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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 *dlx2a*, 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*^{tbx392} (referred to as *syu*) (Schauerte et al., 1998), *slow muscle omitted*^{b641} (referred to as *smu*) (Barresi et al., 2000), *one-eyed-pinhead*^{tz57} (referred to as *oep*) (Hammerschmidt et al., 1996; Schier et al., 1997) and *2.2shh:gfp:ABC#15* (referred to as *shh:GFP*) (Shkumatava et al., 2004).

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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; *con-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 (KAAD-cyclopamine; Toronto Research Chemicals, Canada) was dissolved in ethanol at 70 μM to block Hh signalling and to treat embryos at different intervals up to 32 hpf. Control siblings were vehicle treated.

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 the 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,E′, 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. 1J,J′). In a posteromedial 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

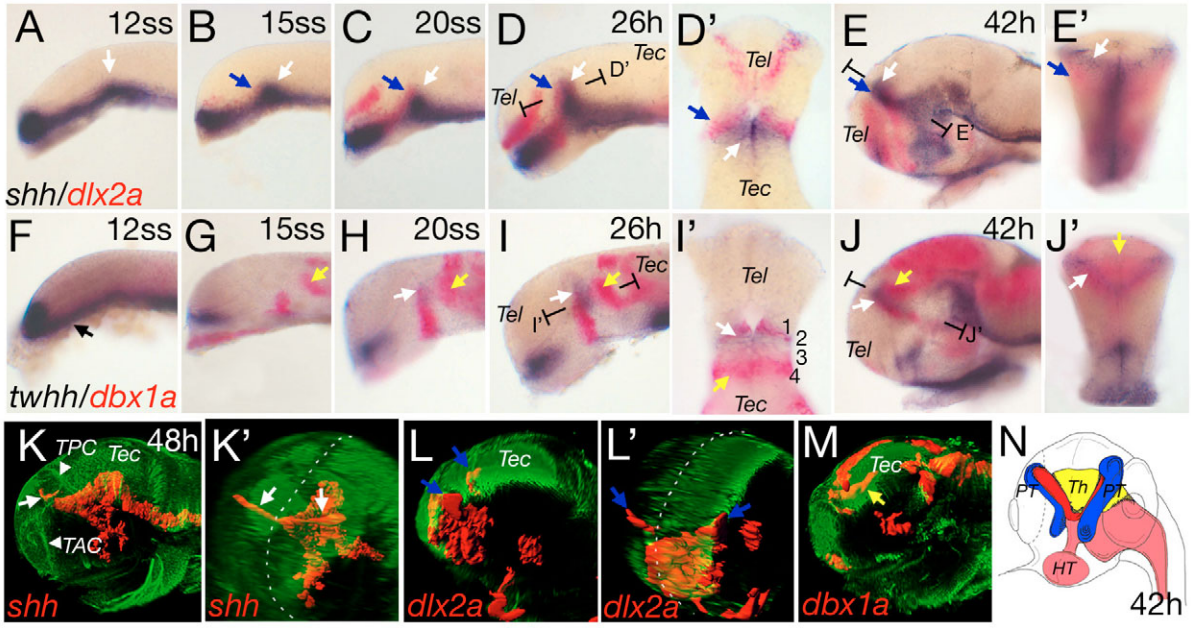

Fig. 1. Anteroposterior differentiation in the mid-diencephalic territory (MDT). Whole-mount double in situ hybridization of wild-type embryos, with *shh* and *dlx2a* (A-E′) and *twhh* and *dbx1a* (F-J′). Section planes of D′,E′,I′,J′ are indicated. (**A**) *shh* expression in the ventral neural tube with the position of the presumptive ZLI indicated (white arrow). At the 15-somite stage, *dlx2a* expression starts in the forebrain (**B**,**C**; blue arrows). Horizontal section of **D** shows the abutting expression domains of *dlx2a* and *shh* (D′). *shh* expression extends to the dorsal and *dlx2a* expression is located more laterally (**E**, blue arrow). A cross-section in (E′) shows the ZLI (white arrow) and the prethalami (blue arrow). Onset of *twhh* expression in the presumptive ZLI is contemporaneous with *shh* (H and I, white arrow). *twhh* expression marks the v-shaped structure of the ZLI at 42hpf (J,J′). At 12 somites, *dbx1a* expression is anteroventral (**F**,**G**). At 15 somites, *dbx1a* is expressed at the base of the future ZLI (G), extending dorsally in later stages (**H-J**) and co-localized with *twhh* [horizontal section at 26 hpf (I′) and cross-section at 42 hpf (J′)]. From 15 somites onwards, a posterior domain of *dbx1a* can be detected, marking the presumptive thalamus (G-J, yellow arrows). At 42 