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Zebrafish *aussicht* mutant embryos exhibit widespread overexpression of *ace* (*fgf8*) and coincident defects in CNS development

Carl-Philipp Heisenberg*, Caroline Brennan and Stephen W. Wilson

Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK
*Author for correspondence (e-mail: c.heisenberg@ucl.ac.uk)

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SUMMARY

During the development of the zebrafish nervous system both *noi*, a zebrafish *pax2* homolog, and *ace*, a zebrafish *fgf8* homolog, are required for development of the midbrain and cerebellum. Here we describe a dominant mutation, *aussicht* (*aus*), in which the expression of *noi* and *ace* is upregulated. In *aus* mutant embryos, *ace* is upregulated at many sites in the embryo, while *noi* expression is only upregulated in regions of the forebrain and midbrain which also express *ace*. Subsequent to the alterations in *noi* and *ace* expression, *aus* mutants exhibit defects in the differentiation of the forebrain, midbrain and eyes. Within the forebrain, the formation of the anterior and postoptic commissures is delayed and the expression of markers within the pretectal area is reduced. Within the midbrain, En and *wnt1* expression is expanded. In heterozygous *aus* embryos, there is ectopic outgrowth of neural retina in the temporal half of the eyes, whereas in putative homozygous *aus* embryos, the ventral retina is reduced and the pigmented retinal epithelium is expanded towards the midline.

The observation that *aus* mutant embryos exhibit widespread upregulation of *ace* raised the possibility that *aus* might represent an allele of the *ace* gene itself. However, by crossing carriers for both *aus* and *ace*, we were able to generate homozygous *ace* mutant embryos that also exhibited the *aus* phenotype. This indicated that *aus* is not tightly linked to *ace* and is unlikely to be a mutation directly affecting the *ace* locus. However, increased Ace activity may underly many aspects of the *aus* phenotype and we show that the upregulation of *noi* in the forebrain of *aus* mutants is partially dependent upon functional Ace activity. Conversely, increased *ace* expression in the forebrain of *aus* mutants is not dependent upon functional Noi activity. We conclude that *aus* represents a mutation involving a locus normally required for the regulation of *ace* expression during embryogenesis.

Key words: Neurogenesis, Forebrain, Optic stalk, *fgf8*, *pax* genes, *acerebellar*, *noisthmus*, Zebrafish, *Danio rerio*

INTRODUCTION

During early vertebrate development, the nervous system becomes subdivided into discrete regions along its anterior-posterior and dorsoventral axes and within the anterior neural plate, domains of cells give rise to the telencephalon, diencephalon, eyes and midbrain. Many genes are known to be involved in early regional patterning of the anterior central nervous system (CNS) and, in a few cases, the genetic pathways underlying regional patterning are beginning to be unravelled. For instance, transplantation and ablation studies have demonstrated that a group of cells located at the midbrain/hindbrain boundary (MHB) are required for patterning the midbrain and cerebellum (Bally-Cuif and Wassef, 1995). The secreted proteins Fgf8 and Wnt1, and the transcription factors Engrailed (En), Pax2/5/8, Gbx2 and Isl3 are all known or strongly suspected to be involved in the production, reception or propagation of signals from the MHB (Crossley and Martin, 1995; Joyner, 1996; Crossley et al., 1996; Brand et al., 1996; Favor et al., 1996; Kikuchi et al., 1997; Reifers et al., 1998; Lun and Brand, 1998; Pfeffer et al., 1998). However, our understanding of how these genes interact and are themselves regulated remains superficial. In this study, we identify a novel mutation in zebrafish which leads to misregulated expression of *ace*/*fgf8* and *noi*/*pax2.1* both at the MHB region and within the rostral forebrain.

Pax2 belongs to the Paired-box family of transcription factor encoding genes related to the *Drosophila* segmentation gene *paired*. Loss-of-function mutations in the zebrafish *pax2* homolog, *noisthmus* (*noi*), result in loss of the midbrain tectum and cerebellum (Brand et al., 1996; Lun and Brand, 1998). *eng* and *wnt1* expression is reduced or absent in *noi* mutants (Brand et al., 1996; Lun and Brand, 1998) and, indeed, studies in mice have shown Pax protein binding sites within the promoter of the *eng2* gene (Song et al., 1996). In addition, *noi* mutants show phenotypic defects in the rostral forebrain. In the developing eye, the choroid fissure fails to close resulting in coloboma, a phenotype that is also observed in mice and humans carrying mutations in the *Pax2* gene (Sanyanusin et al., 1995; Favor et al., 1996; Torres et al., 1996; Macdonald et al., 1997). Phenotypic defects are also observed in the differentiation of the optic stalks and in guidance of axons

across the midline in the postoptic commissure and optic chiasm (Torres et al., 1996; Macdonald et al., 1997).

The phenotype of *noi* embryos is reminiscent of another zebrafish mutant, *acerebellar* (*ace*). Recent studies have shown that *ace* is a probable loss-of-function mutation in zebrafish *fgf8* (Reifers et al., 1998 and see Fürthauer et al., 1997). Fgf8 is a member of the large family of fibroblast growth factors that have been implicated in many aspects of early developmental patterning. In *ace* mutant embryos, the anlage of the cerebellum does not form and midbrain-like tissue is continuous with the medullary region of the hindbrain (Brand et al., 1996; Reifers et al., 1998). A role for Fgf8 in patterning the midbrain and cerebellum is supported by studies in chick that have shown that exogenously applied Fgf8 can mimic many of the effects of the MHB organiser (Crossley et al., 1996). Similar to *noi*, *ace* is also expressed in the rostral forebrain and phenotypic analysis indicates that Ace is also important for patterning this region of the CNS (R. Macdonald, M. Brand, and S. W. W., unpublished observations).

Although *ace* and *noi* are both required for correct development of the MHB region, neither gene is required for the initial induction of the other, as *ace* expression is initially present in *noi* embryos and *noi* expression is initially present in *ace* embryos (Brand et al., 1996; Reifers et al., 1998). However, this changes over time and, within a few hours, *noi* expression is no longer detectable in *ace* mutant embryos and *ace* expression is lost in *noi* mutants. These results suggest that, within the MHB region, *ace* and *noi* are initially activated independently but may later depend upon each other for maintained expression. This conclusion is supported by the observation that both genes are initially expressed in adjacent domains of the neural plate and only later are they co-expressed in cells around the MHB (Reifers et al., 1998). The relationship between Noi and Ace activity in other regions of the embryo has yet to be determined. One further regulator of *noi* expression within the rostral forebrain is the secreted signalling protein Sonic hedgehog (Shh). In mice lacking Hedgehog signalling in the rostral forebrain, *pax2* expression is severely reduced (Chiang et al., 1996) and overexpression of Hedgehog proteins in fish leads to widespread ectopic induction of *noi* expression (Macdonald et al., 1995; Ekker et al., 1995).

In this study, we analyze a mutation named *aussicht* (*aus*) which causes overexpression of both *noi* and *ace*. In *aus* mutant embryos, *ace* is overexpressed at many sites in the embryo while *noi* expression is only upregulated within the rostral forebrain and around the MHB, the only sites in the CNS where the expression domains of *noi* and *ace* overlap. Furthermore, by analyzing embryos double mutant for *aus* and *ace*, we find that the upregulation of *noi* within *aus* mutant embryos is at least in part dependent upon functional Fgf8 activity. In contrast, the analysis of *aus*/*noi* double mutant embryos shows that loss of Noi activity has little consequence upon the upregulation of *ace* in *aus* mutant embryos. Although *noi* expression is responsive to Shh, the overexpression of *noi* in *aus* mutant embryos is unlikely to be due to ectopic Shh activity as we observe no ectopic *shh* expression in *aus* mutants.

Fish that give rise to *aus* mutant embryos also give rise to embryos exhibiting a cell death phenotype suggesting that the *aus* mutation may represent a balanced translocation. Although the *aus* mutation is dominant, the phenotype of *aus* heterozygous and putative homozygous mutants is indistinguishable at early stages of development, with embryos exhibiting a variety of phenotypic alterations including reduced *pax6* expression in the ventral retina, delayed formation of the anterior and postoptic commissures and reduced expression of genes within the pretectal area. During subsequent development, some *aus* heterozygous embryos show ectopic outgrowth of temporal retina while, in suspected homozygous embryos, there is a reduction of ventral retinal tissue and an expansion of the retina towards the midline. In the midbrain of *aus* mutant embryos, the expression of *wnt1* and *eng* is increased. Based upon the known functions of *noi* and *ace*, the majority of the defects in *aus* mutants are consistent with what one might predict from increased activity of these two genes.

