The isthmic organizer links anteroposterior and dorsoventral patterning in the mid/hindbrain by generating roof plate structures

Paula Alexandre and Marion Wassef*

Régionalisation Nerveuse CNRS/ENS UMR 8542, Département de Biologie Ecole normale supérieure, 46 rue d'Ulm, 75005 Paris, France

*Author for correspondence (e-mail: wassef@wotan.ens.fr)

This paper is dedicated to Cuca Alvarado-Mallart who retired recently

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Summary

During vertebrate development, an organizing signaling center, the isthmic organizer, forms at the boundary between the midbrain and hindbrain. This organizer locally controls growth and patterning along the anteroposterior axis of the neural tube. On the basis of transplantation and ablation experiments in avian embryos, we show here that, in the caudal midbrain, a restricted dorsal domain of the isthmic organizer, that we call the isthmic node, is both necessary and sufficient for the formation and positioning of the roof plate, a signaling structure that marks the dorsal midline of the neural tube and that is involved in its dorsoventral patterning. This is unexpected because in other regions of the neural tube, the roof plate has been shown to form at the site of neural fold fusion, which is under the influence of epidermal ectoderm derived signals. In addition, the isthmic node contributes cells to both the midbrain and hindbrain roof plates, which are separated by a boundary that limits cell movements. We also provide evidence that mid/hindbrain roof plate formation involves homeogenetic mechanisms. Our observations indicate that the isthmic organizer orchestrates patterning along the anteroposterior and the dorsoventral axis.

Key words: Mid/hindbrain junction, Isthmic organizer, Mid/hindbrain organizer, Roof plate, Homeogenetic mechanisms, Hensen's node, Dorsoventral patterning, Chick, Quail

Introduction

The isthmic organizer (IO), located at the constriction (or isthmus) that marks the mid/hindbrain (MHB) junction of the developing neural tube, is essential for the growth and patterning of the midbrain and anterior hindbrain (Joyner et al., 2000; Rhin and Brand, 2001; Simeone, 2000; Wurst and Bally-Cuif, 2001). When transplanted ectopically into competent territories, the IO induces the adjacent neuroepithelium to form supernumerary MHB structures (Martinez et al., 1991; Martinez et al., 1995), an activity mediated by FGF8 that is expressed at the MHB boundary (Crossley et al., 1996). The isthmic region is also the site of extensive cell rearrangements (Alvarez Otero et al., 1993; Louvi et al., 2003; Millet et al., 1996). Although much attention has been devoted to the understanding of the genetic interactions that position and maintain the IO (Joyner et al., 2000; Rhin and Brand, 2001; Simeone, 2000; Wurst and Bally-Cuif, 2001), less is known about the morphogenetic mechanisms that it induces. Ectopic IO grafts induce morphological distortions of the host and graft neuroepithelium (Martinez et al., 1999) that contrast with the perfect morphological and genetic integration of other transplanted regions of the midbrain neuroepithelium (Alvarado-Mallart et al., 1990; Martinez and Alvarado-Mallart, 1990; Nakamura et al., 1986). Our previous transplantation experiments (Louvi et al., 2003) indicated that the midline regions of both the midbrain and hindbrain are populated by divergent migrations of cells generated from a restricted dorsal midline region in the caudal midbrain. The roof plate (RP) is a specialized structure that marks the dorsal midline of the neural tube and is involved in dorsoventral patterning (Lee et al., 1998; Liem et al., 1995). At early stages, the expression of two RP markers, *Gdf7* and *Wnt1*, displays singularities at the level of the MHB (Louvi et al., 2003). *Gdf7* expression is delayed in the MHB domain compared to adjacent regions of the neural tube that express the transcript at the 19 somite stage (ss) whereas *Wnt1* is expressed in a wide dorsal MHB domain at the 10-13ss that encircles the neural tube at the level of MHB junction (Bally-Cuif and Wassef, 1994). Experimental manipulations involving rotation or partial ablation of the avian midbrain, although not previously connected to IO activity, were found to affect or to completely prevent the formation or positioning of the roof plate (RP) (Cowan and Finger, 1982; Marin and Puelles, 1994). Finally, the expression of *Wnt1* is deflected in the direction of ectopically transplanted (Bally-Cuif and Wassef, 1994) or FGF8-induced (Crossley et al., 1996) IOs. Altogether these observations suggest a specific behavior of the developing RP at the level of the MHB.

We show that the dorsal IO is arranged around a restricted central node that contributes cells to the midbrain and cerebellar RPs and is both necessary and sufficient for the formation of the midbrain RP. Interfering locally with IO node function and plasticity prevents RP formation in the caudal midbrain. We also present evidence that the induction or maintenance of the expression of RP markers *Gdf7* and *Wnt1*

involve homeogenetic mechanisms. Finally, we show that a stream of cells extends from ectopic, FGF8-induced, IOs to populate the locally induced ectopic RP. These findings demonstrate that the IO is responsible for RP induction and positioning in the caudal midbrain. They indicate that the IO is involved in dorsoventral patterning and in the selection of the symmetry axis of the optic tectum. This process resembles that organized in the ventral spinal cord by the regressing Hensen's node (Catala et al., 1996; Charrier et al., 1999), the avian counterpart of the Spemann organizer. Thus, our observations emphasize that axis organization and contribution to axial structures may represent a common function of secondary organizers in the vertebrate brain.

