Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon

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SUMMARY

Fibroblast growth factors (Fgfs) form a large family of secreted signalling proteins that have a wide variety of roles during embryonic development. Within the central nervous system (CNS) Fgf8 is implicated in patterning neural tissue adjacent to the midbrain-hindbrain boundary. However, the roles of Fgfs in CNS tissue rostral to the midbrain are less clear. Here we examine the patterning of the forebrain in zebrafish embryos that lack functional Fgf8/Ace. We find that Ace is required for the development of midline structures in the forebrain. In the absence of Ace activity, midline cells fail to adopt their normal morphology and exhibit altered patterns of gene expression. This disruption to midline tissue leads to severe commissural axon pathway defects, including misprojections from the eye to ectopic ipsilateral and contralateral targets. Ace is also required

for the differentiation of the basal telencephalon and several populations of putative telencephalic neurons but not for overall regional patterning of forebrain derivatives. Finally, we show that *ace* expression co-localises with anterior neural plate cells that have previously been shown to have forebrain patterning activity. Removal of these cells leads to a failure in induction of *ace* expression indicating that loss of Ace activity may contribute to the phenotypes observed when anterior neural plate cells are ablated. However, as *ace* mutant neural plate cells still retain at least some inductive activity, then other signals must also be produced by the anterior margin of the neural plate.

Key words: Fibroblast growth factor, Ace, Central nervous system, Commissural axons, Telencephalon, Forebrain patterning, Zebrafish

INTRODUCTION

Cells throughout the vertebrate brain acquire regional and cell-type identities during early stages of CNS development and several populations of cells with signalling properties appear to be involved in patterning the forebrain anlage. For instance, extraembryonic endoderm is a source of signals that initiate development of head structures in mouse (Beddington and Robertson, 1999). In addition, signals from axial mesendoderm and midline CNS pattern the ventral forebrain (Blader and Strähle, 1998), while at least in mouse and zebrafish, signals from the margin of the neural plate appear to influence telencephalic development (Houart et al., 1998; Shimamura and Rubenstein, 1997).

Members of the Bone Morphogenetic Protein (BMP) and Fibroblast Growth Factor (FGF) families are proposed to be involved in forebrain patterning activity at the rostral margin of the neural plate. BMP signalling is required for the induction of dorsal forebrain identity as zebrafish embryos lacking Swirl/BMP2b activity do not express telencephalic markers (Barth et al., 1999). Experiments in mice and chick also implicate the BMP family in patterning the dorsal forebrain.

Ectopic expression of chick *bmp4* and *bmp5* leads to a loss of ventral forebrain fates (Golden et al., 1999) whilst in mice, expression patterns of *bmp2*, *bmp5* and *bmp7* all suggest roles in forebrain patterning. Indeed, in vitro, Bmp2/4 can induce dorsal telencephalic identity from tissue fated to form ventral telencephalon (Furuta et al., 1997).

The expression of *fgf8* in the anterior neural ridge (ANR) and later in the telencephalic midline suggests a role for Fgf8 in forebrain patterning. In mammals, a dual role for Fgf8 in regionalisation and differentiation of the forebrain has been suggested. Removal of the ANR results in a loss of telencephalic *Bf1* expression while Fgf8-soaked beads can restore *Bf1* expression in explants denuded of the ANR (Shimamura and Rubenstein, 1997). Gain-of-function and loss-of-function experiments using rat explant cultures also suggest a role for Fgf8 in the specification of forebrain and midbrain dopaminergic neurons (Ye et al., 1998). Further support for a role for Fgf8 in regulating forebrain development comes from analysis of a zebrafish mutant, *aussicht (aus)*, that has elevated levels of *fgf8* transcription and exhibits defects in development of the forebrain midline and optic stalks (Heisenberg et al., 1999).

In this study, we examine the role of Fgf8 in patterning the zebrafish forebrain through analysis of *acerebellar* (*ace*) mutant fish (Brand et al., 1996). The *ace* mutation is a G to A transition in the splice site after the second exon of the *fgf8* gene that leads to incorrect splicing generating a truncated, non-functional protein (Reifers et al., 1998). We show that although a variety of defects are present in the rostral forebrain of *ace⁻* embryos, telencephalic and diencephalic territories are specified, suggesting that Fgf8 activity is unlikely to induce the telencephalon or underlie all the activity of the ANR. *ace⁻* embryos display major defects in commissural axon pathfinding indicating that Ace has a crucial role in patterning midline tissue in the commissural region of the forebrain. Commissural defects are also observed in *no-isthmus* (*noi⁻*) embryos that carry mutations in the *pax2.1* gene (Brand et al., 1996; Macdonald et al., 1997; Lun and Brand, 1998) suggesting that in the forebrain, as at the midbrain/hindbrain boundary, Fgf8 and Pax2.1 may cooperate to pattern CNS tissue.

MATERIALS AND METHODS

Maintenance of fish

Breeding fish were maintained at 28.5°C on a 14-hour light/10-hour dark cycle. Embryos were staged according to Kimmel et al. (1995) or in hours post-fertilisation at 28.5°C beyond prim26 stage. To prevent pigment formation, embryos were raised in 0.2 mM 1-phenyl-2-thiourea after prim10 stage (PTU, Sigma). Carriers of the *ace^{ti282}* mutation were identified by random intercrosses and mutant embryos obtained through heterozygote matings.

Immunohistochemistry

Embryos were anaesthetised in 0.03% MS222 (3-aminobenzoic acid ethyl ester; Sigma), fixed in either 4% paraformaldehyde at 4°C overnight or 2% trichloroacetic acid for embryos older than prim20 stage for 3 hours at room temperature. Standard antibody protocols were used (Wilson et al., 1990). Embryos older than prim20 stage were treated with 0.25% trypsin on ice for 5 minutes. For primary incubations, anti-acetylated tubulin was diluted 1:1000 (Sigma), anti-islet1/2 was diluted 1:500 (Hybridoma bank, Ohio) and anti-Zns2 was diluted 1:1000 (Zebrafish antibody stock centre, Oregon). For plastic sectioning, antibody labelled embryos were dehydrated to 100% methanol, embedded in JB4 resin (Agar Scientific) and sectioned at 5-10 μm using a tungsten knife on a Jung 2055 Autocut.

Analysis of gene expression

Standard methods for in situ hybridisation were used (Macdonald et al., 1995), and details of all probes used are available from the authors. For in situ hybridisation analysis coupled with immunohistochemistry, in situ hybridisation reactions were completed first.

Optic vesicle transplantation and cell transplantations/ablations

Optic vesicle transplantation was performed as in Picker et al. (1999). Retinal nerve projections were visualised using DiO and/or DiI, dissolved in dimethylformamide and pressure injected into the eyes of fixed larvae. Retinal nerve projections were analysed with a Leitz DM IRB confocal microscope equipped with a TCS 4D argon/krypton laser and SCANware 5. Alternatively, fluorescence was photoconverted as described by Wilson and Easter (1991). For cell ablations and transplantations, procedures described in Houart et al. (1998) were followed.

Blocking FgfR activity

Two methods were employed to block Fgf receptor activity. For injection of a truncated form of the FgfR (Amaya et al., 1991), capped RNA was synthesised by in vitro transcription of linearised plasmid (Ambion) and RNA concentration was determined spectrophotometrically. RNA was injected into one cell of one to four cell stage zebrafish embryos using a glass capillary needle attached to a Picospritzer (Griffin et al., 1995).

Alternatively, embryos were treated with the drug SU5204, to block FgfR activity (Mohammadi et al., 1997). For this, embryos at stages from 90% epiboly were incubated in 12 μM SU5204 (dissolved in DMSO) containing embryo medium at 30.5°C for periods of 3 hours (Sigma). After treatment, embryos were transferred to fresh medium and allowed to develop to prim12-18 stage.

RESULTS

ace expression suggests a role in the development of the forebrain

ace is first expressed in the prospective forebrain at bud stage in a one-two cell wide band around the rostral margin of the neural plate (Figs 1A, 2A; Reifers et al., 1998). The homeobox gene *emx1* (Morita et al., 1995) is also expressed in a band of marginal cells and double labelling experiments show that *ace* is expressed within a subset of *emx1*-expressing cells (Fig. 1A,B). Fate mapping experiments (Z. Varga, personal communication; Varga et al., 1999) confirm comparative in situ hybridisation analyses which suggest that the *emx1*-expressing marginal neural plate cells give rise to the telencephalon.

By the 10 somite stage, the rostral neural plate has condensed to form the forebrain and *ace* expression is detected in dorsal cells throughout the telencephalon (Fig. 1B). Over time, *ace* expression becomes restricted to a patch of cells at the midline of the basal telencephalon, just dorsal to the anterior commissure (AC; Fig. 1C,D).

ace is also expressed within the developing optic stalks. This site of expression may initially be continuous with the telencephalic expression during early stages of optic vesicle evagination. However, by the 26 somite stage, a gap between the basal telencephalic and optic stalk expression domains is visible and corresponds to a narrow domain of cells just ventral to the AC. At the midline, *ace* is expressed similarly to the Pax protein Noi (see Macdonald et al., 1997) in cells just dorsal to the postoptic commissure (POC; Fig. 1C). More laterally within the optic stalks, *ace* is expressed diffusely and, unlike Noi, only weakly within the choroid fissure of the eye (not shown).

