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Regionalisation of anterior neuroectoderm and its competence in responding to forebrain and midbrain inducing activities depend on mutual antagonism between OTX2 and GBX2

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SUMMARY

The anterior neural ridge (ANR), and the isthmic organiser (IsO) represent two signalling centres possessing organising properties necessary for forebrain (ANR) as well as midbrain and rostral hindbrain (IsO) development. An important mediator of ANR and IsO organising property is the signalling molecule FGF8. Previous work has indicated that correct positioning of the IsO and *Fgf8* expression in this domain is controlled by the transcription factors *Otx2* and *Gbx2*. In order to provide novel insights into the roles of *Otx2* and *Gbx2*, we have studied mutant embryos carrying different dosages of *Otx2*, *Otx1* and *Gbx2*. Embryos deficient for both OTX2 and GBX2 proteins (*hOtx1^{2}/hOtx1^{2}; Gbx2$^{-/-}$*) show abnormal patterning of the anterior neural tissue, which is evident at the presomite-early somite stage prior to the onset of *Fgf8* neuroectodermal expression. Indeed, *hOtx1^{2}/hOtx1^{2}; Gbx2$^{-/-}$* embryos exhibit broad co-expression of early forebrain, midbrain and rostral hindbrain markers such as *hOtx1*, *Gbx2*, *Pax2*, *En1* and *Wnt1* and subsequently fail to activate forebrain and midbrain-specific gene expression. In this genetic context, *Fgf8* is expressed throughout the entire anterior neural plate, thus indicating that its activation is independent of both OTX2 and GBX2 function. Analysis of *hOtx1^{2}/hOtx1^{2}; Gbx2$^{-/-}$* and *Otx1$^{+/-}$; Otx2$^{+/-}$* mutant embryos also suggests that FGF8 cannot repress *Otx2* without the participation of GBX2. Finally, we report that embryos carrying a single strong hypomorphic *Otx2* allele *(Otx2$^{\lambda}$)* in an *Otx2* and *Gbx2* null background (*Otx2$^{\lambda}$/–; Gbx2$^{-/-}$*) recover both the headless phenotype exhibited by *Otx2$^{\lambda}$/–* embryos and forebrain- and midbrain-specific gene expression that is not observed in *hOtx1^{2}/hOtx1^{2}; Gbx2$^{-/-}$* mutants. Together, these data provide novel genetic evidence indicating that OTX2 and GBX2 are required for proper segregation of early regional identities anterior and posterior to the mid-hindbrain boundary (MHB) and for conferring competence to the anterior neuroectoderm in responding to forebrain-, midbrain- and rostral hindbrain-inducing activities.

Key words: *Otx2*, *Gbx2*, Forebrain, Midbrain, Rostral hindbrain, Regionalisation, Competence, Isthmic organiser, Mouse

INTRODUCTION

Patterning of the vertebrate neural plate is dependent upon signals produced by discrete organising centres. In mouse, signals from the anterior visceral endoderm (AVE) and the node and its derivatives are responsible for the initial induction and early maintenance of anterior patterning (Beddington and Robertson, 1999; Stern, 2001). Subsequently, maintenance and refinement of regionally restricted identities is believed to occur through the formation of compartments where positional identity is maintained by a polyclonal cell population with restricted cell lineages (Lumsden, 1990; Figdor and Stern, 1993; Lumsden and Krumlauf, 1996). Local organising centres with polarising and inductive properties develop within the broadly regionalised neuroectoderm in genetically defined positions and operate to refine local identities (Meinhardt, 1983; Rubenstein et al., 1998; Joyner et al., 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Two signalling centres have been so far identified and correspond to the anterior neural ridge (ANR), at the junction between the most anterior neural plate and the non-neural ectoderm (Shimamura and Rubenstein, 1997; Houart et al., 1998) and the isthmic organiser (IsO), which develops within the neural plate at the mid-hindbrain boundary (MHB) (Martinez et al., 1991). Among other signalling molecules, both centres express *Fgf8*.

In mouse, embryological and genetic evidence suggests that the ANR and *Fgf8* expression in this domain are important for forebrain development (Shimamura and Rubenstein, 1997; Meyers et al., 1998; Ye et al., 1998).

A remarkable amount of data has been collected on the

morphogenetic properties of the IsO and molecules involved in its development. Midbrain and cerebellum-inducing activity that characterises the IsO has hitherto been demonstrated only for FGF8 (Alvarado-Mallart et al., 1990; Martinez et al., 1991; Crossley et al., 1996; Puelles et al., 1996; Martinez et al., 1999). Therefore, the FGF8 molecule is capable of inducing rostral forebrain restricted (ANR) or midbrain- and rostral hindbrain-specific (IsO) gene expression, suggesting the existence of a differential territorial competence in responding to the same signal.

In mouse, by the end of gastrulation, *Otx2* is expressed along the presumptive fore- and midbrain region, with a sharp posterior border adjacent to the anterior border of the *Gbx2* expression domain, which, in turn, defines the prospective anterior hindbrain (Wassarman et al., 1997). Subsequently, at somitogenesis, the transcription factors *En1*, *Pax2*, *Pax5* and *Pax8* and the signalling molecules *Wnt1* and *Fgf8* are transcribed in broad domains across the *Otx2*/*Gbx2* border. Later in development, their expression domains sharpen and refine around the MHB. Specifically, *Wnt1* and *Fgf8* are expressed in two narrow rings within the *Otx2* and *Gbx2* expression domains, respectively, thus defining the anterior and posterior border of the MHB, whilst *En1*, *Pax2*, *Pax5* and *Pax8* are expressed in a wider domain encompassing the MHB as well as the caudal midbrain and rostral hindbrain (Joyner et al., 2000; Simeone, 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Transplantation and FGF8-soaked bead experiments as well as genetic studies in mouse and zebrafish have provided insights into the function and interactions of these molecules in IsO development.

Altogether, these previous studies have indicated that maintenance of IsO activity and transduction of its inducing properties require a positive loop involving *Fgf8*, *Wnt1*, *En1* and *Pax* genes, whilst positioning of the IsO is defined by negative interactions between *Otx2* and *Gbx2* (Joyner et al., 2000; Simeone, 2000; Garda et al., 2001; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Nevertheless, some important questions have not yet been fully addressed. Among these, it is still unknown: (i) how the anterior neural plate develops and whether it retains the ability to express *Fgf8* in the absence of both OTX2 and GBX2 function; and, more importantly, (ii) whether *Otx2* and *Gbx2* are key transcription factors in conferring territorial competence in responding to morphogenetic signals required for regionalisation of the anterior neural plate. The study of these aspects may also provide new insights into the hierarchy of genetic and molecular interactions controlling IsO development.

