Lineage restriction maintains a stable organizer cell population at the zebrafish midbrain-hindbrain boundary

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Summary
The vertebrate hindbrain is subdivided into segments, termed neuromeres, that are units of gene expression, cell differentiation and behavior. A key property of such segments is that cells show a restricted ability to mix across segment borders – termed lineage restriction. In order to address segmentation in the midbrain-hindbrain boundary (mhb) region, we have analyzed single cell behavior in the living embryo by acquiring time-lapse movies of the developing mhb region in a transgenic zebrafish line. We traced the movement of hundreds of nuclei, and by matching their position with the expression of a midbrain marker, we demonstrate that midbrain and hindbrain cells arise from two distinct cell populations. Single cell labeling and analysis of the distribution of their progeny shows that lineage restriction is probably established during late gastrulation stages. Our findings suggest that segmentation as an organizing principle in early brain development can be extended to the mhb region. We argue that lineage restriction serves to constrain the position of the mhb organizer cell population.

Key words: Midbrain-hindbrain boundary, Isthmic organizer, Lineage restriction, Neuromeres, segments, Zebrafish, Mesencephalon, Metencephalon, otx2, gbx1, fgf8

Introduction
The developing central nervous system is compartmentalized at the neural tube stage into discrete units, so-called neuromeres. Based on anatomical comparisons, early anatomists suggested that neuromeres are evidence in favor of a primitive, metameric segmentation of the nervous system that allows the identification of homologous brain parts between vertebrate species (Vaage, 1969).

Work in recent years has revealed that hindbrain neuromeres are evolutionarily conserved units of gene expression, differentiation and cell behavior (Keynes et al., 1990; Puelles, 2001; Cooke and Moens, 2002; Moens and Prince, 2002; Pasini and Wilkinson, 2002). In terms of their behavior, cells are free to mix within a given neuromere, but not across the boundary into the neighboring compartment. This important phenomenon, termed lineage restriction, was discovered in Drosophila wing development (Garcia-Bellido et al., 1973; Crick and Lawrence, 1975). In the vertebrate brain, lineage restriction acts during the formation of hindbrain compartments, the rhombomeres (Fraser et al., 1990). Here, cells acquire distinct adhesive properties that prevent them from mixing between rhombomeres (Mellitzer et al., 1999; Xu et al., 1999).

In the Drosophila wing, the anteroposterior compartment boundary coincides with the position of an organizer, a localized group of cells that controls neighboring cell fate by secreting diffusible signaling proteins. To ensure proper tissue formation and differentiation, the position of such potent organizing cells has to be tightly controlled (Dahmann and Basler, 1999). Organizers also serve important functions during vertebrate brain development (Irvine and Rauskolb, 2001). The organizer situated at the junction of the midbrain and anterior hindbrain – termed midbrain-hindbrain boundary (mhb) organizer or isthmic organizer – serves as a paradigm for organizer activity in the forming nervous system (Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). The mhb organizer forms at the interface between the expression domains of two transcription factors in the neural plate – an anterior otx and a posterior gbx domain (Rhinn et al., 2005). otx and gbx expression domains initially overlap, but subsequently sort out and form a sharp interface (Hidalgo-Sanchez et al., 1999; Garda et al., 2001; Rhinn et al., 2003). Cells on the posterior side of this interface start to express the signaling protein Fgf8 as the key molecule exerting organizer function (Crossley et al., 1996; Reifers et al., 1998). It has been proposed that cells that might cross this boundary readjust their gene expression profile via mutual repression (Jungbluth et al., 2001; Wurst and Bally-Cuif, 2001).

Although lineage restriction in the mhb region has been addressed in previous studies, we still do not know how tightly cell movement is controlled in this brain area. No lineage restriction between the chick mesencephalon and metencephalon was detected using a clonal analysis approach (Jungbluth et al., 2001), while other studies using broader labeling techniques or tissue grafting argue in favor of a cell movement restriction across the mhb (Millet et al., 1996; Alexandre and Wassef, 2003; Louvi et al., 2003). A recent study strongly suggests lineage restriction between the mouse...
midbrain and rhombomere one (Zervas et al., 2004). To determine whether the midbrain-hindbrain boundary is a compartment boundary in the developing vertebrate brain, we analyzed morphological changes, gene expression patterns and cell behavior during the formation of the mhb region in zebrafish with single-cell resolution. To this end, we imaged the developing mhb region in a GFP transgenic line that marks all nuclei. Using a novel combination of antibody staining and continuous single cell tracking, we present strong evidence for the existence of a lineage restriction boundary between the mesencephalon and metencephalon in the zebrafish. Single cell injection and clonal analysis indicate that this boundary is established as early as late gastrulation. We argue that lineage restriction constrains the organizing cell population at the mhb to ensure proper patterning and differentiation of the mhb region.

