Positioning of the midbrain-hindbrain boundary organizer through global posteriorization of the neuroectoderm mediated by Wnt8 signaling

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Summary

The organizing center located at the midbrain-hindbrain boundary (MHB) patterns the midbrain and hindbrain primordia of the neural plate. Studies in several vertebrates showed that the interface between cells expressing Otx and Gbx transcription factors marks the location in the neural plate where the organizer forms, but it is unclear how this location is set up. Using mutant analyses and shield ablation experiments in zebrafish, we find that axial mesendoderm, as a candidate tissue, has only a minor role in positioning the MHB. Instead, the blastoderm margin of the gastrula embryo acts as a source of signal(s) involved in this process. We demonstrate that positioning of the MHB organizer is tightly linked to overall neuroectodermal posteriorization, and specifically depends on Wnt8 signaling emanating from lateral mesendodermal precursors. Wnt8 is required for the initial subdivision of the neuroectoderm, including onset of posterior gbx1 expression and establishment of the posterior border of otx2 expression. Cell transplantation experiments further show that Wnt8 signaling acts directly and non-cell-autonomously. Consistent with these findings, a GFP-Wnt8 fusion protein travels from donor cells through early neural plate tissue. Our findings argue that graded Wnt8 activity mediates overall neuroectodermal posteriorization and thus determines the location of the MHB organizer.

Key words: Midbrain-hindbrain boundary, MHB/isthmic organizer, Posteriorization, Zebrafish, Neural plate, Morphogen, wnt8, gbx1, otx2, gbx2, nodal, fgf

Introduction

The initial subdivision of the neural plate is the first step towards generating cellular diversity in the vertebrate brain. A key question is how this initial subdivision is achieved along the anteroposterior (AP) axis. In the two-step ‘activation-transformation’ model, an activating signal induces the ectoderm to become anterior neural tissue. Inhibition of bone morphogenetic protein (Bmp) activity by factors released from the organizer like chordin, noggin or follistatin is thought to be crucial for this step (Wilson and Hemmati-Brivanlou, 1997). A second, transforming signal is thought to convert part of the neuroectoderm into a more posterior identity (transformation, or ‘posteriorization’). Posteriorization has been difficult to understand because multiple signaling pathways can exert posteriorizing effects. Candidate molecules for posteriorizing signals are fibroblast growth factors (Fgfs), Wnt proteins and retinoic acid, but which signaling protein(s) are acting directly on target cells has been difficult to resolve (Gamse and Sive, 2000).

By the end of gastrulation, cell-type diversity of the neural plate is further refined through activities of local organizing centers. The midbrain-hindbrain boundary (MHB) organizer mediates positional information in the neuroectoderm via secreted molecules such as Fgf8 or Wnt1. Mutant analyses in mouse and zebrafish identified several genes involved in MHB development, e.g. Wnt1, Pax2 and Fgf8, which are involved in the formation and organizing activity of the MHB (for reviews, see Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). Restricted gene expression in the zebrafish neural plate around the MHB is first observed during gastrulation stages (Lun and Brand, 1998; Reifers et al., 1998; Rhinn et al., 2003), and is prefigured by the interface between anterior otx2- and posterior gbx1-expressing cells. As in other vertebrates, this precise alignment suggests that forming the otx2/gbx1 interface is a crucial step for positioning the MHB in the neural plate. Indeed, Otx and Gbx loss- and gain-of-function experiments in different species support the notion that these genes are required to correctly position the MHB in the neural plate (Wassarman et al., 1997; Rhinn et al., 1998; Rhinn et al., 2004; Acampora et al., 1998; Broccoli et al., 1999; Millet et al., 1999; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Kikuta et al., 2003) (M.R. and M.B., unpublished).

These findings raise the issue of how the interface itself is positioned in the early neural plate. Here, we study how the otx2/gbx1 interface is set up in the zebrafish neuroectoderm. Regionally restricted otx2 and gbx1 expression occurs very early during gastrulation stages, and could be linked to the formation of axial mesendoderm. Conflicting results have been obtained concerning the involvement of the axial...
mesendoderm in the AP patterning of the CNS. Studies in Xenopus have indicated that induction of AP neural patterning might involve signals from the involuted dorsal mesoderm to the overlying ectoderm (Ruiz i Altaba and Jessel, 1993). Work in several species suggests that signals from the anterior mesendoderm or notochord might regulate the expression of Engrailed genes in the neural plate (Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Darnell and Schoenwolf, 1997; Shamim et al., 1999). However, these findings are inconsistent with node removal experiments, which reveal correct AP patterning, including En2 expression, in the absence of axial mesendoderm (Darnell et al., 1992), and a role in refining rostrocaudal pattern has been suggested instead (Rowan et al., 1999; Camus et al., 2000). Moreover, in mouse or in zebrafish that lack the notochord, a well-formed neuraxis develops (Ang and Rossant, 1994; Weinstein et al., 1994; Klingensmith et al., 1999; Shih and Fraser, 1996; Saude et al., 2000). To address a possible (even transient) role, we examine the role of axial mesendoderm in positioning the MHB at the time it is first formed in zebrafish. We find that axial mesendoderm is not required for AP positioning of the MHB, but that the likely source of the signal is the blastoderm margin (non-axial mesendoderm). Thus, MHB positioning is related to the more general problem of posteriorization of the neuroectoderm (Woo and Fraser, 1997). Through blocking reception for individual signaling pathways, we identify Wnt signaling, and particularly Wnt8, as being required for initial establishment of otx2 and gbx1 territories, and present evidence that Wnt8 acts directly. We suggest that initial positioning of the MHB organizer is a direct consequence of overall posteriorization of the neuroectoderm, and that Wnt8 protein produced by lateral mesendodermal precursors is necessary to mediate this process directly.

Materials and methods

Strains and developmental conditions
The flh allele used is flh6241 (Odenthal et al., 1996) and the ntl allele used is ntl61 (Odenthal et al., 1996). The Dfw8 mutation (Df(LG14)wnt8bts) was induced by γ irradiation (Lekven et al., 2001). Maternal-Zygotic Oep (M Zacp) fish where generated as described previously (Gritsman et al., 1999). Zebrafish were raised and kept under standard laboratory conditions at 28°C.

