Hedgehog signalling from the zona limitans intrathalamica orchestrates patterning of the zebrafish diencephalon

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Midway between the anterior neural border and the midbrain-hindbrain boundary, two well-known local signalling centres in the early developing brain, is a further transverse boundary with putative signalling properties – the zona limitans intrathalamica (ZLI). Here, we describe formation of the ZLI in zebrafish in relation to expression of sonic hedgehog (shh) and tiggy-winkle hedgehog (twhh), and to development of the forebrain regions that flank the ZLI: the prethalamus and thalamus. We find that enhanced Hh signalling increases the size of prethalamic and thalamic gene expression domains, whereas lack of Hh signalling leads to absence of these domains. In addition, we show that shh and twhh display both unique and redundant functions during diencephalic patterning. Genetic ablation of the basal plate shows that Hh expression in the ZLI alone is sufficient for diencephalic differentiation. Furthermore, acquisition of correct prethalamic and thalamic gene expression is dependent on direct Hh signalling. We conclude that proper maturation of the diencephalon requires ZLI-derived Hh signalling.

KEY WORDS: Regionalization, Forebrain, Shh, Zebrafish, ZLI

INTRODUCTION

During vertebrate brain development, induction and progressive posteriorization of neurectoderm is followed by a phase of regionalization (reviewed by Lumsden and Krumlauf, 1996; Wilson and Houart, 2004; Kiecker and Lumsden, 2005). This may involve specialized groups of cells in ‘signalling centres’ (reviewed by Rhinn and Brand, 2001), the best-characterized of which are the anterior neural border (ANB) (Houart et al., 2002) (reviewed by Wilson and Houart, 2004), the roof plate (reviewed in Chizhikov and Millen, 2004), the floor plate (reviewed in Strähle et al., 2004) and the midbrain-hindbrain boundary (MHB). The cells that release signal molecules from such centres are often located at boundaries between distinct territories, e.g. the MHB forms at the interface between the mesencephalon and anterior rhombencephalon, whereas the ANB lies at the boundary between the telencephalon and anterior epidermal ectoderm. The zona limitans intrathalamica (ZLI), a narrow transverse region between the prethalamus (also known as the ventral thalamus) and the thalamus (also known as the dorsal thalamus) (Kuhlenbeck, 1937; Shimamura et al., 1995) also bears the hallmarks of a signalling centre. Fate mapping experiments in chick have shown that the ZLI is cell lineage restricted at its boundaries and is thus a true developmental compartment (Zeltser et al., 2001; Garcia-Lopez et al., 2004). Furthermore, the ZLI is the only structure in the alar plate that expresses signal molecules of the Hedgehog family (Hh) (Figdor and Stern, 1993; Puelles and Rubenstein, 2003). Well-described functions of Hh signalling from basal and floor plates are ventralization of the neural tube (reviewed by Briscoe and Ericson, 1999; Jessell, 2000), promotion of growth and proliferation (Britto et al., 2002; Ruiz i Altaba et al., 2002), and formation of the hypothalamus (Chiang et al., 1996; Mathieu et al., 2002).

The function of Hh signalling at the ZLI has not been addressed directly in mouse Shh mutants owing to loss of the diencephalon (Ishibashi and McMahon, 2002), but studies in chick have shown that Shh is both necessary and sufficient for thalamic gene induction in vitro (Hashimoto-Torri et al., 2003) and in vivo (Kiecker and Lumsden, 2004).

In zebrafish, two Hh genes are expressed in the ZLI: sonic hedgehog (shh) (Krauss et al., 1993) and tiggy-winkle hedgehog (twhh) (Ekker et al., 1995). Normal development of the ZLI has not been described in zebrafish nor have the mechanisms of its formation been investigated. In addition, it is important to examine whether recent observations in chick (Kiecker and Lumsden, 2004) could be ascribed to an evolutionary conserved mechanism for diencephalic patterning.

Here, we describe how the mid-diencephalic territory (MDT, composed of prethalamus, the ZLI and thalamus) develops in zebrafish. We show that the expression of shh and twhh mark the ZLI, and that ZLI development is accompanied by expression of dbx2a, a marker of the prethalamus, and of dbx1a, a marker of the thalamus. Furthermore, we show that Hh signalling is sufficient for molecular differentiation of both the prethalamus and the thalamus, but is not required for their maintenance. Interestingly, shh and twhh function similarly during prethalamic induction, whereas thalamic induction appears to require Shh signalling exclusively. Finally, we show that the ZLI forms independently of the basal plate and that Hh signalling from the ZLI is sufficient for maturation of prethalamic and thalamic territories while ventral Hh signals are dispensable.