Materials and methods

Fish maintenance

Zebrafish were maintained under standard conditions (Kimmel et al., 1995; Brand and Granato, 2002). Embryos were staged as described elsewhere (Kimmel et al., 1995) or by hours post fertilization at 28°C. The histone H2A.F/Z::GFP transgenic line was a kind gift of J. A. Campos-Ortega (Pauls et al., 2001).

Staining of living embryos

Embryos were stained with the vital dye BODIPY-ceramide (Molecular Probes/Invitrogen) (Cooper et al., 1999), mounted for imaging (Concha and Adams, 1998; Langenberg et al., 2003) and optically sectioned on a Leica confocal microscope.

Analysis of gene expression

Standard methods for whole-mount RNA in situ hybridization were used, with laboratory modifications as described elsewhere (Reifers et al., 1998). DIG probes were developed with Fast Red substrate (Sigma) to yield a fluorescent signal. Embryos were moved to 70% glycerol, mounted and imaged on a Zeiss confocal system with 488/543 nm excitation. The 488 nm excitation gave a sufficient signal to visualize the tissue background. Probes for the following genes were used: otx2 (Mori et al., 1994), gbx2 (Rhinn et al., 2003), wnt1 (Kelly et al., 1993) and fgf8 (Reifers et al., 1998).

Immunohistochemistry

The Orthodenticle/Otx antibody was a kind gift from Antonio Simeone. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, washed and incubated in methanol for at least 30 minutes at −20°C. Embryos were then digested with 0.0025% Trypsin in PBT (PBS + 0.8% Triton) for 5 minutes on ice, postfixed for 30 minutes with 4% PFA, washed and blocked for 2 hours in PBT with 10% heat-inactivated normal goat serum (NGS) and 1% DMSO. Antibody incubations were as follows: overnight in anti-Otx antibody (1:3000) in PBT + 1% NGS; secondary antibody (Jackson ImmunoResearch TRITC coupled anti-goat 1:200) for 2 hours at room temperature. Embryos were postfixed, moved to 70% glycerol, mounted and imaged on a Zeiss confocal system with 488/543 nm excitation.

RNA injection

RNA injections were carried out as described by Reim (Reim and Brand, 2002). Fifty picograms GFP RNA per embryo was sufficient to strongly label donor cells.

Transplantation

Cell transplantations were essentially carried out as described

Results

Morphological changes during midbrain-hindbrain boundary formation

To visualize the formation of the midbrain-hindbrain boundary region in the zebrafish, we stained a series of live embryos with the vital dye BODIPY-ceramide and took confocal optical sections of the brain (Fig. 1A-C). Up to about the 12-somite stage, the mhb region of the neural tube shows no overt signs of morphological segmentation (Fig. 1A). During the formation of the next two to four somites, a small indentation forms in the prospective mhb region (Fig. 1B, arrowheads). This indentation successively deepens and widens until, at 24 hours post fertilization (hpf), it lies within the prominent fold at the mhb (Fig. 1C, arrowhead, dotted line), the isthmus.

To analyze the behavior of individual cells during the folding process, we transplanted cells expressing cytosolic GFP from injected donor embryos into unlabeled wild-type hosts at the onset of gastrulation (6 hpf) and imaged the developing mhb region by confocal time-lapse microscopy between the five-somite stage (1.5 hpf) and 30 hpf (Fig. 1D-F; see Movie 1 in the supplementary material). Cells stretch from the apical (ventricular) to the basolateral surface of the neuroepithelium, forming a pseudo-stratified epithelium (Schmitz et al., 1993) (Fig. 1E, double arrow and see Movie 1 in the supplementary material), while nuclei constantly cycle between the two sides. Upon division, cells round up at the ventricular side (Kimmel et al., 1994; Papan and Campos-Ortega, 1994) (Fig. 2G-I and Movies 1, 2 in the supplementary material). We traced groups of cells divided by the morphological boundary back to separate cells or cell groups at the beginning of the time-lapse (Fig. 1D-F, pseudo-colored cells and see Movie 1 in the supplementary material). Between these groups, a gap of unlabeled cells developed over time (Fig. 1, arrowheads in E, asterisks in E,F). Cell movement between the groups appeared very restricted. These data show that the developing midbrain and anterior hindbrain in the zebrafish go through distinct morphological movements that lead to a folded anteroposterior axis. The position of the late fold can be traced back to a small indentation in the prospective mhb region. We took the behavior of groups of cells on either side of the boundary as...
the first indication of restricted cell mixing between the midbrain and anterior hindbrain.

