INTRODUCTION

The vertebrate forebrain forms through a series of complex morphological and molecular events and interactions. Initial divisions, telencephalon and diencephalon, are further subdivided to produce pallial and subpallial telencephalon and diencephalic derivatives: hypothalamus, ventral thalamus, zona limitans intrathalamica (ZLI), dorsal thalamus and pretectum (Fig. 1A). Eyes and optic stalks are derived as outpocketings from the diencephalon (Puelles et al., 2000; Rubenstein et al., 1998). Comparatively little is known about the molecular regulation of forebrain patterning, although fate mapping, gene expression analysis, and genetic and transgenic analyses have provided some mechanistic insights (Brown et al., 2001).

Forebrain patterning in zebrafish begins during early gastrulation stages (Grinblat et al., 1998), when telencephalic, diencephalic and retinal precursors already occupy distinct domains (Woo and Fraser, 1995). The Hedgehog signalling pathway is required for ventral forebrain development in zebrafish (Varga et al., 2001) and Wnt pathway antagonists, such as hdl, boc and mbl, are required for telencephalon formation (Heisenberg et al., 1996; Kim et al., 2000; Fekany-Lee et al., 2000; Heisenberg et al., 2001). Local patterning within the anterior forebrain depends upon another Wnt pathway antagonist, tlc, which is expressed at the anterior neural boundary (Houart et al., 1998; Houart et al., 2002). Once established, telencephalic and diencephalic subdomains can be distinguished by their gene expression patterns (Wilson and Rubenstein, 2000; Hauptmann et al., 2002).

Fibroblast growth factors (Fgfs) regulate patterning events in the midbrain and hindbrain, and have synergistic actions when expressed in overlapping domains. Notably, Fgf8 and Fgf17, expressed midbrain-hindbrain boundary (isthmus), regulate the patterning and later aspects of development of the adjacent territories (Crossley et al., 1996; Reifers et al., 1998; Shamim et al., 1999; Martinez et al., 1999; Liu et al., 1999; Irving and Mason, 1999; Irving and Mason, 2000; Xu et al., 2000; Reifers et al., 2000; Sato et al., 2001; Irving et al., 2002). There is also a combinatorial role for Fgf8 and Fgf3, from presumptive rhombomere 4, in patterning the hindbrain (Walshe et al., 2002; Maves et al., 2002), and in induction of the adjacent otic placode (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002).

An emergent theme is one of both unique and combinatorial functions for Fgfs in brain patterning. Several Fgfs are expressed within the developing forebrain (Mason et al., 1994; Mahmood et al., 1995; Crossley and Martin, 1995; Mahmood et al., 1996; McWhirter et al., 1997; Reifers et al., 2000; Crossley et al., 2001; Gimeno et al., 2002), and Fgf8 has been assigned a role in forebrain development. Mutant mice carrying hypomorphic fgf8 alleles have smaller forebrains with midline deletions (Meyers et al., 1998). A patterning function is supported by in vitro studies using both chick and mouse tissues (Shimamura and Rubenstein, 1997; Crossley et al., 1998).
2001), by in vivo studies in the mouse (Fukuchi-Shimogori and Grove, 2001), and by analysis of the zebrafish acerebellar (ace) mutant (Shanmugalingam et al., 2000). Although detailed analyses are lacking, fgf3 is also expressed in the forebrain, (Mahmood et al., 1996; Raible and Brand, 2001; Walshe et al., 2002) and its ectopic expression affects the expression of certain forebrain markers (Koshida et al., 2002).

We report a complex and dynamic expression pattern for fgf3 in the zebrasah fish forebrain, which partially overlaps with that of fgf8. Using morpholino oligonucleotides to inhibit Fgf3 and Fgf8, both individually and together, we identify unique functions for Fgf3 in both telencephalon and several regions of the diencephalon, and in combinatorial actions with Fgf8. In addition, we report further roles for Fgf8 in forebrain development.

MATERIALS AND METHODS

Fish stocks
Zebrafish, Danio rerio, of the King’s wild-type (kwt) strain were used throughout these studies. They were maintained at 28°C, and embryos were staged according to Kimmel et al. (Kimmel et al., 1995).

Morpholino oligonucleotide injections
Fgf8, Fgf3 and Fgf control morpholino oligonucleotides (Gene Tools), at a concentration of 6 μg/μl, were injected into zebrafish embryos as previously described (Maroon et al., 2002). Embryos were dechorionated and incubated with the FGFR inhibitor SU5402, as previously described (Maroon et al., 2002), except that SU5402 stock solutions were prepared at 10 mM and diluted to 0.1 mM for use.

RESULTS

fgf8 and fgf3 have unique and overlapping expression domains within the developing zebrafish forebrain

Expression of fgf8 within the developing zebrafish forebrain has been reported previously (Reifers et al., 1998; Shanmugalingam et al., 2000), therefore only a brief description is presented here to facilitate comparison with fgf3 expression. fgf8 transcripts were first detected at the anterior margin of the forebrain primordium at the tailbud stage (Fig. 1B), during early somitogenesis transcripts accumulated within the dorsal telencephalon (Fig. 1C), and by late somitogenesis fgf8 expression was detected within the anterior telencephalon, dorsal diencephalon, optic stalks, posterior hypothalamus and retina (Fig. 1D,E). At 30 hpf transcripts remained in the anterior telencephalon, optic stalks and posterior tuberculum, and were also detected in the epiphysis (Fig. 1F).

Descriptions of fgf3 expression in zebrafish embryos have been reported previously (Phillips et al., 2001), by in vivo studies in the mouse (Fukuchi-Shimogori and Grove, 2001), and by analysis of the zebrafish acerebellar (ace) mutant (Shanmugalingam et al., 2000). Although detailed analyses are lacking, fgf3 is also expressed in the forebrain, (Mahmood et al., 1996; Raible and Brand, 2001; Walshe et al., 2002) and its ectopic expression affects the expression of certain forebrain markers (Koshida et al., 2002).