Materials and methods

White Leghorn chick and Japanese quail embryos, were operated on between somite stages (ss) 9 and 13 and fixed 2 days later between stages 19 and 21 (HH19-21) of Hamburger and Hamilton (Hamburger and Hamilton, 1951). The methods for performing small ablations, homotopic isochronic grafts (Alvarez Otero et al., 1993; Bally-Cuif and Wassef, 1994), bead implantation (Martinez et al., 1999) mesencephalic vesicle rotation including the notochord [type 1A in Marin and Puelles (Marin and Puelles, 1994)] in situ hybridization (Bally-Cuif and Wassef, 1994) and cell death assay using Nile Blue Sulfate (Louvi and Wassef, 2000) were as described with minor modifications. The DiI crystals were inserted into a small slit made in the neuroepithelium using tungsten needles. Heparin acrylic beads were soaked sequentially in FGF8 (R&D, 0.2 mg/ml) and DiI emulsion (Selleck and Stern, 1991) or only in DiI emulsion.

Results

A discontinuity in early cell behavior at the presumptive midbrain beak

Previous quail to chick transplantation experiments have indicated that there is a global convergence of the caudal midbrain neuroepithelium towards the midline (Millet et al., 1996; Louvi et al., 2003). In addition, some medial grafts were observed to elongate markedly more along the dorsal midline [figure 2B,C in Louvi et al. (Louvi et al., 2003)]. This pattern could have been a mere consequence of the presence of a midline boundary deflecting the flow of converging cells in the orthogonal directions, on both sides. Alternatively, and more in line with the interpretation of Millet et al. (Millet et al., 1996), a restricted midline region at the caudal limit of the midbrain could generate the midline structures in an extended anteroposterior domain around the MHB junction. A first series of experiments was thus performed that aimed at better characterizing the origin of the MHB midline. We first inserted small homotopic and isochronic quail-to-chick transplants (Fig. 1A) or DiI crystals (Fig. 1B) laterally in the caudal mesencephalic vesicle of 10-13ss embryos. The operated embryos were analyzed 2 days later, at stage HH 19-21, when the isthmic constriction coincides with the limit between the midbrain and anterior hindbrain (Haleness et al., 1990; Martinez and Alvarado-Mallart, 1989; Millet et al., 1996). The lateral grafts or DiI-labeled cells ended medially without extending much along the anteroposterior axis and both were excluded from a small midline domain (purple arrows in Fig. 1A,B). These observations suggested that a restricted medial region in the dorsal IO differed either in growth or in cell movements from the adjacent neuroepithelium. Quail transplants inserted into this region were rarely recovered 2 days later (see however Fig. 1C). Extensive cell death affecting the MHB midline region at the 14-16ss (Lumsden et al., 1991) (see also Fig. 3F-I) and, possibly, a lower proliferation rate could explain why small quail transplants grafted into the dorsal IO failed to survive. Small medial grafts inserted slightly more caudally often contributed scattered cells to the cerebellar RP [figures 2B and 3 in Louvi et al. (Louvi et al., 2003)]. DiI crystals inserted in the dorsal IO marked a small compact cell population in HH19-21 embryos (Fig. 1D). At this stage, the DiI-labeled cells ended into a protrusion (arrowheads in Fig. 1E,F) that marks the posterior midbrain midline and has been described as a wedge in mouse (Bally-Cuif et al., 1995) or a beak in chick (Millet et al., 1996). Subsequent experiments described below indicated that cells destined to populate the RPs of the midbrain and anterior hindbrain were produced at the periphery of the prospective beak.

