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FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on *Otx2* expression

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SUMMARY

Beads containing recombinant FGF8 (FGF8-beads) were implanted in the prospective caudal diencephalon or midbrain of chick embryos at stages 9-12. This induced the neuroepithelium rostral and caudal to the FGF8-bead to form two ectopic, mirror-image midbrains. Furthermore, cells in direct contact with the bead formed an outgrowth that protruded laterally from the neural tube. Tissue within such lateral outgrowths developed proximally into isthmic nuclei and distally into a cerebellum-like structure. These morphogenetic effects were apparently due to FGF8-mediated changes in gene expression in the vicinity of the bead, including a repressive effect on *Otx2* and an inductive effect on *En1*, *Fgf8* and *Wnt1* expression. The ectopic *Fgf8* and *Wnt1* expression domains formed nearly complete concentric rings around the FGF8-bead, with the *Wnt1* ring outermost. These observations suggest that FGF8 induces the formation of a ring-like ectopic signaling center (organizer) in the lateral wall of the brain, similar to the one that normally encircles the neural tube at the isthmic constriction, which is located at the boundary between the prospective midbrain and hindbrain. This ectopic isthmic organizer apparently sends long-range patterning signals both rostrally and caudally, resulting in the development of the two ectopic midbrains. Interestingly, our data suggest that these inductive signals spread readily in a caudal direction, but are inhibited from spreading rostrally across diencephalic neuromere boundaries. These results provide insights into the mechanism by which FGF8 induces an ectopic organizer and suggest that a negative feedback loop between *Fgf8* and *Otx2* plays a key role in patterning the midbrain and anterior hindbrain.

Key words: Brain patterning, Cerebellum, *En1*, *Fgf8*, Isthmic organizer, Midbrain, Mid/hindbrain organizer, Neuromere boundaries, *Otx2*, *Wnt1*

INTRODUCTION

Regional specification processes partition the neural plate into its principal transverse and longitudinal subdivisions. Anteroposterior (A/P) patterning leads to the establishment of the forebrain, midbrain, cerebellum, hindbrain and spinal cord (reviewed by Lumsden and Krumlauf, 1996; Rubenstein and Beachy, 1998). Early patterning of the prospective midbrain and anterior hindbrain (cerebellum; Cb) can be divided into two phases. During the first phase, the position of this region within the anterior neural plate is determined. This process occurs during gastrulation and early neural plate stages of development and is mediated by signals from the anterior visceral endoderm (reviewed by Beddington and Robertson, 1998) and mesendoderm (Ang and Rossant, 1993; Ang et al., 1994), which establish gene expression domains within the neural plate. Several of the genes that are expressed at early neural plate stages and have been shown by genetic analysis to be required for normal development of the region, encode homeodomain transcription factors, including OTX1 AND OTX2 (see Acampora et al., 1997; Suda et al., 1997; references therein), GBX2 (Wassarman et al., 1997), PAX2 (Favor et al., 1996; Lun and Brand, 1998) and EN1 (Wurst et al., 1994). During the second phase, which begins at early neural plate stages, the patterning process apparently becomes independent of exogenous influences as a potent embryonic ‘organizer’ is formed within the neuroepithelium. Once established, this organizer is co-localized with a morphological feature of the developing neural tube known as the isthmic constriction at the midbrain/hindbrain (m/h) boundary. Because of this association, it has been termed the m/h or isthmic organizer (IsO).

During normal development, patterning signals emanating

from the IsO are thought to control the polarized development of the entire region encompassing the prospective midbrain, isthmus and cerebellum (reviewed by Joyner, 1996; Puelles et al., 1996; Wassef and Joyner, 1997). However, tissue grafting experiments in the chick have shown that territories both posterior and anterior to this region are competent to respond to signals from the IsO (reviewed by Puelles et al., 1996; Wassef and Joyner, 1997). Thus all hindbrain rhombomeres respond in their alar plate to IsO signals (Martinez et al., 1995). Within the forebrain, the competence to respond to IsO activity is restricted to tissue located caudal of the zona limitans intrathalamica (ZL) (Martinez et al., 1991; Bloch-Gallego et al., 1996), a diencephalic region that gives rise to the epithalamus, dorsal thalamus (DT) and pretectum (PT). Although they respond similarly to IsO signals, it is thought that the regions fated to give rise to the DT and the PT correspond to dorsal regions of independent developmental units (neuromeres) called prosomere 2 (p2) and prosomere 1 (p1), respectively. The prosomeres are postulated to be separated from one another and from the prospective midbrain by specialized boundaries (reviewed by Puelles and Rubenstein, 1993; Rubenstein et al., 1994).

Genetic and experimental studies have identified two secreted signaling molecules that function as mediators of IsO activity: WNT1, encoded by a vertebrate homolog of the *Drosophila wingless* gene (Wilkinson et al., 1987; Nusse and Varmus, 1992) and FGF8, a member of the Fibroblast Growth Factor family (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995). At early stages of brain development (~8-15 somites in the mouse and chick) both *Wnt1* and *Fgf8* are expressed in relatively broad, overlapping domains: *Wnt1* is expressed throughout the region that will become the midbrain and *Fgf8* is expressed throughout the region that will form the isthmus and Cb (isthmocerebellum). These gene expression domains subsequently become restricted to transverse rings that encircle the neural tube in the vicinity of the isthmic constriction at the m/h boundary. The ring of *Wnt1*-expressing cells is localized at the caudal end of the prospective midbrain, adjacent and rostral to the ring of *Fgf8*-expressing cells, which are localized in the prospective isthmocerebellum (McMahon et al., 1992; Bally-Cuif and Wassef, 1994; Crossley and Martin, 1995; Mahmood et al., 1995). Gene inactivation studies have indicated that *Wnt1* function is required for development of both the midbrain and isthmocerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992). Further studies have suggested that its major function is to maintain expression of *En1* and *En2* (Danielian and McMahon, 1996). However, it may also play a role in stimulating cell proliferation (Dickinson et al., 1994). Likewise, genetic studies in mice and zebrafish have demonstrated that reduced *Fgf8* expression disrupts development of the m/h region (Meyers et al., 1998; Reifers et al., 1998). Ectopic expression studies in the mouse have suggested that one function of FGF8 produced by the IsO is to stimulate cell proliferation and perhaps also to regulate the rate of neural differentiation from dividing precursor cells (Lee et al., 1997).

In a previous study (Crossley et al., 1996a), we demonstrated that implanting a bead containing FGF8 protein (FGF8-bead) in p2 of the embryonic chick brain induced nearby cells to express *En2*, *Fgf8* and *Wnt1*, thereby forming an ectopic IsO. Like grafts of normal isthmic organizer cells placed in the caudal diencephalon, this ectopic organizer apparently produced signals that induced cells in p2, as well as those more caudal to it in p1, to form a supernumerary midbrain and isthmic tissue, present in opposite orientation to the normal midbrain and isthmus. Cells in the rostral diencephalon (rostral to the ZL) were not affected. Thus signals traveling caudally through the neuroepithelium transformed cells from a diencephalic to a more posterior fate. In the study described here, we investigated the effects of implanting FGF8-beads more caudally, in p1 and the prospective midbrain. The results of this study provide insight into the role of FGF8 in normal IsO function and the mechanism by which the IsO functions to pattern the developing brain.

MATERIALS AND METHODS

Experimental manipulations

Chick embryos were staged according to the method of Hamburger and Hamilton (1951). Bead implantation experiments were performed as previously described (Crossley et al., 1996a) using beads containing recombinant FGF8 protein (variant 1, [Crossley and Martin, 1995], also known as isoform B [MacArthur et al., 1995]). FGF8-beads were prepared by soaking heparin acrylic beads (Sigma, St. Louis, MO) in a solution containing FGF8 protein obtained from Dr C. MacArthur (Washington University, St Louis, MO; ~0.8 mg/ml) or from R&D Systems (Minneapolis, MN; ~0.15 mg/ml) for 30 minutes to 4 hours at room temperature or overnight at 4°C, and washing in phosphate-buffered saline (PBS). Control beads were similarly prepared, but soaked in PBS only (PBS-beads). DiI-labeled FGF8-beads were prepared by soaking the beads for 5 minutes in a saturated solution of DiI (Molecular Probes, Eugene, OR) in ethanol at room temperature, washing them in PBS and then soaking them for 30 minutes in FGF8 (R&D Systems, 1 mg/ml). Control beads were prepared in the same way, but soaked in DiI solution only and then washed in PBS. All beads were used immediately after the PBS wash.