Separate from the optic stalk expression, *ace* is expressed within the neural retina from approximately the 22 somite stage and later in the anterior pituitary and posterior tuberculum (Fig. 1D and see Reifers et al., 1998). *ace* is also expressed in cells of the dorsal diencephalon and in ventral nasal placodal cells from around prim14 (Fig. 1E).

ace expression is regulated by row1 activity and by Ace itself

Removal of cells at the rostral margin of the prospective neural plate (row1) leads to loss of induction of telencephalic genes (Houart et al., 1998). Double labelling experiments showed that row1 cells express *ace* (Fig. 2A). However *ace* expression

extends further caudal along the margins of the neural plate than do the cells that are removed in row1 ablation experiments (data not shown and Houart et al., 1998). To determine if row1 activity was required for induction of *ace* expression, we analysed *ace* expression in embryos in which the row1 cells were removed (Fig. 2B-D). In row1-ablated embryos, *ace* expression was lost both from the territory in which cells were removed and in cells located more posteriorly along the
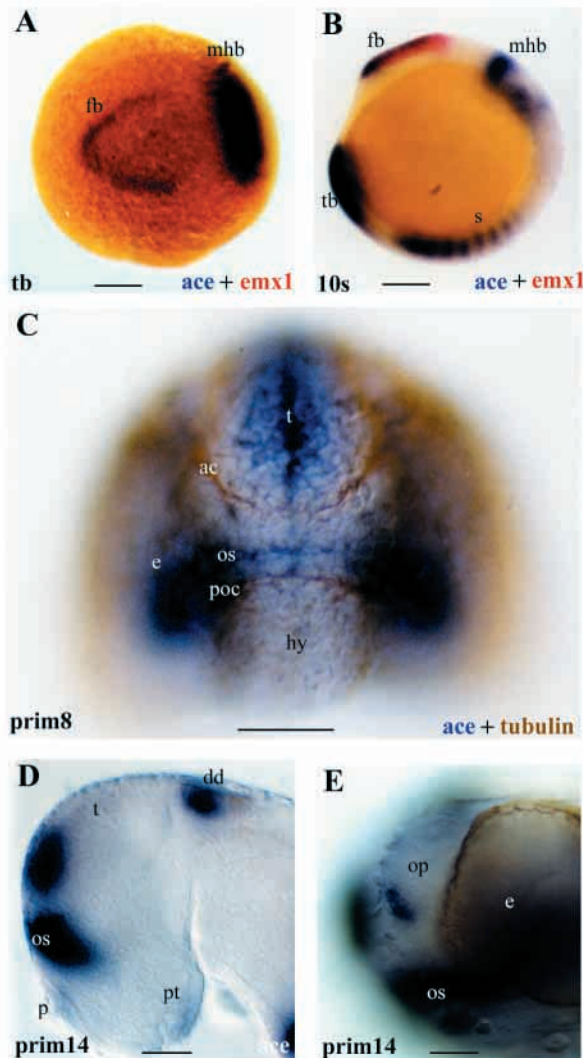
margins of the neural plate. At later stages, *ace* expression was severely reduced or absent from the forebrains of row1-ablated embryos (Fig. 2C,D). As row1 cells only constitute a small

Fig. 1. *ace* expression in the forebrain suggests a role in midline development. Animal pole (A), lateral (B,D,E) and frontal (C) views of wild-type embryos labelled with RNA probes for *ace* (blue), *emx1* (red) and anti-*N*-acetylated tubulin antibody (brown). Anterior is to the left except C. (A) Bud (tb) and (B) 10 somite stages. *ace* is expressed in anterior neural plate cells and later in dorsal forebrain cells within the domain of *emx1* expression. (C) Prim8 and (D,E) Prim-14 stages. *ace* is expressed in medial cells dorsal to the AC in the telencephalon and throughout the ventral optic stalk territory dorsal to the POC. Other areas of *ace* expression include the pituitary, dorsal diencephalon, posterior tuberculum and ventral nasal placode. ac, anterior commissure; dd, dorsal diencephalon; e, eye; fb, forebrain; hy, hypothalamus; mhb, mid-hindbrain boundary; op, olfactory placode; os, optic stalk; p, pituitary; poc, posterior commissure; pt, posterior tuberculum; s, somite; t, telencephalon; tb, tailbud. Scale bars,100 μm (A,B); 50 μm (C-G).
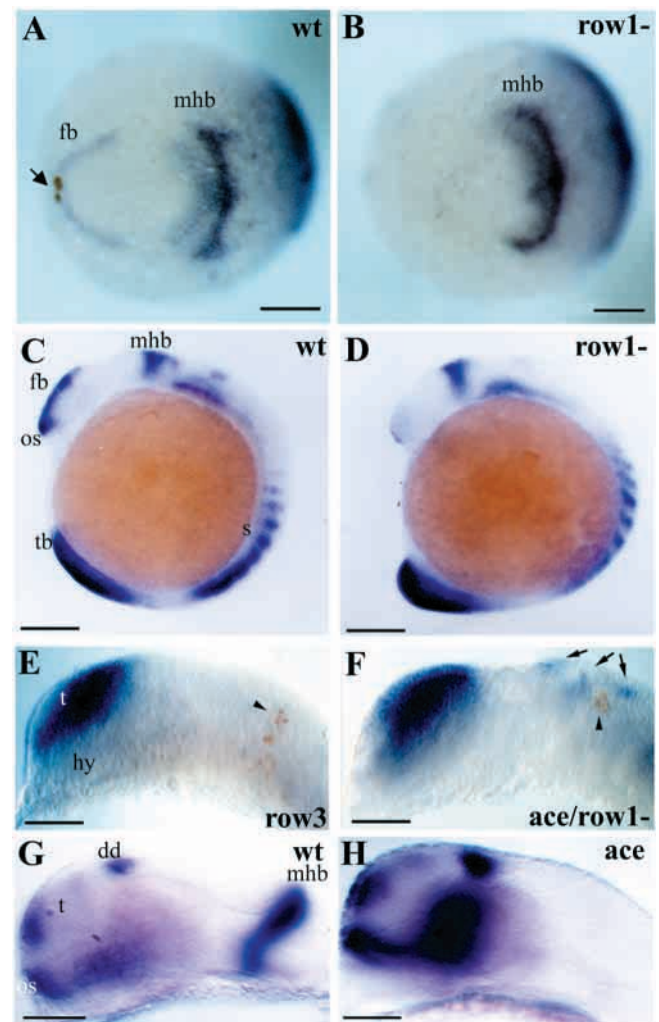

Fig. 2. *ace* expression is regulated by Ace activity and by signals from the rostral margin of the neural plate. (A) Animal pole view of a bud stage embryo in which several row1 (anterior neural plate) cells were injected with dextran (brown, arrow) at 70% epiboly and left in place. The row1 cells and their descendents co-localise with the rostral-most site of *ace* expression at the margin of the neural plate. (B) Animal pole view of a bud stage embryo in which row1 cells were ablated. *ace* expression around the margin of the neural plate is absent. (C,D) Lateral views of 10 somite stage wild-type (C) and row1 ablated (D) embryos. *ace* expression is reduced in the forebrain of the row1 ablated embryo. (E,F) Lateral views of brains from 20 somite stage embryos in which control row3 cells (E) or row1 cells from *ace*⁻ embryos (F) were transplanted to more caudal regions of the prospective neural plate at 70-80% epiboly. Transplanted cells were labelled with dextran (brown). Ectopic spots of *emx1* expression are observed in the vicinity of the *ace*⁻ row1 cells while no ectopic expression is observed near the row3 cells. (G,H) Lateral views of *ace* expression in prim-5 stage wild-type and *ace*⁻ embryos. The level of expression is increased in forebrain domains and absent at the MHB of the *ace*⁻ embryo. dd, dorsal diencephalon; fb, forebrain; hy, hypothalamus; mhb, midbrain-hindbrain boundary; os, optic stalks; s, somites; tb, tailbud; t, telecephalon. Scale bars, 100 μm (A-D); 40 μm (E-F).

fraction of telencephalic cells (Houart et al., 1998), this confirms a cell non-autonomous role for row1 cells in regulation of *ace* expression. These results suggest that loss of Ace activity could underlie the row1 ablation phenotype. However, transplantation of *ace⁻* row1 cells into more caudal neural plate positions of wild-type or *ace⁻* embryos still resulted in ectopic induction of telencephalic markers, indicating that Ace alone does not impart the full activity of row1 cells (Fig. 2E,F and data not shown). Additionally, *ace⁻* row1 cells retain the ability to induce telencephalic markers when transplanted into wild-type hosts in which row1 cells had previously been removed (data not shown).

ace expression in the forebrain is also regulated by Ace itself. Thus the loss of functional Ace activity results in variable upregulation of *ace* expression in *ace⁻* embryos most noticeably within the dorsal diencephalon, basal telencephalon and pituitary (Fig. 2G,H).