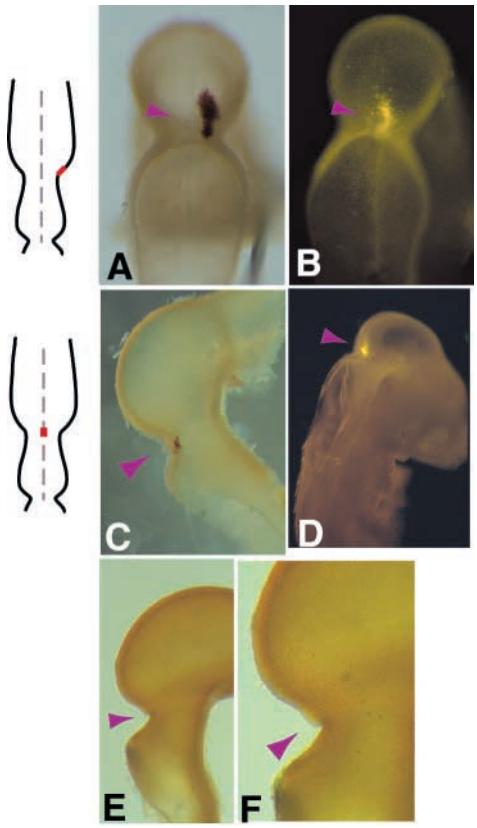

Fig. 1. An immobile pivotal domain in the caudal midbrain. (A,B) Posterior, (C) posterolateral, and (D) lateral views of the dissected (A-C) or whole-mount (D) neural tubes of HH19-21 chick embryos that received a graft or DiI crystals as schematized on the left. Small lateral grafts (A) and lateral DiI crystals (B) all converged towards the midline. They avoided a small medial circular area in the caudal midbrain (arrowheads in A,B). Small grafts (C) or DiI crystals (D) inserted into the center of the caudal mesencephalic vesicle apparently remained still (arrowheads in C and D) and ended in a protrusion or 'beak' that marks the caudal midline of the mesencephalon. This is best seen on lateral views of the dissected neural tube (E,F arrowheads).

Restricted origin of the midbrain and rostral hindbrain roof plates at the isthmic node

In contrast to midline grafts, calibrated paramedial transplants extended long distances anteriorly or posteriorly along the midline (Louvi et al., 2003). The organization of the dorsal midline region of the MHB was therefore analyzed by insertion of small DiI crystals around the central IO region identified earlier. The dye-labeled cells were observed to disperse widely along the midline. Depending on the posterior (Fig. 2A) or anterior (Fig. 2D) position of the DiI crystal, the labeled cells were confined either to the RP of the anterior hindbrain (Fig. 2A-C) or of the midbrain (Fig. 2D-F). DiI labeling of the anterior hindbrain RP was stereotyped: the dye-labeled cells populated its entire length abutting rostrally the beak protrusion (Fig. 2B,C) and reaching the edge of the choroid plexus caudally, with a small bifurcation at the end (Fig. 2B). Although the rostrocaudal extension of midbrain RP labeling was less stereotyped, the DiI-labeled cells in the anterior hindbrain and the midbrain RPs respected the same limit located beneath the beak protrusion (Fig. 2B,C,E,F). Thus, we identified on the midline a sharp limit to cell movements beneath the midbrain beak. We could not detect, however, any indication of a transverse restriction to cell movements (Millet et al., 1996) more laterally, in the form of a discontinuity in the shape of the grafts or the distribution of dye-labeled cells (Fraser et al., 1990). A small pattern difference was noticed between the origin of the midbrain RP, clearly a paired domain interrupted on the midline, and that of the anterior hindbrain RP that straddled the midline.