MATERIALS AND METHODS

Maintenance of fish

Breeding fish were maintained at 28°C on a 14 hour light/10 hour dark cycle (reviewed by Brand et al., 2002). Embryos were staged according to Kimmel et al. (Kimmel et al., 1995). Our data derive from analysis of wild-type (wt) fish and the following homozygous mutant embryos and transgenic fish: sonic you (syu) (referred to as syu) (Schauerte et al., 1998), slow muscle omitted (smu) (referred to as smu) (Barresi et al., 2000), one-eyed-pinhthead (oe) (referred to as oe) (Hammerschmidt et al., 1996; Schier et al., 1997) and 2shh:gfp:ABC#15 (referred to as shh:GFP) (Shkumatava et al., 2004).
Injections
Expression constructs for shh mRNA (Krauss et al., 1993) and for twhh mRNA (Hammond et al., 2003) were generated in vitro (Message Machine Kit, Amersham). mRNA was dissolved in 0.25 M KCl including 0.2% of fluorescein-labelled dextran (Mini Emerald, Molecular Probes) as a lineage tracer. During injection, ~150 pg mRNA was deposited into one cell of a 32-cell stage embryo. For transient knock-down of gene expression, Morpholino-antisense oligomers (MO) were used at a concentration of 0.5 mM as described previously (Nasevicius and Ekker, 2000; Scholpp et al., 2003). twhh morpholinos (twhh-MO; 5'-GCT TCA GAT GCA GCC TTA CGT CCA T-3' (Lewis and Eisen, 2001) were injected into the yolk cell close to the blastomeres at one- to eight-cell stages at a concentration of 0.5 mM. A non-binding morpholino (morpholino-sense twhh oligomer; control-MO) showed no effect on embryos when injected at 0.5 mM.

Transplantation
At the one-cell stage, wild-type embryos were injected with 0.25% rhodamine- or biotin-dextran (Molecular Probes). Thirty to 40 cells from the animal region were grafted into a host embryo at the sphere stage (3.5 hpf) to generate a random distribution of labelled cells. At 32 hpf, embryos were identified by morphology or GFP expression.

Inhibitor treatment
3-Keto-N-aminoethylaminoethylcaproyldihydrocinnamoyl cyclopamine (Inhibitor treatment identified by morphology or GFP expression. Animal region were grafted into a host embryo at the sphere stage (3.5 hpf) rhodamine- or biotin-dextran (Molecular Probes). Thirty to 40 cells from the animal region were grafted into a host embryo at the sphere stage (3.5 hpf) to generate a random distribution of labelled cells. At 32 hpf, embryos were identified by morphology or GFP expression.

Staining procedures and imaging techniques
Whole-mount mRNA in situ hybridization was carried out as described previously (Scholpp et al., 2003), and stained by NBT/BCIP and Fast Red (Roche). Embryos were dissected and mounted in 70% (v/v) glycerol/PBS or further processed for antibody staining. Expression patterns have been described for shh (Krauss et al., 1993), twhh (Ekker et al., 1995), dlx2a (originally described as dlx2) (Akimenko et al., 1994), dbx1a (originally described as hlx1) (Fjose et al., 1994), neurog1 (Blader et al., 1997), lhx5 (originally described as lim5) (Toyama et al., 1995), ptc1 (Concordet et al., 1996), emx1 and emx2 (Morita et al., 1995).

Antibody staining was performed as described by Scholpp and Brand (Scholpp and Brand, 2003). Live transgenic embryos were mounted dorsal upwards in 1% LMP-agarose, and imaged using a Nikon C1 confocal microscope. For the fate mapping experiment (Fig. 5), the following parameters were chosen: pinhole, 30 μm; z-step, 10 μm. Images were acquired by single scan combining red and green channels. Data sets were deconvolved by AutoDeblur X CF (AutoQuant) and further processed using Imaris 4.1.3 (Bitplane AG).

RESULTS
Diencephalic regionalization
The ZLI is first detectable as a narrow stripe of shh-expressing cells at 22 somites (Barth and Wilson, 1995). To follow its development, we mapped expression of the Hedgehog genes shh and twhh (Krauss et al., 1993; Ekker et al., 1995) between 12 somites and 48 hpf. These ZLI markers were mapped relative to expression of the prethalamic marker dlx2a (the homologue of Dlx2 in mouse) (Akimenko et al., 1994) and the thalamic marker dbx1a (the homologue of Dbx1 in mouse) (Fjose et al., 1994) by double in situ hybridization.