In order to address these questions, we took advantage of three different genetic combinations expressing different levels of OTX2 and GBX2 proteins. In particular, we have studied the development of the anterior neural plate and the expression of a number of diagnostic markers for the IsO, forebrain and midbrain regions in $hOtx1^{2}/hOtx1^{2}$; $Gbx2^{-/-}$, $Otx1^{+/-}$; $Otx2^{+/-}$ and $Otx2^{\lambda}/-$; $Gbx2^{-/-}$ double mutants. $hOtx1^{2}$ mice were generated by replacing the *Otx2* locus with the human *Otx1* cDNA (*hOtx1)* (Acampora et al., 1998). In these mutants, *hOtx1* transcription was under the control of the *Otx2* promoter. $hOtx1^{2}/hOtx1^{2}$ homozygous mutants recover the gastrulation defects of the $Otx2^{-/-}$ mutants, but subsequently, at 8.5 days post coitum (d.p.c.), exhibit lack of the fore- and midbrain regions due to the absence of OTX2 protein in the anterior neuroectoderm. The $Otx2^{\lambda}$ allele was generated by inserting 300 bp of the λ phage DNA into the 3′ untranslated region (UTR) of the *Otx2* locus (Pilo Boyl et al., 2001). In $Otx2^{\lambda}$ mutants OTX2 protein level was drastically reduced in the epiblast and epiblast-derivatives. When the $Otx2^{\lambda}$ was combined with an *Otx2* null allele ($Otx2^{\lambda}/-$), OTX2 protein level in the anterior neuroctoderm was decreased by up to 20% of that seen in wild-type embryos and $Otx2^{\lambda}/-$ embryos showed an almost complete head-less phenotype by 9 d.p.c. onwards (Pilo Boyl et al., 2001a).

Here we report that OTX2 and GBX2 proteins are not only required for positioning the IsO, but importantly, they are required for early segregation of forebrain, midbrain and rostral hindbrain identities and, possibly, for conferring territorial competence to the neuroectoderm in responding to forebrain and midbrain inducing activity. At the molecular level, these findings also indicate that (i) neither OTX2 nor GBX2 function is required for initiation of *Fgf8* expression; (ii) FGF8 is unable to repress *Otx2* expression without the contribution of GBX2; (iii) GBX2 activity is not required for ectopic expression of *Fgf8* throughout the midbrain of embryos lacking OTX2 or exhibiting low level of OTX1 and OTX2 proteins, and (iv) *hOtx1* and *Gbx2* abnormal expression observed in $hOtx1^{2}$ and *Gbx2* single and double mutants is not dependent on *Fgf8* expression and may be due to OTX2/GBX2 negative interactions.

MATERIALS AND METHODS

Generation and genotyping of mice

$hOtx1^{2}/hOtx1^{2}$; $Gbx2^{-/-}$ embryos were generated by intercrossing $hOtx1^{2}/Otx2$; $Gbx2^{+/-}$ mice (Wassarman et al., 1997; Acampora et al., 1998); $Otx1^{+/-}$; $Otx2^{+/-}$ embryos were generated by crossing $Otx1^{+/-}$; $Otx2^{+/-}$ males with $Otx1^{+/-}$ females (Acampora et al., 1997); $Otx2^{\lambda}/-$; $Gbx2^{-/-}$ embryos were generated by crossing $Otx2^{\lambda}/Otx2$; $Gbx2^{+/-}$ with $Otx2^{+/-}$; $Gbx2^{+/-}$ mice (Acampora et al., 1995; Wassarman et al., 1997; Pilo Boyl et al., 2001). Genotypes were determined by PCR as previously described (Acampora et al., 1995; Acampora et al., 1997; Acampora et al., 1998; Wassarman et al., 1997; Pilo Boyl et al., 2001).

In situ hybridisation and probes

In situ hybridisation experiments on sections and whole embryos were performed as previously described (Hogan et al., 1994; Thomas and Beddington, 1996; Simeone, 1999). *hOtx1, Fgf8, Otx2, Pax2, En1, Wnt1, Bf1* and *Six3* probes were the same as previously described (Acampora et al., 1997; Acampora et al., 1998).

The *Gbx2* probe employed for the wild-type and the *Gbx2* null allele was a PCR fragment 836 bp long that included 361 bp upstream and 475 bp downstream the ATG. The *Atx* probe is a cDNA fragment 849 bp long including the region between the ATG and the amino acid 283 downstream of the homeodomain.

RESULTS

Genetic and morphological analysis of $hOtx1^{2}/hOtx1^{2}$; $Gbx2^{-/-}$ embryos

$hOtx1^{2}/hOtx1^{2}$; $Gbx2^{-/-}$ embryos were generated by crossing $hOtx1^{2}/Otx2$; $Gbx2^{+/-}$ double heterozygous mice, which were viable and fertile. Parental $hOtx1^{2}/Otx2$ and $Gbx2^{+/-}$ were

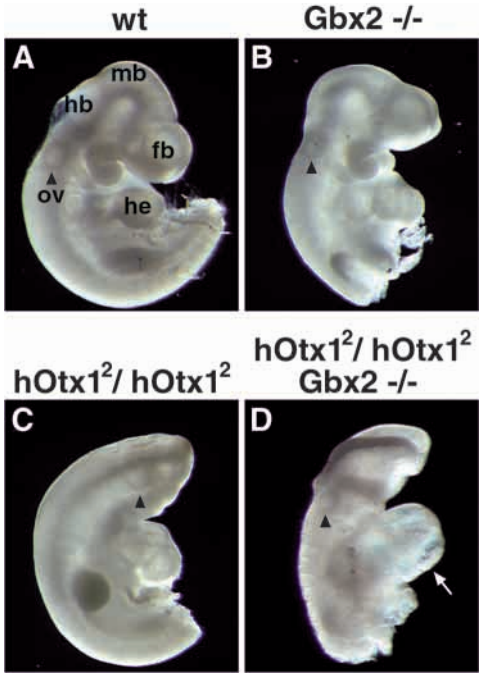

Fig. 1. Morphology of a *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryo at 9.7 d.p.c. (A-D) Compared with wild-type (A) and *Gbx2*$^{-/-}$ (B) embryos, *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ (D) mutants exhibit severe morphological abnormalities affecting the anterior neuroectoderm and the heart (arrow); however, compared with *hOtx1²/hOtx1²* embryos (C), it is evident that the distance between the otic vesicle (arrowhead in C,D) and the rostral tip of the embryo is increased in *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ mutants. fb, forebrain; mb, midbrain; hb, hindbrain; ov and arrowheads, otic vesicle.

generated and maintained on the same genetic background (C57 BL6/DBA2) (Wassarman et al., 1997; Acampora et al., 1998).