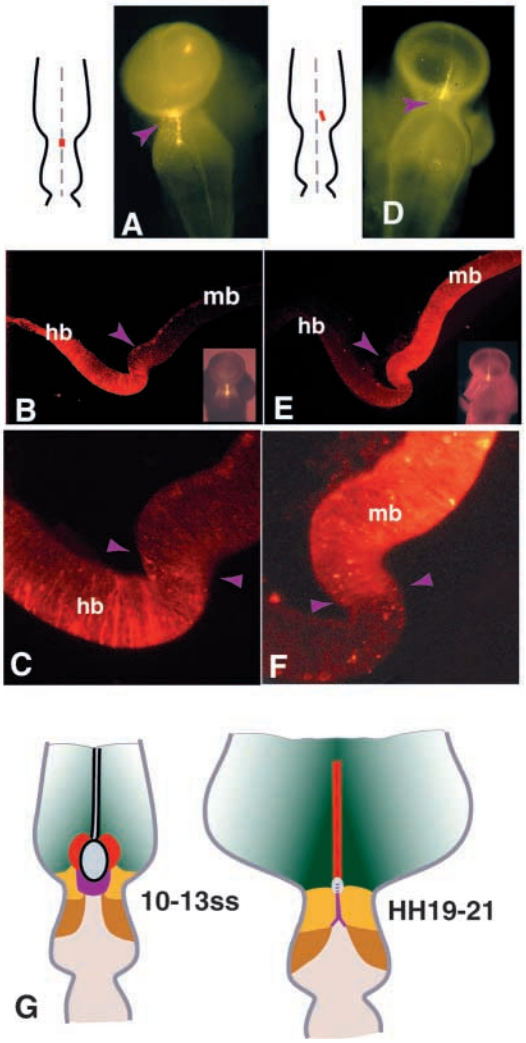

Fig. 2. Isthmic origin of MHB roof plate structures. (A,D) Posterior views of the dissected neural tubes of HH19-21 embryos in which DiI crystals were inserted at the 10-13ss. The site of insertion is schematized on the left. Midline DiI crystals inserted at the level of the isthmic constriction (A) label the entire anterior hindbrain roof plate (RP). Paramedial DiI crystals inserted into the caudal midbrain vesicle (D) label the midbrain RP. (B,C,E,F) Midsagittal cryostat sections of two embryos (shown in the insets) similar to A and D. The DiI-labeled hindbrain (hb) RP ends beneath the beak (B,C). When it extends to the isthmic region (E,F), DiI labeling in the midbrain (mb) RP respects a similar sharp limit beneath the beak. (G) Fate map of the dorsal isthmic region focused on the central IO node. The cells that contribute to the roof plates of the midbrain (in red) and anterior hindbrain (purple) are organized around a central domain (gray) whose relative size decreases between the 10-13ss and HH19-21 stages. They migrate extensively along the midline but respect a common limit in the caudal midbrain. The 10-13ss and HH19-21 stage embryos are not drawn at the same scale.

These observations are schematically illustrated in Fig. 2G. They indicated that the cells destined for the midbrain and anterior hindbrain RPs were generated at the periphery of an immobile midline domain of the IO. We have called this structure the IO node.

The IO node is required for midbrain RP development

To investigate whether the IO node was necessary for the generation of the midbrain RP, small midline ablations were performed in the isthmic region of 10-13ss chick embryos. Two days later, expression of *Gdf7* (and *Wnt1*, not shown), a chick and quail RP marker (Fig. 3A,A′) (Lee et al., 1998), was completely (4/6) or partially (thinner RP, 2/6) regenerated (Fig. 3A,A′,B,B′), consistent with previous reports on the regulative behavior of the isthmic region (Alvarado-Mallart et al., 1990; Alvarez Otero et al., 1993; Marin and Puelles, 1994; Martinez and Alvarado-Mallart, 1990; Nakamura et al., 1986). In contrast, homotopic quail-to-chick grafts inserted into the IO node resulted in marked alterations of the RP (Fig. 3C,C′,D,D′,E). Depending on the stage at operation, *Gdf7* midline expression observed 2 days later was either split or duplicated (Fig. 3C,C′ 14-15ss embryos, 5/5), missing (Fig. 3D, between arrowheads; D′, 13ss embryos, 2/3) or missing with a small *Gdf7*-positive protrusion bulging in the middle of the *Gdf7* gap (Fig. 3E, arrowhead, 10-12ss, 3/4). Very few or no QCPN-immunoreactive quail cells were detected in the isthmic region of the grafted embryos. Indeed extensive cell death was observed on the midline of the mid-hindbrain junction between the 14ss and 22ss (Fig. 3F-I). The transient presence of the small chunk of grafted quail cells could impair the regulation of IO node function that we observed after ablation. The bulge observed in the youngest embryos (Fig. 3E) was interpreted as a tentative regeneration of the IO node. We also noticed that embryos that received a slightly larger transplant including the IO node survived better. We interpret this as the result of better healing of the grafts in these cases. Indeed, it is known that brain growth involves an increase in ventricular pressure that requires both neural tube closure

