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

Tobias Langenberg and Michael Brand*

Department of Genetics, University of Technology, Dresden, c/o Max Planck Institute for Molecular Cell Biology and Genetics, Germany

*Author for correspondence (e-mail: brand@mpi-cbg.de)

Accepted 13 May 2005

Development 132, 3209-3216
Published by The Company of Biologists 2005
doi:10.1242/dev.01862

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 (Westerfield, 1994). Host embryos carrying transplanted cells were imaged on an Olympus BX61 microscope with a Spot RT Slider camera and Metamorph acquisition software.

Iontophoretic single cell injection

Iontophoretic cell labeling was performed as described by Fraser (Fraser, 1996). Embryos were photographed on the above described setup.

Nuclei tracking and plotting

Histone H2A.F/Z:GFP transgenic fish were mounted in 1.5% low melting point agarose in an imaging chamber (Concha and Adams, 1998) and imaged for up to 12 hours with 1.5 μm z- and 3- to 4-minute time resolution on a Nikon/BioRad two-photon confocal system. Image stacks were imported into NIH image, converted to single tiff files, renamed with FileBuddy (SkyTag Software) and imported as 4D stacks into the NIH Image4D version (modified NIH Image by Richard Adams). Nuclei were manually tracked and their positions taken down in Excel files. Calibration and plotting was performed with self-written routines in MatLab (The Mathworks) or with Excel.

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 (11.5 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

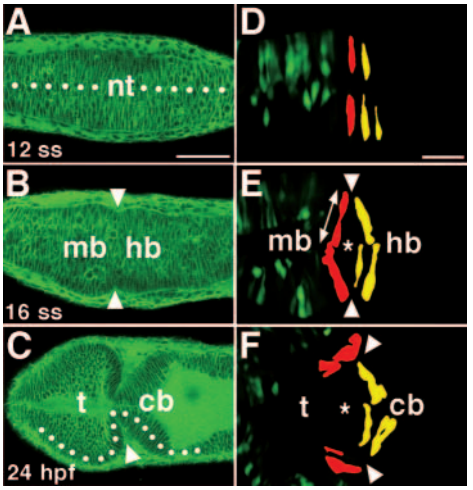

Fig. 1. Morphological changes and cell behavior during mhb formation. (A-C) Confocal optical sections of live embryos, stained with BODIPY-ceramide. From the 12-somite stage (A) to 24 hpf (C), a small indentation forms in the neural tube (nt) between the prospective midbrain (mb) and hindbrain (hb) (B, arrowheads). At 24 hpf (C), the now very deep invagination clearly separates the midbrain tectum (t) from the hindbrain cerebellum (cb). The AP axis folds up in the mhb area (dots in A and C). (D-F) Confocal optical sections of time-lapsed live embryos containing GFP-expressing cells in the mhb area. Cells form a pseudo-stratified epithelium, stretching from apical to basolateral (double arrow in E). Cell groups are pseudocolored according to their final position and traced backwards in the time-lapse: tectal cells are red, cerebellar cells are yellow. No mixing between the cell groups was observed (n=13 time-lapse movies analyzed). The asterisk indicates a gap containing 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.

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 histone-GFP line (Pauls et al., 2001), which expresses GFP in the nuclei of all cells, and matched nuclei positions with Otx antibody staining. By acquiring z-stacks of confocal images over a narrow time interval (3-4 minutes) on a two-photon confocal microscope, we were able to monitor the movement of individual nuclei with very high spatial and temporal resolution.

