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The isthmic organizer signal FGF8 is required for cell survival in the prospective midbrain and cerebellum

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SUMMARY

Numerous studies have demonstrated that the midbrain and cerebellum develop from a region of the early neural tube comprising two distinct territories known as the mesencephalon (mes) and rostral metencephalon (met; rhombomere 1), respectively. Development of the mes and met is thought to be regulated by molecules produced by a signaling center, termed the isthmic organizer (IsO), which is localized at the boundary between them. FGF8 and WNT1 have been implicated as key components of IsO signaling activity, and previous studies have shown that in *Wnt1*$^{-/-}$ embryos, the mes/met is deleted by the 30 somite stage (~E10) (McMahon, A. P. and Bradley, A. (1990) *Cell* 62, 1073-1085). We have studied the function of FGF8 in mouse mes/met development using a conditional gene inactivation approach. In our mutant embryos, *Fgf8* expression was transiently detected, but then was eliminated in the mes/met by the 10 somite stage (~E8.75). This resulted in a failure to maintain expression of *Wnt1* as well as *Fgf17*, *Fgf18*, and *Gbx2* in the mes/met at early somite stages, and in the absence of the midbrain and cerebellum at E17.5. We show that a major cause of the deletion of these structures is ectopic cell death in the mes/met between the 7 and 30 somite stages. Interestingly, we found that the prospective midbrain was deleted at an earlier stage than the prospective cerebellum. We observed a remarkably similar pattern of cell death in *Wnt1* null homozygotes, and also detected ectopic mes/met cell death in *En1* null homozygotes. Our data show that *Fgf8* is part of a complex gene regulatory network that is essential for cell survival in the mes/met.

Key words: Brain patterning, Cerebellum, *Fgf8*, Isthmic organizer, mes/met, Mesencephalon, Metencephalon, Midbrain, Midbrain/hindbrain organizer, Mouse, Rhombomere 1

INTRODUCTION

A major issue in developmental neurobiology is how the vertebrate neural tube becomes regionalized and patterned along its axes. Substantial progress has been made toward understanding the molecular mechanism of specification and early anterior-posterior (AP) patterning of the region that forms the midbrain and cerebellum (reviewed by Liu and Joyner, 2001a; Martinez, 2001; Nakamura, 2001; Wurst and Bally-Cuif, 2001). This region originates from two distinct portions of the early neural tube, the mesencephalon (mes), which gives rise to the midbrain, and the rostral metencephalon (met; rhombomere 1 at the anterior end of the hindbrain), which gives rise to cerebellum dorsally and pons ventrally (Alvarado-Mallart, 1993; Wingate and Hatten, 1999). Development of the mes and met is thought to be regulated and coordinated by molecules produced in a signaling center, known as the isthmic organizer (IsO), which develops at the mes/met boundary, and eventually co-localizes with an anatomical feature called the isthmic constriction.

Mes/met specification and AP patterning begin during gastrulation, with the induction of gene expression that distinguishes the mes/met from the rest of the neuroepithelium [*paired*-related (Pax) and *engrailed*-related (En) transcription factor genes] and the prospective mes from met (*Otx2* and *Gbx2* transcription factor genes, respectively). Genetic analysis in mice has demonstrated that *Pax2* and *Pax5* (Bouchard et al., 2000) and *En1* and *En2* (Hanks et al., 1995), respectively, are functionally equivalent in the mes/met, and that in the absence of either pair of genes, the entire midbrain to cerebellum region is deleted (Schwarz et al., 1997; Wurst et al., 1994) (W. W. and A. Joyner, unpublished observations). Within the mes/met, *Otx2* and *Gbx2* act antagonistically and are required for positioning and function of the IsO (reviewed by Simeone, 2000; Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001; see also Li and Joyner, 2001; Martinez-Barbera et al., 2001).

The patterning activity of the IsO was initially demonstrated in avian embryos by studies showing that grafts of tissue that include the mes/met boundary region could induce cells in the caudal forebrain to develop into an ectopic midbrain and cells in

the posterior hindbrain to develop into ectopic cerebellar structures (reviewed by Puelles et al., 1996; Wassef and Joyner, 1997). Gene expression analysis identified members of the WNT and FGF families of secreted signaling molecules as candidate mediators of IsO activity. In the mouse mes/met, *Wnt1* expression is first detected at the 1 somite stage [embryonic day (E) 8.0] throughout the prospective midbrain, but is soon restricted to a transverse band at its caudal end, and is also expressed along the lateral edges of the neural plate (McMahon et al., 1992; Parr et al., 1993). *Fgf8* expression is first detected at approx. the 3-5 somite stage, and initially extends throughout the prospective cerebellum (rhombomere 1; r1), but is soon restricted to a narrow band at the anterior end of the hindbrain. Thus *Wnt1* and *Fgf8* are expressed in adjacent bands on either side of the midbrain/hindbrain boundary (Crossley and Martin, 1995).

As yet, there is no evidence that WNT1 can mediate IsO patterning activity. However, *Wnt1* is essential for mes/met development, since first prospective midbrain and then cerebellum are deleted at early stages in $Wnt1^{-/-}$ embryos (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990). Further studies have suggested that in the mes/met, *Wnt1* is required to promote cell proliferation (Dickinson et al., 1994; Wurst and Bally-Cuif, 2001), to maintain *En1* expression (Danielian and McMahon, 1996), and to maintain a stable midbrain/hindbrain boundary (Bally-Cuif et al., 1995).

Unlike WNT1, FGF8 can mimic the effects of grafts of the mes/met boundary region in the chick, in some cases inducing diencephalic tissue to form complete ectopic midbrains as well as cerebellar tissue (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). The results of experiments aimed at determining how FGF8 affects cells that are competent to form mes/met tissue have not been entirely consistent, but generally support the hypothesis that it normally stimulates cell proliferation in the mes/met and maintains gene expression required for IsO activity (reviewed by Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001).

The role of FGF8 in mes/met development cannot be investigated in $Fgf8^{-/-}$ mouse embryos because they fail to gastrulate (Sun et al., 1999), but it can be studied in *Fgf8* hypomorphs, which survive to birth. A preliminary analysis has shown that when *Fgf8* expression is reduced, a substantial portion of the midbrain as well as isthmus and cerebellum are deleted (Meyers et al., 1998). However, it is possible that the brain phenotypes in such hypomorphic embryos are secondary to mild defects caused by reduced *Fgf8* expression during gastrulation. To address this concern, we have used a conditional gene inactivation approach to determine the effects of inactivating *Fgf8* in the mes/met without perturbing its expression during gastrulation. We show that this causes extensive cell death in the mes/met before E10, and confirm that *Fgf8* is part of a gene regulatory network that is essential for mes/met development. Together with the results of previous studies, our data suggest that loss of *Fgf8* function may be the cause of the deletions that are also observed in embryos that lack *Wnt1*, *En1/2* or *Pax2/5* gene function.

MATERIALS AND METHODS

Production and genotyping of mutant embryos

All mouse lines used in this study were maintained on a mixed genetic background. To obtain *Fgf8* 'MHB KO mutants', we crossed $En1^{Cre/+}$ mice (Kimmel et al., 2000) to mice heterozygous for $Fgf8^{\Delta 2,3}$ (Meyers et al., 1998), hereafter referred to as $Fgf8^{null}$ or $Fgf8^{-}$. Their $En1^{Cre/+}$,$Fgf8^{null/+}$ male offspring were mated to $Fgf8^{flox/flox}$ (Meyers et al., 1998) females. The desired $En1^{Cre/+}$,$Fgf8^{null/flox}$ progeny were obtained at the expected frequency (~25%). Their $En1^{Cre/+}$,$Fgf8^{flox/+}$or $Fgf8^{flox/null}$ or $Fgf8^{flox/+}$ littermates, which develop normally, were used as control embryos. The $En1^{Cre}$ allele was detected by a PCR assay for cre (Sun et al., 2002). To detect the $En1^{+}$ allele, we used the following PCR primer pair: 5′-CACCGCACCACCAACTTTTTC-3′ and 5′-TCGCATCTGGAG-CACACAAGAG-3′ to amplify a 238 bp fragment representing *En1* sequences replaced by *cre* in $En1^{Cre}$. Standard PCR conditions were used for a 35-cycle run with an annealing temperature of 59°C.