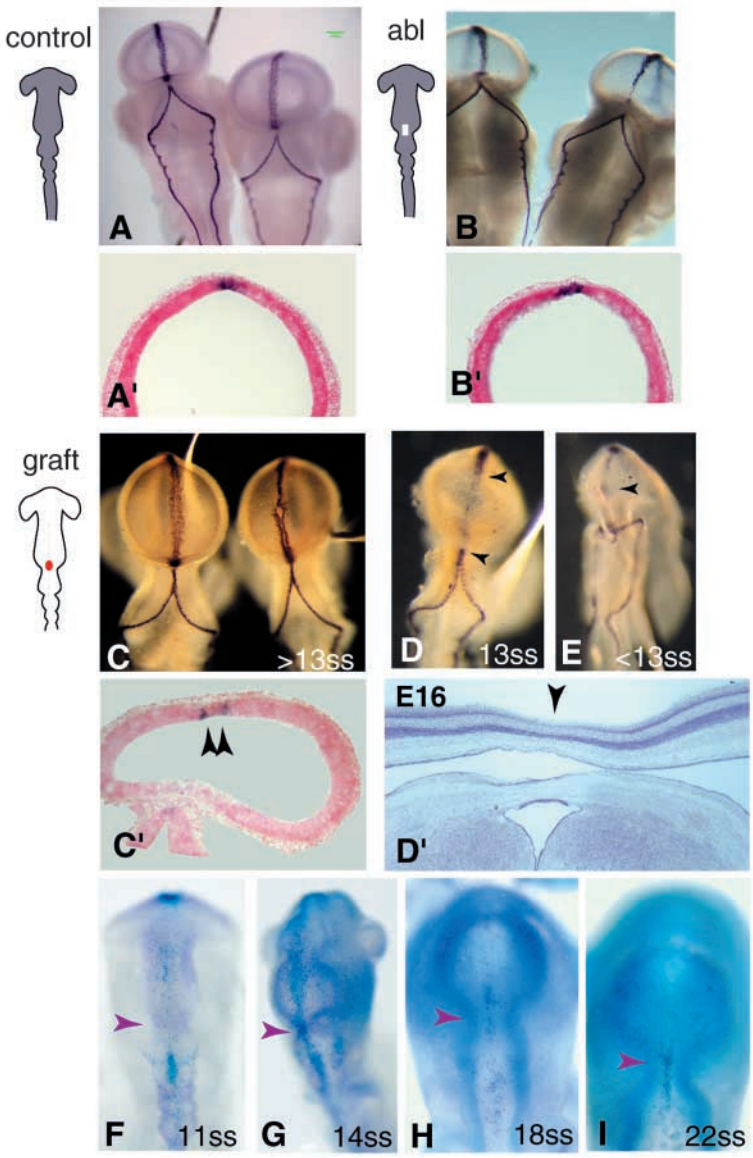

Fig. 3. Expression of midbrain RP markers depends on IO-derived signals. (A-E) Posterior views of the MHB of HH19-21 embryos that were operated on at the 10-15ss, as schematized on the left, and treated for the detection of *Gdf7* transcripts (purple). Corresponding transverse cryostat sections counterstained with nuclear Fast Red are illustrated in A′-C′. (A) In control HH19-21 chick (or quail) embryos *Gdf7* expression marks the RP. (B) *Gdf7* expression is completely (left, 4/6) or partially (right, 2/6) regenerated after ablations of the isthmic dorsal midline. (C-E) Homotopic quail to chick IO node grafts performed at different stages interfere with midbrain RP formation. Graft insertion into the IO node resulted in RP duplication in 14-15ss embryos (C, double arrowhead in C′; 5/5) and in the formation of a gap in *Gdf7* expression in younger embryos (between arrowheads in D; 2/3). A medial bulge that expresses *Gdf7* is observed in 10-12ss embryos (arrowhead in E; 3/4). (D′) Transverse section of an E16 embryo that received a slightly larger graft centered on the IO node at the 12ss. The section was immunostained with QCPN to detect the grafted quail cells and counterstained with Cresyl Violet. Quail cells are detected at more caudal levels but the illustrated section is anterior to the graft limit. Note the absence of RP structure on the midline (arrowhead). Very few quail cells were detected in embryos that received transplants limited to the presumptive IO node (C-E) either because the low proliferation rate interfered with graft integration, or because of the high level of cell death detected by Nile blue sulfate staining on the midline of the isthmic region between the 14ss and 22ss (F-I) (see also Lumsden et al., 1991).

anteriorly and a transient obturation of the ventricular lumen posteriorly (Desmond and Schoenwolf, 1986). The embryos that received small IO node grafts showed a marked growth delay and their aspect is suggestive of ventricular leakage (Fig. 3D,E). Grafts involving the IO node often resulted in the formation, at E16, of a dome-shaped optic tectum that lacked a roof plate on the midline rostrally to the graft (arrowhead in Fig. 3D′). These observations indicated that experimental manipulations that affect a small MHB region, the IO node, interfere with the formation of the midbrain RP. They suggest that the IO node is essential for midbrain RP development.

Homeogenetic mechanisms contribute to midbrain RP formation or maintenance

Whereas RP markers regenerated after ablation of the midline in the posterior midbrain, ablations performed more rostrally (Fig. 4A-F) often resulted in gaps in the expression of *Gdf7* (4/4 in anterior midbrain, not shown, and 1/6 in intermediate midbrain, Fig. 4C,C′). Interestingly, most (4/6) of the intermediate midbrain ablations resulted in partial gaps containing scattered *Gdf7* (Fig. 4B,B′) or *Wnt1* (Fig. 4D) cells at low density that could have migrated from or been induced by the cut ends of the RP. In addition, local perturbations of the midline often resulted in moderate alterations in the expression of RP markers that spread on both sides of the lesion but were more obvious anteriorly (Fig. 4E,F). Also, anteroposterior rotations of the midbrain vesicle that maintained dorsoventral polarity (Marin and Puelles, 1994) and did not directly involve the dorsal midline resulted in the disappearance of RP markers at HH19-21 (Fig. 4G) as well as in the absence of RP structures at later stages (Bally-Cuif and Wassef, 1994; Marin and Puelles, 1994). Thus, a temporary interruption or reversal of anteroposterior (AP) signaling dramatically impaired RP formation. This behavior suggested that homeogenetic mechanisms are involved in the induction or maintenance of RP identity during development. To directly demonstrate that a homeogenetic mechanism operates during RP development, an anteroposterior strip of quail