Analysis of embryos mutant for both *aus* and *noi* and for *aus* and *ace* indicates that *aus* is unlikely to constitute a gain-of-function allele of either *noi* nor *ace*. We therefore conclude that *aus* is likely to represent a mutation affecting a novel locus involved in the regulation of *noi* and *ace* expression during embryonic development of the zebrafish nervous system.

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 collected by natural spawning and were staged according to Kimmel et al. (1995).

Fish lines and genetics

The *aus*^{t294} allele described in this paper was found in a fish whose male parent had been mutagenized with ENU. *aus* represents a mutation with a dominant but not fully penetrant phenotype and we were able to identify adult *aus* heterozygous carriers that appear phenotypically wild type and were fertile in both sexes (for a more detailed description and discussion of the genetics of the *aus* mutation see Results and Discussion). To generate double mutant *aus*/*noi* and *aus*/*ace* embryos, we identified fish carrying both mutations in the progeny of a cross between *aus*/+ and *noi*^{tu29a}/+ and *aus*/+ and *ace*^{ti282a}/+, respectively. Since embryos heterozygous for *aus* and homozygous for *noi* or *ace* showed characteristics of both single mutant phenotypes, we were able to phenotypically identify them from early pharyngula stage onwards.

Where percentages are cited in the results, they are always based upon analysis of more than 200 embryos.

Whole-mount antibody labeling and in situ hybridization

Standard procedures were used for both antibody and in situ labeling as described by Hammerschmidt and Nüsslein-Volhard (1993). For antibody staining, the Vectastain detection kit was used. The anti-Pax6 antibody (Macdonald et al., 1994) was diluted 1:400, the anti-En antibody (Developmental Hybridoma Bank) 1:25 and the anti-acetylated alpha tubulin antibody (Sigma) 1:1000. Antisense digoxigenin-labelled RNA probes were synthesised using the digoxigenin RNA labeling kit (Boehringer Mannheim). As templates, full-length pax2.1, fgf8, islet-1, zash-1b, ephrin-A-l2, ephrin-A-rtk2 and ephrin-A-rtk7 cDNAs, and 0.8 kb netrin-1a, 1.6 kb shh and 0.58 kb wnt-1 cDNA fragments were used. The in situ hybridisation staining was detected using BM-purple substrate (Boehringer Mannheim), embryos were then fixed in 4% paraformaldehyde in phosphate-buffered saline for 1 hour, washed in phosphate-buffered saline, cleared in 70% glycerol and mounted on a glass slide.

Sectioning

Embryos were dehydrated in 100% ethanol, embedded in JB4 resin

(Agar Scientific Ltd), and sectioned (15 μm) using a tungsten knife on a Jung 2055 Autocut.

RESULTS

Genetics of the *aussicht* (*aus*) mutation

aus^{t294} is a dominant mutation with a partially penetrant phenotype in which transcriptional regulation of *noi* (*pax2.1*) and *ace* (*fgf8*) is disturbed (see below). The *aus* phenotype was originally observed in a cross between a fish whose male parent had been mutagenised with ethylnitrosurea and a heterozygous carrier for an unrelated mutation. Subsequent matings of the fish carrying the *aus* mutation to wild-type fish showed that the *aus* phenotype is dominant. In crosses between a carrier of *aus* and a wild-type fish, approximately 12% of embryos show the *aus* phenotype and 18% exhibit extensive cell death in the CNS (Table 1). The cell death phenotype (*degeneration*, *deg*^{t294a}) represents a second dominant mutation invariably carried by fish that possess the *aus* mutation. We were able to raise both female and male fish that carried *aus* to adulthood and, in subsequent generations, these fish also turned out to be carriers of the *deg* mutation. The expression of two separate lethal phenotypes, each in less than 25% of embryos, from viable fertile adult fish is characteristic of a balanced translocation (Morgan et al., 1925; Talbot et al., 1998 – see Discussion).

In crosses between two heterozygous carriers of *aus*, there were four different phenotypes: the *aus* and *deg* phenotypes were each visible in approximately 20% of embryos (Table 1). During subsequent development, *aus* and *deg* mutant embryos could be subdivided into about half showing a phenotype equivalent to embryos originating from crosses between a heterozygous carrier and a wild-type fish, while the other half exhibited a related but more severe phenotype (see below and data not shown). The two additional phenotypes may represent the homozygous conditions for *aus* and *deg* mutations since both phenotypes were only detectable in crosses between two heterozygous carriers but not in crosses between a heterozygous carrier and a wild-type fish. In the following analysis of the *aus* phenotype, we name both putative *aus* heterozygous and homozygous mutant embryos as '*aus* mutant embryos' up until the pharyngula stage since we are not able to phenotypically distinguish between putative heterozygous and homozygous mutants at these stages. At older stages, when

Table 1. Summary of the percentages of phenotypes seen in pharyngula stage embryos of crosses between an *aus* heterozygous fish and a wild-type fish and between two *aus* heterozygous fish

Phenotype	*aus*/+×+/+ %	*aus*/+×*aus*/+ %
Wild type	70	60
Enlarged optic stalks/expanded optic recesses	12	20
Degeneration/collapsed ventricles	18	20

Number of embryos analysed > 200 for each cross.

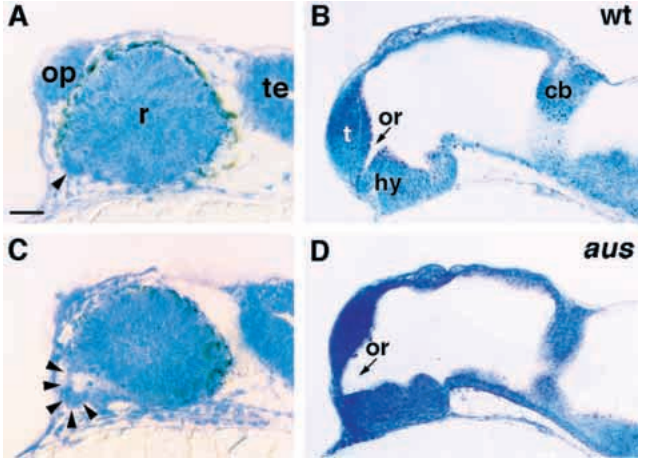

Fig. 1. Optic stalks and optic recesses are enlarged in *aus* mutant embryos. Sections through the head region of prim-12 stage wild-type and *aus* mutant embryos. (A,C) Parasagittal section at the junction between retina and optic stalks. The optic stalks (arrowheads) are larger in the *aus* mutant embryo (C) as compared to a wild-type sibling (A). (B,D) Sagittal section. The optic recess is expanded in an *aus* mutant embryo (D) as compared to a wild-type sibling (B). Abbreviations: cb, cerebellum; hy, hypothalamus; op, olfactory placodes; or, optic recess; r, retina; t, telencephalon; te, tectum; wt, wild type. Scale bar: 25 μm.

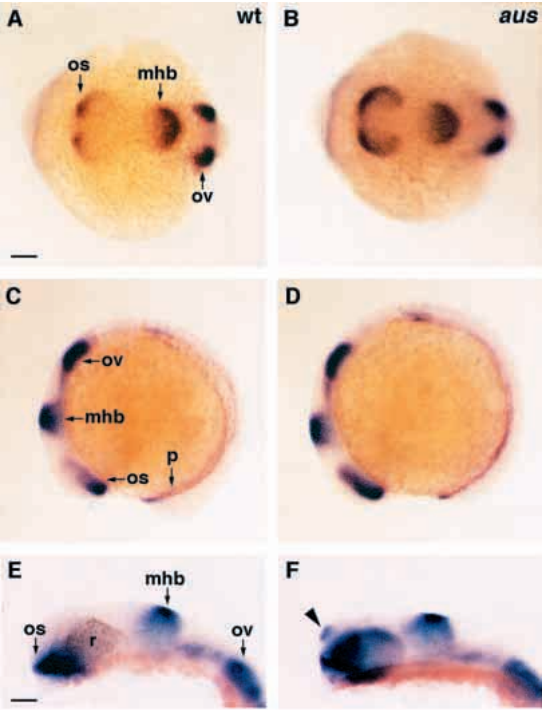

Fig. 2. *noi(pax2.1)* expression is increased in *aus* mutant embryos. Dorsal (A,B) and lateral (C-F) views of whole embryos (A-D) or heads (E,F) with rostral to the left. (A-D) 6-somite-stage embryos. The expression domains of *noi* are expanded within the optic stalks and to a lesser extent at the mhb in the *aus* mutant embryo (B,D) compared to a wild-type sibling (A,C). (E,F) Prim-12 stage embryos. *noi* expression is upregulated within the optic stalk and mhb and ectopically expressed (arrowhead in F) within the telencephalon of the *aus* mutant embryo (F) as compared to a wild-type sibling (E). Abbreviations: d, diencephalon; mhb, midbrain/hindbrain boundary; os, optic stalk; ov, otic vesicle; p, pronephric duct; r, retina; wt, wild type. Scale bars: (A-D) 100 μm; (E,F) 50 μm.