In situ hybridisation
In situ hybridisation reactions were essentially performed as described previously (Shamim et al., 1999; Maroon et al., 2002), except that embryos younger than 24 hours post fertilisation (hpf) were not treated with proteinase K, and the hydrogen peroxide treatment was omitted.

Cell death and division
Dividing cells were detected using an anti-phosphorylated histone H3 (ser 128) antiseurm (Calbiochem), and apoptotic cells were detected using the DeadEnd™ colourimetric detection kit (Promega) as described (Maroon et al., 2002). Numbers of dividing cells, within an area measuring 200 μm by 300 μm encompassing the presumptive forebrain from four embryos at tailbud stage injected with either control morpholinos, Fgf8 morpholinos (Fgf8mo), Fgf3 morpholinos (Fgf3mo), or both Fgf8mo and Fgf3mo, were determined and subjected to a Student’s t-test for statistical analysis.

Fig. 1. Expression of fgf8 (B-F) and fgf3 (G-L) during development of the zebrafish embryo forebrain. Lateral (B-D,F,G,J-L) or dorsal (E,H,I) views with anterior to the left. The eye was removed in F and L to facilitate observation of expression in the brain. (A) Diagram depicting brain subdivisions and structures relevant to this study in a 24 hpf embryo. (B-F) fgf8 expression at tailbud stage (B), during early somitogenesis (C), late somitogenesis (D,E; dorsal view focussed on the hypothalamic region) and 30 hpf (F), (G-L) fgf3 expression. (G) onset at 80% epiboly (arrow). (H,I) Dorsal views at 90% epiboly (H) and 2s (I), showing fgf3 transcripts in the forebrain and in presumptive rhombomere 4. Early (J) and late (K) somitogenesis, and 30 hpf (L). (J) ac, anterior commissure; d, diencephalon; dd, dorsal diencephalon; dt, dorsal thalamus; ep, epiphysis; fb, forebrain; h, hypothalamus; hb, hindbrain; hpf, hours postfertilisation; i, isthmus; os, optic stalk; poc, post-optic commisure; pt, pretectum; r, retina; s, somites; t, posterior tuberculum; tc, tectum; tel, telencephalon; tg, tegmentum; vt, ventral thalamus. Asterisk indicates the zona limitans intrathalamica.
Forebrain were not included in those studies. fgf3 transcripts were first detected in anterior neuroectoderm at 80% epiboly (Fig. 1G), and at 90% epiboly fgf3 expression was detected in cells of the presumptive forebrain and underlying prechordal hypoblast (Fig. 1H) (Phillips et al., 2001). At the beginning of somitogenesis, fgf3 was expressed in cells of the anterior neural boundary (row 1 cells; Fig. 1H,I). At early somite stages, transcripts became confined to the dorsal telencephalon and the polster located anterior to the forebrain (Fig. 1J). At late somite stages, very low level fgf3 expression remained in the telencephalon, while a new expression domain appeared in the ventral hypothalamus (Fig. 1K). fgf3 transcripts remained within the ventral hypothalamus until at least 30 hpf and were additionally present in the optic stalks at this stage (Fig. 1L).

**Morpholino oligonucleotides effectively inhibit Fgf3 and Fgf8 functions but do not result in increased cell death or division in the forebrain**

To analyse the involvement of Fgf3 and Fgf8 in forebrain development, 1- to 4-cell stage zebrafish embryos were injected with morpholino oligonucleotides to inhibit their translation. We have previously shown that these morpholino oligonucleotides compromise Fgf8 and Fgf3 function, phenocopying the ace (fgf8) mutant and rendering Fgf3 protein undetectable, respectively (Maroon et al., 2002; Walshe et al., 2002).

Fgf morpholino injection did not appear to affect cell division in the forebrain at either the tailbud or 10-somite stage (10s; Fig. 2A-H); this was confirmed by Student’s t-test analyses. In addition, no changes in cell death were detected in forebrains at 10s (Fig. 2I-P) or at 24 hpf (data not shown).

**Fgf3 and Fgf8 are required for different aspects of telencephalic regionalisation**

We investigated Fgf3 and Fgf8 function in telencephalic patterning. Embryos were injected with morpholinos and analysed at the tailbud stage for emx1 expression at the anterior margin of the neural plate (Morita et al., 1995). Previous studies have shown that emx1 expression at the tailbud stage is reduced in ace embryos (Shanmugalingam et al., 2000), and this was also observed following Fgf8mo injection (8/8). Injection of Fgf3mo alone (n=10/10), or in combination with Fgf8mo (n=11/11), resulted in a more severe phenotype, in which emx1 expression was very weak or undetectable (Fig. 3A-D). Reduction of emx1 expression was only transitory as later, at 24 hpf, expression was observed in the telencephalons of all embryos injected with Fgf8mo (n=17/17), Fgf3mo (n=12/12), and both Fgf morpholinos (n=13/13). Furthermore, in many cases, expression was expanded into the subpallial domain of the telencephalon (Fig. 3E-H). These results implied that Fgf8 and Fgf3 were not required for specification of the pallial telencephalon, but that both were required for correct specification of the subpallial telencephalon.