At 12 somites, shh expression is confined to the ventral midline of the neural tube (Fig. 1A) (Krauss et al., 1993). The future anteroposterior position of the ZLI is already visible at this stage by a kinking of the head (white arrow). At 15-somites, dlx2a is expressed in the anterior forebrain in a ‘salt-and-pepper’ pattern dorsal to the ventral midline shh expression domain (blue arrow) (Fig. 1B). At 20 somites, the dorsal extension of shh in the future ZLI becomes more pronounced (Fig. 1C, white arrow). We find that spatial progression of dlx2a expression evolves concomitantly with shh expression (Fig. 1D, white and blue arrows), in adjacent but non-overlapping domains (Fig. 1D’). At 42 hpf, shh expression extends transversely in a very narrow domain across the alar plate, prefiguring the ZLI (Fig. 1E, white arrow) (Puelles and Rubenstein, 2003). Notably, the ZLI is the only place in the embryo in which shh is expressed in such a dorsal domain. Owing to the posterior invagination of the dorsal forebrain, the shh expression domain translates mediolaterally, resulting in the typical forked shape, the two prongs of which reflect the position of the ZLI (Fig. 1E’, white arrow). A further consequence of invagination is that the prethalamus becomes located lateral to the ZLI (Fig. 1E, blue arrow).

We then analysed the expression of twhh relative to dbx1a. At 12 somites, twhh has an expression profile similar to that of shh (Fig. 1F) and dbx1a is expressed in the anterior neural ectoderm in a ventral domain overlapping with twhh (Fig. 1F, black arrow). By 15 somites, twhh expression is downregulated ventrally and maintained only in a patch of cells in the ventral telencephalon until at least 42 hpf (Fig. 1G–J). dbx1a expression is first detectable at 15 somites ventrally adjacent to the ZLI (Fig. 1G) as well as posterior to the ZLI (Fig. 1G, yellow arrow). Expression of twhh is first observed in the presumptive ZLI at 20 somites, (Fig. 1H, white arrow) and is accompanied by an extended dorsal domain of the dbx1a expression (Fig. 1I,J, marked by white arrows). The thalamic domain of dbx1a increases in both size and intensity over time (Fig. 1G–J). A horizontal section (Fig. 1I) reveals a stripe-like pattern in which we observed characteristic expression subdomains: an anterior region, where twhh and dbx1a are co-expressed (1); a more posterior twhh-positive stripe (2); a region of low dbx1a expression (3); and a region with strong dbx1a expression (4). At 42 hpf, dbx1a is expressed in the fork-shaped ZLI territory (Fig. 1I,J’). In a postero medial position, dbx1a marks the thalamus, as shown in the section (yellow arrow).

To map gene expression domains onto emergent neuroanatomy at 48 hpf, we visualized the expression domains of shh, dlx2a and dbx1a by fluorescence in situ hybridization, followed by counterstaining with an anti-acetylated tubulin antibody to mark axons.

We analysed these combined patterns in the entire head (Fig. 1K-M) using confocal microscopy and three-dimensional reconstruction software. shh is expressed in a medioventral domain stretching continuously from the anterior hypothalamus through the tegmentum into the hindbrain (Fig. 1K). Interestingly, the shh expression domain in the alar plate appears small in lateral view (K, white arrow), but reveals it full size in a vertical rotation (Fig. 1K’). The fork-shaped ZLI shows its ventral limit by a thin connection to the basal expression domain (Fig. 1K’). In situ hybridization for dlx2a reveals the location of the massive cup-shaped expression domain of the prethalamus lateral to the ZLI on either side (Fig. 1L, blue arrows), from which there is but a very thin connection to the more ventral expression domains of the preoptic region (Fig. 1L’) (reviewed in Puelles and Rubenstein, 2003). In the lateral view, the thalamic expression domain of dbx1a is located posterior to the ZLI and medial in the neural tube. 3D rotation movies can be provided on request that show how the original anteroposterior layout of the MDT becomes translated lateromedially by 48 hpf.

Overexpression of shh increases the size of the MDT
To study local activity of Shh at the ZLI, we generated small Shh-expressing clones by injecting 150 pg shh mRNA into one blastomere of a 32-cell embryo (Fig. 2A–A’). To check efficiency, we analysed a bona fide target gene of Hh signalling – the Hh
receptor patched1 (ptc1) (Concordet et al., 1996). After 28 hpf of normal development, ptc1 is expressed in regions of normal Hh activity, e.g. the hypothalamus, ZLI and floor plate (Fig. 2B,B’). In embryos injected with shh, we detected an increased expression of ptc1 at the sites of endogenous expression, e.g. the ZLI (bracket), and also ectopic sites of ptc1 expression that co-localised with shh-positive clones, e.g. in the forebrain (Fig. 2C,C’). The observed phenotypes cannot be explained by ventralisation of the neural tube in response to increased ventral Shh signalling, rather they suggest a function for shh in anteroposterior regionalization of the neural tube. Although our experimental approach produces Shh-overexpressing cells all over the embryo, the ectopic expression of prethalamic markers was observed only anterior to the ZLI, whereas increased expression of thalamic markers was observed only posterior to the ZLI. This suggests that competence fields anterior and posterior to the ZLI are established independently of Shh. Furthermore, we find that shh influences the acquisition of both prethalamic and thalamic fate, and specifies the size of these territories within these fields.