Whole-mount in situ hybridization and antibody staining
In situ hybridization, probes and antibody staining against EN4D9 (Ntl) was performed as described previously (Brand et al., 1996; Reifers et al., 1998). Probes and wild-type expression patterns have been described elsewhere: otx2 (Mercier et al., 1995), wnt8 (Kelly et al., 1995), pax2.1 (Krauss et al., 1991), eng2 (Ekker et al., 1992; Fjose et al., 1988), wnt1 (Kelly et al., 1993), fgf8 (Reifers et al., 1998), and gbx1 and gbx2 (Rhihn et al., 2003).

DNA construct and RNA synthesis
To generate the C-terminal Wnt8-GFP fusion protein, a gfp-coding sequence (Clontech) was inserted into pCS2+ vector (Rupp et al., 1994) via XbaI/SnaBI. wnt8-coding sequence was amplified by PCR and fused in frame into pCS2+/GFP vector. We synthesized capped mRNA as described previously (Reifers et al., 1998).

RNA and morpholino injections
For RNA injections, embryos were dechorionated using pronase and injected at the one-cell stage. Morpholinos were designed and synthesized by Gene-Tools (Corvallis, OR). They were resuspended in sterile water, stored at –20°C as 10 mg/ml solutions and diluted before use to the appropriate concentration in water, 0.2% Phenol Red. For morpholino injections, embryos were dechorionated using pronase and injected in the yolk with 1-15 ng between the one- and four-cell stage.

Transplantations
Donors embryos were injected with biotin-coupled tetramethylrhodamineextran (M, 10,000, Molecular Probes D-1817) diluted in 0.25 M KCl. Transplantations of donor cells into host embryos were carried out at shield stage using trimmed borosilicate capillaries. Transplanted cells were then visualized by immunocytochemically staining using the Vectastain ABC system (VectorLabs) and the DAB system (Sigma).

Pharmacological inhibition of Fgf signaling
To inhibit Fgf activity, embryos were treated with Su5402 (Mohammadi et al., 1997) (Calbiochem) at 16 µM in E3 medium as described (Reifers et al., 2000).

Results
The blastoderm margin and not the axial mesendoderm is involved in positioning the otx2/gbx1 interface
It has been postulated that, in vertebrate embryos, the axial mesendoderm is involved in specifying different AP segments of the neural axis (see Introduction). To address whether axial mesendoderm is required for correct AP positioning of the MHB primordium in the neural plate, we analyzed the expression of otx2, gbx1 and the MHB markers eng2 and fgg8 in mutants for two transcription factors – no tail (ntl/brachyury) and floating head (flh/Xnot) – that lack notochord differentiation (Halpern et al., 1993; Schulte-Merker et al., 1994; Talbot et al., 1995). otx2, gbx1, eng2 and fgg8 are expressed at their correct AP position in ntl mutant embryos (Fig. 1E-H) and in flh mutant embryos (Fig. II-L) when compared with wild-type embryos (Fig. 1A-D).

Because axial mesendodermal progenitors are present in ntl and flh mutants, we removed these precursors by performing shield ablation experiments. Complete shield ablation was achieved by extirpating the shield plus adjacent territories of dorsal margin, as previously reported (Saude et al., 2000), and results in embryos missing the head and trunk notochord but forming a remarkably well-patterned nervous system (Shih and Fraser, 1996; Saude et al., 2000). The complete absence of dorsal mesendoderm in shield-ablated embryos was confirmed by the absence of axial Ntl staining (Fig. 1M-P). In such embryos, we find that the AP position of the MHB markers otx2 (Fig. 1A,M; n=5/5), gbx1 (Fig. 1B,N; n=3/3), eng2 (Fig. 1C,O; n=3/3), and fgg8 (Fig. 1D,P; n=4/4) is normal. However, in ntl mutants and shield-ablated embryos, gbx1 expression was enhanced in the midline of the neural plate (Fig. 1, insets B’,F’,N’), and particularly in shield-ablated embryos, the midline gap between the neuroectodermal ‘wings’ of fgg8 expression is more narrow (bracket, Fig. 1D,P), suggesting that signals repressing gbx1 in the midline are lost. This finding suggests a possible role for axial mesendoderm in regulating gbx1 expression in the midline neuroectoderm, but not in AP positioning. Together, these findings argue against a role of the
axial mesendoderm in positioning the early MHB primordium in the zebrafish neural plate.

Transplantation experiments in zebrafish suggested that the lateral blastoderm margin is involved in neural posteriorization at pre-gastrula stages (Woo and Fraser, 1997; Koshida et al., 1998). When cells from this region are transplanted into the animal pole of the embryo, hindbrain markers such as krox20 and hoxa1, are induced in the host tissue, suggesting that secreted molecules are involved in this process (Woo and Fraser, 1997; Koshida et al., 1998; Momoi et al., 2003).

In order to analyze a possible involvement of the blastoderm margin in the establishment of the otx2/gbx1 interface, we transplanted cells from the blastoderm margin situated at a defined angular distance from the shield (30-45°), to the animal pole, a region fated to become forebrain. Expression analysis shows that otx2 is repressed (Fig. 2A,A′; n=50/50) (Koshida et al., 1998), whereas gbx1 is induced ectopically (Fig. 2B,B′; n=25/40) around the transplanted clones as early as 2 hours after transplantation. The ability of the blastoderm margin to repress otx2 and to induce gbx1 strongly suggests that the signal for correct positioning of the otx2 and/or the gbx1 expression domain arises from the blastoderm margin.

The establishment of the gbx1 and otx2 expression domains involves putative posteriorization signals

The results above indicate that a signal from the blastoderm margin is able to regulate otx2 and gbx1 expression. Several candidate signals are produced by cells at the blastoderm margin: retinoic acid, Fgfs, Nodals and Wnts. Analysis of gbx1 and otx2 expression in embryos treated with BMS493, a pan-retinoic acid receptor antagonist, shows no changes in initial positioning of gbx1 and otx2 expression domain (Grandel et al., 2002) (data not shown). By contrast, global disruption of all other pathways interferes with normal gbx1 and otx2 expression.