In total, we evaluated three independent movies, covering development of the mhb region between the 5- and 26-somite stages (Table 1 and Movie 2 in the supplementary material). After imaging, embryos were immediately fixed, stained for Otx protein, and optically sectioned on a confocal microscope (Fig. 2A,C). By comparing the last image stack of the time-lapse (Fig. 2B) with the antibody staining, we were able to assign a molecular status (Otx-positive or Otx-negative) to the nuclei at the end of the time-lapse (Fig. 2B,C). Nuclei close to the boundary of Otx expression were then backtracked through the time-lapse (Fig. 2D,E) and their position (xy center and z-level) was noted in intervals of about 1 hour. Using this approach, we assigned a molecular status to and followed nearly all cells at the boundary throughout the whole time-lapse (Table 1). We observed virtually no cell death as a result of photodamage, even under almost continuous scanning. Owing to the high temporal resolution, we were able to track cells through divisions, when nuclei temporarily left their respective groups to divide at the midline (Fig. 2G-I). Because of the working distance of the objective and signal quality issues, the analysis was restricted to a focal depth of 80 μm, corresponding to the dorsal three quarters of the neural tube at the mhb.

To visualize all tracked nuclei from a time-lapse at different time points, we plotted their positions in a three-dimensional coordinate system from which we exported two-dimensional plots (projected along the z-axis), using MatLab routines written for this purpose. As an example for the behavior of tracked nuclei, Fig. 3 shows plots from time-lapse 1: at every time point analyzed, nuclei formed coherent groups with minimal or no overlap (Fig. 3A,B for the start and end, respectively). The term coherent is used here to describe a group of cells whose members are never interspersed with cells from a neighboring group. Only one out of 280 tracked cells behaved differently (Table 1): although located within the second row of the putative Otx-positive cell population at the start of the time-lapse, it gave rise to two Otx-negative cells.

By contrast, nuclei of arbitrarily defined boundaries in the midbrain or cerebellum did not sort out into coherent groups (Fig. 3E,F). Arbitrary boundaries were picked within the midbrain or anterior hindbrain at locations that do not – to our

Table 1. Nuclei tracking summary

		Otx (+)		Otx (–)			
Time-lapse movie	Stage covered	Start	End	Start	End	Lost end*	Neural crest end*
1 (12 hours)	5–24 ss	57	129	53	131	34	20
2 (10 hours)	8–26 ss	36	66	28	56	0	2
3 (8 hours)	10–24 ss	56	77	51	94	1	0
Total		149	272	132	281	35	22
Restricted		149	272	131	279	–	–
Not restricted		0	0	1	2	–	–

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.

