FGF8 can activate *Gbx2* and transform regions of the rostral mouse brain into a hindbrain fate

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Summary

The mid/hindbrain junction region, which expresses *Fgf8*, can act as an organizer to transform caudal forebrain or hindbrain tissue into midbrain or cerebellar structures, respectively. FGF8-soaked beads placed in the chick forebrain can similarly induce ectopic expression of mid/hindbrain genes and development of midbrain structures (Crossley, P. H., Martinez, S. and Martin, G. R. (1996) *Nature* 380, 66-68). In contrast, ectopic expression of *Fgf8a* in the mouse midbrain and caudal forebrain using a *Wnt1* regulatory element produced no apparent patterning defects in the embryos examined (Lee, S. M., Danielian, P. S., Fritzsch, B. and McMahon, A. P. (1997) *Development* 124, 959-969). We show here that FGF8b-soaked beads can not only induce expression of the mid/hindbrain genes *En1*, *En2* and *Pax5* in mouse embryonic day 9.5 (E9.5) caudal forebrain explants, but also can induce the hindbrain gene *Gbx2* and alter the expression of *Wnt1* in both midbrain and caudal forebrain explants. We also show that FGF8b-soaked beads can repress *Otx2* in midbrain explants. Furthermore, *Wnt1-Fgf8b* transgenic embryos in which the same *Wnt1* regulatory element is used to express *Fgf8b*, have ectopic expression of *En1, En2, Pax5* and *Gbx2* in the dorsal hindbrain and spinal cord at E10.5, as well as exencephaly and abnormal spinal cord morphology. More strikingly, *Fgf8b* expression in more rostral brain regions appears to transform the midbrain and caudal forebrain into an anterior hindbrain fate through expansion of the *Gbx2* domain and repression of *Otx2* as early as the 7-somite stage. These findings suggest that normal *Fgf8* expression in the anterior hindbrain not only functions to maintain development of the entire mid/hindbrain by regulating genes like *En1, En2* and *Pax5*, but also might function to maintain a metencephalic identity by regulating *Gbx2* and *Otx2* expression.

Key words: FGF8, Mid/hindbrain patterning, *Gbx2*, *Otx2*, Mouse

INTRODUCTION

Anteroposterior patterning of the vertebrate central nervous system begins to be established early during gastrulation (reviewed by Beddington and Robertson, 1999), and local signaling centers play very important roles in refining the identity of neural tissues at specific anteroposterior positions later during development. Two local signaling centers that have been identified are the anterior neural ridge (ANR, Shimamura and Rubenstein, 1997; Rubenstein et al., 1998) and the junction between the future midbrain and hindbrain (also called the isthmus, see reviews by Joyner, 1996; Wassef and Joyner, 1997). Tissue transplantation experiments between quail and chick embryos have shown that, when isthmic tissue is grafted to ectopic sites in the brain, it not only maintains its own identity but also can induce expression of mid/hindbrain genes such as *En2* and *Wnt1* in surrounding tissues. Later, ectopic development of midbrain structures occurs in the caudal diencephalon, or cerebellar structures in the caudal hindbrain (Gardner and Barald, 1991; Itasaki et al.,

1991; Martinez et al., 1991; Bally-Cuif et al., 1992; Bally-Cuif and Wassef, 1994; Marin and Puelles, 1994). These experiments showed that the isthmic region is determined earlier than adjacent tissue and that it contains signal(s) sufficient to induce ectopic development of midbrain tissue in the caudal forebrain and cerebellum in the caudal hindbrain.

Aspects of the molecular mechanisms underlying this organizer activity and patterning of the midbrain and anterior hindbrain (mesencephalon and metencephalon, respectively, referred to as mes/met) have been revealed by genetic studies of a group of transcription factors, EN1, EN2, PAX2, PAX5, OTX2 and GBX2, and of two secreted factors, WNT1 and FGF8, which are expressed in this region (see Fig. 8) and required for midbrain and cerebellar development (reviewed in Wassef and Joyner, 1997). Mice homozygous for null mutations in *Wnt1*, which is expressed early in the mesencephalon and later in the isthmus, do not develop derivatives of the mesencephalon or metencephalon (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas

and Capecchi, 1990). Mutations in *En1*, which is expressed across the mes/met, cause a similar, but milder phenotype (Wurst et al., 1994), whereas, mutations in *En2* lead to subtle defects in tectum and cerebellum development (Joyner et al., 1991; Millen et al., 1994). However, mice with mutations in both *En* genes lack the entire mes/met (W. Wurst and A. J., unpublished data) and *En2* can rescue the *En1* mutant brain defects when it is expressed in place of *En1* (Hanks et al., 1995, 1998), revealing overlapping functions between the two *En* genes. A similar deletion of the mes/met also was found when both mouse *Pax2* and *Pax5*, or the zebrafish *Pax2* homolog *pax2.1*, are mutated (Schwarz et al., 1997; Urbanek et al., 1997; Krauss et al., 1992; Brand et al., 1996; Lun and Brand, 1998). These *Pax* genes, like the *En* genes, are expressed in most of the mes/met.

In contrast to the similar phenotypes produced by mutations in these mes/met genes, mutations in *Otx2* and *Gbx2* alter the midbrain or hindbrain differentially. *Otx2* expression in neural tissue is restricted to the forebrain and midbrain from presomitic stages (Simeone et al., 1992). The first phenotype

seen in *Otx2* mutants, however, arises during gastrulation and includes deletion of the brain anterior to rhombomere 3 (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). It has been shown in chimera and knock-in studies that this phenotype results from a lack of *Otx2* in the visceral endoderm and that later expression of *Otx2* in the anterior neural tube is essential for maintenance of the forebrain and midbrain (Rhinn et al., 1998; Acampora et al., 1998). A function of *Otx1* and *Otx2* in patterning the forebrain and midbrain was addressed by analyzing $Otx1^{+/-}$; $Otx2^{+/-}$ (Suda et al., 1997) or $Otx1^{-/-}$; $Otx2^{+/-}$ (Acampora et al., 1997) mutant mouse embryos. Strikingly, in such mutants, the *Gbx2* domain is expanded anteriorly and the midbrain and caudal forebrain are transformed into a cerebellar fate. A mutation in *Gbx2*, which is expressed posterior to *Otx1* and *Otx2*, causes a complementary phenotype in which the anterior hindbrain (r1-r3) is deleted and the *Otx2* domain and tectum are expanded caudally, suggesting that *Gbx2* might be involved in maintaining the fate of the anterior hindbrain (Wassarman et al., 1997; Millet et al., 1999). The *Gbx2* and *Otx2* mutant
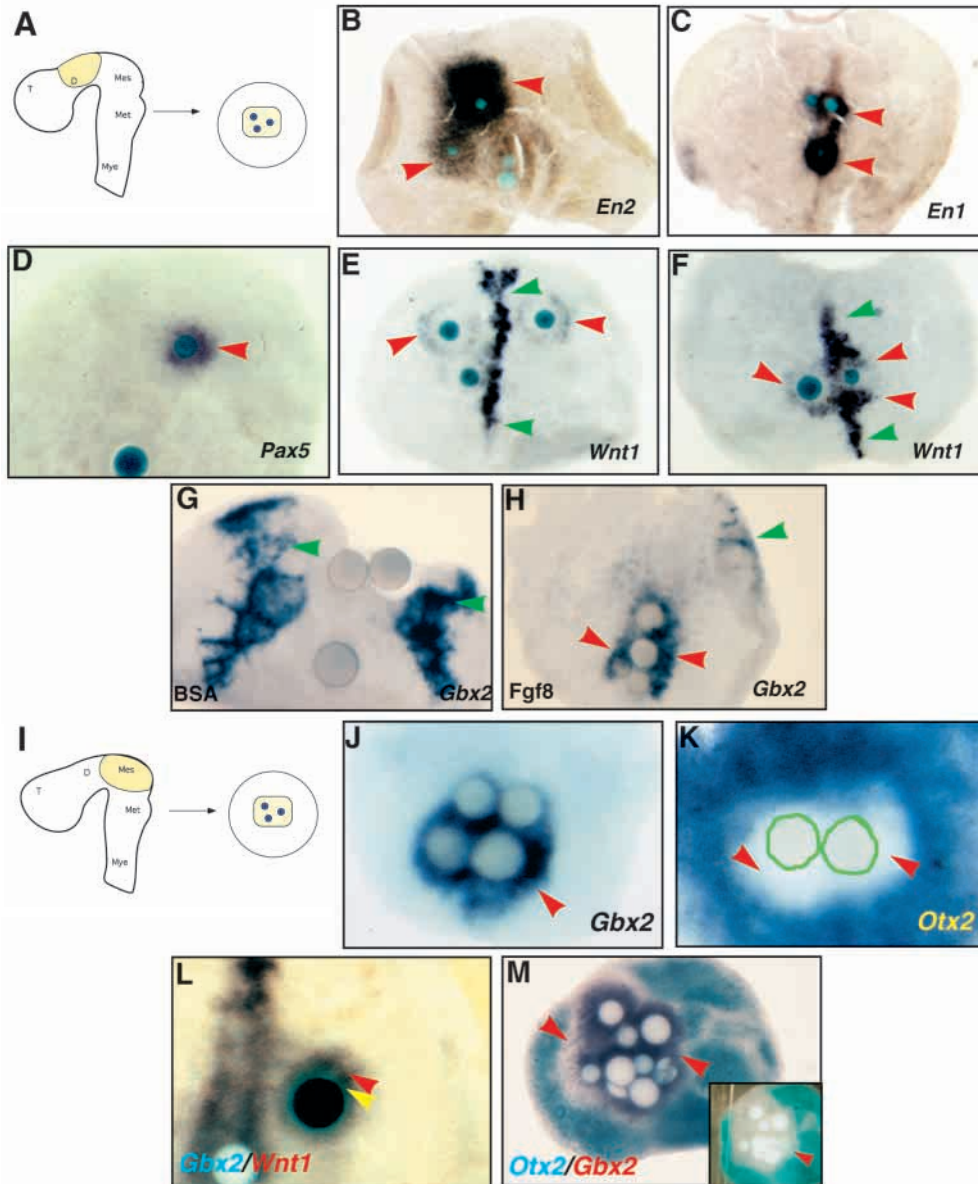