Genotypic analysis of embryos collected between 8.5 and 9.5 d.p.c. showed that *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ mutant embryos were generated approximately at the expected Mendelian frequency (Table 1). However, when the progeny of double heterozygous intercrosses was analysed at 10 –10.5 d.p.c. no double *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ homozygous embryos were found (n=84). Therefore the *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ mutant was embryonic lethal by 10 d.p.c.

At 9.75 d.p.c. the morphology of the anterior neural plate in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ mutants (Fig. 1D) was different from that of *hOtx1²/hOtx1²* embryos (Fig. 1C). Indeed, in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos the distance between the otic vesicle and the rostral tip of the embryo appeared greater (arrowhead in Fig. 1C,D). However, abnormalities affecting the neural tube, branchial arches and heart and an overall reduction of the body size were detected in double mutant embryos. The cranial neural folds were wide-open and failed to close along the dorsal midline (exencephaly). Telencephalic and optic vesicles, as well as the isthmic constriction, were not recognisable, and non-neural anterior structures, such as the branchial arches, olfactory and optic placodes, were absent (Fig. 1D). The heart was dilated and displayed an abnormal morphology. Since exencephalic embryos can develop until late gestation, we suspected that the heart defect was the most likely reason for the embryonic lethality observed in the *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ by 10 d.p.c.

Neural patterning in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos

Previous data indicated that *hOtx1²/hOtx1²* embryos showed no detectable OTX protein in the neuroectoderm and failed to maintain forebrain and midbrain identity. This was revealed by absence of the fore- and midbrain markers *hOtx1* and *Bf1* in the anterior neural plate, and a shift of the MHB markers *Gbx2*, *Fgf8*, *Pax2*, *En1* and *Wnt1* to the rostral tip of the neuroectoderm in these mutants (Fig. 2H-N) (Acampora et al., 1998). Conversely, *Gbx2*$^{-/-}$ embryos lacked the anterior hindbrain and displayed an abnormal posterior expansion of the midbrain preceded by the caudalisation of *Otx2*, *Gbx2*, *Fgf8, Pax2, En1* and *Wnt1* expression domains (Fig. 2O-T) (Wassarman et al., 1997). Forebrain and *Bf1* expression were unaffected in these mutants (Fig. 2U).

Expression of the *hOtx1* and *Gbx2* transcripts in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos was compared with that of *hOtx1²/hOtx1²* and *Gbx2*$^{-/-}$ single mutants at 8.7 d.p.c. In double mutant embryos, *hOtx1* transcripts were abundantly detected along the anterior neural plate with a caudal border approximately corresponding to the caudal limit of *Otx2* expression in the *Gbx2*$^{-/-}$ mutant littermates (compare Fig. 2V,V′ to O). In *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ mutants, *Gbx2* expression was detected along the anterior neural plate (Fig. 2W,W′), thus indicating that *Gbx2* and *hOtx1* were co-expressed in these mutants. Notably, this expression analysis revealed the presence of a graded and opposite decrease in the expression of *hOtx1* posteriorly, and *Gbx2* anteriorly (Fig. 2V,V′,W,W′). This suggests that factor(s), other than *Otx2* and *Gbx2* might be active in antagonising anteriorly and posteriorly the full spread of *Gbx2* and *Otx2* expression, respectively. *Fgf8* was actively transcribed throughout the anterior neural plate in the same domain that expressed *hOtx1* and *Gbx2* transcripts

Table 1. Frequency of genotypes observed by intercrossing *hOtx1²/Otx2; Gbx2*$^{+/-}$ mice

Genotypes	Number of embryos	Expected frequency (%)	Observed frequency (%)
hOtx1²/hOtx1²; *Gbx2*$^{-/-}$	14	6.2	4.9
hOtx1²/hOtx1²; *Gbx2*$^{+/-}$	24	12.5	8.4
hOtx1²/Otx2; *Gbx2*$^{-/-}$	30	12.5	10.5
hOtx1²/Otx2; *Gbx2*$^{+/-}$	74	25	26
hOtx1²/hOtx1²; *Gbx2*$^{+/+}$	21	6.2	7.3
hOtx1²/Otx2; *Gbx2*$^{+/+}$	44	12.5	15.4
Otx2/Otx2; *Gbx2*$^{-/-}$	14	6.2	4.9
Otx2/Otx2; *Gbx2*$^{+/-}$	42	12.5	14.7
Otx2/Otx2; *Gbx2*$^{+/+}$	22	6.2	7.7
Total	285		