Telencephalic patterning was further analysed by investigating the expression of two T-box transcription factors expressed in postmitotic neurons of the posterior dorsal telencephalon, eomesodermin (eom; eomes – Zebrafish Information Network) and T-brain 1 (tbr1) (Mione et al., 2001), as Fgfs have been implicated in the regulation of T-box transcription factors in other embryological contexts (Smith et al., 1991; Griffin et al., 1998; Rossant and Cross, 2001). Expression of eom was greatly reduced or undetectable in the forebrains of embryos injected with either Fgf8mo (n=13/13) or Fgf3mo (n=12/12), or both morpholinos (n=18/18; Fig. 3M-P). By contrast, telencephalic tbr1 expression expanded anteriorly following injection of Fgf8mo (n=12/23), but was

![Fig. 2. Cell death and division in the forebrain are unaffected in embryos injected with Fgf morpholinos.](image-url)
reduced following Fgf3mo injection (n=15/18). When both morpholinos were injected the effects of the Fgf3 morpholino were dominant, with reduction of **tbr1** expression (n=18/22; Fig. 3I-L). **dlx2** is expressed in the zebrafish telencephalon and ventral thalamus from 13 hpf (Akimenko et al., 1994), and is required for the development of specific telencephalic neurons in mice (Qiu et al., 1995; Anderson et al., 1997). Injection of Fgf3mo resulted in variable reduction of **dlx2** expression in the telencephalon at 24 hpf (n=6/9), but complete loss of expression by 28 hpf (n=22/23), whereas Fgf8mo-injection had no effect on telencephalic **dlx2** expression at either 24 hpf (n=11/11) or 28 hpf (n=21/21; Fig. 3I-L,Q-T). Double in situ hybridisation results demonstrated that expanded **tbr1** expression in the subpallial telencephalon following Fgf8mo injection was largely anterior and lateral to the more medially-located **dlx2** domain in that region (Fig. 3J).  

**Fig. 3.** Fgf3 and Fgf8 regulate telencephalic gene expression. Dorsal (A-D,M-P) or lateral (E-L,Q-T) views with anterior to the left.  
(A-D) **emx1** expression at the anterior margin of the forebrain primordium at tailbud stage. (A) **emx1** transcripts are detected in the presumptive anterior forebrain in control embryos but are reduced in embryos injected with Fgf8mo (B), and are undetectable in embryos injected with either Fgf3mo (C), or Fgf8mo and Fgf3mo (D). (E-H) **emx1** expression at 24 hpf. (E) **emx1** expression is limited to the pallial telencephalon in control embryos. Arrow indicates expression boundary. **emx1** transcripts are detected in both pallial and subpallial (arrow in F) telencephalon in embryos injected with Fgf8mo (F), Fgf3mo (G), or Fgf8mo and Fgf3mo (H). (I-L) **tbr1** (red) and **dlx2** (blue) expression at 24 hpf. (I) A control embryo: **dlx2** transcripts are found in the ventral thalamus and subpallial telencephalon, and extend into the posterior region of the pallial telencephalon. **tbr1** transcripts occupy the pallial telencephalon. (J) **tbr1** expression extends into the anterior subpallial telencephalon, partially overlapping **dlx2** expression, in embryos injected with Fgf8mo (compare arrows in I and J). **tbr1** and **dlx2** expression is reduced following injection with either Fgf3mo (K), or Fgf8mo and Fgf3mo (L). (M-P) Telencephalic **eom** expression at 24 hpf. (M) **eom** transcripts are detected in the telencephalon in control embryos. **eom** transcripts in the telencephalon are reduced or undetectable in embryos injected with Fgf8mo (N), Fgf3mo (O), or Fgf8mo and Fgf3mo (P). (Q-T) 28 hpf embryos, bisected along the AP axis, and mounted to show the internal brain surface following detection of **fgf3** (blue) and **dlx2** (red) transcripts. (Q) Control embryos express **dlx2** in the telencephalon and ventral thalamus, and **fgf3** in hypothalamus (and isthmus). (R) **fgf3** transcripts (arrow) are upregulated in the telencephalon in the absence of Fgf8. Loss of Fgf3 (S), or both Fgf3 and Fgf8 (T), results in the absence of **dlx2** expression. d, diencephalon; tel, telencephalon.
the telencephalon at 28 hpf, whereas the absence of Fgf3 protein did not produce such an effect. Upregulated fgf3 transcripts were not detected in the telencephalons of embryos lacking both Fgf8 and Fgf3, although normal expression was detected in the hypothalamus \( (n=23/23); \text{Fig. 3I-L}) \). Taken together, our data suggest that these two factors perform both combinatorial and unique functions in telencephalic patterning.

**Patterning of the diencephalon requires Fgf3 and Fgf8 function**

We examined the effects of Fgf3 and Fgf8 inhibition on regional diencephalic development using markers of the hypothalamus \([shh, nk2.1b (tiff1b – Zebrafish Information Network), fgf3]\), ventral thalamus \([nk2.1b, shh, twhh, dlx2, pax6.1 (pax6a – Zebrafish Information Network)]\), dorsal thalamus \([pax6.1, zona limitans intrathalamica (shh)]\) and optic stalks \([pax2.1; pax2a – Zebrafish Information Network]\).

**Hypothalamus**

shh is expressed in the hypothalamus, as well as in other regions of the brain at 30 hpf (Fig. 4A) (Krauss et al., 1993; Mathieu et al., 2002). Expression was substantially reduced in embryos injected with both Fgf8 and Fgf3 morpholinos \( (n=14/20) \), but not in embryos injected with either morpholino alone (Fig. 4A-D). By contrast, hypothalamic fgf3 expression was unaffected in embryos injected with the Fgf8mo and/or Fgf3mo (Fig. 3Q-T). nk2.1b, required for ventral forebrain development in mice (Kirimura et al., 1996), is expressed in the zebrafish hypothalamus, as well as in the anterior ventral thalamus and subpallial telencephalon at 30 hpf (Fig. 4E). Previous studies showed reduced expression in the forebrains of zebrafish embryos deficient in shh signalling (Rohr et al., 2001). Unexpectedly, embryos co-injected with Fgf8mo and Fgf3mo had normal nk2.1b expression in the hypothalamic (and telencephalic) regions (Fig. 4H), despite shh being greatly reduced when both Fgf8 and Fgf3 were inhibited (Fig. 4D). These results suggest that residual Shh in embryos injected with both morpholinos may have been sufficient to regulate nk2.1b, or that expression of the latter was dependent upon earlier Shh signalling.