Fig. 1. FGF8b can induce *Gbx2* and alter *Wnt1* in both midbrain and caudal forebrain explants and repress *Otx2* in midbrain explants. (A) Schematic diagram showing the caudal alar plate forebrain tissue, p1, p2, taken at E9.5 and cultured in vitro with FGF8b protein-soaked beads in experiments shown in B-H. (B-D) After 40 hours in culture, the expression of *En1, En2* and *Pax5* (red arrowhead) was seen induced around the FGF8b-soaked beads (blue). (E,F) *Wnt1* expression is always several cell diameters away from the beads (E,F) and endogenous midline expression (green arrowheads) was rearranged when the beads were placed near the *Wnt1* domain (F). (G) Endogenous *Gbx2* is not repressed by BSA beads. (H) Endogenous *Gbx2* is repressed whereas ectopic *Gbx2* is induced in cells near FGF8b beads. (I) Schematic diagram showing the mesencephalic tissue taken at E9.5 and cultured in vitro with FGF8b protein-soaked beads in experiments shown in J-M. (J) After 40 hours in culture, expression of *Gbx2* (blue) was induced in cells around the FGF8b-soaked beads (pale yellow). (K) *Otx2* expression (blue) was repressed (arrowheads) in cells surrounding the FGF8b-soaked beads (outlined in green). (L) *Wnt1* (purple, red arrowhead) is induced outside the *Gbx2* domain (blue, yellow arrowhead). (M) *Gbx2* (purple) is induced in cells where *Otx2* (blue) is repressed. Inset shows the same explant after *Otx2* staining and before *Gbx2* staining.
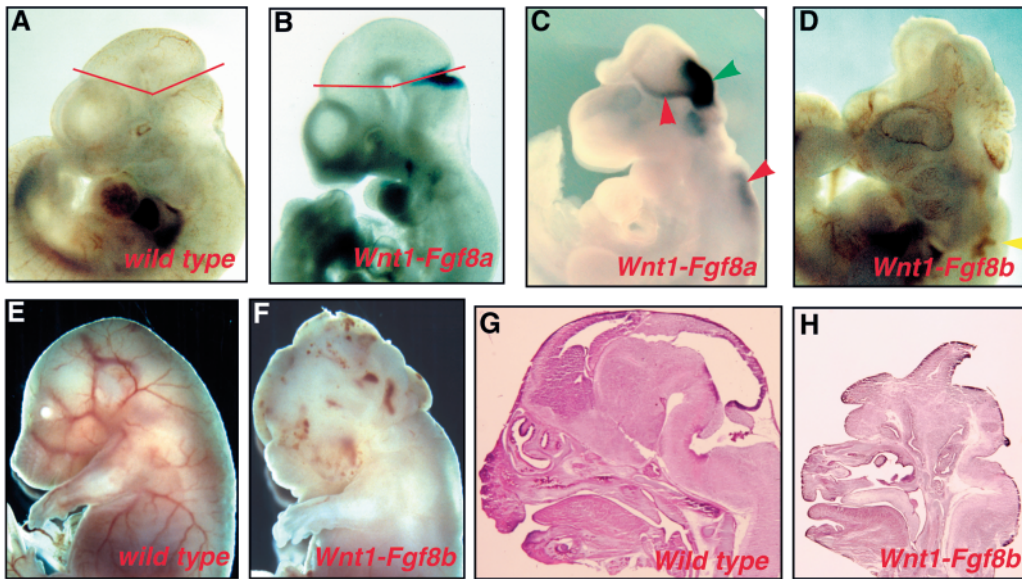
Fig. 2. Ectopic expression of *Fgf8b* in transgenic mice produces a more extreme phenotype than *Fgf8a*. (A-D) E10.5 embryos representing a (A) wild type, (B) *Wnt1-Fgf8a* transgenic with the most prevalent phenotype, an overgrowth of the di/mes region (structures above the red line). An *En2-lacZ* transgene is also present in this embryo marking the isthmus with β-galactosidase expression (blue), (C) *Wnt1-Fgf8a* transgenic embryo showing the exencephaly phenotype and ectopic *En2* expression in the lateral edges of the midbrain and dorsal midline of the hindbrain and spinal cord (red arrowheads). Green arrowhead points to the *En2* expression across the mes/met. (D) *Wnt1-Fgf8b* transgenic, showing extreme exencephaly with an open neural tube anterior to rhombomere 4. Yellow arrowhead points to the otic vesicle. (E,F) Lateral view of the whole head of an E15.5 wild-type (E) and a *Wnt1-Fgf8b* (F) embryo. (G,H) Mid-sagittal section of the same E15.5 wild type (G) and transgenics (H).
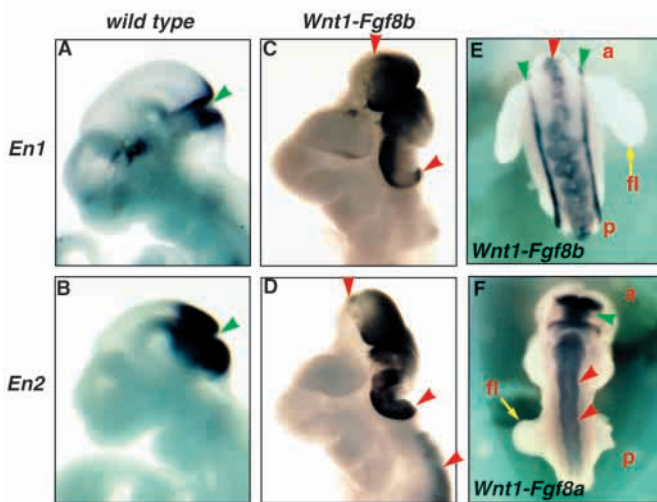
Fig. 3. Both *En-1* and *En-2* are expressed ectopically in E10.5 *Wnt1-Fgf8b* transgenic embryos. (A,B) Wild-type embryos showing that expression of both *En* genes is restricted to the mes/met, with *En1* occupying a narrower domain (green arrowhead). (C,D) A *Wnt1-Fgf8b* embryo was bisected in half at the midline and each side is shown in lateral view. (C) *En1* and (D) *En2* expressions were detected by whole-mount RNA in situ analysis. Expression of both *En* genes was extended rostrally and caudally and along the dorsal (lateral in exencephalic regions) brain and spinal cord (red arrowheads). (E) *En1* expression is shown to be induced in the dorsal spinal cord of an E10.5 *Wnt1-Fgf8b* embryo (red arrowhead). (F) *En2* expression is shown to be induced in an E10.5 *Wnt1-Fgf8a* embryo that had exencephaly. In both E and F, anterior (a) is up and posterior (p) is down, green arrowheads point to endogenous *En* expression and yellow arrows indicate the forelimbs (fl).
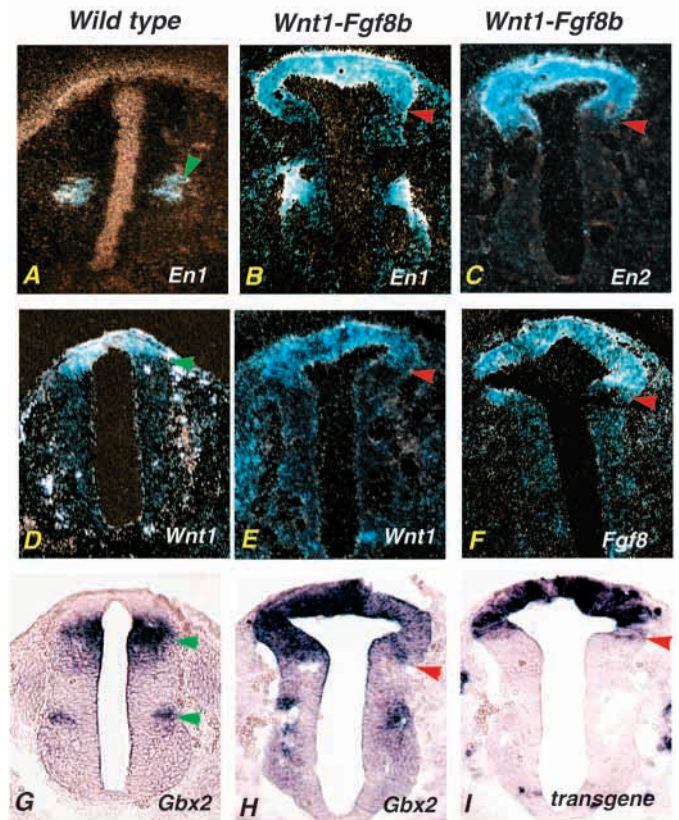
Fig. 4. Ectopic *Fgf8* expression produces abnormal morphology, expanded *Wnt1* expression and ectopic induction of *En1, En2* and *Gbx2* expression in the dorsal spinal cord at E10.5. Cross-sections at the lumbar level are shown. Green arrowheads in A, D and G point to endogenous gene expression. Red arrowheads in B, C, E, F, H and I point to the boundary of gene expression in dorsal spinal cord.