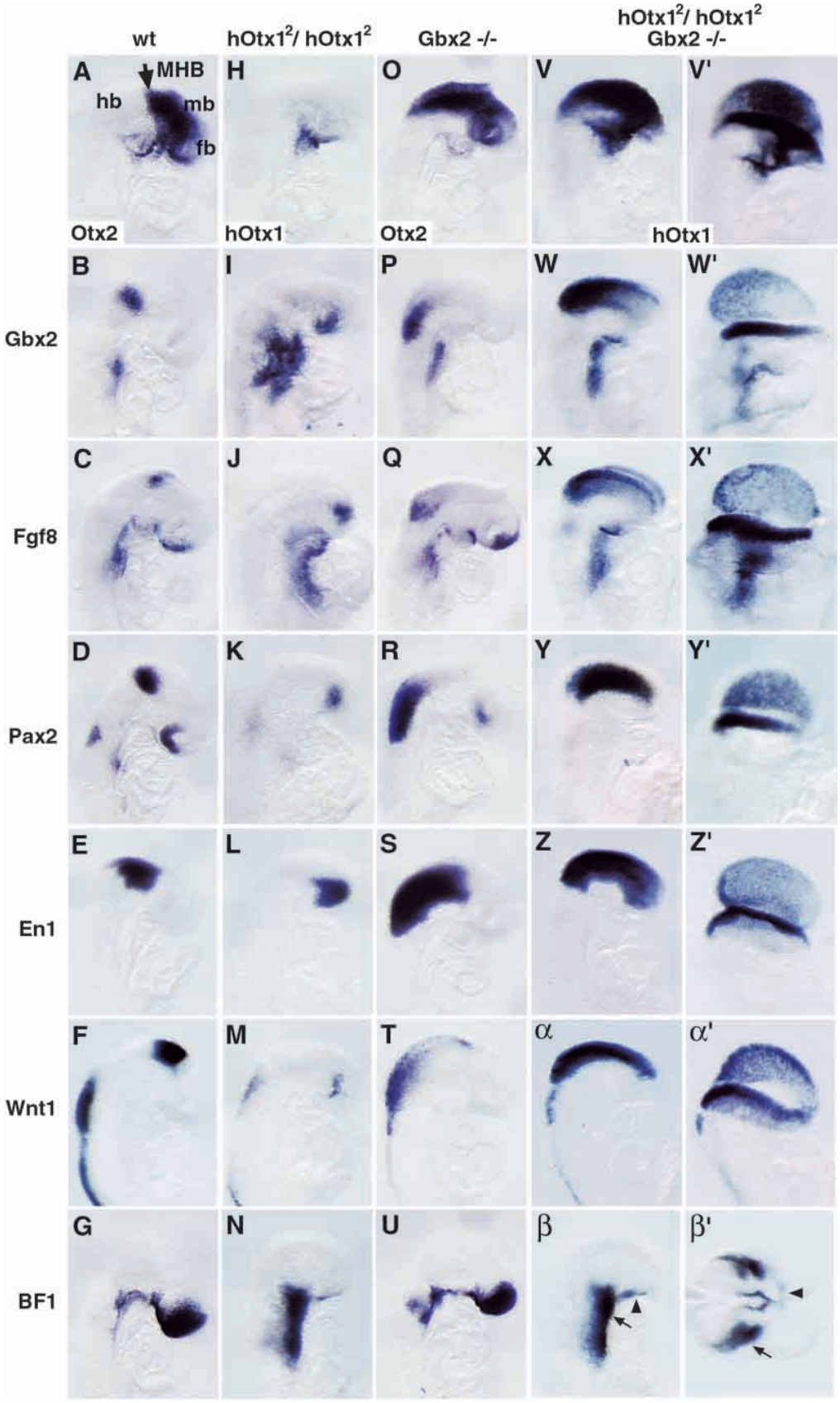

Fig. 2. Neural patterning in *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ embryos. Whole-mount in situ hybridisation of 8.7 d.p.c. wild-type (A-G), *hOtx1²/hOtx1²* (H-N), *Gbx2*$^{-/-}$ (O-U) and *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ (V-β′) embryos with *Otx2* (A,O), *hOtx1* (H,V,V′), *Gbx2* (B,I,P,W,W′), *Fgf8* (C,J,Q,X,X′), *Pax2* (D,K,R,Y,Y′), *En1* (E,L,S,Z,Z′), *Wnt1* (F,M,T,α,α′) and *Bf1* (G,N,U,β,β′) probes shows that *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos exhibit widespread expression of *hOtx1, Gbx2, Fgf8, Pax2, En1* and *Wnt1* genes whilst the forebrain specific gene *Bf1* is not transcribed in the anterior neuroectoderm. A-β are sagittal and V′-β′ are dorsolateral views. Abbreviations as in previous figure plus, MHB, midbrain-hindbrain boundary. The arrow and the arrowhead in β-β′ indicate the corresponding regions in non-neural tissue.

(Fig. 2X,X′). This indicates that absence of both OTX2 and GBX2 functions does not prevent *Fgf8* activation, rather it affects the restriction of *Fgf8* expression at the MHB. It is important to note that in the *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos FGF8 was unable to repress *hOtx1 (Otx2)* expression, as both genes were co-expressed in the anterior neural plate of the double mutants (see below).

Expression of other neural markers, such as *Pax2*, *En1*, *Wnt1*, *Bf1* and *Six3* was also analysed in the *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ double mutants. In *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos, *Pax2*, *En1* and *Wnt1* transcripts were broadly co-expressed with *hOtx1*, *Gbx2* and *Fgf8* along the anterior neural plate (compare Fig. 2V-X′ with Y-α′). Notably, *Pax2* was not transcribed along the lateral/dorsal edge of the neural plate (Fig. 2Y,Y′). At this stage, the forebrain marker *Bf1* was expressed normally in *Gbx2*$^{-/-}$ mutants, but undetectable in *hOtx1²/hOtx1²* homozygous embryos (Fig. 2N,U). No neural expression of *Bf1* was

