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 protein expression within the ZLI is induced in a characteristic ventral-to-dorsal progression from the basal plate into the ZLI. Thus, 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 dorsal Shh is 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 lunar 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 Fetz) and Fezf2 (formerly known as Fezfl) gene functions, and fish deficient for fezfl 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, otxl1 and otxl2 (collectively referred to as otxl1/2) – are expressed in a highly regulated spatiotemporal pattern (Mercier et al., 1995). During gastrulatation, 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...
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 prethalamic, characteristics, such as expression of GABAergic markers, suggesting that Otx genes drive glutamatergic thalamic fate (Puuelles et al., 2006).

Another regionally expressed group of genes in the early neural plate is the Iroquois 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; Itho et al., 2002). In contrast to the Otx genes, expression of the Irgx genes is upregulated by Wnt signalling (Itho 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 persistent expression is a prerequisite for the formation of the ZLI, because lack of Otx1/2 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). In contrast to the Otx genes, expression of the Irgx genes is upregulated by Wnt signalling (Itho 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.

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 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 *dlx1a* 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; *Pt*, pretectum; *Pth*, prethalampus; *pZLI*, presumptive ZLI limits intrathalamica; ss, somite stage; *Th*, thalamus; *ZLI*, zona limits 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 (hp); 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’). *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’). 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 *shha*-positive; the anterobasal part of the ZLI is additionally *dbx1a*-positive; the thalamus is *otx2*- and *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
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**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.
- **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-I) At prim-15, in control siblings, the expression domains of **pax6a** and **epha4a** become downregulated in the mid-diencephalon (marked by arrowheads); this structure is not detectable in OtxH embryos. 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; expression is indistinguishable from OtxH embryos at the same stage (F).

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).

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**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, whereas in OtxH embryos, neither the ZLI nor the PC is detectable (D,F,H). Consequently, the 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).
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 is expanded similarly to the thalamus: absence of these proteins led to transformation of the ZLI/thalamus into pretectum and, to a lesser extent, into basal thalamus: absence of these proteins led to transformation of the ZLI/thalamus into pretectum and, to a lesser extent, into basal thalamus.

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. 4J). At prim-10, we found that this Shh-independent irx1b expression domain was still downregulated in OtxH embryos (Fig. 4L). Moreover, expression of the Shh-dependent thalamic marker genes enx2 and dbx1a was absent (Fig. 4G,H) and does not show 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 prepectal 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). 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 irx1b 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 Otx1l2 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 thalamus (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 Otx1l2 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 Otxl- 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).
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 prethalamic-thalamic boundary (Fig. 5D,F,H). In control (con) embryos, shha 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, shha expression can be rescued in the presumptive MDT (H,H'), and a cross-section reveals that the induction is independent of the basal shha expression domain (H'). The rescue of shha 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', yellow arrowhead), as shown by a co-localisation of the red-lineage tracer of the Otx2* cells with the green GFP expression. The inx1b expression profile was assessed in the same embryo (K-K*). When Otx2* cells are located within the presumptive thalamic territory, they express inx1b (white arrowhead), shown by co-localisation of the red lineage tracer with inx1b detection (blue). (K*) Merged picture of Shh-GFP expression, the red lineage tracer and inx1b expression. prim-10, primordium stage 10; Th, prethalamus; PTh*, rescued prethalamus; Th, thalamus; ZLI, zona limitans intrathalamica; ZLI*, rescued zona limitans intrathalamica.
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 caudoventral 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 Otx11/2-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. 41-1′). 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 Otx11/2 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 Otx11/2 are required for the induction of the ZLI and the thalamic anlage. However, although Otx11 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 zebrarfish 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 *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
In the diencephalon, we found that ZLI formation, including enlarged cerebellum (Acampora et al., 1997; Foucher et al., 2006), resulting in an anterior shift of the MHB and formation of an presumptive midbrain territory into an extended rhombomere 1, and the territory of the Shh-positive ZLI is restricted by the thalamic expression of \( \text{inx1b} \) (red T-brackets).

**Complex relationship between Shh and Otx in the neural tube**  
The \( \text{Otx}2^{+/} \text{Otx1}^{-/-} \) 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 \( \text{shha} \) induction, is dependent on proper Otx1/2 function. Mice embryos with reduced Otx1/2 transcripts show a similar lack of \( \text{Shh} \) 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 Otx1/2 proteins. By contrast, a recent study suggests that Otx2 protein is also able to restrict \( \text{Shh} \) 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 \( \text{Shh} \) 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 \( \text{shh} \) 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 \( \text{Foxg1} \) in the prechordal neural plate and \( \text{En1/2} \) 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 \( \text{Six3} \) and \( \text{Irx3} \) 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 \( \text{Six3} \)-deficient mice, excluding an inductive role for \( \text{Six3} \) (Lagutin et al., 2003). Finally, our data show that \( \text{irx1b} \) 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 \( \text{Fezf1} \) and \( \text{Fezf2} \) genes with an unknown epichordal factor (Hirata et al., 2006; Jeong et al., 2007). \( \text{Fezf1} \)/\( \text{Fezf2} \)-deficient mice lack Shh expression in the ZLI, due to mis-specification of the prethalamus. Interestingly, mis-expression of \( \text{Fezf2} \) 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 Otx1/2 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-

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**Fig. 7. Summary.** Regionalisation of the diencephalon. At mid-somitogenesis stages, Otx1/2 mark the presumptive ZLI (p-ZLI, light blue) and the thalamic anlage (Th, yellow), whereas \( \text{irx1b} \) is expressed only in the thalamic anlage. Shh marks the floor plate and basal plate (red) and starts to be induced in the Otx1/2-positive pZLI (green arrows). At pharyngula stages, \( \text{shh} \) is expressed in the mature ZLI (red) and the territory of the Shh-positive ZLI is restricted by the thalamic expression of \( \text{inx1b} \) (red T-brackets).
localises with the stripe of wnt8b expression. Thus, this Otx1/2/-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; Zeltzer 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 otx1l2-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 Otx1cre; Otx2flox mice shows small patches of cells deficient for Otx1/2 function in the thalamic anlage (Puelles et al., 2006). These patches show characteristics of inhibitory GA Baergic 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 GA Baergic 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 GA Baergic 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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