1) fgf3, fgf8 and fgf24 are expressed in the margin during gastrulation (Fürthauer et al., 1997; Reifers et al., 1998; Raible and Brand, 2001; Draper et al., 2003). Indeed, gbx1 and otx2 expression is altered in wild-type embryos in which Fgf signaling has been blocked pharmacologically using Su5402 (Mohammadi et al., 1997), a potent inhibitor that blocks all Fgf receptor signaling (Fig. 2D,H). Su5402 treatment causes a posterior shift of otx2 expression mediadorsally (Fig. 2D), probably reflecting the enhanced ventralization after loss of Fgf expression.

![Fig. 1. Expression of MHB markers is normal in mutants with defects in notochord formation and in shield-ablated embryos. (A-D) In situ hybridization and Ntl immunostaining (brown) of control embryos, (E-H) ntl mutant embryos, (I-L) flh mutant embryos and (M-P) shield-ablated embryos at tailbud stage. Probes were applied: otx2 (A,E,I,M), gbx1 (B,F,J,N), eng2 (C,G,K,O), fgf8 (D,H,L,P). In B′, no Ntl immunostaining has been performed. No changes in the AP position of the different MHB markers analyzed is observed in ntl mutants, in flh mutants or in shield-ablated embryos when compared with wild-type embryos. (D,P) Brackets indicate the reduced midline width following shield ablation. (A-O) Lateral views, anterior upwards; (D,H,L,P,B′,F′,N′) dorsal views, anterior upwards.

![Fig. 2. The blastoderm margin is the source of signals important for otx2 and gbx1 expression. (A-B′) Lateral blastoderm margin transplantations into the animal pole of a wild-type host embryo. (A,B) Embryo containing cells from the blastoderm margin derived from injected wild-type embryos with a lineage tracer (brown). (A) The white arrowhead indicates a clone of non-otx2-expressing cells in the otx2 domain of a transplanted embryo. (A′) Close-up of the transplanted cells. otx2 is repressed around the transplanted cells. (B) The black arrowhead indicates a clone of gbx1-expressing cells in the otx2 domain. (B′) Close-up of the transplanted cells. gbx1 expression is induced around the transplanted cells. (C-J) gbx1 and otx2 expression in the absence of FGF, Nodal or Wnt signaling at 60%.

(C) Control embryo stained for Ntl and Ntl protein (brown). (D) In Su5402-treated embryos, otx2 is strongly reduced and expands up to the margin at the level of the midline. (E) In MZoep embryos, otx2 expands up to the margin at the level of the midline. (F) In dkk1-injected embryos (100 pg), a posterior shift of otx2 is visible laterally (compare brackets in C and F). (G) Control embryo stained for gbx1. gbx1 is expressed dorsolaterally and absent in the margin ventrally in Su5402-treated embryos (H). (I) In MZoep embryos, gbx1 expands up to the margin. (J) dkk1-injected embryos (100 pg) do not express gbx1, showing the Wnt-dependent activation of gbx1. The black arrowheads indicate the margin. (C-J) Dorsal views, anterior upwards; (C,D,E,F) right corner, lateral views, dorsal towards the right; (G-I) right corner, animal pole views, dorsal downwards.}
function (Fürthauer et al., 2004). In addition, ventral gbxl expression is strongly reduced in Su5402-treated embryos (Fig. 2H insets), presumably owing to the lack of induction of prospective vegetal neural fate (Kudoh et al., 2004; Rentzsch et al., 2004).

(2) Nodal-related factors are required for mesendoderm induction, and also affect gbxl and otx2 expression, as studied in Maternal-Zygotic one-eyed-pinhead (MZoep) mutants that are unresponsive to Nodal signaling (Gritsman et al., 1999; Schier, 2001). MZoep mutants show an expansion of gbxl expression up to the margin (Fig. 2I, arrowhead) and a posterior shift of otx2 expression mediadorsally (Fig. 2E), presumably owing to the involvement of Nodal in the specification of dorsal mesendoderm.

(3) dickkopf-1 (dkk1) is a secreted inhibitor of Wnt signaling (Glinka et al., 1998; Hashimoto et al., 2000). dkk1 mRNA injection causes complete absence of gbxl expression at 60% of epiboly (Fig. 2J). However, by 80% of epiboly, gbxl expression recovers to nearly its normal size, although its location shifts more posteriorly (data not shown). Simultaneously, a posterior shift of otx2 expression is observed in dkk1-injected embryos, severely reducing the gap between the posterior border of otx2 and the blastoderm margin (Fig. 2F). In summary, establishment of gbxl and otx2 expression is strongly affected when Nodal, Fgf or Wnt signals are inhibited in whole embryos, but these experiments do not distinguish direct from indirect action of these pathways.

Wnt signaling directly defines the posterior border of otx2

To test how direct the action of these factors is, we examined whether clones of cells that are ‘blind’ to Fgf, Nodal or Wnt proteins – owing to disrupted signal reception – can respond to AP positional information in an otherwise wild-type environment. A dominant-negative Xenopus Fgf receptor, XFD (Amaya et al., 1991) makes cells unresponsive to Fgf signaling when overexpressed. XFD mRNA-injected donor embryos show the same defects in otx2 and gbxl expression as Su5402-treated embryos and fail to activate the Fgfr8-target gene sprouty4 (Fürthauer et al., 2001) (Fig. 3O,P). We transplanted cells from XFD-injected donors into wild-type host embryos and monitored their fate. The transplanted cells showed otx2 and gbxl expression appropriate to their location (Fig. 3A-D; otx2, 40/40; gbxl, 30/30) [for otx2 see also Koshida et al. (Koshida et al., 1998)], demonstrating that Fgf signaling is not directly involved in defining the otx2/gbxl interface. Likewise, MZoep donor-derived cells that are unresponsive to Nodal signaling show otx2/gbxl expression appropriate to their location (Fig. 3E-H; otx2, 70/70; gbxl, 60/60), showing that Nodals are also not directly involved.