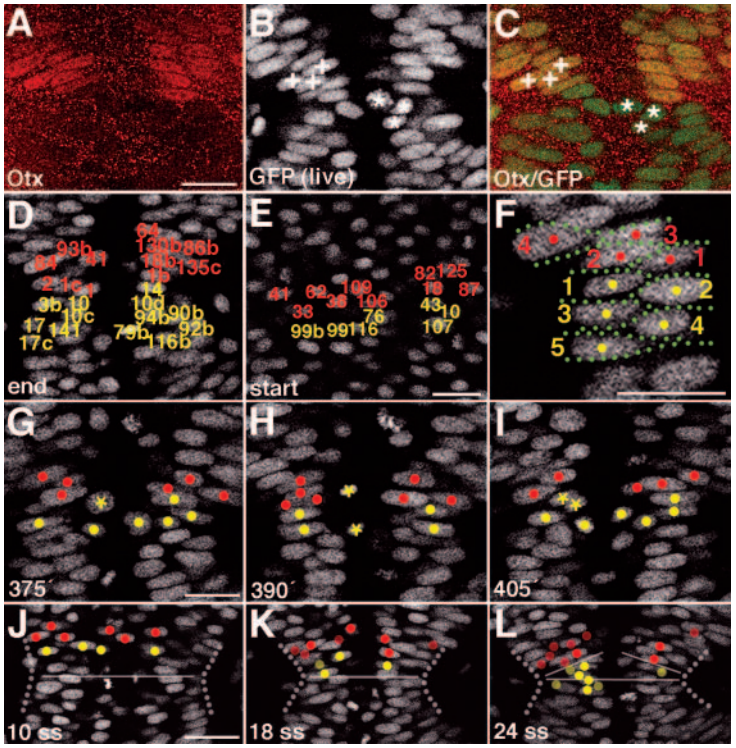

Fig. 2. Single nucleus tracking procedure. (A-C) Nuclei can be identified in both the anti-Otx staining and the live image. (A) One plane of an Otx antibody staining stack. (B) One plane of the last live stack of a time-lapse. (B,C) Three Otx-positive nuclei are marked by plus signs, three Otx-negative nuclei (histone-GFP positive) are marked by asterisks. Owing to the fixation and mounting, the embryo is slightly compressed in C, therefore more nuclei are visible in C than in B. (D,E) Individual nuclei are assigned a status (red, Otx-positive), numbered and tracked backwards to the start of the time-lapse. Imaged embryos displayed a normal morphology with only minor distortions because of the agarose embedding procedure (data not shown). (F) Cell position can be measured in rows distance from the Otx interface. (G-I) Nuclei can be tracked over divisions. Daughter cells of the dividing Otx-negative cell (asterisks) move a significant distance apart (H) but sort back into the Otx-negative domain (I). Elapsed time is indicated in minutes. (J-L) Morphological and lineage boundary do not match. Shown are stills from Movie 2 in the supplementary material. A strip of nuclei at the boundary and their progeny were marked throughout the time-lapse. Nuclei out of focus are marked by translucent dots. The morphological boundary (horizontal line) is always posterior to the lineage restriction boundary (tilted anterior lines in L, compare with Figs 1, 5). Gray dots indicate the borders of the neuroepithelium. All panels show dorsal views, anterior is towards the top. Scale bars: 20 μm.

knowledge – correspond to any gene expression or morphological boundaries. At these arbitrary boundaries, we consistently found violation of the artificial boundary by a large proportion of the tracked nuclei (Fig. 3E,F). We also examined artificial boundaries shifted just one cell row towards posterior or anterior from the detected boundary, and found no lineage restriction at this sharply defined interface (Fig. 3C,D shows an anterior shift as an example). This demonstrates that the movement of cells forming the mhb is specific to this boundary, and that the observed lineage restriction is not due to a general behavior of cells in this region of the neural keel and tube.

Fig. 3 further illustrates the behavior of cells near the mhb: 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