hpf, the *dbx1a*-positive thalamus lies in a medial position, posterior to the ZLI (cross-section in J′). (**K-M**) Lateral and pseudo-frontal views of 3D reconstructions of confocal stacks of in situ hybridization combined with anti-acetylated tubulin staining. Broken white lines indicate the midline (K′ and L′). At 48 hpf, *shh* is expressed in the ZLI in two prongs (K,K′, white arrows). *dlx2a* expression in the prethalami is located ventral and lateral to the ZLI (L,L′, blue arrows). *dbx1a* is expressed posterior and dorsal to the *shh* expression domain (M, yellow arrow). (**N**) Scheme of the subdomains of the MDT based on marker expression at 42 hours: red, ZLI; blue, prethalami; yellow, thalamus; pink, *shh*-positive basal plate. HT, hypothalamus; PT, prethalamus; Th, thalamus; TAC, tract of the anterior commissure; Tec, tectum; Tel, telencephalon; TPC, tract of the posterior commissure. Embryos are oriented dorsal side to the top and anterior towards the left in all figures, except when indicated.

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′, arrows).

Subsequently, we investigated expression of the prethalamic markers *dlx2a* and *lhx5* (Fig. 2D-G′) (Akimenko et al., 1994; Toyama et al., 1995). Ectopic expression of *shh* mRNA resulted in the expression of *dlx2a* (Fig. 2E,E′) as well as of *lhx5* (Fig. 2G,G′) being extended anteroposteriorly, with a higher intensity compared with non-injected siblings (Fig. 2D,D′,F,F′; bracket). In addition, *dlx2a* expression was induced in ectopic locations (Fig. 2E,E′, marked by arrow). Ectopic *shh* resulted in similar changes to expression of the thalamic markers *dbx1a*, *emx2* and *neurog1* (Fig. 2H-K′; see Fig. S1 in the supplementary material) posterior to the ZLI. Here, mosaic overexpression of Shh led to the anteroposterior expansion of both *dbx1a* (Fig. 2F,F′, bracket) and *emx2* (Fig. 2G,G′, yellow arrow).

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 (Incardona 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 *dlx2a* (Fig. 3A,C; blue brackets) and *dbx1a* (Fig. 3A,C; yellow brackets) compared with control siblings, while *ptc1* expression was downregulated, as for the earlier treatments (Fig. 3C′). Cyclopamine 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 *smu* mutant embryos, *dlx2a and lhx5* expression was reduced or undetectable at 32 hpf (Fig. 3E-F′; blue arrows). In addition, expression of *dbx1a*, *emx2* and *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.