Histological and in situ hybridization analyses

Embryos were collected at the stages indicated and the brain was either dissected from the head or left in situ. Samples for histological analysis were fixed in Clarke's solution (75% EtOH, 25% glacial acetic acid), embedded in wax, sectioned at 10 μm and mounted in three series. The first series (which included the 1st, 4th, 7th etc. section) was stained with Cressyl violet; the second (which included the 2nd, 5th, 8th etc. section) and the third (which included the 3rd, 6th, 9th etc. section) were processed for immunohistochemistry using anti-Calretinin or anti-Calbindin polyclonal antibodies (Swant Swiss Antibodies, Bellinzona, Switzerland), respectively. Immunoreactivity was detected using the ABC-Elite system and biotinylated secondary antibodies purchased from Vector Labs (Burlingame, CA) according to the manufacturer's instructions.

Samples for whole-mount RNA in situ hybridization were fixed in 4% paraformaldehyde at 4°C, and then processed essentially as described by Henrique et al. (1995). Antisense riboprobes for in situ hybridization were prepared using previously published chick sequences: *En1* (Logan et al., 1992), *Fgf8* (Crossley et al., 1996b), *Wnt1* (Bally-Cuif and Wassef, 1994) and *Otx2* (Bally-Cuif et al., 1995b).

RESULTS

FGF8 induces cerebellar as well as isthmus and midbrain development

An FGF8-bead was implanted into the neural tube of chick embryos at stages 9-12 (7-17 somites). The bead was lodged in an incision made at one of three positions along the A/P axis: 'a', in the mesencephalon (mes; prospective midbrain), 'b', in the vicinity of the boundary between the mes and p1 (the prospective PT), and 'c', most likely within p1 (but possibly

within p2, the prospective DT) (see Fig. 1A). A total of 42 embryos incubated for up to 14 days after bead implantation (i.e. to stages 34-42) were examined histologically. There were no obvious differences in the types of tissues formed in response to FGF8-beads implanted at different positions. In 8/42 embryos (19%), the FGF8-bead had no effect. In 12/42 embryos (29%), the effect was similar to what was previously observed when an FGF8-bead was implanted in p2: tissue surrounding and caudal to the bead was transformed into an ectopic midbrain and isthmic tissue in mirror-image orientation to the normal midbrain and isthmus (data not shown; see Crossley et al., 1996a).

In 19/42 embryos (45%), the FGF8-bead had a more dramatic effect. Upon external examination the brain of most of these embryos was found to contain two large abnormal vesicles situated between the midbrain and the telencephalon (see Fig. 1B-E). An abnormal small vesicle was also present between the two larger ones (arrow in Fig. 1D). Histological analysis of sagittal sections of these 19 brains elucidated the nature of the morphogenetic changes caused by the FGF8-bead (Fig. 1F and data not shown). In the normal embryo, the dorsal wall (optic tectum, Tc) of the midbrain displays a distinct rostrocaudal gradient of cytoarchitectonic maturation, with rostral regions containing superficial layers that have not yet formed more caudally (Fig. 1G; LaVail and Cowan, 1971). In 18/19 cases, the Tc was present and morphologically normal except at its rostral end, where it was smoothly joined to a mirror-image supernumerary Tc (marked by a single asterisk in Fig. 1). Rostral to this abnormal Tc (hereafter referred to as Mb*), we observed a second supernumerary Tc in the same orientation as the normal one (marked by two asterisks in Fig. 1; hereafter referred to as Mb**). Thus, the morphologically caudal ends of Mb* and Mb** were present in close proximity and mirror-image orientation to one another (Fig. 1F). In the remaining 1/19 cases (bead implanted in the mes), it appeared that an Mb* and Mb** were present, but development of the normal midbrain was almost completely inhibited (data not shown).

In addition to the morphogenetic effects on the alar plate described above, we detected effects on the basal plate in 10 of these 19 brains. Ventrally, in the region where the morphologically caudal ends of Mb* and Mb** were located, we observed pairs of nuclei that resembled, in shape and topological relationship to one another, the oculomotor nuclei (IIIn) normally located at the caudal end of the ventral midbrain (tegmentum) (Fig. 1H). Interestingly, strictly sagittal sections through the region containing these ectopic nuclei (Fig. 1H) look remarkably similar to transverse sections through the caudal region of the normal tegmentum (Fig. 1I). In transverse sections of the normal caudal midbrain, IIIn flank the floor plate (ventral midline), which at this stage consists of Calretinin-producing radial glia (see Fig. 1K). The similarities between sagittal sections of the experimental brains and transverse sections of the normal caudal tegmentum were further demonstrated by Calretinin immunohistochemistry. In the experimental brains, Calretinin-producing cells were detected in the region between the ectopic nuclei, as well as in the regions ventral to them (Fig. 1J). In the normal brain, Calretinin is detected in the floor plate and the ventral tegmental area (VTA) (Fig. 1K). These data suggest that, in addition to inducing two ectopic Tc, FGF8 can induce floor and basal plate-like midbrain structures, which are oriented perpendicular to the normal ventral tissues. Analysis of serial sagittal sections of the experimental samples confirmed that the FGF8-induced floor plate-like structure extended laterally from the normal ventral CNS (shown diagramatically in Fig. 1O).

In 14/19 (74%) of experimental brains with Mb* and Mb**, we found that more lateral sections contained structures resembling isthmic nuclei, including the nucleus semilunaris, the nucleus parvocellularis and the nucleus isthmo-opticus, whose axons project to the isthmo-optic tract (Fig. 1L-N, and data not shown), as well as a distinctive structure that we identified as cerebellum-like (the evidence for this is described below). From a serial section analysis, it was apparent that the ectopic isthmic nuclei were found near the proximal end of the small vesicle that protrudes laterally between the two ectopic midbrains (see Fig. 1D), in the region where this vesicle joined the wall of the brain. In contrast, the cerebellum-like structure apparently extended from the proximal part of the lateral outgrowth to its distal tip. In parasagittal sections, which cut transversely through the lateral outgrowth, this cerebellum-like structure was found to be symmetrical around a central cavity (Figs 1L,M, 2A). The topographic relationship of the ectopic Tc, isthmic and cerebellar tissue, and floor and basal plate-like midbrain structures to the normal structures in the brain is summarized in Fig. 1O.

The conclusion that the distinctive structure in the lateral outgrowths was cerebellum-like was based on the results of a morphological and immunohistochemical analysis. At stage 42, the most advanced stage examined, this structure did not display the characteristic foliated morphology of the normal cerebellum (Fig. 2A,B), perhaps because of its relatively small size. However, numerous mitotic figures were detected in the superficial cell layers (data not shown), a characteristic of the external granular layer (EGL) of the normal cerebellum but not of any other superficial cell layers in the developing avian CNS. Underlying the densely packed superficial layers of darkly stained small cells was a region of relatively sparse cells with large cell bodies (Fig. 2C), resembling the Purkinje cell layer of the normal cerebellum. These cells stained with an antibody against Calbindin (CaBP) (Fig. 2D), which in the normal cerebellum specifically stains the Purkinje cell layer (Fig. 2E). The topological arrangement of the CaBP-positive cells in the induced cerebellum-like structure was very similar to that of Purkinje cells in the normal cerebellum, an arrangement not observed in any other region of the brain. Furthermore, the CaBP-producing cells in the ectopic cerebellum-like structure had axons that projected to structures resembling deep cerebellar nuclei (Fig. 2D), just as Purkinje cells project to deep cerebellar nuclei in the normal anterior cerebellum (Fig. 2E). From this analysis, we conclude that FGF8-beads can induce the formation of ectopic cerebellar tissue.

The remaining 3/42 experimental brains (14%) were clearly different from the others in that they contained an apparently normal midbrain, but had an isthmocerebellar lateral outgrowth located between the caudal end of the midbrain and the normal isthmus. In all of these embryos, the FGF8-bead was implanted in the mes. We speculate that it induced the formation of an ectopic IsO just rostral to the normal IsO, which caused the formation of the lateral outgrowth.