The mhb is a lineage restriction boundary

To address cell behavior at the single cell level, we designed a high resolution imaging approach to cell movement in the mhb region, in correlation with a canonical midbrain marker. To this end, we imaged the developing mhb region using a transgenic yellow. No mixing between the cell groups was observed (Fig. 3A,F). Arbitrary boundaries were picked within the unlabeled cells that forms between the cell groups. All images are dorsal views, anterior is towards the left. Arrowheads indicate the position of the early indentation (B,E) and late fold (C,F) in the mhb area. Scale bars: 50 µm.

<table>
<thead>
<tr>
<th>Time-lapse movie</th>
<th>Stage covered</th>
<th>Otx (+)</th>
<th>Otx (–)</th>
<th>Neural crest end*</th>
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<tr>
<td></td>
<td></td>
<td>Start</td>
<td>End</td>
<td>Lost end*</td>
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<tr>
<td>1 (12 hours)</td>
<td>5–24 ss</td>
<td>57</td>
<td>129</td>
<td>53</td>
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<td>2 (10 hours)</td>
<td>8–26 ss</td>
<td>36</td>
<td>66</td>
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<td>3 (8 hours)</td>
<td>10–24 ss</td>
<td>56</td>
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<td>Total</td>
<td></td>
<td>149</td>
<td>272</td>
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<td>Restricted</td>
<td></td>
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Out of the successfully tracked nuclei, nearly 100% are found within the lineage restricted populations. Stage covered, start and end developmental stage of the time-lapsed embryo; lost, nuclei that could not be tracked; neural crest, putative neural crest cells, located very dorsally and leaving the neuroepithelium during the movie.

*Only the final number of nuclei is given.
we determined the position of nuclei in rows distance from their common boundary at the start and at the end of the time-lapse, with the rows making up the interface receiving the number one (Fig. 2F). This is possible because neuroepithelial cells form a pseudo-stratified epithelium at these stages (Papan and Campos-Ortega, 1994). By plotting the difference between these values, the relative movement of cells can be determined with respect to their interface: only a fraction of the cells moves towards the boundary, the majority displays a movement away from it or remains stationary (Fig. 3G,H).

In summary, these data demonstrate clearly that Otx-positive and Otx-negative cells at the mhb of the 1-day old zebrafish embryo derive from cells that were spatially separated for the last 12 hours of development, arguing that lineage restriction is established and maintained from at least the five-somite stage onwards.

**Single cell lineage analysis by iontophoretic injection**

Having found that two lineage restricted cell populations are established from early somitogenesis stages onwards, we wanted to determine the onset of lineage restriction between the midbrain and hindbrain. Because large-scale morphogenetic movements make imaging and continuous tracking throughout gastrulation stages less precise, we decided to test the early mhb for lineage restriction by labeling individual cells and analyzing their distribution at later developmental stages.

In the zebrafish, the expression domains of the transcription factors *otx2* and *gbx1* (the functional homolog of *gbx2* in the mouse) become mutually exclusive towards the end of gastrulation, at the 80% epiboly stage (Rhinn et al., 2003). We therefore expected this period to be important for cell behavior at the *otx2*/*gbx1* interface, and labeled single cells by iontophoretic injection of a fluorescent dye at successive gastrulation stages (Fig. 4). In addition, we transplanted single cells from GFP-injected donors to wild-type unlabeled hosts at the shield stage (onset of gastrulation). Ability of the clonal descendants to cross the mhb was determined at 24 hpf (Fig. 4) and 36 hpf (data not shown).