**Hh signalling is required for specification of the MDT**

To complement our gain-of-function approach, we analysed embryos treated with the Hh signalling inhibitor cyclopamine (Icardona et al., 1998), and those carrying a mutation in the Hh co-receptor smoothened (slow muscle omitted, smu), in which all Hh signalling is blocked (Varga et al., 2001).

To elucidate the timing of the Hh requirement, we blocked Hh signalling with cyclopamine for different durations up to 30 hpf (Fig. 3A-D’). Blocking Hh signalling from 10 somites leads to a phenotype similar to that observed in smu mutant embryos with respect to diencephalic development: a strong downregulation of dlx2a and dbx1a (Fig. 3B). To verify our experimental procedure, we studied ptc1 expression in embryos of the same batch and found
a severe downregulation, consistent with a blockade of Hh signalling (Fig. 3B’). Inhibition from 20 somites onwards results in a partial downregulation of \( \text{dlx2a} \) (Fig. 3A,C; blue brackets) and \( \text{dbx1a} \) (Fig. 3A,C; yellow brackets) compared with control siblings, while \( \text{ptc1} \) expression was downregulated, as for the earlier treatments (Fig. 3C’). Cycloamine treatment at 30 hpf produced only very subtle defects in the MDT by comparison with controls (Fig. 3D, blue and yellow brackets). Thus, we find that Hh signalling is required to induce prethalamic and thalamic markers during the normal induction phase between 10 somites and 24 hpf, and thereafter becomes dispensable for maintenance of marker expression.

In \( \text{smu} \) mutant embryos, \( \text{dlx2a} \) and \( \text{lhx5} \) expression was reduced or undetectable at 32 hpf (Fig. 3E-F’; blue arrows). In addition, expression of \( \text{dbx1a}, \text{emx2} \) and \( \text{neurog1} \) was also undetectable, showing a similar requirement for Hh signalling (Fig. 3G-H’, yellow arrows; see Fig. S1 in the supplementary material). Together with the overexpression data, these results show that Shh signalling is required for proper differentiation of the MDT, as manifested by induction of marker gene expression in prospective prethalamic and thalamic regions.

**Distinct roles of Shh and Twhh in mid-diencephalic development**

Because two Hh genes are expressed in the forming ZLI (Fig. 1), we analysed the individual contribution of Shh and Twhh to diencephalic development in a series of loss-of-function experiments: analysis of the phenotype of \( \text{sonic-you} \) embryos, carrying a mutation in the \( \text{shh} \) gene (\( \text{syu} \)) (Schauerte et al., 1998) and/or morphant embryos created by an antisense morpholino targeting \( \text{twhh} \) mRNA (Fig. 4A-E,H-L) (Lewis and Eisen, 2001).

First, we studied the endogenous efficiency of Shh and Twhh by analysis of the expression width and strength of the bona fide target genes \( \text{ptc1} \) and \( \text{nksx2.2} \) in various loss-of-function combinations (Fig. 4A-D; data not shown) at the ZLI flanking region at 28 hours. The cells anterior and posterior the ZLI, which receive Hh signalling above the threshold required for \( \text{ptc1} \) induction, can be used as an indirect readout of the relative efficiency of the Hh signals. \( \text{ptc1} \) is expressed in a total width of 16 cells in wild-type embryos (\( n=42; \) Fig. 4A, bracket). In a knock-down analysis for \( \text{twhh} \), we detect \( \text{ptc1} \) expression all along the ZLI, but find the range of \( \text{ptc1} \) expression is moderately reduced to 14 cells (7/15; Fig. 4B, bracket). Interestingly, in \( \text{syu} \) mutants, \( \text{ptc1} \) expression resembles the expression pattern from the remaining \( \text{twhh} \) gene: positive in the ZLI, but missing in the adjacent ventral part (Fig. 4C, compare with Fig. 1I). The width of the expression domain of \( \text{ptc1} \) decreases to five cells (\( n=12; \) bracket). To generate a combined \( \text{shh/twhh} \) mutant/knock-down, we injected \( \text{twhh-MO} \) into \( \text{syu} \) mutant embryos, \( \text{dlx2a} \) and \( \text{lhx5} \) expression was reduced or undetectable at 32 hpf (Fig. 3E-F’; blue arrows). In addition, expression of \( \text{dbx1a}, \text{emx2} \) and \( \text{neurog1} \) was also undetectable, showing a similar requirement for Hh signalling (Fig. 3G-H’, yellow arrows; see Fig. S1 in the supplementary material). Together with the overexpression data, these results show that Shh signalling is required for proper differentiation of the MDT, as manifested by induction of marker gene expression in prospective prethalamic and thalamic regions.