The non-operated (contralateral) side of the brain appeared grossly normal in all but one embryo. In that case, only the dorsal area of the PT was abnormal (data not shown). Control experiments were performed in which PBS-beads were implanted in incisions at positions ‘a’ (n=7), ‘b’ (n=1) or ‘c’ (n=10), and the embryos were incubated for ~8 days. In all but

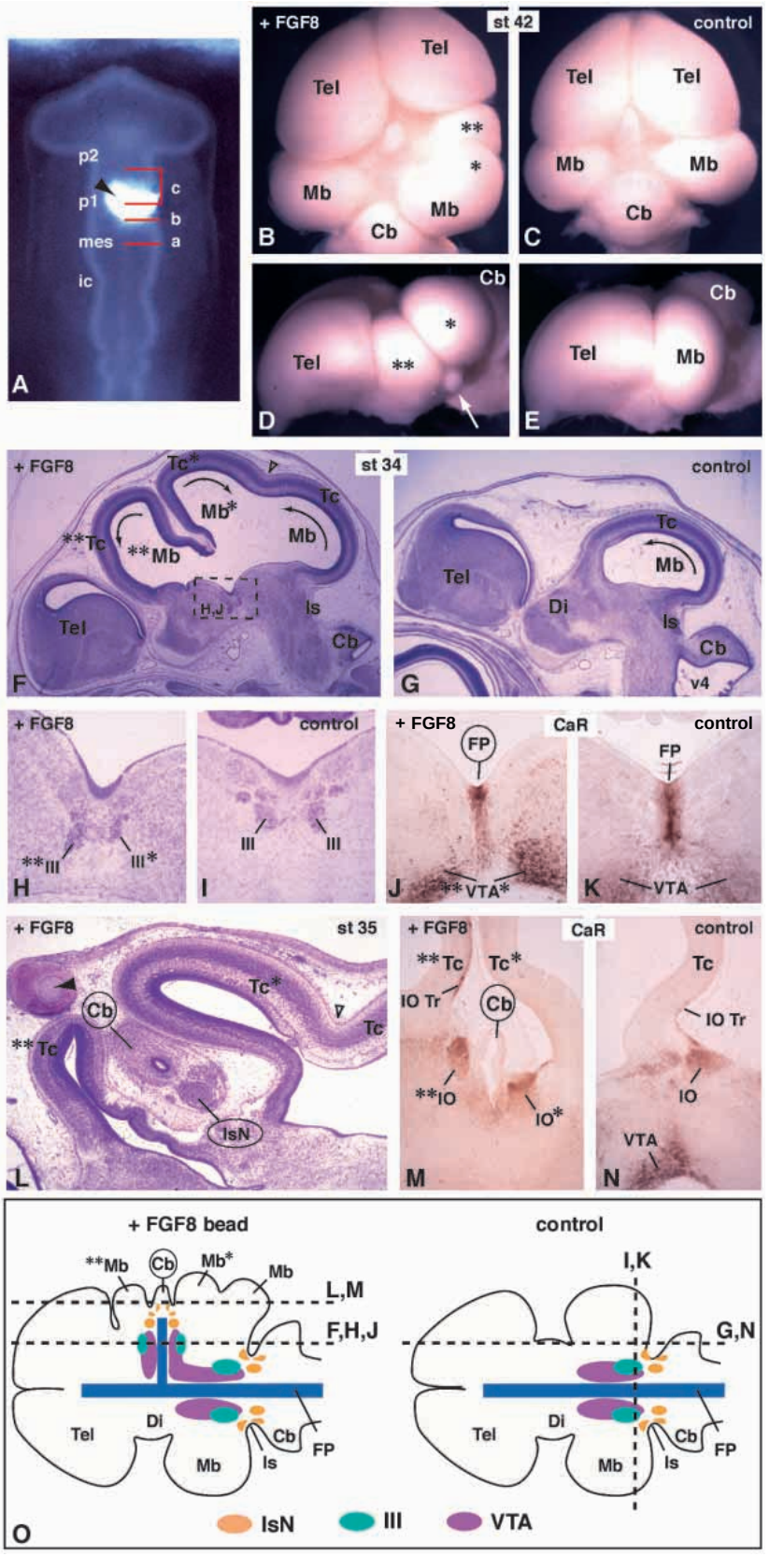

Fig. 1. Morphogenetic changes induced by implanting an FGF8-bead. (A) Dorsal view of a stage 10 chick embryo illustrating the positions at which incisions through the neural tube were made for implantation of an FGF8-bead: position 'a', in the middle of the mes; position 'b', in the vicinity of the p1/mes boundary; position 'c', in the region between the middle of p1 and the p1/p2 boundary. In the embryo shown, an FGF8-bead (arrowhead) was inserted into an incision made at position 'b'. (B-E) External appearance of the brain from stage 42 experimental and control (non-operated) embryos. (B,C) Dorsal and (D,E) lateral views of the brains from (B,D) an embryo in which the FGF8-bead was implanted at stage 11^+ in an incision at position 'b' on the right side of the brain, and (C,E) a control embryo. Asterisks indicate the large ectopic vesicles detected between the midbrain (Mb) and the telencephalon (Tel). The arrow in D points to an abnormal lateral outgrowth (small vesicle) between the two larger ectopic vesicles. Note that in all panels showing lateral views or sagittal sections of the brain, anterior is to the left. In order to conform with this convention photographs taken of the right side of an intact brain were printed in reverse orientation (as for example in D and E).
(F-N) Histological analysis of the experimental brains. Paramedial sagittal sections of the brain from: (F) an embryo in which an FGF8-bead was implanted at stage 11^+ in an incision at position 'b'; (G) a control embryo at stage 34. Note the rostrocaudal gradient of cytoarchitectonic maturation in the optic tectum (Tc) of the normal midbrain (indicated by a curved arrow that points rostrally) and the presence in the experimental brain of two ectopic tecta in opposite orientations. The dashed box in F indicates the midbrain basal plate region shown at higher magnification in H and J. (H,J) Sagittal sections of the ventral region of the experimental brain shown in F; (I,K) transverse sections through the caudal end of a normal midbrain at stage 35 (H,I) Nissl stain.
(J,K) Immunohistochemical analysis using an anti-Calretinin (CaR) antibody. CaR is detected in floor plate (FP) cells in the normal brain and in a cell population similar in shape and location in the experimental brain.
(L) Lateral sagittal section of the brain from an experimental embryo incubated to stage 35 in which an FGF8-bead was implanted at stage 11^- in an incision at position 'c'. Note the presence of an ectopic cerebellum-like structure (Cb) and an isthmic nucleus (IsN), which resembles the nucleus semilunaris. The filled arrowhead points to the FGF8-bead, which at this stage is lodged in the mesenchyme overlying the region between the two ectopic tecta. (M,N) Immunohistochemical analysis using an anti-CaR antibody of lateral sagittal sections through: (M) the caudal ends of the two ectopic midbrains in a stage 35 brain in which an FGF8-bead was implanted at stage 10^- in an incision at position 'b'; (N) the caudal end of a normal midbrain at stage 35. Note the CaR-positive cells of the nucleus isthmo-opticus (IO), whose axons project to the isthmo-optic tract (IO Tr) in the normal brain, and similar ectopic nuclei in the experimental brain.
(O) Schematic diagrams representing ventral views of (left) a stage 42 brain in which an FGF8-bead was implanted, and (right) a control brain. The experimental brain has two ectopic midbrains and a lateral outgrowth containing an ectopic cerebellum-like structure. The horizontal dashed lines indicate the plane of section shown in the panels indicated; the vertical dashed line shows the plane of section through the control brain in panels I, K. In all panels, when ectopic structures are present in duplicate, a single asterisk (*) designates the one located more caudally, a double asterisk (**) designates the one located more rostrally. When a single ectopic structure is present, the abbreviation designating it is circled. The open arrowheads in F and L indicate the region in which the rostral end of the normal tectum (Tc) is joined to the morphologically rostral end of an ectopic tectum (Tc*). Additional abbreviations: Di, diencephalon; ic, isthmic constriction; Is, isthmus; v4, fourth ventricle; VTA, ventral tegmental area.