**Ventral thalamus**

The ventral thalamus is situated between the ventral telencephalon and hypothalamus (Fig. 1A). nk2.1b was expressed in the ventral thalamus in both control and Fgf8mo-injected embryos at 30 hpf (Fig. 4E,F), but was reduced or absent in embryos lacking Fgf3 \( (n=16/16) \), or both Fgf8 and Fgf3 \( (n=17/17) \), whereas other sites of expression were largely unaffected (Fig. 4G,H). To further examine regionalisation of the anterior ventral thalamus, we analysed embryos at 30 hpf for the expression of tiggywinkle hedgehog \( (twhh) \), which is normally expressed in the ventral midline of the developing neural tube, including that region of the ventral thalamus located between the optic stalks (Ekker et al., 1995). We found that embryos co-injected with Fgf3mo and Fgf8mo lacked twhh expression in the ventral thalamus \( (n=22/23) \), whereas expression in embryos injected with either Fgf8mo \( (n=20/20) \) or Fgf3mo \( (n=22/23) \) was unaffected (Fig. 4I-L). Although these results clearly demonstrated a requirement for both fgf8 and fgf3 for twhh expression in the ventral thalamus, they differed from a previous study suggesting that loss of Fgf8 function alone was sufficient to reduce levels of twhh transcripts (Shanmugalingam et al., 2000). However, others have previously noted subtle differences between ace and Fgf8 morpholino-injected zebrafish (Draper et al., 2001).

To examine patterning of the posterior ventral thalamus, dlx2 and pax6.1 were analysed. As in telencephalon, injection of Fgf3mo resulted in reduced expression of dlx2 in the ventral thalamus at 24 hpf \( (n=6/9) \), and complete loss of expression by 28 hpf \( (n=23/23) \), whereas Fgf8mo injection had no effect at either 24 hpf \( (n=11/11) \) or 28 hpf \( (n=21/21) \) (Fig. 3I-L,Q-T). pax6.1 expression marks posterior ventral thalamus, dorsal thalamus and pretectum at 28 hpf (Fig. 4M) (Püschel et al., 1992; Nornes et al., 1998; Hauptmann et al., 2002). pax6.1 transcripts were detected in the diencephalons of all embryos at 28 hpf; however, the extent and pattern of expression was altered in embryos lacking either Fgf3, or both Fgf3 and Fgf8. In these embryos, ventral thalamic and dorsal thalamic domains of expression were reduced, and there was no clear separation between them, which is suggestive of defects in the ZLI (Fig. 4M-P). Overall, we found patterning defects in both the anterior and posterior ventral thalami in embryos lacking Fgf3, with more severe defects in the anterior ventral thalami in embryos lacking both Fgf8 and Fgf3.

**Zona limitans intrathalamica**

The ZLI expresses shh, may pattern the adjacent ventral and dorsal thalami, and, in chick, has been identified as a lineage-restricted compartment (Zeltser et al., 2001). Embryos injected with either Fgf3mo \( (n=14/16) \), or both Fgf3mo and Fgf8mo \( (n=20/20) \), had substantially reduced expression of shh in the ZLI region at 30 hpf. In particular, expression was undetectable in the dorsal ZLI (Fig. 4A-D,A’-D’).

**Optic stalks**

The optic stalks are transitory structures through which retinal axons extend to the diencephalon. pax2.1 is expressed in the optic stalks and is required for their development (Krauss et al., 1991; Macdonald et al., 1997). At 18 hpf, two well-separated lateral domains of pax2.1 expression corresponding to the optic stalks were present in embryos lacking either Fgf8 \( (n=13/13) \) or Fgf3 \( (n=15/15) \). By contrast, when both Fgf8 were depleted these expression domains were fused at the midline \( (n=10/14) \), providing further evidence for a patterning defect in the ventral thalamic midline and a potential problem with separation of the eye field (Fig. 4Q-T), although there was no evidence of cyclopia.

In summary, diencephalic patterning defects were observed in the ventral thalamus in the absence of Fgf3, and were more severe in the absence of both Fgf3 and Fgf8. Additional Fgf3-dependent defects were found in the ZLI, where shh was reduced and adjacent thalamic pax6.1 domains merged.