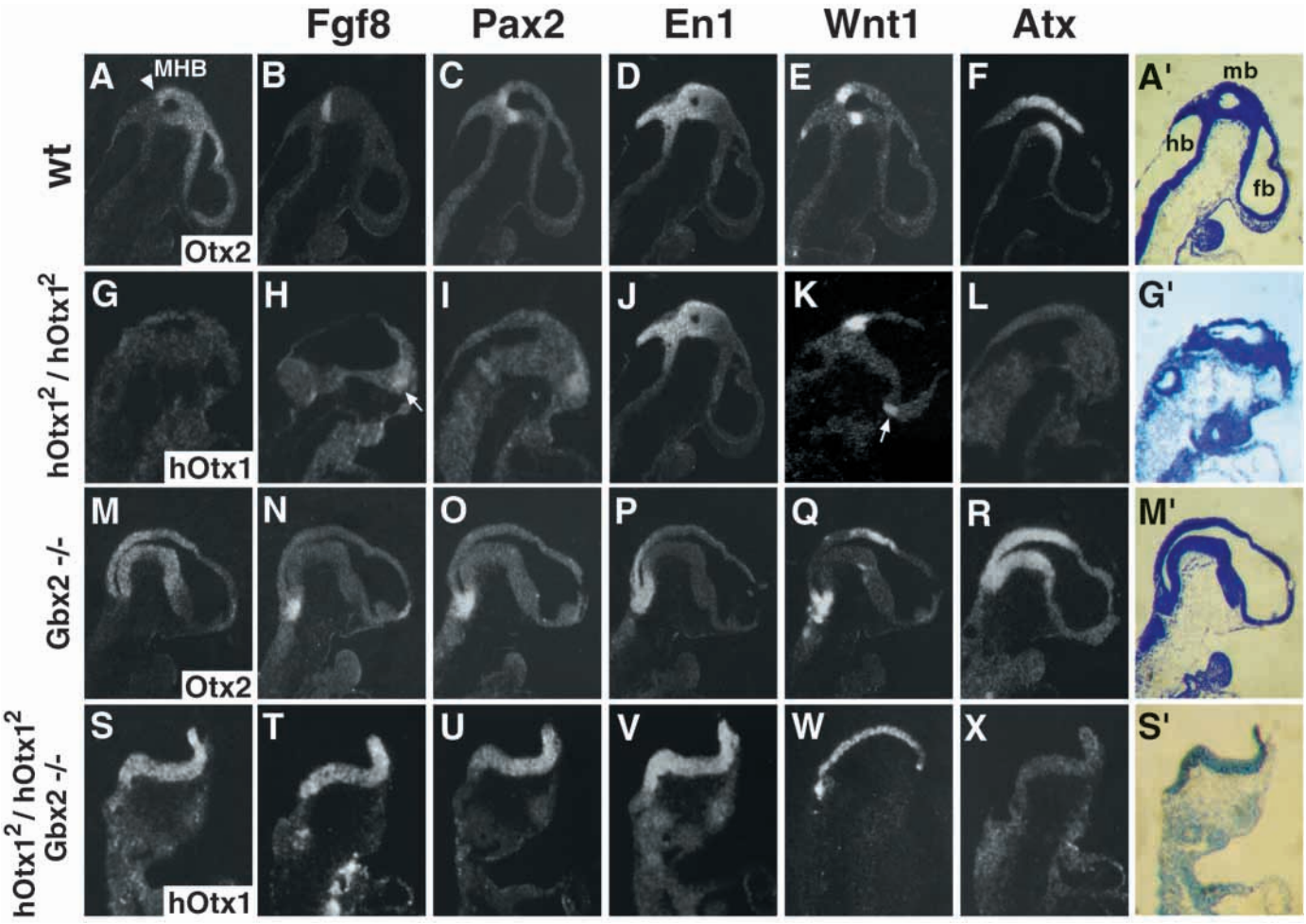

Fig. 3. Failure in regionalisation of the anterior neural plate in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos. (A-X) In situ hybridisation of sagittal sections of 9.7 d.p.c. wild-type (A-F), *hOtx1²/hOtx1²* (G-L), *Gbx2*$^{-/-}$ (M-R) and *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ (S-X) embryos, with *Otx2* (A,M), *hOtx1* (G,S), *Fgf8* (B,H,N,T), *Pax2* (C,I,O,U), *En1* (D,J,P,V), *Wnt1* (E,K,Q,W) and *Atx* (F,L,R,X) probes reveals that in *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ embryos widespread co-expression of *hOtx1, Fgf8, Pax2, En1* and *Wnt1* is maintained and that the midbrain-specific gene *Atx* is not transcribed. Abbreviations as in previous figures. A′,G′,M′,S′ are bright-field images of the sections I A,G,M and S.

detectable in three *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ mutants analysed (Fig. 2β,β′).

Neural patterning in *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ embryos was also analysed at 9.7 d.p.c. Compared with wild-type littermates (Fig. 3A-E), *hOtx1, Otx2, Fgf8, Pax2, En1* and *Wnt1* were undetectable (*hOtx1*) (Fig. 3G) or restricted to the rostral tip of the neural tube of *hOtx1²/hOtx1²* mutants (*Fgf8, Pax2, En1* and *Wnt1*) (Fig. 3H-K), but were expanded posteriorly up to the presumptive rhombomere 4 (r4) in *Gbx2*$^{-/-}$ embryos (Fig. 3M-Q) (Wassarman et al., 1997). In *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ embryos these genes were co-expressed along the anterior neural plate (Fig. 3S-W).

Therefore, these data indicate that co-expression of these neural markers was not a transient feature of *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ mutants. At 9.7 d.p.c., *Six3* and *Bf1* expression was not detected in four *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ mutant embryos (data not shown).

An interesting question raised by lack of forebrain-specific markers and the expanded *Fgf8* expression profile was whether, despite the broad co-expression of early mid- and rostral hindbrain markers in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ embryos, midbrain-specific gene expression could be induced in these mutants.

To address this question we studied the expression of *Atx*, a novel homeobox-containing gene that is activated at 8.5 d.p.c. in the presumptive pretectal area and subsequently, between 9 and 9.5 d.p.c., in the midbrain of mouse embryos (R. G., F. S., A. S. and A. L., unpublished data). Indeed, at 9.7 d.p.c., in wild-type embryos, *Atx* transcripts were restricted to the midbrain and pretectum with a caudal limit coincident with the posterior border of *Otx2* expression at the MHB (Fig. 3F) (R. G., F. S., A. S. and A. L., unpublished data). Compared with wt littermates, *Atx* expression was undetectable in *hOtx1²/hOtx1²* homozygous embryos (Fig. 3L) and posteriorly expanded in *Gbx2*$^{-/-}$ mutants (Fig. 3R). Importantly, *Atx* was not expressed in *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ mutant embryos (Fig. 3X).

Altogether this expression analysis suggests that *Otx2* and *Gbx2* are both required for conferring regional identity (either fore and midbrain or rostral hindbrain, respectively) within the anterior neural plate, since absence of both gene products leads the anterior neuroectoderm to adopt a mixed identity.

