Development 133, 2905-2913 (2006) doi:10.1242/dev.02460

Positive and negative regulations by FGF8 contribute to midbrain roof plate developmental plasticity

Paula Alexandre*, Isabelle Bachy, Morgane Marcou and Marion Wassef†

The roof plate (RP) of the midbrain shows an unusual plasticity, as it is duplicated or interrupted by experimental manipulations involving the mid/hindbrain organizer or FGF8. In previous experiments, we have found that FGF8 induces a local patterning center, the isthmic node, that is essential for the local development of a RP. Here, we show that the plasticity of the midbrain RP derives from two apparently antagonistic influences of FGF8. On the one hand, FGF8 widens beyond the neural folds the competence of the neuroepithelium to develop a RP by inducing the expression of LMX1B and WNT1. Ectopic overexpression of these two factors is sufficient to induce widely the expression of markers of the mature RP in the midbrain. On the other hand, FGF8 exerts a major destabilizing influence on RP maturation by controlling signaling by members of the TGFβ superfamily belonging to the BMP, GDF and activin subgroups. We show in particular that FGF8 tightly modulates follistatin expression, thus progressively restraining the inhibitory influence of activin B on RP differentiation. These regulations, together with FGF8 triggered apoptosis, allow the formation of a RP progress zone at some distance from the FGF8 source. Posterior elongation of the RP is permitted when the source of FGF8 withdraws. Growth of the posterior midbrain neuroepithelium and convergent extension movements induced by FGF8 both contribute to increase the distance between the source of FGF8 and the maturing RP. Normally, the antagonistic regulatory interactions spread smoothly across the midbrain. Plasticity of midbrain RP differentiation probably results from an experimentally induced imbalance between regulatory pathways.

KEY WORDS: Isthmic organizer, Mid/hindbrain organizer, Roof plate, FGF8, Follistatin, Activin, BMP7, GDF7, Chick, Quail

INTRODUCTION

The roof plate (RP) is a specialized structure that extends on the dorsal midline of the neural tube along its entire anteroposterior axis. The RP forms at the site of neural fold closure during development (Liem et al., 1995). It constitutes a signaling center that influences dorsoventral patterning of the neural tube, specification of dorsal neuronal types, and axonal guidance across the dorsal midline (Liem et al., 1997; Lee et al., 1998). RP development has been best studied in the spinal cord, where its specification relies on interactions between inductive signals of the TGFβ family produced by the adjacent epidermal ectoderm and intrinsic homeodomain transcription factors (Liem et al., 2000; Chizhikov and Millen, 2004b; Chizhikov and Millen, 2004c). Less is known about RP development in the anterior brain, where modulations of intrinsic or extrinsic factors involved in neural tube patterning along the AP axis could influence local RP differentiation (Bach et al., 2003). In chick embryos, the RP of the midbrain is highly plastic. Experimental perturbations of the midbrain neuroepithelium that do not directly involve the dorsal midline result in the disappearance of the RP at later stages or in its reorientation or bifurcation (Marin and Puelles, 1994; Bally-Cuif and Wassef, 1994; Crossley et al., 1996; Alexandre and Wassef, 2003).

Growth and patterning of the midbrain-hindbrain (MH) domain of the neural tube depend on the activity of a signaling center called the isthmic organizer (IsO), located at the constriction or isthmus that links the midbrain and hindbrain. Fibroblast growth factor 8 (FGF8), a diffusible molecule secreted by the IsO, is one of the major mediators of its organizing and growth-inducing properties (Crossley et al., 1996; Martinez et al., 1999). Insertion of a FGF8-soaked bead in the MH domain or the posterior forebrain induces the formation of a supernumerary IsO, the development of an ectopic MH junction and also of an ectopic RP. The influence of the IsO, whether endogenous or FGF8 bead induced, on the midbrain RP is puzzling. It seems to promote RP elongation and maturation when an ectopic source of FGF8 is inserted into the midbrain (Bally-Cuif and Wassef, 1994; Crossley et al., 1996) or to impair its differentiation when rotation of the midbrain vesicle brings the anterior RP closer to the IsO or when the isthmic node is ablated (Marin and Puelles, 1994; Alexandre and Wassef, 2003).

In the spinal cord, BMP signaling controls RP formation through the induction of the competence factors *Lmx1a* and *Lmx1b* (Chizhikov and Millen, 2004a; Chizhikov and Millen, 2004b; Chizhikov and Millen, 2004c). *Wnt1* has also been implicated in later aspects of RP differentiation (Shimamura et al., 1994; Amoyel et al., 2005). *Lmx1b* and *Wnt1* are also targets of FGF8 signaling widely expressed in the caudal midbrain. At later stages of development, *Lmx1b* and *Wnt1* expression becomes confined to the RP and floor plate and to a ring of cells marking the MH boundary. A cue to the unexplained behavior of the midbrain RP may therefore reside in crossregulatory interactions between the BMP and FGF pathways on common downstream genes essential for RP development. The aim of the present study was to understand better these interactions. We find that FGF8 orchestrates midbrain RP differentiation by finely adjusting BMP, GDF and activin signaling in time and space.

MATERIALS AND METHODS

Animals

White Leghorn chick (Morizeau) and Japanese quail (La Caille de Chanteloup) embryos were operated on between somite stages (ss) 9 and 14, and fixed 1-3 days later between Hamburger and Hamilton stages 14 and 23

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

*Present address: Department of Anatomy and Developmental Biology, UCL, Gower Street, London, WC1E 6BT, UK
†Author for correspondence (e-mail: wassef@wotan.ens.fr)

Accepted 25 May 2006

(HH 14-23) (Hamburger and Hamilton, 1951). Some embryos were also examined after long survival, mainly 4.5, 7 and 12 days after surgery. The methods for performing small ablations, homotopic and heterotopic isochronic grafts (Bally-Cuif and Wassef, 1994; Alexandre and Wassef, 2003), bead implantation (Martinez et al., 1999), in situ hybridization, immunocytochemistry and cryostat sections (Bally-Cuif and Wassef, 1994) were as previously described with minor modifications. Rabbit anti-active caspase 3 (Promega) was diluted 1/250, mAb QCPN (Developmental studies hybridoma bank) was diluted 1/10 and mouse anti-AFP (Q-biogene) was diluted 1/2000.

Vectors

The following vectors were used for in situ hybridization: *ChWnt1* and *QWnt1* are species specific, although they weakly crossreact (Bally-Cuif and Wassef, 1994); *Gdf7* (Lee et al., 1998) (gift from K. Lee), *Lmx1b* (Matsunaga et al., 2002) (gift from N. Nakamura), *follistatin* (Graham and Lumsden, 1996) (gift from F. Giudicelli), *noggin* (Hirsinger et al., 1997) (gift from C. Freitas), *Id3* (Kee and Bronner-Fraser, 2001) (gift from M. Bronner-Fraser), and *ActA* and *ActB* (Merino et al., 1999) (gift from J. M. Hurle).