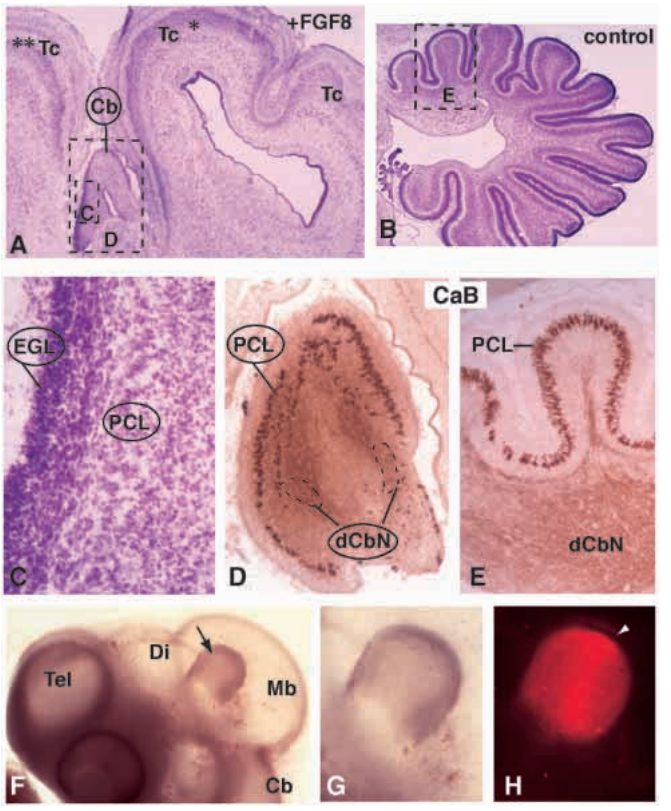

Fig. 2. Cerebellar differentiation induced by an FGF8-bead. (A-E) Histological comparison of an FGF8-induced cerebellum-like structure with the cerebellum of a control (non-operated) embryo. (A) Lateral sagittal section through the brain of a stage 42 embryo in which an FGF8-bead was implanted in a stage 10^{-} embryo in an incision at position 'c'. The dashed boxes indicate the regions shown at higher magnification in C and D, respectively. (B) Medial sagittal section through the normal cerebellum at stage 42. The dashed box indicates the region shown at higher magnification in E. (C) Higher magnification view of the ectopic cerebellum-like structure shown in A. Note the superficial cells and the underlying cell layer, which resemble the external granular layer (EGL) and the Purkinje cell layer (PCL) of the normal cerebellum, respectively. (D,E) Immunohistochemical analysis with an anti-Calbindin (CaB) antibody of a section through (D) the ectopic cerebellum-like structure shown in A, and (E) the normal stage 42 cerebellum shown in B. The Purkinje cells, marked by high levels of CaB, have axonal projections to the ventrally located deep cerebellar nuclei (dCbN). (F-H). Analysis of the relationship between outgrowth and contact with the FGF8-bead. (F) Lateral view of the brain of an embryo in which a DiI-labelled FGF8-bead was implanted at stage 10 in an incision at position 'c', and which was then incubated for 3 days. The arrow points to the lateral outgrowth formed in response to the inductive signal from the bead. (G,H) Higher magnification views of the lateral outgrowth viewed with (G) bright-field or (H) fluorescence illumination. The arrowhead in H points to the position at which the bead, which was removed during dissection, was found in the mesenchyme surrounding the outgrowth. Note the relatively sharp boundary between the DiI-positive and -negative cells, which is localized at the region where the base of the lateral outgrowth joins the wall of the brain. Abbreviations and symbols as in Fig. 1.

two cases, the brain appeared unaffected. In the two affected cases, there were malformations but no ectopic structures (data not shown).

The data on all 42 experimental brains examined histologically are summarized in Table 1. Together these results indicate that, in addition to inducing the development of midbrain and isthmic nuclei, as previously reported (Crossley et al., 1996a), FGF8 can also induce cerebellar

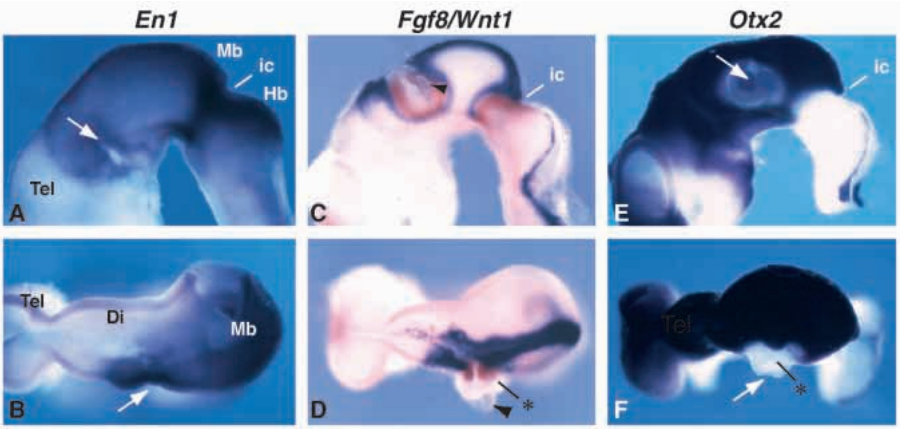

Fig. 3. Changes in gene expression induced by implanting an FGF8-bead. Gene expression was assayed by whole-mount RNA in situ hybridization of isolated experimental brains. During dissection of the head the FGF8-bead was sometimes removed with the mesenchyme. (A,B) Induction of *En1* expression. *En1* RNA was detected ~24 hours after an FGF8-bead was implanted (at stage 12, incision at position 'b'). Arrow points to the site where the FGF8-bead was located. (A) Lateral view of the operated side of the experimental brain. Note the normal domain of *En1* expression with highest levels of RNA in the vicinity of the isthmic constriction (ic) at the boundary between the developing midbrain (Mb) and hindbrain (Hb). (B) Dorsal view of the same brain. Note the absence of *En1* RNA in the diencephalon (Di) on the non-operated, contralateral side of the brain (top), and its abundance in tissue surrounding the site at which the FGF8-bead was located. (C,D) Ectopic expression of *Wnt1* and *Fgf8*. *Fgf8* (red stain) and *Wnt1* (blue stain) RNAs were detected ~48 hours after an FGF8-bead was implanted (at stage 10^{-}, incision at position 'b'). Arrowhead points to the FGF8-bead, which is still lodged in the neuroepithelium. (C) Lateral view of the operated side of the experimental brain. Note the normal domains of *Wnt1* and *Fgf8* expression in transverse rings in the vicinity of the ic; *Wnt1* is also expressed along the dorsal midline of the CNS. (D) Dorsal view of the same brain. Note the absence of *Fgf8* and *Wnt1* RNA in the prominent lateral outgrowth (marked by an asterisk), and the almost completely circular ectopic expression domains of both genes. (E,F). Repression of *Otx2* expression. *Otx2* RNA was detected ~48 hours after an FGF8-bead was implanted (at stage 9^{+}, incision at position 'b'). Arrow points to the site where the FGF8-bead was located. (E) Lateral view of the operated side of the experimental brain. Note the sharp caudal boundary of the normal *Otx2* expression domain in vicinity of the ic. *Otx2* RNA was not detected in the region surrounding the site at which the bead was implanted, although this is not readily evident in the photograph shown because the brain is semi-transparent and the *Otx2* RNA on the non-operated, contralateral side is visible through the negative region. (F). Dorsal view of the same brain, in which the absence of *Otx2* RNA in the prominent lateral outgrowth (asterisk) is readily visible. Additional abbreviations as in Fig. 1.

Table 1. Summary of ectopic structures formed following implantation of an FGF8-bead

Position of bead implant	No. samples	Ectopic midbrains	Ectopic Cb tissue	Ectopic Isthmic tissue	Ectopic tegmental structures
a (mes)	1◊	Mb** and Mb*	–	–	–
	3	Mb*	–	–	–
	3	–	+¶	+¶	–
	2	–	–	–	–
Subtotal	**9**		**3**	**3**	**0**
b (p1/mes)	5	Mb** and Mb*	+	+	+
	1	Mb** and Mb*	+	+	–
	2	Mb** and Mb*	–	+	+
	2	Mb*	–	–	–
	1	–	–	–	–
Subtotal	**11**		**6**	**8**	**7**
c (p1)	3	Mb** and Mb*	+	+	+
	5	Mb** and Mb*	+	+	–
	2	Mb** and Mb*	–	–	–
	1	Mb*	+	+	+
	1	Mb*	+	+	–
	3	Mb*	–	+	–
	1	Mb*	–	–	+
	1	Mb*	–	–	–
	5	–	–	–	–
Subtotal	**22**		**10**	**13**	**5**
Total	**42**		**19**	**24**	**12**

Mb**, same orientation as the normal midbrain.
Mb*, orientation opposite that of the normal midbrain.
◊In this embryo, there appeared to be little if any normal midbrain tissue present.
¶In these embryos, a lateral outgrowth containing ectopic isthmocerebellar tissue was located just rostral to the normal isthmocerebellum.

development, and that the prospective DT, PT and midbrain are competent to respond to its inductive effect.