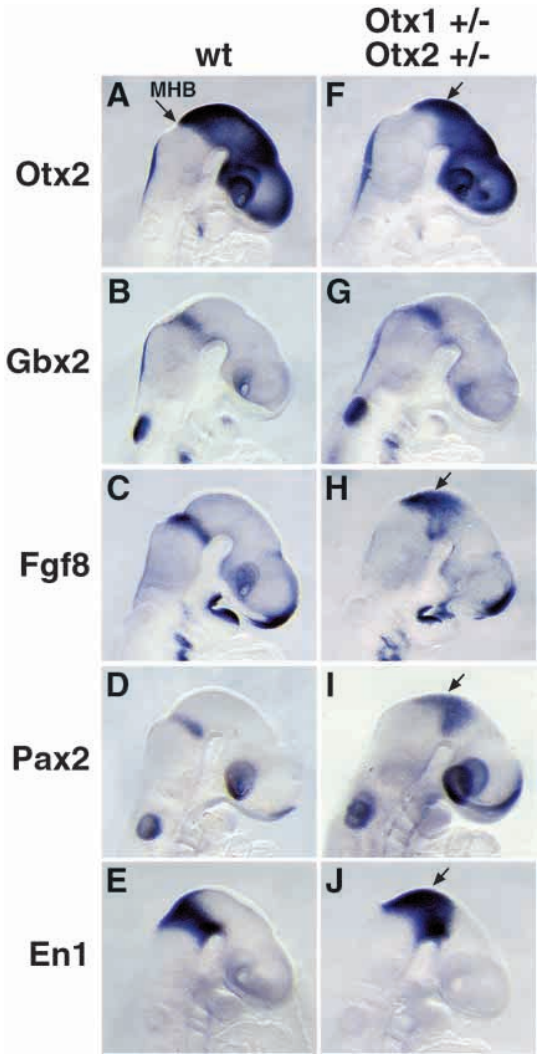

Fig. 4. *Fgf8* is unable to repress *Otx2* in the dorsal midbrain of *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ embryos. (A-J) Whole-mount in situ hybridisation of wild-type (A-E) and *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ (F-J) embryos with *Otx2* (A,F), *Gbx2* (B,G), *Fgf8* (C,H), *Pax2* (D,I) and *En1* (E,J) probes shows that in double heterozygous embryos *Fgf8* (arrow in H), *Pax2* (arrow in I) and *En1* (arrow in J) are ectopically co-expressed in the dorsal midbrain where *Otx2* is also transcribed (arrow in F), whilst the *Gbx2* stripe of expression (G) is only slightly thickened. Abbreviations as in previous figures.

Moreover, our analysis suggests that at least *Otx2* is also required for anterior neuroectoderm to acquire the territorial competence in responding to forebrain and midbrain inducing activities.

This is based primarily on the fact that in the absence of OTX2, the anterior neuroectoderm fails to activate forebrain- (*Bf1*) and midbrain- (*Atx*) specific gene expression (see also below). However, it is possible that *Fgf8* or other signalling molecules may be defective quantitatively or qualitatively at the molecular level and therefore unable to perform their role.

FGF8 inability in repressing *Otx2* expression and differential sensitivity of MHB genes to the OTX2 repressive effect

Our data indicated that FGF8 was unable to repress *hOtx1* transcription and that GBX2 was not required for the expansion of the *Fgf8* expression domain in the *hOtx1*^{2}*/hOtx1*^{2}; *Gbx2*$^{-/-}$ mutants. One possible explanation is that FGF8 could not repress *hOtx1* expression without the contribution of GBX2.

However, it was also possible that the *Fgf8* responding element in the *Otx2* locus had been deleted or mislocated in the *hOtx1*^{2} targeted allele, thus rendering the *hOtx1* transcription unresponsive to FGF8 (Acampora et al., 1998). To test these two possibilities, we analysed *Otx2*, *Gbx2* and *Fgf8* expression in *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ double heterozygous embryos, which retained one copy of the wild-type *Otx2* allele.

At 9.7 d.p.c., *Otx2* was expressed on the mesencephalic side while *Gbx2* and *Fgf8* were expressed on the metencephalic side of the MHB in wild-type embryos (Fig. 4A-C). In *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ embryos, the posterior border of the *Otx2* expression domain was slightly displaced anteriorly in the lateral region of the caudal midbrain (Fig. 4F). In a complementary manner, *Gbx2* expression at the MHB was slightly expanded into the neural tissue deprived of *Otx2* transcripts (Fig. 4G). In contrast, *Fgf8* expression was expanded broadly into most of the dorsal midbrain (Fig. 4H). Therefore, in *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ embryos *Fgf8* and *Otx2* were co-expressed along the dorsal midbrain in a wide region free of *Gbx2* transcripts (arrow in Fig. 4F,H). Later in development, at 10.5 d.p.c. and 12.5 d.p.c., co-expression of *Fgf8* and *Otx2* in a midbrain territory, which did not express *Gbx2*, was confirmed (data not shown). We have previously reported that embryos carrying only one single functional copy of *Otx2* (*Otx1*$^{-/-}$*; Otx2*$^{+/-}$), displayed co-ordinated anterior shift of *Fgf8*, *Gbx2*, *Pax2* and *Wnt1* expression domains and posterior repression of *Otx2* (Acampora et al., 1997). Altogether, these results suggest two important conclusions. Firstly, FGF8 was unable either to repress *Otx2* or to activate ectopically *Gbx2* when the *Otx* gene dosage was above one single functional copy of *Otx2*. Secondly, directly or indirectly, *Fgf8* and *Gbx2* exhibit a differential sensitivity to the *Otx2* repressive effect, *Gbx2* being more sensitive to this effect than *Fgf8*.

Expression of *En1* and *Pax2* was analysed in *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ because it has been shown that they can regulate *Fgf8* expression (Urbanek et al., 1997; Lun and Brand, 1998; Okafugi et al., 1999; Shamim et al., 1999). When compared with wild-type littermates (Fig. 4D,E), *Pax2* (Fig. 4I) and *En1* (Fig. 4J) expression domains were clearly expanded anteriorly into the dorsal midbrain where they colocalised with ectopic *Fgf8* transcripts (arrow in Fig. 4H-J).

Therefore, ectopic expression of *Fgf8* might be explained by a positive effect of PAX2 and/or EN1 transcription factors in activating *Fgf8* transcription in the territory where the OTX protein level is permissive. These data also imply that *Pax2*, *En1* and *Fgf8* exhibit similar sensitivity to the OTX2 repressive effect.