Beads

Heparin acrylic (Sigma) or Affigel Blue (BioRad) beads were soaked in a drop of recombinant protein (R&D, 16-20 beads/μl, unless otherwise specified) at the following concentrations: FGF8, 0.1, 0.2 and 0.4 mg/ml; BMP7, 1 mg/ml; GDF7, 0.5 mg/ml; noggin, 1 mg/ml; follistatin, 0.5 and 1 mg/ml; FGF8/GDF7, 0.2/0.5 mg/ml; activin A, 1 and 2.5 μg/ml). Formate-derivatized AG-1 X2 beads (BioRad) were used to deliver the FGFR1 inhibitor SU5402 (Promega, 4 mg/ml in DMSO); control beads were soaked in PBS or DMSO. The beads were incubated overnight in a moist chamber at 4°C and rinsed three times before insertion. When heparin acrylic beads soaked in PBS were inserted in the caudalmost region of the midbrain, they sometimes induced a relocation of the MH boundary or widened the *Wnt1* expression domain, thus mimicking the effect of an FGF8 bead. We interpreted this behavior as resulting from a change in local FGF8 signaling resulting from the diffusion of heparin, which is known to be a potent co-factor of FGF8, or from a modification of the shape and time course of the gradient of FGF8, which may be accumulated and released by the heparin bead. PBS-soaked heparin acrylic beads had no noticeable effect elsewhere.

Explant culture

The heads of HH14-15 embryos were isolated from the rest of the body at the level of the otic vesicles then separated into two halves by cutting the dorsal and ventral midlines. The two halves remained together, one left unperturbed as control. The caudal part of explants, including the isthmic region, was ablated to remove the endogenous source of FGF8. FGF8 signaling was also modulated through implantation of FGF8, PBS, SU5402 or DMSO beads in the caudal midbrain. The explants were placed ventricular side down on floating membranes (de Diego et al., 2002) and cultured for 6 or 18 hours before fixation and processing for in situ hybridization.

Electroporation

The chick *Lmx1b* expression vector [pMiw-*Lmx1b* (Matsunaga et al., 2002); a gift from K. Nakamura] was derived from the pMiwIII vector containing regulatory sequences from the Rous sarcoma virus enhancer and chicken ß-actin promoter. In ovo electroporation on stage HH10 chick embryos was performed as described (Funahashi et al., 1999). A GFP-GPI expression vector (Keller et al., 2001) (gift of D. Henrique) derived from pEGFP-N1 (Clontech) was co-electroporated with pMiw-LMX1B to check for the efficiency of transfection.

RESULTS

In the midbrain, an extensive reorganization of RP patterning may be induced by seemingly unrelated experimental conditions, as schematically represented in Fig. 1. We have shown previously (Louvi et al., 2003; Alexandre and Wassef, 2003) that FGF8 beads inserted in the midbrain may induce locally a short RP segment. The hypothesis that FGF8 derived from the IsO may initiate development

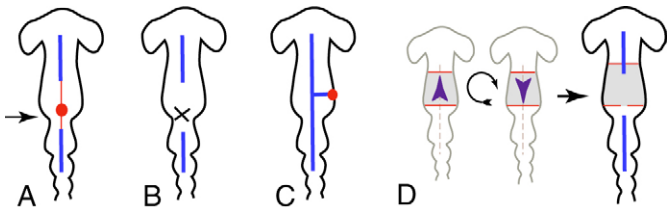

Fig. 1. Background on midbrain roof plate properties: schematic representation of previous experiments that disclosed unexpected properties of the RP near the isthmic organizer (IsO). (**A-C**) For simplicity, although the midbrain RP is not yet differentiated from the neural folds at stage HH10, the experimental manipulation performed at stage HH10 and its outcome on RP patterning 1 or 2 days later are both represented on the same outline of a stage HH10 chick neural tube. (A) Cells from a restricted isthmic domain (red circle isthmic node, Isnode) extend to populate the midline on both sides of the MH junction, marked by an arrow (Millet et al., 1996; Louvi et al., 2003; Alexandre and Wassef, 2003). (B) Interfering with local patterning at the Isnode prevents RP formation on the adjacent midline (Alexandre and Wassef, 2003). (C) A lateral source of FGF8 created by insertion of a FGF8-soaked bead or of an IsO transplant induces RP duplication/bifurcation (Bally-Cuif and Wassef, 1994; Crossley et al., 1996). (**D**) The RP fails to develop on the dorsal midline of midbrain vesicles whose AP axis has been inverted by a 180° rotation (Marin and Puelles, 1994; Alexandre and Wassef, 2003).

of the endogenous RP is, however, difficult to reconcile with the observation that expression on the midline of several BMP family members is transiently downregulated at the level of the IsO (Louvi et al., 2003). In the midbrain, BMP signaling is still required after neural tube closure for RP differentiation. Beads impregnated with the BMP inhibitor noggin inserted in the dorsal midbrain of HH10 embryos prevent *Gdf7* expression on the midline and RP development (7/16, Fig. 2A-A″). However, even in the most affected embryos (Fig. 2A′,A″), a short RP segment still develops at both ends of the midbrain. Thus other factors may compensate for BMP downregulation at the anterior and posterior poles of the midbrain. Local factors on the midline may also potentiate RP marker induction by FGF8. In general, the ectopic RP segment induced by FGF8 links the bead to the dorsal midline (Fig. 2B). When the FGF8 induced RP is reduced to a short segment near the bead (Alexandre and Wassef, 2003) (arrows in Fig. 2C), a small deflection is also induced opposite the bead on the midline RP (arrowhead in Fig. 2C). This indicates that, even if dispensable, co-factors on the midbrain dorsal midline potentiate the inductive activity of FGF8 on RP differentiation. To identify genetic factors involved in midbrain RP development, we examined another experimental situation where an ectopic RP is induced.

Acquisition of RP competence by ectopic transplants

We examined grafts of naive dorsal midbrain neuroepithelium implanted across the host midline. We have shown previously (Alexandre and Wassef, 2003) that ectopic RP segments were induced in these transplants as extensions of the host RP. The induced RP formed a solid row of cells usually of uniform width. RP induction could be initiated both anteriorly and posteriorly in the graft (16/63, Fig. 3A,A′,A″). In general, the large dorsal midbrain transplants grafted perpendicular to the midline did not grow much (Louvi et al., 2003). Most transplants did not develop a RP structure. In these transplants, *Wnt1* expression was maintained or induced in a wide domain comprising the whole graft (9/26, Fig. 3B,B′) or a

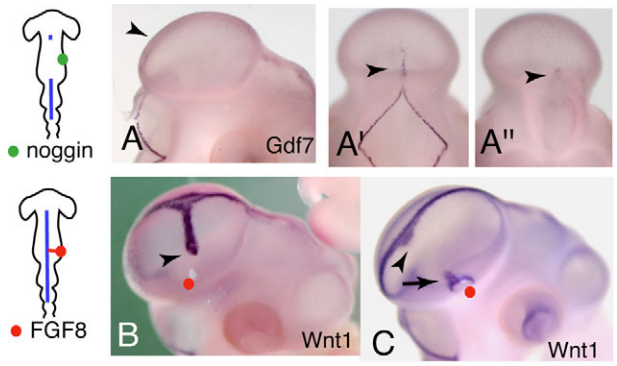

Fig. 2. Bipolar differentiation of the midbrain roof plate. (**A-A″**) BMP signaling is required for RP differentiation later than stage HH10. Lateral (A), posterior (A′) and dorsal (A″) views of the same embryo fixed 2 days after insertion of a noggin-soaked bead in the midbrain and labeled for *Gdf7* transcripts. The midbrain RP is mostly missing (A, arrowhead) except for a faint labeling at its caudal (A′, arrowhead) and rostral (A″, arrowhead) ends. (**B**,**C**) Local and distant effects of FGF8 beads. Lateral views of embryos labeled for *Wnt1* 2 days after implantation of FGF8 beads. In most cases, the induced RP extends from the dorsal midline to the bead (B, arrowhead). In cases where the FGF8 bead induces locally a small RP segment (C, arrow), a small deflection of the endogenous RP also points in the direction of the bead (C, arrowhead).

large part of the graft (11/26, Fig. 3,C,C′,D,D′,D″). *Lmx1b* expression was upregulated in the grafts (10/10). In the transplants, the domain of *Lmx1b* expression was always included within the *Wnt1* domain (4/4, Fig. 3B). *Gdf7* and *Lmx1b* expression coincided in most cases (4/6, not shown). Sometimes (2/6) part of the *Lmx1b* domain contained only scattered cells expressing *Gdf7* (not shown). Induction of an ectopic RP in the transplant was accompanied by a downregulation of this widespread expression of *Wnt1* and *Lmx1b*. Thus, ectopic transplantation stabilizes the expression of the RP competence factors *Lmx1b* and *Wnt1* which is normally transient in the lateral midbrain. These factors have been shown essential for RP differentiation in the spinal cord (Chizhikov and Millen, 2004a; Chizhikov and Millen, 2004b; Chizhikov and Millen, 2004c).