$Fgf8^{neo/neo}$ and $Fgf8^{neo/null}$ embryos were obtained from appropriate crosses of $Fgf8^{neo/+}$ and $Fgf8^{null/+}$ mice, and genotyped as previously described (Meyers et al., 1998; Sun et al., 2002). Their $Fgf8^{neo/+}$, $Fgf8^{null/+}$ and $Fgf8^{+/+}$ littermates were used as controls. Mice carrying a *Wnt1* null allele on a Swiss Webster background were kindly provided by A. McMahon. $Wnt1^{+/-}$ mice were crossed inter se to obtain null mutant homozygotes. Genotyping by PCR was performed as previously described (McMahon and Bradley, 1990). Their heterozygous and wild-type littermates were used as control embryos. To obtain *En1* null homozygotes, $En1^{Cre/+}$ mice were crossed inter se. $En1^{Cre/+}$ and $En1^{+/+}$ littermates were used as controls. Mice carrying the R26R (Soriano, 1999) Cre reporter gene were kindly provided by P. Soriano.

Embryos were collected at various stages of gestation, and dissected free of maternal tissues in phosphate-buffered saline (PBS) before use in specific assays. Noon of the day of vaginal plug detection was designated embryonic day (E) 0.5. Prior to E10, embryos were staged by determining somite number (Jacobson and Tam, 1982). In the mouse, a new pair of somites is generated every 1.5 to 2 hours (Tam, 1986). DNA for genotype analysis was prepared from embryonic or yolk sac tissue.

Histological analysis, immunolocalization and in situ hybridization assays

E17.5 embryonic brains were left in situ, but the overlying epithelium was dissected open to allow penetration of Clarke's fixative (75% ethanol, 25% glacial acetic acid). Heads were fixed for 14-24 hours at room temperature, stored in 70% ethanol, then embedded in paraffin wax and sectioned at 12 μm in the sagittal and horizontal planes. Serial sections were stained with Cressyl Violet or processed to detect tyrosine hydroxylase (TH). Sections were incubated overnight in anti-TH antibody (Inst. Jacques BOY S.A., Reims, France), following the manufacturer's recommendations. Antibody staining was detected using biotinylated anti-rabbit IgG and streptavidin-conjugated with peroxidase (Vector Labs, Burlingame, CA). Parallel sections were processed without primary antibody as controls for the specificity of the immunolabelling.

Whole-mount immunohistochemistry was performed essentially as described by O'Connor et al. (O'Connor et al., 1999) using 2H3 antibody (Dodd et al., 1988) supernatant (Developmental Studies Hybridoma Bank, U. Iowa) and an HRP-linked sheep anti-mouse secondary antibody (Amersham Pharmacia NA931). Samples were post-fixed, dehydrated and cleared in 1:2 benzyl benzoate:benzyl alcohol.

Whole-mount RNA in situ hybridization was performed on embryos fixed in 4% PFA (in PBS), and processed essentially as described by Neubuser et al. (Neubuser et al., 1997). Digoxigenin-labeled ribroprobes were used to detect expression of *En1* (Danielian and McMahon, 1996), *Fgf8*-exon3 (Lewandoski et al., 2000), full-length *Fgf8* (Crossley and Martin, 1995), *Fgf17* and *Fgf18* (Maruoka et al., 1998), *Gbx2* (Wassarman et al., 1997), *Hoxa2* (Wilkinson et al., 1989), *Otx2* (Simeone et al., 1993), *Pax2* (Dressler et al., 1990), *Spry2* (Minowada et al., 1999) and *Wnt1* (McMahon et al., 1992). For

simultaneous detection of *Otx2* and *Hoxa2* RNA, the probes were mixed in a 1:2 ratio. β-Galactosidase was assayed essentially as described previously (Logan et al., 1993).

Assays for cell death

For whole-mount Nile Blue Sulfate (NBS) staining, embryos were washed in PBS and incubated at 37°C in filtered NBS solution [10 μg/ml NBS (Sigma N-5632) in PBS containing 0.1% Tween 20 (PBT)]. Incubation times varied with embryo stage: 0-10 somite stages, 15 minutes; 10-30 somite stages, 20 minutes; >30 somite stage, 40 minutes. Embryos were then washed in PBS at room temperature and photographed immediately.

Whole-mount TUNEL was performed with the 'In Situ Cell Death Detection, POD' kit (Roche) following a modified protocol (A. Strickler, personal communication). Embryos were fixed in 4% PFA and stored in methanol, then rehydrated into PBT and treated with proteinase K (20 μg/ml in PBT) for 2 to 10 minutes. They were then post-fixed in 4% PFA/0.2% glutaraldehyde for 20 minutes, incubated for 1 hour in 3% H_2O_2 in methanol to inactivate endogenous peroxidases, then permeabilized in 0.1% sodium citrate/0.1% Triton X-100 for 5 minutes on ice. The treated embryos were then incubated in Roche kit TUNEL Reaction Mix for 1 hour at 37°C, and washed in PBT. To block nonspecific antibody binding, embryos were next incubated in KTBT buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM KCl, 1% Triton X-100) containing 2% blocking reagent (Roche 1096176) and 20% sheep serum. Embryos were then incubated in Roche kit converter POD for 30 minutes at 37°C, and washed in PBT. Finally, specimens were reacted in diaminobenzidine for 30 to 90 minutes.

RESULTS

Inactivation of *Fgf8* in the mes/met by Cre-mediated recombination

To knock out (KO) *Fgf8* in the mes/met at early stages of brain development, we generated $En1^{Cre/+}$,$Fgf8^{flox/null}$ embryos (hereafter referred to as 'MHB KO mutants') using the cross described in the Materials and Methods. In these embryos, $Fgf8^{flox}$, an allele with wild-type activity, is converted by Cre-mediated recombination into $Fgf8^{null}$ (Meyers et al., 1998) (see Fig. 1A), resulting in homozygosity for $Fgf8^{null}$ in cells that produced Cre, and all of their descendants. The timing of *Fgf8* inactivation in MHB KO mutants depends on when Cre protein is produced by $En1^{Cre}$, a null allele in which the cre gene partially replaces EN1 coding sequence (Kimmel et al., 2000) (Fig. 1A). Based on what is known about the pattern of *En1* expression (Davis et al., 1991; Davis and Joyner, 1988) (Fig. 1B, and data not shown), we anticipated that $En1^{Cre}$ might produce sufficient Cre protein throughout the midbrain/hindbrain boundary region to eliminate *Fgf8* function by 3 somite stage, when *Fgf8* expression normally commences in a subset of *En1*-expressing cells.

To assess Cre function, we examined $En1^{Cre/+}$ embryos carrying the R26R reporter gene (Soriano, 1999) at ~E8.0-E8.5. Cre activity, as reflected by β-galactosidase activity (Fig. 1A), was first detected in only a few cells at the 2 somite stage, more cells at the 5 somite stage, and in most or all cells by the 7 somite stage, in a domain encompassing the mes/met (Fig. 1C). We also assayed MHB KO mutants for functional *Fgf8* RNA using a probe that hybridizes to exon 3 sequence that is deleted by Cre-mediated recombination of $Fgf8^{flox}$ and is therefore absent from the $Fgf8^{null}$ allele (Meyers et al., 1998). *Fgf8* RNA was detected in the mes/met at the 3-7 somite stage, but at a lower level than in control embryos. At the 8 somite stage, the level was even lower in mutant embryos, and from approximately the 10 somite stage it was not detected in mutants but was readily detected in control embryos (Fig. 1D, and data not shown). Together, the data suggest that in our MHB KO mutants, functional *Fgf8* RNA is expressed normally at approx. the 3-5 somite stage, but subsequently its level decreases progressively, such that by about the 10 somite stage, it is no longer expressed in the mes/met.

Deletion of mes/met derivatives increases in severity with progressive loss of *Fgf8* function

Previously, we reported that homozygosity for an *Fgf8* hypomorphic allele, $Fgf8^{neo}$, caused deletion of posterior midbrain and cerebellar tissue. In $Fgf8^{neo/null}$ compound heterozygotes, larger deletions were observed. Reduced *Fgf8* function in these mutant embryos also caused other defects, including absence of the olfactory bulbs in both genotypes, and severe forebrain, craniofacial, heart and kidney abnormalities in $Fgf8^{neo/null}$ embryos (Meyers et al., 1998). In contrast, in E17.5 MHB KO mutants, we found that the midbrain, isthmus and cerebellum was deleted, but olfactory bulb, forebrain and craniofacial development appeared normal (Fig. 2A-B′), and we detected no obvious abnormalities in other organs (M. Lewandoski and G.R.M., unpublished observations). These data demonstrate that inactivating *Fgf8* using $En1^{Cre}$ causes defects specifically in structures that develop from the mes/met.