The lateral outgrowth is derived from cells in contact with the FGF8-bead

In the histological analysis described above, the FGF8-bead was detected in approximately half the affected brains, usually in the adjacent mesenchyme (see Fig. 1L). In almost all cases with obvious lateral outgrowths, the FGF8-bead was found in the most lateral sections of the brain, at or near the distal end of the outgrowth. When brains with lateral outgrowths were examined at earlier times after bead implantation (48-72 hours), the bead was likewise almost always found in the mesenchyme overlying the outgrowth, at or near its distal end (see Fig. 3D). To determine whether the lateral outgrowths were derived from cells in direct contact with the bead, we implanted beads soaked in both FGF8 and a carbocyanine dye, DiI, at stage 10, and the examined the embryos after ~72 hours incubation (to ~stage 23). Because there is no intercellular transfer of DiI, all labeled cells must either have been in contact with the bead or be descendants of such cells.

In 6/22 brains (27%), we observed a relatively large lateral outgrowth of the neuroepithelium, with the DiI-FGF8-bead at or near its distal tip (Fig. 2G,H). When viewed by fluorescence microscopy, the entire outgrowth was labeled and there was a sharp boundary between labeled and non-labeled cells at or near the junction of the outgrowth with the wall of the brain (Fig. 2H). In 8/22 (36%) brains, the diencephalon and rostral midbrain were enlarged but there was no pronounced localized outgrowth, and, in 8/22 (36%) brains, the bead had no obvious effect (data not shown). These data demonstrate that at 3 days after implanting an FGF8-bead, the lateral outgrowths that apparently assume an isthmocerebellar fate, are derived from cells that have been in contact with the FGF8-bead.

FGF8 induces ectopic expression of *En1*, *Fgf8* and *Wnt1*, and represses *Otx2* expression

Midbrain and isthmocerebellar development requires the function of both *En1* and *En2* (reviewed by Joyner, 1996). In our previous study, we demonstrated that an FGF8-bead implanted in p2 induces *En2* expression in the neuroepithelium near the bead (Crossley et al., 1996a). Here we assayed for *En1* RNA ~24 hours after implanting an FGF8-bead in p1. *En1* expression was detected in the vicinity of the bead in 6/8 brains, as well as in its normal domain in a double gradient that decreases caudally and rostrally from a high point in the vicinity of the m/h boundary (Fig. 3A; see also Gardner et al., 1988; Davis et al., 1991; Millet and Alvarado-Mallart, 1995). The presence of *En1*-expressing cells on the operated side and the absence of such cells on the contralateral non-operated side of the diencephalon is shown in Fig. 3B. In 2/8 cases, which displayed little or no ectopic *En1* expression, the bead had apparently dislodged from the neuroepithelium and was located within the ventricle (data not shown). Ectopic *En1* expression was also readily detected in 6/6 brains assayed ~48 hours, and in 8/9 brains assayed 5 days after an FGF8-bead was implanted. When lateral outgrowths were present, they always contained *En1*-expressing cells (data not shown). These results indicate that FGF8 can induce *En1* expression in the prospective caudal diencephalon/rostral midbrain.

Midbrain and isthmocerebellar development is also dependent on *Wnt1* and *Fgf8* gene function, which together produce IsO activity. These genes are normally expressed in adjacent transverse rings of cells at the m/h boundary, and

Wnt1 expression is also detected in cells along the dorsal midline of the prospective caudal forebrain, midbrain, hindbrain and spinal cord (Fig. 3C,D; see Bally-Cuif and Wassef, 1994; Mahmood et al., 1995; Crossley et al., 1996a). When *Fgf8* and *Wnt1* expression was assayed 24 or 41-48 hours after an FGF8-bead was implanted in p1 or mes, expression of both genes was detected in the neuroepithelium surrounding the bead and also in their normal domains (Fig. 3C,D and data not shown). A striking feature of the ectopic *Fgf8* expression domain in samples incubated for up to 48 hours (n=7) was that it extended in an almost complete circle around the FGF8-bead. *Wnt1* expression was likewise detected in an almost complete circle, concentric to and further from the bead than the *Fgf8* expression domain (Fig. 3C). When a lateral outgrowth was present, the *Fgf8* expression domain was localized at its junction with the wall of the brain and *Fgf8* RNA was not detected more distally in the outgrowth (Fig. 3D). These observations suggest that when an FGF8-bead is implanted in p1 or mes, it induces a nearly circular isthmic signaling center, which can send patterning signals through the neuroepithelium both rostral and caudal of the bead, thereby accounting for the formation of two ectopic midbrains with opposite polarity. Consistent with this conclusion, we found that, in brains in which the bead could be detected at stages 34-42, it was always situated in the region between the two ectopic midbrains (see Fig. 1L).

Otx2 also plays a key role in the development of the m/h region (see Discussion). In the normal brain, *Otx2* RNA is detected in regions of the telencephalon, and throughout the prospective diencephalon and midbrain, with a sharp boundary separating *Otx2*-positive and -negative cells at the m/h boundary, just rostral to the *Fgf8* expression domain (Fig. 3E; see Millet et al., 1996, and references therein). When *Otx2* expression was assayed 24 hours after implantation of an FGF8-bead in p1 or mes, a small *Otx2*-negative region was detected in the tissue surrounding the bead (n=2; data not shown). In brains collected 45 or more hours after bead implantation, an *Otx2*-negative region was likewise detected within the normally *Otx2*-positive neuroepithelium (n=6; Fig. 3E,F, and data not shown). In samples that contained a prominent lateral outgrowth, *Otx2* RNA was not detected in the outgrowth, and a sharp boundary was observed between the *Otx2*-expressing cells in the wall of the brain and the *Otx2*-negative cells in the lateral outgrowth (see Fig. 3F). In control experiments with PBS-beads, *Otx2* expression was not affected (n=8; data not shown). These results indicate that implantation of an FGF8-bead represses *Otx2* expression, and that *Otx2* is not expressed in the lateral outgrowths that apparently develop into isthmocerebellar tissue.

Prosomere boundaries inhibit rostral spread of an IsO signal

The data described above indicate that signals from the ectopic IsO induced by an FGF8-bead can spread both rostrally and caudally through the neuroepithelium. There appears to be no inhibition of the caudal spread of the IsO signal, since the caudal-most ectopic structure formed in response to a bead implanted in p2 or p1 (i.e. Mb*) was always smoothly joined to the normal midbrain (see Fig. 1L; Crossley et al., 1996a). In order to explore the extent to which the signal from an ectopic IsO spreads rostrally, we examined each of the 34 experimental brains in which there had clearly been a morphogenetic response to the FGF8-bead (see Table 1) and determined the extent to which tissue rostral to the bead was affected. To assess the effect on PT (p1), we examined every section of the brain in each experimental embryo and scored for the presence or absence of specific nuclei found in the PT, which can be distinguished by their unique shape and location. Likewise, we scored each sample for the presence of specific nuclei found in the DT (p2) and ventral thalamus (VT; p3). The locations of these diencephalic nuclei are illustrated in Fig. 4A.

Each of the experimental brains could be classified in one of three categories: (I) all of the PT, DT and VT marker nuclei were present (Fig. 4B-D), (II) none of the PT but all of the DT and VT marker nuclei were present (Fig. 4E-G), and (III) none of the PT or DT but all of the VT marker nuclei were present (Fig. 4H-J). Thus, in each case, there was a quantal effect: when a prosomere was affected, all of it appeared to be transformed to a more posterior fate (midbrain or isthmocerebellum). The prosomere immediately rostral to it was either likewise affected or completely normal.

The category in which an experimental brain was classified was related to the position in which the FGF8-bead was implanted (Table 2). Thus, all (7/7) of the brains in which the FGF8-bead was implanted at in an incision at position ‘a’ (in the mes) were classified in category I (PT, DT, and VT unaffected).