The roof plate marker *Gdf7* is induced in the midbrain by ectopic expression of *Lmx1b*

Lmx1b and *Wnt1* are competence factors for RP development in the spinal cord (Chizhikov and Millen, 2004a; Chizhikov and Millen, 2004b; Chizhikov and Millen, 2004c). Both are targets of FGF8 and, in addition to their dorsal (and ventral) midline expression, are expressed in a wide domain of the caudal midbrain (Fig. 4A,B). We wondered whether expression of *Gdf7* could be induced by *Lmx1b* on the anterior midbrain midline. Overexpression of *Lmx1b* in the dorsal midbrain was obtained by electroporation. Confirming the report of Matsunaga et al. (Matsunaga et al., 2002), *Wnt1* but not *Gdf7* was already strongly induced 10 hours after *Lmx1b* electroporation (Fig. 4D,D′,D″). *Gdf7* was induced in a few scattered cells 24 hours after *Lmx1b* electroporation in the midbrain (Fig. 4D). High levels of *Gdf7* expression were not detected before 34 hours after electroporation, when *Gdf7* was expressed in large cell strands or patches in the lateral midbrain (Fig. 4E,F). Interestingly, *Gdf7* induction by *Lmx1b* was less efficient in the caudal midbrain (arrowheads in Fig. 4E,E′). Thus, initiation of *Gdf7* expression on the anterior midbrain midline could be triggered by *Lmx1b* and *Wnt1*, which are both present much earlier on the midline.

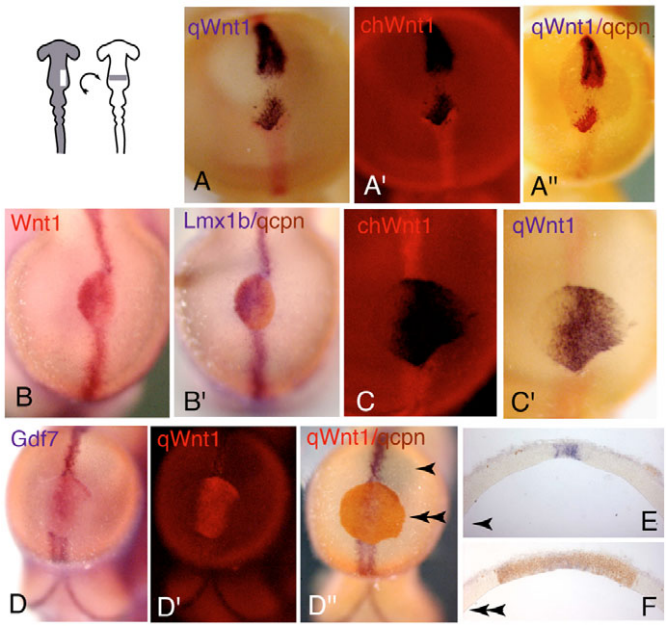

Fig. 3. Expression of RP competence factors in rotated dorsal midbrain transplants. Dorsal views of four HH22-23 quail-chick chimeras (**A-A″**,**B**,**B′**,**C**,**C′**,**D-D″**) that received a 90° rotated dorsal midbrain transplant 3 days earlier at stage HH10, as schematized on the left (black, quail donor). The embryos were treated for the detection of *Wnt1*, *Lmx1b* or *Gdf7* transcripts, as indicated together with the color of detection (red or purple) at the top of each picture. Species-specific probes were used in A-A″,C-D″ to discriminate between chick and quail *Wnt1* transcripts. After using the second probe, the quail transplant was identified by QCPN immunocytochemistry (A″,B′,D″). RP-like structures developed in the graft in contact with one or both ends of the host RP in about 25% of the transplants (16/63, A-A″). In more than 70% of cases, *Wnt1* remained widely expressed in the transplants (B-D′). *Lmx1b* was expressed ectopically in part of the *Wnt1* expression domain (4/4, B′). Except in the smallest transplants, *Wnt1* expression was regulated along the DV axis, leading to its reorganization in a wide band that flanked the host midline (C′,D′). In these cases, the transplant straddles the midline (D′, see also **F**, a transverse section through the transplant, double arrowhead) without differentiating a RP (compare with **E**, a section through the host RP, arrowhead).

Influence of the isthmic organizer and FGF8 on the expression of RP maturation markers

In addition to *Gdf7*, the expression of other markers of the mature roof plate [*Bmp5*, *Bmp7* (Louvi et al., 2003), *noggin* (Fig. 5A) and *Id3* (Fig. 5B)] is delayed in the caudal midbrain. Insertion of a FGF8 bead dorsally in the caudal midbrain prolonged this transient downregulation of *Gdf7* expression (Fig. 5C-E). This suggests that, in situ, FGF8 not only promotes RP formation but also inhibits its maturation. To test if this dual influence could also be detected during the process of ectopic RP induction by FGF8 beads, we examined the expression of early (*Wnt1*) and late (*Gdf7*) RP markers 24 and 30 hours after FGF8 bead insertion. One day after bead implantation, a row of cells prefiguring the ectopic RP expressed *Wnt1* along its entire length (in red in Fig. 5F,F′) bridging the midline to the FGF8 bead. *Gdf7* expression was intense near the midline but became fainter more distally (in purple in Fig. 5F). In slightly older embryos (Fig. 5G), both *Wnt1* and *Gdf7* expressions in the induced RP reached the bead. Thus, maturation of the induced and endogenous RP was similarly delayed near the source of FGF8,

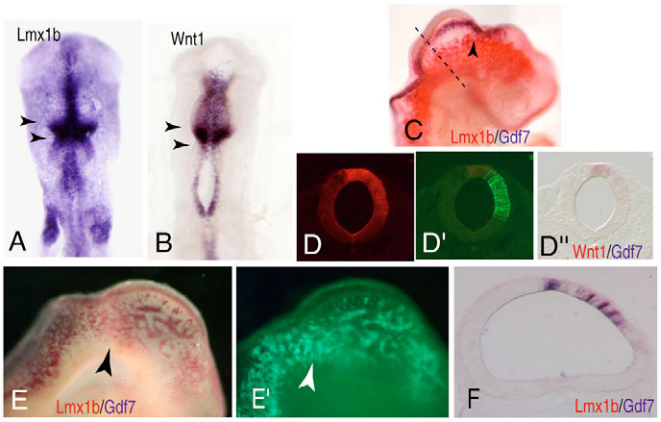

Fig. 4. LMX1B and WNT1 slowly induce GDF7 expression. *Lmx1b* and *Wnt1* are competence factors for RP differentiation. At stage HH10-11, *Lmx1b* (**A**) and *Wnt1* (**B**) are expressed both on the dorsal midline and widely in the caudal midbrain (between arrowheads). Lateral views (**C**,**E**,**E′**) and transverse sections of the midbrain (**D-D″**,**F**) of embryos fixed 10 (D-D″), 24 (C) and 34 (E,E′,F) hours after co-electroporation of *Lmx1b* and *Egfp* expression vectors. *Lmx1b* (C,E,F) or *Wnt1* (D-D″) transcripts are detected in red, *Gdf7* transcripts in purple (C,D″,E,F) and EGFP in green (D′,E′). Twenty-four hours after electroporation, very few scattered cells express *Gdf7* ectopically (C). *Gdf7* expression is strongly induced 34 hours after electroporation (E,F). Except in the caudal midbrain (arrowhead in E) and at the midbrain-forebrain junction, the purple staining for GDF7 partly obscures that for *Lmx1b* (E,F). This fainter induction of GDF7 does not result from a difference in the efficiency of *Lmx1b* electroporation around the constrictions, as the co-electroporated EGFP reporter is expressed at high levels in the same regions (arrowhead in E′).