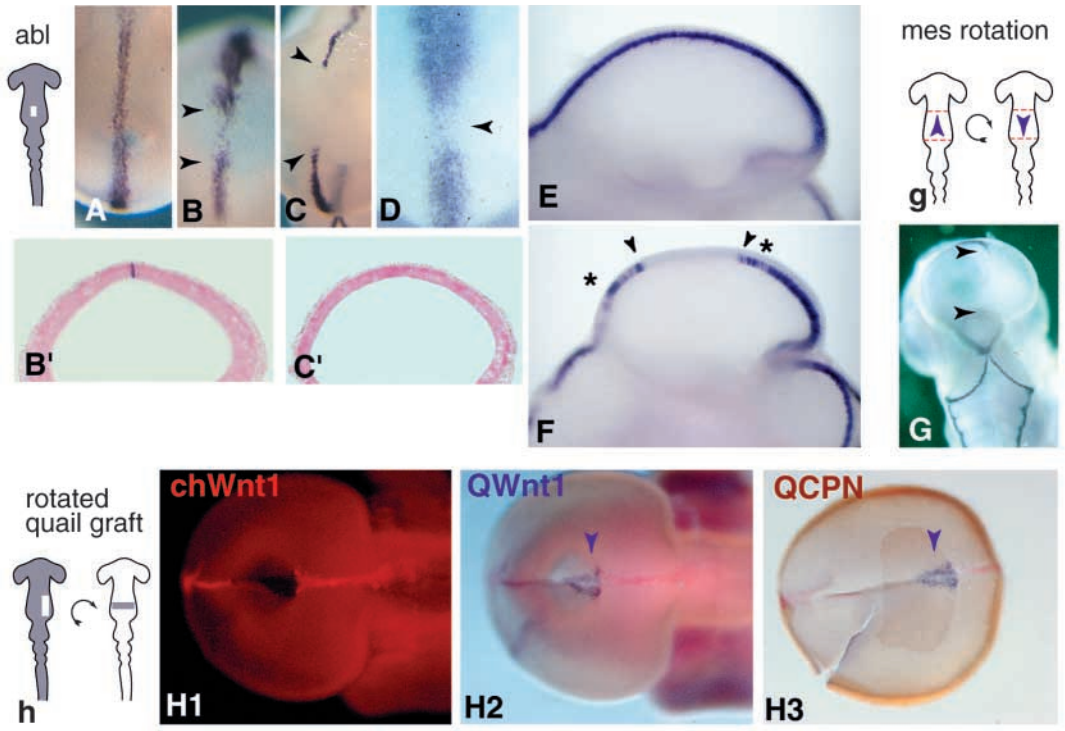

Fig. 4. Homeogenetic signaling induce the expression of RP markers. (A-D) Dorsal view of the midbrain region of HH19-21 embryos that received a small midbrain midline ablation at the 10-12ss as schematized on the left. Corresponding transverse cryostat sections counterstained with Nuclear Fast Red are illustrated in B′,C′. The embryos were treated for the detection of *Gdf7* (A-C) or *Wnt1* (D) transcripts. The ablation resulted in complete regeneration (A, 1/6), or in the formation of partial (B, 4/6) or complete (C, 1/6) gaps in the midbrain *Gdf7* expression domain. Scattered cells in the partial gaps expressed *Gdf7* (B) or *Wnt1* (D). (E,F) Lateral views (anterior is to the left) of *Gdf7* midline expression in control (E) and ablated (F) embryos. *Gdf7* expression is perturbed (*) on both sides of the ablation (delimited by the arrowheads). (G) Posterior view of a HH19 embryo illustrating the lack of *Gdf7* expression (between arrowheads) on the midline of the mesencephalic vesicle after inversion of its anteroposterior axis as schematized in g. (H) Dorsal view of the midbrain of a 4-day-old chimera. An anteroposterior strip of quail midbrain neuroepithelium was transplanted at HH10 perpendicular to the host midline as schematized in h. H1-H3 illustrate the same chimera. The host roof plate (RP), labeled with chWnt1 (Fast Red), is seen in red using fluorescent optics (H1); the induced RP (purple arrowheads in H2 and H3), labeled with QWnt1 (NBT/BCIP), appears purple under bright-field optics (H2). After dissection of the dorsal midbrain, a faint QCPN labeling delineates the quail transplant (H3). Anterior is to the right.

mesencephalon was rotated 90° and grafted orthogonal to the mesencephalic midline of a chick host (Fig. 4h). To distinguish between the host and graft RPs, we took advantage of species-specific chick and quail Wnt1 probes (Bally-Cuif et al., 1994). The row of ChWnt1 expressing host RP cells (Fig. 4H1), abutted a stripe of QWnt1-labeled cells within the graft (Fig. 4H2, H3). The induced quail RP develops in continuity with the host roof plate, orthogonally to the presumptive AP axis of the graft. This clearly demonstrates that homeogenetic mechanisms are involved in roof plate development.

