FGF17b and FGF18 have different midbrain regulatory properties from FGF8b or activated FGF receptors

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Summary

Early patterning of the vertebrate midbrain and cerebellum is regulated by a mid/hindbrain organizer that produces three fibroblast growth factors (FGF8, FGF17 and FGF18). The mechanism by which each FGF contributes to patterning the midbrain, and induces a cerebellum in rhombomere 1 (r1) is not clear. We and others have found that FGF8b can transform the midbrain into a cerebellum fate, whereas FGF8a can promote midbrain development. In this study we used a chick electroporation assay and in vitro mouse brain explant experiments to compare the activity of FGF17b and FGF18 to FGF8a and FGF8b. First, FGF8b is the only protein that can induce the r1 gene *Gbx2* and strongly activate the pathway inhibitors *Spry1/2*, as well as repress the midbrain gene *Otx2*. Consistent with previous studies that indicated high level FGF signaling is required to induce these gene expression changes, electroporation of activated FGFRs produce similar gene expression changes to FGF8b. Second, FGF8b extends the organizer along the junction between the induced *Gbx2* domain and the remaining *Otx2* region in the midbrain, correlating with cerebellum development. By contrast, FGF17b and FGF18 mimic FGF8a by causing expansion of the midbrain and upregulating midbrain gene expression. This result is consistent with *Fgf17* and *Fgf18* being expressed in the midbrain and not just in r1 as *Fgf8* is. Third, analysis of gene expression in mouse brain explants with beads soaked in FGF8b or FGF17b showed that the distinct activities of FGF17b and FGF8b are not due to differences in the amount of FGF17b protein produced in vivo. Finally, brain explants were used to define a positive feedback loop involving FGF8b mediated upregulation of *Fgf18*, and two negative feedback loops that include repression of *Fgfr2/3* and direct induction of *Spry1/2*. As *Fgf17* and *Fgf18* are co-expressed with *Fgf8* in many tissues, our studies have broad implications for how these FGFs differentially control development.

Key words: *Fgf8*, *Fgf17*, *Fgf18*, Mid/hindbrain organizer, FGF receptors, *Sprouty*, Mouse, Chick

Introduction

Early patterning of the vertebrate presumptive midbrain and rhombomere 1 (r1), which dorsally gives rise to the cerebellum, is regulated by a local organizer situated at the mid/hindbrain junction (reviewed by Joyner et al., 2000; Liu and Joyner, 2001a; Wurst and Balley-Cuif, 2001). FGF8, a member of the fibroblast growth factor (FGF) family, is expressed in r1 adjacent to the mid/hindbrain junction and has organizer activity. FGF8 can induce the patterned expression of many midbrain/r1 genes and the formation of ectopic midbrain or cerebellar structures depending on the cellular environment and isoform (a or b) of FGF8 protein used. Furthermore, loss-of-function studies in mouse and zebrafish have shown that *Fgf8* is required for normal development of the midbrain and cerebellum (Meyers et al., 1998; Reifers et al., 1998; Chi et al., 2003). *Fgf17* and *Fgf18*, which encode proteins more closely related to FGF8 than the other FGF family members, are also expressed in the mid/hindbrain junction region in broader domains than *Fgf8*, including the posterior midbrain (Maruoka et al., 1998; Xu et al., 1999). In biochemical and cell culture assays FGF17b and FGF18 have similar receptor binding properties and ability to induce proliferation when compared with FGF8b (Xu et al., 1999; Xu et al., 2000). In zebrafish, mRNA injection experiments indicate that *Fgf8* and *Fgf17* have similar effects on gastrulation (Reifers et al., 2000). Loss of *Fgf17* in mouse results in truncation of the posterior midbrain (inferior colliculus) and reduced proliferation of the anterior cerebellum (Xu et al., 2000), whereas *Fgf18* does not appear to be required for midbrain or cerebellum development (Liu et al., 2002; Ohbayashi et al., 2002). There is clearly overlap in function between at least *Fgf8* and *17*, as removal of one copy of *Fgf8* on an *Fgf17* mutant background leads to an exaggerated cerebellum phenotype (Xu et al., 2000). The exact functions of each FGF protein therefore are not clear.

Fgf8 mRNA is differentially spliced to generate multiple protein isoforms. FGF8a and FGF8b are the primary isoforms expressed in r1 (Sato et al., 2001) and they differ by only 11 amino acids that are included in FGF8b. Surprisingly, we have shown that these two FGF8 isoforms produce very different phenotypes when mis-expressed in transgenic mouse embryos

(Liu et al., 1999). Ectopic expression of the *a* isoform of *Fgf8* in the midbrain and caudal forebrain results in both expansion of the midbrain and ectopic expression of *En2*, but not other genes expressed in the midbrain and r1 (Lee et al., 1997; Liu et al., 1999). The EN transcription factors alone cannot mediate the midbrain expansion, as similar ectopic expression of *En1* does not induce the same phenotype (Lee et al., 1997), and *Fgf8a* produces midbrain expansion even in *En2* mutants (D. Song and A.L.J., unpublished data). In contrast to FGF8a, the b isoform produces exencephaly and a rapid transformation of the midbrain and diencephalon into an anterior r1 fate (Liu et al., 1999) that includes repression of the midbrain gene *Otx2*, expansion of the hindbrain gene *Gbx2* and an anterior shift in organizer genes (*Fgf8/Wnt1*). A further study showed that GBX2 and EN1/2 are both required for FGF8b to regulate some midbrain/r1 genes (Liu and Joyner, 2001b).

Recently, the functions of FGF8a and b also were elegantly compared in chick following electroporation of different concentrations of DNA expression constructs. Similar to what was observed in mouse, *Fgf8a* causes expansion of the midbrain and *Fgf8b* transforms the midbrain into a cerebellum based on early gene expression changes and later morphology (Sato et al., 2001). Interestingly, the initial effect of FGF8b is to reduce growth of the midbrain. Thus, FGF8a and b have distinct activities, both on growth and regulation of gene expression. Of relevance, 100 times lower levels of *Fgf8b* induce an expanded midbrain. These results, and other studies (Martinez et al., 1999; Liu et al., 1999), have led to the suggestion that a high level of FGF8 signaling induces cerebellum development and a lower level induces midbrain development. If this is the case, then strongly inducing the FGF pathway using activating mutations in FGFRs should mimic the effects of FGF8b. Furthermore, given the dual functions of FGF8 proteins in midbrain and cerebellum development, it is important to determine whether FGF17 and 18 are similar to FGF8a or b.

FGF signaling is mediated by fibroblast growth factor receptor (FGFR) proteins, which belong to a family of tyrosine kinase-containing transmembrane proteins that bind to FGF molecules and mediate FGF signaling (reviewed by Powers et al., 2000). Loss-of-function studies in mouse have demonstrated that various FGFRs are essential in processes such as gastrulation, limb outgrowth and lung morphogenesis (reviewed by Liu and Joyner, 2001a). In vitro studies have indicated that in the presence of heparin, all three FGFs present in the mid/hindbrain region bind to the c isoforms of FGFR2 and FGFR3 with high affinity, but not to FGFR1 (Blunt et al., 1997; Xu et al., 1999). Interestingly, in mouse and chick embryos *Fgfr2* and *Fgfr3* are not expressed near the mid/hindbrain organizer and *Fgfr1* is expressed at low levels (Ishibashi and McMahon, 2002; Walshe and Mason, 2000), raising the question of whether FGFR2/3 mediate FGF signaling from the organizer. Indeed, a recent study of mice lacking *Fgfr1* specifically in the midbrain and r1 showed that *Fgfr1* is the primary FGF receptor required in midbrain and cerebellum development (Trokovic et al., 2003).

The Sprouty (Spry) family of proteins are antagonists of multiple tyrosine kinase-containing receptors including those for epidermal growth factor and FGF. In *Drosophila*, *spry* is expressed in cells receiving Fgf signals, and loss of *spry* phenocopies gain-of-function mutations in *fgf* (*breathless*) or *fgfr* (*branchless*) (Hacohen et al., 1998). There are multiple Spry members in the vertebrates, two of which (*Spry1* and *Spry2*) are expressed in the mid/hindbrain region of mouse and chick embryos and induced by FGF (4 or 8b)-soaked beads in chick embryos (Chambers et al., 2000; de Maximy et al., 1999; Minowada et al., 1999). Thus, similar to other signaling pathways, FGF induces a negative feedback loop, and a fine balance between activating and suppressing signaling must be required for proper midbrain and cerebellum development.