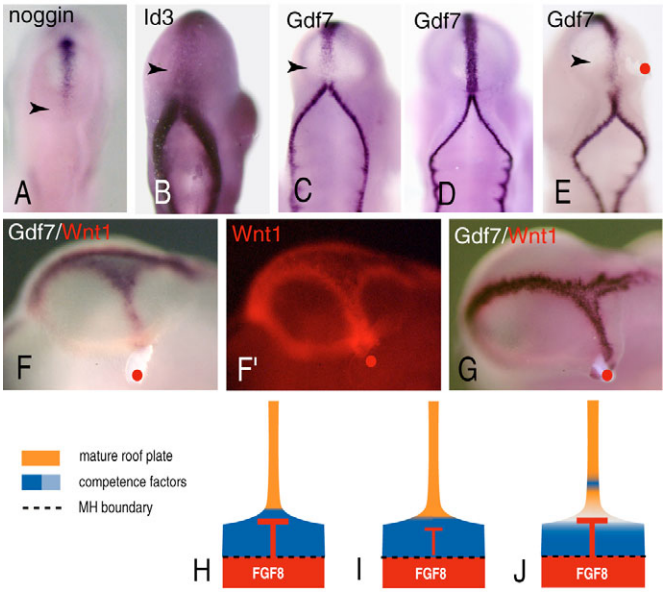

Fig. 5. FGF8 delays RP maturation. Posterior (**A-E**) and lateral (**F-G**) views of HH14-15 (A-C), HH16-17 (D-F′) and HH18 (G) chick embryos treated by in situ hybridization for the detection of RP markers. The expression of *noggin* (A), *Id3* (B) and *Gdf7* (C) is transiently downregulated in the caudal midbrain at stage HH14-15 (arrowheads). A mature expression pattern of *Gdf7* is observed in the caudal midbrain, beginning at stage HH16 (D). Insertion of a FGF8-soaked bead (E, red dot) in the caudal midbrain prolongs *Gdf7* downregulation beyond HH17. (F,G) The same delay in *Gdf7* expression is observed on the ectopic RP induced by FGF8 beads (red dots in F,G). Embryos fixed 24 (F,F′) or 30 (G) hours after FGF8 bead insertion were treated for the detection of *Gdf7* in purple and *Wnt1* in red. (F,F′) Same embryo shown in bright and dark field. At 24 hours, an ectopic preRP is already labeled for *Wnt1* (F,F′), but *Gdf7* expression lags behind (F). At 30 hours *Gdf7* and *Wnt1* are co-expressed in the induced RP (G). (**H-J**) The hypothetical consequences of an imbalance between the positive and negative influences of FGF8 on RP differentiation. The competence factors *Lmx1b* and *Wnt1* (purple) are targets of FGF8 signaling; their expression is sufficient to induce slowly maturing RP markers (see Fig. 4). Conversely, high levels of FGF8 signaling (red) inhibit the expression of RP maturation markers. (H) A balance between these two influences of FGF8 maintains a progress zone of RP maturation (orange). (I) An imbalance in favor of competence factors should result in RP widening, whereas (J) increasing the inhibitory activity of FGF8 at the expense of competence results in the formation of gaps in the developing RP. We propose that the lack of a RP structure in the caudal part of rotated midbrain vesicles (Alexandre and Wassef, 2003; Marin and Puelles, 1994) is the consequence of this last configuration.

suggesting that FGF8, in situ, regulates the caudal progression of RP differentiation. As mentioned above, *Gdf7* induction by ectopic electroporated *Lmx1b* was less efficient in the caudal midbrain than elsewhere, which also suggests the existence of an isthmus-derived inhibitory influence that prevents or delays *Gdf7* induction by *Lmx1b*.

Thus, FGF8 both promotes RP differentiation by inducing a wide expression of the competence factors *Lmx1b* and *Wnt1* and delays RP differentiation. As illustrated in Fig. 5H, any imbalance between these two FGF8 activities could result in enlargements or gaps in the developing RP structure. The short-range mechanisms of RP auto induction described below may partly obviate this problem.

Regulation of roof plate differentiation by BMP signaling

Because BMP signals are involved at successive stages of RP development, we wondered if a BMP family member could mediate this RP homeogenetic signal.

Insertion of BMP7-soaked beads at stage HH9-10 affected the medial domain of the RP but did not induce *Gdf7* (Fig. 6A) or *Wnt1* (Fig. 6B,B′) locally, indicating that BMP7 is not directly involved in the homeogenetic behavior of the RP. The thin sheet of cells that develops between the two RP halves resembled the neural component of the choroid plexus but it did not express choroid plexus markers such as transthyretin (TTR, Fig. 6B,B′) or BMP5 (not shown). Downregulation of BMP signaling through the insertion of a noggin-soaked bead locally impaired RP differentiation (Fig. 6C,C′), but did not result in the formation of a choroid plexus.

In contrast to BMP7, GDF7-soaked beads acted locally. *Gdf7* expression was induced (Fig. 6D,E) at high (4/16) or moderate (9/16) levels, 24 hours after insertion of a GDF7 bead in the midbrain. The induction could involve the dorsal midline (Fig. 6E) or consist of smaller lateral cell patches (Fig. 6D). Interestingly, ectopic expression of *Gdf7* was never detected at 48 hours, indicating that the induction was transient. *Lmx1b* was faintly or not induced by GDF7 beads (not shown), which could perhaps explain the lack of maintenance of ectopic *Gdf7*. Thus, autoregulation of *Gdf7* could be an important component of the homeogenetic mechanism that promotes the extension of a pre-existing RP (Alexandre and Wassef, 2003).

We asked whether FGF8 could widen the competence domain for GDF7 auto-activation. *Gdf7* expression was widely induced, though at a low level, by beads soaked in a mixture of FGF8 and GDF7, and the refinement of RP differentiation into a compact linear structure was prevented (Fig. 6G,H). Overexpression of FGF8 and GDF7 together also induced a large number of activated caspase 3-immunoreactive cells (7/7; Fig. 6I), whereas beads soaked in GDF7 (1/5; Fig. 6F) or FGF8 (not shown) did not induce an increase in cell death. Thus, FGF8 signaling, by inducing cell death, precludes a rapid progression of the front of *Gdf7*-expressing cells, therefore stabilizing it [see the pattern of cell death in the caudal midbrain in Alexandre and Wassef (Alexandre and Wassef, 2003); Fig. 3].