**Axon tract formation in the forebrain is disrupted in embryos lacking Fgf3 and Fgf8**

At 16 hpf neurons appear as bilateral clusters within the forebrain (Wilson et al., 1990; Ross et al., 1992). A pair of ventrostral clusters, positioned ventral to the optic stalks within the diencephalon, extend axons towards the midline at 18 hpf to form the post-optic commissure (POC). Soon after, a pair of dorsorostral clusters within the telencephalon extend axons to form the anterior commissure (AC; Fig. 5A-C).
Fig. 4. Diencephalic gene expression is altered in the absence of Fgf8 and Fgf3. Lateral views of embryos bisected along the AP axis and with eyes removed (A-D,E-P), frontal (A’-D’) or dorsal (Q-T) views. (A-D) shh expression at 30 hpf. Embryos mounted to show internal surface of the brain. shh is expressed in the ventral midline of the brain, anterior hypothalamus and ZLI in control embryos (A). shh expression is unaffected in embryos injected with Fgf8mo (B), is slightly reduced after injection of Fgf3mo (C), but is reduced throughout the brain, especially in the hypothalamus (arrow) and ZLI, when both Fgf3mo and Fgf8mo are injected (D). (A’-D’) As A-D, but frontal views with the ZLI in focus. Arrows indicated the dorsal extent of shh expression. shh expression in the ZLI extends into the dorsal diencephalon in control (A’) and Fgf8mo-injected (B’) embryos. Dorsal shh expression in the ZLI is severely reduced in embryos injected with either Fgf3mo (C’), or both Fgf8mo and Fgf3mo (D’). (E-H) nk2.1b expression at 30 hpf. nk2.1b is expressed in the subpallial telencephalon, anterior ventral thalamus and hypothalamus in control embryos (E). Expression is unaffected in embryos injected with Fgf8mo (F). Ventral thalamic expression is reduced in embryos injected with Fgf3mo (arrowhead; G), and absent (arrowhead) in embryos injected with both Fgf8mo and Fgf3mo (H). (I-L) twhh expression at 30 hpf. twhh is expressed in the anterior ventral thalamus (arrowhead) in control embryos and those injected with either Fgf8mo (J) or Fgf3mo (K), but not in those co-injected with Fgf8mo and Fgf3mo (arrowhead; L). (M-P) pax6.1 expression at 28 hpf. In control embryos pax6.1 is expressed in the telencephalon, posterior ventral thalamus and dorsal thalamus (M). Expression is unaltered following injection with Fgf8mo (N). pax6.1 expression domains are smaller and merged following injection with either Fgf3mo (O), or both Fgf8mo and Fgf3mo (P). (Q-T) pax2.1 expression in developing optic stalks (and isthmic region) at 18 hpf. Two well-separated optic stalks are present in control embryos (Q). A slight reduction in pax2.1 expression is detected in embryos injected with Fgf8mo (R), whereas expression is relatively normal in embryos injected with Fgf3mo (S). The pax2.1 expression domains are fused (arrowhead) in embryos injected with both Fgf8mo and Fgf3mo (T). dt, dorsal thalamus; h, hypothalamus; os, optic stalk; vt, ventral thalamus; zli, zona limitans intrathalamica.
Because morpholino injections affected forebrain midline gene expression patterns, we examined the formation of these commissures. The AC and POC were visualised using an acetylated β-tubulin antibody at 34 hpf, following Fgf morpholino injection (Fig. 5D-F). The AC did not form properly in embryos injected with Fgf8mo (n=8/19), and axons with abnormal trajectories were observed in the space between the two commissures, which is normally devoid of axons, in agreement with previous results (Shanmugalingam et al., 2000). The POC developed normally in the majority of these embryos (n=18/19; Fig. 5G-I). Commissure formation was more severely affected in embryos injected with Fgf3mo: both the AC (n=14/16) and POC (n=12/16) failed to form, and axons projected abnormally. In less severely affected embryos, the AC and POC were situated abnormally close together at the midline (Fig. 5J-L). Both commissures failed to form and axons projected abnormally after co-injection of Fgf3mo and Fgf8mo (n=9/12), and in the most severe cases no axons were observed in the midline (Fig. 5M-O).

**Fgf3 is required for differentiation of forebrain neurons**

Failure of commissure formation could occur as a result of a defect in the neurons themselves, or in the territory through which the axons extend, or both. To investigate whether dorsorostral and ventrorostral cluster neurons differentiated correctly, we analysed Fgfm0-injected embryos for neuronal gene expression during early and late stages of differentiation. *zashla* (*asha – Zebrafish Information Network*), a member of the *achaete-scute* family of transcription factors, is expressed in the zebrafish ventral forebrain from 9 hpf, and by 18 hpf it is expressed in dorsorostral and ventrorostral clusters, and in presumptive epiphysis. *zashla* is proposed to have a proneural function, and expression precedes neuronal differentiation (Allende and Weinberg et al., 1994). We examined *zashla* expression at 18 hpf when the first forebrain neurons differentiate. A minority of embryos injected with Fgf8mo had reduced expression in the dorsorostral and ventrorostral clusters, with increased expression of *zashla* in presumptive epiphysis (n=5/13). Injection of Fgf3mo (n=13/13), or Fgf3mo and Fgf8mo (n=15/15), resulted in a more dramatic phenotype; dorsorostral and ventrorostral cluster expression was severely reduced or absent, whereas epiphysial expression was expanded in these embryos (Fig. 6A-D). These results indicated a potential problem with neuronal specification in dorsorostral and ventrorostral clusters in embryos lacking functional Fgfs, in particular in embryos lacking Fgf3.

**Fig. 5.** Lack of Fgf8 or Fgf3 results in aberrant axon trajectory and failure of commissure formation in the forebrain. (A-C) Diagrams depicting neuronal clusters (light brown) and axon tracts (dark brown) during zebrafish forebrain development (from information and diagrams in Wilson et al. and Ross et al. (Wilson et al., 1990; Ross et al., 1992]). Lateral (D,G,J,M) or frontal (E,F,H,I,L,N,O) views focussed on the anterior and postoptic commissures following immunocytochemistry at 34 hpf with acetylated β-tubulin antibodies. (D-F) Control embryos showing anterior and postoptic commissures. (G-I) Anterior commissure formation is defective in embryos injected with Fgf8mo. In some cases there is a complete failure of anterior commissure formation (H), and in other cases axons with abnormal trajectories (arrowhead) extend towards the midline (I). (J-L) Formation of both commissures is defective in embryos injected with Fgf3mo. In some cases, axons extend across the midline (arrowhead), partially forming commissures that are positioned abnormally close together (L). (M-O) There is a pronounced failure of commissure formation in the absence of both Fgf8 and Fgf3 (N). In severe cases, no axons enter the midline (O), ac, anterior commissure; drc, dorsorostral cluster; ep, epiphysis; npc, nuclei of the posterior commissure; poc, post-optic commissure; vcc, ventrocaudal cluster; vrc, ventrorostral cluster.
We also analysed the effects of inhibiting Fgf function on gene expression in differentiated neurons of the dorsorostral and ventorostral clusters at 30 hpf. Homeobox genes *isl1* and *lim1* are expressed by subsets of dorsorostral and ventorostral cluster cells, and by other neurons within the zebrafish forebrain (Korzh et al., 1993; Toyama and Dawid, 1997). At 30 hpf, dorsorostral and ventorostral expression of *isl1* and *lim1* was unaffected in embryos lacking Fgf8 (*isl1*, *n* = 20/20; *lim1*, *n* = 18/18), but was greatly reduced in embryos lacking either Fgf3 (*isl1*, *n* = 14/15; *lim1*, *n* = 14/19) or both Fgfs (*isl1*, *n* = 18/20; *lim1*, *n* = 17/21; Fig. 6E-L). These results suggest that Fgf3 is required for expression of both early and late neuronal markers in the dorsorostral and ventorostral clusters, and that abnormal neuronal specification may contribute to the commissural defects observed in embryos lacking functional Fgf3.