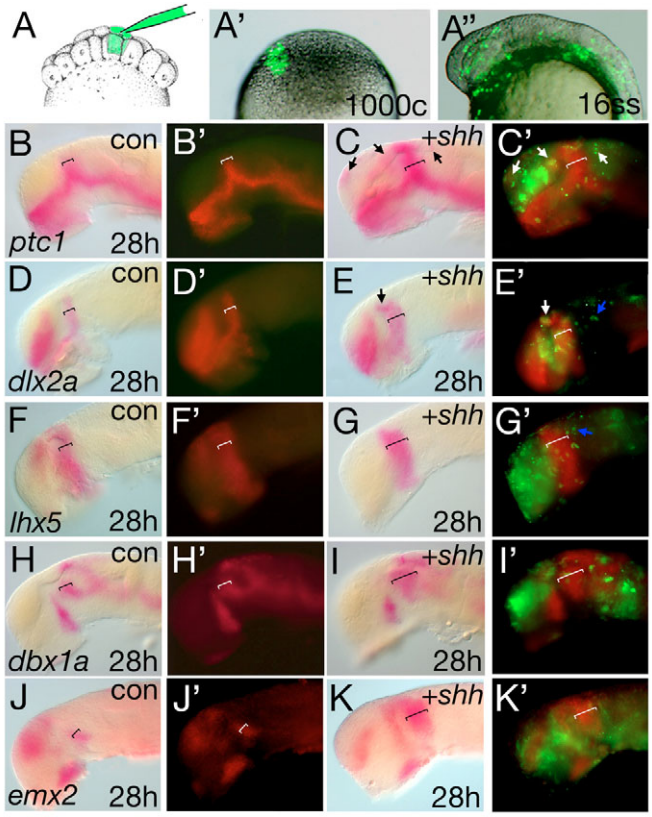

Fig. 2. Mis-expression of *shh* results in an increase of the MDT. *shh* mRNA (150 pg) was injected into one of 32 cells to generate randomly distributed *shh*-positive cell clones, shown by an FITC-coupled lineage tracer (**A-A′′**). Mis-expression of *shh* leads to expansion of *ptc1* (**B-C**′, bracket) and induces *ptc1* positive clones at ectopic sites (arrows). *dlx2a* as well as *lhx5* are expanded following *shh* mRNA mis-expression (**D-G**′, bracket). *dlx2a* is induced anterior to the ZLI (white arrow), but not posterior (blue arrow). *dbx1a* and *emx2* expression increases in size after *shh* mRNA mis-expression (**H-K**′, brackets).

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 *sonic-you* embryos, carrying a mutation in the *shh* gene (*syu*) (Schauerte et al., 1998) and/or morphant embryos created by an antisense morpholino targetting *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 *ptc1* and *nkx2.2* 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 *ptc1* induction, can be used as an indirect readout of the relative efficiency of the Hh signals. *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 *twhh*, we detect *ptc1* expression all along the ZLI, but find the range of *ptc1* expression is moderately reduced to 14 cells (7/15; Fig. 4B, bracket). Interestingly, in *syu* mutants, *ptc1* expression resembles the expression pattern from the remaining *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 *ptc1* decreases to five cells (n=12; bracket). To generate a combined *shh/twhh* mutant/knock-down, we injected *twhh*-MO into *syu* mutant

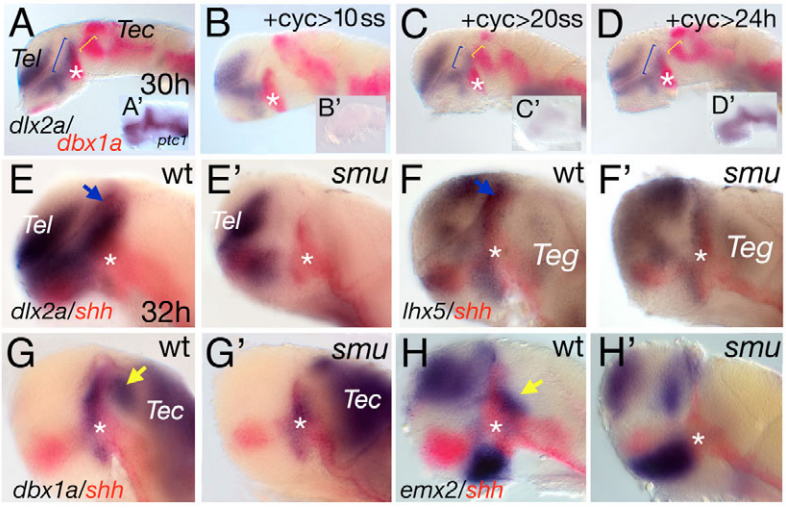

Fig. 3. The MDT is reduced in embryos deficient for Hh signalling. (**A**) Inhibition of Hh signalling by cyclopamine treatment (70 μm) from 10-somites to 30 hours leads to a strong reduction of *dlx2a* expression anterior to the ZLI and *dbx1a* posterior to the ZLI (**B**). *ptc1* expression is not detectable in these embryos (B′). Weaker phenotype observed with treatment between 20 somites and 30 hours (**C**; *dlx2a*-positive prethalamus is marked by blue bracket and *dbx1a* by yellow bracket), although *ptc1* expression is still undetectable (C′). After 24 hpf, inhibition of Hh signalling has no detected effect (**D**) compared with wild-type siblings (A). Asterisks indicate the ZLI (A-D). Analysis of the *smu* phenotype reveals Hh dependency for gene expression in the MDT (**E-H**′). *dlx2a* is not detectable in the prethalamus (E,E′, blue arrow), *lhx5* expression is downregulated (F,F′, blue arrow), *dbx1a* is absent form the thalamus (G,G′; yellow arrow), in contrast to the ZLI (asterisk). Similarly, *emx2* is downregulated (H,H′; yellow arrow). Tec, tectum; Teg, tegmentum; Tel, telencephalon.