Increase in activin signaling interferes with RP patterning and maintenance

Activins belong to the activin/nodal/TGFβ subgroup of the TGFβ superfamily. At the difference of BMPs, which signal through SMADs1/5/8, the members of the activin subgroup signal through SMAD2/SMAD3. Two observations point to a possible function of activins in dorsal midbrain development. First, the neural folds of the MH domain express high levels of active SMAD2 in E8.5-E9.5 mouse embryos (de Sousa Lopes et al., 2003). Second, follistatin a high-affinity activin inhibitor, is expressed in a dynamic pattern in the MH domain (see below). We first examined if the pattern of expression of activins between HH9 and HH18 is consistent with a possible function in dorsal midbrain patterning and RP differentiation. Although activin A expression was not detected above background levels, we found that activin B is expressed in the midbrain-forebrain domain with a progressive anterior shift in its domain of expression. Activin B is first expressed at low level throughout the midbrain vesicle at HH10 (Fig. 7A). Its expression increases and becomes restricted to the mid-forebrain junction at stage HH12-13 (Fig. 7B). By stage HH18, activin B expression becomes confined to the pretectum (Fig. 7C). Overexpression of activin through the insertion of activin A-soaked beads induced a local increase in *Lmx1b* expression 7 and 24 hours later (Fig. 7D,E). Although clear, the induction of *Lmx1b* expression by activin does not compare with its widespread and extremely rapid (less than 3 hours) upregulation by FGF8 (Adams et al., 2000). Two apparently opposite modifications of the RP were observed 2 days after activin bead insertion. In about half of the cases (8/18, Fig. 7F,H,H′), a thin RP bifurcation was induced between the bead and dorsal midline. In 3/18 embryos, the expression of RP markers was destabilized in the caudal midbrain (Fig. 7G). Both phenotypes could be observed in the same embryo (Fig. 7H). At later stages (E7.5, 6 days after bead insertion), most embryos lacked a RP in the posterior midbrain and the locally induced RP could no longer be detected (not shown). Thus, activin could be a major player in midbrain RP lability, because increasing activin signaling positively modulates RP extension but also results in its destabilization.

FGF8 tightly controls the expression of the activin antagonist follistatin

Follistatin binds activins A and B with 1000- and 100-fold higher affinities, respectively, than BMPs (Thompson et al., 2005), and inhibits their binding to activin receptors. At HH11, a small region at the midbrain forebrain junction is delineated by two follistatin domains (Fig. 7I), flanking an intense midline spot of *Gdf7* expression (Fig. 7J). Follistatin expression progresses posteriorly in a decreasing gradient between stages HH11 and HH18 (Fig. 7K). Overexpression of follistatin at HH9-11 did not impair RP differentiation. Affigel Blue beads soaked in follistatin and inserted on the midline slightly widened the *Gdf7* expression domain (8/24; Fig. 7L). This modest effect of follistatin overexpression contrasts with that of noggin beads and indicates that follistatin has little influence on BMP signaling in the midbrain. The anterior high/posterior low gradient of expression of follistatin is suggestive

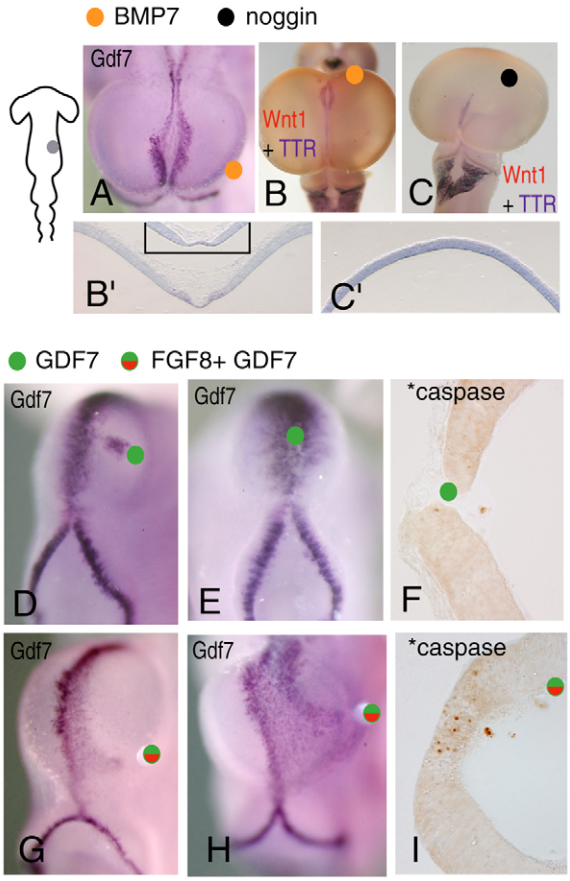

Fig. 6. BMP family members and RP autoinduction. Whole embryos treated for the detection of *Gdf7* transcripts (A,D,E,G,H) or WNT1 (purple) and TTR (black) (B,C) 3 (**A**), 5 (**B**,**B′**,**C**,**C′**) and 1 (**D-I**) day(s) after implantation of beads soaked in BMP7, noggin, GDF7 or a mixture of GDF7+FGF8 recombinant proteins (see color code). Dorsal (A-C) or posterior (D,E,G,H) views. (B′,C′) Transverse sections through B and C, respectively. The inset in B′ shows a comparable section through a control embryo. (F,I) Longitudinal sections through the midbrain of embryos similar to D and G, respectively. The sections were immunostained for active caspase 3 (*caspase). (A,B,B′) BMP7 beads did not induce RP markers locally. They affected RP patterning on the host midline, inducing the formation of a sheet of cells between the two widened *Gdf7* (A) or *Wnt1* (B,B′) positive RP halves. This cell sheet expressed neither the roof-plate marker WNT1 nor the choroid plexus marker TTR (B,B′). (C,C′) Noggin beads induced the formation of a dome-shaped tectum that lacked both WNT1 and TTR expression. (D,E; 13/16) GDF7 beads induced *Gdf7* expression in a cell patch isolated from the midline (D) or through widening the midline *Gdf7* domain (E). Cell death was not markedly increased by GDF7 overexpression: *caspase immunoreactive cells were barely detectable (F, a single immunoreactive cell at most per section in one out of five embryos). Beads releasing GDF7 and FGF8 together induced a widely scattered expression of *Gdf7* (G,H) and impaired the condensation of *Gdf7* expression into the characteristic structure of the FGF8-induced ectopic RP. Cell death was increased near the midline (I) in all cases (7/7, at least three sections containing more than four *caspase immunoreactive cells).