To determine more precisely the consequences of reducing or eliminating *Fgf8* function in the mes/met, we performed a histological analysis of brains from mutants expressing different amounts of functional *Fgf8* RNA (*n*=6 of each genotype). Since $Fgf8^{neo/neo}$, $Fgf8^{neo/null}$ and MHB KO mutants die soon after they are born, we carried out this analysis at E17.5, shortly before birth. Despite the mixed genetic background of the mutant embryos, we observed relatively little variation in the phenotype of the midbrain to cerebellar region among individuals of a given genotype.

In $Fgf8^{neo/neo}$ embryos, in which the level of functional *Fgf8* RNA has been roughly estimated to be ~40% of the amount in wild-type embryos (Meyers et al., 1998), the rostral midbrain appeared normal, but caudal regions of the midbrain, including a substantial portion of the posterior superior colliculus and the entire inferior colliculus were missing, as was the isthmus. Dorsal anterior hindbrain tissue (cerebellum) was largely deleted, except in the most lateral regions. The locus ceruleus, a marker of ventrolateral anterior hindbrain tissue (the pons), was present but reduced in size (Fig. 2C-D′, and data not shown). This suggests that when *Fgf8* expression is moderately reduced, at least part of the midbrain and intermediate and basal anterior hindbrain still develops.

In $Fgf8^{neo/null}$ embryos, in which functional *Fgf8* RNA is presumably reduced to approximately half the level in $Fgf8^{neo/neo}$ embryos, the extent of the deletion was greater. Rostrally, all midbrain tissue except the posterior pretectal nucleus (PPT) was absent. Although its name implies that the PPT is of forebrain origin, comparative anatomical studies indicate that it is a midbrain structure derived from the rostral mesencephalon (Lagares et al., 1994). Caudally, no cerebellar tissue was observed, and the locus ceruleus was absent. However, the pontine nucleus, which is derived primarily from

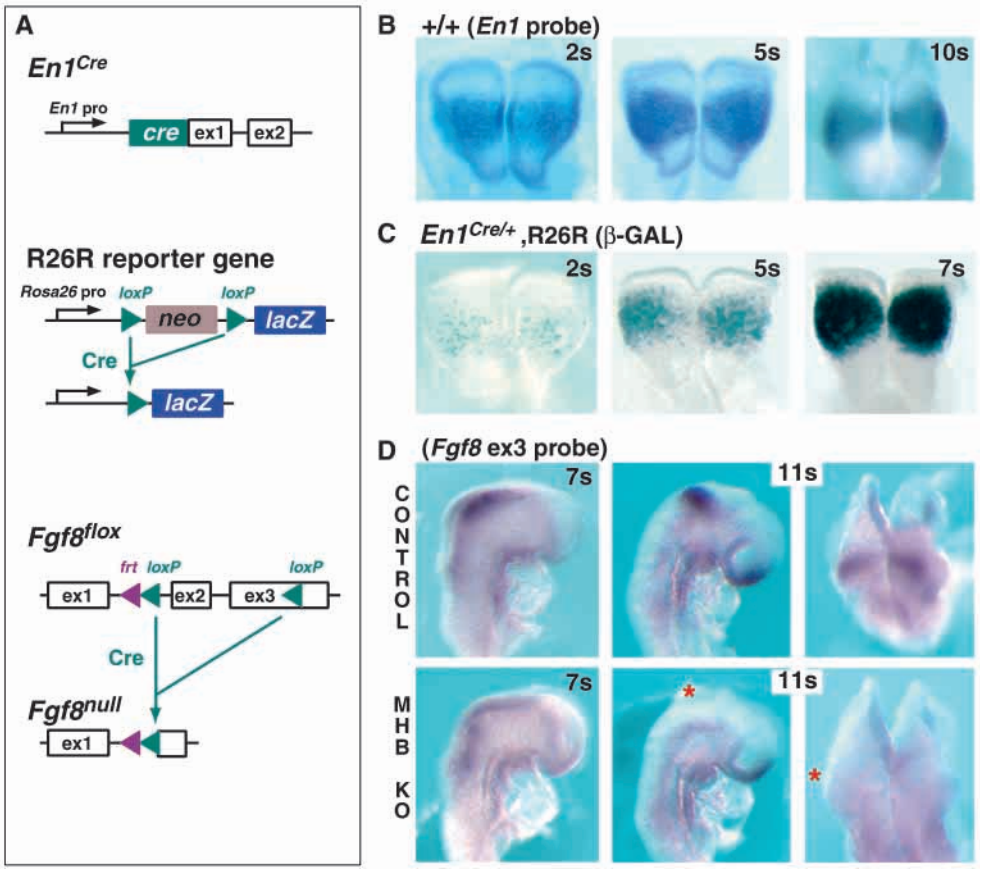

Fig. 1. Analysis of Cre-mediated inactivation of *Fgf8* in MHB KO mutants. (A) Schematic representation of the mutant alleles carried by $En1^{Cre/+}$, $Fgf8^{flox/null}$ embryos (MHB KO mutants) and a reporter gene used to detect Cre activity. Cre-mediated recombination of the R26R reporter gene deletes the *neo* sequences flanked by *loxP* sites, and allows expression of *lacZ*. Cre-mediated recombination of $Fgf8^{flox}$ deletes crucial exons, creating the $Fgf8^{null}$ allele. (B,C) Dorsal views of embryos at the somite stages (s) indicated, assayed for *En1* expression by RNA whole-mount in situ hybridization (B), or for Cre-mediated recombination by staining for β-galactosidase (β-GAL) activity (C). (D) Control and MHB KO mutants hybridized with a probe that detects *Fgf8* exon 3. Embryos at the 7 somite stage are shown in lateral view only; those at the 11 somite stage are shown in both lateral (left) and dorsal (right) view. Red asterisks indicate the regions in which *Fgf8* expression is detected in control, but not in mutant embryos. *frt*, frt site present in the $Fgf8^{flox}$ and $Fgf8^{null}$ alleles, as described by Meyers et al. (Meyers et al., 1998); pro, promoter; s, somite stage.

tissue caudal to the prospective cerebellum, was present and appeared normal (Fig. 2E,E′, and data not shown).

MHB KO mutants exhibited the most severe phenotype. The entire midbrain, including the PPT, and the anterior hindbrain were deleted, but the pontine nucleus was normal (Fig. 2F,F′, and data not shown). The posterior commissure, which comprises axons of pretectal (forebrain) nuclei, appeared abnormally extended (Fig. 2F), perhaps because of the lack of caudal tissue to confine it. These data show that *Fgf8* is essential for development of the mes/met, and that even a moderate decrease in the level of functional *Fgf8* RNA results in deletions of posterior midbrain and most cerebellar tissue. A more substantial decrease results in the absence of almost the entire midbrain, except for the PPT, and all anterior hindbrain structures. Finally, elimination of *Fgf8* function results in complete deletion of the territory spanning from the rostral end of the midbrain through the cerebellum, whereas tissues derived from adjacent anterior or posterior regions are intact. However, because *Fgf8* MHB KO mutants are heterozygous for a null allele of *En1*, we cannot formally rule out the possibility that reduced *En1* dosage played some role in the deletion of the PPT in those mutants.

To study the phenotype at an earlier stage, we performed immunohistochemistry using an anti-neurofilament antibody that detects the cranial nerves (n) and their associated ganglia (g) that mark derivatives of specific portions of the mes/met by ~E10.5 (reviewed by Cordes, 2001; Fig. 2G). In E10.5 $Fgf8^{neo/neo}$ embryos, nIV (trochlear), which marks the isthmus, was absent, but other markers appeared normal (2/2; Fig. 2H, and data not shown). In contrast, in both $Fgf8^{neo/null}$ (2/2) and MHB KO (3/3) embryos, not only nIV, but also nIII (oculomotor), which marks the ventral midbrain, and the r1-derived component of the trigeminal nV were either absent or severely truncated. Cranial nerves and ganglia originating from r2 and more caudal territory, including the trigeminal gV, n/gVII (facial), and n/g VIII (acoustic), appeared normal (Fig. 2I,J, and data not shown). These data are consistent with the results of our histological analysis at E17.5, in that they show a correlation between the amount of functional *Fgf8* expression and the amount of mes/met-derived tissue present. Furthermore, since the trigeminal and more posterior ganglia appeared to be intact in all three mutants, the results indicate that the effects of loss of *Fgf8* function in the midbrain/hindbrain boundary region do not extend caudally beyond r1. In addition, these data demonstrate that the deletions, including loss of ventral structures, occur by ~E10.5.