phenotypes together suggest that these genes also are involved in positioning and normal functioning of the organizer.

Fgf8 was first suggested to play a role in patterning the midbrain and cerebellum in vertebrates based on its expression in the isthmic region (Heikinheimo et al., 1994; Crossley and Martin, 1995) and then shown to be capable of mimicking isthmic transplants in the diencephalon (Crossley et al., 1996a). In the chick diencephalon, beads soaked in FGF8b or FGF4 can induce ectopic tectum development, as well as ectopic expression of *En2, Fgf8* and *Wnt1*, whereas in the hindbrain, FGF8b could not induce these genes or cause morphological changes. In contrast to the chick experiments, *Wnt1-Fgf8a* transgenic mouse embryos expressing *Fgf8a* from a *Wnt1* enhancer in the mesencephalon and the roof plate were reported to have an enlarged midbrain and caudal diencephalon, without an obvious morphological transformation, although *En2* expression was expanded in the dorsal midbrain and hindbrain (Lee et al., 1997). Loss-of-function studies have shown that FGF8 is required for development of both the midbrain and cerebellum. A *Fgf8* mutation in zebrafish thought to produce a hypomorphic allele results in loss of the cerebellum (Brand et al., 1996; Reifers et al., 1998). A hypomorphic mutation in mouse *Fgf8* leads to a more severe deletion with much of the midbrain and cerebellum lost, while a null mutation causes an earlier gastrulation defect (Meyers et al., 1998).

Many alternatively spliced isoforms of mouse *Fgf8* mRNA have been characterized (Tanaka et al., 1992; Crossley and Martin, 1995), and all seem to be found in the various embryonic regions where *Fgf8* is expressed. However, cell transformation assays and receptor affinity assays have indicated that isoform b has the strongest activity (MacArthur et al., 1995a,b; Blunt et al., 1997). Thus, one possible explanation for the difference between the in vivo gain-of-function studies in chick and mouse is that different isoforms of FGF8 were used. Indeed, short-term explant studies using 3- to 5-somite mouse anterior brain tissue indicated that FGF8b could induce *En2* in posterior regions of the explants within 24 hours (Shimamura and Rubenstein, 1997). We have explored the roles of FGF8a and FGF8b in anteroposterior patterning of the mouse midbrain and cerebellum using both in vitro explant assays and in vivo transgenic analysis. In explant assays, we found FGF8b could not only induce expression of the mid/hindbrain genes *En1, En2* and *Pax5* in the caudal diencephalon, but it could also induce the hindbrain gene *Gbx2* and alter the expression of *Wnt1* in both midbrain and caudal forebrain tissues. We also show that FGF8b could repress the anterior gene *Otx2* in midbrain explants. To examine whether ectopic expression of *Fgf8b* is sufficient in vivo to re-specify the central nervous system along the anteroposterior axis, we used the *Wnt1* enhancer to generate a new domain of *Fgf8a* or *Fgf8b* expression in the midbrain at early somite stages and in the isthmus and roof plate of the hindbrain and spinal cord at later stages. Our results show that early ectopic expression of *Fgf8a* or *Fgf8b* in the mesencephalon can cause exencephaly and is capable of inducing expression of mes/met genes in the brain and spinal cord. Furthermore, instead of inducing midbrain structures, FGF8b transformed the midbrain and caudal diencephalon into a metencephalic fate, which correlated with the induction of *Gbx2* and repression of *Otx2,* as early

as E8.5. A model is proposed to account for the various chick and mouse gain-of-function studies with FGF8.

MATERIALS AND METHODS

Explant culture

One package (25 µg, in 1.25 mg BSA) of FGF8b protein (R&D) was reconstituted in PBS (Cellgro) to a final concentration of 1 mg/ml FGF8b. Blue gel affi-gel beads (Pharmacia) or Heparin acrylic beads (Sigma) were rinsed in 50 volume PBS 4-6 times and then about 100 beads were soaked in 12 µl FGF8b solution for 1 hour at 37°C or overnight at 4°C for each type of bead, respectively (Ye et al., 1998; Furuta et al., 1997). The beads were then rinsed in PBS three times and two or three beads were applied to each explant. Control beads were soaked in 50 mg/ml BSA (Sigma) in PBS in the same manner.

Outbred Swiss Webster (SW, Taconic) mouse embryos were dissected in ice-cold L15 medium (Cellgro) at embryonic day 9.5 (E9.5). Explants were made by cutting out the alar plate of either the diencephalic or mesencephalic region along with the overlying ectoderm and mesoderm and placing it on a Micropore filter membrane (Costar) floating on Dulbecco's Modified Eagle's Medium (DMEM, Cellgro) supplemented with 20% Fetal Bovine Serum (Gibco) and 1× Non-essential Amino Acid (100× stock from Gibco). The beads were then added and the explants cultured at 37°C, 10% CO_2 for 16-40 hours.

At the end of culture, the membranes were transferred into fresh 4% paraformaldehyde to let the explant float off the membrane. Then the explants were fixed in 4% paraformaldehyde at 4°C overnight, washed with PBS, 2× 5 minutes, dehydrated through 25%, 50% and 75% methanol and stored in methanol at −20°C.

Fgf8 misexpression transgenes

A full-length *Fgf8b* cDNA (kind gift from Dr MacArthur of Washington University at St Louis; Tanaka et al., 1992) was subcloned into the *Wexp3* vector (*Wnt1*-misexpression vector, Dr McMahon, Harvard University, Echelard et al., 1994). The resulting *Wexp3-Fgf8b* construct, referred to as *Wnt1-Fgf8b*, was released from the plasmid using *Sal*I digestion. The *Wexp3-Fgf8a* construct (Lee et al., 1997), referred to as *Wnt1-Fgf8a*, was a kind gift from Dr McMahon and it was also released from the plasmid with *Sal*I.

Production of transgenic embryos

Transgenic mice were made as described by Hogan et al. (1994). SW mice (Taconic) were used to generate the fertilized eggs. Injected eggs were cultured overnight in M16 medium and transferred at the 2-cell stage into the oviduct of a pseudopregnant female mouse that had been mated the night before. The day when embryos were transferred was designated E0.5. Staged transgenic and wild-type embryos were dissected at E8.5, E9.5 and E10.5 into PBS and fixed in 4% paraformaldehyde overnight, rinsed with PBS and photographed using a M10 stereo photomicroscope (Leica) using either a Polaroid Digital Microscopic camera or Kodak 64T film. Embryos were then dehydrated through 25%, 50% and 75% methanol in PBS and stored in 100% methanol at −20°C.

The genotypes of embryos were determined by PCR analysis of genomic DNA extracted from embryonic yolk sacs. The primers used for *Wnt1-Fgf8b* were directed against the *lacZ* sequences present in the transgene construct; 5′ primer, CCG AAC CAT CCG CTG TGG TA and 3′ primer, CAT CCA CGC GCG CGT ACA TC. The following primers were used to identify the *Wnt1-Fgf8a* transgene; 5′ primer, CTG CTC CCT CAC ATG CTG TG and 3′ primer, CTT ATT AGA GCC AGC CTG G 3′. The PCR program included 30 cycles of: 94°C, 1 minute; 61°C, 1 minute; 72°C, 1 minute. PCR products were visualized on ethidium-bromide-stained agarose gels.

To determine whether the transgene was expressed in the CNS as

expected, the tail regions of E9.5-E10.5 embryos were used to do whole-mount RNA in situ hybridization using *lacZ*- or *Fgf8*-specific RNA probes (see below). For the *Wnt1-Fgf8b* transgene, of a total of 225 embryos obtained between E8.5 and E10.5, 75 were transgenic and of these, 38 expressed the transgene, all of which showed the reported phenotype. For the *Wnt1-Fgf8a* transgene, a total of 219 E10.5 embryos were dissected and, of these, 32 carried the transgene and 11 embryos expressed the transgene. 4 of the 11 showed the exencephaly phenotype and the other 7 showed an expanded midbrain and diencephalon.

Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization of embryos was performed as described (Matise and Joyner, 1997). Antisense riboprobes used for in situ hybridization were prepared using previously published mouse sequences. *En1, En2* (Millen et al., 1995), *Fgf8* (Crossley and Martin, 1995), *Gbx2* (Bouillet et al., 1995), *Wnt1* (Parr et al., 1993), *Pax2, Pax5, Pax8* (Asano and Gruss, 1992), *Pax6* (Grindley et al., 1997) and *Otx2* (Simeone et al., 1993). Embryos were fixed, washed and dehydrated as described above. The embryos were rehydrated immediately before whole-mount RNA in situ hybridization was performed. After hybridization, embryos were washed in PBT (0.1% Tween-20 in PBS) overnight at 4°C, refixed in 4 % paraformaldehyde overnight at 4°C and stored in 70% glycerol in PBS at 4°C. Modifications were made for double in situ as described by Hecksher-Sorensen et al. (1998).

Section RNA in situ hybridization

Frozen sections were hybridized with ^{35}S-labeled antisense riboprobes using a protocol similar to the one described by Hui and Joyner (1993). The probes used were prepared using previously published mouse sequences; *Wnt7b* (Parr et al., 1993) and *Meis-2* (Toresson et al., 1999), and those used for whole-mount analysis. ^{35}S-labeled sections were counterstained with Toluidine Blue and mounted. Digoxigenin-labeled sections were counterstained with Nuclear Fast Red and mounted. Pictures were taken using a DR MXE microscope (Leica) and Polaroid Digital Camera.

RESULTS

FGF8b protein can induce *Gbx2* and alter *Wnt1* in both mesencephalic and diencephalic explants and repress *Otx2* expression in midbrain explants

As one approach to test whether FGF8b was capable of inducing mes/met genes in the mouse embryonic forebrain, we performed a pilot experiment in which FGF8b-soaked beads were placed in diencephalic explants taken at E8.5, E9.5 and E10.5, and analyzed for *En2* expression after 40 hours. At all stages *En2* was found to be induced (data not shown). We therefore performed additional explant assays at E9.5 when the morphological landmarks between telencephalon, diencephalon, mesencephalon and metencephalon are clearly visible as constrictions and the nature of the explanted tissue can be confirmed by checking the expression of marker genes specific for each domain. Explants were taken from the dorsal and lateral part of the diencephalon, such that they consisted mostly of prosomere 1 (p1) and p2 (Fig. 1A). To confirm that no midbrain tissue was included in the explants, RNA in situ analysis was done on parallel control explants that were not cultured using the mesencephalic marker gene *Meis-2* (Cecconi et al., 1997; Oulad-Abdelghani et al., 1997, data not shown).

Whole-mount RNA in situ hybridization was done after

culturing each explant with two or three FGF8b beads for 16 or 40 hours. After 40 hours of culture, expression of the mes/met genes *En1* (in 24 explants), *En2* (in 17 out of 18 explants) and *Pax5* (in 4 explants) were found to be induced in the cells adjacent to the FGF8b beads (Fig. 1B-D). However, no induction of *Pax2* (in 12 explants) or *Pax8* (in 5 explants) could be detected (data not shown). Surprisingly, we failed to see *Fgf8* expression either after 16 hours (in 6 explants) or 40 hours (in 6 explants) of culture (data not shown). We did however, find that *Wnt1* expression was altered by FGF8b beads (in 3 explants). Interestingly, in contrast to the other genes examined, *Wnt1* expression was only seen in cells several cell diameters away from the beads (Fig. 1E,F). When beads were placed at a distance from the midline, the induced *Wnt1* expression seemed to be separate from the endogenous one, leaving the endogenous expression undisturbed (Fig. 1E). In contrast, when the beads were placed adjacent to the endogenous midline *Wnt1*-expressing cells (green arrows in Fig. 1E,F), the endogenous expression was altered such that the cells adjacent to the beads were always devoid of *Wnt1* expression and *Wnt1*-positive cells were induced in a 'halo' around the beads, several cell diameters from them (Fig. 1F).

We also examined the expression of *Gbx2* and *Otx2* in diencephalic explant. In control explants *Gbx2* was found to be expressed in the lateral region of the explants after 40 hours in culture (in 5 explants, data not shown), consistent with the fact that *Gbx2* is normally expressed in the dorsal thalamus at E11.5 (Bouillet et al., 1995). This endogenous expression was not altered by BSA-soaked beads (Fig. 1G, in 2 explants) but was greatly reduced (Fig. 1H, in 2 explants) or abolished (6 explants, not shown) in the presence of FGF8b-soaked beads. In addition, FGF8b induced ectopic *Gbx2* expression around the beads (Fig. 1H). Endogenous *Otx2* expression in control diencephalic explants was variable with some explants showing *Otx2* in patches and there were *Otx2*-negative cells scattered among *Otx2*-positive cells (9 explants examined, not shown). Similar results were obtained when the explants were cultured with BSA-soaked beads (in 3 explants, not shown). The expression of *Otx2* after 40 hours in culture with FGF8b beads was more variable. In some of the explants (7/21, not shown), *Otx2* expression was similar to the control explants, although the expression seemed a little weaker around the beads. In other explants (14/21, not shown), *Otx2* expression seemed to be greatly downregulated in most cells in the middle of the explant, but this downregulation did not seem to be restricted to the vicinity of the beads. Because of the variable expression of *Otx2* in control and FGF8b-treated explants, it is not clear whether FGF8b can regulate *Otx2* in the diencephalon at this stage.

We also analyzed the expression of *Otx2, Gbx2* and *Wnt1* in mesencephalic explants (Fig. 1I); the expression of *En1, En2* and *Pax5* was not examined because they are normally expressed in the caudal midbrain. The expression of *Gbx2* (in 7 out of 8 explants) and *Wnt1* (in 6 explants) in response to FGF8b beads was similar to that in the diencephalon; both genes were induced, *Gbx2* adjacent to the beads (Fig. 1J) and *Wnt1* at a distance (data not shown). *Otx2* was found to be maintained in control cultures (10 explants with BSA beads) and repressed in cells adjacent to FGF8b-soaked beads (Fig. 1K, in 8 explants). Double in situ hybridization showed that the *Wnt1* domain was adjacent to the *Gbx2* domain, although

Fig. 5. *Wnt1-Fgf8b* transgenics lack a midbrain at E9.5. (A-D) Adjacent sagittal sections of E10.5 wild-type (A,C) and transgenic (B,D) embryos showing that *En2* and *Pax6* expression abut each other in *Wnt1-Fgf8b* transgenics (arrowheads in B and D) rather than being separated as in wild-type embryos by a rostral midbrain region that does not express either gene (between arrowheads in A and C). (E-H) RNA in situ analysis in adjacent sagittal sections of E9.5 wild-type (E,G) and transgenic (F,H) embryos using either a *Pax6* probe (E,F) or a *Gbx2* probe