Early telencephalic gene expression is affected in *ace⁻* embryos

Shortly after *ace* is first expressed at the anterior margin of the neural plate, we detected altered gene expression in prospective midline tissue. In about 25% of bud stage embryos from a cross between two heterozygous *ace* carriers, *emx1* and *anf* (Kazanskaya et al., 1997) expression was reduced (Fig. 3A-D), most noticeably at the rostral midline of the neural plate. This position is a local highpoint of *ace* expression (Reifers et al., 1998) suggesting that Ace may have a role in patterning this region of the prospective forebrain midline. As the marginal neural plate condensed to form the telencephalon, it became difficult to assess if *anf* and *emx1* were still altered in *ace⁻* embryos. However, as shown below, alterations in telencephalic gene expression do persist at later stages.
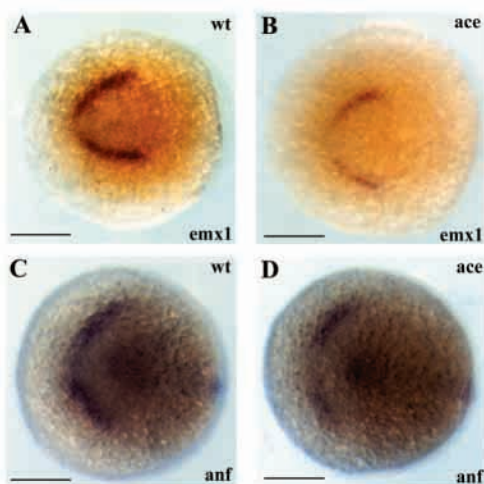

Fig. 3. Early telencephalic gene expression is perturbed in *ace⁻* embryos. Animal pole views of bud stage wild-type (A,C) and putative *ace⁻* (B,D) tailbud embryos showing *emx1* (A,B) or *anf* (C,D) expression. In wild-type embryos, *emx1* and *anf* are expressed in a horseshoe of cells that will give rise to the telencephalon. In putative *ace⁻* embryos, expression of both genes is reduced laterally and absent at the rostral midline of the neural plate. This is unlikely to be due to a general developmental delay in the mutants as *anf* is normally expressed in a more broad territory at earlier stages (Kazanskaya et al., 1997). Scale bars, 50 μm.

ace is required for patterning the AC and POC

The rostral tip of the neural plate is believed to give rise to the basal telencephalon and commissural region (Eagleson et al., 1995; Houart et al., 1998). To examine whether pathfinding errors occur in this region of *ace⁻* embryos, we performed axon labelling studies.

ace⁻ embryos showed defects of variable severity in the establishment of the AC and POC at the rostral tip of the developing forebrain. Instead of the directed extension of pioneering axons towards the midline in wild-type embryos, axons meandered as they approached the midline of *ace⁻* embryos (Fig. 4A,B). At slightly later stages, axons wandered between the two commissures around the base of the optic stalks, a territory that normally excludes axons (Fig. 4C-H). Unlike in *noi⁻* embryos (see Macdonald et al., 1997), axons frequently misprojected quite lateral to the midline and in about two thirds of prim20 stage *ace⁻* embryos, one, or more commonly both, commissures failed to form altogether (Fig. 4D,F). Although axons showed severe pathfinding defects between the two commissures, they did not make navigational errors into midline tissue either dorsal to the AC or ventral to the POC.

The severity of the axon guidance phenotype in *ace⁻* embryos suggested that midline tissue itself might be disrupted in mutant embryos. We therefore examined the morphology of the midline cells located between the two commissures in *ace* mutants. In wild-type embryos, there are about 9-10 cells between the AC and POC, the majority of which have a highly characteristic cuboidal morphology (Fig. 4I), reminiscent of the floorplate in more caudal regions. Some of these cells express *noi* and are likely to differentiate as primitive glial cells (Macdonald et al., 1997). In *ace⁻* embryos, cuboidal midline cells were absent and the optic recess was occluded (Fig. 4J), indicating that midline tissue between the two commissures fails to develop properly in the absence of Ace function.

As Fgfs are a large family of genes, it is likely that other Fgfs retain activity in the forebrain of *ace⁻* embryos. To attempt to address the effects of more global reduction in Fgf activity, we examined axon pathfinding in embryos overexpressing a truncated Fgf receptor (XFD; Amaya et al., 1991) and in embryos treated with the SU5204 compound. This drug is an efficient in vitro FgfR inhibitor (Mohammadi et al., 1997) and in vivo has proved to be a useful reagent for reducing Fgf signalling activity (McCabe et al., 1999; Reifers et al., 2000). As expected, widespread overexpression of XFD led to embryos that lacked posterior structures (Griffin et al., 1995) reflecting the requirement for Fgf signalling during mesoderm and tail formation. More rostrally, the brain phenotypes of injected embryos frequently resembled *ace⁻* embryos, with the development of the AC and POC invariably disrupted (Fig. 4K). Although the in vivo specificity of XFD is uncertain, this observation does suggest that it may interfere with signalling via Ace or another Fgf with a similar function.

To address when Fgf signalling might be required for patterning the commissures, we treated embryos with SU5204 for periods of 3 hours from 90% epiboly stage to the 30 somite stage. Early treatment (from 90% epiboly) with SU5204 led to a more severe phenotype than *ace⁻*, in which commissures were absent and the eyes and brain were smaller than controls

(Fig. 4L). In contrast, treatment from the 6 somite stage gave commissural phenotypes similar to *ace⁻* without severe alterations to overall forebrain development (Fig. 4M). Finally, treatment from the 18 somite stage produced embryos that exhibited milder defects in which both the AC and POC were present but axons wandered ectopically between the two commissures (Fig. 4N), reminiscent of *noi⁻* embryos (Macdonald et al., 1997).

AC and POC defects were therefore detected in *ace⁻*, XFD- and drug-treated embryos confirming a key role for Fgf signalling in commissure formation. However, the increased severity of embryos treated with SU5204 at early developmental stages suggests that Fgfs other than Ace may also have early roles in patterning forebrain tissue. As a caveat, it has not been proved that SU5204 only targets Fgf receptor activity in fish embryos and so it remains a possibility that other signalling pathways are also disrupted in treated embryos.

ace⁻ embryos exhibit severe defects in formation of the optic chiasm

About 12 hours after the POC is established, the first retinal axons cross the midline establishing the optic chiasm, directly adjacent and rostral to

the POC (Fig. 5A; Burrill and Easter, 1994). To determine if Ace is required for establishment of the optic chiasm, we examined retinal projections in *ace⁻* embryos.

In *ace⁻* embryos, retinal axons frequently misprojected both ipsilaterally and rostrally to the telencephalon (Fig. 5 and see Picker et al., 1999). Axons showed no obvious defects within the eye (Fig. 5C), and unlike *noi⁻* embryos, only mild coloboma was observed in the retinae of *ace⁻* mutants (not shown). Although the earlier commissural axons made