the phenotypes are distinct, we refer to the likely homozygous mutants as 'putative homozygous *aus* mutant embryos'.

noi (*pax2.1*) and *ace* (*fgf8*) are mis-regulated in *aus* mutant embryos

The most obvious aspects of the *aus* phenotype in living embryos and sectioned tissue were enlarged optic stalks and expanded optic recesses (Fig. 1C,D). We have previously shown that the zebrafish Pax2 ortholog, Noi, is required for correct differentiation of the optic stalks (Macdonald et al., 1997) and so we examined expression of this gene in *aus* mutant embryos. *noi* expression in the optic stalks and at the midbrain-hindbrain boundary (MHB) of *aus* mutants was expanded into the retina and the posterior midbrain respectively (Fig. 2 and see Fig. 8). Moreover, in some *aus* mutants, there was an ectopic patch of *noi* expression in the anterior telencephalon (Fig. 2F). Other domains of *noi* expression (spinal cord and pronephric duct) appeared normal in *aus* embryos.

The *ace* mutation has some similar phenotypic consequences to mutations in *noi* (Brand et al., 1996) and recent analysis has shown that *ace* is a mutation in the *fgf8* gene (Reifers et al., 1998). We therefore examined the expression of *ace* in *aus* mutant embryos. Similar to *noi*, *ace* expression in the optic stalk area and around the MHB of *aus* embryos was expanded into the anlage of the retina and the posterior midbrain, respectively, from early somite stages (Fig. 3D,F). However, in contrast to *noi*, *ace* expression was elevated at other sites at which *ace* is normally expressed in *aus* mutants (Fig. 3D,F). The degree of upregulation varied substantially depending on the site of *ace* expression. While *ace* expression is expanded in the tailbud, there is little or no upregulation within the somites (Fig. 3D). The tailbud appeared broader while the somites were morphologically unaffected and there was no obvious change in *her1*, *myoD* and Myosin expression in the somites of *aus* mutant embryos (Fig. 3D and data not shown). On the basis of morphology alone, we were not able to identify *aus* mutant embryos until late somite stages, but from as early as gastrula stages, some embryos showed increased *ace* expression with respect to similarly labelled siblings (Fig. 3A,B). We presume that these embryos would be likely to exhibit the *aus* phenotype at later stages of development. Beyond the pharyngula stage, levels of *ace* expression gradually returned to normal in *aus* mutants (Fig. 3G,H) indicating that there is recovery in this aspect of the phenotype over time.

Since *noi* and *ace* are both upregulated in the optic stalk region and around the MHB of *aus* mutant embryos, we focussed our subsequent analysis upon the development of the eyes and MHB of *aus* embryos.

The *aus* mutation affects differentiation of the eye

In crosses between heterozygous *aus* carriers and wild-type fish, approximately 70% of mutant embryos that exhibited large optic stalks and an expanded optic recess at pharyngula stage, showed a temporal outgrowth of retinal tissue during subsequent development (Fig. 4B). Sections through these eyes showed that the retinal outgrowth is an outfolding of an otherwise normally layered neural retina (data not shown). The remaining ~30% of embryos that had shown enlarged optic stalks and expanded optic recesses at earlier stages were later superficially indistinguishable from wild-type siblings, again indicating that the *aus* phenotype can recover over time.

Crosses between two heterozygous *aus* carriers gave rise to mutants exhibiting two differing eye phenotypes. Of the embryos that showed expanded optic recesses at pharyngula stage, about 45% later showed a temporal outgrowth of retinal tissue as described above (Fig. 4B). In a further 45% of the mutant embryos, the eyes and, in particular, the ventral/nasal part of the retina, were reduced (Fig. 4C) and at later stages pigmented retina extended from the back of the eye towards the midline (Fig. 4D,E). This was always accompanied by a failure of the choroid fissure to close (coloboma; Fig. 4G). Since this phenotype was only detectable in mutant embryos from crosses between two heterozygous *aus* carriers, we believe that it represents the homozygous *aus* phenotype whereas the temporal outgrowth of the retina also seen in crosses between heterozygous *aus* carriers and wild-type fish is likely to represent the heterozygous *aus* phenotype. The remaining 10% of the embryos that showed expanded optic recesses at pharyngula stage, recovered over time and were later indistinguishable from wild-type embryos.

Somewhat surprisingly, 10% of embryos from crosses between two heterozygous *aus* carriers, which were phenotypically wild type at pharyngula stage, showed slightly smaller eyes in which retinal tissue extended out of the back of the eye during subsequent development (data not shown). As this phenotype was also not observed in crosses between *aus* carriers and wild-type fish, it may represent a weaker expressivity of the homozygous phenotype that is only detectable at later stages of development.

Altered gene expression in the retinas of *aus* mutant embryos

To address the alterations in gene activity that might underlie the morphological abnormalities in the eyes of *aus* mutants, we examined the expression of various genes known or suspected to be involved in patterning of the eye. *Islet1* is expressed in the first neurons that differentiate in the developing eyes (Dorsky et al., 1996). In *aus* mutant embryos, there was a reduction of *islet1* expression within the ventral/nasal half of the retina suggesting that neuronal differentiation is delayed (Fig. 5A,G). Within the developing optic vesicles, *noi* and *pax6* are expressed in complementary domains with *noi* expression restricted to the optic stalks and cells lining the choroid fissure and *pax6* expression restricted to the retina (Macdonald et al., 1995, 1997). In *aus* mutant embryos, ectopic *noi* expression within the ventral/nasal part of the eyes (Fig. 5B,H) was accompanied by a downregulation of *pax6* expression in a similar region (Fig. 5C,I), indicating an involvement of *aus* in the regulation of both of these Pax genes.

Eph receptors and their ligands, the ephrins, are expressed in discrete domains of the developing eye and have been shown to be involved in cell-to-cell interactions required for patterning various tissues in the embryo (Drescher et al., 1997). *ephrin-A-l2*, a GPI-linked ephrin, is expressed in nasal retina, whereas the Eph receptor *eph-A-rtk2* is expressed in the temporal retina of wild-type embryos. In some of the *aus* mutant embryos showing much enlarged optic recesses, *ephrin-A-l2* expression within the nasal retina was upregulated and expanded into the temporal half of the eye whereas *eph-A-rtk2* expression within the temporal retina was reduced (Fig.

5D,E,J,K). These observations raise the possibility that *aus* may be involved in regulation of nasotemporal patterning within the developing retina.

The netrins are a family of secreted proteins that are involved in axonal guidance within the CNS. Within the eye, Netrin activity is required for guidance of axons out of the choroid fissure (Deiner et al., 1997) and we have previously shown that *netrin* expression in the retina is dependent upon functional Noi (Macdonald et al., 1997). In all *aus* mutant embryos, there was an increase in *net1a* expression around the choroid fissure and ectopic expression throughout much of the ventral retina (Fig. 5F,L).

As mentioned above, some of the *aus* mutant embryos that exhibit visible phenotypic defects at early stages later recover and are superficially indistinguishable from wild-type siblings. Supporting the conclusion that the *aus* phenotype can recover over time, we found that changes in the expression pattern of *islet1*, *pax6*, *ephrin-A-l2*, *eph-A-rtk2* and *net1a* became less prominent over time (data not shown).

aus mutant embryos exhibit commissural pathway defects in the rostral forebrain

We have previously shown that commissural axonal guidance is perturbed in embryos lacking functional Noi protein (Macdonald et al., 1997). As *noi* expression is upregulated in *aus* mutant embryos, we examined axonal patterning and gene expression in the vicinity of the rostral commissures in mutant embryos. Staining embryos with an axonal differentiation marker shows that both the anterior and postoptic commissure have not formed in *aus* mutants by the early pharyngula stage (Fig. 6A,B). Other pathways in the forebrain and midbrain appeared relatively normal, although there was some axonal disorganization in the hindbrain (not shown). Over time, the rostral commissural defects show recovery such that by prim-25 stage, axons have crossed the midline in both commissures (Fig. 6C,D). By 3 days postfertilization (protruding mouth stage), the anterior commissure appeared relatively normally positioned and fasciculated whereas the postoptic commissure/optic chiasm was defasciculated in some of the heterozygous and homozygous *aus* mutant embryos (Fig. 6E-H). In addition, retinal ganglion cell axons were less tightly fasciculated in the retinas of putative homozygous *aus* mutant embryos, a phenotype that is also observed in *noi* mutant embryos (Fig. 6G,H) (Macdonald et al., 1997).