By strong contrast, blocking Wnt-signal reception in receiving neuroectodermal cells by Dntcf3 mRNA injection provides evidence for a direct involvement of Wnt signaling. Dntcf3 encodes a N-terminal deletion of Tcf3 that lacks the β-catenin-binding domains, but retaining its repressor activity, and thus renders overexpressing cells unresponsive to Wnt signaling (Molenaar et al., 1996). Embryos injected with Dntcf3 mRNA do not express gbxl at 60% of epiboly (data not shown) like dkk1-injected embryos (Fig. 2J), and expression is strongly diminished of eng2, a known β-catenin/Tcf signaling target (Kim et al., 2000) (Fig. 3Q,R). We transplanted cells from Dntcf3-injected embryos into wild-type embryos and monitored the fate of the transplanted cells. Importantly, and in contrast to cells blind to Nodals or FGFs, cells blind for Wnt show ectopic otx2 expression outside the endogenous otx2 expression domain (Fig. 3E-H; 34/34). Similarly, when located within the gbxl domain, these cells do not express gbxl (Fig. 3L-N; 12/20). Together these data argue that the requirement of Fgf and Nodal signaling is indirect, whereas Wnt signaling may act directly to position otx2 and gbxl expression in the neural plate.

Expression of gbxl and otx2 is regulated by Wnt8

Among several Wnt genes expressed in the blastoderm margin, wnt8 shows the closest correlation in spatiotemporal expression to the expected signal with an onset in the blastoderm margin at 50% of epiboly and exclusion from the shield (Kelly et al., 1995; Lekven et al., 2001; Erter et al., 2001). To test a possible role of wnt8 for activation of gbxl
expression, we expressed \textit{wnt8} mRNA in all cells by injecting into one-cell stage zebrafish embryos. Depending on the amount of injected \textit{wnt8} mRNA, different levels of \textit{gbx1} expression are induced. Compared with the normally posteriorly restricted domain of \textit{gbx1} expression, low doses (1-5 pg) enlarge the \textit{gbx1} domain, but leave the animal pole area free of \textit{gbx1} expression (Fig. 4A-B). Intermediate doses of \textit{wnt8} mRNA (10-50 pg) induce high levels, whereas high doses (200-400 pg) induce lower levels of \textit{gbx1} expression throughout the neuroectoderm (Fig. 4A,C,D). Conversely, \textit{otx2} repression is apparent at low doses (1-5 pg) but more complete at intermediate (10-50 pg) and high doses (200-400 pg) of \textit{wnt8} mRNA (Fig. 4G-J). In these experiments, we observed that \textit{gbx1} can be induced both in prospective neural and non-neural ectoderm (Fig. 4A-D). We therefore asked if neural induction precedes \textit{gbx1} induction by \textit{wnt8} ectopic expression in prospective non-neural ectoderm, by investigating whether the non-neural markers \textit{foxi1} and \textit{pd63}, or the pan-neural markers \textit{sox31} and \textit{zic2.2} (Kudoh et al., 2004; Rentzsch et al., 2004) are induced or suppressed, respectively. We observe that the non-neural markers tested are repressed, and that neural markers are expanded throughout the ectoderm (see Fig. S1 in supplementary material). This is also true when \textit{Wnt8} is expressed from transplanted clones of cells (see Fig. 6O-Q'). These findings suggest that \textit{wnt8} gain-of-function is associated with induction of neural tissue. Further studies will need to address how direct this effect is and/or which other molecules may cooperate in promoting induction of prospective neural fate under these conditions (see also Baker et al., 1999). We also found that \textit{Wnt8} can act independently of Nodal signaling (Fig. 4F,L), but does require Fgf signaling for \textit{gbx1} expansion, though not for \textit{otx2} repression (Fig. 4E,K), probably reflecting the ventralization of the neuroectoderm following Fgf inhibition (Fürthauer et al., 2004). As expected, inhibition of Fgf signaling through \textit{Su5402}, or of Nodal signaling in \text{MZoep} embryos, eliminates or strongly diminishes \textit{wnt8} mRNA expression dorsally (Fig. 4M-O).

**Loss of Wnt8 shifts the position of the MHB primordium posteriorly**

A crucial test is to determine whether \textit{Wnt8} loss-of-function causes mispositioning of the \textit{gbx1/otx2} interface. We extended previous \textit{wnt8} loss-of-function studies (Erter et al., 2001; Lekven et al., 2001) to investigate the dependency of \textit{gbx1} and \textit{otx2} on \textit{Wnt8}. We knocked-down \textit{wnt8} either by injecting antisense morpholino oligonucleotides or by using \textit{Df(LG14)wnt8wh} (\textit{Df(wnt8)}) mutants, which carry a deletion of a region of linkage group 14 containing the \textit{wnt8} locus and other genes (Lekven et al., 2001). In both loss-of-function situations, \textit{gbx1} expression is absent from the ectoderm until 65-70% of epiboly (Fig. 5A-C). During later epiboly stages, \textit{gbx1} expression initiates, but in an inappropriate, more posterior position at the margin itself, overlapping with the Ntl expression domain (Fig. 5D-F). Because at its very onset \textit{gbx1} is expressed transiently at the margin (Rhinne et al., 2003), we suggest that in the absence of \textit{wnt8} function, this earlier stage of \textit{gbx1} expression may be retained. These observations suggest that \textit{Wnt8} is required for \textit{gbx1} activation and positioning during early gastrulation. Furthermore, the requirement for \textit{Wnt8} diminishes during later gastrulation stages, suggesting that additional signal(s) are capable of activating \textit{gbx1} expression at stages beyond 65% of epiboly.