Ectopic IOs contribute cells to a locally induced RP

In vivo, the relative contribution to RP formation of the IO and of epidermal ectoderm-derived signals cannot be assessed since the position of the MHB RP corresponds with the location of neural fold fusion. We thus examined the capacity of the IO to induce RP formation in a different location. We have previously observed (Bally-Cuif and Wassef, 1994) that ectopic quail-to-chick IO grafts induced the formation of a row of *Wnt1*-expressing cells linking the host midline to the graft. Because *Wnt1* is expressed both on the dorsal midline and in an isthmic ring, the RP identity of the IO-induced cells could not be determined. No directed migration of quail cells outside the IO graft was observed in these experiments. Nevertheless, on the basis of the IO node midbrain location determined in the present study, we would expect midbrain RP-directed migration to involve host chick cells rather than quail cells. To examine the behavior of IO cells in an ectopic environment, we induced the formation of an ectopic IO by inserting heparin acrylic beads soaked sequentially in FGF8 and in DiI solutions into the lateral mes- or diencephalon (Crossley et al., 1996). DiI served to mark the cells of the FGF8-induced ectopic IO. The embryos were photographed 2 days after bead implantation under rhodamine fluorescence optics, then processed for *Gdf7* or *Wnt1* transcript detection. Fragments of control beads soaked only in DiI labeled a patch of cells (Fig. 5A) but did not modify the morphology of the neural tube or the pattern of *Gdf7* (Fig. 5A′) or *Wnt1* expression (not shown). In contrast, a dorsally directed row of DiI-labeled cells was always detected streaming from the FGF8-induced cerebellum (Fig. 5B,C) that develops as a bulge around the FGF8/DiI-soaked bead fragments (Crossley et al., 1996; Martinez et al.,

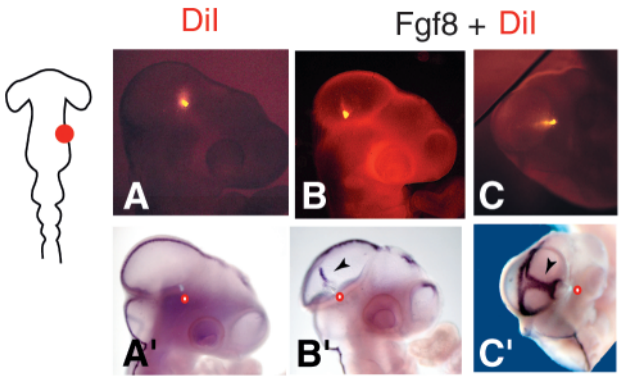

Fig. 5. Cell migration from the FGF8-induced IO marks the position of the ectopic roof plate (RP). Lateral views of three HH19-21 undissected embryos, 2 days after implantation in the lateral midbrain of DiI (A,A′), or FGF8/DiI (B,B′,C,C′) -soaked beads as schematized on the left. The same embryos photographed under rhodamine fluorescence optics in (A-C) were processed for the detection of *Gdf7* (A′,B′) or *Wnt1* (C′) transcripts (purple). (A,A′) Control embryo; a patch of fluorescent cells surrounds the DiI-labeled bead fragment (A). Midline *Gdf7* (or *Wnt1*, not shown) expression is not perturbed (A′). In embryos that have received a FGF8/DiI bead fragment, a row of DiI-labeled cells (B,C) migrated from the FGF8-induced cerebellum in the direction of the host midline. These cells marked the position of ectopic RP structures labeled with *Gdf7* (B′) or *Wnt1* (C′). The ectopic RP (arrowheads) either remained confined to the vicinity of the bead (B′) or reached the endogenous midline (C′). The position of the bead is indicated with a red circle.

1999). The row of DiI-labeled cells prefigured an ectopic row of *Gdf7* (Fig. 5B′) or *Wnt1* (Fig. 5C′) expressing cells that in many cases extended further dorsally (Fig. 5C′) to reach the endogenous RP. Thus, the site of neural fold fusion is not required by the IO to induce an ectopic RP and to contribute cells to it, suggesting that RP determination and positioning is a robust property of the IO.

Discussion

The dorsal part of the MHB junction produces two major sensory structures, the optic tectum and the cerebellum. The development of a smoothly polarized optic tectum structure is a prerequisite to the establishment of ordered retinotectal projections and to the accurate representation of the visual field. It has been shown that signals derived from the IO are necessary in chick and zebrafish (Nakamura, 2001; Picker et al., 1999) for the development of anteroposterior polarity in the optic tectum. We show that the IO also governs the development and positioning of the RP, a structure involved in dorsoventral signaling.

Involvement of the IO in dorsoventral patterning

It is generally believed that when the neural tube forms by fusion of the dorsal tips of the neural folds, the RP and neural crest are generated at its dorsal midline under the influence of TGFβ-related signals of the BMP family produced by the epidermal ectoderm (Liem et al., 1995). After neural tube closure, neural crest cells emigrate from the dorsal neural tube. At the same time, BMP expression is lost from the epidermal ectoderm and induced in the RP converting the initially lateromedial TGFβ signals into dorsoventral signals. Our observations indicate that, while dorsal signals may be required to establish the competence to form RP structures, precise positioning of the MHB RP depends on signaling from the IO. Indeed, we have observed that expression of members of the BMP/GDF family is downregulated at the MHB junction (Louvi et al., 2003).