(G,H). (I,J) Double in situ hybridization of an E9.5 wild-type (I) and transgenic (J) embryo using both a *Pax6* (purple/red) and a *Gbx2* (blue) probe.
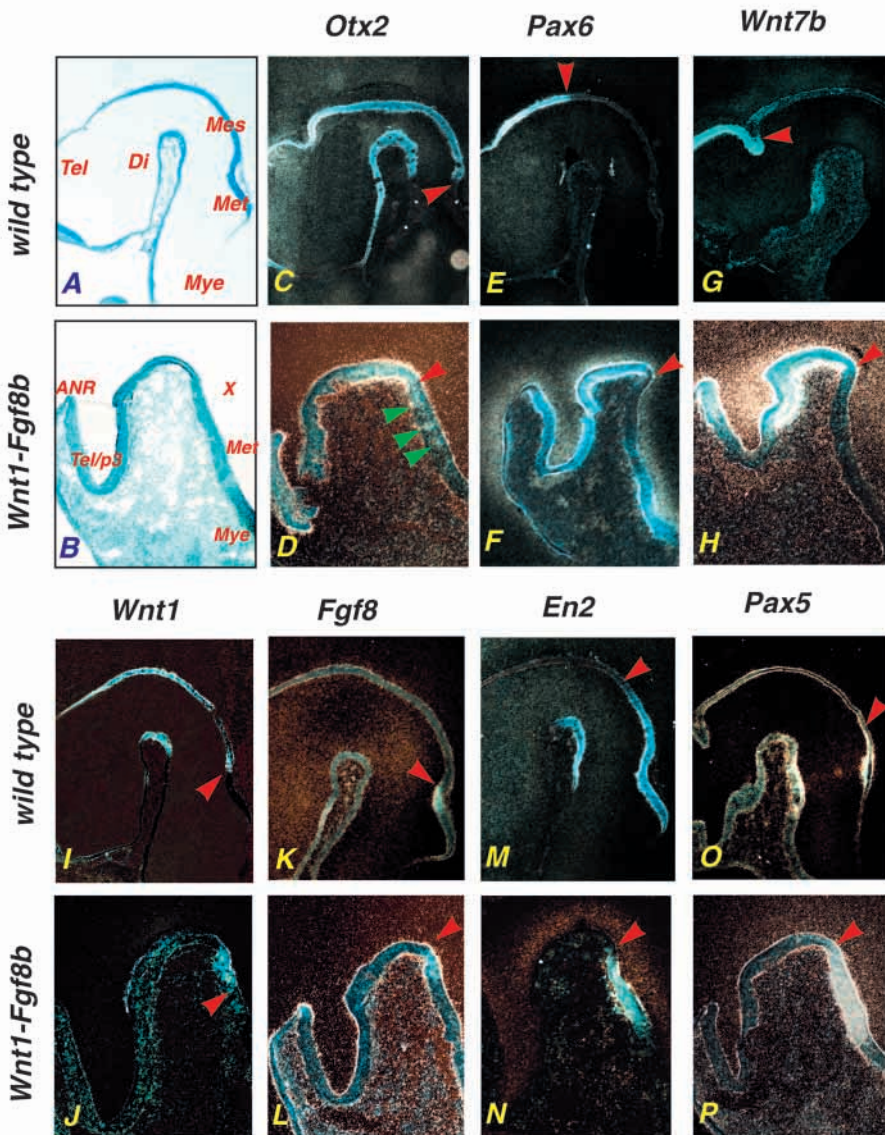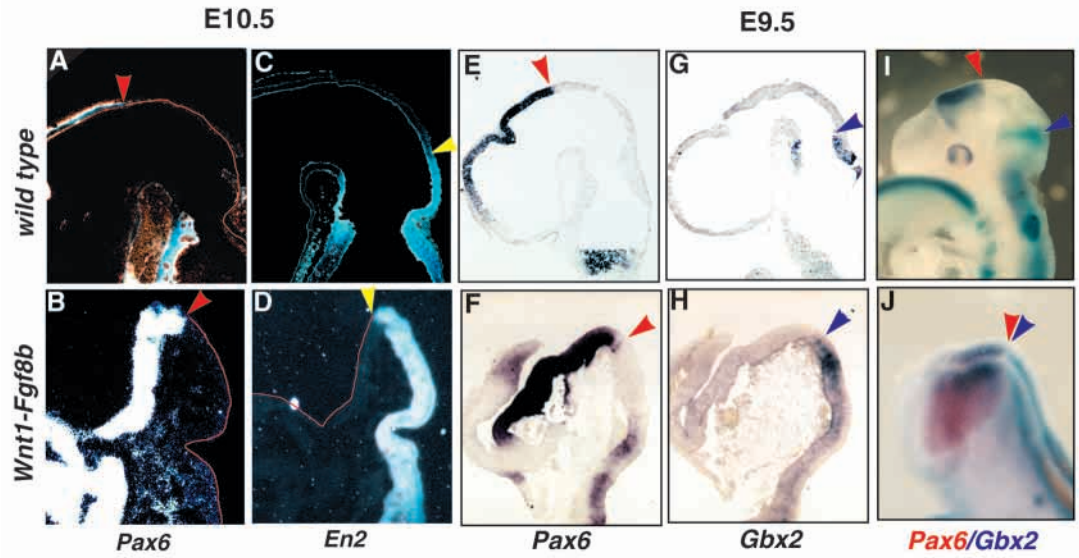

the boundary between the two domains was not very sharp (Fig. 1L). Double in situs also showed that *Gbx2* was strongly induced only in *Otx2*-negative cells, but there did not appear to be a complete complementarity in their expression, as there were cells that appeared not to express either gene (Fig. 1M).

Ectopic expression of *Fgf8b* produces exencephaly and abnormalities in the spinal cord

In contrast to our explant studies with FGF8b, when *Fgf8a* was ectopically expressed in the midbrain using a *Wnt1* enhancer, no disturbance of anteroposterior patterning or ectopic mes/met gene induction was observed in the embryos examined, except for a loss of tectum polarity at late stages. However, 4 embryos at E10.5 were reported to have a more severe non-specific phenotype that was suggested to be due to widespread activation of the transgene (Lee et al., 1997). To determine whether different isoforms of FGF8 account for the different

Fig. 6. *Wnt1-Fgf8b* transgenic embryos lack a posterior forebrain at E9.5. Radioactive RNA in situ hybridization of sagittal sections of E9.5 wild-type embryos (C,E,G,I,K,M,O) and *Wnt1-Fgf8b* transgenic embryos (D,F,H,J,L,N,P). (A,B) Toluidine-blue-stained sections of wild-type (A) and transgenic (B) embryos. Red arrowheads in C-J point to the caudal boundary of the expression domain of *Otx2, Pax6, Wnt7b* and *Wnt1* and red arrowheads in K-P point to rostral boundary of the expression domains of *Fgf8, En2* and *Pax5*. Green arrowheads in D point to patchy *Otx2* expression posterior to the new boundary formed in the caudal forebrain.
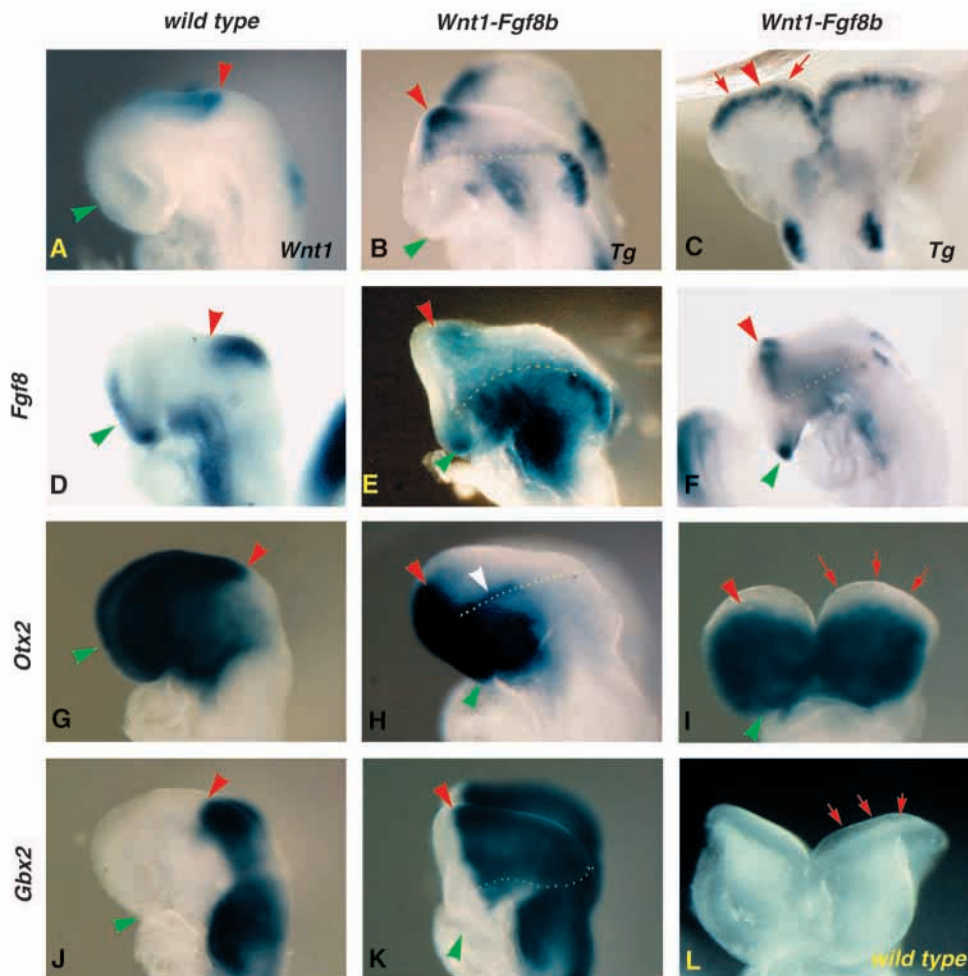
Fig. 7. The midbrain and posterior forebrain are transformed into a hindbrain fate at early somite stages. Green arrowheads in A,B,D-K point to the ANR. Red arrows in C,I,L highlight the dorsal profiles of the brains shown in dorsal or frontal view. Dotted lines in B,E,F,K indicate the lateral edges of neural plates. Red arrowheads in A,D,G,J point to the mid/hindbrain junction as defined by the caudal border of *Wnt1* and *Otx2* expression, and the rostral border of *Fgf8* and *Gbx2* expression. Red arrowheads in B,C,E,F,H,I,K point to the new boundary formed in the caudal forebrain marked by a sharp bend and the rostral border of transgene, *Fgf8*, and *Gbx2* expression, as well as the caudal border of *Otx2* expression. (A)*Wnt1* expression in a 7-somite-stage wild-type embryo. (B, lateral view; C, dorsal view) *Wnt1-Fgf8b* transgene (Tg) expression in a 7-somite transgenic embryo. (D,E) *Fgf8* expression in a 5-somite wild-type (D) and a transgenic (E) embryo. (F) *Fgf8* expression in a 13-somite-stage transgenic embryo. (G-I) *Otx2* expression in a 7-somite wild-type (G) and a transgenic (H, lateral view; I, ventral view) embryo. (J,K) *Gbx2* expression in a 7-somite-stage wild-type (J) and a transgenic (K) embryo. (L) Frontal view of a 7-somite-stage wild-type embryo showing the V-shaped dorsal profile.

results in vitro and in vivo and to further study the role of *Fgf8* in mouse embryonic CNS development, we made transgenics expressing *Fgf8b* from the same *Wnt1* enhancer and also made *Wnt1-Fgf8a* transgenic embryos for comparison of the phenotypes.