In morphants and \textit{Df(wnt8)} mutants, the posterior border of \textit{otx2} expression is shifted posteriorly at the ventrolateral level at all stages of development investigated (Fig. 5G-L, red arrowheads). This posterior shift is apparent already when \textit{gbx1} and \textit{otx2} still overlap (60% of epiboly), and the mutually repressive interactions between them are presumably not yet operating (Rhinne et al., 2003). This argues that at 60% of epiboly, \textit{Wnt8} also positions the posterior border of the \textit{otx2} expression domain. Interestingly, however, even in the \textit{Df(wnt8)} mutants a small gap persists between the posterior border of the \textit{otx2} and the Ntl expression domain (Fig. 5G-L), suggesting that additional factor(s) are involved in positioning the posterior border of \textit{otx2}. These additional factor(s) are unlikely to be other redundant members of the \textit{Wnt} family.

**Fig. 4. gbx1 and otx2 expression in Wnt8 gain-of-function experiments.** (A) Control embryo stained for \textit{gbx1} at 60%. (B-D) Injected embryos with \textit{wnt8} RNA 1-5 pg (B), 10-50 pg (C) and 200-400pg (D) stained for \textit{gbx1}. Overexpression of \textit{wnt8} induces ectopic \textit{gbx1} expression throughout the epiblast in a dose-dependant way. Overexpression of \textit{wnt8} (100 pg) does not induce ectopic \textit{gbx1} in \textit{Su5402}-treated embryos (E) but does so in \textit{MZoep} embryos (F). (G) Control embryo stained for \textit{otx2} at 60%. (H-J) Injected embryos with \textit{wnt8} RNA 1-5 pg (H), 10-50 pg (I) and 200-400 pg (J) stained for \textit{otx2}. Overexpression of \textit{wnt8} inhibits \textit{otx2} expression. Overexpression of \textit{wnt8} (100 pg) in \textit{Su5402}-treated embryos (K) and in \textit{MZoep} embryos (L) inhibits \textit{otx2} expression. (M-O) \textit{wnt8} expression in wild type at 60% (M), in \textit{Su5402}-treated embryos (N) and in \textit{MZoep} embryos (O). (A-L) Animal pole views. (M-O) Dorsal views, anterior upwards.
based on three pieces of evidence. First, injection of antisense morpholino oligonucleotides against wnt3a (Buckles et al., 2004), another Wnt gene expressed in the margin slightly later than wnt8, does not affect otx2 positioning; and co-injection with the wnt8 morpholino does not increase the extent of the otx2 domain towards the margin (data not shown). Second, no other Wnt from the canonical pathway has been shown to be expressed in the correct time and place to have an effect on otx2 and/or gbx1. Third, injection of dkk, as a general inhibitor of all Wnts signaling through the canonical pathway, does not generate a stronger phenotype than that observed in wnt8 morphants or the Df(Wnt8) mutants (compare Fig. 2F with Fig. 5H,I), suggesting that no other Wnt molecules are involved.

Wnt8 can repress otx2 expression independently of gbx1 and mesendoderm

To reveal how direct the effect of Wnt8 on gbx1 and otx2 is, we transplanted wnt8-overexpressing cells into wild-type host embryos at pre-gastrula stages, and analyzed by in situ hybridization for induction of gbx1 and repression of otx2. Host embryos carrying such clones showed ectopic gbx1 expression (Fig. 6A-F,R), both in the transplanted cells and in the surrounding host tissue (Fig. 6B,D,E). Fig. 6F summarizes all locations in which Wnt8-expressing clones were able to induce gbx1 expression in host cells. Although the Wnt8-expressing clones were distributed essentially randomly, it appears to be more ‘difficult’ to activate gbx1 expression close to the margin (zone II in Fig. 6F), probably because the level of endogenous Wnt8 in zone II is too high to allow gbx1 induction (akin to the suppression observed at high doses of injected wnt8 in Fig. 4D). Similarly, fewer clones were found dorsoanteriorly, probably owing to the activity of extracellular Wnt inhibitors in the anterior neural plate (Wilson and Houart, 2004) (see below). Within the otx2 domain, transplanted Wnt8-expressing cells were able to repress otx2 expression in the host over about five cell diameters (Fig. 6G,H,R). This repression was never observed in control embryos transplanted with cells from donor embryos injected with lacZ mRNA (Fig. 6G). Contrary to a previous report (Agathon et al., 2003), we never observed wnt8 induction in neighboring non-transplanted host cells, arguing that wnt8 expression does not ‘self-induce’ (see Fig. S2 in supplementary material). To further confirm this result, we showed that repression of otx2 around a wnt8-expressing transplanted clone of cells does not require wnt8 function in the host embryo, as tested in embryos homozygous for Df[wnt8] (see Fig. S2 in supplementary material).

Considering the importance of mutual repression between otx2 and gbx1 at later stages, we sought to investigate if the repression of otx2 by Wnt8-expressing clones requires gbx1 function. In embryos transplanted with wnt8-overexpressing cells we do not observe gbx1 induction when otx2 is suppressed around the transplanted clone (Fig. 6I,J), arguing that the repression of otx2 by Wnt8 is not mediated through gbx1. Given the previously reported Wnt8-function in specifying ventrolateral mesodermal fate (Christiaen et al., 1991; Leckeler et al., 2001), could Wnt8 indirectly alter otx2 and gbx1 expression through inducing mesoderm secondarily? Probably not, because gbx1 expression is induced throughout the ectoderm in wnt8-mRNA injected embryos, whereas Ntl expression is only slightly broadened at the blastoderm margin (Fig. 6M,N) (Kelly et al., 1995). Moreover, otx2 inhibition or gbx1 induction is not linked with mesendoderm induction, as seen in double in situ hybridization on transplanted embryos with a nil and an otx2 probe (Fig. 6K,L,R), in which we never detected ectopic nil expression around the transplanted clone. Therefore, otx2 inhibition and gbx1 activation around clones of Wnt8-expressing cells are more likely due to a direct effect of Wnt8.