In this study, we compared the activity of FGF17b and FGF18 to FGF8 in midbrain/cerebellum development using the chick electroporation assay. Strikingly, mis-expression of *Fgf17b* or *Fgf18* at similar levels to *Fgf8* induced expansion of the midbrain and regulation of midbrain genes similar to FGF8a. Of significance, among the four FGF proteins tested, only FGF8b induces *Gbx2* and represses *Otx2* producing a broad *Gbx2+/Otx2–* domain that abuts the *Otx2* positive cells in the remainder of the midbrain. Interestingly, FGF8b induces organizer genes at the new *Gbx2/Otx2* border, whereas FGF8a induces *Fgf8* in scattered cells in the midbrain. In addition, only FGF8b strongly induces the feedback inhibitors *Spry1* and *Spry2*, and we show that *Spry1* is a direct target of FGF8 signaling. Consistent with the idea that FGF8b induces a higher level of signaling, mis-expression of activated FGFRs leads to induction of *Gbx2* and *Spry1/2* and repression of *Otx2* similar to FGF8b, although the induction is in scattered cells and does not produce a late phenotype of cerebellum induction.

Materials and methods

Techniques

Mouse brain explant culture, RNA in situ hybridization of whole-mount tissue and sections was carried out as previously described (Liu and Joyner, 2001b; Liu et al., 1999). In ovo electroporation in chicken embryos was performed as described previously (Timmer et al., 2001) with some modifications. Specifically, cDNAs for mouse *Fgf8a* (Lee et al., 1997), *Fgf8b* (Liu et al., 1999), *Fgf17b* (Xu et al., 1999) and *Fgf18* (Maruoka et al., 1998), and human mutant FGFR genes were cloned into a chicken expression vector *pMiwIII* (Muramatsu et al., 1997) such that they are under the control of a chicken *β-actin* promoter. Two mutant forms of human FGFR1, N546K and K656E (M. Mohammadi, unpublished), as well as one mutant form of human FGFR2, C342Y (Mansukhani et al., 2000), were used in this study. The expression constructs were injected into the midbrain ventricles of stage 9-12 chicken embryos (Hamburger and Hamilton, 1992) and two electrodes were placed on either side of the rostral brain. Five rectangular electric pulses of 10 volts, for 50 mseconds were then delivered.

Reagents

Human FGF17b protein was kindly provided by Shaun K. Olsen and M. Mohammadi. An in situ probe for chicken *Gbx2* was generated by RT-PCR from stage 18 chicken brain RNA according to chicken sequences published in GenBank. A probe for chicken *Otx2* was made by Dado Boncinelli. The chick *Fgf8* probe was from Brigid Hogan, and *Spry1* and *Spry2* probes were from Gail Martin. The chick *Wnt1* probe was from Marion Wassef.

Results

FGF8b can regulate expression of mouse *Fgf18, Spry1 and 2, Fgfr2 and 3*

Previous studies in mouse showed that *Fgf8*, *Fgf17* and *Fgf18*

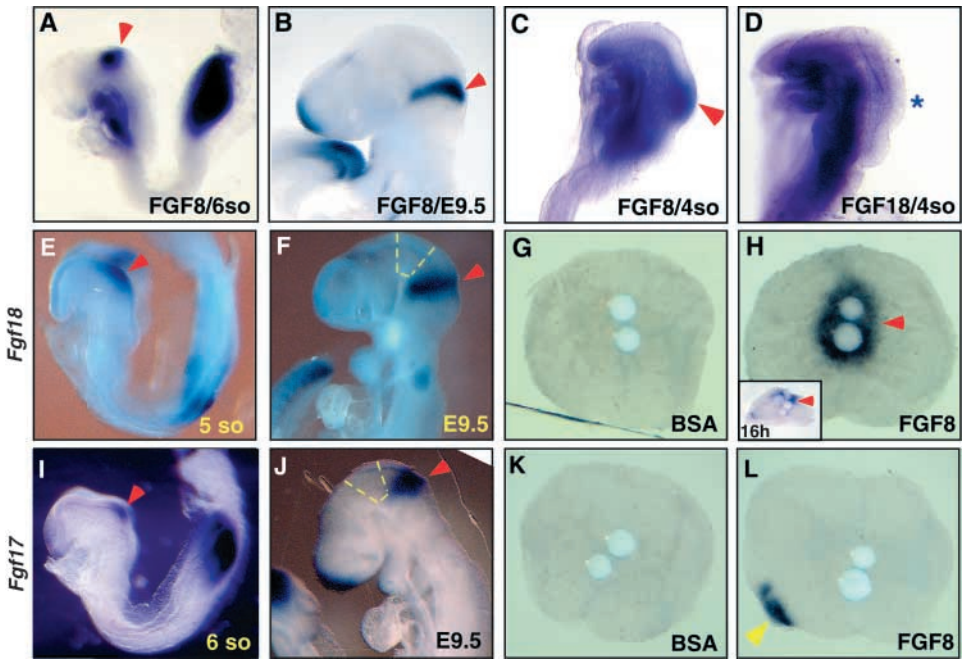

Fig. 1. FGF8 begins to be expressed in the mouse mid/hindbrain region prior to *Fgf18* and can induce *Fgf18* expression in mouse midbrain explants. (A-C) *Fgf8* is first expressed (arrowheads) in the mid/hindbrain region at the four-somite stage (C). (D-F) *Fgf18* is first expressed in the mid/hindbrain region at the five-somite stage (arrowhead in E) and becomes restricted to a narrow transverse band straddling the isthmus by E9.5 (arrowhead in F; asterisk in D indicates the presumptive mid/hindbrain junction region). (G,H) *Fgf18* is induced by FGF8b-soaked beads (arrowhead in H) by 48 hours, but not by BSA-soaked beads (G). Inset in H shows that *Fgf18* is induced by FGF8b by 16 hours. (I,J) *Fgf17* expression is first detectable in the mid/hindbrain region at the six-somite stage and at E9.5 it is in a broad domain on both sides of the mid/hindbrain junction (arrowheads). (K,L) *Fgf17* is not induced after 48 hours by either the BSA-soaked or FGF8-soaked beads in rostral midbrain explants. Arrowhead in L indicates the endogenous *Fgf17* expression sustained in the explant. Broken lines in F and J indicate the tissues used for the explant assays.

are all expressed in the isthmus region of eight- to nine-somite mouse embryos with *Fgf8* in the broadest domain (Xu et al., 2000). To determine the temporal sequence of mid/hindbrain expression of the three Fgf genes, we examined gene expression in whole mouse embryos. *Fgf8* expression was first seen at the four-somite stage (Fig. 1A,C), whereas *Fgf18* expression was not detected until slightly later at the five-somite stage (Fig. 1D,E and data not shown). *Fgf17* expression was first detected in the midbrain/r1 region at the six-somite stage (Fig. 1I and data not shown). At E9.5, both *Fgf17* and *Fgf18* were strongly expressed in domains encompassing the posterior midbrain and anterior r1 (Fig. 1F,J), with the *Fgf17* expression domain being broadest. *Fgf8* expression, by contrast, was restricted to a small domain at the anterior border of r1 (Fig. 1B).

As *Fgf8* expression precedes that of *Fgf17* and *Fgf18*, we investigated whether FGF8 can regulate the expression of *Fgf17* and *Fgf18* in the mouse brain. Explants were taken from the anterior midbrain at E9.5 and cultured with FGF8b-soaked beads as described previously (Liu et al., 1999). By 40 hours, *Fgf18* was induced by FGF8b-soaked beads (n=4/4, Fig. 1H) but not by BSA-soaked beads (n=0/4, Fig. 1G). By contrast, *Fgf17* was not induced by either FGF8b-soaked beads (n=0/4, Fig. 1L) or BSA-soaked beads (n=0/4, Fig. 1K). A time course of *Fgf18* induction was then performed. *Fgf18* mRNA was not detected in midbrain explants after 8 hours of exposure to FGF8b (n=4), but was present by 16 hours (n=3) (inset in Fig. 1H and data not shown). We have previously shown that *Fgf8* is not induced by FGF8 in the same assay (Liu et al., 1999). Taken together, these studies demonstrate that *Fgf8* is the first Fgf expressed in the mid/hindbrain region and suggest that it, in turn, induces *Fgf18* (directly or indirectly) in surrounding cells.