Fig. 4. *ace* is required for patterning the anterior and postoptic commissures. Frontal (A-F,K-N) and lateral (G,H) views and parasagittal sections (I,J) of prim-6 (A,B) or prim-20 (others) stage wild-type (A,C,G,I), *ace⁻* (B,D-F,H,J), XFD-injected (K) and SU5204-treated (L-N) embryos labelled with anti-*N*-acetylated tubulin antibody. (A-B) In the wild-type embryo pioneering axons have established the POC as a discrete fascicle of axons and the first AC axons would soon have reached the midline. In the *ace⁻* embryo, axons that should pioneer the AC and the POC meander and are defasciculated as they near midline tissue. (C-H) In wild-type embryos (C,G), axons cross the midline of the rostral forebrain in the AC and the POC. In *ace⁻* embryos, axons wander inappropriately between the two commissures (E,H), or one or both commissures are absent (D,F) with axons forming ectopic longitudinal pathways (arrows, D). (I,J) In the wild-type embryo, cuboidal cells are present at the midline between the two commissures (arrows). Cells do not adopt this morphology in the *ace⁻* embryo and the optic recess is occluded. (K) In this XFD-injected embryo, both the AC and POC are disturbed leading to axonal disorganisation in midline tissue. (L-N) Embryos treated with SU5204 for 3 hours at various times. The embryo treated from 90% epiboly (J) lacks commissures and has reduced forebrain size. The embryo treated from 6 somites also has severe commissural defects but less reduction in forebrain size. The embryo treated from 18 somites (L), exhibits a milder phenotype in which both commissures are present but axons wander ectopically at the midline. ac, anterior commissure, e, eye; hy, hypothalamus; or, optic recess; os, optic stalk, pc, posterior commissure; poc, postoptic commissure; t, telencephalon; tpoc, tract of the postoptic commissure. Scale bars, 25 µm (A-F);10 µm (G,H).
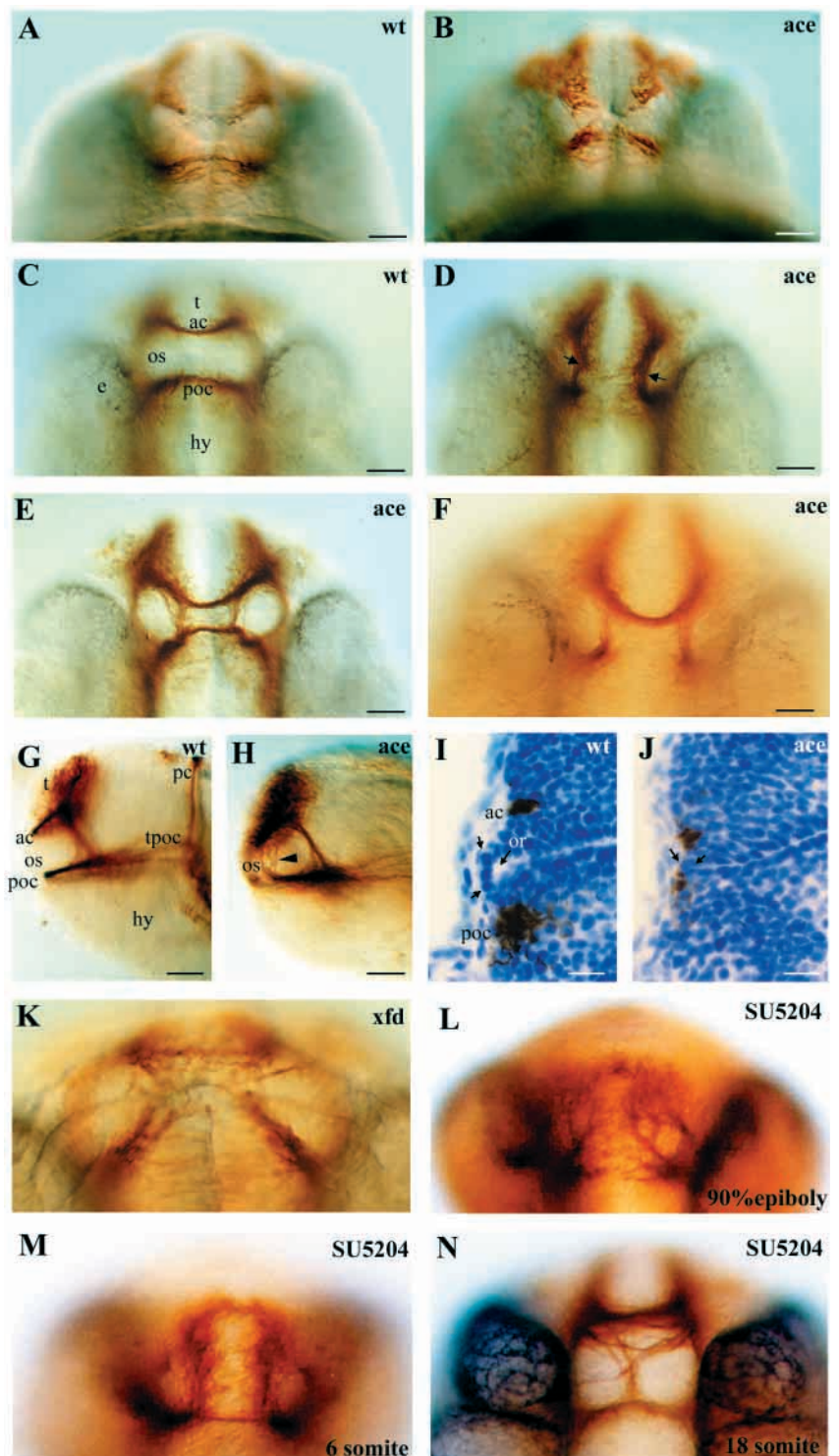
pathfinding errors in this same region, retinal axons formed ectopic projections between the AC and POC that were separate from the other commissural axons (Fig. 5D). Indeed, despite the severe axonal disorganisation at the midline, retinal axons remained tightly fasciculated with other retinal axons, even when they formed inappropriate projections (Fig. 5B,C).

Within the brain, retinal axons often navigated appropriately to tectal targets in *ace*⁻ embryos (see Picker et al., 1999). However in some cases, retinal axons projected ectopically both rostrally and dorsally into the telencephalon (Fig. 5B,C)

where they appeared to form axonal terminals at later stages (Fig. 5I,J). Although the commissural and chiasmal *ace*⁻ phenotypes were variable, they were usually bilaterally symmetrical (Fig. 5B,C) suggesting that the fault may lie in the midline tissue through which the axons attempt to navigate.

The retinal misprojections in *ace* mutants are due to a requirement for Ace in the brain, not the eye

As *ace* is expressed both in the eye and in the brain (Reifers et al., 1998), the defects in retinotectal pathfinding in *ace* mutants could be due to a requirement for Ace activity in either or both locations. To address this issue, we examined the patterning and differentiation of the retina in *ace* mutants and assessed pathfinding following transplantations of wild-type and *ace*⁻ eyes to wild-type and *ace*⁻ host brains.

In *ace*⁻ embryos, retinal differentiation superficially proceeds as in wild type such that by 2 days, all layers of the retina are visible and photoreceptors have started to differentiate (Fig. 5D,E). Furthermore, most, but not all, retinal ganglion cells acquire appropriate positional identity as assayed by their termination patterns within the midbrain (Picker et al., 1999). Therefore overall retinal differentiation does not appear to be significantly disturbed in *ace*⁻ eyes.

Transplantation experiments confirmed that axon pathfinding defects in *ace*⁻ embryos are primarily due to defects in midline CNS development. Wild-type and *ace*⁻ eyes formed normal chiasmata when transplanted onto wild-type host brains (Fig. 5G,H). Conversely, both wild-type and *ace*⁻ eyes formed ectopic ipsilateral and contralateral projections when transplanted onto host *ace*⁻ brains (Fig. 5I,J).

Midline gene expression is altered in *ace* mutants

The defects described above indicate that patterning of midline tissue in the commissure-forming region of the forebrain is disrupted in *ace*⁻ embryos. To elucidate the
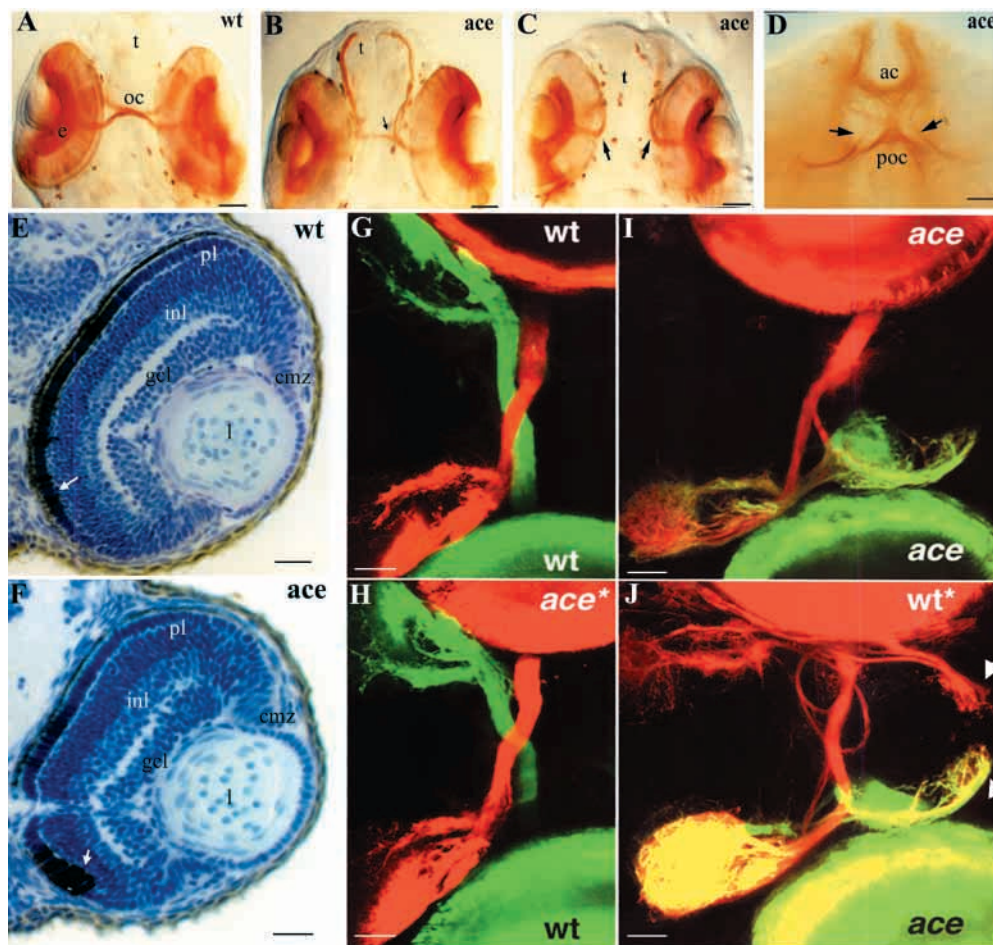