Upon labeling or transplanting at the beginning of gastrulation (shield stage to 60% epiboly), about one quarter of clones had descendant cells on both sides of the boundary at 24 hpf and 36 hpf, in agreement with earlier fate-mapping studies (Woo and Fraser, 1995). The proportion of two-sided clones decreased significantly when cells were injected during later gastrulation stages (80-90% epiboly and tailbud to one-somite stage, Fig. 4B-E), with no clear two-sided clones after labeling at the tailbud stage (summary Fig. 4F). These findings argue for the establishment of the lineage restriction boundary between the prospective midbrain and hindbrain during late gastrulation stages, when the expression domains of *otx2* and *gbx1* become mutually exclusive.

**Gene expression domains at the midbrain-hindbrain boundary**

During hindbrain segmentation, a number of genes are
Lineage restriction at the mhb

expressed in a segmental manner, i.e. they respect the lineage restriction boundaries between rhombomeres as expression borders. Our data show that Otx2 protein expression as a marker for the extent of the midbrain and the lineage restriction boundary coincide. To characterize the expression domains of further known regulatory genes with respect to the identified lineage restriction boundary at the mhb, we analyzed gene expression by fluorescent in situ hybridization and subsequent confocal microscopic imaging.

Fig. 5 illustrates that the expression domains of several genes important for mhb development conform with the morphogenetic changes during mhb development and seem to respect the lineage restriction boundary as either their anterior or posterior expression border. The posterior expression border of otx2 (Fig. 5A-C) exemplifies this: during the formation of the first indentation (16-somite stage, Fig. 5A) it is perpendicular to the neural axis. Towards 24 hpf, the border becomes tilted with respect to the AP axis of the embryo (Fig. 5B,C compare with Fig. 1A-C). Nevertheless, the posterior otx2 expression border abuts the anterior expression limit of gbx2 and fgf8 (Fig. 5D,E show a mid-tectal level), and wnt1 expression extends up to the same posterior limit as otx2 (data not shown and Fig. 5F).

These data show that the lineage restriction boundary coincides with the expression borders of several important regulatory genes in mhb development. Of particular importance, the cell population expressing fgf8 as the key organizer gene (Crossley et al., 1996; Lee et al., 1997; Reifers et al., 1998; Martinez et al., 1999), appears to respect the lineage boundary at its anterior end.

Discussion

In this study we have shown that the mesencephalon and metencephalon of the zebrafish embryo are separated by a lineage restriction boundary. Our conclusions are based on the following results.

The detailed analysis of the movement of hundreds of individual nuclei (summarized in Table 1) shows that two lineage restricted cell populations are established at least from the five-somite stage onwards. By comparing the final nuclei position with an anti-Otx staining (Fig. 2), we were able to assign a status to the tracked nuclei, demonstrating that the lineage restriction boundary lies between an Otx-positive and an Otx-negative cell population. Because the molecular status of the tracked cells was determined only at the end of the time-lapse analysis, it remains a formal possibility that the cells could switch their molecular status during the time period analyzed. However, we consider this possibility unlikely, because Otx-expressing and Otx non-expressing cells form, also at earlier stages, a sharp interface (Fig. 2).
The expression domains of important regulatory genes in the mhb genetic cascade, among them otx2, have been studied in several vertebrate model organisms (Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Raible and Brand, 2004). After a phase of small overlap between the mesencephalic and metencephalic expression domains, mesencephalic genes become restricted to the otx-positive domain, while metencephalic genes become confined to the gbx-positive domain. In agreement with these data, we have shown that several mhb genes share a common expression border that coincides with the lineage restriction boundary and follows the changes in morphology during mhb development (Figs 1, 5). Interestingly, as in the chick (Millet et al., 1996), the otx expression border, and thus the lineage restriction boundary, does not correlate with the morphological indentation in the mhb area, but is situated slightly anterior to it (Fig. 2J-L and see Movie 2 in the supplementary material).

We believe that our technique of combining single cell tracking and marker staining offers a powerful way to generate high resolution fate-maps of developing embryos. A direct application will be the search for further lineage restriction boundaries in the developing zebrafish brain. With the development of computer-based, automatic cell tracking and the steady improvement of laser-scanning microscopes, a 'continuous fate-map' of the whole brain or even embryo seems within reach.