Induction of *Spry1* and *Spry2* by FGF-soaked beads has been shown to occur more rapidly in chick embryonic brains than other midbrain/r1 genes such as *En* and *Wnt1* (Chambers et al., 2000; Minowada et al., 1999). We therefore sought to determine whether the *Spry* genes are direct targets of FGF8b using our mouse brain explant assay, as protein synthesis inhibitors can be added to the medium. First we examined whether expression of the mouse Spry genes are similarly controlled by FGF signaling, using explants from prosomere 1 (p1), where neither *Spry* is expressed (Fig. 2A,B). Similar to in the chick, we found that *Spry1* and *Spry2* were rapidly induced within 4 hours by FGF8b-soaked beads (n=4/4 for each gene, Fig. 2D,H and data not shown), but not BSA-soaked beads (n=0/4 for each gene, Fig. 2C), in p1 explants. Next we added 50 μg/ml cyclohexamide or ethanol to the medium, and found that *Spry1* is not induced by this treatment (Fig. 2E,G), whereas *Spry2* is induced by cyclohexamide alone (data not shown). Regulation of *Spry1* by FGF8b in the presence of the protein synthesis inhibitor was then tested, and indeed *Spry1* was found to be induced (n=6/6; Fig. 2F). By contrast, the induction of *En1*, *En2* and *Gbx2* by FGF8b-soaked beads was efficiently blocked by cyclohexamide (data not shown), consistent with our observation that it takes at least 8 hours for these genes to be induced by FGF8b-soaked beads (Liu and Joyner, 2001b). These results show that induction of *Spry1*, but not of *En1*, *En2* and *Gbx2* by FGF8b is direct.

As *Fgfr2* and *Fgfr3* are not expressed in the cells surrounding the *Fgf8* domain in r1 (Walshe and Mason, 2000; Ishibashi and McMahon, 2002) (Fig. 3A-F), this raises the question of whether FGF8b regulates these receptors as well as *Fgf18*. We therefore examined the effects of FGF8b on the expression of Fgfr genes in p1 brain explants. *Fgfr1* was expressed at low levels in these explants and the expression was not altered by FGF8b-soaked beads after 40 hours (Fig. 3G,H; n=4/4). *Fgfr2* expression was maintained in p1 brain

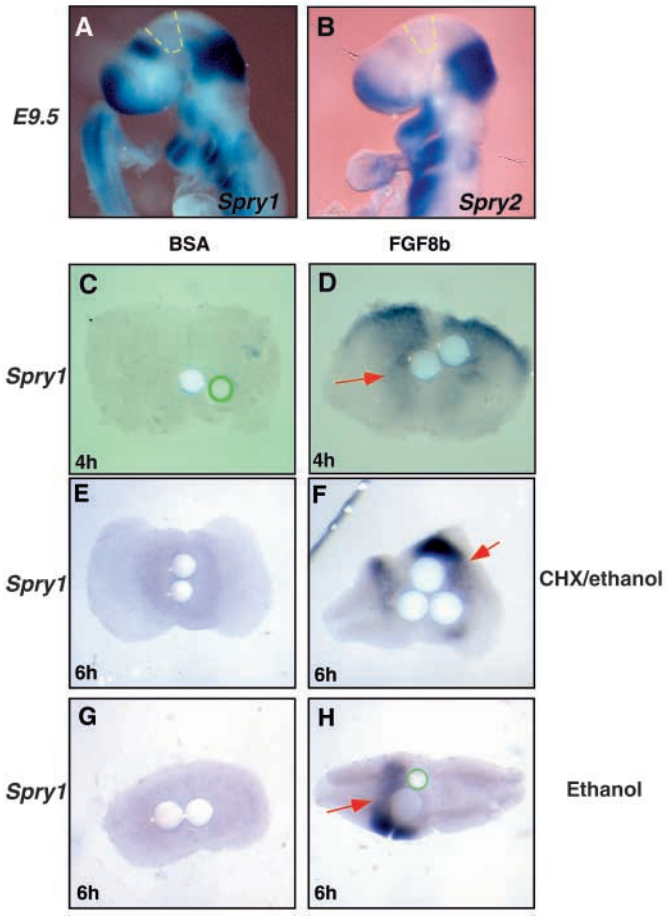

Fig. 2. *Spry1* is induced by FGF8b-soaked beads in prosomere 1 explants in the absence of protein synthesis. *Spry1* (A) and *Spry2* (B) are expressed in broad domains of the mid/hindbrain region at E9.5 *Spry1* is induced by FGF8b-soaked beads within 4 hours of culture (D), but not by BSA-soaked beads (C). (E-H) *Spry1* is induced within 6 hours by FGF8 in the presence or absence of 50 μg/ml cyclohexamide (CHX) in explants grown in medium containing 0.1% ethanol, but not by BSA-soaked beads. Red arrows in D,F,H indicate induced gene expression. Green circles in C and H indicate beads that were lost during processing of the tissues. Broken lines in A and B indicate the tissues used for the explant assays.

explants in the presence of BSA-soaked beads (*n*=4/4), with the highest level being along the dorsal midline (Fig. 3I). Significantly, FGF8b-soaked beads downregulated *Fgfr2* expression in the surrounding cells (Fig. 3J; *n*=4/4). *Fgfr3* expression was also maintained in control p1 brain explants (Fig. 3K; *n*=4/4), and was repressed by FGF8b-soaked beads (Fig. 3L; *n*=4/4). The repression of *Fgfr3* by FGF8b seemed to be more efficient than repression of *Fgfr2*, consistent with *Fgfr3* expression being more restricted to the rostral midbrain at E9.5 than *Fgfr2*.

FGF17b and FGF18 have a similar activity to FGF8a

Given that *Fgf17* and *Fgf18* are expressed in the midbrain, as well as in r1, it is important to determine the activity of these FGFs compared with FGF8a and FGF8b. A previous study using beads soaked in FGF18 and placed in the caudal diencephalon showed that it can induce *Fgf8* and apparently an ectopic midbrain after 3 days (Ohuchi et al., 2000), but did not address whether it can induce a cerebellum or regulate other genes. The function of FGF17 in the midbrain/r1 has not been explored in such a gain-of-function assay. Only one isoform of FGF18 has been described and it contains a 12 amino acid insert in the same position that FGF8b has an 11 amino acid insert compared to FGF8a (Xu et al., 1999). Three isoforms of FGF17 have been described and one (referred to as FGF17b) has an 11 amino acid insert in a similar position to FGF8b, whereas FGF17a lacks this insert. FGF17c has a stop codon that truncates the protein before the conserved FGF domain. To compare the activity of FGF17b and FGF18 with FGF8a/b in midbrain and cerebellum induction we used the electroporation assay described by Sato et al. (Sato et al., 2001) in which FGF8a induces midbrain development and FGF8b represses midbrain development and later induces a cerebellum. Expression constructs containing mouse cDNAs for *Fgf17b* or *Fgf18* (see Materials and methods) were electroporated into the midbrain and caudal forebrain region of chick embryos (Fig. 4A) at stages 9-12 (Hamburger and Hamilton, 1992) at a concentration of 1 μg/μl, and examined for changes in midbrain morphology and midbrain/r1 gene expression.

As a control for our experiments, we repeated the experiments of Sato et al. (Sato et al., 2001) by electroporating mouse *Fgf8a* and *Fgf8b* expression vectors at different concentrations (Table 1). The transfection efficiency was monitored by visualizing the expression of co-electroporated GFP (Fig. 4B) and by examining the transgene RNA using section in situ hybridization (see Fig. 5). Similar to recent studies (Sato et al., 2001), when *Fgf8b* was electroporated into the midbrain and caudal forebrain at a concentration of 0.1 μg/μl to 3 μg/μl, midbrain development was repressed and the region was probably transformed into an ectopic cerebellum (*n*=22/22; Fig. 4D, compare with the control in 4C, and data not shown). By contrast, electroporation of 0.01 μg/μl *Fgf8b* (*n*=6/6, data not shown), or 1-2 μg/μl *Fgf8a* (*n*=5/5; Fig. 4E) led to expansion of the midbrain and transformation of the diencephalon into a midbrain. Analysis of expression of the *Fgf8a* and *Fgf8b* mRNA produced by the expression vectors showed that similar levels and patterns of expression were obtained when 1-2 μg/μl of the DNA was used (inset in Fig. 5A,B). As expected, no *Fgf8* mRNA was detected when 0.01 μg/μl *Fgf8b* was used (data not shown).