Wnt8 does not act via a relay mechanism to inhibit otx2 expression

To test this notion further, we examined if Wnt8 directly inhibits otx2 and activates gbx1, rather than doing so through other factors (‘relay mechanism’), using three different approaches. First, we transplanted Wnt8-expressing cells ectopically into host ectoderm that cannot receive Wnt8 signaling, because they express Fzb1-gpi (Momoi et al., 2003), a competitive secreted inhibitor that binds Wnts and thus should block the effects of Wnt8 clones on otx2 and gbx1 expression if signaling is direct. The gpi anchor of Fzb1-gpi prevents Fzb1 from diffusing into the transplanted clone, and should thus allow them to produce Wnt8. In such embryos, otx2 repression is limited to the transplanted cells, and is not
observed in surrounding host cells (Fig. 7A-A'). Likewise, gbx1 is induced only in transplanted cells and not in host cells (Fig. 7B-B'). In a few (5/80) cases, gbx1 is expressed in one-two cells around the transplanted clone, probably reflecting a slight variability in the Fzb1-gpi inhibition.

Second, we transplanted cells in which the Wnt pathway is activated in a ligand-independent way, and analyzed if these clones phenocopy the effects of a wnt8-expressing clone. In the absence of Wnt signaling, Glycogen synthase kinase-3β (Gsk-3β) is part of a multiprotein complex containing Axin, APC and β-catenin that phosphorylates β-catenin to target it for ubiquitination and subsequent degradation. A lysine-to-arginine exchange in the ATP-binding domain generates a ‘kinase-dead’ mutant of Xgsk3, resulting in a dominant-negative variant that suppresses Wnt signal transduction (Xgsk-3K→R) (Pierce and Kimelman, 1996). Consistent with the involvement of Wnt signal transduction, embryos injected with Xgsk-3K→R mRNA do not express otx2 at 60% of epiboly (Fig. 7E,F) and show ectopic gbx1 expression (Fig. 7G,H) as do wnt8-injected embryos (Fig. 4A-D,G-J). If secondary secreted molecules were involved, otx2 should be repressed not only in the transplanted cells, but also in the cells around a cell clone expressing Xgsk-3K→R. Alternatively, if Wnt8 directly represses otx2, the repression should be observed cell-autonomously, i.e. restricted to the transplanted area. 

Fig. 6. Wnt8 can induce gbx1 and repress otx2. (A-E) Embryos containing cells derived from embryos injected with a lineage tracer (brown) and wnt8 RNA (400 pg) stained for gbx1 expression. (A) Lateral view and (B) close-up of the transplanted cells seen in A; the arrow indicates gbx1 expression in the transplanted cells. (C) Lateral animal pole view of a transplanted embryo and (D) close-up of the transplanted cells. gbx1 is induced in the host tissue but also in the transplanted cells (arrow). (E) Strong induction of gbx1 expression in the host embryo at the AP level of the endogenous gbx1 domain. (F) Summary of all transplanted cells clones that induced gbx1 expression. The embryo can be subdivided into a wnt8-responsive part (I) and non-responsive part (II). (G) wnt8-overexpressing cells (brown) transplanted into wild-type embryos and stained for otx2. (H) Close-up of the transplanted cells. otx2 is repressed in the transplanted cells and in the host cells. (G') Control lacZ-overexpressing cells (brown) transplanted in wild-type embryos and stained for otx2 expression. No repression in the transplanted cells and host cells is observed. (I) wnt8-overexpressing cells transplanted into wild-type embryos and stained for otx2 (red) and gbx1 expression (blue); (J) close-up of the transplanted cell after biotin staining (brown). No ectopic gbx1 expression is seen in the transplanted area where otx2 is repressed. (K) wnt8-overexpressing cells (brown) transplanted into wild-type embryos and stained for otx2 (red) and ntl expression (blue); (L) close-up of the transplanted cells. No ectopic ntl expression is seen in the transplanted area where otx2 is repressed. (M) Control embryo at 60% stained for gbx1 (blue) and Ntl protein (brown). (N) Overexpression of wnt8 induces ectopic gbx1 throughout the epiblast and Ntl expression is expanded from three or four rows of cells. (O-Q') wnt8-expressing clones correlate with loss of non-neural, and gain of neural marker induction. (O-O') foxl1 expression; (O) animal pole view. (P-P') sox31 expression, (Q-Q') zic2.2 expression. (P,Q) Lateral views. Close-up of the transplanted cells (O',P',Q') before biotin staining and (O'',P'',Q'') after biotin staining. foxl1 repression occurs in the transplanted cells and sox31 and zic2.2 are induced. (R) Observed ectopic induction of gbx1, repression of otx2 and induction of Ntl when wnt8-overexpressing cells were transplanted into wild-type host embryos. In the last column, only cell clones within the endogenous gbx1 domain and the animal pole, the area I (see Fig. 6F), are scored. n, number of transplanted embryos.
cells. We find that *otx2* repression and *gbx1* induction is now limited to the transplanted cells (Fig. 7C-C‴, *otx2*, 24/25; Fig. 7D-D‴, *gbx1*, 18/20). Importantly, and in contrast to the transplantation of *wnt8*-injected cells, we now observe *gbx1* induction also more frequently in dorsal positions (Fig. 7D). In the transplanted cells expressing ectopic Xgsk-3K→R mRNA, the Wnt pathway is thought to be activated independently of Wnt molecules. Secreted Wnt-inhibitors from the animal pole and acting dorsally (Wilson and Houart, 2004) are therefore presumably not effective in quenching the effects of ectopic Wnt8.

Third, we tested if Wnt8 protein is found around expressing clones at an appreciable distance. We visualized Wnt8 protein around a transplanted clone by tagging it with GFP. A C-terminal Wnt8-GFP fusion protein is biologically active, as judged by its ability to induce *gbx1* and repress *otx2* following global injection (data not shown). We transplanted clones of *wnt8-gfp* overexpressing cells at pre-gastrula stages into wild-type hosts that were previously injected at the one-cell stage with a palmitoylated RFP mRNA (Iokka et al., 2004) to label all cell membranes (Fig. 7I), and analyzed Wnt8 localization by confocal microscopy. We observed GFP-fluorescent puncta in host cells around the transplanted cells, representing accumulated Wnt8-GFP protein (Fig. 7I, white arrows), mostly associated with the cell surface or extracellular matrix (Fig. 7K). Members of the Wnt family, including Wingless, are thought to tightly associate with membranes and heparan sulfate proteoglycan (Nusse, 2003). We can visualize accumulated Wnt8-GFP at a distance of about two cell diameters away from the donor cell clones, which is generally consistent with the genetically determined non-autonomous characteristics of Wnt8 (see Discussion). Because we observe *otx2* inhibition two to five cell diameters away from transplanted cell clones expressing Wnt8, it is likely that levels of Wnt8 protein below the detection threshold act at distances further away.