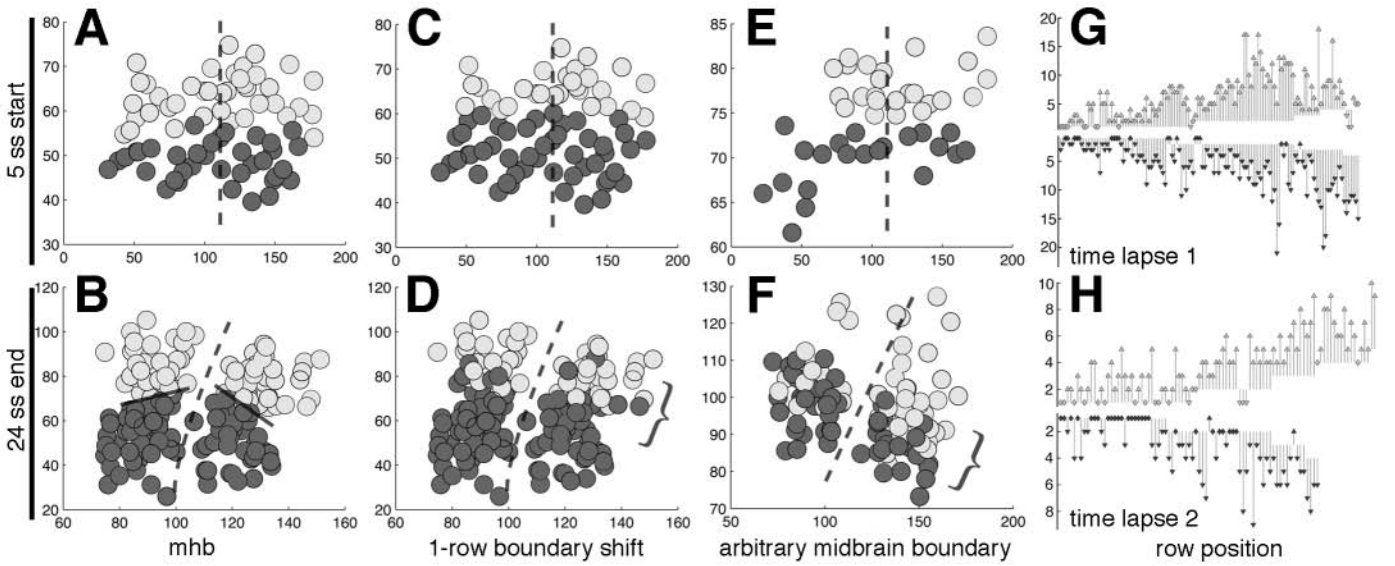

Fig. 3. Summary plots of nuclei positions in time-lapse 1. (A,B) Otx-positive and negative nuclei form coherent groups at the end (B, 24-somite stage) and at the beginning (A, 5-somite stage) of the time-lapse. The lines in B indicate the mhb on the basis of the posterior Otx2 boundary. (C,D) Upon shifting the Otx-positive/Otx-negative interface artificially by about one cell row anterior at the five-somite stage (C), the sharp interface of the two cell populations is lost at the 24-somite stage (D). The bracket indicates the zone of overlap. (E,F) Example of cell behavior at an arbitrary boundary in the middle of the tectum. Even though the cell populations are well separated at the five-somite stage (E), they show a marked zone of overlap at the 24-somite stage (F). (G,H) Plots showing the relative movement of cells with respect to their interface at the mhb from the beginning to the end of the time-lapse, expressed in rows (*y*-axis). The arrows indicate the position at the start of the time-lapse to the position at the end. Diamonds stand for cells that do not change their row position. Upper light-gray arrows represent Otx-positive cells, lower dark-gray arrows represent Otx-negative cells. Each daughter cell is treated as a separate cell (*x*-axis). (G) Cell movement over 10 hours. (H) Cell movement over 12 hours. Notice that cells move 10 and more cell rows away from their common interface. Most non-moving cells are found in the first cell rows (both Otx positive and Otx negative). Otx-positive nuclei are light grey, Otx-negative nuclei dark grey. Broken lines show the midline. (A-F) Dorsal views, anterior is towards the top, units are in μm in A-F. For ease of understanding, only a subset (leaving out the most dorsal and ventral planes) of the data of time-lapse 1 is shown in A-D. The plots are 2D projections along the *z*-axis and nuclei sizes are not drawn to scale.

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 (Figs 2, 3) and thus between mesencephalic and metencephalic cells. 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).

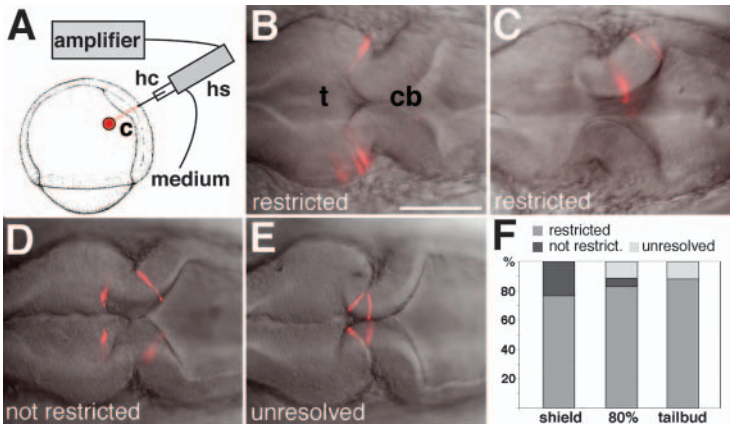

Fig. 4. Clonal analysis of single cell injections. (A) Schematic drawing of the iontophoretic single cell injection: current flows through an electrochemical Ag/AgCl half-cell (hc), a dye-filled glass capillary (c), by dye-flow (red) into the target cell, through the embryo, through the medium and back to the amplifier's headstage (hs). Current intensity is controlled via the amplifier. (B,C) Embryos at 24 hpf bearing labeled cells on one side of the morphological mhb, the midbrain or cerebellum, respectively. (D) An embryo with a clear two-sided label at 24 hpf. (E) Individual cases could not be resolved at the morphological level when cells were located directly in the boundary region. (F) Summary chart of single cell injections and transplantations. The number of two-sided clones decreases from shield stage (n=11/47), 80% epiboly (n=2/35) to tailbud stage (n=0/25). Shield stage statistics show a combination of single cell injections and transplantations. (B-E) Dorsal views, anterior is towards the left. Scale bar: 100 μm. t, tectum opticum; cb, cerebellum.