To better understand the changes in midline patterning that might underlie the commissural defects, we examined the expression of *noi*, *ace* and several other genes potentially involved in midline patterning in pharyngula stage *aus* mutant embryos. *noi* is normally expressed in a group of cells ventral to the optic recess which are directly dorsal to the position at which the postoptic commissure forms (Macdonald et al., 1997). In *aus* mutant embryos, *noi* expression extended dorsal to the optic recess and into the ventral telencephalon (Fig. 7A,B). Furthermore, *ace* expression, normally present in a group of cells similar to those that express *noi* ventral to the optic recess, also crossed the optic recess and was expanded throughout much of the ventral telencephalon of *aus* mutants (Fig. 7C,D).

net1a encodes a secreted axon guidance protein that is strongly expressed dorsal to the optic recess within the ventral telencephalon. In *aus* mutant embryos, *net1a* is ectopically expressed in the optic stalks and levels of transcripts are increased throughout much of the rostral diencephalon (Fig. 7E,F).

Shh encodes a secreted signalling protein that has previously been shown to promote *noi* expression in the optic vesicles in fish (Macdonald et al., 1995; Ekker et al., 1995) and be required for *pax2* expression in this location in mice (Chiang et al., 1996). However, there was little if any ectopic *shh* expression in *aus* mutant embryos suggesting that the *aus* mutation may lead to ectopic *noi* expression via a Shh-independent pathway. We also examined the expression of several other genes (*eph-A4, eph-A-rtk2, eph-A-rtk7, ephrin-A-l2* and *ephrin-B2*) that all showed no major change in their expression pattern in this region of the embryonic brain (data not shown).

The failure to establish the anterior and postoptic commissures at pharyngula stage in *aus* mutants therefore coincided with an expansion of the expression domains of *noi*, *ace* and *net1a* at the commissure-forming region of the midline neuroepithelium.

aus mutants exhibit patterning defects within the midbrain and pretectal area of the forebrain

Both *noi* and *ace* mutations were originally identified on the basis of patterning defects around the MHB. As *ace* is upregulated in this region in *aus* embryos, we examined whether there are any alterations in patterning of this territory in mutant embryos.

In the midbrain, we examined the expression of *noi*, En and *wnt1* in *aus* mutant embryos. Both *noi* and En expression is slightly expanded and levels of *wnt1* transcripts appear to be increased in the midbrain, the MHB and the hindbrain of pharyngula stage *aus* mutants (Fig. 8A-F). One caveat is that En expression does normally change over time at the MHB and so if *aus* affects temporal aspects of midbrain patterning, this could also contribute to the alterations in En expression in *aus* mutants.

In *noi* mutant embryos, pretectal gene expression is altered (M. Brand and others, personal communication) and so we examined expression of four genes characteristic of this region of the diencephalon. In late pharyngula stage *aus* mutants, the pretectal expression domain of *zash1b* is absent and *eph-A4* and *eph-A-rtk7* expression in this same region is reduced (Fig. 8G-J and data not shown). In contrast, pretectal expression of *pax6* appeared unchanged in pharyngula stage *aus* mutant embryos (data not shown). Despite these alterations in gene expression, the nucleus of the posterior commissure located in the pretectum appeared to be normal in mutant embryos (data not shown).

aus is unlikely to be a mutation in the *noi* or *ace* genes

The observation that *aus* is a dominant mutation and that *noi* and *ace* are overexpressed in *aus* mutants raised the possibility that *aus* represented a gain-of-function allele of one of these genes. To test this possibility, we determined if it is possible to generate embryos mutant for both *aus* and *noi* and for both *aus* and *ace*. If double mutant embryos for *aus* and *noi* or *aus* and *ace* can be generated this would indicate that *aus* is not tightly linked to *noi* or *ace* and therefore unlikely to be a gain-of-function allele of one of these genes.

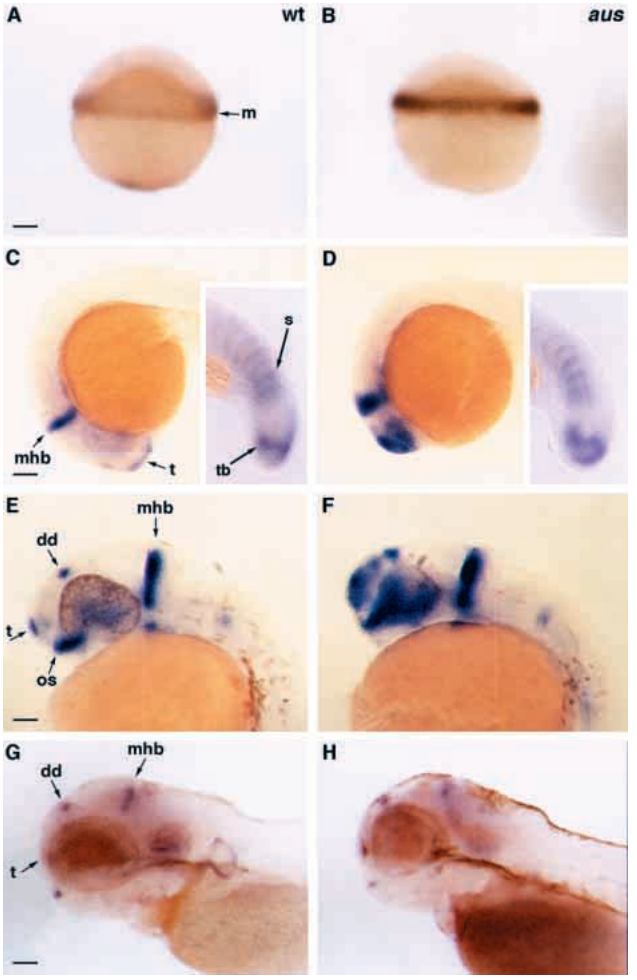

Fig. 3. *ace(fgf8)* expression is upregulated in *aus* mutant embryos. Lateral views of whole embryos. (A,B) Sphere stage. *ace* expression in the blastula margin is upregulated in the putative *aus* mutant embryo (B) as compared to a putative wild-type sibling (A). (C-F) *ace* expression is upregulated at many sites of expression in 18-somite stage (C,D) and prim-12 stage (E,F) *aus* mutant embryos (D,F) as compared to wild-type siblings (C,E). Inset panels in C,D are close-up pictures of *ace* expression in the somites and tailbud. (G,H) By the fourth day of development, there is no apparent difference in the expression levels of *ace* in the *aus* mutant embryo (H) as compared to a wild-type sibling (G). Abbreviations: dd, dorsal diencephalon; m, margin; mhb, midbrain/hindbrain boundary; os, optic stalks; s, somites; t, telencephalon; tb, tailbud; wt, wild-type. Scale bars: (A-D) 125 μm; (E-H) 100 μm.

In crosses of a double carrier for *aus* and *ace* with a single carrier for *ace*, approximately 7% of embryos showed a forebrain phenotype (expanded optic recesses and eye defects) similar but weaker to *aus* and a cerebellar defect similar to *ace* (Fig. 9E-H). Similarly, in crosses of a double carrier of *aus* and *noi* with a single carrier for *noi*, about 7% of embryos looked similar to *aus* mutants in the forebrain and lacked the MHB as seen in *noi* embryos (see below). These results suggest that *aus* is unlinked to *noi* and *ace* and is therefore unlikely to represent a gain-of-function allele of either *noi* or *ace*.

The increased expression of *noi* and *ace* in *aus* mutants raised the possibility that an increase in the activity of one gene product mediates the increase in the other. To test this possibility, we examined if there is still an increase of *noi* expression in *aus;ace* double mutants and an increase in *ace* expression in *aus;noi* double mutants. Expression of *ace* in *aus;noi* double mutant embryos was expanded in the eyes and forebrain to a degree similar to *aus* single mutants (Fig. 9A-D). In contrast, there was only a weak upregulation of *noi* expression in the forebrain of *aus;ace* double mutants as compared to *aus* single mutant embryos (Fig. 9I-L). This indicates that in the forebrain, *aus* functions independently of *noi* in upregulating *ace* expression but is partially dependent on the presence of functional Ace protein for the upregulation of *noi*. By pharyngula stage, in the midbrain of *aus* mutants, *ace* expression is lost in the absence of Noi and *noi* expression is lost in the absence of Ace, similar to the situation in *ace* and *noi* single mutant embryos (Reifers et al., 1998).