**Forebrain patterning requires Fgf signalling at multiple developmental stages**

Morpholinos can interfere with gene function from the time of their injection. Therefore, to provide an indication of the temporal requirement for Fgf signals, and also an independent assay of Fgf function, embryos were treated at different stages with the FGFR inhibitor SU5402 (Mohammadi et al., 1997) and subsequently analysed for changes in gene expression corresponding to those observed with the Fgf morpholinos. Embryos were treated from 50% epiboly until 80% epiboly [corresponding with *fgf* expression in the shield (Walshe et al., 2002), and prior to expression in the presumptive forebrain], from 80% epiboly until tailbud (when only *fgf3* is expressed in the presumptive forebrain), continuously from 50% epiboly until tailbud, from tailbud until 8s (when *fgf3* and *fgf8* expression overlaps in the telencephalon), or from 13s until 18s (when distinct *fgf3* and *fgf8* expression begins in the diencephalon). Sister embryos were taken from each batch and analysed for *erm* expression immediately following treatment. *erm* is a transcription factor downstream of MAPK that is dependent upon Fgf signalling for its transcription in the zebrafish embryo (Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). In all cases, *erm* expression was eliminated from the forebrain (and other regions) of the embryo following SU5402 treatment, which indicated the effective inhibition of Fgf receptor (Fgfr) signalling (Fig. 7A-H; data not shown).

Expression of *eom* in the posterior telencephalon at 24 hpf was reduced when either Fgf3 or Fgf8 activity was compromised (see Fig. 3A-D). Embryos treated with SU5402 at different stages were therefore analysed at 24 hpf to identify when Fgfr signalling was required to establish normal *eom* expression. A small proportion of embryos treated between

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**Fig. 6.** Expression of neuronal markers is altered in embryos lacking Fgf8 and Fgf3. Lateral views of whole-mounted embryos with anterior to the left. (A-D) *zash1a* expression at 18 hpf. In control embryos, *zash1a* expression is detected in the dorsorostral cluster in the telencephalon and in the ventorostral cluster in the diencephalon, as well as in the presumptive epiphysis (A). Injection of Fgf8mo results in a slight reduction of *zash1a* expression in neurons of the ventral diencephalon. By contrast, expression within the presumptive epiphysis is expanded (B). *zash1a* expression is lost from dorsorostral and ventorostral clusters, but is expanded in the presumptive epiphysis in embryos injected with either Fgf3mo alone (C), or with Fgf8mo and Fgf3mo (D). (E-H) *isl1* expression at 30 hpf. In control embryos, *isl1* is expressed in a subset of neurons within dorsorostral and ventorostral clusters (E). There is very little effect on *isl1* expression following Fgf8mo injection (F). *isl1* expression is reduced in the dorsorostral and ventorostral clusters in embryos injected with Fgf3mo (G), and reduced in the dorsorostral cluster and virtually absent in the ventorostral cluster in embryos injected with both Fgf8mo and Fgf3mo (H). (I-L) *lim1* expression at 30 hpf. *lim1* is expressed in a subset of neurons within the dorsorostral and ventorostral clusters (I), and is unaffected following Fgf8mo injection (J). Injection of Fgf3mo results in reduced *lim1* expression in many neuronal populations of the brain, including dorsorostral and ventorostral clusters (K), whereas *lim1* expression is lacking in most neuronal populations, including the dorsorostral and ventorostral clusters, following injection with both Fgf8mo and Fgf3mo (L). d, diencephalon; drc, dorsorostral cluster; ep, presumptive epiphysis; tel, telencephalon; vrc, ventorostral cluster.
50% and 80% epiboly \((n=3/19)\), or between 80% and tailbud stages \((n=3/17)\), failed to express *eom* in the telencephalon. The expression domain of *eom* in the remaining embryos was expanded anteriorly. A higher proportion of embryos had reduced or undetectable *eom* expression at 24 hpf when treatment covered the entire gastrulation period (50% epiboly to tailbud stage; \(n=11/21\)), or early somitogenesis \((n=8/24)\), whereas treatment at later stages failed to affect *eom* expression (Fig. 7I-L; data not shown). These results imply that Fgf signalling can regulate *eom* expression in the developing telencephalon throughout gastrulation and early somitogenesis.