Inactivation of *Fgf8* causes extensive cell death in the mes/met

Although previous studies have suggested that *Fgf8* functions as a proliferation factor in the mes/met (Lee et al., 1997; Xu et al., 2000), in several other developmental contexts *Fgf8* has been found to be required for cell survival (Moon et al., 2000; Storm et al., 2003; Sun et al., 2002; Trumpp et al., 1999). To determine if cell death might be a cause of the deletions described above, we assayed for cell death in MHB KO mutants at E8.0-E10.0 by staining with Nile Blue Sulfate (NBS), which specifically marks dying cells (Tone et al.,

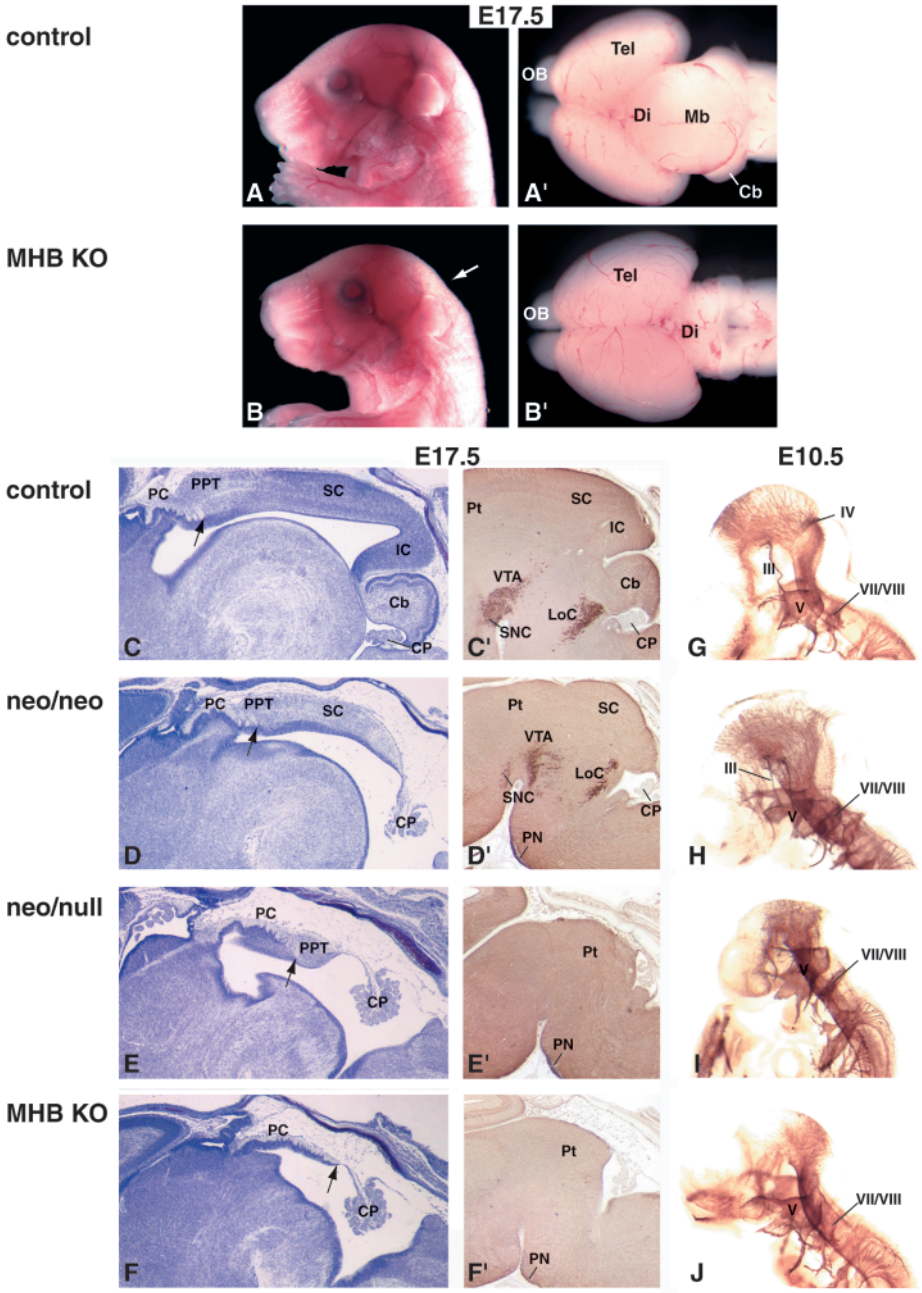

Fig. 2. Analysis of mes-met derivatives in *Fgf8* loss of function mutants. (A-F) Analysis of midbrain, isthmus and cerebellum phenotypes at E17.5. **(**A,B) Lateral views of control embryos and MHB KO mutants at E17.5. The arrow in B points to the part of the head that is misshapen due to the absence of the midbrain and cerebellum. Note that craniofacial development appears normal in the mutant embryo. (A′,B′) Dorsal views of the brains from the embryos shown in A and B. (C-F) Paramedial sagittal sections of control, $Fgf8^{neo/neo}$, $Fgf8^{neo/null}$ and MHB KO mutant brains at E17.5, stained with Cressyl Violet. The arrow in each panel points to the caudal limit of the posterior commissure, which marks the rostral limit of the midbrain. The bulk of the dorsal midbrain (tectum) is subdivided into the superior colliculus (rostral) and inferior colliculus (caudal). (C′-F′) Lateral sagittal sections of the same brains, assayed by immunohistochemistry with an antibody against tyrosine hydroxylase, which specifically stains nuclei in the ventrolateral midbrain (tegmentum) and anterior hindbrain. The pontine nuclei are present in all samples, but are outside of the field in the control. (G-J) Embryos at ~E10.5, assayed by whole-mount immunohistochemistry with an anti-neurofilament antibody to label the cranial nerves and ganglia. III, oculomotor nerve; IV, trochlear nerve; V, trigeminal nerve and ganglia; VII, facial nerve and ganglia; VIII, acoustic nerve and ganglia; Cb, cerebellum; CP, choroid plexus; Di, diencephalon; IC, inferior colliculus; LoC, locus ceruleus; Mb, midbrain; OB, olfactory bulb; PC, posterior commissure; PN, pontine nuclei; PPT, posterior pretectal nucleus; Pt, pretectum; SC, superior colliculus; SNC, substantia nigra pars compacta; Tel, telencephalon; VTA, ventral tegmental area.

1983), and by using the TUNEL assay, which detects apoptotic cells. Because some cell death is a normal part of brain development (Weil et al., 1997), dying cells were observed in control embryos at all stages assayed (Fig. 3, and data not shown). At the 2-5 somite stage (~E8.0-E8.25), the number and distribution of NBS-positive (NBS^+) cells appeared similar in MHB KO mutants (n=5) and control littermates (n=13). However, by the 7-9 somite stage (~E8.5), a small amount of ectopic cell death was detected in the mes/met of all MHB KO mutants assayed (NBS, n=6 mutants, 21 controls; TUNEL, n=1 mutant, 1 control; Fig. 3A, and data not shown), and by the 11-13 somite stage (~E8.75), the amount of ectopic cell death was dramatically increased throughout the mes/met (NBS, n=9 mutants, 17 controls; TUNEL, n=2 mutants, 3 controls). Interestingly, the dying cells appeared to be localized primarily in the alar plate (dorsal portion) (Fig. 3B, and data not shown). It seems unlikely that this is an artifact of the whole mount staining procedure because the mes/met section of the neural tube is open at these early stages, and therefore dorsal and ventral regions should have been equally accessible to NBS stain. Thus, our data show that cell death in the prospective mes/met increased as the level of functional *Fgf8* RNA decreased (Fig. 1D).