DISCUSSION

Genetics of the *aus* mutation

In this study, we show that *aus* is a partially penetrant dominant mutation affecting the regulation of *ace* and *noi* expression. All

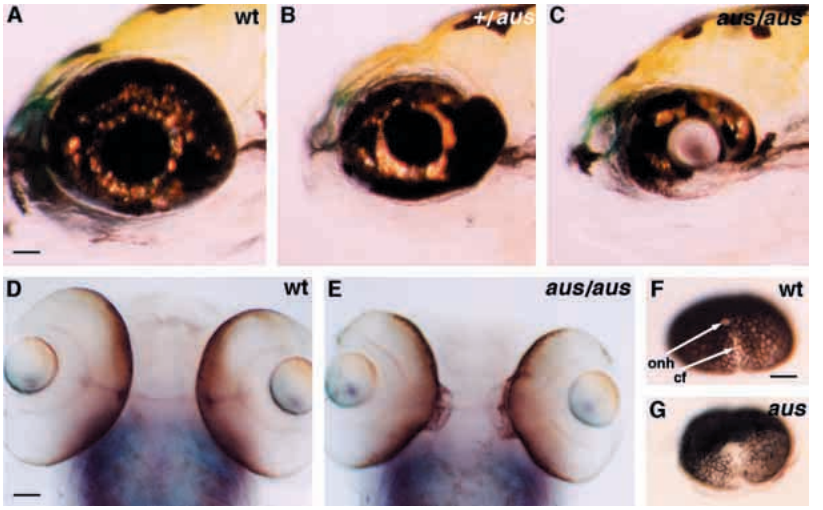

Fig. 4. Differentiation of the eyes is perturbed in *aus* mutant embryos. Lateral (A-C) and ventral (D,E) views of the eyes. (A-C) In heterozygous *aus* mutant embryos at early larval stage, there is ectopic outgrowth of the temporal retina (B) and, in putative homozygous *aus* mutant embryos, the ventral retina is reduced (C) as compared to wild-type siblings (A). (D,E) Ectopic outgrowth at the back of the retina is observed in the *aus* mutant embryo at early larvae stage (E) as compared to a wild-type sibling (D) (the faint blue staining is *noi* expression within the eyes, optic nerves and midline). (F,G) In the putative homozygous *aus* mutant embryo at prim-20 stage (G) there is incomplete closure of the optic fissure as compared to a wild-type sibling (F). See also Fig. 6G,H. Abbreviations: cf, choroid fissure; onh, optic nerve head; wt, wild type. Scale bars: (A-C) 50 μm; (D-G) 25 μm.

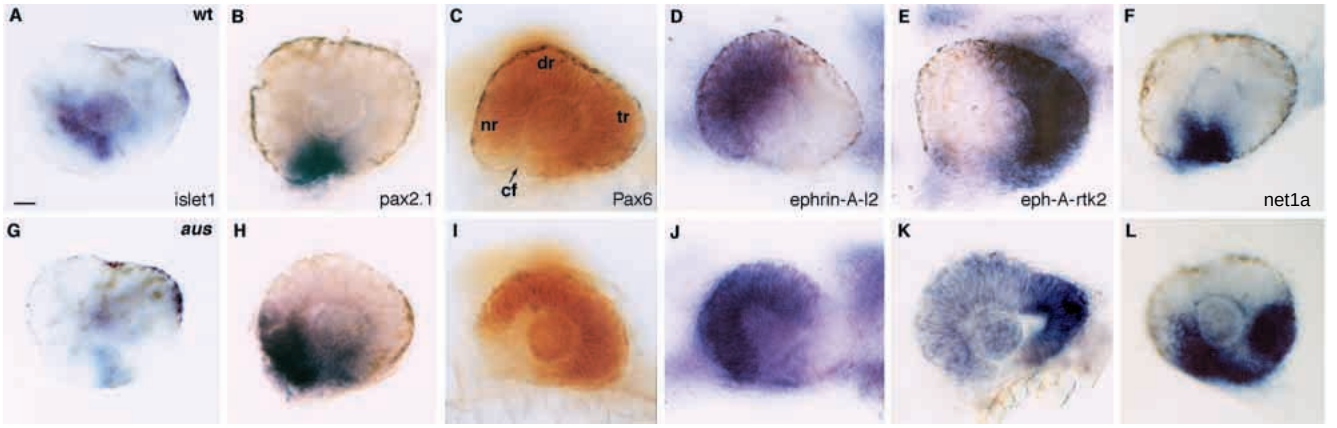

Fig. 5. Gene expression is altered in the retinae of *aus* mutant embryos. Lateral views of eyes of wild-type (top row) and *aus* mutant (bottom row) prim-12 stage embryos. (A,G) *islet1*. Expression is reduced in the *aus* mutant embryo. (B,H) *noi* (*pax2.1*). Expression is expanded in the ventral retina of the *aus* mutant embryo. (C,I) Pax6. Expression is reduced in the ventral retina of the *aus* mutant embryo. (D,J) *ephrin-A-l2*. Expression is expanded into the temporal retina of the *aus* mutant embryo. (E,K) *eph-A-rtk2*. Expression is reduced in the temporal retina of the *aus* mutant embryo. (F,L) *net1a*. Expression is expanded in the ventral retina of the *aus* mutant embryo. Abbreviations: cf, choroid fissure; dr, dorsal retina; nr, nasal retina; tr, temporal retina. Scale bar: 25 μm.

adult carriers of the *aus* mutation were also carriers of a second dominant mutation, *deg*, which gives rise to an extensive cell death phenotype. This indicates that fish that carry both mutations are preferentially viable compared to carriers of either mutation alone. This pattern of inheritance of the *aus* and *deg* mutations is characteristic of a reciprocal translocation between two chromosomes (Talbot et al., 1998). In such a scenario, the adult carrier of the *aus* and *deg* mutations would be a balanced translocation heterozygote that possessed both translocation chromosomes. Gametes from this adult could either inherit both translocation chromosomes (and be a viable carrier of *aus* and *deg*), inherit neither and be wild type, inherit one translocation chromosome (leading to the *aus* phenotype) or the other (leading to the *deg* phenotype). It has recently been shown that the cyc^{b213} allele represents a reciprocal translocation between LG2 and LG12. Similar to *aus*, the second phenotype resulting from the translocation is widespread cell death (Talbot et al., 1998). Chromosomal inversions can also sometimes lead to a pattern of inheritance similar to a reciprocal translocation (Klug and Cummings, 1991) and detailed mapping of the *aus/deg* mutations will be necessary to resolve the genetic defects in carriers of the mutation. Although the *aus* mutation may involve a chromosomal segment encompassing more than one gene, the specificity of the phenotype suggests that a single affected locus may be responsible for much of the phenotype as appears