At E10.5, 64% (7/11) of transgenic embryos that expressed the *Wnt1-Fgf8a* transgene (11/32) showed a slightly enlarged midbrain and caudal diencephalon compared to wild-type littermates (Fig. 2B), similar to the phenotype previously reported (Lee et al., 1997). However, the remaining transgenics (4/11) showed a variable degree of exencephaly (Fig. 2C) and these embryos appeared to show the strongest transgene expression in the dorsal spinal cord based on whole-mount RNA in situ hybridization of tail regions using a transgene-specific *lacZ* RNA probe (data not shown, see Materials and Methods). However, no expression was observed outside of the CNS, indicating that the severe phenotype was not a result of widespread activation of the transgene.

In contrast to the *Wnt1-Fgf8a* transgenics, all *Wnt1-Fgf8b* transgenic embryos that expressed the transgene (10/17) had exencephaly at E10.5 and a more severe phenotype than any of the *Wnt1-Fgf8a* transgenics (Fig. 2D). Again, the tail region from all the *Wnt1-Fgf8b* transgenic embryos examined were probed with a *lacZ* RNA in situ probe and no non-specific transgene expression was found outside the dorsal neural tube

where the transgene was expected to be expressed. Gross observation of *Wnt1-Fgf8b* embryos revealed that, instead of the alar plate of the brain folding inward to form a neural tube, it folded outward such that the neural tube anterior to the otic vesicle was open. An additional striking morphological defect in transgenics was that the head seemed to be truncated. In addition, the branchial arches of *Wnt1-Fgf8b* embryos seemed underdeveloped, possibly resulting from abnormal development of neural crest cells, since the *Wnt1* enhancer expresses in neural crest precursors (Echelard et al., 1994). At E15.5, the exencephaly persisted (Fig. 2F,H) and no eye structure was formed. However, nasal and oral structures were present at this stage (compare Fig. 2G and H).

Ectopic *Fgf8b* expression in the hindbrain and spinal cord resulted in a morphological abnormality, in contrast to the chick FGF8b bead experiments in which the hindbrain was not altered (Crossley et al., 1996b). A similar spinal cord phenotype also was seen in some (6/11) of the *Wnt1-Fgf8a* transgenic embryos. The spinal cord had a wavy appearance in dorsal view (Fig. 3E,F). In cross sections, it could be seen that the dorsal neural tube was overgrown such that the central canal that normally assumes a narrow oval shape had either a 'T' shape or an inverted 'L' shape (Fig. 4). At E15.5, the spinal cord of transgenics appeared normal in ventral regions, but overgrown dorsally (data not shown).

Mes/met genes are induced by ectopic *Fgf8* expression in the brain and more posterior regions

To determine whether ectopic expression of *Fgf8a* or *Fgf8b* in the brain leads to alterations in anteroposterior patterning, transgenic brains were analyzed for mes/met gene expression using whole-mount RNA in situ hybridization. To compare the spatial relationship between the expression of the two *En* genes, E10.5 *Wnt1-Fgf8b* embryos (*n*=2) were bisected along the mid-sagittal plane, followed by whole-mount RNA in situ hybridization using *En1*- or *En2*-specific probes on either half of the embryo. Expression of both *En* genes was clearly induced ectopically in *Wnt1-Fgf8b* embryos (Fig. 3). Normally, *En2* is only expressed in a wide band covering the caudal part of mesencephalon and in the metencephalon (Fig. 3B). *En1* is expressed in a similar, but narrower band than *En2* at E10.5 (Fig. 3A). In addition, *En1* is also expressed in two ventrolateral stripes of postmitotic cells in the hindbrain and spinal cord, as well as in non-neural tissues including the somites and ventral limb ectoderm (Fig. 4A). In transgenic embryos, we found that the anterior border of *En1* expression was the same as that of *En2*, indicating there is at least a rostral expansion of *En1* (compare Fig. 3C and D). Another striking ectopic domain of both *En1* and *En2* expression was along the entire dorsal part of the hindbrain and spinal cord caudally to at least the lumbar level, and rostrally joining the mes/met domain (Fig. 3C-E). The expression of *En1* in the hindbrain appeared more restricted to the dorsal lateral part of the opened neural tissue than *En2*, which appeared to have a wider domain (compare Fig. 3C and D). However, when cross sections were taken at the lumbar level of the same embryo and processed for radioactive RNA in situ hybridization, there seemed to be no apparent difference between the expression domains of *En1* and *En2* (Fig. 4B,C).

Expression of *Wnt1*, *Fgf8*, the transgene (*lacZ* probe), *Pax5* and *Gbx2* in the spinal cord was also examined in cross sections using RNA in situ analysis. A *Wnt1* probe that detects both *Wnt1* and transgene-derived transcripts detected expression in a much wider band of cells than *Wnt1* is normally seen in wild types (compare Fig. 4E with D). This could be due to over-proliferation of the dorsal spinal cord cells or induction by FGF8b, although *Fgf8* and the transgene appeared to be expressed in the same cells as *Wnt1* (Fig. 4F,I). *Pax5*, which normally is expressed only in postmitotic cells in the spinal cord (Asano and Gruss, 1992), was induced in the same dorsal ventricular spinal cord cells that expressed *Wnt1*, *Fgf8*, *En1* and *En2* (data not shown). Interestingly, *Gbx2*, which is normally clear from the roof plate of the spinal cord (Fig. 4G), was induced in the most dorsal region of the spinal cord of the transgenics (Fig. 4H). The expression of *Pax3* in the dorsal spinal cord was not altered in the transgenics (data not shown), indicating that dorsal genes are not repressed by FGF8.

In E10.5 *Wnt1-Fgf8a* transgenic embryos examined for *En2* expression, 2/2 with exencephaly and 2/4 without exencephaly had ectopic expression in the dorsal midbrain and posterior roof plate of the hindbrain and spinal cord (Fig. 2C), similar to *Wnt1-Fgf8b* transgenics. However, two differences in the expression pattern were observed. First, the ectopic roof plate expression domain of *En2* was separate from the normal mes/met expression domain. Second, the morphological abnormality of the spinal cord appeared milder in these *Wnt1-Fgf8a* transgenic embryos compared to *Wnt1-Fgf8b*

transgenics and *En2* expression seemed to fade off posteriorly at the level of the forelimbs (Fig. 3F).

The midbrain is missing by E9.5 in *Wnt1-Fgf8b* transgenics

To explore the neural identity of the cells in the anterior region of *En1* and *En2* expression, we performed RNA in situ analysis on sagittal sections of E10.5 embryos. *Pax6* is normally expressed at this stage in the dorsal forebrain with a posterior boundary at the diencephalon/mesencephalon (di/mes) border, as well as in the ventrolateral hindbrain and spinal cord (Gruss and Walther, 1992). In adjacent sagittal sections of wild-type embryos, the *Pax6* dorsal expression domain is separated from that of *En2* by a rostral mesencephalic domain which is negative for both genes (compare Fig. 5A and C). In contrast, the transgenic embryos analyzed had strong *En2* expression in a domain that abutted the *Pax6* expression domain in the dorsal forebrain (Fig. 5B,D). This indicates that *En2* expression is induced in the rostral region of the mesencephalon or that the region is lost in *Wnt1-Fgf8b* transgenic embryos.

The extreme deformity of the transgenic embryos at E10.5 made it difficult to characterize the anteroposterior patterning defects in the CNS, based on histology or marker gene analysis. Further analysis of expression of *Pax6* and *Gbx2*, which mark different brain regions, was therefore carried out at E9.5 by examining gene expression in adjacent sections and double staining of whole embryos. *Pax6* was used as a marker for the forebrain and di/mes and r2/r3 boundaries (Fig. 5E,I). *Gbx2*, which is most strongly expressed as a ring in the anterior hindbrain, was used to mark the mes/met border and the metencephalon (Fig. 5G,I). In E9.5 transgenics, *Pax6* was expressed in the most rostral part of the CNS with a sharp caudal boundary, as seen at E10.5 (Fig. 5F,J). *Gbx2* was detected in two broad dorsolateral bands extending caudal from the posterior border of Pax6 to the otic vesicle (Fig. 5H,J). This observation strongly suggested that the midbrain was absent in *Wnt1-Fgf8b* transgenics at E9.5.