*nk2.1b* expression in the telencephalon was also examined in treated embryos. Whereas telencephalic *nk2.1b* expression was not dependent upon Fgf3 or Fgf8 function (see Fig. 4E-H), transcripts were absent in embryos treated with SU5402 between 80% and tailbud \((n=21/21)\), or tailbud and 8s

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**Fig. 7.** FGF signalling is required for forebrain patterning during gastrulation and somitogenesis stages. Dorsal (A,D,E,H) or lateral (B,C,F,G,J- T) views of whole-mounted control embryos (A-D,I,M,Q), or embryos treated with SU5402 (E-H,J-L,N-P,R-T) during the developmental periods indicated. (A-H) Sister embryos taken from each batch immediately following treatment with either DMSO (A-D) or SU5402 (E-H), and analysed for expression of *erm*, an FGF-responsive gene, to test the effectiveness of inhibition. Forebrain *erm* expression is efficiently blocked by SU5402 following treatment from 80% to tailbud stage (A,E), tailbud to 8s (B,F) and 13s to 18s (C,D,G,H). After treatment, remaining embryos were allowed to develop to 24 hpf (I-L) or 30 hpf (M-T). Control embryos express *eom* in the pallial telencephalon (I), SU5402 treatment from 50% epiboly to tailbud stage eliminates *eom* expression (J), whereas treatment from 80% epiboly to tailbud results in expansion of *eom* expression into the subpallial telencephalon (K; compare arrowheads in I and K). SU5402 treatment during somite stages does not alter the *eom* expression boundary (arrowhead; L). *nk2.1b* expression in the telencephalon and diencephalon at 30 hpf in a control embryo (M). SU5402 treatment from 80% epiboly to tailbud stage (N), or from tailbud to 8s (O), completely blocks expression of *nk2.1b* in the telencephalon and reduces expression in the ventral thalamus, whereas hypothalamic expression is less affected. Treatment from 13s to 18s results in reduced *nk2.1b* expression in the telencephalon and ventral thalamus but does not affect hypothalamic expression (P). *isl1* expression in the forebrain at 30 hpf in a control embryo (Q). Although *isl1* expression in the dorsal diencephalon is expanded, all other sites of expression are reduced or eliminated following SU5402 treatment from 80% epiboly to tailbud (R), or tailbud to 8s (S). There is little effect on *isl1* expression following treatment from 13s to 18s (T). fb, forebrain; hb, hindbrain.
We investigated Fgf3 and Fgf8 function during zebrafish forebrain development, and found that complex and dynamic spatiotemporal expression underlies requirements throughout the forebrain. Following their inhibition, either singly or in combination, defects in expression of genes associated with early patterning functions were observed in telencephalon, hypothalamus, ventral thalamus, dorsal thalamus, ZLI and epiphysis (Fig. 8). Subsequent neuronal differentiation and axon pathfinding were also found to be abnormal.

Studies of murine Fgf8 function indicated roles in forebrain regionalisation (Shimamura and Rubenstein, 1997; Meyers et al., 1998; Fukuchi-Shimogori and Grove, 2001). However, although Fgf8 is also expressed dynamically in avian and murine forebrains (Mahmood et al., 1995; Mahmood et al., 1996), no forebrain defects have been described in Fgf8-null mice, although detailed analyses have not been reported (Mansour et al., 1993).

In the zebrafish telencephalon, Fgf8 appears to be primarily involved in establishing anteroposterior (AP) polarity, with loss of some subpallial (anterior) markers and expansion of pallial (posterior) markers when inhibited. Thus, *sema3D* and *net1* (*ntn1* – Zebrafish Information Network) (Shanmugalingam et al., 2000) are downregulated in *ace* mutants, whereas *emx1*, *tbr1* and *fgf3* expand anteriorly (this study). Overall, these data are consistent with functions ascribed to Fgf8 in mouse embryos. In addition, our results with SU5402 show that subpallial expression of *nk2.1b* is also dependent upon Fgf signalling. This may be mediated in part by Fgf8, as others report slightly downregulated expression in the *ace* mutant and/or morpholino-injected embryos (Shanmugalingam et al., 2000; Shinya et al., 2001), however, it was unaffected in our study.

In most respects, inhibition of Fgf8 with morpholinos in this study reproduced effects on the forebrain that had been previously reported by others (Shanmugalingam et al., 2000). Notable exceptions were the failure of morpholinos to downregulate the expression of transcripts for *twhh* and *nk2.1b*. Although other explanations are possible, this may indicate incomplete inhibition of Fgf8 by the morpholino and raises the
possibility of dose-dependent requirements for this ligand in forebrain development. Unfortunately, this could not be investigated further as higher concentrations of the morpholino proved to be lethal at gastrulation stages (I.M., unpublished).

We also find roles for Fgf signalling in posterior telencephalic development, with Fgf3 required for pallial tbr1 and dlx2 expression, while Fgf3 and Fgf8 are required together for eom expression.

We and others have examined roles of Fgf signalling in telencephalic development using dominant-negative Fgfrs (dnFgfrs), Fgfr inhibitors or dominant-negative Ras isoforms, an effector of Fgfr signalling (this study) (Shinya et al., 2001). Inhibition of Ras activity results in a loss of telencephalic dlx2 and nk2.1b, and anterior expansion of emx1, eom and tbr1 (Shinya et al., 2001). This is not entirely consistent with the inhibition of Fgf3 and Fgf8, either singly or together: in the absence of Fgf8, tbr1 and emx1 expand anteriorly, whereas eom is downregulated by both Fgf morpholinos. Although Ras may function downstream of other receptors, anterior expansion of tbr1 was also seen following ectopic expression of dnFgfr1iiic or dnFgfr4 (also a iiic isoform) (Shinya et al., 2001), which is consistent with a loss of Fgf8 function, but not a loss of Fgf3, or of both Fgf8 and Fgf3. This might reflect the specificity of Fgf3 for iiib Fgfr isoforms (Kiefer et al., 1996; Ornitz et al., 1996); because dnFgfrs are thought to function as ligand-dependent inhibitors, it is possible that the iiic Fgfr isoforms would not have inhibited the Fgf3 signalling required for telencephalic tbr1 expression. Thus, the complexity of Fgf3 and Fgf8 functions in forebrain development may reflect not only to their dynamic expression patterns, but also Fgfr ligand specificity and expression dynamics. All Fgfrs are expressed in the developing zebrafish forebrain but data is unavailable for individual Fgfr isoforms, although we have reported iiib and iiic isoforms during avian forebrain development (Thissie et al., 1995; Walshe and Mason, 2000; Sleptsova-Friedrich et al., 2001; Tonou-Fujimori et al., 2002).