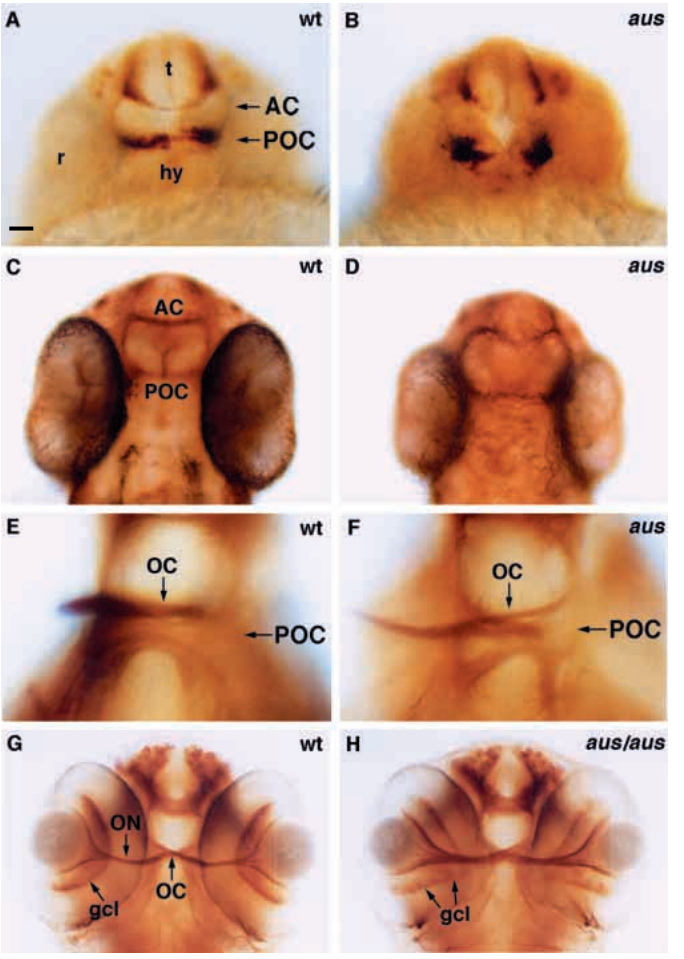

Fig. 6. Commissure formation is delayed and perturbed in *aus* mutant embryos. Frontal/ventral views of whole-mount embryos stained with an antibody against acetylated tubulin focussed at the level of the anterior and postoptic commissures. (A,B) Prim-5 stage embryos. The anterior commissure and postoptic commissure are not formed in the *aus* mutant embryo. (C,D) Prim-25 stage embryos. By this stage, some axons have crossed the midline in both commissures in the *aus* mutant embryo. (E,F) Protruding-mouth stage embryos. The postoptic commissure and optic chiasm are defasciculated and slightly disorganised in the *aus* mutant embryo. (G,H) Protruding-mouth stage embryos. The optic axons are less tightly fasciculated as they exit the eye of the putative homozygous *aus* mutant embryo as compared to the wild-type sibling. The failure of the choroid fissure to fully close (coloboma) results in the retinal ganglion cells protruding towards the midline. Abbreviations: AC, anterior commissure; gcl, ganglion cell layer; hy, hypothalamus; OC, optic chiasm; ON, optic nerve; POC, postoptic commissure; r, retina; t, telencephalon. Scale bar: (A-D,G,H) 25 μm; (E,F) 10 μm.

to be the case for several *cyclops* alleles and other zebrafish mutations generated by gamma ray mutagenesis (Fritz et al., 1996; Fisher et al., 1997). Conversely, the severity of the *deg* phenotype suggests severe genetic deficiencies in *deg* mutant embryos.

aus function in eye development

The *aus* mutation leads to abnormal development of the eyes in both heterozygous and putative homozygous conditions. At early stages of development, the optic stalks are enlarged while the prospective retina appears morphologically almost normal. However, in putative homozygous *aus* mutants, the ventral-nasal retina subsequently fails to develop and the choroid fissure remains open causing coloboma. Defects in the ventral eye are less apparent in heterozygous *aus* mutants in which the most noticeable morphological defect is outgrowth in the temporal retina. We believe that these alterations are at least in part due to expanded Ace and Noi activity within the developing eye as both of these genes are upregulated and ectopically expressed in the developing retina of *aus* mutants. Furthermore, morphological defects are reduced in the eyes of *aus* mutants that are homozygous for the *ace* mutation indicating a partial dependence of the phenotype upon functional Ace protein.

The reduction of Pax6 expression within the ventral/nasal half of the retina in *aus* mutants may underlie the reduced ventral retinal development observed in some mutant embryos. Pax6 is essential for retinal development, as mice lacking Pax6 activity form an optic vesicle but this vesicle subsequently fails to differentiate to form the retina (Hill et al., 1991). The reduction in Pax6 expression in the ventral retina of *aus* mutants may be due to expanded Noi activity in this same region of the retina. These two Pax proteins are normally expressed in mutually exclusive domains of the optic vesicle with Noi expression restricted to the optic stalks and Pax6 restricted to the retina (Macdonald et al., 1995). We have previously suggested that Noi might be involved in the suppression of *pax6* expression allowing medial optic vesicle tissue to differentiate as optic stalk instead of retina (Macdonald et al., 1995). In support of this possibility, overexpression of *shh* leads to upregulation of *noi* throughout much of the optic vesicle, and this is accompanied by suppression of *pax6* expression and inhibition of retinal development (Macdonald et al., 1995, Ekker et al., 1995). Furthermore, in mice lacking Pax2 function, retinal tissue appears to spread into the optic stalks, again raising the possibility that Pax2 suppresses *pax6* expression (Torres et al., 1996). Finally, regulation of *pax6* by Noi/Pax2 could be direct as Pax protein binding sites are present in the promoter of the mouse *pax6* gene (Plaza et al., 1993).

Analysis of Eph receptor and ligand expression in *aus* mutant embryos suggests that *aus* may interfere with assignment of nasotemporal identity in the retina. In some *aus* mutants, expression of *ephrin-A-l2* is expanded from the nasal half of the retina into the temporal retina whereas, conversely, the expression domain of *eph-A-rtk2* within the temporal retina is reduced. As Eph family proteins have been implicated in the assignment of nasotemporal retinal identity (Drescher et al., 1997), these alterations in gene expression suggest that there is an expansion of nasal identity at the expense of temporal identity in *aus* mutants. The upregulation of *fgf8* expression in the rostral CNS of *aus* mutant embryos may be responsible for the altered character of nasal and temporal retinal tissue as some *ace* mutant embryos also have some disruption to naso-temporal patterning (A. Picker, C. B., N. Holder and M. Brand, unpublished data).

One aspect of the *aus* phenotype that we do not yet understand is the expansion of retinal pigment epithelium (RPE) out of the back of the eye. We have previously observed that expansion of *noi* expression following overexpression of *shh* is correlated with reduced development of both RPE and neural retina fates (Macdonald et al., 1995). This does not seem to be the case in *aus*, although we do not know whether the expanded RPE of *aus* mutants represents cells that have adopted abnormal fates or alternatively whether RPE cells have overproliferated or migrated inappropriately. An expanded RPE phenotype is also seen in embryos in which signalling via the Eph-A-rtk1 receptor is compromised (Xu et al., 1996). In this case, it has been suggested that the phenotype might arise through an abnormal contribution of diencephalic cells to the optic vesicle. A similar phenotype is also observed in zebrafish embryos homozygous for the *blowout* mutation though once again, the phenotype is not well understood (Karlstrom et al., 1996).

aus function in axonal guidance in the forebrain

In *aus* mutant embryos, few or no axons cross the midline in the postoptic and anterior commissures at the stages when *noi* and *ace* show the greatest degree of overexpression in the forebrain. At later stages, there is substantial recovery of this phenotype such that by 2 days, the anterior commissure appears relatively normal and both the postoptic commissure and optic chiasm are present, although axons remain somewhat disorganised.

Within the forebrain, commissures appear to be established at the boundaries between domains of neuroepithelial cells that express different combinations of regulatory genes. We have previously proposed that one reason that commissures are established at such locations is because domains of cells on each side of the boundary express different combinations of proteins that repulse growth cones (Wilson et al., 1993, 1997). In this way, axons may preferentially extend along each other and form a tightly fasciculated commissure at the interface between the two domains. Analysis of embryos lacking Noi function supports a role for this transcription factor in regulating repulsive properties of a narrow domain of cells dorsal to the postoptic commissure (Macdonald et al., 1997). In *noi* mutant embryos, growth cones extend among the midline cells that lack Noi activity with the result that axons fail to form a tightly fasciculated commissure and cross the midline in aberrant locations. If Noi does regulate the expression of proteins that confer growth cone repulsive properties to expressing cells, then this could provide an explanation for the delay in commissure formation observed in *aus* mutants. In wild-type embryos, *noi* expression is restricted to a narrow band of cells dorsal to the postoptic commissure whereas, in *aus* mutants, *noi* is widely ectopically expressed throughout the midline territory within which the anterior and postoptic commissures would normally form. We suggest that this ectopic *noi* expression may lead to ectopic expression of growth cone repulsive molecules, which render the entire midline tissue of *aus* mutants impassable to the early

commissural axons. At later stages, ectopic expression of *ace* and *noi* decreases and more normal midline domains of gene activity may be re-established. Unfortunately, to date there are no identified growth cone repulsive proteins that are known to be downstream of Noi in midline forebrain tissue. Noi does appear to regulate *netrin* expression within the eye, but perhaps not within the forebrain as midline *net1a* and *net1b* expression appear to be unaffected in the absence of Noi activity (Macdonald et al., 1997). While the changes in Noi activity are likely to contribute to the commissural defects of *aus* mutants, it is also likely that other regulatory pathways will be disturbed. For instance, *ace* mutant embryos have commissural defects more severe than *noi* mutants (R. Macdonald, M. Brand and S. W. W., unpublished observations) indicating that Ace may also influence midline commissure formation via Noi-independent pathways.