Fig. 5. *ace*⁻ embryos exhibit severe defects in formation of the optic chiasm. (A-C) Ventral views of 72hpf wild-type (A) and *ace*⁻ (B,C) embryos in which the retinotectal projection is visualised through anterograde labelling with DiI. In the wild-type embryo, the optic nerves form a contralateral projection crossing the midline in the optic chiasm directly rostral to the POC. In the *ace*⁻ embryos, many retinal axons fail to cross the midline and project ipsilaterally to both normal and ectopic telencephalic targets. (D) Ventral view of a 48hpf *ace*⁻ embryo labelled with anti-acetyelated tubulin antibody. The retinal axons (arrows) enter the brain rostral to the POC axons and do not fasciculate along them. (E,F) Coronal sections of 48hpf wild-type (E) and *ace*⁻ (F) embryos stained with an anti-Fret43 antibody. No obvious reduction in retinal layering or photoreceptor differentiation (arrowheads) is observed in the *ace*⁻ eye. (G-J) Ventral views of chimaeric embryos in which wild-type eyes were transplanted into wild-type (G) or *ace*⁻ (J) host embryos or *ace*⁻ eyes were transplanted into wild-type (H) or *ace*⁻ (I) host embryos and retinal pathfinding revealed with DiI (red) and DiO (green) labelling. In the embryos in which *ace*⁻ or wild-type eyes are transplanted onto wild-type hosts, normal retinal pathfinding occurs to the contralateral tectum (G,H). However when wild-type or *ace*⁻ eyes are transplanted onto *ace*⁻ host embryos (I,J), ectopic projections are observed, including to the dorsal telencephalon (arrowheads). Anterior is to the right in these panels with the genotype of the transplanted eye indicated top right and the host bottom right. cmz, ciliary marginal zone; e, eye; gcl, ganglion cell layer; inl, inner nuclear layer; l, lens; oc, optic chiasm; pl, photoreceptor cell layer; t, telencephalon. Scale bars, 25 μm (A-C); 20 μm (D-I).
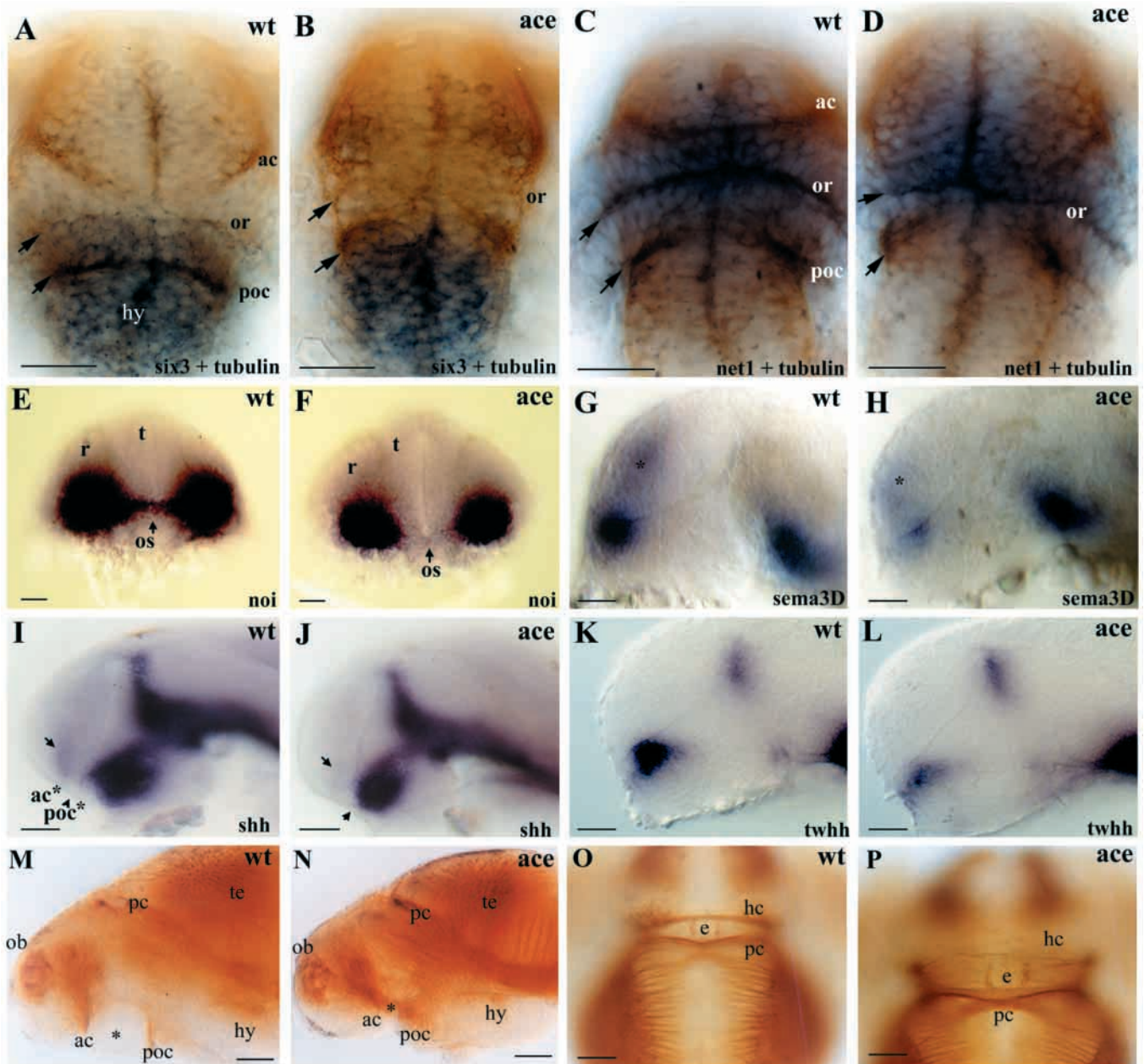
Fig. 6. Midline gene expression changes, late morphological abnormalities and dorsal diencephalic pathfinding defects in *ace⁻* embryos.
(A-F) Frontal views of gene expression in prim10 (A-D) and 18 somite (E-F) stage wild-type and *ace⁻* embryos. (A,B) In the wild-type
embryo, *six3* is expressed in cells ventral to the POC (labelled brown with a *N*-acetylated tubulin antibody) and in cells between the POC and
optic recess. In the *ace⁻* embryo, there is no *six3* expression dorsal to the commissurally projecting axons. The arrows in this and other panels
indicate the positions of the POC axons and the optic recess. (C,D) In the wild-type embryo, *net1* is expressed in cells ventral to the optic recess
and dorsal to the POC. This expression is reduced in the *ace⁻* embryo. (E,F) In the wild-type embryo, *noi* is expressed strongly in the optic
stalks and weakly in midline cells just dorsal to the POC (see Macdonald et al., 1997). In the *ace⁻* embryo, *noi* expression at the midline is lost.
(G-L) Lateral views of gene expression in prim10 (G,H,K,L) and prim14 (I,J) wild-type and *ace⁻* brains. (G,H) *sema3D* is expressed adjacent to
the optic recess and in the basal telencephalon (asterisk) of the wild-type embryo. Both sites of expression are reduced in the *ace⁻* embryo.
(I,J) *shh* is expressed up to the POC and in the basal telencephalon in the wild-type embryo (see Macdonald et al., 1997). Diencephalic
expression is expanded slightly towards the optic recess in the *ace⁻* embryo and expression in the telencephalon is absent. The asterisks mark
the positions of the AC and the POC and the arrows indictate the positions of the telencephalic *shh* expression domain and the optic recess.
(K,L) *twhh* is expressed in cells adjacent to the optic recess and POC in the wild-type embryo. This domain of expression is reduced in the *ace⁻*
embryo. (M-P) Lateral (M-N) and dorsal (O,P) views of 72hpf wild-type and *ace⁻* embryos labelled with *N*-acetylated tubulin antibody.
(M-N) The preoptic area (marked by an asterisk), bordered by the AC and POC in the wild-type embryo is reduced or absent in the *ace⁻*
embryos. (O,P) In the wild-type embryo, axons cross the midline in the habenular commissure in proximity to a site of *ace* expression (see Fig.
1). This commissural projection is reduced in the *ace⁻* embryo. ac, anterior commissure; e, epiphysis; hc, habenular commissure; hy,
hypothalamus; ob, olfactory bulb; oc, or, optic recess; os, optic stalk; pc, posterior commissure; poc, postoptic commissure; r, retina, te, tectum;
t, telencephalon. Scale bars, 25 µm (A-F); 50 µm (G-L); 30 µm (M-P).