Caudal regions of the forebrain are not present at E9.5 in *Wnt1-Fgf8b* transgenic embryos

To further analyze the CNS anteroposterior patterning defects in *Wnt1-Fgf8b* transgenic embryos, RNA in situ hybridization was performed on adjacent sagittal sections at E9.5. Consistent with the results described above with whole-mount and section RNA in situ analysis, *Pax6* expression (Fig. 6F) abutted the *Fgf8* expression domain (Fig. 6L), providing further evidence that the midbrain was largely missing in transgenics. Most strikingly, *Wnt7b*, a gene with strongest expression in the dorsal telencephalon and most rostral diencephalon (p3, Fig. 6G), had a posterior boundary in transgenic embryos which was very similar to the posterior boundary of *Pax6*, abutting the anterior boundary of expression of *Fgf8*, *Wnt1*, *Pax5* and *En2* (compare Fig. 6H to F,J,L,N,P). This finding suggests that the caudal diencephalic region including p1 and p2 is absent in transgenics.

We next compared the expression domains of genes that normally have borders at the mes/met junction (*Otx2*) or are expressed in the isthmus (*Wnt1/Fgf8*), in order to determine whether an 'organizer' was preserved. *Otx2*, which normally labels the entire forebrain and midbrain at this stage (see Fig. 6C), was found to be expressed strongly overlapping with *Pax6*

(compare Fig. 6D and F). However, different from *Pax6*, patchy expression of *Otx2* was seen posterior to the *Pax6* domain, overlapping with transverse bands of *Wnt1* and *Fgf8* expression (Fig. 6D). In contrast to the normal spatial relationship of *Wnt1* and *Fgf8* in adjacent domains, *Fgf8* appeared to be expressed only in the *Wnt1* domain, presumably driven by the *Wnt1* enhancer in the transgene. These studies at E9.5 suggest that the new forebrain caudal border (*Pax6/Wnt7b* caudal limit) in transgenics does not directly abut hindbrain tissue, but there is a border region with cells that express both midbrain (*Otx2, Wnt1*) and hindbrain (*Gbx2, Fgf8*) genes (summarized in Fig. 8). If the organizer is normally defined as the isthmic region that expresses *Wnt1* and *Fgf8* in adjacent regions, where *Wnt1* overlaps with the caudal domain of *Otx2* and *Fgf8* the anterior domain of *Gbx2* (see Fig. 8), then our gene expression analysis demonstrates that the organizer formed in *Wnt1-Fgf8b* transgenics is not a normal one.

Caudal forebrain and midbrain are transformed into hindbrain by early somite stages

In order to determine when a phenotype could first be detected in *Wnt1-Fgf8b* transgenics and to determine whether the di/mes is deleted or transformed into metencephalic tissue, we analyzed transgenic embryos at early somite stages, 12 hours after transgene expression is initiated (Echelard et al., 1994). A morphological difference between wild-type embryos and *Wnt1-Fgf8b* transgenic embryos was already obvious at 5-7 somites. At this stage, the anterior neural plate normally has begun to invaginate in wild-type embryos (Fig. 7A,L). In contrast, in transgenic embryos, the alar plate of the anterior CNS folded out instead of in (Fig. 7B,C,I), and a sharp bend was seen in the anterior neural plate (red arrowhead in Fig. 7B,E,H,K).

To examine anteroposterior patterning in the transgenic embryos, we examined the expression patterns of *Gbx2, Otx2, Fgf8* and the *Wnt1-Fgf8b* transgene using a *lacZ* probe (see Materials and Methods) in early somite stage embryos. At 7 somites, the transgene was strongly expressed in the dorsal hindbrain and spinal cord, similar to *Wnt1* expression in wild-type embryos (compare Fig. 7A and B). Transgene expression in more anterior brain regions was restricted to a transverse band in the caudal forebrain region, as well as two thin lines of weak expression along the lateral edges of the neural folds extending caudally from the transverse band (Fig. 7B). Wild-type embryos, in contrast, express *Wnt1* in a transverse band in the caudal region of the midbrain adjacent to the mid/hindbrain junction (Fig. 7A). *Fgf8* expression was detected in a domain similar to the transgene, as well as in the domains of endogenous *Fgf8* in the ANR and mesoderm (Fig. 7E,F). Strikingly, *Otx2*, which normally is strongly expressed in the forebrain and mesencephalon in wild-type embryos (Fig. 7G), was expressed only in the anterior most forebrain of transgenic embryos at 7 somites (Fig. 7H,I). The only *Otx2* expression left in the caudal brain was two weak, thin bands along the lateral edges of the neural plate (Fig. 7H). *Gbx2* expression, which normally has a rostral border at the mid/hindbrain junction (Fig. 7J) was also dramatically altered in the transgenic embryos. The *Gbx2* domain was expanded such that its rostral border was shifted to the caudal forebrain region (Fig. 7K), overlapping with the anterior border of the transgene and *Fgf8* expression and abutting the caudal *Otx2*

border (Fig. 7B,E,H,K). Since it is unlikely that FGF8 could alter proliferation to such an extent to account for the changes seen in the *Otx2* and *Gbx2* expression domains within 12 hours, these studies indicate that, in *Wnt1-Fgf8b* transgenics, the midbrain and caudal forebrain are transformed into an anterior hindbrain fate during the early somite stages.

DISCUSSION

In this study, we explored the role of mouse FGF8 in patterning the CNS along the anteroposterior axis during early embryonic development using both in vitro explant culture experiments and in vivo transgenic techniques. FGF8b was found to induce expression of *En1, En2* and *Pax5*, but not *Fgf8* in E9.5 diencephalic explants. It also induced *Gbx2* and altered *Wnt1* expression in both midbrain and diencephalic explants and repressed *Otx2* in midbrain explants. When *Fgf8b* was ectopically expressed using a *Wnt1* regulatory element, a phenotype much more severe than that of *Wnt1-Fgf8a* was observed. All the transgenic embryos showed exencephaly and a mild morphological change could be recognized as early as the 7-somite stage. A morphological abnormality was also observed in the roof plate of the caudal hindbrain and spinal cord at E10.5. Genes like *En1, En2*, *Pax5* and *Gbx2* that normally mark the mes/met were induced in the dorsal hindbrain and spinal cord. More strikingly, the midbrain and caudal forebrain were not present in transgenic embryos at E9.5, and tissue expressing the hindbrain gene *Gbx2* was adjacent to the remaining forebrain. This phenotype seems to arise from a transformation of the mesencephalon and diencephalon (at least p1 and p2) into metencephalic tissue at early somite stages, since *Gbx2* expression was expanded anteriorly and the *Otx2* domain reduced within 12 hours of transgene expression. At the new border region between *Otx2* and *Gbx2* expression, organizer genes (*Fgf8* and *Wnt1*) were expressed, but not in their normal relative positions. Furthermore, *Fgf8* was unable to induce the more rostral brain tissue to form a midbrain and only *Gbx2*-expressing hindbrain tissue was maintained posterior to the organizer.

FGF8b has been shown in tissue culture systems to have a stronger cell transformation activity and higher affinity to the known FGF receptors than FGF8a (MacArthur et al., 1995a,b; Blunt et al., 1997). Therefore, it has been suggested that the two isoforms might have different functions in vivo and be responsible for the different results obtained in chick and mouse gain-of-function studies in the limb or brain. In our study, the biological functions of the two isoforms were directly compared by expressing them from the same *Wnt1* regulatory element. Many factors, such as different protein stability, different affinity for the same receptor or the existence of an unknown receptor, could underlie the functional differences of the two FGF8 isoforms. Our studies, however, strongly indicate that the main difference between the proteins is quantitative, because some *Wnt1-Fgf8a* transgenics showed exencephaly and a spinal cord phenotype similar to, but milder than, *Wnt1-Fgf8b* transgenics. Consistent with our studies, a recent comparison of the phenotypic consequences of placing beads soaked in FGF8a or FGF8b in the chick brain reported that both can induce similar changes (Shamim et al., 1999).

## Gene expression patterns in E9.5 *Wnt1-Fgf8b* transgenic embryos
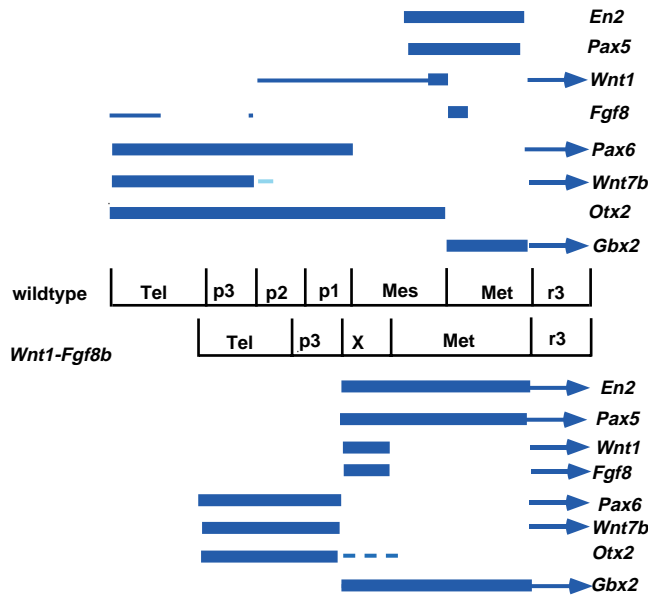
Fig. 8. Summary of gene expression analyses at E9.5. The thick lines represent expression across most or all of the dorsal/ventral extent of the neural tube and thin lines indicate restricted domains of expression. Weak expression is represented by light blue line. Patchy expression is represented with a dashed line.

