh-expressing cells as it matures into the ZLI (Fig. 7) (Zeltzer et al., 2001).

Independent positioning of the anterior and posterior ZLI boundaries

A striking similarity between the ZLI and the MHB organisers is their location at the interface between an Otx-positive and an Otx-negative territory. Besides the involvement of Otx proteins, the two signalling centres also each release a combination of signal molecules (ZLI: Wnt8b and Shh; MHB: Wnt1 and Fgf8). The boundary between the midbrain and hindbrain has lineage-restriction properties (Zervas et al., 2004; Langenberg and Brand, 2005), whereas the ZLI is a narrow stripe of cells defined by both an anterior and a posterior lineage-restriction boundary (Larsen et al., 2001). Therefore, because the ZLI is a zone rather than an interface, we considered whether the position of its prethalamic border is set independently of its thalamic border. In addition to the requirement for an *otx1l/2*-positive tissue for the formation of the ZLI, and concomitant establishment of its anterior limit, we uncovered an *irx1b*-dependent regulation setting the posterior limit of the ZLI. The striking shape of the *irx1b*-expressing domain suggests that it might give the Shh-expressing cell population its characteristic tapering shape. In chick, *Irx3* has been shown to mediate thalamic competence and, furthermore, *Irx3* has a repressive function on ZLI formation (Kiecker and Lumsden, 2004); however, chick loss-of-function data are missing, preventing direct comparison with our fish results. Neither *irx3a* nor *irx3b*, the fish orthologues of *Irx3*, are expressed directly adjacent at the future ZLI boundary, whereas Irx1 in mouse and Irx1b in fish abut the ZLI (Bosse et al., 1997; Cohen et al., 2000; Lecaudey et al., 2005). By knock-down of Irx1b function, we have been able to show that the expression domains of the Shh genes – *shha* and *shhb* – expand posteriorly at the expense of thalamic tissue, while the anterior boundary is unaltered. Complementarily, a recent study has shown that Fezf proteins, expressed in the presumptive prethalamus and ZLI during early segmentation stages, are required for ZLI formation. Otx proteins need to interact with another protein inside the anterior diencephalon, because we showed that Otx proteins are able to induce the ZLI solely inside the diencephalic territory anterior to the presumptive thalamus. We therefore propose that Fezf could be such a partner. The nature of the proposed Otx/Fezf relationship in the rostral neural tube therefore needs to be addressed. Thus, we conclude that the establishment of the anterior and posterior boundaries of the ZLI are independent events and require different mechanisms.

Otx requirement for thalamic identity

In OtxH embryos, we observed that cells located at the anteroposterior position of the thalamic anlage expressed pretectal markers such as *pax7* (Fig. 4). The $Otx1^{cre/-}$; $Otx2^{flox/-}$ mouse shows small patches of cells deficient for Otx1/2 function in the thalamic anlage (Puelles et al., 2006). These patches show characteristics of inhibitory GABAergic neurons in the thalamic anlage, although the thalamus normally contains mostly excitatory glutamatergic cells. Due to characteristic pretectal *pax3* and *pax7* expression and the fact that the adjacent pretectum generates mainly GABAergic neurons, the authors claim that the lack of *Otx1/2* transcripts leads to induction of pretectal fate. We found that thalamic tissue, which is marked by *neurogenin 1* expression and is a prerequisite for the generation of glutamatergic neurons, is not specified in OtxH embryos. Interestingly, a recent mouse study showed that *gsh1* expression is required for the formation of GABAergic neurons in the spinal cord (Mizuguchi et al., 2006). We found that pretectal *gsh1* expression was shifted to the location of the thalamic anlage in OtxH embryos. Due to the fact that neither cell proliferation nor cell death is dramatically increased in OtxH embryos (Foucher et al., 2006), we suggest a fate change of these cells from a thalamic to a pretectal identity, in agreement with the finding in mouse (Puelles et al., 2006).

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