Otx/Gbx2 abnormal expression precedes *Fgf8* activation in *hOtx1*^{2}*/Gbx2* single and double mutants

Previous studies have shown that the anterior shift of *Gbx2* expression and the rostral repression of *hOtx1* transcripts were evident in *hOtx1*^{2}/*hOtx1*^{2} embryos at 8.5 d.p.c. (Acampora et al., 1998). At this stage, it has also been reported that the posterior expansion of *Otx2* expression observed in *Gbx2*$^{-/-}$ embryos correlates with *Gbx2* repression in the rostral hindbrain (r1-r3) (Fig. 2) (Wassarman et al., 1997). However,

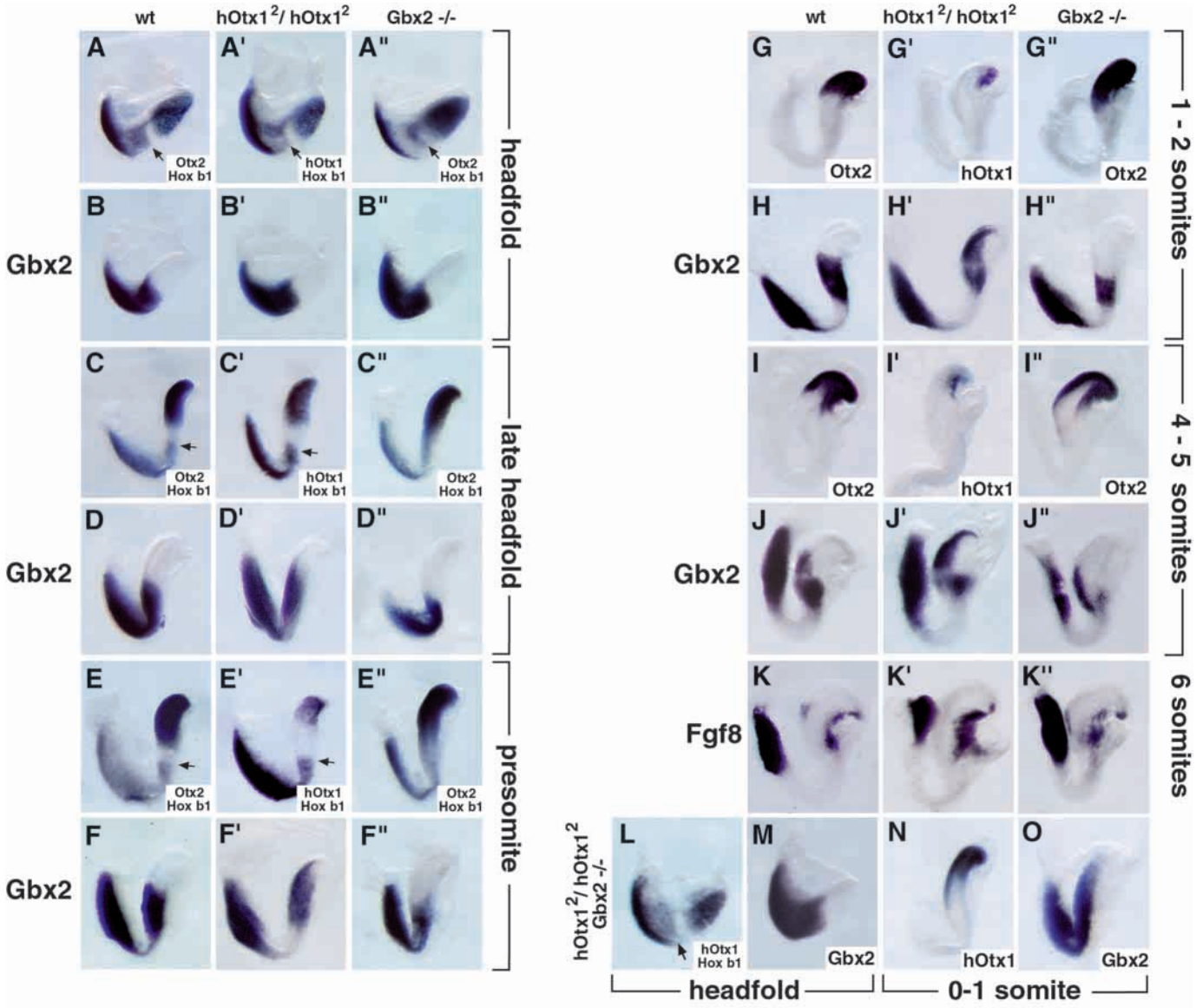

Fig. 5. Onset of the *Otx/Gbx2* abnormal expression occurs at the late headfold-presomite stages and precedes *Fgf8* activation. (A-M) Whole-mount in situ hybridisation of wild-type (A-K) *hOtx1²/hOtx1²* (A′-K′), *Gbx2*$^{-/-}$ (A″-K″) and *hOtx1²/hOtx1²*; *Gbx2*$^{-/-}$ (L-O) embryos with *Otx2*/*Hoxb1* (A,C,E,A″,C″,E″), *Otx2* (G,I,G″,I″), *hOtx1*/*Hoxb1* (A′,C′,E′,L,), *hOtx1* (G′,I′,N), *Gbx2* (B-B″,D-D″,F-F″,H-H″,J-J″ and M,O) and *Fgf8* (K-K″) probes at the stages indicated on the right side of the panel. The arrows indicate the anterior border of *Hoxb1* expression in wild-type (A,C,E) and *hOtx1²/hOtx1²* (A′,C′,E′). In *Gbx2*$^{-/-}$ embryos at late headfold and presomitic stages this border could not be identified, being fused to the posterior expansion of *Otx2* expression (C″,E″).

when this repatterning process takes place during embryonic development is largely unknown. In order to determine the precise onset of *hOtx1* and *Gbx2* abnormal expression and whether this process precedes the neuroectodermal activation of *Fgf8* expression, a detailed expression analysis was performed at early stages in *hOtx1²* and *Gbx2* single as well as *hOtx1²/ hOtx1²; Gbx2*$^{-/-}$ double mutants.

The expression patterns of *hOtx1*, *Otx2*, *Gbx2*, *Hoxb1* and *Fgf8* were assessed at late-streak, early headfold, late headfold, presomite and early somite stages. For the earlier stages, we decided to perform *Otx2/Hoxb1* or *hOtx1/Hoxb1* double hybridisation to better describe eventual alterations of *Otx2* or *hOtx1* expression patterns.