Our finding that, in *Wnt1-Fgf8b* transgenic embryos, the midbrain and caudal forebrain are transformed into a metencephalon, on the surface appears to be different from the results obtained when FGF8b-soaked beads were implanted into the chick diencephalon and ectopic midbrain structures were induced (Crossley et al., 1996). One difference between the two experiments is that ectopic FGF8b was applied at different stages during development. The beads were placed in the chick diencephalon at HH (Hamburger and Hamilton, 1992) stage 9-12, a period when the anterior neural tube is closing, which should correspond to E9 in mouse embryos. In *Wnt1-Fgf8b* transgenic embryos, transgene expression is initiated in the mesencephalon around E8, an earlier stage during neurogenesis. However, this difference does not likely account for the different phenotypes observed, since we demonstrated that FGF8b-soaked beads can induce *Gbx2* expression and repress *Otx2* expression in mouse mesencephalic explants taken at E9.5.

A more likely explanation for the phenotypes seen in chick and mouse is that FGF8 was provided in different manners. In the chick experiments, FGF8b beads provided a focal source of FGF8b which likely induced *Gbx2* and repressed *Otx2* in cells locally, creating an ectopic mes/met border. Similar to our explant results, *Wnt1* was likely activated in the ectopic mes/met border region and, *En*, *Pax5* and *Fgf8* induced more broadly, creating a new organizer region (Fig. 9A). Consistent with this, since the submission of our paper, it has been reported that FGF8b beads can repress *Otx2* expression locally in the caudal diencephalon and mesencephalon and that cells closest to the beads can take on cerebellar characteristics (Martinez et al., 1999). Based on our results, the cells that form the cerebellum likely express *Gbx2* and the cells that form ectopic midbrains express *Otx2*. In contrast, in our studies, FGF8 was initially expressed in most of the midbrain and induced *Gbx2* and repressed *Otx2* broadly. It is plausible that the transgene then induced its own expression in more anterior forebrain regions through induction of the *Wnt1* regulatory elements in the transgene, since we found that FGF8 protein can induce *Wnt1* expression in diencephalic explants. Both *Wnt1* and *Fgf8* became

A
Fgf8 beads provide a point source of Fgf8
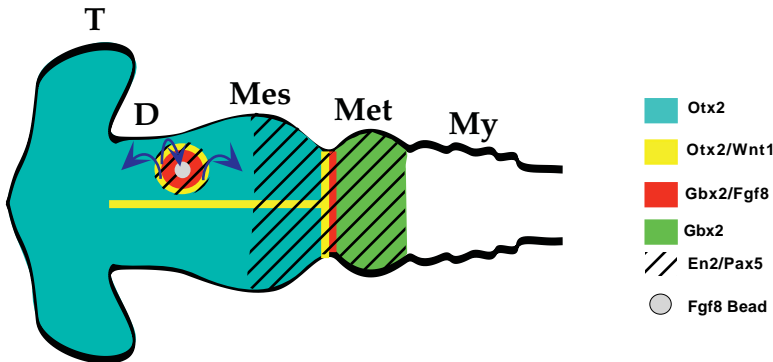
B
Wnt1-Fgf8 Tg expresses *Fgf8* throughout the midbrain
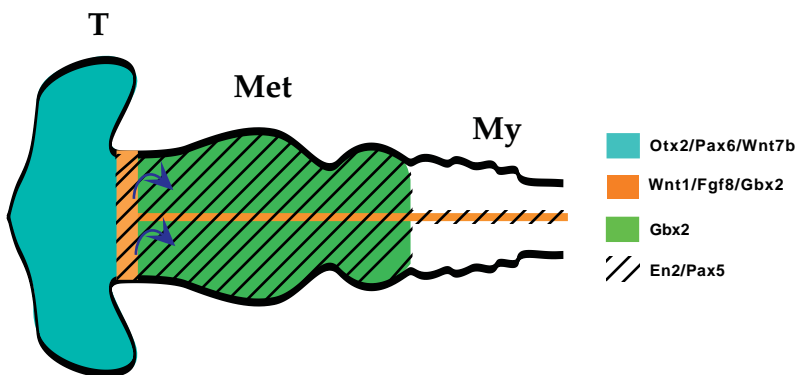
Fig. 9. Application of FGF8b as a point source or in a broad domain leads to different phenotypes. (A) A FGF8b bead provides a point source of FGF8, which induces *Gbx2, En2* and *Pax5* in adjacent cells, *Wnt1* at a distance and represses *Otx2* locally. A new organizer is formed which induces cerebellar tissue in the central *Gbx2*-positive cells and midbrain tissue in the surrounding *Otx2*-expressing cells. (B) The *Wnt1-Fgf8b* transgene (Tg) expresses *Fgf8* throughout the midbrain and caudal forebrain, thus inducing *Gbx2* and repressing *Otx2* broadly. There is no competent *Otx2*-positive tissue in which the organizer can induce a midbrain.

restricted anteriorly, possibly to the prospective ZLI, in *Wnt1-Fgf8* transgenics leaving *Gbx2*-positive metencephalic tissue caudal to the *Fgf8* domain (Fig. 9B). The position of the new *Otx2/Gbx2* boundary might be set by the position of forebrain tissue (p3 and telencephalon), which cannot adopt a mes/met fate in response to FGF8 induction, based on isthmic transplants and FGF8 bead experiments in chick and mouse (Bloch-Gallego et al., 1996; Crossley et al., 1996; Shimamura and Rubenstein, 1997). Since the rostral forebrain cannot respond to FGF8b to form a midbrain, only metencephalic tissue could be induced in *Gbx2*-expressing cells posterior to *Fgf8/Wnt1* in *Wnt1-Fgf8* transgenics.

The patchy expression of *Otx2* in regions posterior to the sharp *Pax6* caudal boundary at E9.5 in *Wnt1-Fgf8b* transgenics, and the overlap in expression of midbrain and hindbrain genes in this region, indicate the organizer region is not normal. It is noteworthy that, in *Gbx2* mutant embryos, a similar abnormal organizer is formed with overlapping expression of *Wnt1, Fgf8* and *Otx2* (Wassarman et al., 1997). In *Wnt1-Fgf8b* transgenics, the organizer region might be compromised by altered gene regulation (i.e. *Fgf8* expression under the control of a *Wnt1* regulatory element), such that the spatial relationship among different mes/met genes differs from that in wild type.

It is interesting that the phenotype that we observe in early *Wnt1-Fgf8b* transgenics has similarities to that seen in *Otx1+/−* *Otx2+/−* (Suda et al., 1997) or *Otx1−/− Otx2+/−* double mutants (Acampora et al., 1997); an early induction of *Gbx2* and repression of *Otx2* in the midbrain and caudal forebrain. In *Otx1−/−*; *Otx2+/−* embryos, an anterior expansion of *Fgf8* expression was found to precede an anterior shift of *Wnt1* and *En1* expression and an anterior retraction of *Otx2* expression (Acampora et al., 1997). The *Otx* mutant studies suggest a certain level of *Otx2* expression is necessary to repress expression of *Fgf8* in the midbrain and forebrain, and our results suggest that, in addition, expanded *Fgf8* expression could contribute to repression of *Otx2* expression in the midbrain. A reciprocal negative regulation between *Otx2* and *Fgf8* might therefore normally contribute to maintaining the *Otx2* caudal boundary and positioning the organizer.

Finally, we showed for the first time that *Fgf8* can induce *Gbx2* in an explant assay and in *Wnt1-Fgf8b* transgenic embryos. Recent studies show that, in *Wnt1-Gbx2* transgenic embryos in which *Gbx2* is expressed in the mesencephalon at early somite stages, *Otx2* expression in the caudal midbrain is repressed by early somite stages and *Fgf8* and *Wnt1* expression is shifted anteriorly to the new *Otx2/Gbx2* border (Millet et al., 1999). These latter gain-of-function studies demonstrate that *Gbx2* can directly, or indirectly, repress *Otx2*. Consistent with this, in *Gbx2* mutant embryos, *Otx2* expression is expanded caudally into what would normally be the *Gbx2* domain at early somite stages (Millet et al., 1999). Taken together with our present studies, these results suggest that in *Wnt1-Fgf8b* transgenics *Gbx2* could mediate the repression of *Otx2* by FGF8b.

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