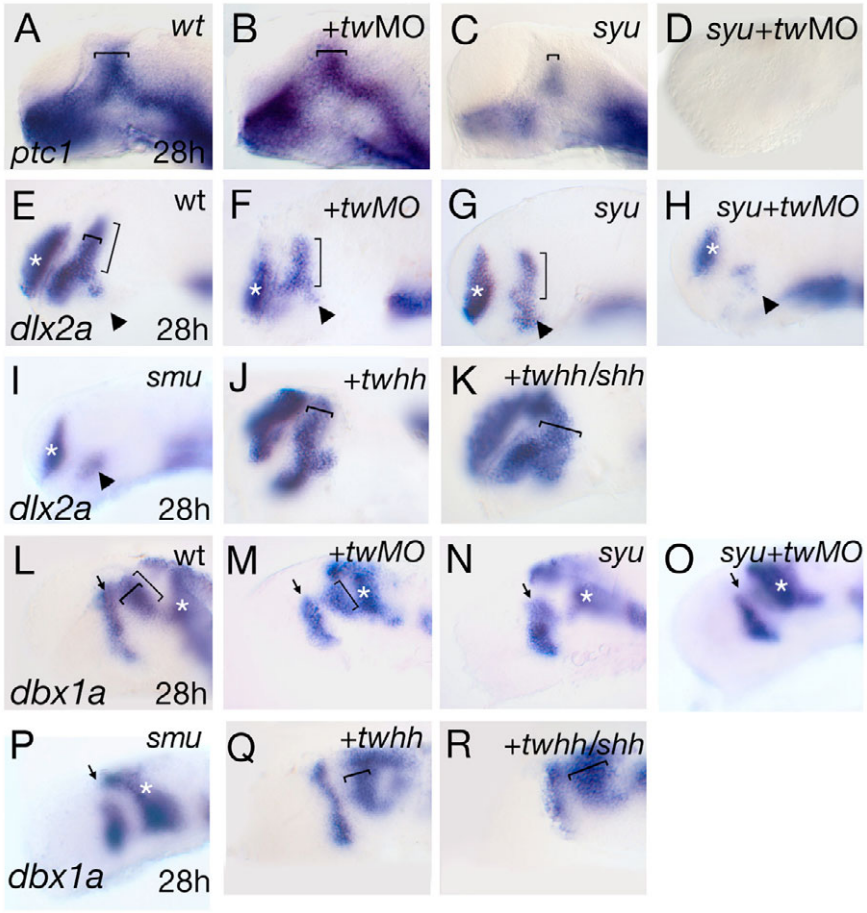

Fig. 4. Shh and Twhh have both redundant and unique function during MDT differentiation. Wild-type embryos or *syu* mutant embryos were injected with *twhh*-MO (0.5 mM), *twhh*-mRNA or both *shh*- and *twhh*-mRNAs. In wild type, *ptc1* is expressed in hypothalamus, ZLI and basal plate (**A**). The width 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* mutants additionally knocked-down for *twhh*, *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**).

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/knock down 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.

To support our loss-of function analysis, we performed a mosaic *twhh* 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 *twhh* 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 *twhh* (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.

twhh morphant embryos display a phenotype similar to controls, allowing but a minor role for Twhh in *dbx1a* induction (Fig. 4L,M; square brackets). Analysis of *syu* mutant embryos, however, revealed a strong reduction of *dbx1a* (Fig. 4N), suggesting that, unlike the situation for the prethalamus, *twhh* 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 *twhh* in the *syu* mutant background led to the complete loss of *dbx1a* 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 *twhh* led to a slight increase of the *dbx1a*-expression domain (10/34; Fig. 4L,Q), whereas *shh* was able considerably to 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 *twhh* 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 *dbx1a*.