At the 14-15 somite stage (~E8.75-E9.0), the domain of ectopic cell death extended along the entire rostrocaudal length of the mes/met, and the prospective midbrain appeared smaller than normal (NBS, n=9 out of 10 mutants, 23 controls; TUNEL, n=1 mutant, 1 control; Fig. 3C, and data not shown). By the 19-20 somite stage (~E9.25), the mutant midbrain appeared to have been deleted, whereas the prospective

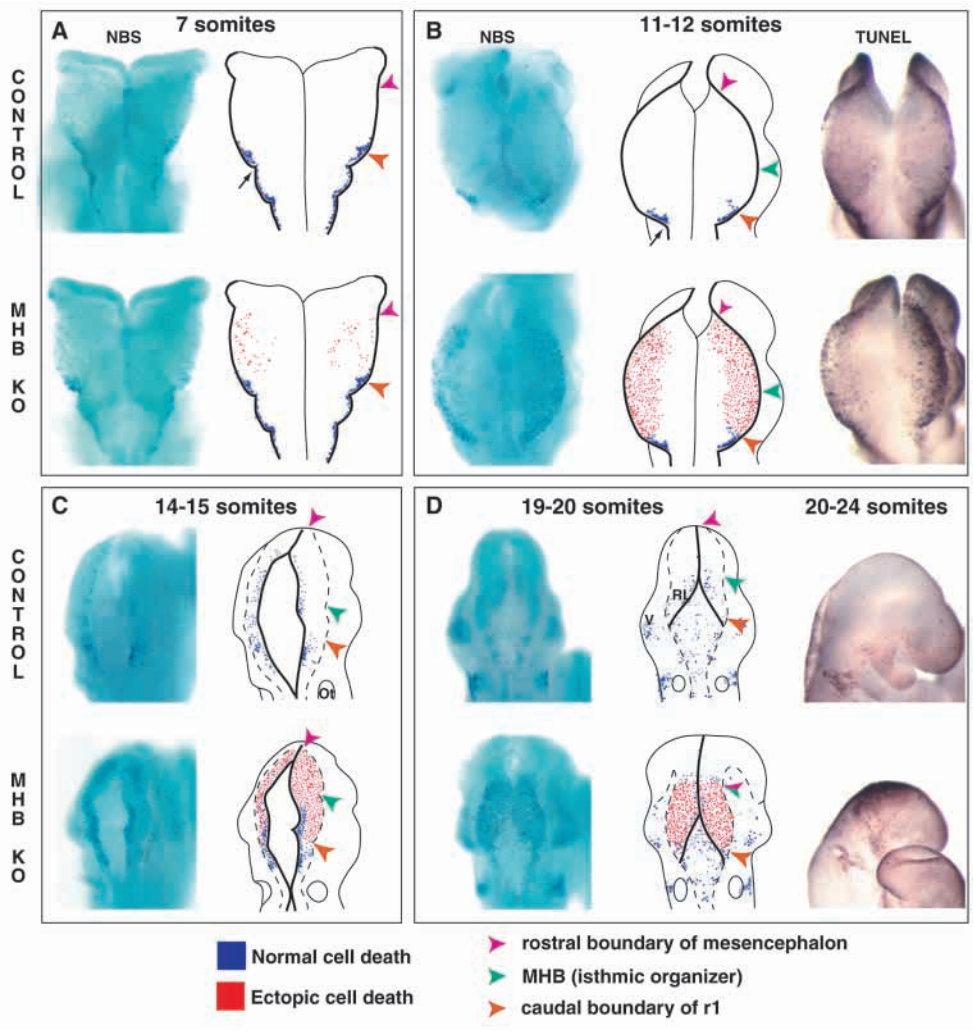

Fig. 3. Analysis of cell death in *Fgf8* MHB KO mutants. (A-D) Whole-mount control and MHB KO embryos assayed for cell death by staining with Nile Blue Sulfate (NBS) or by TUNEL at the stages indicated. All embryos are shown in dorsal view, except for the TUNEL-stained mutant (20 somite stage) and control (24 somite stage) embryos, which are shown in lateral view. Many dying cells are detected in control embryos, particularly at the lateral edges of the neural plate, which will fuse to form the dorsal midline of the neural tube. The schematic diagrams summarize the results of the assays. Areas of normal and ectopic cell death are indicated in blue and red, respectively. Pink and orange arrowheads indicate the approximate rostral border of the mesencephalon and caudal border of the anterior metencephalon (r1), respectively. These borders were determined by referring to morphological landmarks in the developing brain, including the preotic sulcus at the r2/r3 boundary at the 7-12 somite stage (arrow), as well as the locations of the descendants of *En1*-expressing cells, as marked by *lacZ* expression in $En1^{Cre/+}$ embryos carrying the R26R reporter gene (Fig. 1C, and data not shown). The green arrowhead indicates the approximate location of the isthmic organizer at the midbrain/hindbrain boundary, which was identified by referring to gene expression patterns in control and mutant embryos (Fig. 5, and data not shown). At the 19-20 somite stage, the midbrain vesicle is obvious in the NBS stained control embryo (between the pink and green arrowheads), but appears to be entirely deleted in the *Fgf8* MHB KO embryo (indicated by half pink, half green arrowhead). In the mutant embryos, the remaining mes/met tissue, in which ectopic cell death is readily detected, can be identified as rostral metencephalon by the presence of the rhombic lips. Note that at the 14-15 and 19-20 somite stages, the views of the mutant and control embryos are not strictly equivalent, because the curvature of the brain is decreased in the mutants as a result of tissue loss. Consequently, the rostral end of the mesencephalon is not visible in the photographs of the control embryos. Moreover, although the distance between the green and orange arrowheads is essentially the same in mutant and control embryos, it appears different because they are viewed from different angles. Ot, otocyst; RL, rhombic lip; V, trigeminal ganglia.

cerebellum was still present and contained many dying cells not observed in control embryos (NBS, *n*=5 mutants, 9 controls; Fig. 3D, and data not shown). During the period between the 21 and 29 somite stages, ectopic cell death continued to be detected by NBS staining in what remained of the mutant anterior hindbrain (*n*=32 mutants, 60 controls; Fig. 4C, and data not shown). Section analysis of embryos TUNEL-labeled in whole mount (Fig. 3B,D) showed that dying cells were localized in the neuroepithelium, primarily in the alar plate (not shown). By the 30 somite stage (~E10.0), the mutant prospective cerebellum appeared to have been deleted, and subsequently (30-40 somite stage), ectopic cell death was no longer detected (NBS, *n*=10 mutants, 12 controls; data not shown).

We observed a similar pattern of ectopic cell death in the mes/met of $Fgf8^{neo/null}$ embryos. However, as expected, these mutants differed from the MHB KO embryos in that ectopic cell death was also detected in other regions of the brain (Storm et al., 2003), as well as in other tissues (data not shown). No ectopic cell death was detected in $En1^{Cre/+}$,$Fgf8^{flox/+}$ control embryos, indicating that the effects observed in the MHB KO mutants cannot be attributed to cre expression. Together, our data show that complete loss of *Fgf8* function causes extensive cell death in the mes/met, and that the prospective midbrain is deleted before the prospective cerebellum.

Cell death in the mes/met of $Wnt1^{-/-}$ and $En1^{-/-}$ embryos

Deletions of midbrain and cerebellum have been observed following loss of function of several other genes, including *Wnt1* and *En1*. Although the phenotype of $Wnt1^{-/-}$ embryos can vary, tissue loss similar in extent to what we observed in