Strikingly, electroporation of 1 μg/μl *Fgf17b* (*n*=15/15; Fig. 4F and Table 1) or *Fgf18* (*n*=17/17; Fig. 4G and Table 1) led to expansion of the midbrain, similar to the phenotype seen with *Fgf8a*. This phenotype was not due to reduced levels of expression of the constructs compared to the *Fgf8b* (or *Fgf8a*), as similar levels of mRNA were produced by the *Fgf17b* and *Fgf18* vectors (inset in Fig. 5I,M). To analyze the phenotype in more detail, chick embryos were processed for section RNA in situ analysis 24 hours after electroporation and analyzed for midbrain/r1 gene expression. Sato et al. (Sato et al., 2001) found that FGF8b induces *Gbx2*, *Pax2/5*, *En1/2* and represses *Otx2* and *Pax6*, whereas FGF8a only induces *En1/2*. Thus, a key distinction between the activity of FGF8a and FGF8b is that only FGF8b induces *Gbx2* and represses *Otx2* (compare Fig. 5A,B with 5E,F). Consistent with the similar phenotype produced by FGF17b, FGF18 and FGF8a, neither FGF17b nor FGF18 induced *Gbx2* or repressed *Otx2* (Fig. 5I,J,M,N). We

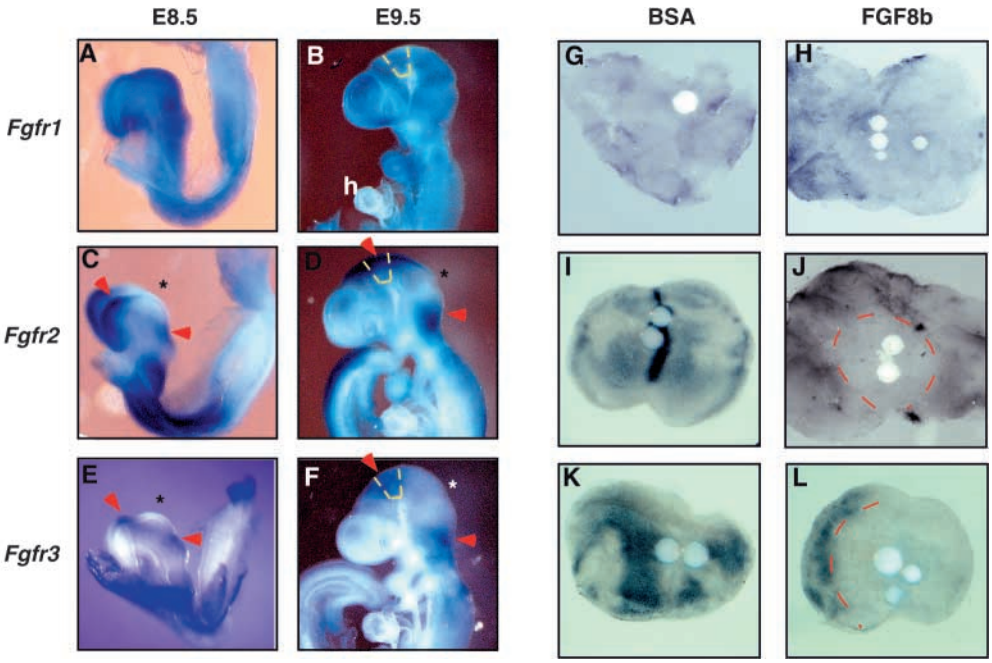

Fig. 3. *Fgfr2* and *Fgfr3* expression is excluded from the mid/hindbrain junction and repressed by FGF8b. (A,B) *Fgfr1* is weakly expressed throughout the embryo at the five-somite stage and E9.5, with the exception of the heart (h in B) at E9.5. (C,D) *Fgfr2* is expressed in the brain at E8.5 and E9.5 in the forebrain, rostral midbrain and part of the posterior hindbrain (arrowheads), but excluded from the mid/hindbrain region (asterisks). (E,F) In the brain, *Fgfr3* is expressed weakly in the caudal forebrain and part of the posterior hindbrain (arrowheads), but not in the mid/hindbrain region (asterisks) at the five-somite stage and E9.5. Note the strong expression in the extra-embryonic tissues at E8.5. (G,H) Weak and patchy *Fgfr1* expression is seen in p1 explants after 48 hours and this expression is not altered by FGF8b-soaked beads. (I) In control p1 explants, strong *Fgfr2* expression is limited to the dorsal midline, whereas weak expression is maintained in the rest of the explants. (J) *Fgfr2* expression is downregulated by FGF8b-soaked beads. (K) *Fgfr3* is maintained in BSA-treated p1 explants, whereas *Fgfr3* is repressed by FGF8b-soaked beads (L). Broken lines in B,D,F indicate the tissues used for the explant assays.

found that electroporation of a low level of *Fgf8b* vector (0.01 μg/μl) also did not alter *Gbx2* and *Otx2* expression (data not shown). The response of the endogenous *Fgf8* gene to the four FGFs was very interesting. FGF8b (1 μg/μl) was found to induce *Fgf8* in a sharp band of cells at the new *Gbx2/Otx2* boundary in the dorsal and lateral midbrain (Fig. 5G; see Fig. 7A,B). By contrast, FGF8a induced *Fgf8* in scattered cells in the midbrain (Fig. 5C), whereas FGF17b and FGF18 or low FGF8a did not induce detectable levels of *Fgf8* (Fig. 5K,O and data not shown). With all expression vectors, *En2* was upregulated broadly (data not shown). Finally, we examined the induction of *Spry1* and *Spry2*, as we found that *Spry1* is a direct target of FGF8b signaling. Only FGF8b strongly induced the *Spry* genes in the midbrain, and FGF17b or FGF18 only weakly induced *Spry1* in some experiments (Fig. 5H,L,P and data not shown). Taken together, the gene expression studies and morphological analysis demonstrate that when *Fgf17* and *Fgf18* are expressed at similar mRNA levels, they enhance midbrain development similar to *Fgf8a*.

Given that FGF17b and FGF18 have similar FGF receptor-binding affinities to FGF8b, at least for FGFR2 and FGFR3 in a tissue culture assay (Xu et al., 2000), it might be expected that the three proteins activate FGF signaling to the same level. One possible reason why electroporation of *Fgf17b* and *18* expression constructs does not produce similar changes in gene expression and proliferation to *Fgf8b* is that production or secretion of FGF17b and FGF18 protein is less efficient than FGF8b. To determine whether the same concentrations of FGF17b or FGF8b have identical activities, we used our mouse brain explant assay to compare the changes in gene expression induced by beads soaked in FGF17b compared with FGF8b. Similar to the in vivo results, FGF17b was found to induce *En1*

Table 1. Electroporation of chicken embryos with FGFs and FGF receptors

Construct		Number of embryos recovered	Stage of embryos	Phenotype
Fgf8b	3 μg/μl	4	E4.5-10.5	Repression of midbrain development
	1 μg/μl	7	E4.5	Repression of midbrain development
	0.1 μg/μl	12	E4.5-7.5	Repression of midbrain development
	0.01 μg/μl	6	E5.5-8.5	Large midbrain
Fgf8a	1 μg/μl	8	E5.5-7.5	Large midbrain
Fgf17b	1 μg/μl	15	E3.5-12.5	Large midbrain
Fgf18	1 μg/μl	17	E3.5-12.5	Large midbrain
$FGFR1^{n546K}$	3 μg/μl	6	E4.5-10.5	Large midbrain
	1 μg/μl	2	E8.5-10.5	Large midbrain
$FGFR1^{k656E}$	3 μg/μl	5	E9.5-10.5	Non-specific death of brain cells
	1 μg/μl	6	E4.5-9.5	Large midbrain
$FGFR2^{C342Y}$	3 μg/μl	5	E7.5-10.5	Large midbrain
	1 μg/μl	4	E5.5-7.5	Large midbrain

(*n*=7/7) and only weakly induce *Spry1* (*n*=6/6) in midbrain explants (Fig. 6I,L; data not shown). Furthermore, *Gbx2* was induced in only three out of 12 midbrain explants and FGF17b did not repress *Otx2* (*n*=8/8) in midbrain explants (Fig. 6C,F; data not shown). In the same experiments, FGF8 strongly induce *En1* (*n*=5/5), *Spry1* (*n*=3/3) and *Gbx2* (*n*=9/9), and repressed *Otx2* (*n*=3/7) (Fig. 6B,E,H,K; data not shown). In all experiments, beads soaked in BSA had no effect on gene expression (Fig. 6A,D,G,H; *n*≥2). Thus, the difference in activity of FGF17b compared with FGF8b is most likely to be due to intrinsic differences in their ability to induce the FGF signaling pathway.