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 (Shkutmatava 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

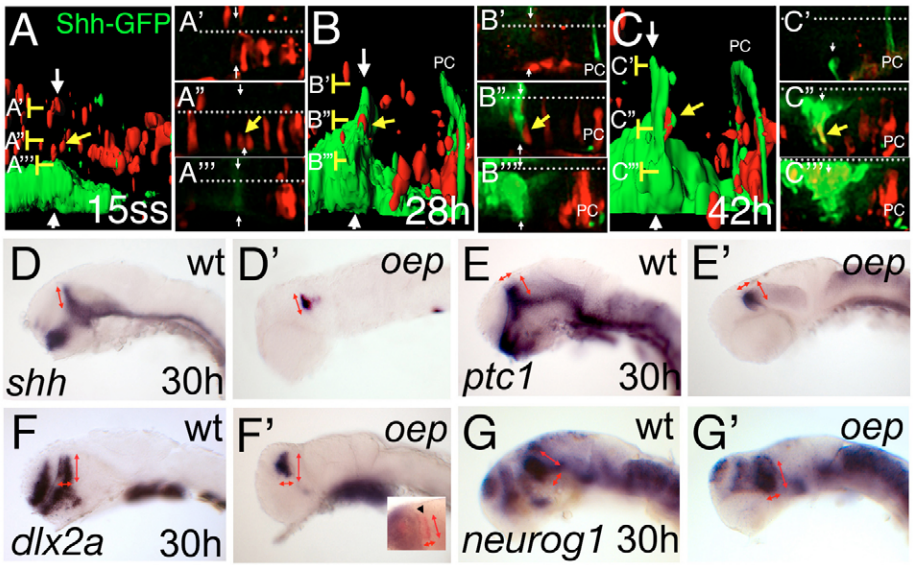

Fig. 5. The basal plate is dispensable for ZLI formation and function. Rhodamine-dextran labelled cells (red) from a Shh-GFP transgenic embryo have been grafted into an embryo with the same genetic GFP-background (green) at sphere stage and further examined from 15 somites to 42 hpf (24 hours) on a confocal microscope. (**A-C**) Lateral views of the MDT of the same embryo at indicated stages after 3D reconstruction and surface rendering. (A′-A′′′, B′-B′′′, C′-C′′′) High magnification of the original dorsal sections of the scan at the indicated levels (brackets). White arrows indicate the ZLI and white dots indicate the midline. An indicated cell (yellow arrow) lies dorsal to the ZLI (A,A′′). At 28 hpf, the cells are adjacent to the GFP positive ZLI (B,B′′) and contribute to the ZLI at 42 hpf, shown by interdigitated red and green surfaces (C), and by a yellow overlay of the red rhodamine label with the GFP expression in the corresponding section (C′′). PC marks the posterior commissure, marked by some shh-GFP positive axons. At 30 hours, *oep* mutant embryos were stained by indicated marker to analyse formation and function of the ZLI in embryos lacking the basal plate. In wild-type embryos, *shh* is expressed in the ventral neural tube as well as the ZLI (**D**). In *oep* mutants, *shh* can be detected only in the ZLI and the ventral hindbrain (D′). The dorsoventral extend of the ZLI is similar in wild-type and *oep* mutants (red arrows). *ptc1* has a similar width to that of wild-type siblings in the diencephalon (**E**,E′; red arrows). *dlx2a* 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).

anterior basal plate, we were able to study mid-diencephalic development in the complete absence of the ventral source of Hh signalling.