Table 2. Summary of effects of FGF8-beads on the diencephalon

Position of bead implant	No. samples	Ectopic midbrains	Structures chacteristic of VT	DT	PT	Number of samples displaying all markers of
a	1	Mb** and Mb*	+	+	+	■ VT,DT,PT: 7/7 (100%)
(mes)	3	Mb*	+	+	+	
	3	–	+	+	+	
b	3	Mb** and Mb*	+	+	+	■ VT,DT,PT: 5/10 (50%)
(p1/mes)	2	Mb*	+	+	+	■■ VT,DT: 4/10 (40%)
	4	Mb** and Mb*	+	+	–	■■■ VT: 1/10 (10%)
	1	Mb** and Mb*	+	–	–	
c	9	Mb** and Mb*	+	+	–	■■ VT,DT: 14/17 (82%)
(p1)	5	Mb*	+	+	–	■■■ VT: 3/17 (18%)
	1	Mb** and Mb*	+	–	–	
	2	Mb*	+	–	–	

■ category I; ■■ category II; ■■■ category III.

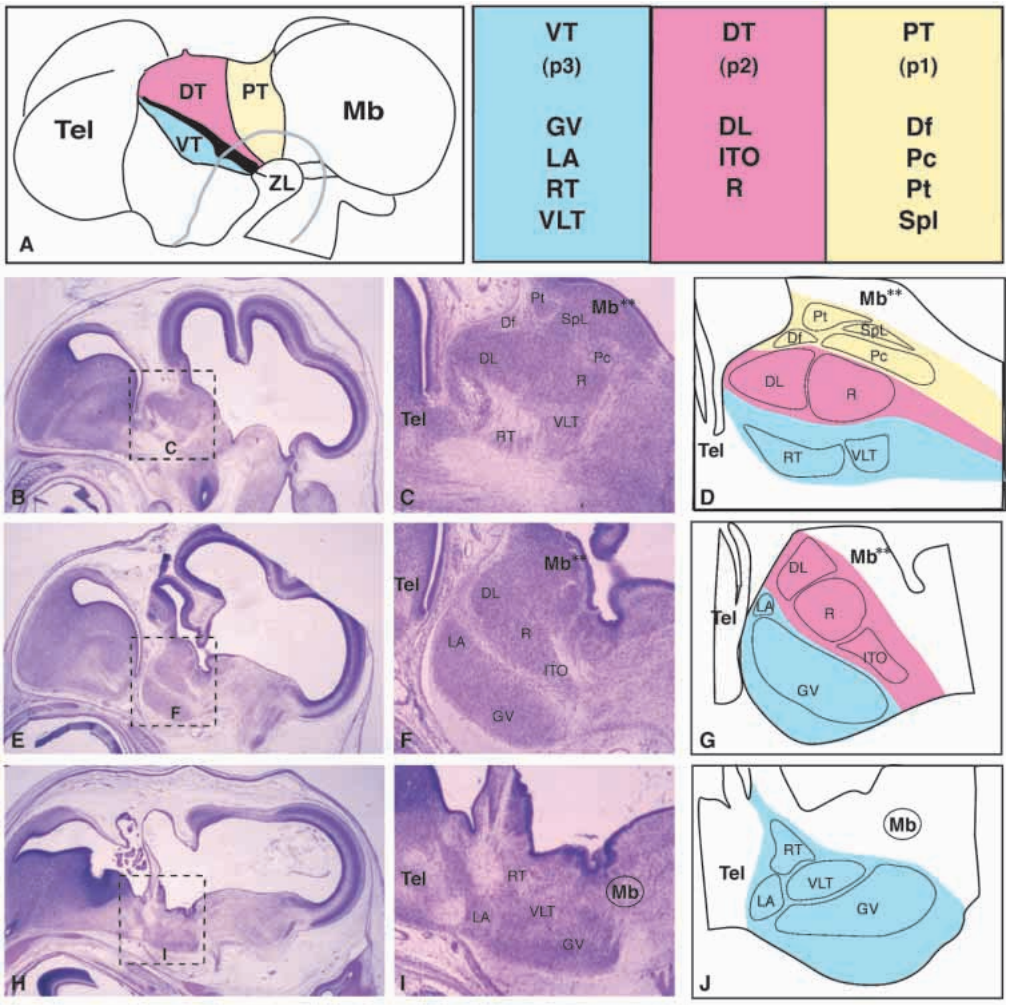

Fig. 4. Effects of inductive signals from an FGF8-bead on development of the diencephalon. (A) Schematic diagram of the brain at stage 35, indicating the locations of the ventral thalamus (VT), dorsal thalamus (DT) and pretectum (PT). The boxes on the right list the nuclei that were used as markers for normal development of each of these regions. (B-D) Example of an experimental brain classified in category I (see text): VT (p3), DT (p2) and PT (p1) unaffected. (B) Low magnification view of a stage 34 brain (sagittal section) in which an FGF8-bead was implanted at stage 11^{+} in an incision at position 'a' (same as brain shown in Fig. 1F). The dashed box indicates the region shown at higher magnification in C, in which the marker nuclei detected are labeled. The diagram in D is a schema of a camera-lucida drawing of the section shown in C, with the outlines of the marker nuclei indicated. Note that not all nuclei used as markers for a particular region are detected in a single section. The conclusions about the presence or absence of a particular prosomere were therefore drawn on the basis of an examination of multiple sections of the experimental brain. (E-G) Example of an experimental brain classified in category II: VT (p3) and DT (p2) unaffected, PT (p1) affected. (E) Low magnification view of a stage 34 brain (sagittal section) in which an FGF8-bead was implanted at stage 12^{-} in an incision at position 'c'. The dashed box indicates the region that is shown at higher magnification in F, in which the marker nuclei detected are labeled. The diagram in G is a schema of a camera-lucida drawing of the section shown in F, with the outlines of the marker nuclei indicated. (H-J) Example of an experimental brain classified in category III: VT (p3) unaffected, DT (p2) and PT (p1) affected. (H) Low magnification view of a stage 35 brain (sagittal section) in which an FGF8-bead was implanted at stage 11 in an incision at position 'c'. The dashed box indicates the region that is shown at higher magnification in I, in which the marker nuclei detected are labeled. The diagram in J is a schema of a camera-lucida drawing of the section shown in I, with the outlines of the marker nuclei indicated. Abbreviations: Df, nucleus dorsofrontalis; DL, nucleus dorsolateralis thalami; GV, nucleus geniculatus lateralis, pars ventralis; ITO, nucleus interstitialis tractus opticus; LA, nucleus lateralis anterior thalami; Pc, nucleus precommissuralis principalis; Pt, nucleus pretectalis principalis; R, nucleus rotundus; RT, nucleus reticularis thalami; SpL, nucleus spiriformis lateralis; VLT, nucleus ventrolateralis thalami. Other abbreviations as in Fig. 1.

In contrast, the brains in which the FGF8-bead was implanted in an incision at position 'c' (most likely in p1, possibly in p2) were almost always (14/17) classified in category II (PT absent, DT and VT unaffected), or infrequently (3/17) in category III (PT and DT absent, but no effect on the VT). When the FGF8-bead was implanted in an incision at position 'b' (near the p1/mes boundary), 5/10 were classified in category I, 4/10 in category II and 1/10 in category III. The simplest explanation of these data is that the signal from an ectopic IsO does not spread rostrally across neuromere boundaries. The strongest evidence for this hypothesis is the observation that when beads were implanted in the mes (position 'a'), the neuroepithelium rostral to the mes (i.e. the diencephalon) was not affected, despite the fact that it is competent to respond to signals from an IsO induced when an FGF8-bead is implanted in p1 or p2.

DISCUSSION

We describe here the consequences of implanting an FGF8-bead in p1 or mes of a stage 9-12 chick embryo. The 'maximal' response, observed in a substantial proportion of cases, can be described as follows. By 24 hours after the FGF8-bead was implanted, *Otx2* expression was repressed and *En1*, *Fgf8* and *Wnt1* were ectopically expressed in cells near the bead. The net result of these changes was the establishment of an ectopic IsO. By 48 hours, the caudal diencephalon and rostral midbrain had expanded and there was local lateral outgrowth of neuroepithelium in contact with the bead. The ectopic IsO functioned as a source of signals that repatterned the neuroepithelium. The regions rostral and caudal to the bead developed into ectopic midbrains in opposite orientations,

whereas the lateral outgrowth developed into an isthmocerebellum. The alar plate was always affected, but in some cases basal plate derivatives were also repatterned and an ectopic floor plate flanked by tegmental structures developed perpendicular to the A/P axis of the experimental brain. These data demonstrate that cells in the caudal diencephalon and midbrain are competent to develop as midbrain, isthmic tissue or cerebellum, and that local application of FGF8 is sufficient to initiate the processes that determine their fate and control their subsequent development. Interestingly, our data provide evidence that, although the inductive signal can spread rostrally within the neuromere in which the FGF8-bead was implanted, it does not readily spread to a rostrally adjacent neuromere.