alterations in gene activity that might underlie the patterning defects, we examined the expression of various genes in the affected territories.

noi is expressed throughout the optic stalk and in a narrow band of cells at the midline just dorsal to the POC (Fig. 6E; Macdonald et al., 1997). In *ace⁻* embryos, midline expression of *noi* was reduced or absent although more lateral expression was not obviously affected (Fig. 6F). *six3* is a homeobox-containing gene expressed in the same domain as *noi* and additionally in cells ventral to the POC (Fig. 6A; Kobayashi et al., 1998). In *ace⁻* embryos, *six3* expression was reduced in cells adjacent to the optic recess (Fig. 6B). *net1* and *sema3D* are genes involved in axon guidance that are expressed in the commissure-forming territory (Fig. 6C,G; Strähle et al., 1997; Halloran et al., 1999). Expression of both genes is reduced or absent in cells dorsal to the POC in *ace⁻* embryos (Fig. 6D,H). *net1, net2* and *sema3D* genes also showed reduced expression in the basal telencephalon (Fig. 6D,H and data not shown). Together these observations indicate that midline tissue just dorsal to the POC is either mis-specified and/or reduced in *ace⁻* embryos.

Examination of other genes revealed further alterations in gene expression in midline tissue of *ace⁻* embryos. Diencephalic *sonic hedgehog* (*shh*) expression was relatively normal but expression in the basal telencephalon, just dorsal to the AC, was reduced or absent in *ace⁻* embryos (Fig. 6J). Unlike *shh*, expression of *Tiggywinkle hedgehog* (*twhh*), a second hedgehog gene (Ekker et al., 1995), was reduced in diencephalic cells adjacent to the POC in *ace⁻* embryos (Fig. 6K,L). *twhh* expression did not extend rostrally to the optic recess in *ace⁻* embryos as might be expected if midline tissue was absent as opposed to being inappropriately patterned. Several other genes expressed in this territory including *nk2.1a*, a putative target gene of the Hedgehog pathway, showed no obvious changes in expression in *ace⁻* embryos (data not shown).

The preoptic area and dorsal diencephalon are affected in *ace⁻* embryos

The most prominent defect in the forebrain of young *ace⁻* embryos is in the patterning of midline tissue between the commissures. This region of the brain is presumed to give rise to preoptic territories at the interface between telencephalon and diencephalon. Although there was initially no major change in morphogenesis of this region of the brain in *ace⁻* embryos, by three days, a gross distortion in brain shape was visible around the commissures (Fig. 6M,N). Caudal to this region, hypothalamic tissue appeared superficially normal (Fig. 6M,N) and there was no obvious alteration in hypothalamic TH neurons (data not shown). Rostral and dorsal to the preoptic region, the telencephalon was smaller in *ace⁻* embryos, but there was usually good separation of the olfactory bulb from other telencephalic structures (Fig. 6M,N).

In addition to the POC, AC and optic chiasm, forebrain axons also cross the midline in the habenular commissure (HC) in the dorsal diencephalon. In *ace⁻* embryos, the HC was reduced or absent, consistent with Ace also being required for formation of this dorsal commissure (Fig. 6O,P). Although *ace* is expressed in the dorsal diencephalon, we cannot be certain that it is required in this region for HC formation. The origin of HC axons is uncertain in zebrafish but in other species,

neurons of the preoptic region contribute axons to the commissure (Jones, 1985). It is therefore possible that defects in the HC are secondary to defects in other forebrain regions of *ace⁻* embryos.

Basal telencephalic patterning and neurogenesis are affected in *ace⁻* embryos

At prim stages, telencephalic morphology appears normal in *ace⁻* embryos and many markers of telencephalic domains (*bf1, dlx2, eph-A4d, arx1, unc4, eome*) show no obvious alterations in expression (Fig. 7A,B and data not shown) indicating that overall induction and patterning of the telencephalon is not severely affected. However, similar to *shh*, telencephalic expression of *nk2.1b* is reduced in *ace⁻* embryos indicating a requirement for Ace in the patterning of basal telencephalic derivatives (Fig. 7C,D).

Markers of neuronal differentiation are also disrupted in *ace⁻* embryos. *lim1* and *lim6* are expressed in complementary clusters of cells in the rostral telencephalon (Fig. 7E,G). While the identity of these cells is currently unknown, it is likely that combinatorial expression of Lim genes defines different populations of neurons in the telencephalon as it does elsewhere in the CNS (Lumsden 1995; Toyama and Dawid, 1997). In *ace⁻* embryos, expression of both *lim1* and *lim6* in the telencephalon was reduced or absent (Fig. 7F,H). In contrast, the Lim genes *isl1* and *lim5* were expressed in similar clusters of telencephalic cells in both wild-type and *ace* mutants (data not shown).

The olfactory system is disrupted in *ace⁻* embryos

In wild-type embryos, olfactory neurons project axons to the telencephalon and form terminations that prefigure glomeruli of the mature olfactory bulb (Fig. 8A,C; Whitlock and Westerfield, 1998). In *ace⁻* embryos, there were fewer axonal condensations and those that did form were typically present in the medial bulb (Fig. 8B,D). As with the retinotectal defects, the olfactory axon defects of *ace⁻* embryos were variable but usually bilaterally symmetrical.

To further characterise the defects within the olfactory bulb, we looked at the expression of cell-specific markers in *ace⁻* embryos. *dlx2*, a marker of olfactory bulb interneurons in mice, appears superficially unaffected in *ace⁻* embryos but markers for putative projection neurons, such as *emx1* and *zeome* (Porteus et al., 1994; Bulfone et al., 1995; Gulisano et al., 1996), show severe perturbation (Fig. 8E,F and data not shown). By 72hpf, *emx1* is expressed in focal patches in the olfactory bulb that correlate with the positions of the glomeruli. In *ace* mutants, expression is reduced and there is less localisation to the glomeruli (Fig. 8E,F).

As there are reciprocal interactions between the olfactory placode and olfactory bulb (Graziedei and Monti-Graziedei, 1992), the defects in olfactory bulb development could at least in part be due to defects in olfactory placode development. The olfactory placodes arise from tissue in the vicinity of the anterior neural plate expression domain of *ace* (Whitlock and Westerfield, 1998) and at later stages *ace* is expressed in the placodes themselves (Reifers et al., 1998). There were no obvious defects in the development of the nasal epithelium as assessed by histological sectioning, and early olfactory placode markers including *eyab, ngn1* and *neuroD* were not obviously altered (data not shown). However, although the nasal

Fig. 7. Telencephalic neurogenesis is affected in *ace⁻* embryos. Lateral views of prim-14 stage wild-type and *ace⁻* brains labelled with various probes. (A,B) *dlx2* is expressed in similar domains in wild-type and *ace⁻* embryos. (C,D) *nk2.1b* expression is reduced in the basal telencephalon (arrowheads) but maintained in the caudal hypothalamus of the *ace⁻* embryo. (E,F) *lim1* is expressed in two clusters of cells dorsally and ventrally near the midline of the telencephalon of the wild-type embryo. The ventral domain of expression is absent and the dorsal domain reduced (arrowheads) in the *ace⁻* embryo. (G,H) *lim6* is expressed in a group of cells between the two lim1 expressing clusters in the wild-type embryo (arrows). This site of expression is severely reduced in the *ace⁻* embryo. d, diencephalon; hy, hypothalamus; t, telencephalon. Scale bars, 30 μm.
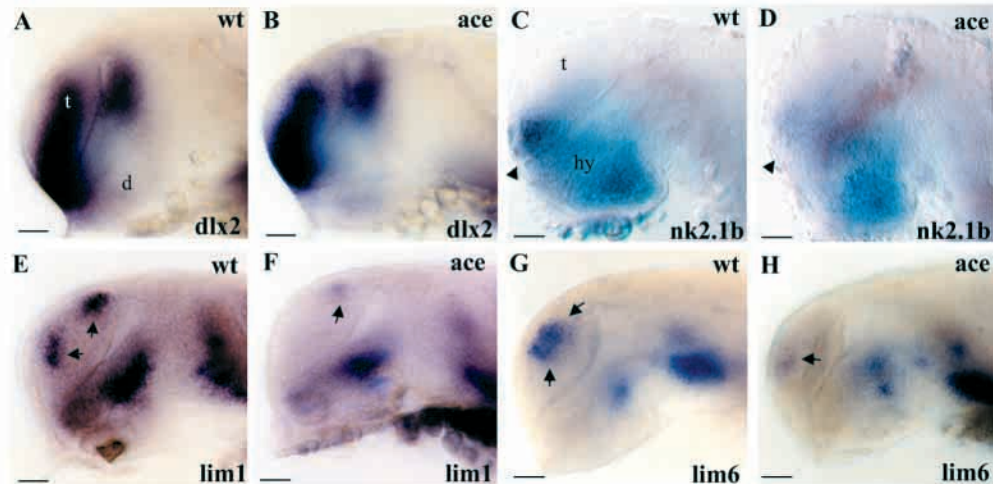
epithelium was present, differentiation of some neurons appeared to be disturbed in *ace⁻* embryos. Thus examination of expression of three odorant receptors showed that *or2.0* was reduced or absent in mutant embryos while *or2.4* and *13.0* appeared unaffected (Fig. 8G,H and data not shown).