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).

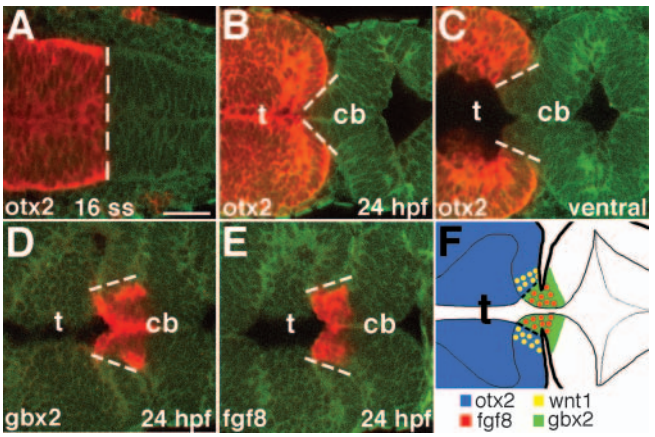

Fig. 5. Gene expression domains at the mhb share a common border that coincides with the lineage restriction boundary. (A,B) The posterior gene expression boundary of *otx2* shifts from perpendicular to the neural axis to oblique between the 16-somite stage and 24 hpf, reflecting morphological changes during mhb formation (compare with Fig. 1). (B,C) From dorsal to ventral, the opening (broken line) in the *otx2* expression domain becomes broader. (D,E) Similar to *otx2*, the anterior *gbx2* and *fgf8* expression domain borders become oblique with respect to the neural axis at 24 hpf. Further ventrally, the expression domains of *gbx2* and *fgf8* extend more anteriorly, probably filling the opening in the *otx2* expression domain (D,E, compare with C). (F) Summary scheme of gene expression boundaries in the mhb area at 24 hpf. Dots indicate co-expression. The lineage restriction boundary between the *otx2* and *gbx2* territories is marked with broken black lines. All panels show dorsal views, anterior is towards the left. In situ probes were detected with fluorescent FastRed substrate and embryos optically sectioned on a Zeiss confocal microscope. Scale bar: 50 μm.

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 by a Wnt1 promoter, was crossed to a *lacZ* driver line. After induction, the vast majority of cells was detected in the mesencephalon. However, independent of the time of labeling, some *lacZ*-positive cells were found within the cerebellum. This may be due to the previously described early activation of Wnt1 on both sides of the mhb (Bally-Cuif et al., 1995) or due to a certain leakiness of the lineage restriction mechanism (Birgbauer and Fraser, 1994). Several studies in chick embryos suggest lineage restriction between 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 to adopt the fate of the target tissue. Studies carried out in chick and mouse embryos (Alexandre and Wassef, 2003; Louvi et al., 2003) suggest that roof plate cells may escape a lineage restriction mechanism. In our study, very dorsally located cells also seemed to violate the lineage restriction boundary (Table 1). However, all of these cells either left the neuroepithelium (18/22) during the imaged time period or moved very fast and over long distances (4/22), classifying them as putative neural crest cells. Therefore, we argue that there is no contribution of midbrain cells to anterior hindbrain structures and vice versa in the zebrafish and that, although we did not address the molecular status of these cells, lineage restriction probably extends to roof plate structures.

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.