Effects on gene expression mediated by an FGF8-bead

A fundamental conclusion of this and our previous study (Crossley et al., 1996a) is that the observed morphogenetic effects are due to the establishment of an ectopic IsO in which both *Fgf8* and *Wnt1* are expressed. However, an important difference between the two studies is that previously, when an effort was made to implant the FGF8-bead within p2 close to the ZL (i.e. near the p3/p2 boundary), a single ectopic midbrain and isthmic tissue in mirror-image orientation to the normal midbrain and isthmus developed. In contrast, in the present study, when the FGF8-beads were implanted more caudally, in p1 or mes, two ectopic midbrains and an ectopic isthmocerebellum were formed. An explanation for these different outcomes is suggested by a comparison of the domains of ectopic *Fgf8* and *Wnt1* expression observed in the two studies. When the bead was implanted near the ZL, ectopic *Fgf8* and *Wnt1* expression was readily detected in cells caudal, but in few cells rostral to the bead (Crossley et al., 1996a), either because the inductive signal from the FGF8-bead did not spread rostrally across the ZL, or because the region rostral to the ZL is not competent to form an IsO. Consequently, the ectopic IsO that formed was localized caudal to the bead. In contrast, when the bead was implanted in p1 or mes, ectopic *Fgf8* and *Wnt1* expression was usually detected in almost complete concentric rings around the bead, with the *Wnt1* ring outermost. In such cases, the bead was presumably implanted sufficiently far from a neuromere boundary to allow spread of the inductive signal both rostral and caudal to the bead, thus permitting the establishment of an ectopic IsO that can send patterning signals in all directions. The difference in the topology of the ectopic IsO also suggests an explanation for the observation of cerebellar development only in the present study. We speculate that a lateral outgrowth of the neuroepithelium (in which cerebellar differentiation occurs) can form only when the cells are almost completely surrounded by concentric rings of *Fgf8*- and *Wnt1*-expressing cells.

A striking feature of the structures induced by implanting an FGF8-bead is their distinct polarity. This is particularly evident in the ectopic midbrains, which display rostrocaudal gradients

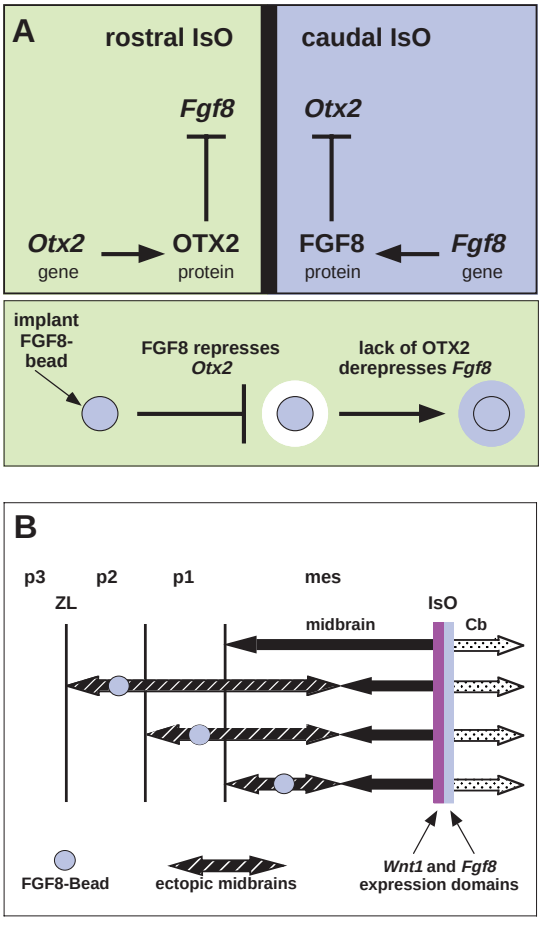

Fig. 5. Mechanisms of gene regulation and spread of inductive signals in the mid/hindbrain region. (A) Schematic representation of the region containing the IsO showing the proposed dosage-sensitive negative-feedback loop between FGF8 and OTX2 at the m/h boundary, and the mechanism by which an FGF8-bead induces *Fgf8* expression. (Top panel) Cells in the rostral IsO, on the rostral side of the m/h boundary (thick vertical line) express *Otx2* (green shading) and *Wnt1* (not illustrated). The OTX2 protein functions at high concentrations to repress *Fgf8* gene expression within the cells that produce it. Cells in the caudal IsO, on the caudal side of the m/h boundary express *Fgf8* (purple shading) and *Gbx2* (not illustrated). The FGF8 protein functions at high concentration to repress *Otx2* gene expression in the cells that produce it and in nearby cells. The net result of these reciprocal interactions, as well as the effects of other genes (see text), is the maintenance of a sharp m/h boundary. Although not illustrated here, a similar negative-feedback loop may exist between FGF8 and OTX1. (Bottom panel) When an FGF8-bead is implanted in p2, p1, or the mes, which are all *Otx2*-expressing territories, FGF8 from the bead locally represses *Otx2* expression. The consequent reduction in the level of OTX2 protein relieves the repressive effect on the *Fgf8* gene, which is then expressed in the *Otx2*-negative cells. (B) Diagram illustrating the presumed spread of inductive signals from the normal IsO and from the ectopic IsO induced by implanting an FGF8-bead. In the normal m/h region, signals from the IsO spread rostrally and caudally to establish the rostrocaudal polarity of the midbrain and anterior hindbrain (which contains the cerebellum [Cb]), respectively. The arrows representing the signals spreading caudally from the IsO are stippled to indicate that they may be quantitatively or qualitatively different from those spreading rostrally. When an FGF8-bead is implanted in p2 the signals produced by the induced IsO spread within and caudal to p2, but not rostrally across the p3/p2 boundary (ZL). When an FGF8-bead is implanted in p1 the signals spread within and caudal to p1, but not rostrally across the p2/p1 boundary. Likewise, when an FGF8-bead is implanted in the mes the signals spread within and caudal to the mes, but not rostrally across the p1/mes boundary.

of cytoarchitectonic maturation, but it is also observed in the lateral outgrowths, in which cells near the base develop into rostral structures (isthmic nuclei), whereas cells at the distal end develop into a more caudal structure (cerebellum). It seems likely that such patterning effects are due, at least in part, to induction within 24 hours of implanting an FGF8-bead of both *En1* (reported here) and *En2* (Crossley et al., 1996a) expression. These are thought to be important downstream target genes through which the IsO exerts its patterning effects (reviewed by Joyner, 1996; Rétaux and Harris, 1996; Wassef and Joyner, 1997).

Our results, as well as those of Ye et al. (1998), showing that local application of FGF8 can induce the expression of *En1* and *En2* in the prospective caudal diencephalon and rostral midbrain, differ markedly from the findings of a study of transgenic mice in which *Fgf8* was ectopically expressed under the control of regulatory elements from the *Wnt1* gene (Lee et al., 1997). In those mice, induction of *En1* expression was not detected and ectopic expression of *En2* was found only in the prospective rostral midbrain. Furthermore, there was no evidence for the development of ectopic structures. There are several plausible explanations for the different outcomes, but we think the most likely explanation lies in the difference between the experimental approaches. When a source of FGF8 protein is implanted in the lateral walls of the neural tube, there is an opportunity for an IsO, with discrete domains of *Fgf8* and *Wnt1*-expressing cells, to become established. In contrast, when *Fgf8* is expressed under the control of the regulatory elements that drive normal *Wnt1* gene expression, *Fgf8* and *Wnt1* are co-expressed in the same cells. This might preclude the formation of a functional IsO, whose activity may depend on the presence of juxtaposed rings of *Fgf8*- and *Wnt1*-expressing cells.

One important question is how does an FGF8-bead induce expression of *Fgf8* in p2, p1 or mes? One possibility is that FGF8 directly induces its own expression. We suggest that this process may involve repression of *Otx* genes, since we found that local application of FGF8 represses *Otx2* expression. Furthermore, studies of $Otx1^{-/-}$;$Otx2^{+/-}$ (Acampora et al., 1997) and $Otx1^{+/-}$;$Otx2^{+/-}$ (Suda et al., 1997) embryos, suggest that the *Otx* genes repress *Fgf8* expression in the prospective midbrain and diencephalon in a dosage-dependent manner. Together these data raise the possibility that FGF8 and OTX1/2 function in a negative feedback loop (Fig. 5A). If such a loop exists, then implantation of a bead containing high levels of FGF8 protein in p2, p1 or the mes would have a repressive effect on the *Otx* genes that are normally expressed there; this would result in a decrease in the amount of OTX protein present, thereby relieving the OTX inhibitory effect on the *Fgf8* gene (Fig. 5A). Moreover, since the proposed negative feedback loop between *Otx* and *Fgf8* gene is apparently dosage sensitive, this could explain why the domain of ectopic *Fgf8* expression is restricted to the vicinity of the FGF8-bead.