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embryos (Fig. 4H). In the shh/twhh mutant/knock-down, we find that ptc1 expression is strongly reduced and absent at the ZLI (Fig. 4D), arguing that twhh and shh are the only Hh genes acting in the ZLI. We conclude that at the ZLI, Twhh signalling is less effective than Shh signalling.

On the basis of these results, we looked for a possible influence on the development of the MDT in these loss-of-function situations. Thus, we further analysed development of the prethalamus by dlx2a expression when either twhh or shh, or both, are knocked down or absent. Knock down of twhh led to a slight reduction of dlx2a expression in the prethalamus compared with controls (Fig. 4E,F; square brackets). In addition, we found a mild reduction of dlx2a expression in the presumptive hypothalamus (arrowhead). In syu mutant embryos, we observed a similar reduction of dlx2a expression in the prethalamus (Fig. 4G; square brackets). By contrast to the twhh morphant embryos, syu mutants lost expression of dlx2a in the anterior hypothalamus, whereas the posterior domain appeared nearly unaffected (arrowhead), suggesting a Shh-independent regulatory upstream signal or maternal contribution. Combined shh/twhh mutant/knockdown resulted in a stronger phenotype, where dlx2a expression is lost from the prethalamus and anterior hypothalamus and reduced in the posterior domain (arrowhead). To verify our experimental procedure, we analysed the smu mutant phenotype, in which there is a complete absence of all Hh signalling (Fig. 4I) (Varga et al., 2001). As expected, the smu phenotype resembles the combinatorial loss of Shh/Twhh function phenotype: absence of the prethalamic expression of dlx2a and a strong reduction in hypothalamic expression (arrowhead), consistent with the complete blockade of Twhh translation in a syu mutant background, and with twhh and shh being the only Hh genes acting in the ZLI territory.

Fig. 4. Shh and Twhh have both redundant and unique function during MDT differentiation. Wild-type embryos or syu mutant embryos were injected with twhh-MO (0.5 mM), twhh-mRNA or both shh- and twhh-mRNAs. In wild type, ptc1 is expressed in hypothalamus, ZLI and basal plate (A). The width of the expression of ptc1 anterior and posterior to the ZLI is around 16 cells (bracket). twhh morphants show an overall reduction of ptc1 expression and the width of ptc1 domain is reduced to 14 cell diameters (B, bracket). In the syu mutants, ptc1 is down-regulated (C), and its domain at the ZLI shrinks to 5 cell diameters (bracket). In syu mutant/knockdown ptc1 expression is strongly reduced at the ZLI (D). (E) dlx2a expression in wild-type embryo at 28 hours. twhh morphants show a reduction of dlx2a in the prethalamus (F). Similarly, dlx2a is reduced in syu mutant embryos and the anterior ventral domain is not detectable (G, arrowheads). dlx2a expression is absent in the shh/twhh mutant/knockdown embryos (H), as in smu mutant embryos (I). Mis-expression of twhh-mRNA leads increased dlx2a expression in the prethalamus (J, bracket) and can be further enhanced by synchronous mis-expression of shh-mRNA (150 pg) (K, bracket). twhh morphants show similar thalamic expression of dbx1a compared with wild-type siblings (L,M). By contrast, syu mutant embryos show no detectable dbx1a in the thalamus (N), whereas expression in the tegmentum (asterisks) and ZLI (black arrow) seem unchanged. In shh/twhh mutant/knockdown embryos dbx1a expression is absent from the thalamus (O). smu mutants phenocopy the shh/twhh mutant/knockdowns (P). Mis-expression of twhh leads to weak expansion of thalamic dbx1a expression (Q), whereas combined shh/twhh mis-expression leads to a strong increase in the anteroposterior direction (R).
To support our loss-of-function analysis, we performed a mosaic twwh overexpression experiment, as with shh (Fig. 2) (Hammond et al., 2003). We analysed the embryos at 28 hpf by in situ hybridization for dlx2a (Fig. 4J,K). Injection of twwh mRNA (150 pg) led to a moderate expansion of dlx2a in the prethalamus \((n=14/42;\) Fig. 4E,J). Misexpression of shh mRNA (150 pg) led to a strong anteroposterior expansion \((20/43;\) Fig. 2D,D′). Similarly, a combination of shh and twwh (150 pg each) also led to expansion of the prethalamic domain \((48/70;\) Fig. 4K). Thus, both Hh genes are able to influence the formation of the prethalamus by upregulation of prethalamic gene expression.