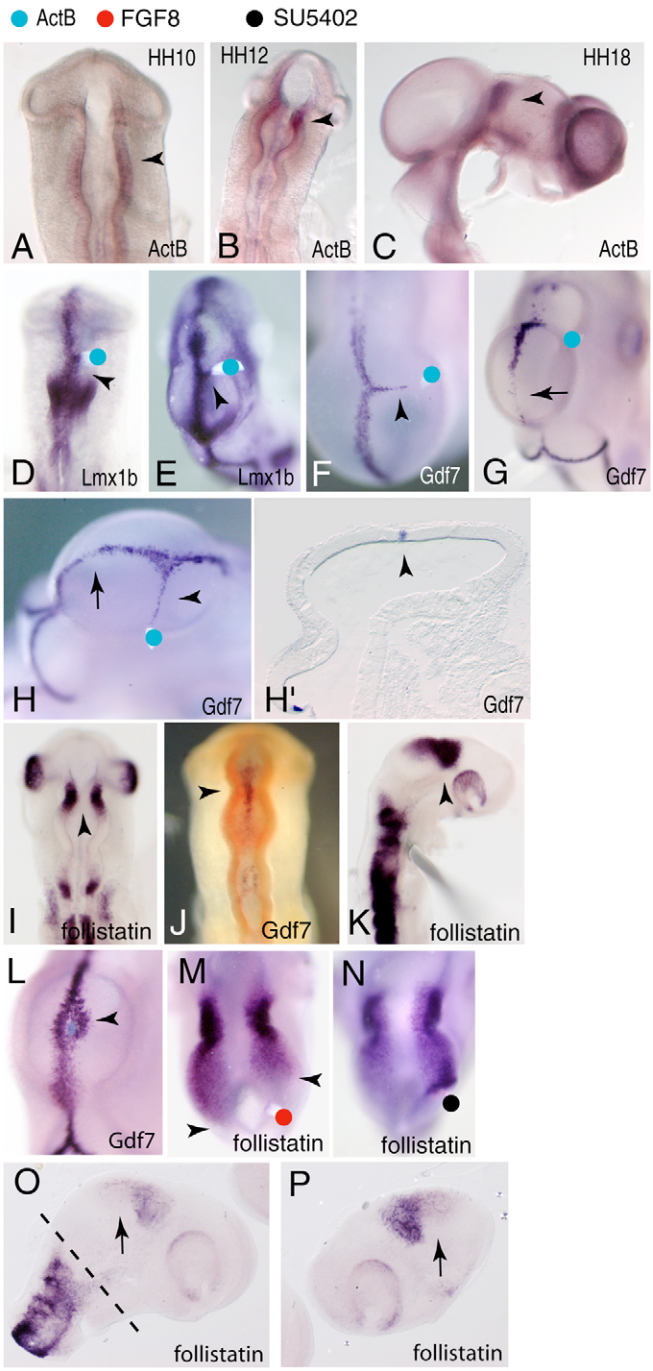

Fig. 7. FGF8 regulates the activity of activin, a potent modulator of RP development. (**A-C**) Dynamic expression of activin B (arrowheads) in the midbrain (A,B) and caudal forebrain (B,C) of HH10, HH12 (A,B, dorsal views) and HH18 (C, lateral view) embryos. (**D-H'**) Overexpression of activin through bead insertion (blue dots) destabilizes the midbrain RP. (D,E) Activin beads induce ectopic LMX1B expression 7 (D) and 23 (E) hours after bead insertion. At 48 hours (F-H'), a thin row of GDF7-expressing cells links the activin bead to the endogenous RP (arrowheads in F,H,H'; H' is a sagittal section through H). Activin also interferes with GDF7 expression in the endogenous caudal midbrain RP (arrows in G,H). (**I-P**) *follistatin* expression is modulated by FGF8 signaling. At stage HH11 (I,J), *follistatin* is expressed in a bilateral domain that straddles the midbrain-forebrain junction and flanks (arrowhead in I) the site of initiation of *Gdf7* expression on the dorsal midline of the anterior midbrain (arrowhead in J). (K) Lateral view of a stage HH14 embryo illustrating the subsequent posterior extension of *follistatin* expression in the midbrain (posterior is leftwards, the arrowhead indicates the midbrain-forebrain junction). In contrast to noggin-soaked beads (Fig. 2A-A''), dorsal follistatin-soaked beads (L, arrowhead) did not impair RP differentiation. Compared with the contralateral control side (arrowheads in M), *follistatin* expression was down- or upregulated, respectively, near beads soaked in FGF8 (red dot in M) or the FGF8 signaling inhibitor SU5402 (black dot in N). (O,P) Stage HH13-14 heads were separated on the midline into two halves. To remove the endogenous source of FGF8, the isthmic region was removed from one half (as indicated by the broken line in O). The control (O) and ablated (P) halves were cultured side by side on floating membranes for 6 hours and treated together for the detection of *follistatin* transcripts. The level of expression of *follistatin* was higher on the ablated side (arrows in O and P).

of a negative modulation by FGF8. In order to test this, FGF8 signaling in the midbrain was modulated in several ways. When FGF8 beads were inserted in the midbrain at stage HH10, the caudal progression of follistatin expression was prevented around the beads (Fig. 7M). At later survival times, follistatin expression was, however, normally turned on caudally beyond the circular range of action of the FGF8 bead (not shown). Downregulation of FGF8 signaling was obtained through the use of SU5402, a FGF signaling inhibitor. Follistatin expression was increased on the side of SU5402-soaked beads inserted in vivo at stage HH10 (Fig. 7N). The downregulation of follistatin expression around the FGF8 beads could reflect the transformation of the midbrain neuroepithelium into cerebellum and isthmus. In order to manipulate FGF8 signaling in older embryos, we used MH explants. The MH region of the neural tube of stage HH14-15 embryos was cut on the dorsal and ventral midlines, the two halves were cultured side by side on floating membranes, one serving as control. Follistatin expression was increased compared with the control side 6 hours after ablation of rhombomere 1, which suppressed the posterior source of FGF8 (Fig. 7O,P). Insertion of beads soaked in SU5402 or DMSO in the posterior midbrain of explants gave similar results as in vivo, although less consistent. Thus, follistatin expression responds rapidly to variations in FGF8 signaling, allowing it to control activin signaling indirectly.

DISCUSSION

We have previously identified (Alexandre and Wassef, 2003) a patterning mechanism depending on the IsO and mimicked by an FGF8 source that locally induces the formation of a short RP segment. However, a posterior trigger for midbrain RP differentiation was not easy to reconcile with the initial anterior expression of markers of RP maturation. In addition, the long-range RP duplications induced by FGF8 beads or IsO grafts or the failure of RP differentiation observed after 180° rotation of the midbrain vesicle remained unexplained. The aim of the present study was to characterize new regulations involved in midbrain RP formation that could relate to its plasticity. We show here that activin dynamically expressed at the midbrain-forebrain junction acts as a potent modulator of RP differentiation.

FGF8 influence on RP differentiation: taking precedence over the BMPs

RP differentiation (Liem et al., 1995; Liem et al., 1997; Furuta et al., 1997; Bach et al., 2003) (present work noggin bead treatment) and the dorsal midline expression of *Lmx1b* (Chizhikov and Millen,

2004b; Liu et al., 2004) generally depend on BMP signals. However, FGF8 controls the expression of genes such as *Lmx1b* and *Wnt1*, which act as competence factors and general markers of the RP. Changes in FGF8 signaling rapidly up- or downregulate *Lmx1b* expression in the caudal midbrain. In chick, we confirmed the observation of Adams et al. (Adams et al., 2000) that FGF8-soaked beads broadly increased *Lmx1b* in less than 3 hours. Conversely, in zebrafish, a 4 hour treatment with SU5402 completely abolished expression of *Lmx1b* in the caudal midbrain including the dorsal midline (O'Hara et al., 2005). Midline expression of *Lmx1b* was not affected in the anterior midbrain. Thus, on the caudal midbrain midline, FGF8 is the major regulator of *Lmx1b* expression which, in addition, is not maintained by BMP signaling. This is consistent with the observation that the level of expression of several ligands and targets of the BMP pathway is transiently downregulated on the caudal midbrain midline (Louvi et al., 2003) (this work). Taken together, these observations indicate that expression of *Lmx1b* in the caudal midbrain is under the control of FGF8, which locally abolishes the neural folds competence for RP differentiation. Thus, similar to the action of ectopic FGF8 beads, endogenous FGF8 may direct the differentiation of the caudal midbrain RP at the center of the field it organizes, independently of the site of early neural tube closure. It is interesting to note that the same behavior, pattern reorganization around the bead and RP duplication, is also induced by FGF8 beads inserted into the forebrain (Crossley et al., 2001). This strengthens the idea that regulatory mechanisms involved in FGF8 patterning may be co-opted for axis formation.