We thank the late J. A. Campos-Ortega for the histone-GFP transgenic line, A. Simeone and G. Corte for anti-Otx antibody, J. Clarke and M. Landolfa for tips on iontophoretic labeling, K. Khairy for help with MatLab programming, and A. Oates, C.-P. Heisenberg, A. Siekmann and A. Picker for valuable comments on the manuscript. This work was supported by funds from the EU (Zf models), the Dresden University of Technology and the Max Planck Society to M.B.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/14/3209/DC1

References

Alexandre, P. and Wassef, M. (2003). The isthmic organizer links anteroposterior and dorsoventral patterning in the mid/hindbrain by generating roof plate structures. *Development* **130**, 5331-5338.

Bally-Cuif, L., Cholley, B. and Wassef, M. (1995). Involvement of *Wnt-1* in the formation of the mes/metencephalic boundary. *Mech. Dev.* **53**, 23-34.

Birgbauer, E. and Fraser, S. E. (1994). Violation of cell lineage restriction compartments in the chick hindbrain. *Development* **120**, 1347-1356.

Brand, M. and Granato, M. (2002). Keeping and raising zebrafish. In *Zebrafish: A Practical Approach* (ed. C. Nüsslein-Volhard and R. Dahm). Oxford: Oxford University Press.

Concha, M. L. and Adams, R. J. (1998). Oriented cell divisions and cellular morphogenesis in the zebrafish gastrula and neurula: a time-lapse analysis. *Development* **125**, 983-994.

Cooke, J. E. and Moens, C. B. (2002). Boundary formation in the hindbrain: Eph only it were simple. *Trends Neurosci.* **25**, 260-267.

Cooper, M. S., D'Amico, L. A. and Henry, C. A. (1999). Analyzing morphogenetic cell behaviors in vitally stained zebrafish embryos. *Methods Mol. Biol.* **122**, 185-204.

Crick, F. H. C. and Lawrence, P. A. (1975). Compartments and polyclones in insect development. *Science* **189**, 340-347.

Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68.

Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet.* **15**, 320-326.
Fraser, S. E. (1996). Iontophoretic dye labeling of embryonic cells. *Methods Cell Biol.* **51**, 147-160.
Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.
Garcia-Bellido, A., Ripoll, P. and Morata, G. (1973). Developmental compartmentalisation of the wing disk of Drosophila. *Nat. New Biol.* **245**, 251-253.
Garda, A. L., Echevarria, D. and Martinez, S. (2001). Neuroepithelial co-expression of *Gbx2* and *Otx2* precedes *Fgf8* expression in the isthmic organizer. *Mech. Dev.* **101**, 111-118.
Hidalgo-Sanchez, M., Millet, S., Simeone, A. and Alvarado-Mallart, R. M. (1999). Comparative analysis of *Otx2*, *Gbx2*, *Pax2*, *Fgf8* and *Wnt1* gene expressions during the formation of the chick midbrain/hindbrain domain. *Mech. Dev.* **81**, 175-178.
Hirose, Y., Varga, Z. M., Kondoh, H. and Furutani-Seiki, M. (2004). Single cell lineage and regionalization of cell populations during Medaka neurulation. *Development* **131**, 2553-2563.
Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu. Rev. Cell Dev. Biol.* **17**, 189-214.
Jungbluth, S., Larsen, C., Wizenmann, A. and Lumsden, A. (2001). Cell mixing between the embryonic midbrain and hindbrain. *Curr. Biol.* **11**, 204-207.
Kelly, G. M., Lai, C. J. and Moon, R. T. (1993). Expression of *wnt10a* in the central nervous system of developing zebrafish. *Dev. Biol.* **158**, 113-121.
Keynes, R., Cook, G., Davies, J., Lumsden, A., Norris, W. and Stern, C. (1990). Segmentation and the development of the vertebrate nervous system. *J. Physiol. (Paris)* **84**, 27-32.
Kimmel, C. B., Warga, R. M. and Kane, D. A. (1994). Cell cycles and clonal strings during formation of the zebrafish central nervous system. *Development* **120**, 265-276.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
Langenberg, T., Brand, M. and Cooper, M. S. (2003). Imaging brain development and organogenesis in zebrafish using immobilized embryonic explants. *Dev. Dyn.* **228**, 464-474.
Lee, S. M., Danielian, P. S., Fritzsch, B. and McMahon, A. P. (1997). Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. *Development* **124**, 959-969.
Louvi, A., Alexandre, P., Metin, C., Wurst, W. and Wassef, M. (2003). The isthmic neuroepithelium is essential for cerebellar midline fusion. *Development* **130**, 5319-5330.
Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development *via* a repressive effect on *Otx2* expression. *Development* **126**, 1189-1200.
Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.
Millet, S., Bloch-Gallego, E., Simeone, A. and Alvarado-Mallart, R. M. (1996). The caudal limit of *Otx2* gene expression as a marker of the midbrain/hindbrain boundary: a study using in situ hybridisation and chick/quail homotopic grafts. *Development* **122**, 3785-3797.
Moens, C. B. and Prince, V. E. (2002). Constructing the hindbrain: insights from the zebrafish. *Dev. Dyn.* **224**, 1-17.
Mori, H., Miyazaki, Y., Morita, T., Nitta, H. and Mishina, M. (1994). Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res. Mol. Brain Res.* **27**, 221-231.
Papan, C. and Campos-Ortega, J. A. (1994). On the formation of the neural keel and neural tube in the zebrafish *Danio (Brachydanio) rerio*. *Roux's Arch. Dev. Biol.* **203**, 178-186.
Pasini, A. and Wilkinson, D. G. (2002). Stabilizing the regionalisation of the developing vertebrate central nervous system. *BioEssays* **24**, 427-438.
Pauls, S., Geldmacher-Voss, B. and Campos-Ortega, J. A. (2001). A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for in vivo studies of embryonic development. *Dev. Genes Evol.* **211**, 603-610.
Puelles, L. (2001). Brain segmentation and forebrain development in amniotes. *Brain Res. Bull.* **55**, 695-710.
Raible, F. and Brand, M. (2004). Divide et impera – the midbrain-hindbrain boundary and its organizer. *Trends Neurosci.* **27**, 727-734.
Reifers, F., Böhli, H., Walsh, E. C., Crossley, P. H., Stainier, D. Y. R. and Brand, M. (1998). *Fgf8* is mutated in zebrafish *acerebellar* mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. *Development* **125**, 2381-2395.
Reim, G. and Brand, M. (2002). Spiel-ohne-grenzen/pou2 mediates regional competence to respond to *Fgf8* during zebrafish early neural development. *Development* **129**, 917-933.
Rhinn, M. and Brand, M. (2001). The midbrain–hindbrain boundary organizer. *Curr. Opin. Neurobiol.* **11**, 34-42.
Rhinn, M., Lun, K., Amores, A., Yan, Y. L., Postlethwait, J. H. and Brand, M. (2003). Cloning, expression and relationship of zebrafish *gbx1* and *gbx2* genes to *Fgf* signaling. *Mech. Dev.* **120**, 919-936.
Rhinn, M., Lun, K., Luz, M., Werner, M. and Brand, M. (2005). Positioning of the midbrain-hindbrain boundary organizer through global posteriorization of the neuroectoderm mediated by *Wnt8* signaling. *Development* **132**, 1261-1272.
Schmitz, B., Papan, C. and Campos-Ortega, J. A. (1993). Neurulation in the anterior trunk region of the zebrafish *Brachydanio rerio*. *Roux's Arch. Dev. Biol.* **202**, 250-259.
Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (Gallus domesticus). *Ergebnisse der Anatomie und Entwicklungsgeschichte* **41**, 3-87.
Westerfield, M. (1994). *The Zebrafish Book*. 2.1 edn. Oregon: University of Oregon Press.
Wingate, R. J. and Lumsden, A. (1996). Persistence of rhombomeric organisation in the postsegmental hindbrain. *Development* **122**, 2143-2152.
Woo, K. and Fraser, S. E. (1995). Order and coherence in the fate map of the zebrafish nervous system. *Development* **121**, 2595-2609.
Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat. Rev. Neurosci.* **2**, 99-108.
Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-271.
Zervas, M., Millet, S., Ahn, S. and Joyner, A. L. (2004). Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. *Neuron* **43**, 345-357.