Putative homozygous *aus* mutants also exhibit defects in axon guidance out of the eye. Retinal axons still coalesced towards the choroid fissure in the ventral retina but remained defasciculated as they exited the eye. This phenotype is similar to that observed in mouse and fish embryos lacking Pax2/Noi activity (Torres et al., 1996; Favor et al., 1996; Macdonald et al., 1997). The optic nerve head at which retinal axons coalesce as they exit the eye is lined by Pax2/Noi-expressing glial cells and it is likely that defects in these glial cells underlie both the coloboma and axon guidance defects observed in the eyes of *noi/pax2* mutant fish and mice. *net1a* and *net1b* are expressed in similar cells to Noi around the choroid fissure and expression of these genes is severely reduced in *noi* mutants suggesting that guidance of axons out of the eye is disrupted due to compromised Netrin signalling. This possibility gains support from analysis of mice in which Netrin activity is compromised and axons do indeed have problems exiting the eye (Deiner et al., 1997). It is surprising that *aus* mutants, in which both *noi* expression and *net* expression are expanded throughout the ventral retina, exhibit phenotypes resembling the loss of function of these genes. However, it may be essential to correctly localise Netrin guidance cues at the optic nerve head and the likely disorganisation of such cues in *aus* mutants may underlie the fasciculation defects.

aus function in the midbrain

The *aus* mutation leads to upregulation of *ace* and, to a lesser extent, *noi* in the midbrain and from analysis of embryos carrying mutations in *noi* and *ace*, it is known that both these genes are required for correct development of the midbrain and cerebellum (Brand et al., 1996). En genes are also important regulators of midbrain and cerebellar development, and it is believed that their graded activity contributes to the polarity of this region of the CNS (Itasaki and Nakamura, 1996). In the absence of Noi function, En expression is reduced or absent (Brand et al., 1996; Lun and Brand, 1998), a result consistent with the presence of two essential Pax protein binding sites in the mouse En-2 gene (Song et al., 1996). En expression is also eventually lost in *ace* mutant embryos supporting ectopic expression studies in mice and chicks which have shown that ectopic Fgf8 can induce En expression (Crossley et al., 1996; Brand et al., 1996; Lee et al., 1997; Reifers et al., 1998). From these results, it appears that Ace/Fgf8 and Noi/Pax2/5/8 are upstream of En genes in the midbrain and this provides a likely explanation of why En expression is enhanced in *aus* mutants.

Wnt1 is also required for development of this region of the CNS and this gene is also upregulated in some *aus* mutants. *Wnt1* is required for survival of En-expressing cells in the midbrain (McMahon et al., 1992) and it is believed that it may also be involved in the regulation of *fgf8* expression in the rostral metencephalon (Lee et al., 1997). Although these studies suggest that Wnt1 is upstream of *fgf8*, it is also true that ectopic Fgf8 can induce *wnt1* (Crossley et al., 1996). Therefore, it is again possible that the increased *wnt1* expression of *aus* mutants could be due to increased Ace activity. One surprising observation was that *wnt1* expression also appeared to be increased in the rostral hindbrain of *aus* mutants. While this could be an independent effect of the *aus* mutation, Reifers et al. (1998) have recently shown that the rostral hindbrain is the initial site of *ace* expression during gastrulation.

In addition to the midbrain alterations in *aus* mutants, we also observed a reduction or absence of transcripts of several genes expressed in the pretectal region of the diencephalon. As yet we do not know if this is due to *aus* having activity in the pretectum or alternatively whether it is secondary to the effects in the midbrain. However, changes in pretectal gene expression are observed in *noi* mutant embryos (M. Brand and others, personal communication), raising the possibility that alterations in midbrain patterning could affect the caudal diencephalon.

aus is unlikely to be a mutation in the *ace* gene

The observation that *aus* is a dominant mutation in which *ace* is overexpressed at many of its sites of expression raised the possibility that *aus* could be a gain-of-function allele of *ace*. However, crosses between a carrier of *ace* and *aus* and a carrier of *ace* alone generate embryos that exhibit the mutant phenotypes for both *aus* and *ace* suggesting that *aus* is not tightly linked to *ace.* For a single embryo from such a cross to exhibit both phenotypes, one could propose that transheterozygous *aus/ace* embryos exhibit the double mutant phenotype. This is not the case, however, as crosses between a heterozygous carrier of *ace* and a heterozygous carrier of *aus* never generate the double mutant phenotype. Thus one can conclude that embryos exhibiting both phenotypes must carry two ace^{ti282a} alleles and at least one *aus* allele. If *aus* is a translocation allele of the *ace* gene, then it is indeed possible that single embryos could be $ace^{ti282a}/ace^{ti282a}$ and $ace^{aus}/+$. For this genotype to generate the observed double mutant phenotype, one would have to propose that expression of *ace* from the translocated *aus* allele is unable to rescue the midbrain phenotype but is sufficient to cause the *aus* forebrain phenotype. Furthermore, if *aus* represented a balanced translocation of the *ace* gene, then some embryos from a cross between two carriers of *aus* should inherit both deletion chromosomes, lack both copies of the *ace* gene and have no expression of *ace* RNA. A loss of *ace* expression was never observed in such crosses. While we cannot completely exclude the possibility of a translocation of the *ace* gene itself, a more parsimonious explanation of our results is simply that *aus* is not an allele of *ace*. In this scenario, double mutant embryos exhibit the *aus* phenotype in combination with the loss of function of Ace. The weaker expressivity of the *aus* phenotype in such double mutants is entirely consistent with the *aus* phenotype being partly dependent upon functional Ace protein.

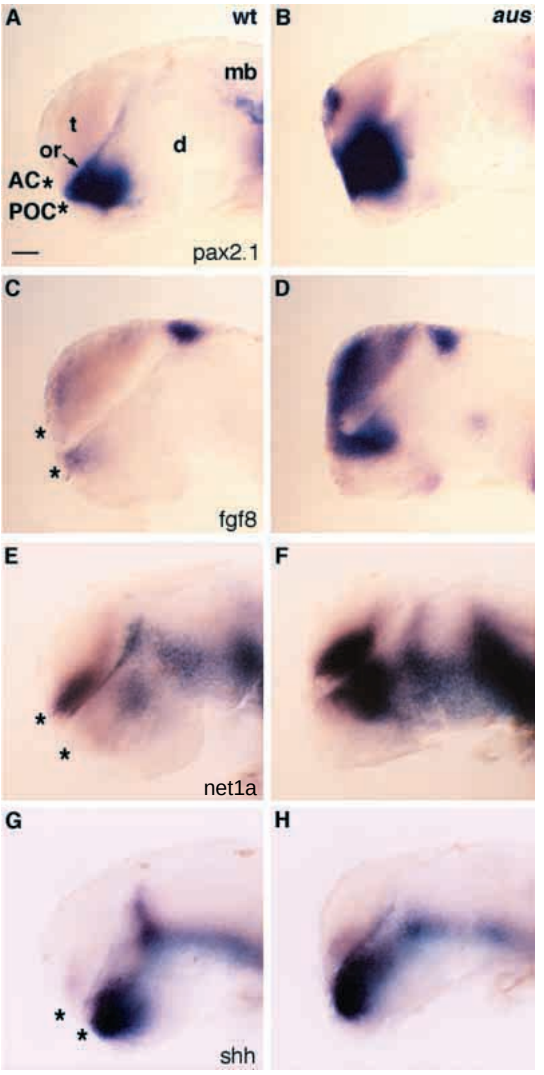

Fig. 7. Midline gene expression is expanded in *aus* mutant embryos. Lateral views of gene expression in wild-type (A,C,E,G) and *aus* mutant (B,D,F,H) dissected prim-12 stage brains with rostral to the left. The asterisks indicate the positions of the anterior and postoptic commissures. (A,B) *noi/pax2.1*. Expression around the optic recess is expanded and there is ectopic expression within the telencephalon of the *aus* mutant embryo. (C,D) *ace/fgf8*. Expression is upregulated throughout much of the rostral/dorsal forebrain of the *aus* mutant embryo. (E,F) *net1a*. Expression is upregulated in the *aus* mutant embryo, particularly around the optic recess. (G,H) *shh*. Expression domains are slightly disrupted in the *aus* mutant but generally similar to the wild-type embryo. Abbreviations: AC, anterior commissure; d, diencephalon; mb, midbrain; POC, postoptic commissure; t, telencephalon; wt, wild type. Scale bar: 30 μm.