First, we analysed *shh* and *twhh* expression in these mutant embryos at the 20-somite stage and 30 hpf. As expected, the ventral expression domain in the diencephalon is absent in these mutants (Fig. 5D,D′; see Fig. S1 in the supplementary material). At 20 somites, we saw the first expression of *shh* in the dorsalmost part of the normal *shh* expression domain in the alar plate (by definition, the ZLI) in *oep* mutants (see Fig. S1 in the supplementary material). In all mutant embryos analysed, the presumptive ZLI is similar in size to that of wild-type siblings (n=63; Fig. 5D′, red arrows) (Schier et al., 1997). This shows that formation of the ZLI and its Shh expression domain are independent of the basal plate, and specifically of ventral Hh. To compare the effective range of Hh signalling, we analysed the expression of *ptc1*. Owing to the lack of *Hh* expression ventrally, *ptc1* becomes reduced in the majority of ventral regions. By contrast, expression of *ptc1* astride the ZLI shows the same anteroposterior and dorsoventral extent as in wild-type embryos, suggesting that Hh signalling from the ZLI is unaffected in *oep* mutant embryos (Fig. 5E,E′).

In further experiments, we analysed the formation of the MDT in *oep* mutants. Prethalamic *dlx2a* expression and thalamic *neurog1* expression in *oep* mutants is similar to that in wild-type embryos (Fig. 5F-G', red arrows) indicating that Hh signalling from the ZLI alone is sufficient to induce the characteristic expression of markers in the MDT with no requirement for Hh signalling from the ventral region of the forebrain.

Wt cells are able to receive a Hh signal and display correct expression profile in *smu* mutant embryos

To address the issue of whether Hh signalling is necessary and sufficient to induce prethalamic and thalamic fate at a distance, we grafted wild-type cells into a *smu* mutant background either anterolateral or posterior to the presumptive ZLI at sphere stage. At 32 hpf, we performed double in situ hybridization with *shh,* as a marker for the ZLI, and a marker for either the prethalamus (*dlx2a*) or thalamus (*dbx1a*). In addition, we stained the embryos with FITC-conjugated streptavidin to detect biotin-containing transplanted wild-type cells.

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.

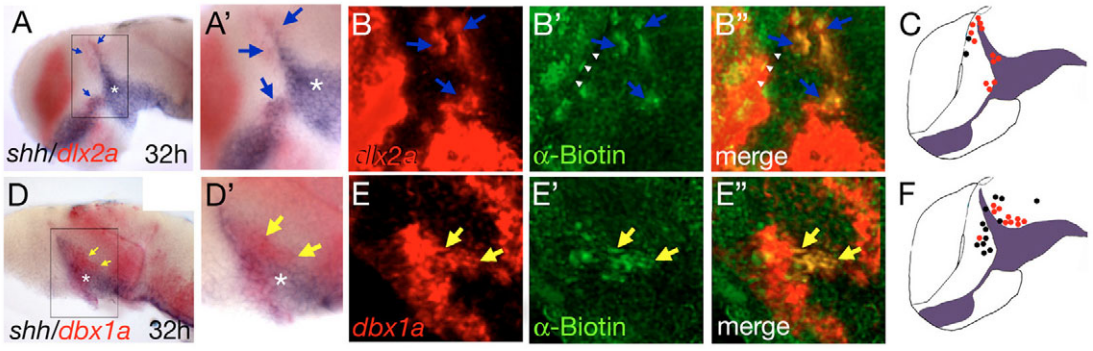

Fig. 6. Direct Hh signalling is required for acquisition of proper MDT gene expression. Wild-type embryos were injected with a biotin-coupled lineage tracer and grafted into a *smu* mutant embryo at sphere stage. At 32 hpf, the embryos were fixed and stained for *shh/dlx2a* or *shh/dbx1a* by double in situ hybridization, followed by fluorescence detection of the biotin-containing wild-type cells. *dlx2a*-positive cell clones are detectable anterolateral to the ZLI (**A**, higher magnification in A′; blue arrows). A section of the same embryo shows co-localization between the red *dlx2a* with the green biotin (**B**-B′′, blue arrows). *dbx1a*-positive cell clones are seen posterior to the ZLI (**D**, higher magnification in D′; yellow arrows). A section of the same embryo shows co-localization between the red *dbx1a* with the green biotin (**E**-E′′, yellow arrows). (**C**,**F**) Summaries of all transplantation experiments showing *dlx2a* 14 wild-type clones, positive (12 red dots) and negative (2 black dots); and *dbx1a* 20 wild-type clones, positive (9 red dots) and negative (black dots, 7 anterior and 4 posterior to the ZLI).

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 dispensible.

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 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 posterization 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
http://dev.biologists.org/cgi/content/full/133/5/855/DC1

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