Slowing down RP differentiation or breaking it up

As schematized in Fig. 5H and Fig. 8A, the induction of a wide RP competent domain in the caudal midbrain is counterbalanced by the inhibitory activity of FGF8 on a variety of RP differentiation markers. The sharp progression front of RP maturation illustrated in Fig. 5H and Fig. 8A, however, is an oversimplification. Individual RP markers are downregulated at variable distances from the FGF8 source at the MH junction (Louvi et al., 2003). FGF8 induces a battery of negative regulators (reviewed by Thisse and Thisse, 2005) that could slow down the normal process of differentiation in the RP, as it does in the adjacent neuroepithelium. The complex behavior of the midbrain RP suggests that midbrain-specific targets of FGF8, such as *E1/E2*, which are known to be potent inhibitors, or *Pax2*, which acts as activator or inhibitor depending on cellular context, could preferentially affect specific steps of RP maturation. First expressed independently of FGF8, *Pax2* is later regulated by FGF8 signaling (Crossley et al., 1996) and it is required for *Lmx1b* induction by FGF8 (O'Hara et al., 2005). Pax2 is known to interact directly with LMX1B (Marini et al., 2005), which could explain why induction of *Gdf7* by *Lmx1b* is inhibited in the caudal midbrain.

Extending RP differentiation in the caudal midbrain

In several systems (Sun et al., 2002; Dubrulle et al., 2001; Delfini et al., 2005; Akai et al., 2005; Mathis et al., 2001; Diez del Corral et al., 2002; Delfino-Machin et al., 2005), a local pool of undifferentiated progenitors is maintained by high levels of FGF signaling, while the establishment of the polarity of the neuroepithelium and the progression of cell differentiation are synchronized by the decreasing gradient in FGF signaling (Lee et al., 1997). RP differentiation seems to be linked to the establishment or maintenance of planar cell polarity in the midbrain. Convergent extension movements induced by FGF8 are observed at the midline (Millet et al., 1996; Louvi et al., 2003; Alexandre and Wassef, 2003). They may be important to prevent discontinuities between the *Lmx1b* expression domains controlled by FGF8 and BMPs. Convergent extension also repels the *Gdf7*-expressing cells beyond the influence of high FGF8 signaling, thus rescuing them from death. It is interesting to note that discontinuities in midbrain polarity induced by neuroepithelium rotation are rapidly regulated in the

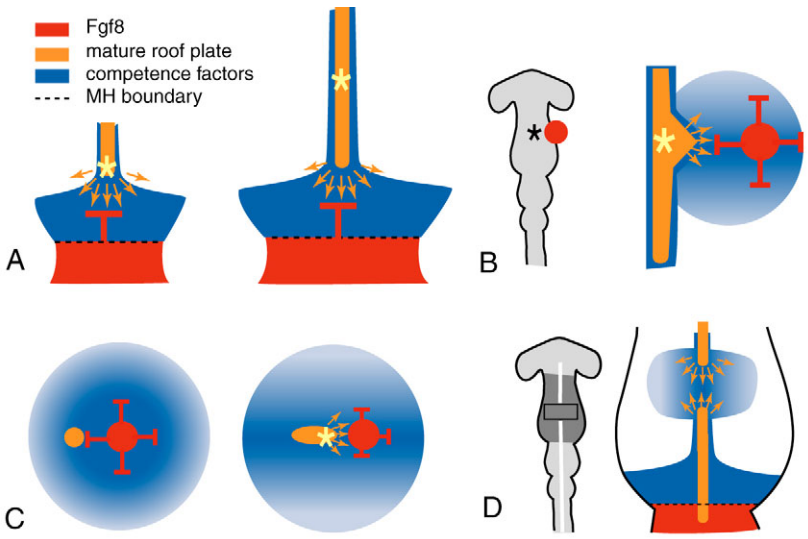

Fig. 8. Summary and interpretation. The left diagrams indicate the initial condition; the right diagrams indicate how it evolves with time. (**A**) In the caudal midbrain, FGF8 maintains expression of *Lmx1b* and *Wnt1* in a wide domain that is therefore competent to differentiate into RP (Chizhikov and Millen, 2004b). However, FGF8 prevents RP maturation by inhibiting the transcription of maturation markers or by inducing cell apoptosis. Conversely, short-range homeogenetic mechanisms (arrows), mediated in part by GDF7, induce RP extension. When the system equilibrates, a RP progress zone forms at short distance from the FGF8 source. Our previous studies (Louvi et al., 2003; Alexandre and Wassef, 2003) indicate that growth, but also convergent extension, may increase the distance between the FGF8 source and the progress zone, leading to RP extension posteriorly. Interestingly, although elsewhere in the neural tube RP differentiation is confined to the competent neural folds, FGF8 releases RP progression from the neural fold environment by inducing a RP competent territory. (**B**) Lateral extension of the RP under the influence of a FGF8-soaked bead depends on similar mechanisms. (**C**) The same interactions could induce a short local RP segment near FGF8 beads. FGF8 signaling rapidly induces high levels of *Lmx1b* and *Wnt1* before decreasing. Because *Gdf7* expression is slowly induced by *Lmx1b* and *Wnt1*, a patch of *Gdf7*-expressing cells may be induced whose extension would follow the FGF8 decreasing gradient or respond to convergent extension. (**D**) We find that changing the polarity of dorsal midbrain fragments results in the maintenance of high levels of *Wnt1* and induction of *Lmx1b* expression in the transplants. The transplant thus becomes competent for RP homeogenetic induction from the adjacent host RP. Long-range DV regulations probably also contribute to RP induction by reorganizing tissue polarity in the transplant and upregulating dorsally the expression of competence factors. The asterisks indicate the sites where homeogenetic RP induction was initiated.

isthmic region (Martinez and Alvarado-Mallart, 1990), but that elsewhere the transplants tend to adopt or maintain the signature of the isthmic midbrain [*Lmx1b* and *Wnt1*, this work; *Pax2* (Vieira et al., 2006)], which is considered to be less differentiated, and thus become competent to develop a RP (see Fig. 8C). However, signals from the host RP, possibly mediated by GDF7, are essential to initiating the formation of an ectopic RP.

Follistatin and activin function in the dorsal midbrain

Follistatin is often considered to be an inhibitor of BMP signaling, but it plays distinct roles, depending on the context, and interacts with members of several groups of TGFβ family ligands, including BMPs, GDFs and activins. Interestingly, we find that BMPs, GDF and activin ligands all play distinct roles in midbrain RP development. BMPs are involved as RP competence factors, GDF7 in auto-activation of RP differentiation, and activin B in RP expansion and stabilization. Muscle development is controlled by BMP7 and myostatin, both of which are modulated by follistatin, but in different ways. Follistatin binds BMP7 reversibly with low affinity. It converts the muscle growth-inhibiting effect of BMP7 into a strong stimulatory one that is blocked by noggin. Therefore, as follistatin does not prevent BMP7 binding to its receptor (Iemura et al., 1998), it has been suggested that follistatin influences BMP7 binding to its receptors. Follistatin binding to myostatin/GDF8 seems to completely prevent receptor activation (Amthor et al., 2002; Amthor et al., 2004). Because follistatin binds TGFβ ligands with distinct affinities and differentially affects interaction with their receptors, its progressive expansion across the midbrain may be important to modulate their function. Follistatin binds to activin with a much higher affinity than to other TGFβ ligands. The dynamic expression patterns of follistatin and activin suggest that they tightly regulate each other's availability in the midbrain. It remains unclear if the destabilization of the posterior midbrain RP that we observe after insertion of activin beads relates to an increase in activin signaling or whether sequestration by activin impairs other functions of follistatin.