Taken together, our findings support a model where Wnt8 regulates, in a concentration-dependent manner, the location of the *otx2/gbx1* interface and hence, the position of the MHB organizer, via direct action of secreted Wnt8 emanating from the endomesodermal primordium at the blastoderm margin (Fig. 7L).

**Discussion**

**Positioning of the MHB organizer is a direct consequence of posteriorization**

In several vertebrates, including zebrafish, the position of the future MHB in the neuroectoderm is marked by the interface between cells expressing the Otx and Gbx transcription factors (Wassarman et al., 1997; Broccoli et al., 1999; Millet et al., 1999; Rhinn et al., 2003), raising the issue of how this interface is set up in turn. Here, we reported that AP positioning of the two expression domains of *gbx1* and *otx2* depends on
signals from lateral mesendodermal precursors, but not from axial mesendoderm. Explant cultures in chick previously suggested an involvement of axial mesendoderm in positioning MHB marker gene expression (Ang and Rossant, 1993; Shamim et al., 1999). In our studies, abolishing the formation of axial mesendoderm either in mutants or by ablation of its primordium, the shield, revealed however that the axial mesendoderm is not involved (Fig. 1). By contrast, lateral blastoderm margin transplants reveal that the marginal cells can induce gbx1 and suppresses otx2 in the surrounding tissue. This is in agreement with previous work, showing that cells from the lateral margin can stimulate hindbrain gene expression (Woo and Fraser, 1997; Koshida et al., 1998; Sagerstrom et al., 1996; Momoi et al., 2003). Our work extends these findings to the earliest stages of neuroectoderm subdivision and positioning of the MHB organizer, and suggests that these events are directly linked to overall posteriorization of the neural primordium by signal(s) from the blastoderm margin.

The wealth of inductive interactions in early gastrulation stages makes it difficult to dissect the contribution of the various signaling pathways involved in posteriorization of the neural plate. This is clearly evident also for the formation of the gbx1 and otx2 expression domains in our loss-of-function experiments of the Nodal, Fgf or Wnt signaling pathways, all of which are active in the early blastoderm margin (Fig. 2). Specific interference with signal reception for individual signaling pathways in combination with a transplantation assay allowed us, however, to distinguish their contribution. Examination of cell clones that are ‘blind’ to any of the three signals enabled us to address whether such cells can respond with correct otx2/gbx1 expression to positioning cues (Fig. 3). We find that Wnt signaling is crucially involved in setting up the otx2 and gbx1 expression domains, because cells that are ‘blind’ to Wnt signaling do not respond to AP positional information, causing otx2 to be ectopically expressed in more posterior domains of the embryo. This is not the case when Nodal or Fgf signal reception is blocked, arguing that these signals have a different role. A role for Wnt molecules in repressing the otx2 domain has been suggested previously in different experimental systems. Caudal chick neural plate cells revert to a rostral forebrain character when grown in vitro in the absence of a Wnt signal (Nordstrom et al., 2002). In Xenopus, treatment of animal caps with XWnt8 leads to a progressive posteriorization and a repression of anterior markers, including otx2 (Kiecker and Niehrs, 2001). By contrast, Fgf and Nodal signaling seem to act in a more global context, because individual cells that cannot receive Fgf or Nodal signals respond correctly to AP positional signals. We suggest that Fgf and Nodal signaling are more indirectly involved in generating the otx2/gbx1 interface: Nodal proteins, through their role in mesoderm formation (reviewed by Schier, 2001); and Fgfs, through their involvement in the induction of neural fate of vegetal ectoderm (Kudoh et al., 2004; Rentzsch et al., 2004) and in ventralization of the neuroectoderm (Fürthauer et al., 2004).

The posteriorizing molecule Wnt8 mediates positioning of the MHB

Both our loss- and gain-of-function studies show that Wnt8, normally expressed in the blastoderm margin, is involved in the onset and correct positioning of the gbx1 expression domain, and for the establishment of the posterior border of the otx2 expression domain (Figs 4-6). Although we have focused here on gbx1 and otx2 as the most critical components for MHB development, it is likely that other target genes with posterior-specific expression would respond similarly to Wnt8, e.g. the Cdx genes. Our findings raise the issue of whether Wnt8 is directly involved in positioning the MHB primordium. We addressed this issue by injecting increasing amounts of wnt8 mRNA, and by employing wnt8-overexpressing clones, which we find can repress otx2. Our clonal analysis in a Fzb1-gpi background, the cell-autonomous activation of the Wnt pathway by Xgsk-3K→R clonal analysis and our visualization of Wnt8-GFP all support the argument that Wnt8 regulates otx2 and gbx1 directly in a non-cell-autonomous manner (Fig. 7).

As in other vertebrates, mutually repressive interactions are thought to exist in zebrafish between Otx and Gbx genes (Rhinn and Brand, 2001; Rhinn et al., 2003) (M.R. and M.B., unpublished). Importantly, several findings argue that feedback regulation is preceded by a phase of direct regulation of both otx2 and gbx1 via Wnt8. First, we found that Wnt8 can regulate both genes independently, because it can induce gbx1 and regulate otx2 prior to the establishment of the feedback loop between them around 70% epiboly. In the Df(wnt8) mutants and in wnt8 morphants, posterior expansion of otx2 is evident prior to the 70% stage when these mutually repressive interactions become evident. Second, wnt8-overexpressing clones can repress otx2 without inducing gbx1, and Wnt ‘blind’ cells ectopically express otx2. Analysis of wnt8 showed that the gene is crucially involved in the patterning of mesoderm and neural ectoderm (Christian et al., 1991; Lekven et al., 2001; Erter et al., 2001). gbx1 activation coincides with the involution of the forming mesendoderm (Rhinn et al., 2003), raising the possibility that vertical signaling from the involuted mesendoderm to the overlying ectoderm could also be involved in this process. Our results argue against this possibility. First, in MZoeo embryos, where no dorsolateral mesendoderm involution occurs, gbx1 is induced and otx2 expansion does not extend further posteriorly, towards the lateral margin. Second, gbx1 expression throughout the complete epiblast is observed when wnt8 is expressed ectopically with a limited upregulation of mesendodermal markers (Kelly et al., 1995) (Fig. 6M,N). This suggests that the whole embryo can respond to wnt8 signaling to induce gbx1 in the absence of mesendoderm. Third, we found that in transplantations of wnt8-expressing cells, gbx1 is induced and otx2 is repressed without new mesoderm induction, consistent with previous findings that Wnt signaling can induce posterior neural markers in the absence of mesendoderm (McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001). Altogether these findings suggest that wnt8 plays a key role in the activation of gbx1 and repression of otx2, independent of its role in mesoderm patterning.