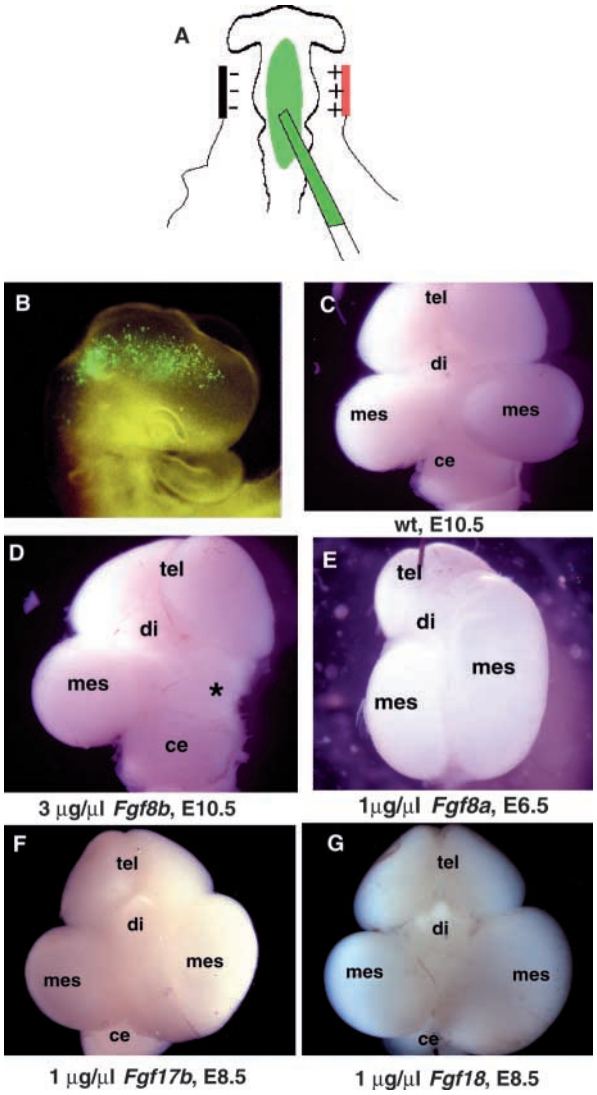

Fig. 4. FGF8b represses midbrain development whereas FGF8a, FGF17b and FGF18 promote midbrain development. (A) Schematic diagram showing the in ovo electroporation experiments. DNA (green) is injected into the midbrain by a glass needle and five electric pulses are applied. DNA is driven toward the anode and transfected only on the right side of the brain, whereas the left side serves as an internal control. (B) Co-transfection of a GFP expression vector with the experimental vector serves to show that most cells on the right side of the brain, including the mid/hindbrain region and caudal forebrain, are transfected. (C) Dorsal view of a wild-type E10.5 chicken brain. (D) Dorsal view of an E10.5 chicken brain electroporated with 1μg/μl *pMiw-Fgf8b*; the asterisk indicates lack of midbrain (mes) on the transfected right side. (E) Dorsal view of an E6.5 chicken brain electroporated with 1 μg/μl *pMiw-Fgf8a*; the midbrain on the transfected side is larger than the one on the control side. (F) Dorsal view of an E8.5 chicken brain electroporated with 1 μg/μl *pMiw-Fgf17b*; the midbrain on the transfected side is larger than the one on the control side. (G) Dorsal view of an E8.5 chicken brain electroporated with 1 μg/μl *pMiw-Fgf18*; the midbrain on the transfected side is larger than the one on the control side. tel, telencephalon; di, diencephlon; mes, mesencephalon; ce, cerebellum.

Activated FGFRs regulate gene expression similar to FGF8b

If the differential effects of FGF8a, 17b and 18 versus FGF8b mis-expression on midbrain development are because FGF8b can activate the FGF pathway more efficiently than the other FGFs, then ectopic expression of activated Fgfr genes should have the same effect as *Fgf8b*. To test this, we electroporated human FGFR constructs containing activating mutations into the midbrain and caudal forebrain and examined gene expression after 24-36 hours and brain morphology at later stages. We chose three mutant forms of FGFRs to test: one containing a mutation in the extracellular domain (C342→Y) of FGFR2, which leads to receptor activation possibly by inducing spontaneous dimerization of the receptors (Mangasarian et al., 1997); and two containing mutations in the tyrosine kinase domains of FGFR3 [N540→K (Bellus et al., 1995)] or [K650→E (Tavormina et al., 1995)]. As the kinase domains are very well conserved among different FGFRs, and *Fgfr1* but not *Fgfr3* is expressed in the midbrain/r1 region, activated forms of FGFR1 containing the N546K and K656E mutations were used.

We examined gene expression changes in embryos electroporated with 1 μg/μl of the $FGFR2^{C342Y}$ or the $FGFR1^{N546K}$ vector. Similar to FGF8b, *Gbx2*, *Fgf8* and *Spry1* and *2* were induced and *Otx2* repressed in the midbrain by $FGFR2^{C342Y}$ (Fig. 5Q,R,T and data not shown). Different from the homogeneous alterations in gene expression produced by electroporation of *Fgf8b*, the expression of the activated *FGFR* induced *Gbx2*, *Fgf8* and *Spry* and repressed *Otx2* in patches of cells mainly in the ventricular zone. This is probably due to the cell autonomous function of the activated FGFR compared with the secreted FGF8b protein, as the level and pattern of expression of the $FGFR2^{C342Y}$ mRNA was similar to the mouse *Fgf8* cDNA (inset in Fig. 5Q). The $FGFR1^{N546K}$ vector produced similar results, when assayed for *Fgf8* (*n*=2/3) and *Gbx2* (*n*=3/3) expression by whole-mount RNA in situ analysis (data not shown).

We next determined the long-term phenotype of transiently expressing activated FGFRs in the midbrain. Unlike FGF8b, the three activated FGFRs ($FGFR1^{N546K}$, $FGFR1^{K656E}$ and $FGFR2^{C342Y}$) produced enlargement of the midbrain and diencephalon (Fig. 7A-C; Table 1 and data not shown). At a DNA concentration of as high as 3 μg/μl, ectopic expression of $FGFR2^{C342Y}$ or $FGFR1^{N546K}$ caused a similar phenotype to that obtained with 1 μg/μl DNA (Fig. 7B and data not shown). The $FGFR1^{K656E}$ mutant at 3 μg/μl led to a non-specific loss of the entire brain region including midbrain and cerebellum, preventing a morphological or marker gene analysis (Table 1 and data not shown). Histological analysis of sections through E8-10 chicken embryos (*n*=2) electroporated with $FGFR2^{C342Y}$