twwh morphant embryos display a phenotype similar to controls, allowing but a minor role for Twhh in dlx1a induction (Fig. 4L,M; square brackets). Analysis of sys mutant embryos, however, revealed a strong reduction of dlx1a (Fig. 4N), suggesting that, unlike the situation for the prethalamus, twwh is not able to compensate for the lack of shh posterior to the ZLI. Interestingly, the presumptive ZLI appeared to be slightly broadened (Fig. 4N; arrows). Knock down of twwh in the sys mutant background led to the complete loss of dlx1a expression in the thalamus (Fig. 4O) similar to the smu mutant phenotype (Fig. 4P), lending further support to Shh and Twhh being the only Hh proteins acting at the ZLI. Similar results were observed in the analysis of neurog1, another marker of thalamic differentiation (data not shown).

In a further gain-of-function analysis, we examined the embryos at 28 hpf by in situ hybridization using the thalamic marker dbx1a (Fig. 4Q,R). Misexpression of twwh led to a slight increase of the dbx1a-expression domain \((10/34;\) Fig. 4L,Q), whereas shh was able to considerably expand the expression domain of dbx1a posteriorly \((14/18;\) Fig. 2H,H′). Misexpression of both mRNAs resulted in a phenotype indistinguishable from that of shh overexpression alone \((24/70;\) Fig. 4R), suggesting that shh is required for the induction of the dbx1a expression domain, whereas twwh does not augment the effect of shh under these conditions.

In summary, we find that Shh and Twhh act in an additive way in prethalamic development, whereas thalamic development requires a much greater contribution from Shh. Induction of prethalamic dlx2a expression thus requires less overall Hh signalling from the ZLI compared with thalamic dlx1a.

**Basal plate is dispensable for the formation of the MDT**

From gastrulation stages, the ventral forebrain continuously expresses hh transcripts (Fig. 1) (Krauss et al., 1993), allowing the possibility that ventral Shh-expressing cells could contribute to the formation of the ZLI by dorsalward cell migration or by ‘bucket-brigade’ Hh signalling.

To test these possibilities, we traced randomly distributed alar cells in a transgenic shh:GFP background (Shkutntavata et al., 2004) and followed their movement from 15 somites until 42 hpf. We found that ventral-to-dorsal cell movement in the alar plate is rather minor (Fig. 5A) and that cells keep their dorsoventral position for at least 24 hours until 42 hpf. In addition, alar plate cells are able to switch on Shh expression if they are located at the correct anteroposterior position of the presumptive ZLI (Fig. 5B,C; yellow arrow). These observations suggest that active cell movement is unimportant.

Another possibility is that Hh signalling from ventral regions is required for induction of the ZLI, as Hh signalling is needed for dorsoventral patterning in other regions of the CNS (reviewed by Jacob and Briscoe, 2003). In chick, this early ventral expression has been held responsible for Shh expression within the ZLI itself (Zeltser, 2005). We asked, therefore, whether hh expression in the ventral neural plate plays a role in the formation of the ZLI and in directly regulating development of the prethalamus and thalamus. By studying embryos carrying a mutated form of the EGF-CFC nodal co-receptor one-eyed pinhead (oep) (Hammerschmidt et al., 1996; Schier et al., 1997; Gritsman et al., 1999), which lack the expression in the prethalamus has the same anteroposterior as well as dorsoventral extend in wild-type siblings as in oep mutants \((F,F′;\) red arrows), as does neurog1 expression in the thalamus \((G,G′;\) red arrows). Double in situ hybridization for the telencephalic marker emx1 in blue and dlx2a in red distinguishes the diencephalic dlx2a expression domain (inset in F′, arrowhead).
Transplanted wild-type cells in anterolateral proximity to the ZLI expressed *dlx2a* (12 clones in three embryos, Fig. 6A,B′, blue arrows; Fig. 6C, red dots). However, wild-type cells that lay at a greater distance from the ZLI (more than 10 cell diameters, close to the telencephalic boundary: two clones in one embryo, Fig. 6C, black dots) did not stain for *dlx2a*, suggesting that they are unable to receive proper Hh signalling. Similarly, wild-type cells in posterior proximity to the ZLI expressed *dbx1a* (Fig. 6D–D′, yellow arrows; Fig. 6E–E′, yellow arrows; nine cell clones in four embryos; Fig. 6F, red dots). By contrast, cells located at a distance to the Hh source (more than 10 cell diameters) were unable to express the thalamic marker (three cell clones in two embryos, Fig. 6C and F, black dots). In summary, we find that cells located close to the ZLI are able to respond to Hh signalling and subsequently acquire prethalamic or thalamic fate but cells that require longer range Hh signalling are not able to respond appropriately in *smu* mutants, suggesting an inductive relay mechanism or a requirement for a modified trafficking for Hh molecules to reach cells on the both sides of the ZLI. Thus, we find induction of *dlx2a* only anterolateral (12 cell clones) and *dbx1a* only posterior to the ZLI (nine cell clones, except one cell clone — see Discussion), consistent with our earlier finding that a competence pre-pattern is established in parallel with, or upstream of, functional Hh signalling.