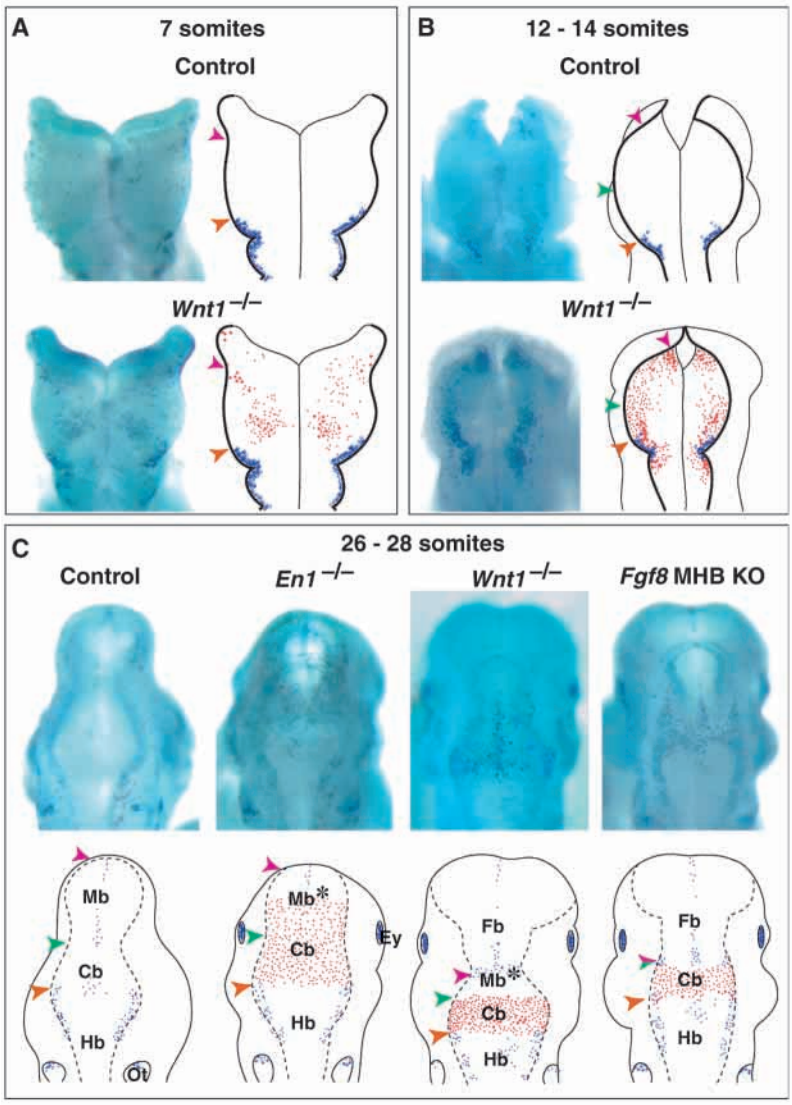

Fig. 4. Analysis of cell death in *Wnt1*$^{-/-}$, *En1*$^{-/-}$ and *Fgf8* MHB KO mutants. (A-C) Dorsal views of control and mutant embryos of the genotypes indicated, stained in whole mount with NBS. Schematic diagrams and arrowheads are as described in Fig. 3. In C, note that the midbrain appears partially truncated in the *En1*$^{-/-}$ embryo, mostly deleted in the *Wnt1*$^{-/-}$ embryo, and completely absent in the *Fgf8* MHB KO embryo (indicated by half pink, half green arrowhead). *, partial deletion; Fb, forebrain; Hb, hindbrain; Mb, midbrain.

Fgf8$^{neo/null}$ or MHB KO mutants has been observed (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Interestingly, it was previously reported that ectopic cell death was detected in the rostral dorsal metencephalon of *Wnt1*$^{-/-}$ embryos at the 20-30 somite stage, but not in the prospective midbrain at earlier stages (Serbedzija et al., 1996). However, we detected ectopic cell death throughout the mes/met of *Wnt1*$^{-/-}$ embryos at the 7-8 somite stage (n=8 mutants, 5 wild-type controls; Fig. 4A, and data not shown) and the 12-14 somite stage (n=6 mutants, 4 controls; Fig. 4B, and data not shown). At the 26-28 somite stage, most of the mutant midbrain was deleted, and ectopic cell death continued to be detected in what remained of the rostral metencephalon (n=3 mutants, 3 *Wnt1*$^{+/+}$ or *Wnt1*$^{+/-}$ controls; Fig. 4C, and data not shown).

Deletions of posterior midbrain and cerebellar tissue were also observed in embryos that lack Engrailed gene function. On some genetic backgrounds, *En1*$^{-/-}$ embryos have an altered midbrain and cerebellum phenotype (Wurst et al., 1994) similar to that of *Fgf8*$^{neo/neo}$ embryos at E17.5, whereas loss of function of both *En1* and *En2*, which are co-expressed in the mes/met, results in a more extensive deletion (Joyner, 1996) (W.W. and A. Joyner, unpublished observations), similar to what we observed in *Fgf8* MHB KO mutants. We detected an abnormal number of NBS^{+} cells in both the prospective posterior midbrain and cerebellum of embryos homozygous for *En1*^{Cre} (hereafter referred to as *En1*$^{-}$) at the 22-28 somite stage (n=9 mutants, 13 *En1*$^{+/-}$ or *En1*$^{+/+}$ controls; Fig. 4C, and data not shown). Together our data suggest that cell death is a likely cause of the midbrain and cerebellum deletions observed in embryos that lack *Wnt1* or *En1* function. Moreover, the pattern and timing of cell death in *Wnt1*$^{-/-}$ embryos is remarkably similar to what we observed in *Fgf8* MHB KO mutants, with deletion of the prospective midbrain occurring before deletion of the prospective cerebellum.

FGF8 is required to maintain gene expression in the mes/met

Gain-of-function studies have demonstrated that FGF8 can induce ectopic expression of several genes that are normally expressed in the mes/met, raising the possibility that FGF8 regulates their expression during normal brain development (reviewed by Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001). However, to definitively determine whether *Fgf8* is required to induce or maintain the expression of specific genes in the mes/met, loss-of-function experiments are required. We assayed gene expression in our MHB KO mutants at the 7-9 somite stage, when the level of functional *Fgf8* RNA was very reduced compared to normal (Fig. 1D), but before much ectopic cell death was detected (Fig. 3A, and data not shown). Four genes were found to be negatively affected by the loss of *Fgf8* function. *Wnt1* expression is normally upregulated and detected in a band at the caudal end of the prospective midbrain at the 7-9 somite stage (Parr et al., 1993) (Fig. 5A). In MHB KO mutants, no band of *Wnt1* expression was observed, although *Wnt1* RNA was detected at normal levels along the lateral edges of the open neural tube (Fig. 5B). Similarly, *Gbx2* RNA, which is normally detected in a band at the rostral end of the metencephalon (Bouillet et al., 1995; Fig. 5C), was not detected in this domain in MHB KO mutants (Fig. 5D). In contrast, *Gbx2* RNA was readily detected in the posterior hindbrain of both MHB KO mutants and control embryos (Fig. 5C,D). *Fgf17* and *Fgf18* expression, which is normally detected in the midbrain/hindbrain boundary region (Maruoka et al., 1998; Xu et al., 2000) (Fig. 5E,G) was either barely detected (Fig. 5F, and data not shown) or not detected (Fig. 5H)

in MHB KO mutants. At a later stage (12-15 somite stage), no expression of these genes was detected (data not shown). Together with studies of the normal expression patterns of these genes, our data suggest that FGF8 is required to induce or maintain *Fgf18* and to maintain *Wnt1, Gbx2* and *Fgf17* expression in the mes/met.

The specificity of these effects was demonstrated by experiments showing that cells in the midbrain/hindbrain boundary region of the mutant embryos were capable of expressing other genes at the 7-9 somite stages. For example, using a probe containing sequences present in all *Fgf8* transcripts in our embryos, we detected *Fgf8* RNA at the same level in MHB KO mutants as in their normal littermates (Fig. 5I,J, and data not shown). These data indicate that FGF8 is not required for transcription of *Fgf8* in the midbrain/hindbrain boundary region, similar to what has been observed in the limb (Sun et al., 2002). Likewise, we detected little difference between MHB KO mutants and control embryos in the levels of *Pax2* (Fig. 5K,L) or *Spry2* (data not shown) RNAs, whereas the level of *En1* RNA appeared to be slightly lower in the mutant than in control embryos (Fig. 5M,N). However, when the assays were performed several somite stages later, after *Fgf8* had been completely inactivated (see Fig. 1D), expression of these genes was detected in MHB KO mutants at very low levels, in small patches (Fig. 5Q-V, and data not shown) that may represent a remnant of the ventral mes/met or the rostral met. Thus, at later stages (13-16 somites), the reduction in *Pax2*, *Spry2*, and *En1* expression could be either due directly to lack of FGF8 activity or a secondary consequence of cell death.