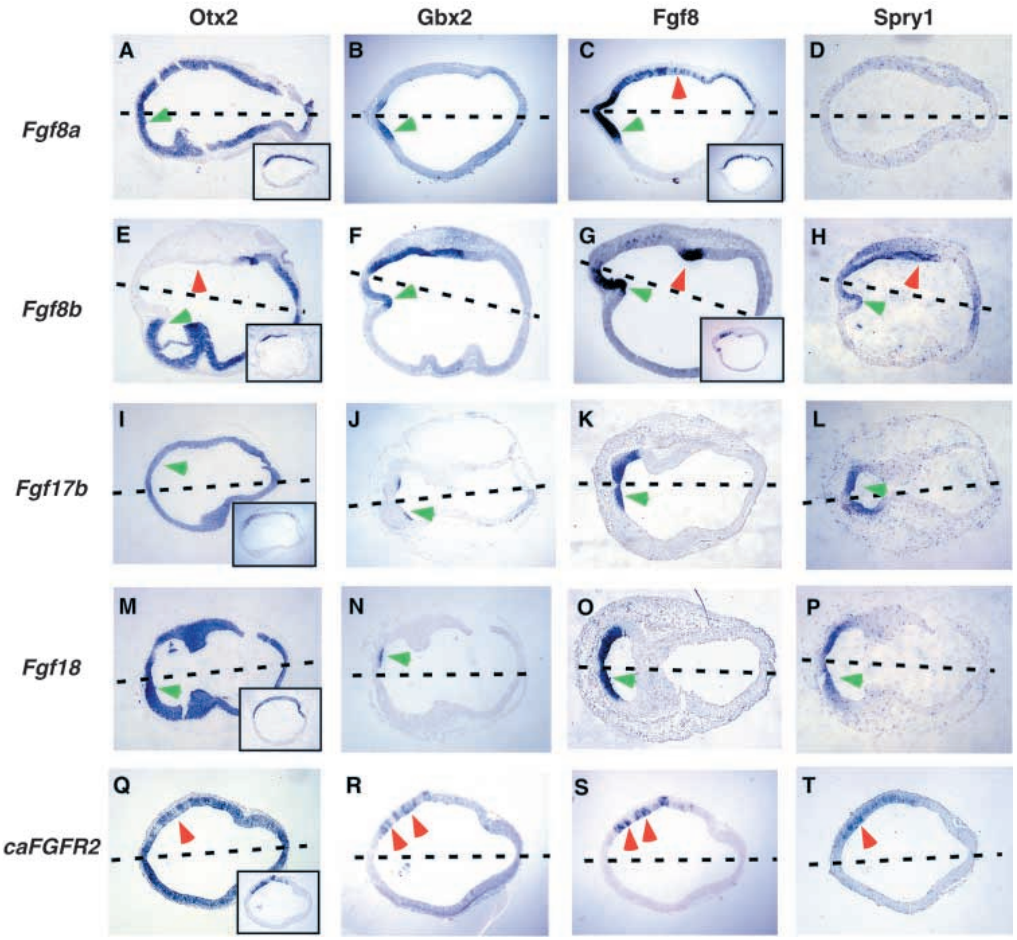

Fig. 5. FGF8b activates more molecular pathways than FGF8a, FGF17b and FGF18. (A-D) 1 μg/μl *pMiw-Fgf8a* induces *Fgf8* (C), but does not induce *Gbx2* (B) or *Spry1* (D), or repress *Otx2* (A). (E-H) 1 μg/μl *pMiw-Fgf8b* induces *Gbx2* (F), *Fgf8* (G) and *Spry1* (H) and represses *Otx2* (E). (I-L) 1 μg/μl *pMiw-Fgf17b* fails to induce *Gbx2* (J), *Fgf8* (K) or *Spry1* (L), or to repress *Otx2* (I). (M-P) 1 μg/μl *pMiw-Fgf18* fails to induce *Gbx2* (N), *Fgf8* (O) or *Spry1* (P), or to repress *Otx2* (M). (Q-T) 1 μg/μl *pMiw-caFGFR2* induces *Gbx2* (R), *Fgf8* (S) and *Spry1* (T) in scattered cells. *Otx2* is repressed on the electroporated side but scattered *Otx2*-expressing cells still exist (Q). In all panels, coronal or near coronal sections are shown with the anterior end towards the right. The broken lines indicate the midline with the electroporated side above the line and the control side below. In all panels, the red arrowheads indicate ectopic gene expression on the electroporated side and the green arrowheads indicate endogenous expression on the control side except for E and Q where the red arrowheads indicate the electroporated side where *Otx2* expression is repressed (completely in E and incompletely in Q). Insets in A,C,E,G,I,M,Q show expression of the mouse or human genes electroporated into the right side of the brain.

confirmed that the long-term phenotype of activated FGFRs is an enlarged midbrain, as the electroporated side showed the same histological features of the midbrain as on the control side (data not shown).

One possible reason why transient mis-expression of the FGFRs leads to expansion of the midbrain is that the upregulation of *Gbx2* and *Spry1/2*, and repression of *Otx2* does not happen in a sufficient number of cells to transform the midbrain into a cerebellum. Indeed, Sato et al. (Sato et al., 2001) showed that co-electroporation of an *Otx2* expression vector (1 μg/μl DNA) with an *Fgf8b* expression vector (0.1 μg/μl DNA) results in expansion of the midbrain, indicating that *Otx2* positive cells that receive an FGF8b signal respond by expanding the midbrain, whereas *Gbx2* positive cells form a cerebellum. Alternatively, or in addition, FGF8b may eventually lead to transformation of the midbrain into a cerebellum because the organizer is extended along the new *Gbx2/Otx2* border and it maintains the transformation. In order to explore these ideas further, we analyzed whole-mount embryos mis-expressing *Fgf8b* or $FGFR2^{C342Y}$ for expression of genes normally expressed near the organizer region. In embryos electroporated with the *Fgf8b* vector, the normal rings of *Fgf8* (*n*=4), *Wnt1* (*n*=4) and *En1* (*n*=3) at the mid/hindbrain junction were repressed on the electroporated side and both genes were induced along the dorsal midline and in a transverse band in the caudal diencephalon, probably adjacent to the induced *Gbx2* domain (Fig. 8A-D; *n*=4 for *Fgf8*, *n*=3 for *En1*). By contrast, expression of the activated *FGFR* vector induced *Fgf8*, *En1* and *Wnt1* only in patches of cells in the midbrain and caudal diencephalon (Fig. 8E,F and data not shown). The resolution of the experiment is not such that we can determine whether these genes are induced at new *Gbx2/Otx2* borders. Taken together, these studies are consistent with the idea that a high level of FGF signaling is required to induce *Gbx2* and repress *Otx2* and that transformation of a broad region into *Otx2-/Gbx2+* cells is required for late cerebellum formation.

Discussion

FGF signaling regulates positive and negative feedback loops

We show here that FGF8b can positively regulate FGF signaling by inducing *Fgf18* expression in brain explants, and that *Fgf18* expression is initiated slightly later than *Fgf8* in the mouse mid/hindbrain junction region. *Fgf17* is not expressed until later and is not induced by FGF8 in mouse brain explants. Thus, FGF8 could normally be required to induce expression of *Fgf18*. Furthermore, although the three FGFs have overlapping spatial distributions after the six somite stage, *Fgf17* and *Fgf18* should not be able to compensate for a loss

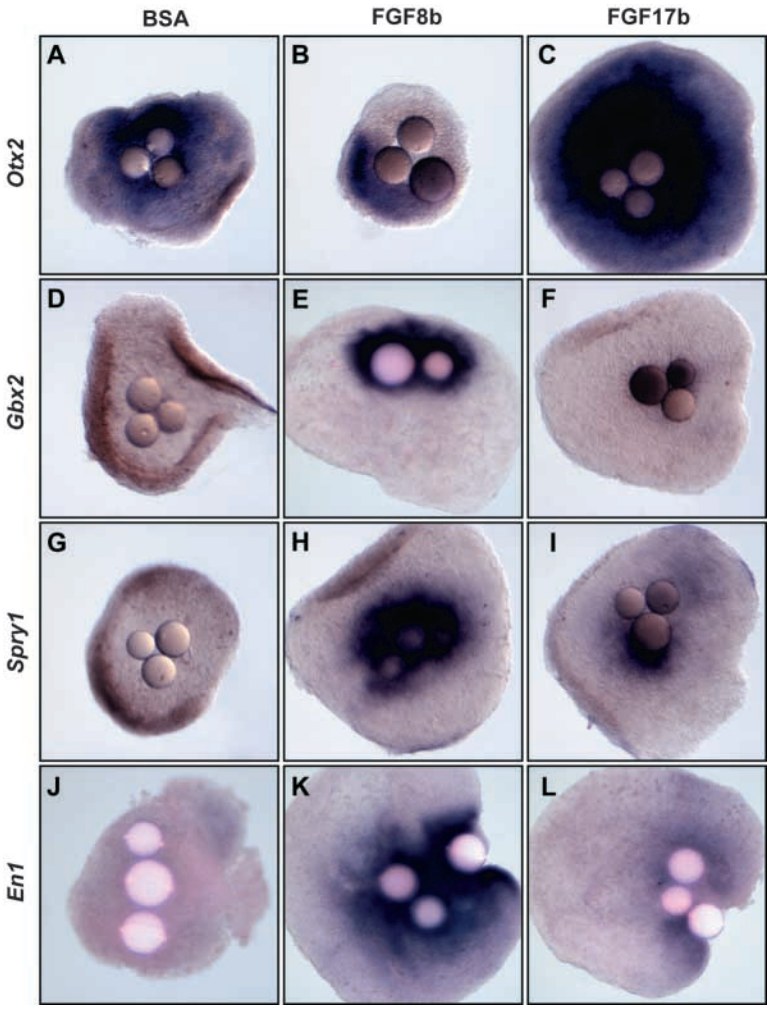

Fig. 6. FGF8b and FGF17b proteins differentially regulate genes in mouse brain explants. Beads soaked in BSA, mouse FGF8b or human FGF17b, as indicated, were placed in midbrain explants and cultured for 48 hours. Whole-mount RNA in situ analysis was then performed with the indicated probes. FGF8b strongly induces *Gbx2* (E), *Spry1* (H) and *En1* (K), and represses *Otx2* (B); FGF17b weakly induces *En1* (L) and *Spry1* (I).

of FGF8 protein because they are expressed too late and *Fgf18* expression is dependent on FGF8 function. A recent study of mice lacking *Fgf8* function specifically in the midbrain/r1 region after the five somite stage showed that both *Fgf17* and *Fgf18* are actually dependent on *Fgf8*, as their expression is greatly reduced at the seven- to nine-somite stage and gone by the 12- to 15-somite stage in such mutants (Chi et al., 2003). Thus, *Fgf8* mutants are equivalent to *Fgf8/17/18* triple mutants, and determination of the normal requirement for *Fgf17/18* in the midbrain and cerebellum will await analysis of *Fgf17/18* double mutants.