The data reported here, as well as the results of a variety of transplantation studies in the chick, have demonstrated that the prospective caudal diencephalon can be transformed to a midbrain, isthmic or cerebellar fate by signals from an IsO (Martinez et al., 1991; Marin and Puelles, 1994; Bloch-Gallego et al., 1996; reviewed by Puelles et al., 1996; Wassef and Joyner, 1997). An important question is what determines the response of cells to signals from the IsO? Genetic studies have suggested that the *Otx1* and *Otx2* genes, whose normal expression domains include most of the prospective forebrain and the midbrain and have a sharp caudal limit at the m/h boundary, play a key role in regulating the choice between a midbrain vs. cerebellar fate (Acampora et al., 1997; Suda et al., 1997). This hypothesis is based on the finding that reduction in *Otx* gene dosage results in a rostral shift of the location of the IsO. Tissue caudal to this misplaced IsO, which normally expresses both *Otx1* and *Otx2* and develops into p2, p1 and mes, develops instead as an enlarged cerebellum. In view of these results, our finding that FGF8 not only induces the formation of an ectopic IsO composed of *Fgf8*- and *Wnt1*-expressing cells, but that it also represses the expression of *Otx2*, suggests an explanation for the different fate transformations observed in response to implantation of an FGF8-bead: cells in which *Otx2* expression has been repressed by FGF8, for example, those in the lateral outgrowths, respond to the signals generated by the ectopic IsO by undergoing isthmocerebellar differentiation, whereas cells in which *Otx2* expression has not been affected by the FGF8-bead, i.e. those in the wall of the brain, respond by undergoing midbrain differentiation.

Inhibition of the rostral spread of morphogenetic signals across prosomere boundaries

The boundaries between neuromeres delineate domains of gene expression (reviewed by Rubenstein et al., 1994; Rubenstein and Puelles, 1994; Lumsden and Krumlauf, 1996) and specialized cellular behavior. For example, in the hindbrain, clones of neuroepithelial cells within an individual neuromere (rhombomere) generally do not cross the boundaries between rhombomeres (Fraser et al., 1990; Birgbauer and Fraser, 1994). Likewise, clones of cells within a specific forebrain neuromere (prosomere) do not mix with the cells in adjacent neuromeres (Figdor and Stern, 1993). Such boundaries contain extracellular matrix with special characteristics, and cells with distinct patterns of gene expression and a slow rate of proliferation (Guthrie et al., 1991; Heyman et al., 1995, and references therein). Moreover, boundary cells display little gap junctional communication with adjacent cells (Martinez et al., 1992).

The data described here provide evidence that the morphogenetic signals from an ectopic IsO do not readily pass rostrally across mes/p1, p1/p2, or p2/p3 boundaries. In contrast, they appear to be readily transmitted caudally across the p2/p1 and p1/mes boundaries (Crossley et al., 1996a) (Fig. 5B). A similar conclusion was reached by Bloch-Gallego et al. (1996), who examined the spread of inductive signals from grafts of tissue with IsO activity by assaying for the induction of *En2* expression. A somewhat different conclusion was drawn from a study of the ability of IsO signals to cross rhombomere boundaries caudally (Martinez et al., 1995). The results of those experiments suggested that signals from an IsO do not spread either rostrally or caudally through the boundaries between rhombomeres 3 and 7.

Together these results suggest an explanation for the observation that the patterning signals from the normal IsO apparently influence only the development of the midbrain, isthmus and cerebellum, even though the entire region extending caudally from the ZL (p3/p2 boundary) through the hindbrain (to r7) is competent to respond to them. Thus signals from the IsO may be prevented from influencing cells in the prospective caudal diencephalon because they are unable to spread rostrally through

the mes/p1 boundary (see Fig. 5B). On the contrary, signals from the IsO spreading caudally may be prevented from influencing cells in the posterior hindbrain (r3-r7) because they are unable to spread caudally through the r2/r3 boundary. At present, the mechanisms by which the spread of inductive signals is limited are unknown. One possibility is that the specialized cells at neuromere boundaries function to block signal transmission between neuromeres, perhaps due to discontinuities in gap junction connectivity (Martinez et al., 1992).

Role of FGF8 in normal mid/hindbrain development

The results reported here and by others provide some insights into the functions that FGF8 produced in the IsO performs. First, it is likely to play a key role in stimulating cell proliferation. Evidence for this hypothesis includes our finding that the lateral outgrowths that form when a diI-labeled FGF8-bead is implanted in the caudal diencephalon are derived from cells in contact with the bead. In addition, Lee et al. (1997) have shown that ectopic expression of *Fgf8* under the control of *Wnt1* regulatory elements results in excess proliferation of neural precursors, resulting in dramatic hyperplasia of the midbrain and caudal diencephalon.

Second, FGF8 may regulate rostrocaudal polarity in the developing m/h region. One way in which FGF8 might influence this process is via its proposed effect on cell proliferation, which in turn may regulate the ability of the cells to respond to the factors that control their differentiation. According to this hypothesis (Lee et al., 1997), cells closest to the source of FGF8 protein in the IsO are stimulated to divide rather than differentiate, whereas those further away from the IsO cease dividing and differentiate, thus producing the normal rostrocaudal cytoarchitectonic developmental gradient. FGF8 may also regulate rostrocaudal polarity via an inductive effect on the expression of *En1* and *En2* (data reported here, and by Crossley et al., 1996a; Lee et al., 1997; Ye et al., 1998).

Third, FGF8 may influence fate decisions in the m/h region via a repressive effect on the *Otx* genes, which apparently regulate the choice between midbrain and anterior hindbrain fate (Acampora et al., 1997; Suda et al., 1997). In turn, OTX proteins produced in p2, p1 and the mes may serve to repress *Fgf8* gene expression, thus preventing the *Fgf8* expression domain from spreading anteriorly (Fig. 5A). However, it is likely that other molecules are involved in stabilizing the location of the IsO. For example, *Wnt1* might function, by virtue of its co-expression with *Otx* genes on the rostral side of the m/h boundary, to reduce the sensitivity of the cells to the repressive effects FGF8. Conversely, *Gbx2*, which is co-expressed with *Fgf8* on the caudal side of the m/h boundary (Bouillet et al., 1995; Wassarman et al., 1997), may function to increase the sensitivity of cells to the repressive effects of FGF8. In support of these suggestions, it has been observed that mouse embryos homozygous for a hypomorphic allele of *Wnt1* (Bally-Cuif et al., 1995a) or a null allele of *Gbx2* (Wassarman et al., 1997) display abnormalities in *Otx2* expression and an inability to establish a normal m/h boundary during early development.

Critical tests of these hypotheses about FGF8 function will require analysis of the consequences of loss of gene function in the m/h region. One approach is to employ inhibitors of FGF signaling, as for example in the study by Ye et al. (1998), which indicated that FGF8 is necessary for the development of midbrain dopaminergic and hindbrain serotonergic neurons. Genetic studies have recently become feasible in zebrafish since *Fgf8* was identified as the gene responsible for the *acerebellar* mutation (Reifers et al., 1998). Genetic analysis of the functions of FGF8 in brain development in higher vertebrates is potentially hampered by the finding that *Fgf8* is required for gastrulation in mice, and that the brain does not develop in *Fgf8* null mutant homozygotes (X. Sun and G. R. M., unpublished observations). However, the availability of mice carrying a hypomorphic allele of *Fgf8* has made possible genetic analysis of the role of *Fgf8* in mouse brain development (Meyers et al., 1998). A preliminary study of the mutant embryos has suggested that relatively small reductions in *Fgf8* expression result in a failure to form the isthmocerebellum and posterior midbrain, and that greater reductions have a more severe effect on midbrain development (E. Meyers, S. M. and G. R. M., unpublished observations). Further studies of these mutant embryos, as well as embryos in which *Fgf8* gene function is completely eliminated in specific regions of the developing embryo, should help to determine precisely how FGF8 contributes to establishment and function of the IsO.

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