At early neural plate stages, *Otx2* is normally expressed in a domain that stretches from the rostral end of the prospective forebrain to the caudal end of the prospective midbrain (Fig. 2O). Numerous studies have suggested that *Gbx2* expressed in the rostral metencephalon negatively regulates *Otx2* expression and restricts its caudal border to the midbrain/hindbrain boundary (Li and Joyner, 2001; Li et al., 2002; Martinez-Barbera et al., 2001), and that FGF8 may play a role in this process by regulating *Gbx2* expression in r1 (Garda et al., 2001; Irving and Mason, 2000; Liu et al., 1999; Martinez et al., 1999; Sato et al., 2001). In order to determine if the posterior limit of the *Otx2* expression domain was altered in MHB KO mutants, we assayed for *Otx2* RNA in combination with a probe for *Hoxa2*, which is strongly expressed in r3. At the 7-9 somite stage, we detected no obvious difference between the distance from the caudal end of *Otx2* expression domain to r3 in control and MHB KO mutants (Fig. 5O,P). However, by the 12-13 somite stage stage, this distance appeared to be slightly reduced in MHB KO as compared with control embryos (Fig. 5W,X), possibly reflecting a posterior shift in the caudal limit of *Otx2* expression, or a small amount of tissue loss.

DISCUSSION

We have shown that when the *Fgf8* gene is inactivated in the mes/met at early somite stages, the entire midbrain, isthmus and cerebellum are absent at E17.5, whereas tissues that

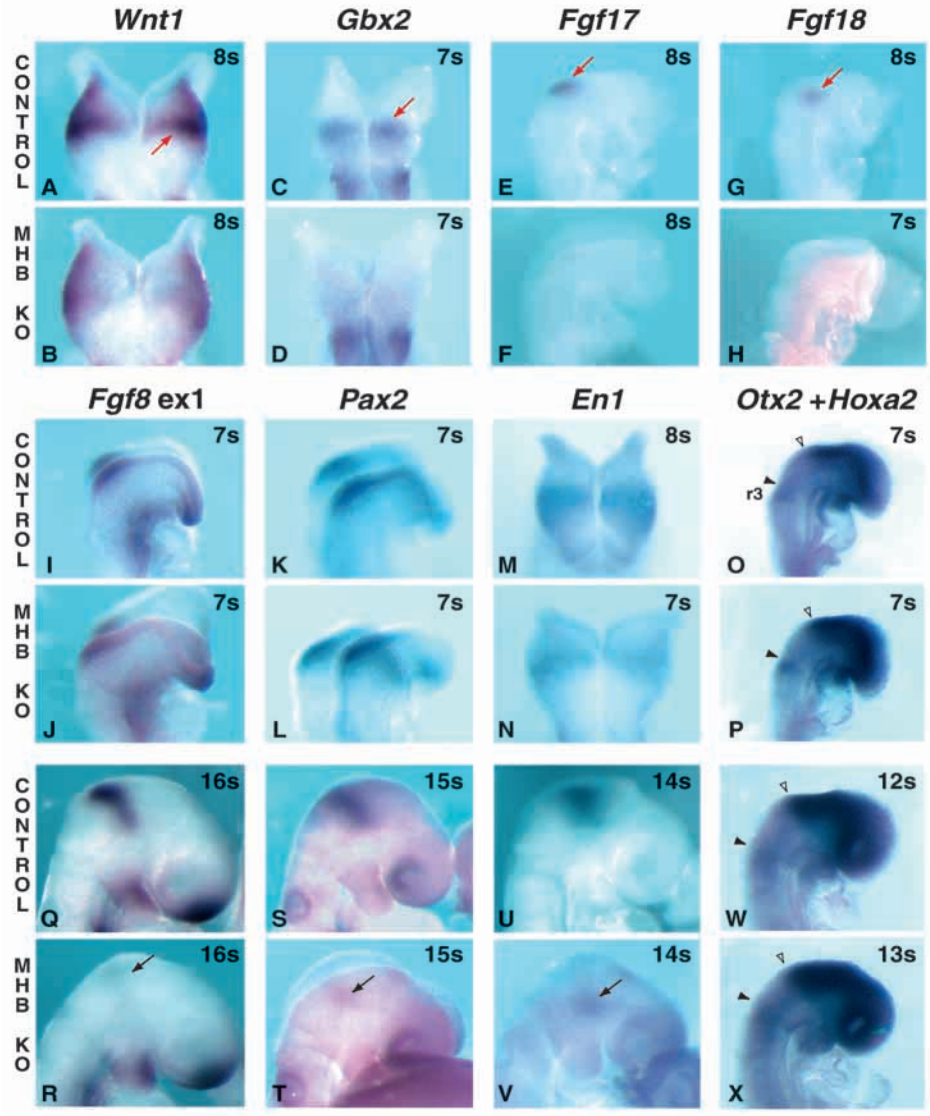

Fig. 5. Analysis of gene expression in *Fgf8* MHB KO mutants. Control embryos and MHB KO mutants at the somite stages indicated (s) were assayed by whole-mount RNA in situ hybridization using the probes indicated. Red arrows in A,C,E,G indicate the regions of gene expression in control embryos that are affected in MHB KO mutants. In M and N, the control and mutant embryos are both heterozygous for *En1*^{cre}. In O,P,W,X, note the distance between the caudal limit of the *Otx2* expression domain (open arrowhead) and the r2/3 border (black arrowhead), adjacent to the *Hoxa2* expression domain in r3. Black arrows in R,T,V indicate regions in MHB KO mutants in which a small amount of gene expression is detected.

originate just rostral and caudal to this region are essentially unaffected. The loss of these structures occurs prior to E10.0, when many of their cellular progenitors undergo apoptosis. There is a distinct temporal pattern to this cell death: the prospective midbrain is deleted approximately half a day before the cerebellum. The extent of tissue loss appears to be *Fgf8* dosage-dependent. Although the region encompassing the midbrain and cerebellum is not affected in $Fgf8^{+/-}$ embryos in which *Fgf8* is presumably expressed at ~50% of the normal level, slightly less *Fgf8* expression results in deletion of the dorsal posterior midbrain (posterior superior colliculus and inferior colliculus) and much of the cerebellum. Further reduction leads to a bigger deletion, with only the rostral-most midbrain derivative remaining. When *Fgf8* expression is completely eliminated, even that is absent. The *Fgf8* MHB KO phenotype closely resembles that of embryos lacking *Wnt1*, En or Pax gene function (discussed below), consistent with the hypothesis that these genes are involved in complex cross-regulatory interactions during mes/met development. Indeed, we show that *Fgf8* is required to maintain *Wnt1* expression in the prospective midbrain. Together, our data demonstrate that *Fgf8* expressed in the early neuroectoderm is an essential regulator of gene expression and cell survival during mes/met development.

Deletions of the midbrain and cerebellum in *Fgf8*, *Wnt1*, En and Pax mutant mice may be due to cell death

In our *Fgf8* MHB KO mice, those portions of the brain normally derived from the mes/met are deleted. Likewise, on certain genetic backgrounds, similar deletions are found in *Wnt1* (McMahon and Bradley, 1990), *En1/2* (Joyner, 1996) (W.W. and A. Joyner, unpublished observations) and *Pax2* (Bouchard et al., 2000) mutant mouse embryos. In zebrafish embryos, loss of function of a *Pax2* ortholog likewise causes deletion of the midbrain to cerebellum (Brand et al., 1996; Lun and Brand, 1998), whereas homozygosity for *ace*, a mutant allele of *Fgf8*, causes deletion of only the isthmus and cerebellum (Brand et al., 1996; Reifers et al., 1998). One possible reason why the midbrain is intact in *ace* mutants is that *ace* is a hypomorphic rather than a null allele (Draper et al., 2001). Alternatively, the midbrain may be rescued by some other FGF family member that is expressed in the zebrafish midbrain/hindbrain boundary region and is functionally redundant with *ace*.

Our data show that cell death is a major cause of the midbrain to cerebellum deletion in *Fgf8* MHB KO mutants, and that the cells die soon after they are deprived of FGF8. However, since FGF8 has been shown to stimulate cell proliferation in the mes/met (Lee et al., 1997; Xu et al., 2000), it is possible that some of the tissue is lost because the cells do not proliferate. Indeed, a failure to proliferate might possibly be the cause of the cell death we observed. Our data are consistent with previous studies showing that loss of *Fgf8* function results in cell death in the first branchial arch (Trumpp et al., 1999), the developing limb bud (Moon et al., 2000; Sun et al., 2002), and the early forebrain (Storm et al., 2003). There has been no evidence that cell death is the cause of the deletions in Pax or En mutant mouse embryos, but in $Wnt1^{-/-}$ embryos cell death was observed in the rostral metencephalon (Serbedzija et al., 1996). We show here that in $Wnt1^{-/-}$ embryos, there is also extensive cell death in the prospective midbrain, and that overall the pattern of ectopic cell death and deletion is very similar to the one we observed in *Fgf8* MHB KO mutants, except that in the *Wnt1* mutants a small portion of the prospective midbrain survived. This presumably reflects the variability that has been observed in the extent of the midbrain and anterior hindbrain deletion in $Wnt1^{-/-}$ embryos (McMahon and Bradley, 1990; Thomas and Capecchi, 1990).