Consistent with the expression patterns of *Fgfr1*, *Fgfr2* and *Fgfr3*, we found that FGF8b is sufficient to repress expression of *Fgfr2* and *Fgfr3* in caudal forebrain explants. A study of zebrafish *ace* mutants that have a mutation in *fgf8* showed that FGF8 is also required to restrict *fgfr3* from the mid/hindbrain junction, because in *ace* mutants *fgfr3* is mis-expressed in the midbrain and r1 (Sleptsova-Friedricha et al., 2001). Thus, *Fgf8* negatively regulates FGF signaling by repressing two FGF receptors. Although *Fgfr1* is the key receptor that mediates FGF signaling in r1 and the caudal midbrain (Trokovic et al., 2003), the other two FGF receptors might play a role in mediating a low level of FGF signaling in anterior regions of the midbrain.

Expression of two negative regulators of FGF signaling, *Spry1* and *Spry2*, in a broad domain surrounding the mouse mid/hindbrain junction region has indicated that FGF signaling is attenuated by SPRY proteins in this region. Interestingly, we found that only FGF8b strongly induces expression of *Spry1* and *Spry2* in the chick anterior midbrain or mouse brain explants. Furthermore, using a brain explant assay we demonstrated that *Spry1* (and probably *Spry2*) is a direct downstream target of FGF8b signaling. This indicates that *Spry1* expression can be used as a read-out for FGF signaling. Consistent with this, in mouse embryos lacking *Fgf8* in the midbrain/r1 after the six-somite stage, *Spry2* is maintained at the 7- to 9-somite stage, but greatly reduced by the 13-16 somite stage (Chi et al., 2003).

Taken together, our studies and others show that in mouse FGF8b regulates at least three components of the FGF signaling pathway. First, FGF8b induces expression of another FGF protein, FGF18. FGF8b also directly induces two negative modulators of the pathway (SPRY 1/2), and thus produces a negative-feedback loop. Furthermore, our finding that FGF8b also represses *Fgfr2* and *Fgfr3* demonstrates that a second

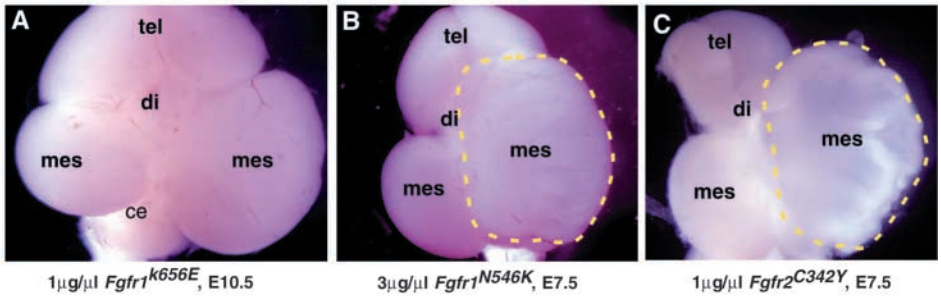

Fig. 7. Activated FGFR1 and FGFR2 produce over-proliferation of the midbrain. (A) Dorsal view of an E10.5 chicken brain electroporated with 1 μg/μl *pMiw-Fgfr1*^{K656E}, the midbrain on the transfected side is larger than the one on the control side. (B) Dorsal view of an E7.5 chicken brain electroporated with 3 μg/μl *pMiw-Fgfr1*^{N546K}, the midbrain on the transfected side is larger than the one on the control side. (C) Dorsal view of an E7.5 chicken brain electroporated with 1 μg/μl *pMiw-Fgfr2*^{C342Y}, the midbrain on the transfected side is larger than the one on the control side. In all panels, the right sides are the experimental sides and the left sides serve as controls. Broken outline indicates the expanded midbrain.

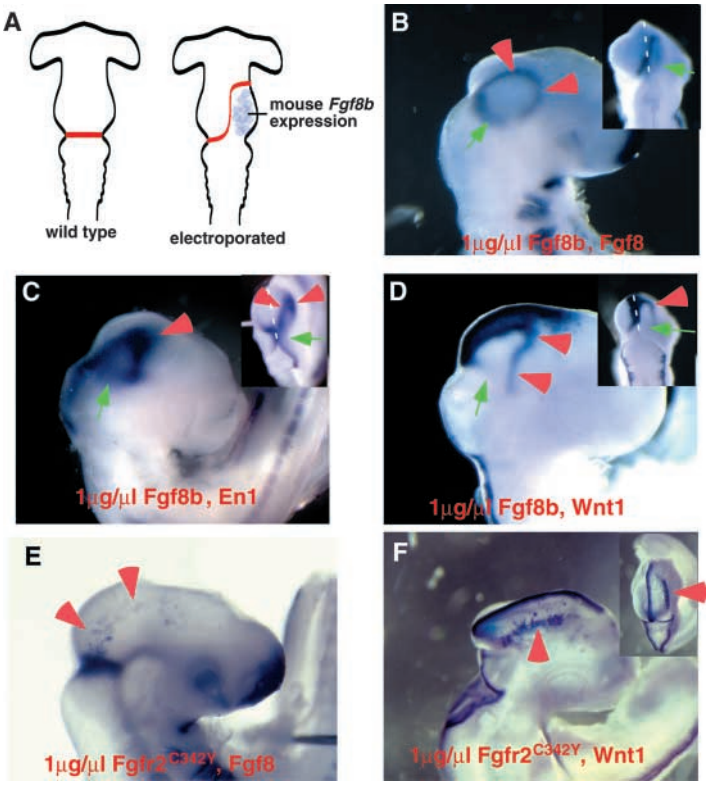

Fig. 8. FGF8b transforms the midbrain into a cerebellum and shifts the mid/hindbrain organizer rostrally. (A) Experiments shown in B, C and D. 24 hours after 1 μg/μl *pMiw-Fgf8b* was electroporated into the midbrain, *Fgf8* expression is shifted into the caudal forebrain region on the experimental side, as well as in a thin band along the dorsal midline (red lines in A and arrowheads in B) that connects the ectopic *Fgf8* domain to the endogenous *Fgf8* domain on the control side. Green arrow shows the down regulation of *Fgf8* expression on the transfected side in the isthmus. Inset shows the rear view of the same embryo. (C) 24 hours after 1 μg/μl *pMiw-Fgf8b* is electroporated into the chicken midbrain *En1* expression is shifted rostrally on the electroporated side, and seen in the most dorsal cells in the midbrain and anterior hindbrain (red arrowheads), whereas the endogenous expression surrounding the isthmus (green arrow) is downregulated. Inset shows a rear view of the same embryo, note the normal expression on the control (left) side. (D) 24 hours after 1 μg/μl *pMiw-Fgf8b* is electroporated into the chicken midbrain, the endogenous *Wnt1* expression in the isthmus (green arrow) is downregulated, whereas ectopic expression is induced near the dorsal midline and in a transverse band in the rostral midbrain. Inset shows a rear view of the same embryo, note the normal expression on the control (left) side. (E) Scattered expression of *Fgf8* is induced in the midbrain and caudal forebrain (arrowheads) by ectopic expression of activated FGFR2. Note that the endogenous *Fgf8* expression is not repressed. (F) Scattered expression of *Wnt1* is induced in the midbrain and caudal forebrain (arrowheads) by ectopic expression of activated FGFR2. Inset shows a rear view of the same embryo.

negative feedback loop contributes to fine regulation of the level of FGF signaling in r1 and the midbrain to ensure appropriate patterning and growth.