Secondary organizers and neural tube axis formation

We describe here an IO-dependent positioning and development of the RP that resembles, in many respects, the control of floor plate development (Catala et al., 1996; Charrier et al., 1999; Placzek et al., 1993) by the regressing Hensen's node (Fig. 6). Both the IO and Hensen's node behave as neural organizers and induce convergent extension cell movements. They both initiate the formation of the neural tube midline signaling structures by directly contributing cells to them. The identity of both ventral and dorsal midline structures is propagated and reinforced by homeogenetic mechanisms (Placzek et al., 1993). A major difference between the two organizers is that the Hensen's node actively moves posteriorly whereas the IO seems to remain still. We suggest that the IO node is immobilized by the opposing forces that result from the progression of two divergent axial structures. This is consistent with the observation that in *En1*^{Otx2} mutants the midbrain roof plate extends more caudally in the absence of a cerebellar roof plate (Louvi et al., 2003). The formation of the beak protrusion could also result from these constraints exerted on the central neuroepithelium of the IO node.

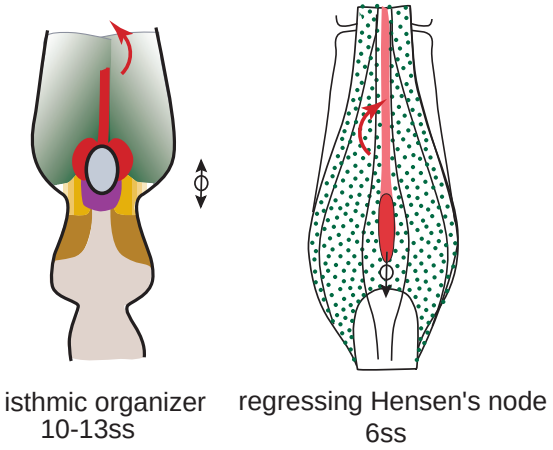

Fig. 6. The immobile IO node and the regressing Hensen's node. Schematic drawing comparing the organizing properties of the IO node (left, same colour code as in Fig. 2G) and the regressing Hensen's node [right, the dotted area represents the neural plate; modified from Charrier et al. (Charrier et al., 1999)]. The IO and Hensen's node organize similar convergent extension cell movements and produce cells destined for axial structures. In both cases, the differentiation of the definitive midline neural structures depends on homeogenetic mechanisms (curved arrows). The stability of the IO node contrasts with the active caudalward movement of the regressing Hensen's node that leads to extension of the neural tube. The opposing centrifugal migrations arising from the IO node may explain why it remains still. Black arrows point to the direction of cell movements.

Positioning the optic tectum symmetry axis

Why rely on the IO rather than on ectoderm-derived signals to position the MHB RP? It may be argued that maintaining the symmetry in size and structure of the two optic tecta before the establishment of the retinotectal connection is of particular physiological importance to prevent asymmetry in the representation of the visual world. Visual lateralization exists in birds. It relies on asymmetric visual signals that depend on the prehatch position of the embryo so that the right eye is exposed to light through the translucent shell (Vallortigara et al., 2001). This asymmetry is part of the functional adaptation of the brain to the visual environment and does not preclude the basic need for isotropy in the visual representation. Two kinds of developmental mechanisms, those involved in symmetry breaking and those controlling midbrain growth could interfere with the development of left-right symmetric midbrain structures. Several signals involved in symmetry breaking are operating at successive developmental stages around the MHB domain. FGF8 (Boettger et al., 1999) and FGF18 (Ohuchi et al., 2000), two FGF family members, as well as SHH (Levin et al., 1995; Pagan-Westphal and Tabin, 1998) are expressed asymmetrically in the Hensen's node. FGF8 (Crossley et al., 1996; Joyner et al., 2000; Martinez et al., 1999; Rhin and Brand, 2001; Simeone, 2000; Wurst and Bally-Cuif, 2001) and SHH (Zhang et al., 2000) are known to affect later stages of MHB development and their asymmetric expression could lateralize the early MHB. Also, the anterior midbrain abuts the dorsal diencephalon that expresses clear morphological asymmetries affecting in particular the habenula and the pineal organ in zebrafish (Concha et al., 2000). The development of these morphological asymmetries is preceded by the asymmetric expression of several genes in the left and right dorsal diencephalon (Concha et al., 2000). Asymmetric signaling in the prosencephalon could indirectly affect midbrain development. Finally, compared to the neighboring structures, the MHB grows enormously. Even a transient increase in FGF8 protein has been shown to produce long lasting growth stimulation in the mesencephalic neuroepithelium (Shamim et al., 1999). Thus, any difference in FGF8 signaling between the left and right IO would result in marked asymmetries in the mature optic tecta, had the two midbrain halves been delimited before the IO is fully active. Postponing the positioning of the roof plate and linking it to the source of growth signals may aid to circumvent the consequences on dorsal midbrain development of the symmetry-breaking processes.

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