DISCUSSION

Although Fgfs have been extensively studied in a multitude of developmental processes, relatively little work has addressed their roles in patterning the vertebrate forebrain. Previously, explant studies have suggested that Fgfs may play an inductive role in forebrain development (Shimamura and Rubenstein, 1997; Ye et al., 1998) and lowered Fgf8 activity in mice leads to a reduction in size of the forebrain (Meyers et al., 1998). Here we show that in zebrafish, Fgf8/Ace regulates specific aspects of rostral forebrain development. In the absence of Ace activity, patterning defects are associated with severe disruptions to commissural axon pathway formation, to patterning of the basal telencephalon, to specification of putative telencephalic neuronal populations and to differentiation of the olfactory system. Furthermore, the loss of *ace* expression in embryos in which the anterior margin of the neural plate is ablated suggests that Ace contributes to the patterning activity of these cells. However, such ablations lead to more severe phenotypes than removal of Ace function and *ace⁻* marginal cells retain inductive properties indicating that other signals must also be produced from the ANR.

Ace is required for axon guidance through the POC and AC

In contrast to the caudal CNS, there are very few locations in the forebrain at which axons cross the midline and those commissures that do form are established at invariant locations by tightly fasciculated bundles of axons. At these locations, axons must be both attracted to the midline yet also inhibited from decussating at inappropriate locations, suggesting that a combination of both attractive and inhibitory guidance cues are required. We have previously suggested that axons may be funneled across the midline at the interface between two domains of cells, both of which discourage growth cone exploration (Macdonald et al., 1997). Support for this hypothesis has come from analysis of *noi⁻* embryos. Noi is normally expressed in a territory adjacent to the POC and in *noi⁻* embryos, axons invade this territory (Macdonald et al., 1997). Thus Noi may normally regulate the expression of cell surface and/or secreted proteins that discourage axons from entering the territory adjacent to the POC as they approach the midline.

In *ace⁻* embryos, there are severe defects in the establishment of both the POC and the AC such that both commissures are usually initially absent and fused at later stages. This indicates that Ace has a crucial role in the development of commissural neuroepithelium. In support of this, we observed alterations in expression of midline genes encoding proteins that are likely to directly influence axon extension (*net1*, *sema3D*; Strähle et al., 1997; Halloran et al., 1999), and in genes more likely to indirectly influence guidance cues (*noi*, *six3*, *twhh*).

The reduction or loss of *noi* expression at the midline of *ace⁻* embryos suggests that at least some of the commissural defects of *ace⁻* embryos might be attributed to loss of Noi activity. However, the *ace⁻* phenotype is more severe than complete loss-of-function *noi* alleles and so Ace must also regulate midline patterning through Noi-independent pathways. Indeed, in *noi⁻* embryos, the morphogenesis of midline tissue initially appears relatively normal whereas in *ace⁻* embryos, the cuboidal cells characteristic of midline tissue between the commissures fail to differentiate properly.

The disruption to midline development could occur if midline tissue is deleted in *ace⁻* embryos, and indeed, by 3 days, the preoptic area is greatly reduced in size. However, the distance between AC and POC axons as they approach the midline (10 cell diameters or so) is not obviously affected in early prim stage *ace⁻* embryos and only some of the genes expressed adjacent to the POC showed spatial (and quantitative) alterations in expression. This suggests that
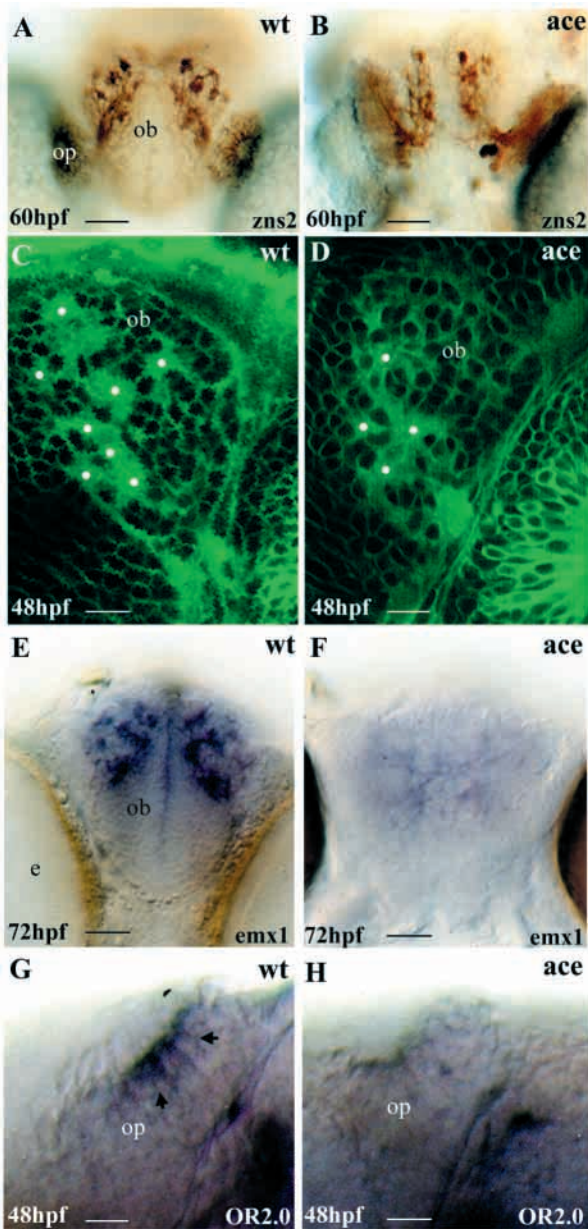
Fig. 8. Disruption of olfactory axon projections and olfactory bulb and odorant receptor differentiation in *ace⁻* embryos. Frontal views of the olfactory bulb (A-F) and ventral views of the olfactory placode (G,H) of wild-type and *ace⁻* embryos. (A,B) Wild-type and *ace⁻* embryos labelled with the zns2 antibody that reveals a transient group of pioneer olfactory axons that form termini in the developing olfactory bulb. In the *ace⁻* embryo, fewer termini are detected and those that are present are located in the medial olfactory bulb. (C,D) Confocal images of living embryos labelled with bodipy ceramide revealing glomerular structure and organisation. The *ace⁻* olfactory bulb is more disorganised with fewer glomeruli. White dots indicate putative glomeruli. (E,F) *emx1* expression in wild-type and *ace⁻* embryos. In the wild-type embryo, *emx1* is expressed discrete cells of the olfactory bulb that may be projection neurons. Expression is reduced and disorganised in the *ace⁻* embryo. (G,H) *odorant receptor 2.0* (*or2.0*) expression in olfactory neurons (arrows) in wild-type and *ace⁻* embryos. *or2.0* expression is reduced throughout the olfactory placode in the *ace⁻* embryo. e, eye; ob, olfactory bulb; op, olfactory placode. Scale bars: 25 μm (A,B,E,F); 10 μm (C,D); 5 μm (G,H).

midline cells are initially mis-specified rather than deleted in *ace* mutants and that the late reduction in preoptic tissue may secondarily derive from early mis-specification of this territory.

Analysis of Hh pathway mutations indicates that this signalling pathway is also required for patterning of midline tissue around the forebrain commissures (Schaurte et al., 1998; Karlstrom et al., 1999). Indeed *noi* expression is widely expanded in response to ectopic Hh expression (Ekker et al., 1995; Macdonald et al., 1995) and mice lacking Shh activity lack *pax2* expression (Chiang et al., 1996) suggesting that *noi/pax2* expression is normally regulated by Hh signalling. As *noi* expression and midline development are also affected in *ace⁻* embryos, this raises the possibility that the Fgf and Hh pathways may cooperate to pattern rostral midline tissue in the forebrain.