Conclusion

Similar mechanisms are involved in RP differentiation in the anterior midbrain and in the spinal cord. FGF8 signaling prevents their normal deployment in the caudal midbrain through its modulation of the function of several members of the TGFβ superfamily. The need for a progressive transition between these two modes of regulation may increase the vulnerability of RP differentiation to experimental manipulation.

We thank L. Bally-Cuif for critical reading of the manuscript, Boris Barbour for checking the English, Rosette Goiame for technical help and many colleagues for providing plasmids or antibodies. The QCPN monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank. This work was supported by ARC and ACI research grants to M.W. P.A. was supported by fellowships from FCT, Fondation des Treilles and FRM, and I.B. was supported by ARC.

References

Adams, K. A., Maida, J. M., Golden, J. A. and Riddle, R. D. (2000). The transcription factor Lmx1b maintains *Wnt1* expression within the isthmic organizer. *Development* **127**, 1857-1867.

Akai, J., Halley, P. A. and Storey, K. G. (2005). FGF-dependent Notch signaling maintains the spinal cord stem zone. *Genes Dev.* **19**, 2877-2887.

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.

Amoyel, M., Cheng, Y. C., Jiang, Y. J. and Wilkinson, D. G. (2005). Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain. *Development* **132**, 775-785.

Amthor, H., Christ, B., Rashid-Doubell, F., Kemp, C. F., Lang, E. and Patel, K. (2002). Follistatin regulates bone morphogenetic protein-7 (BMP-7) activity to stimulate embryonic muscle growth. *Dev. Biol.* **243**, 115-127.

Amthor, H., Nicholas, G., McKinnell, I., Kemp, C. F., Sharma, M., Kambadur, R. and Patel, K. (2004). Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Dev. Biol.* **270**, 19-30.

Bach, A., Lallemand, Y., Nicola, M. A., Ramos, C., Mathis, L., Maufras, M. and Robert, B. (2003). Msx1 is required for dorsal diencephalon patterning. *Development* **130**, 4025-4036.

Bally-Cuif, L. and Wassef, M. (1994). Ectopic induction and reorganization of Wnt-1 expression in quail/chick chimeras. *Development* **120**, 3379-3394.

Chizhikov, V. V. and Millen, K. J. (2004a). Mechanisms of roof plate formation in the vertebrate CNS. *Nat. Rev. Neurosci.* **5**, 808-812.

Chizhikov, V. V. and Millen, K. J. (2004b). Control of roof plate development and signaling by Lmx1b in the caudal vertebrate CNS. *J. Neurosci.* **24**, 5694-5703.

Chizhikov, V. V. and Millen, K. J. (2004c). Control of roof plate formation by Lmx1a in the developing spinal cord. *Development* **131**, 2693-2705.

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

Crossley, P. H., Martinez, S., Ohkubo, Y. and Rubenstein, J. L. (2001). Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. *Neuroscience* **108**, 183-206.

de Diego, I., Kyriakopoulou, K., Karagogeos, D. and Wassef, M. (2002). Multiple influences on the migration of precerebellar neurons in the caudal medulla. *Development* **129**, 297-306.

Delfini, M. C., Dubrulle, J., Malapert, P., Chal, J. and Pourquie, O. (2005). Control of the segmentation process by graded MAPK/ERK activation in the chick embryo. *Proc. Natl. Acad. Sci. USA* **102**, 11343-11348.

Delfino-Machin, M., Lunn, J. S., Breitkreuz, D. N., Akai, J. and Storey, K. G. (2005). Specification and maintenance of the spinal cord stem zone. *Development* **132**, 4273-4283.

de Sousa Lopes, S. M., Carvalho, R. L., van den Driesche, S., Goumans, M. J., ten Dijke, P. and Mummery, C. L. (2003). Distribution of phosphorylated Smad2 identifies target tissues of TGF beta ligands in mouse development. *Gene Expr. Patterns* **3**, 355-356.

Diez del Corral, R., Breitkreuz, D. N. and Storey, K. G. (2002). Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. *Development* **129**, 1681-1691.

Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001). FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-232.

Funahashi, J.-I., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H. (1999). Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. *Dev. Growth Differ.* **41**, 59-72.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124**, 2203-2212.

Graham, A. and Lumsden, A. (1996). Interactions between rhombomeres modulate Krox-20 and follistatin expression in the chick embryo hindbrain. *Development* **122**, 473-480.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquie, O. (1997). Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124**, 4605-4614.

Iemura, S.-I., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9337-9342.

Kee, Y. and Bronner-Fraser, M. (2001). The transcriptional regulator Id3 is expressed in cranial sensory placodes during early avian embryonic development. *Mech. Dev.* **109**, 337-340.

Keller, P., Toomre, D., Diaz, E., White, J. and Simons, K. (2001). Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat. Cell Biol.* **3**, 140-149.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev.* **12**, 3394-3407.

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.

Liem, K. F., Jr, Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Liem, K. F., Jr, Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Liem, K. F., Jr, Jessell, T. M. and Briscoe, J. (2000). Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127**, 4855-4866.
Liu, Y., Helms, A. W. and Johnson, J. E. (2004). Distinct activities of Msx1 and Msx3 in dorsal neural tube development. *Development* **131**, 1017-1028.
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.
Marin, F. and Puelles, L. (1994). Patterning of the embryonic avian midbrain after experimental inversions: a polarizing activity from the isthmus. *Dev. Biol.* **163**, 19-37.
Marini, M., Giacopelli, F., Seri, M. and Ravazzolo, R. (2005). Interaction of the LMX1B and PAX2 gene products suggests possible molecular basis of differential phenotypes in Nail-Patella syndrome. *Eur. J. Hum. Genet.* **13**, 789-792.
Martinez, S. and Alvarado-Mallart, R. M. (1990). Expression of the homeobox Chick-en gene in chick/quail chimeras with inverted mes-metencephalic grafts. *Dev. Biol.* **139**, 432-436.
Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. R. 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.
Mathis, L., Kulesa, P. M. and Fraser, S. E. (2001). FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nat. Cell Biol.* **3**, 559-566.
Matsunaga, E., Katahira, T. and Nakamura, H. (2002). Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development. *Development* **129**, 5269-5277.
Merino, R., Macias, D., Ganan, Y., Rodriguez-Leon, J., Economides, A. N., Rodriguez-Esteban, C., Izpisua-Belmonte, J. C. and Hurle, J. M. (1999). Control of digit formation by activin signalling. *Development* **126**, 2161-2170.
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.
O'Hara, F. P., Beck, E., Barr, L. K., Wong, L. L., Kessler, D. S. and Riddle, R. D. (2005). Zebrafish Lmx1b.1 and Lmx1b.2 are required for maintenance of the isthmic organizer. *Development* **132**, 3163-3173.
Shimamura, K., Hirano, S., McMahon, A. P. and Takeichi, M. (1994). Wnt-1-dependent regulation of local E-cadherin and alpha N-catenin expression in the embryonic mouse brain. *Development* **120**, 2225-2234.
Sun, X., Mariani, F. V. and Martin, G. R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* **418**, 501-508.
Thisse, B. and Thisse, C. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev. Biol.* **287**, 390-402.
Thompson, T. B., Lerch, T. F., Cook, R. W., Woodruff, T. K. and Jardetzky, T. S. (2005). The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. *Dev. Cell* **9**, 535-543.
Vieira, C., Garcia-Lopez, R. and Martinez, S. (2006). Positional regulation of Pax2 expression pattern in mesencephalic and diencephalic alar plate. *Neuroscience* **137**, 7-11.