Surprisingly, we also found that loss of Fgf8 upregulated telencephalic fgf3, whereas loss of Fgf3 did not affect expression, and loss of both Fgf3 and Fgf8 together resulted in an absence of ectopic fgf3. These data suggest a complex interplay of Fgf activities in regulating fgf expression. This appears to be a common theme in the developing brain as Fgf3 and Fgf8 regulate the transcription of each other in the hindbrain (Walshe et al., 2002), and Fgf8 regulates its own transcription in the forebrain (Shanmugalingam et al., 2000).

We found that Fgf3 and Fgf8 are required for the patterning of multiple diencephalic derivatives. In ventral thalamus, the most striking defects occurred in embryos lacking both Fgf8 and Fgf3. These embryos lacked transcripts for nk2.1b, tolhh and dlx2, and shh expression was reduced, with midline expression of pax2.1 expanded. Although shh expression was greatly reduced, the presence of nk2.1b transcripts [Shh-dependent in the mouse (Shimamura and Rubenstein, 1997; Ericson et al., 1995)] and pax2.1 [Shh-dependent in zebrafish (Macdonald et al., 1995)], indicated either that forebrain Shh function was not fully compromised or that Shh activity was required at an earlier developmental stage. Our results also confirm a previous study, which identified a role for Fgf8 in patterning the ventral thalamic midline (Shanmugalingam et al., 2000), and extend those data to provide evidence that Fgf3 is also required to pattern that tissue.

Shh expression is a characteristic of the ZLI, which develops as a compartment between dorsal and ventral thalami (Larsen et al., 2001). Because of its tightly regulated compartmentation, and expression of genes such as shh and the Wnt genes (Garda et al., 2002), the ZLI is postulated to be a signalling centre within the diencephalon. Our results show that Fgf3 function is required for the expression of shh in dorsal ZLI, and for the separation of adjacent ventral and dorsal thalamic pax6.1 expression domains. Taken together, these results suggest that ventral and dorsal ZLI formation may be differentially regulated, and that Fgf3 function is required for dorsal ZLI formation. However, it seems unlikely that Fgf3 directly regulates dorsal ZLI formation as it is only detected in the ventral diencephalon at relevant stages, instead factors crucial for ZLI formation may depend upon earlier Fgf3 function for their expression or function.

Both fgf8 and fgf3 come to be expressed in the hypothalamus during somitogenesis. However, shh expression was reduced, but not abrogated, following injection with both Fgf8mo and Fgf3mo, and all other markers were unaffected, which suggested that fgf8 and fgf3 were not essential for most aspects of hypothalamic development. This was supported by the Fgfr inhibition studies.

The midline tissue of the ventral thalamus and subpallial telencephalon provides an important conduit for axons. pax2.1 transcripts were upregulated in the ventral thalamic midline between the optic stalks in the absence of Fgf8 and Fgf3, a phenotype also observed in embryos after elevation of Shh, resulting in midline tissue with optic stalk morphology (Macdonald et al., 1995). Optic stalk pax2.1 expression begins at 6-7s (MacDonald et al., 1997), when fgf3 and fgf8 are expressed in adjacent telencephalic tissue. Thus, Fgf3 and Fgf8 may serve to antagonise Shh, and to repress inappropriate pax2.1 expression in midline tissue.

We found that, in the absence of Fgf8, formation of the anterior commissure was severely compromised, whereas formation of the post-optic commissure was less affected. This is consistent with a role for Fgf8 in patterning midline tissue of the subpallial telencephalon through which the anterior commissure forms. Others also identified a requirement for Fgf8 in the formation of the anterior commissure, and proposed that this was probably because of defects in the midline tissue rather than in the axons themselves (Shanmugalingam et al., 2000). In support of this, our analyses showed that lack of Fgf8 had little effect on neuronal gene expression in either ventral or dorsal rostral clusters. Loss of Fgf3 affected both commissures, consistent with a role for Fgf3 in patterning both subpallial telencephalon and ventral thalamus. However, a lack of Fgf3 also resulted in reduction or loss of proneural gene expression and differentiation markers in dorsorostral and ventorostral clusters, but not in the loss of the clusters themselves, indicating that a problem with neuronal specification may have contributed to the commissural defects. Embryos deficient in both Fgf8 and Fgf3 exhibited more extreme commissural phenotypes but showed similar effects on neuronal markers as Fgf3mo alone.

Whereas axons extended within the midline territory following inhibition of Fgf3, inhibition of both Fgf3 and Fgf8 frequently resulted in a complete absence of axons from the midline territory.

Dynamic spatial and temporal expression of fgf3 and fgf8,
coupled with both unique and combinatorial actions in forebrain development, suggested that Fgf signalling is required during multiple stages of forebrain morphogenesis. This was investigated by pharmacological inhibition of Fgfr activity during different periods of development. Our results confirmed a requirement for Fgfr activity in forebrain from at least the beginning of gastrulation until 18s. This contrasts with studies that indicate that Fgf patterning activities in hindbrain, isthmus and otic induction only require signalling during a brief, 2 hour period from late epiboly (Walshe et al., 2002; Maroon et al., 2002). It should be noted that SU5402 also produced some additional effects on gene expression and embryo morphology not observed when Fgf3 and Fgf8 were specifically inhibited. These are indicative of additional functions of Fgf signalling in forebrain development, most likely mediated by other Fgf ligands.

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REFERENCES