**Graded activity of Wnt8 signaling in the early neural plate**

How does Wnt8 participate in positioning of the MHB organizer? wnt8 is expressed in the marginal cells and hypoblast and two receptors, f8c and f9, are detected in both hypoblast and epiblast (Momoi et al., 2003). Conceivably, Wnt8 is transmitted in a planar fashion through the
neuroectoderm. This idea is supported by the clonal analysis of wnt8 overexpressing cells: gbx1 is activated in the host activity one or two cells distant from the transplanted cells, and otx2 is repressed four or five cells distant from the transplanted cells (Fig. 6). In unmanipulated neuroectoderm, the onset of gbx1 expression occurs close to the wnt8 domain with little or no overlap, and the otx2 expression domain is situated eight to ten cell diameters away from the wnt8 domain at 60% of epiboly. Thus, the wnt8 expression domain is appropriately located to generate a graded morphogenetic Wnt8 signal that regulates the expression of gbx1 and otx2 genes in vivo. This finding is more generally consistent with the ability of Wnt molecules to form gradients and to activate target genes in a concentration-dependent manner, as in the Drosophila wing imaginal disc, where expression of wingless target genes like neuralized, distalless and vestigial depends on the distance from wingless-expressing cells (Zecca et al., 1996; Strigini and Cohen, 2000). Similarly, in the unmanipulated zebrafish neuroectoderm, the otx2 and the gbx1 domains are located at different distances from the Wnt8 source at the lateral blastoderm margin. Following global misexpression experiments, different Wnt8 doses can differentially regulate otx2 and gbx1 expression: wnt8 ectopic expression can induce gbx1 expression at low/intermediate doses, but represses at high doses. Conversely, otx2 is increasingly repressed with increasing wnt8 concentration. Similarly, around wnt8-expressing clones, gbx1 is induced at a distance of one or two cells around the clone, whereas otx2 is repressed at a distance of four or five cells. This suggests that a lower Wnt8 concentration is needed to repress otx2 than to induce gbx1. Altogether, these observations suggest that Wnt8 has properties of a morphogen whose activity is required to correctly position the otx2/gbx1 interface, and probably other target genes in the forming neural plate. Our observation of secreted Wnt8-GFP protein emanating from clones of producing cells is generally consistent with this possibility. Distribution of another signaling molecule in the early neural plate, Fgf8, is carefully controlled by endocytosis (Scholpp and Brand, 2004). It will be interesting to determine if Wnt8 protein is indeed distributed in a graded fashion, and which mechanisms control this distribution. In mice, Wnt8 is expressed in the posterior epiblast of early primitive streak-stage embryos (Bouillet et al., 1996); although its function is unknown, Wnt8 may therefore serve a similar function as proposed here.

Other studies also suggest that a Wnt/β-catenin signaling gradient may underlie AP patterning in the neuroectoderm. In Xenopus gastrula, Wnt activity declines in the neural plate from high caudal to low rostral levels (Kiecker and Niehrs, 2001). In favor of graded Wnt activity are also observations in chicken where neural plate explants express different regional markers in response to different concentrations of Wnt-conditioned medium (Nordstrom et al., 2002). In zebrafish, results by Momoi and collaborators (Momoi et al., 2003) did not detect graded Wnt activity. These authors observed at 70% of epiboly stage a nuclear localization of β-catenin along the AP axis, but only in wnt8-expressing cells in the blastoderm margin. However, given the timing of gbx1/otx2 onset and the genetic requirement for Wnt8 documented here, we predict that the Wnt gradient is generated in the neural plate already prior to 70% of epiboly. Conceivably, a low level of β-catenin nuclear translocation might suffice to transmit the Wnt8 signal that was not detectable in the assay by Momoi et al. (Momoi et al., 2003). In summary, our data argue that Wnt8 acts without a relay mechanism in the regulation of otx2 and gbx1, and we hence strongly favor the idea that during the early and mid-gastrulation stages in zebrafish, similar to Xenopus and Drosophila, there is a graded Wnt8 signal that is generated from the blastoderm margin (see also Dorsky et al., 2003). The recent description of Tcf4-binding sites in the enhancers driving Otx2 expression in the mouse anterior neural plate suggests that one level of this regulation occurs at the level of the Otx2 promoter (Kurokawa et al., 2004).

The suggested Wnt8 action from the lateral blastoderm margin, located at the posterior edge of the forming neural plate, may be antagonized from the anterior side forming at the animal pole, through inhibition that is required for assigning cell fate in the forebrain neural plate (Kim et al., 2000; Onai et al., 2004; Wilson and Houart, 2004). One possibility is that these interactions start quite early when the distances between cells of the future anterior and posterior neural plate are still small. The Wnt8 signal is then integrated to process neural AP patterning and positioning of the MHB primordium at 60% of epiboly (Fig. 7L). Positioning of the future MHB organizer is thus achieved by Wnt8 setting up the complementary gbx1 and otx2 expression domains before mutual interaction between them sharpens the interface, around 70-80% of epiboly (Rhinn et al., 2003), and before the establishment of the complex regulatory cascade conferring to the MHB cells their organizing capacity (Fig. 7L).

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Supplementary material
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