To determine the onset of the lineage restriction mechanism, we injected single cells at three developmental stages: at the beginning, middle and end of the gastrulation period (Fig. 4). This comprises the time window during which the expression domains of otx2 and gbx1, whose interface correlates with the position of the mhb, become mutually exclusive. Our results obtained from this approach suggest that lineage restriction is established already by the end of gastrulation, at around 80% epiboly (Fig. 4). Setting up lineage restriction at this early time point may be important to prevent mixing between two cell populations that start to express secreted patterning molecules shortly thereafter: mesencephalic, otx2 and wnt1-expressing cells; and metencephalic, gbx and fgf8-expressing cells (Hidalgo-Sanchez et al., 1999; Rhinn et al., 2003). The fgf8-positive cells are thought to constitute the organizer in the mhb region (Crossley et al., 1996; Lee et al., 1997; Reifers et al., 1998; Martinez et al., 1999). We argue that it is of high importance for the developing embryo to limit fgf8 expression to a defined compartment in the brain to ensure proper patterning and differentiation of the mhb region.

Our detailed analysis of cell movement further shows that nuclei can move a significant distance away from the Otx expression boundary (Fig. 1, Fig. 3G,H, see Movie 1 in the supplementary material). This principal capability of cells to move within the neuroepithelium further underscores the importance of restricting movement across the mesencephalon-metencephalon (mes-met) interface: cells expressing the secreted organizer molecule Fgf8 would probably move far
into the midbrain in the absence of a cell-sorting mechanism and cause disorganization of mhb development. The putative mechanism that establishes and maintains the lineage restriction boundary between the mesencephalon and metencephalon will need to both restrict mixing across the mes-met interface and to allow mixing within the groups of cells on either side. We consider a mechanism based on differential adhesion between mesencephalic and metencephalic cells as the most likely possibility.

Lineage restriction at the midbrain-hindbrain boundary has been addressed in other vertebrate model systems. A recent study in mouse (Zervas et al., 2004) postulates lineage restriction boundaries in the mhb region, one of them situated at the dorsal mes-met interface. In this study, a mouse strain carrying a tamoxifen-inducible Cre recombinase, controlled at the dorsal mes-met interface. In this study, a mouse strain been addressed in other vertebrate model systems. A recent study in chick embryos suggests lineage restriction at the mesencephalon and metencephalon (Millet et al., 1996; Alexandre and Wassef, 2003; Louvi et al., 2003). In one study, one or two founder cells were labeled, and in this study no lineage restriction was observed (Jungbluth et al., 2001). In neither of these studies has cell movement in the midbrain-hindbrain boundary area been followed directly. By examining the movement of individual cells within the mhb region of the zebrafish, we have detected only two cells (out of 551) within a few cell diameters of the boundary that were clearly located in the neural tube, and that did not respect the lineage restriction boundary (Table 1). These two Otx-negative cells were traced backwards and derived from the same founder cell within the future Otx-positive domain at the start of the time-lapse. Possible explanations for this exception to the rule are as follows. (1) We may have wrongly assigned these cells or mistracked them repeatedly. However, given our overall high accuracy of tracking, we consider this unlikely. (2) Initially lineage restricted cells may move across during later differentiation, e.g. by neuronal migration in the mantle layer, as reported in the chick hindbrain (Wingate and Lumsden, 1996). (3) The restriction mechanism may be leaky, as suggested for rhombomere boundaries (Birgbauer and Fraser, 1994). Escapers would need to readjust their gene expression, which may affect their fate.

Our results are consistent with a study of clonal dispersion after single cell injections in the brain of another fish species, Medaka (Oryzias latipes) (Hirose et al., 2004). Data derived from 150 single cell injections were fitted onto a model of a developing Medaka embryo. Although the position of cells relative to putative lineage restriction boundaries or genetic markers was not followed in this study, reduced mixing between all examined brain regions at the transition from developmental stage 16+ to 17 was observed, which corresponds approximately to the time we observe onset of restriction at the tailbud stage in zebrafish.

In summary, we suggest that the midbrain-hindbrain boundary separates two neuromeres in the developing zebrafish brain, raising the possibility that the neuromeric organization of the vertebrate brain extends to this part of the neural tube. Further studies will show whether the anterior neural tube is compartmentalized in general, similar to the rhombencephalon. A picture emerges where cell populations secreting organizing molecules are flanked by neuromere boundaries. The reverse conclusion can apparently not be drawn, as several rhombomere boundaries and the diencephalon-mesencephalon border are not (yet) known to be associated with organizers. Studies with cellular resolution of the type reported here may help to determine the relationship between organizing cell populations and lineage restriction boundaries, a link discovered and well studied in the fly (Crick and Lawrence, 1975; Dahmann and Basler, 1999), but poorly characterized in vertebrate brain development.

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Supplementary material
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