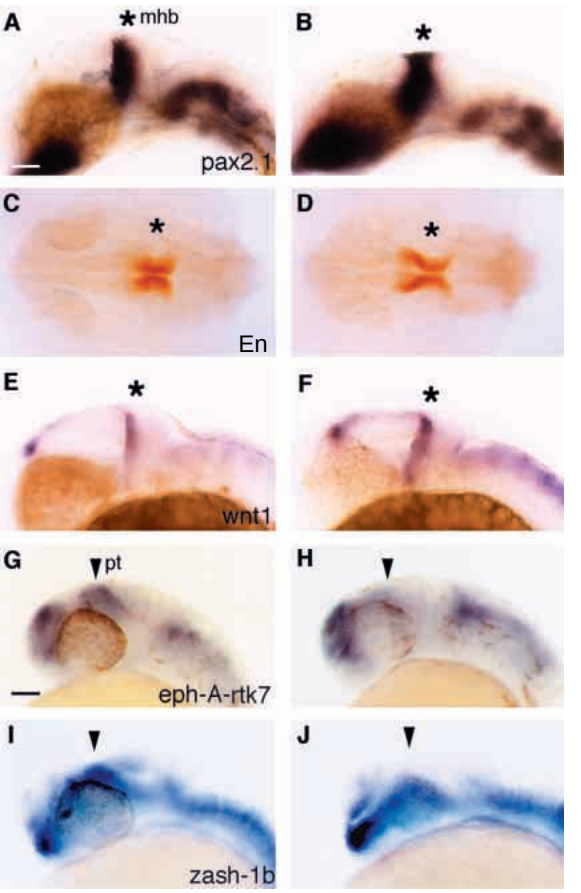

Fig. 8. Midbrain and pretectal gene expression is altered in *aus* mutant embryos. Lateral (A,B,E-J) and dorsal (C,D) views of prim-12 stage wild-type (A,C,E,G,I) and *aus* mutant (B,D,F,H,J) embryos. (A,B) *noi* (*pax2.1*). Expression is slightly expanded in the midbrain of the *aus* mutant embryo, particularly in ventral regions. (C,D) En. Expression appears expanded in the midbrain of the *aus* mutant embryo. (E,F) *wnt1*. Levels of transcripts are enhanced in the caudal diencephalon and mhb of the *aus* mutant embryo. Expression also appears to be increased in the dorsal hindbrain. (G,H) *eph-A-rtk7*. Pretectal expression (arrowhead) of *eph-A-rtk7* is reduced in the *aus* mutant embryo. (I,J) *zash-1b*. Pretectal expression (arrowhead) of *zash-1b* is reduced in the *aus* mutant embryo. Abbreviations: mhb, midbrain/hindbrain boundary (*); pt, pretectum; wt, wild type. Scale bar: 50 μm (A-F); 60 μm (G-J).

Given the arguments stated above, we believe that *aus* is most likely to involve a locus that is normally required for transcriptional regulation of the *ace* gene. One of the simplest ways in which one might envisage *aus* affecting *ace* expression at all of its sites of expression is if Ace activity normally modulates its own expression and that the *aus* locus is normally involved in this autoregulation. A mutation involving the *aus* locus would interfere with this feedback and might lead to deregulation of *ace* expression. Analysis of *ace* mutant embryos supports the idea that Ace is indeed involved in a feedback loop to regulate its own transcription (Reifers et al., 1998; R. Macdonald, M. Brand and S. W. W., unpublished observations). Although clearly important, *ace* is not the only gene upon which *aus* must act, as embryos lacking Ace activity still exhibit aspects of the *aus* phenotype indicating that *aus* must affect some genes via an Ace-independent route.

noi expression in the forebrain is less severely affected in *aus* mutants that lack functional Ace indicating that much of the upregulation of *noi* is dependent upon Ace activity. This dependence upon Ace may also explain why *noi* is only upregulated at sites where its expression overlaps with *ace* and not in regions of the embryo where the two genes are likely to be independent of each other's activity (such as spinal neurons and pronephros). Conversely, the increase in *ace* expression in the forebrain does not appear to be dependent upon functional Noi suggesting that *ace* is upstream to *noi* in the forebrain of

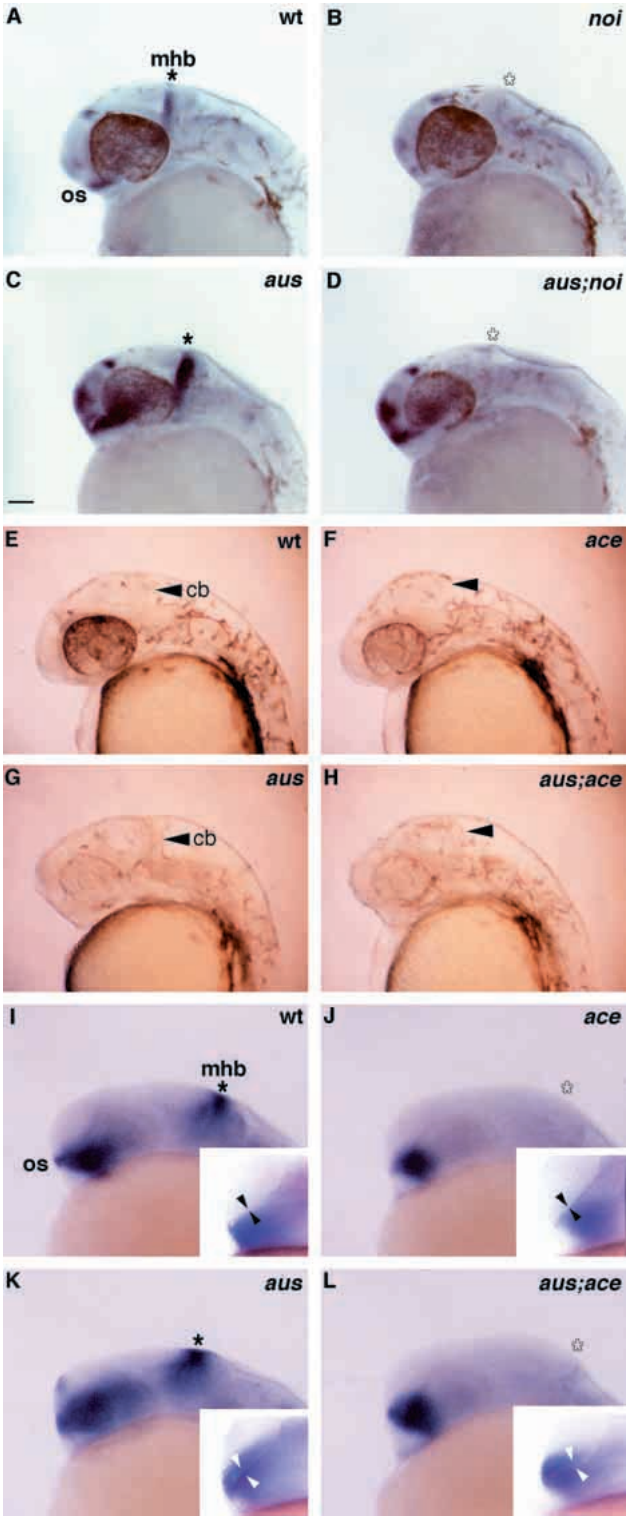

Fig. 9. *aus* is unlikely to represent a mutation in the *ace* or *noi* genes. Lateral views of prim-12 (A-H) and prim-5 stage (I-L) embryos with rostral to the left. Asterisks indicate the position of the mhb. (A-D) Analysis of *ace* expression in wild-type (A), *noi* (B), *aus* (C) and *aus;noi* double mutant (D) embryos. *ace* expression is absent at the mhb in the absence of functional Noi and is upregulated in the forebrain both in *aus* and *aus;noi* double mutant embryos. (E-H) Appearance of wild-type (E), *ace* (F), *aus* (G) and *aus;ace* double mutant (H) embryos. In the *aus;ace* double mutant, the rostral brain looks similar to the *aus* phenotype while the absence of cerebellum (arrowheads) is characteristic of the *ace* phenotype. (I-L) Analysis of *noi* expression in wild-type (I), *ace* (J), *aus* (K) and *aus;ace* double mutant (L) embryos. *noi* expression is absent at the mhb in the absence of functional Ace. The *aus* dependent upregulation of *noi* in the eyes and forebrain (K) is much reduced in the absence of functional Ace (L). The inset panels in I-L show the width of the optic recess (arrowheads) used as a landmark to infer the genotype the embryos – phenotypic morphological differences are much more visible in living embryos prior to the in situ protocol (see E-H). Abbreviations: cb, cerebellum; mhb, midbrain/hindbrain boundary; os, optic stalk. Scale bar: 50 μm.

aus mutants. However, *noi* expression is not lost altogether in the forebrain of *ace* mutants or *ace;aus* double mutants indicating that, while Ace is required for expansion of *noi* expression in the forebrain of *aus* mutants, it is not required for induction of *noi* expression.

The relationship between *noi* and *ace* in the midbrain is likely to be different to that in the forebrain. In this region, *ace* expression is lost in the absence of Noi activity and *noi* expression is lost in the absence of Ace activity (Reifers et al., 1998) suggesting mutual dependence. However, Reifers et al. (1998) have shown that the two genes are initially activated independent of each other and the late loss of expression could be due to cell fate alterations in the mutant embryos.

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