**DISCUSSION**

We have described marker gene expression in the ZLI and examined the signalling function of the ZLI during mid-diencephalic regionalization. We find that *shh* and *twhh* are the only Hh genes expressed in the ZLI and that *Twhh* acts in a more restricted domain than does Shh. In the absence of Hh signalling, genes marking the prethalamus and the thalamus, two major subdivisions of the forebrain that flank the ZLI, are not expressed. In addition, we show that the ZLI forms in the absence of ventral Hh-expressing neuroepithelium and that signalling from the ZLI is necessary and sufficient to induce gene expression in the MDT in the absence of ventral signalling. Finally, we show that acquisition of correct regional identity in the MDT is dependent on direct responses to Hh signalling.
Formation of the MDT

We find that from the 12 somites onwards, *shh* and *twhh* expression in the ZLI extends dorsally into the alar plate from a ventral origin. Cell tracing experiments (Fig. 5) suggest that a progressive activation of genes is required for acquisition of ZLI identity rather than it being formed by a stream of cells migrating from the floor plate. However, we cannot exclude that migration takes place at an earlier stage (before 15-somites) or that single cells from the floor plate move dorsally. A 5-hour lag between the detection of GFP in the ZLI and detection of *shh* mRNA by in situ hybridization provides further evidence for progressive maturation (Shkumatava et al., 2004).

Studies in chick have suggested that Shh is required for the formation of the ZLI (Kiecker and Lumsden, 2004; Zeltser, 2005). However, we find that Hh expression in the zebrafish ZLI is independent of Hh, or indeed any ventral signals. Thus, absence of the ventral midline region of the neural tube, as in the *oep* mutant, does not interfere with establishment of the ZLI. This has been observed previously in other nodal mutants such as *cyclops* (Barth and Wilson, 1995). Furthermore, we can conclude that Hh signalling is dispensable for the formation of the ZLI, as shown in the *smu* mutant embryos (Fig. 3) (Varga et al., 2001). Although the ZLI appears narrower in *smu* mutants when compared with wild-type siblings, the grafting assay shows that Hh from the ZLI is still able to regionalize the territory appropriately (Fig. 6). One possible explanation for the difference between chick and zebrafish is that the experimentally induced reduction of ventral Hh signalling in chick causes the ZLI to mature more slowly. Alternatively, the positive feedback autoregulatory mechanism for Hh expression, a plausible mechanism in chick (Kiecker and Lumsden, 2004), is less evident in fish. The persistence of *shh* expression in the ZLI of *smu* embryos argues that positive feedback autoregulation is indeed of little importance in zebrafish.

In the absence of dorsalward cell migration from the basal plate, we propose that the ZLI is formed by process of progressive maturation of alar plate cells, reflected by the activation of Hh from ventral to dorsal. This observation could be explained by existence (and decay) of an inhibitory signal from the roof plate. (Zeltser, 2005) (F. Guinazu, C. Kiecker and A. Lumsden, unpublished). The ventral limit of *twhh* expression in the ZLI coincides with the ventral border of *shh* expression in *oep* mutant embryos, where the basal plate is genetically depleted. Based on this observation, we can define the dorsoventral extent of the ZLI in zebrafish.

In vitro studies have claimed that thalamic development is partially dependent on signals from the basal plate, although expression patterns of prethalamic or thalamic markers have not been investigated directly (Hashimoto-Torii et al., 2003; Zeltser, 2005). By contrast, focal blockade of Hh signal reception has suggested that horizontal Hh signalling is more important than vertical (Kiecker and Lumsden, 2004). Our findings now offer further evidence for this: embryos initially lacking the ventral Hh signal are able to induce prethalamic and thalamic expression similar to wild type (Fig. 5). We conclude, therefore, that the MDT requires direct Hh signalling solely from the ZLI and that the ventral contribution of Hh signal for induction of prethalamic and thalamic tissue is dispensable.