We also detected ectopic cell death in both the prospective midbrain and cerebellum of $En1^{-/-}$ embryos. This is consistent with the phenotype of *En1* mutants described by Wurst et al. (Wurst et al., 1994), in which the posterior midbrain and much of the cerebellum was deleted. Although an analysis of cell death in the mes/met has not yet been reported in mouse Pax mutants, death of a large block of predominantly dorsal cells has been observed in the zebrafish *Pax2.1* mutant midbrain (Brand et al., 1996). Together, the data indicate that the midbrain to cerebellum deletions caused by loss of *Fgf8*, *Wnt1*, En and Pax gene function can be explained, in large measure, by a failure of cell survival.

Interestingly, our data indicate that the cell death that occurs as a result of loss of *Fgf8* function in the mes/met is localized primarily in the dorsal portion of the neural plate/tube. However, we also found that as early as E10.5, some ventral structures are absent. At present we do not know why dorsal and ventral mes/met cells respond differently to a loss of *Fgf8* function, or what causes the loss of ventral structures.

Loss of *Fgf8* function may be the cause of midbrain and cerebellum deletion phenotypes in other mutants

It has been suggested that loss of function of genes involved in specifying the mes/met very early in neuroectoderm development, such as Pax and En, causes cells to assume the wrong fate, to fail to develop normally, and therefore to die (Wurst et al., 1994). An alternative possibility is that the loss of function of such genes results in a failure to induce and/or maintain *Fgf8* expression, which is required for cell survival in the mes/met. This hypothesis is supported by the finding that *Fgf8* expression is not induced in $Pax2^{-/-}$ embryos (Ye et al., 2001), and is detected but then rapidly down-regulated in *En1/2* double null homozygotes (Liu and Joyner, 2001b). Thus loss of *Fgf8* function in both Pax and En mutant embryos occurs before there is any significant loss of mes/met tissue. In $Wnt1^{-/-}$ embryos, *Fgf8* expression appears normal at the 6 somite stage but is rapidly down-regulated (Lee et al., 1997), raising the possibility that the ectopic cell death that we observed in *Wnt1* null homozygotes is also due to loss of *Fgf8* function.

However, the tissue deletions observed in all these mutant might be due to a loss of *Wnt1* function, since the midbrain domain of *Wnt1* expression is not maintained in the absence of FGF8. There is ample evidence that both the FGF and WNT signaling pathways can regulate cell survival (reviewed by Feig and Buchsbaum, 2002; Patapoutian and Reichardt, 2000), so either FGF8 or WNT1, or both, could be the essential factor(s) required for cell survival in the mes/met. One intriguing possibility, consistent with their different domains of expression, is that *Wnt1* is required for cell survival in the prospective midbrain whereas *Fgf8* is required in the prospective cerebellum. If so, it should be possible to rescue

the cerebellum in *Wnt1*$^{-/-}$ embryos by expressing an *Fgf8* transgene, and the midbrain in *Fgf8* MHB KO mutants by expressing a *Wnt1* transgene in the midbrain/hindbrain boundary region.

Effects of loss of *Fgf8* function on gene expression in the mes/met

The observation that the various mutations that cause midbrain and cerebellum deletions also cause extensive cell death makes it difficult to interpret the significance of gene expression data in such mutants, except at the earliest stages of mes/met development. In our *Fgf8* MHB KO mutants, the stages at which we could perform a useful analysis were further restricted by the fact that *Fgf8* was transiently expressed before it was inactivated. Nevertheless, we were able to determine that in addition to maintaining *Wnt1* expression, *Fgf8* function is also required to maintain *Gbx2* expression in the rostral metencephalon, consistent with what has been concluded from gain-of-function experiments (Garda et al., 2001; Irving and Mason, 2000; Liu et al., 1999; Martinez et al., 1999; Sato et al., 2001). The expression of other genes thought to be regulated by *Fgf8*, such as *Pax2*, *En1*, and *Spry2* (reviewed by Liu and Joyner, 2001a; Wurst and Bally-Cuif, 2001), was not significantly reduced in *Fgf8* MHB KO embryos at early stages, when *Fgf8* expression is very low but before many cells have begun to die. This was particularly surprising for *Spry2*, since previous loss-of-function studies have demonstrated that *Spry2* expression is dependent on FGF signaling (Minowada et al., 1999). Presumably the low level of *Fgf8* expression at the 7 somite stage was sufficient to maintain *Spry2* expression.

The results of our analysis of *Otx2* expression in *Fgf8* MHB KO embryos are consistent with the extensive body of evidence that *Gbx2* expression in the rostral metencephalon determines the posterior limit of *Otx2* expression in the mes/met (Li and Joyner, 2001; Martinez-Barbera et al., 2001; Li et al., 2002). In the absence of *Gbx2* expression in r1, we detected a small decrease in the distance between the posterior limit of *Otx2* expression and r3, as marked by *Hoxa2* expression, in our mutant embryos at 13 som, which might reflect a caudal expansion of the *Otx2* expression domain. However, we cannot exclude the possibility that this effect is due to cell death.

One hypothesis about the role of FGF8 in mes/met development is that it functions to define the rostral limit of *Hoxa2* expression and restrict it to the r1/r2 boundary (Irving and Mason, 2000). Support for this hypothesis comes from an elegant series of studies in the chick embryo, including the demonstration that blocking FGF8 function in r1 by applying anti-FGF8 antibody resulted in anterior expansion of the *Hoxa2* expression domain into r1. We made an effort to test this hypothesis in our *Fgf8* MHB KO embryos. Unfortunately, although *Hoxa2* expression is relatively strong in chicken r2, it is extremely weak in mouse r2, and we were unable to determine whether it was expressed to a more rostral limit than normal in the mutant embryos we assayed at the 7 and 13 somite stages.

Interestingly, we found that FGF8 is essential for maintaining the expression of two other FGF family members, *Fgf17* and *Fgf18*, in the mes/met (Maruoka et al., 1998). These two FGF genes are closely related to *Fgf8* and encode proteins with similar biochemical and inductive properties (Ohuchi et al., 2000; Xu et al., 2000). It remains to be determined whether *Fgf17* and/or *Fgf18* are functionally redundant with *Fgf8* in mes/met development. Analysis of the phenotypes of *Fgf17*$^{-/-}$ and *Fgf17*$^{-/-}$,*Fgf8*$^{+/-}$ mice showed that *Fgf17* contributes to midbrain and cerebellar development, but abnormalities were not detected until ~E11.5, two days after the IsO is thought to function. This led Xu et al. (Xu et al., 2000), to propose that FGF genes have both an early and a late function in midbrain and cerebellar development: prior to E10, they contribute to IsO activity, and after E10, they maintain precursors of the cerebellar vermis in a proliferative, undifferentiated state. As yet, there is no evidence that *Fgf18* is required for midbrain or cerebellar development, since the brain appears normal in *Fgf18*$^{-/-}$ embryos (Liu et al., 2002; Ohbayashi et al., 2002). Analysis of *Fgf17/18* double null homozygotes should help to clarify this point. It would also be interesting to know if, in addition to its function at early stages, *Fgf8* also plays a role at a later stage. This could be determined by varying the timing of cre expression so that *Fgf8* is inactivated after E10.

Concluding remarks

The finding that a bead soaked in recombinant FGF8 can induce one or even two nearly complete, ectopic midbrains, as well as cerebellar tissue, led to the hypothesis that FGF8 functions to pattern the mes/met region. However, another interpretation of this observation is that FGF8 induces these structures only because it is capable of initiating the genetic cascade that leads to the formation of an ectopic IsO. Our data show that without FGF8 the entire mes/met fails to survive. Whether FGF8 directly participates in patterning this region remains unknown. Methods that enable cells to survive in the absence of FGF8 will need to be developed, to fully explore the role that FGF8 plays in mes/met development.

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