FGF8b has a distinct activity from FGF8a, FGF17b and FGF18 in the midbrain

To gain insight into how three Fgf genes orchestrate midbrain and cerebellum development, we explored the activity of FGF17b and FGF18 in comparison to FGF8a and FGF8b in their ability to regulate cell proliferation and gene expression when mis-expressed in the midbrain. Of the four proteins, only FGF8b has the ability to transform the midbrain into a cerebellum. Associated with this unique activity, only FGF8b can induce *Gbx2* and repress *Otx2* when expressed in the midbrain. Furthermore, and likely of crucial importance for maintaining the transformation, only FGF8b induces an ectopic organizer region at the new *Gbx2/Otx2* border in the midbrain. By contrast, FGF8a, FGF17b and FGF18 induce expansion of the midbrain, and do not alter *Gbx2* or *Otx2* expression. *Spry1/2* is strongly induced by FGF8b and only weakly by FGF17b and FGF18, whereas endogenous *Fgf8* is only induced locally by FGF8a. This different activity of FGF8b protein can not be due to a higher level of expression of the *Fgf8b* construct, as it is only at a 100-fold lower DNA concentration at which the mouse *Fgf8b* mRNA can not even be detected that FGF8b induces a midbrain. As *Fgf17*and *18* are expressed in the midbrain, although *Fgf8* is restricted to r1, *Fgf17* and *Fgf18* could be the main FGFs that normally directly regulate growth and patterning of the midbrain.

Mouse mutant analyses have shown that *Fgf17* is more important in the midbrain than *Fgf18*, because only *Fgf17* mutants have a truncation of the posterior midbrain (Xu et al., 2000; Liu et al., 2002; Ohbayashi et al., 2002). Our comparison of the activities of FGF17 and FGF18 show that *Fgf18* could also function with *Fgf17* in regulating midbrain development. Loss-of-function studies have also shown that *Fgf17* plays a role, along with *Fgf8*, in regulating late proliferation of the anterior cerebellum (Xu et al., 2000). Our finding that FGF17b and FGF18 have such distinct activities from FGF8b in the midbrain are in contrast to previous tissue culture studies that indicated the proteins have similar binding affinities to FGFR2c and FGFR3c and similar functions in regulating proliferation (Xu et al., 1999; Xu et al., 2000). One possibility was that FGF17b and FGF18 proteins are not produced or secreted as efficiently as FGF8b in the chick midbrain. We have ruled out this possibility by showing that when similar concentrations of FGF17b and FGF8b protein are compared in mouse brain explant assays, they differentially regulate gene expression similar to the electroporation experiments. Thus, the intrinsic activity of FGF8b is different from that of FGF17b, possibly because the 11 amino acid inserts in the two proteins are distinct. Our study demonstrates the importance of testing the activity of proteins in vivo where they normally function. Finally, although *Fgf8* alone encodes two proteins sufficient for directing development of both the midbrain and cerebellum, *Fgf17* and *Fgf18* probably augment the proliferative and midbrain inducing ability of *Fgf8a* or a low level of *Fgf8b*.

Activated FGFRs regulate midbrain/r1 genes similar to FGF8b

It is possible that the difference in the phenotypes produced by mis-expression of *Fgf8a* versus *Fgf8b* is quantitative, because in vitro studies have shown that FGF8b has a much higher affinity for FGFRs than FGF8a. Consistent with this, electroporation of a low concentration of *Fgf8b* expression vector has similar effects to high concentrations of *Fgf8a* (Sato

et al., 2001), and some *Wnt1-Fgf8a* transgenics have phenotypes similar to *Wnt1-Fgf8b* transgenics (Liu et al., 1999). By contrast, FGF17b and FGF18 have similar binding affinities and proliferation activities to FGF8b in vitro (Xu et al., 1999; Xu et al., 2000), but do not behave like FGF8b when mis-expressed in the midbrain or applied to brain explants. However, the biochemical studies were carried out using FGFR2 and FGFR3, which are not the major receptors that mediate midbrain/r1 patterning (Trokovic et al., 2003). It is possible that there are qualitative differences in the way FGF8b interacts with FGFR1, that allow FGF8b to activate the downstream pathway more efficiently. We addressed this possibility by asking whether high level FGF signaling is sufficient to induce *Gbx2* and repress *Otx2* using activating mutations in FGFR1 and FGFR2. Indeed, the activated FGFRs regulate key target genes similar to FGF8b. Of significance, the activated FGFRs strongly induce *Spry1/2* and *Gbx2* and repress *Otx2*.

Given the changes in gene expression induced by activated FGFRs, it was perhaps surprising that the long-term phenotype of transient expression of activated FGFRs is expansion of the midbrain. We suggest that in transient mis-expression studies such as electroporations, *Gbx2* must be induced in a homogeneous domain so that a new organizer can form along the extended *Gbx2/Otx2* border, and the organizer can then maintain the long-term transformation of the midbrain into a cerebellum. In support of this idea, when *Gbx2* is electroporated into the midbrain, *Otx2* is only transiently repressed in scattered cells in the anterior midbrain, and although the isthmus is expanded anteriorly, no ectopic cerebellum forms (Katahira et al., 2000). As electroporation produces mosaic gene expression, the secreted protein FGF8b, but not the activated FGF receptor, can induce *Gbx2* throughout the electroporated region. In addition, although the activated FGFRs can induce *Fgf8* as well as *Wnt1* and *En1* in the midbrain, it is in patches of cells because of the cell-autonomous nature of the receptors. As the response of *Otx2*-expressing midbrain cells to FGF8b is proliferation of the midbrain (Sato et al., 2001), and there are *Otx2*-positive cells present on the side of the midbrain electroporated with the activated FGFRs, this could account for the later expansion of the midbrain.

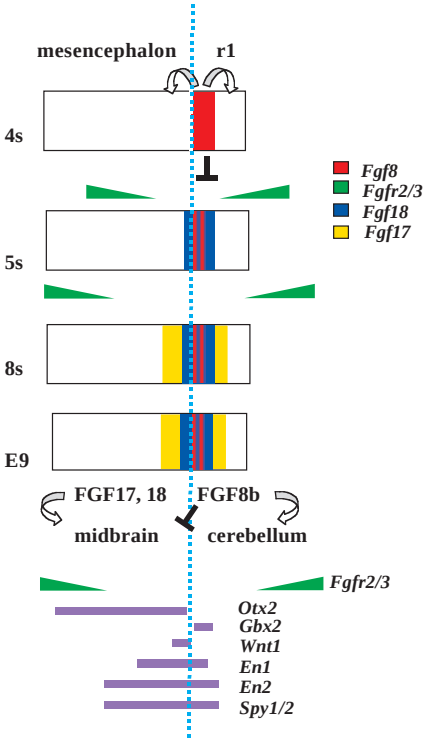

Fig. 9. FGF signaling is autoregulated at multiple levels and multiple FGF proteins regulate midbrain and cerebellum development. In the mouse, FGF8 expression in the isthmus at the four-somite stage represses the expression of *Fgfr2* and *Fgfr3* and activates the expression of *Fgf18* at the five-somite stage. *Fgf17* expression is initiated in a broader domain slightly later, and by E9 the three Fgfs are expressed in overlapping gradients radiating from the isthmus, whereas *Fgfr2* and *Fgfr3* are absent in this region. *Spry1/2* genes are upregulated by FGFs. FGF8b is required to maintain a cascade of gene expression that includes absence of Otx2 in r1, allowing cerebellum development to occur. FGF17 and FGF18, and possibly FGF8a and a low level of FGF8b regulate growth and *En* expression in the midbrain.

Conclusions

Based on our studies and those of others, we suggest the following steps in midbrain and cerebellum development in mouse (Fig. 9). At the four-somite stage, *Fgf8* is induced in the presumptive r1 territory by an unknown factor. *Pax2* is required for this induction (Ye et al., 2001) and OTX2 inhibits *Fgf8* from being induced in the midbrain (Li and Joyner, 2001; Martinez-Barbera et al., 2001). FGF8b then induces *Fgf18* in the surrounding cells, producing a larger domain and gradient of *Fgf* mRNA that extends into the midbrain. FGF8b also maintains two negative feedback loops by inducing *Spry1* and *Spry2* expression and inhibiting *Fgfr2* and *Fgfr3*. *Fgf17* is then induced by an unknown mechanism that is dependent on *Fgf8* (Chi et al., 2003) in a broader domain than *Fgf18*, further extending the gradient of Fgf mRNA expression. FGF17 and FGF18 protein, and possibly FGF8a and a low level of FGF8b, then regulate proliferation of the midbrain and cerebellum and *En* expression. The narrow domain where *Fgf8* is expressed becomes the isthmus because of the activity of FGF8b (Li et al., 2002), and the adjacent *Otx2*-negative r1 cells become the cerebellum. We have recently shown that by the 15-somite stage *Gbx2* is not required in r1 for cerebellum development, but is required earlier to specify r1 (Li et al., 2002). Thus, once *Fgf8* expression in r1 is stabilized, perhaps by a secreted factor from the midbrain (Irving and Mason, 1999), a key function of high level signaling by FGF8b is to maintain a cascade of gene expression in the midbrain/r1 that maintains an *Otx2*-negative domain in r1 in which the cerebellum develops.

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