Establishment of diencephalic subdivisions

We show that Hh signalling from the ZLI is directly required for induction or maintenance of *dlx2a* and *lhx5* in the prethalamus and for induction of *dbx1a*, *emx2* and *neurog1* in the thalamus. All of these are pro-neural genes. It is well known that Shh is needed in the spinal cord and hindbrain for the induction of specific neuronal progenitor identities (reviewed by Jessell, 2000). Recently, it was shown that Hh signalling can actively direct cell-cycle exit and lead cells to differentiation (Shkumatava and Neumann, 2005); our description of the maturation of the diencephalon serves as a further example of this. Thus, we suggest that Hh signalling is important for regionalization of the MDT and the subsequent generation of various neuronal identities. Interestingly, we find that a regional pattern is already established in the diencephalon before expression of Hh genes at the ZLI. In our grafting experiments as well as in our overexpression analysis, we show that Hh signalling is able to induce expression profiles of various neuronal subtypes appropriate to their position relative to the ZLI. These findings confirm by experiments in chick, which have shown that the initial pattern is set by an interaction of the competence factors Six3 and Irx3 in the anterior and posterior diencephalon, respectively (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004).

Timing and concentration of Hh signalling

Cyclopamine treatment arrests *dlx2a* and *dbx1a* expression at the time of treatment (Fig. 3). Therefore, persistent Hh signalling is necessary between 12 somites and 24 hpf to induce the full extent of adjacent expression domains. After 24 hpf, Hh signalling is dispensable with respect to marker expression. Therefore, we conclude that the timing of Hh signalling has to be tightly controlled. In addition, it has been shown that patterning within the thalamus reflects dose-dependent Hh signalling for the induction of *Sox14* (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004), and we find that knocking down one Hh gene reveals another concentration-dependent mechanism anterior to the ZLI. A further example is served by the spinal cord, where Shh induces concentration-dependent changes in ventral genes (Kohtz et al., 1998).

Differences of activity range anterior and posterior to the ZLI

Prethalamic gene expression is activated in a ventral-to-dorsal direction, accompanying the dorsal extension of the ZLI, whereas the thalamus matures from anterior to posterior. This could be explained by the topography of the two territories: the prethalamus forms lateral to the ZLI, such that it remains close to the source. By contrast, the thalamus lies in a medial position and stretches far posterior, such that the distance from the Hh source is comparatively greater. This would lead first to the induction of the anterior part of the thalamus, followed by progressively more posterior tissues. Different activity ranges of Hh signalling around the ZLI could be explained by different propagation mechanisms for Hh anterior and posterior to the ZLI.

Interestingly, we find that Twhh acts in prethalamic development, whereas it is virtually dispensable for thalamic gene induction, suggesting different concentrations of the Hh signals at the ZLI or different effectivity of the two proteins. A further possibility would be that *dlx2a* in the prethalamus is induced at a lower threshold compared with *dbx1a* in the thalamus. Therefore, Shh is able to replace Twhh in most functions, but not vice versa. This would also explain no *twhh* mutants have been discovered.

In the absence of Hh signalling, the *dbx1a* expression domain at the ZLI seems slightly broadened compared with wild type (Fig. 3). Our data show that the expression of *dbx1a* at the ZLI is Hh independent. It has been suggested that, in addition to functional Hh signalling, Wnts could play a role during mid-diencephalic regionalization (Garda et al., 2002; Braun et al., 2003).
Direct acquisition of diencephalic specification.

To acquire the correct genetic profile, single cells in the MDT have to integrate Hh signalling directly. Blocking reception of the Hh signal in small cell clones has a similar effect (Kiecker and Lumsden, 2004). In addition, the latter experiment suggests that there is an Hh-independent pre-pattern in the tissue as discussed previously. We found a single exception where one cell clone located anterior to the ZLI switched on a thalamic marker. This could be due to a mislocated tectal cell clone or it could be that mechanical stress of grafting caused the induction of signals leading to local posteriorization of these cells (Storey et al., 1998; Scholpp et al., 2003) (reviewed in Chiquet et al., 2003).

In summary, we have described the ZLI as a new signalling centre in the zebrafish embryo and have further explored its organising role during development of the MDT. Although we have elucidated certain aspects of its formation and function, these findings raise further questions. What are the consequences of lacking marker gene expression in the prethalamus or thalamus? How does the absence of dlx2a or dbx1a interfere with (e.g.) neuronal composition of these territories? In addition, it has been proposed that other signalling molecules such as Wnts and Fgfs, play roles during the development of this territory (Braun et al., 2003; Echevarria et al., 2003).

However, studies that range across different vertebrate models could reveal the existence of a common basic mechanism leading to the correct positioning, differentiation and function of the ZLI through vertebrate evolution.

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Supplementary material

Supplementary material for this article is available at the development website.

References