One of the striking aspects of the *ace⁻* midline phenotype is that commissural axons project inappropriately quite distant to the midline suggesting that midline tissue loses some of its attractive properties. Midline tissue expresses a complex combination of guidance cues with both attractive and repulsive growth cone activities elicited by members of the Netrin, Semaphorin and Ephrin families (Cook et al., 1998). Reduced *net1* and *sema3D* expression is likely to contribute to the failure of many axons to reach the midline and the wayward trajectories of those axons that do cross (Deiner and Sretavan, 1999). Furthermore, although axons projected inappropriately between the AC and POC, they were never observed to project more ventrally, consistent with maintained expression of growth cone repelling Ephrins in the hypothalamus of *ace⁻* embryos (data not shown).

Ace is required for formation of the optic chiasm

In *ace⁻* embryos, retinal axons frequently fail to establish the optic chiasm and instead project to the ipsilateral tectum and/or telencephalon. This defect is due to a requirement for Ace within the brain and not within the eye, as transplanted *ace⁻* eyes form normal chiasmata in wild-type brains and wild-type eyes misproject in the brain of *ace* mutants.

The pathfinding errors made by the retinal axons in *ace⁻* embryos are not due to fasciculation along earlier misprojecting AC or POC axons as the retinal axon fascicles are discrete from other commissural axons. Indeed, in wild-type embryos, retinal axons cross the midline in channels lined by immature glial cells that prevent contact with existing POC axons (Burrill and Easter, 1995; Marcus and Easter, 1995). Thus although retinal, AC and POC axons all make comparable errors in *ace⁻* embryos, each set of axons probably initially makes these errors independent of other axonal populations.

In wild-type embryos, the retinal axons form a tight fascicle of axons extending along the surface of ventrally positioned Noi-expressing cells that later differentiate as reticular astrocytes. We have previously suggested that these astrocytes may funnel the contralaterally directed retinal axon fascicles directly across the midline (Macdonald et al., 1997). In *noi⁻* embryos, the astrocytes fail to differentiate and the subsequent failure to compartmentalise the optic nerves may lead to the ipsilateral projections frequently observed in *noi⁻* embryos (Burrill and Easter, 1995; Macdonald et al., 1997). As *noi* expression is reduced at the midline of *ace⁻* embryos, a failure of midline glial cell differentiation probably also occurs and this may contribute to the retinal pathfinding defects. Midline

patterning defects may then allow optic nerve axons access to pathways previously unavailable with the consequence that there are frequently projections both ipsilaterally and to the telencephalon in *ace* mutants.

Once within the diencephalon, retinal axon guidance is not obviously affected in *ace* mutants and the majority of retinal axons project to their normal target, the midbrain tectum (Picker et al., 1999). Indeed, even axons that initially misproject to the telencephalon often correct their trajectories back towards the tectum. Curiously, those axons that failed to correct their trajectory most often projected to the dorsal telencephalon where they appeared to form ectopic terminal arbors.

In addition to contact-mediated glial guidance, other cues are probably important in retinal axon pathfinding across the midline. For instance, retinal axons display a DCC receptor-mediated response towards Netrin in vitro, and in vivo, Net1 is required for retinal axon guidance out of the eye (Deinier et al., 1997; de la Torre et al., 1997). Although *netrin* expression is reduced in the eyes of *noi* mutants, it is relatively normal in *ace* mutants correlating with the lack of any substantial pathfinding defect in this position in *ace* mutants. However, the altered *netrin* expression more medially in *ace⁻* embryos may, as with POC and AC axons, help explain why retinal axons fail to approach the midline.

Fgfs have also been proposed to directly influence retinal axon outgrowth and inhibition of Fgf activity can lead to delayed axon outgrowth and defects in target recognition (McFarlane et al., 1995, 1996). No direct role for Fgfs in guidance through the chiasm has previously been shown, but as retinal ganglion cells do express Fgf receptors (McFarlane et al., 1995), a failure to detect Ace signals in the optic stalk and midline could contribute to the retinal axon phenotype of *ace* mutants.

Ace is required for forebrain patterning but not induction

Previous studies have suggested that Fgf8 signalling from the anterior neural ridge is required for induction of forebrain fates (Shimamura and Rubenstein, 1997). In support of such a role for Fgf8, loss of *ace* signalling leads to patterning defects at the front of the neural plate and reduction of *shh* and *nk2.1b* expression in the basal telencephalon. The eventual fate of basal telencephalic territories has not been determined in fish although in mice, telencephalic *shh* and *nkx2.1* expression is restricted to the medial ganglionic eminence (Sussel et al., 1999). Most broad telencephalic sub-domains do, however, appear to be present in *ace⁻* embryos suggesting a more restricted role for Ace in the patterning of telencephalic derivatives rather than in their induction. A similar situation is observed in the midbrain of *ace⁻* embryos, where markers of the midbrain territory are induced but not maintained (Reifers et al., 1998).

Although most telencephalic cells appear to acquire regional identities in *ace* mutants, we did observe defects in Lim gene expression within certain telencephalic domains. Putative dorsoanterior neuronal groups defined by expression of *lim1* and *lim6* (Toyama and Dawid, 1997), were reduced or lost in *ace⁻* embryos. These cell populations are both in close proximity to the dorsal midline expression domain of *ace*, suggesting that local activity of Ace may influence their

differentiation. Such a cell fate specification role for Fgfs is not unprecedented and indeed Fgf8 itself regulates Lim gene expression in the pituitary and branchial arches (Ericson et al., 1998; Tucker et al., 1999).

ace is regulated by signals from the margin of the neural plate and by Ace itself

Both in mice (Shimamura and Rubenstein., 1997) and in zebrafish (Houart et al., 1998), signals from the anterior margin of the neural plate appear to be involved in the patterning of rostral forebrain territories. The localisation of *ace* expression to the cells with patterning activity raises the possibility that Fgf8/Ace itself might be a key signal produced by the cells. Indeed, in explants of mouse tissue, Fgf8 can reconstitute telencephalic *bf1* expression in the absence of the anterior neural ridge.

Our results in zebrafish suggest that Fgf8 is unlikely to account for all of the activity of marginal neural plate cells. *ace* expression is only detectable shortly after the marginal cells acquire signalling properties and the loss of Ace function leads to a milder phenotype than loss of the anterior margin of the neural plate. Other Fgfs may act together with Ace and although we are unaware of any Fgfs expressed as early as marginal neural plate cells appear to have signalling activity, anterior expression of *fgf3* is maintained in *ace⁻* embryos (R. Mahmood and M. B. Mason, personal communication; F. R. and M. B., unpublished observations). Other signalling pathways are also likely to be involved in signalling from the margin of the neural plate. For instance, we have recently shown that reduction of BMP activity leads to a loss of telencephalic gene expression (Barth et al., 1999) suggesting that BMPs may be involved either in the establishment of the marginal neural plate cells or indeed in their signalling activity.

Although Ace may not be the first or only signal produced by marginal neural plate cells, anterior *ace* expression is reduced or lost when marginal neural plate cells are removed. This loss of expression includes cells that lie distant to the site of cell ablation suggesting that marginal neural plate cells non-autonomously induce *ace* expression. In support of this, transplantation of rostral marginal cells to more caudal regions leads to induction of *ace* expression (unpublished results). These observations indicate that the loss of *ace* expression is likely to be a contributing factor to the phenotype caused by removal of cells at the margin of the neural plate.

There is variable upregulation of *ace* transcription in the forebrain of *ace⁻* embryos indicating that Fgf8 negatively regulates its own transcription. A recently characterised mutation *aussicht* leads to upregulation of *ace* at most of its sites of expression raising the possibility that the *aussicht* mutation interferes with Fgf-mediated regulation of *ace* transcription (Heisenberg et al., 1999).

Ace and the development of the olfactory system

ace⁻ embryos exhibit defects in the differentiation of the olfactory bulb and in the projections of olfactory axons to the bulb. The primary site of activity of Ace in patterning the olfactory system is uncertain as *ace* is expressed both in the placode and in the telencephalon and defects are present in both locations. Indeed, as proliferation in, and differentiation of, the olfactory bulb is dependent upon innervation by

olfactory axons (Graziadei and Monti-Graziadei, 1992; Gong et al., 1995), it will be necessary to inactivate Ace independently in each location to determine how and where Ace functions in olfactory development.

The loss of olfactory receptor expression in ace^- embryos may have a direct bearing upon the development of the olfactory projection as odorant receptors are directly responsible for pathfinding of olfactory sensory neuron axons to invariantly positioned glomeruli in the bulb (Wang et al., 1998). We have shown that at least some zns2+ axons do enter the bulb in ace^- embryos, but they fail to establish a full complement of terminal projections. Whether this is due to there being fewer projections or due to less arborisations by a normal number of neurons remains to be determined. Although we have not quantified the numbers of olfactory neurons in ace^- embryos, early neurogenic gene expression in the placode and the overall differentiation of the olfactory epithelium did not appear to be significantly affected. Mice carrying hypomorphic alleles of $fgf8$ also appear to have olfactory system defects (Meyers et al., 1998) and detailed analyses of the phenotypes both in fish and mice will more precisely determine the roles of Fgf8 and how conserved these roles are between different vertebrates.

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