No abnormalities were observed in the expression patterns of *Gbx2*, *hOtx1/Hoxb1* and *Otx2/Hoxb1* at late-streak and early headfold stages in *hOtx1²/hOtx1²*, *Gbx2*$^{-/-}$ and *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ mutants when compared with wild-type littermates (Fig. 5A,A′,A″,B,B′,B″ and L,M). However, at the late headfold and presomitic stages, *hOtx1*, *Otx2* and *Gbx2* expression was clearly altered in these mutants. In *hOtx1²/hOtx1²* embryos, *Gbx2* transcripts (Fig. 5D′,F′) were gradually expanded into the *hOtx1* expression domain and *hOtx1* (Fig. 5C′,E′) was gradually repressed at the posterior side of its expression domain where the gap with the anterior border of *Hoxb1* expression enlarged. Conversely, in *Gbx2*$^{-/-}$ embryos, *Otx2* expression expanded posteriorly into the *Gbx2*

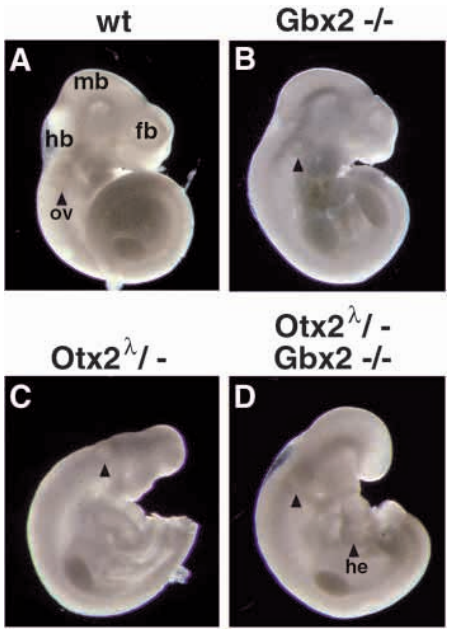

Fig. 6. Morphology of an *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ embryo at 10.5 d.p.c. Compared with wild-type (A) and *Gbx2*$^{-/-}$ (B) embryos, *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ embryos (D) show compromised development and severe abnormalities of the anterior CNS; while, compared with the head-less phenotype of *Otx2*$^{\lambda}$*/–* embryos (C) it is evident that the anterior neural tube of *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ embryos (D) develop morphological similarities having midbrain and forebrain. Moreover, the heart (D) reveals normal morphology when compared with *hOtx1*^{2}*/hOtx1*^{2}; *Gbx2*$^{-/-}$ embryos (Fig. 1D). Abbreviations as in previous figures plus, he, heart.

domain and fused to the *Hoxb1* expression domain (Fig. C″,E″) while the anterior domain of *Gbx2* expression was silenced (Fig. 5D″,F″).

Subsequently at the 1-2 somite stage in *hOtx1*^{2}*/hOtx1*^{2} embryos, *hOtx1* expression was restricted at the rostral tip of the embryo (Fig. 5G′) and *Gbx2* was ectopically expressed along the anterior neural plate almost reaching the most rostral tip (Fig. 5H′), while in *Gbx2*$^{-/-}$ embryos *Otx2* transcripts were detected along the presumptive anterior hindbrain (Fig. 5G″) where *Gbx2* was repressed (Fig. 5H″). At the 4- to 5-somite stage the repatterning process was complete. In *hOtx1*^{2}*/hOtx1*^{2} embryos, *Gbx2* was expressed throughout the anterior neural plate (Fig. 5J′) and *hOtx1* was barely detectable in the neuroectoderm, although strong *hOtx1* expression was evident in the foregut (Fig. 5I′). *Gbx2*$^{-/-}$ embryos showed posterior expansion of *Otx2* expression into the rostral hindbrain and concomitant repression of *Gbx2* in this domain (Fig. 5I″,J″).

In wild-type embryos *Fgf8* expression in the presumptive MHB was firstly detected at the 5- to 6-somite stage (Fig. 5K). In *hOtx1*^{2}*/hOtx1*^{2} embryos, its expression was detectable at the same stage but, interestingly, *Fgf8* activation occurred at the most rostral tip (Fig. 5K′). In *Gbx2*$^{-/-}$ embryos, *Fgf8* was transcribed in a broader area adjacent to the posterior border of *Otx2* expression (Fig. 5K″).

Finally, we analysed the expression of *hOtx1* and *Gbx2* in *hOtx1*^{2}*/hOtx1*^{2}*; Gbx2*$^{-/-}$ double mutants at 0- to 1-somite stage. Interestingly, the overlap between *hOtx1* and *Gbx2* genes was detected throughout most of the anterior neural plate (Fig. 5N,O).

These data suggest that *hOtx1/Otx2* and *Gbx2* expression profiles are altered at the presomite-early somite stage, a few hours before the onset of *Fgf8* expression in the neuroectoderm.

Altogether these findings also suggest that (i) *Otx/Gbx2* transcriptional repatterning is independent of *Fgf8* expression; (ii) *hOtx1* and *Gbx2* repression may be mediated by negative reciprocal interaction between OTX2 and GBX2 proteins during presomitic stages. In this respect, it is likely that this GBX2/OTX2 antagonism is required to maintain the initial anterior-posterior patterning of the neural plate, which is induced earlier during gastrulation.

Forebrain and midbrain fates require OTX2 protein and depend on mutual antagonism between OTX2 and GBX2

Analysis of the *hOtx1*^{2}*/hOtx1*^{2}; *Gbx2*$^{-/-}$ double mutants suggested that regional segregation of early mid-hindbrain markers and competence of anterior neural tissue in responding to fore- and midbrain inducing activities may be a consequence of an early basal prepatterning that requires OTX2 and GBX2 functions. In particular, absence of these two factors resulted in failure to activate the expression of forebrain- and midbrain-specific markers such as *Bf1* and *Atx*. In this context, we decided to assess whether in a *Gbx2*$^{-/-}$ background, the presence of a particularly low level of OTX2 protein was sufficient to rescue both the expression of these markers and a more normal positioning of *Fgf8* and *Gbx2* expression. To address this issue, mutant embryos carrying a single hypomorphic *Otx2* allele (*Otx2*$^{\lambda}$) in an *Otx2* and *Gbx2* null background (*Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$) were analysed.

As mentioned above (see Introduction), embryos carrying the *Otx2*$^{\lambda}$ hypomorphic allele in an *Otx2* null background (*Otx2*$^{\lambda}$*/–*) showed a remarkable reduction of OTX2 protein in epiblast and neuroectoderm. This significant reduction leads to an almost head-less phenotype (Fig. 6C) (Pilo Boyl et al., 2001). In contrast, *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ double mutants exhibited an evident morphological rescue of the anterior defects observed in the *Otx2*$^{\lambda}$*/–* mutants even though head and brain development still appeared compromised (Fig. 6D). Moreover, as compared with *hOtx1*^{2}*/hOtx1*^{2}*; Gbx2*$^{-/-}$ embryos (Fig. 1D), *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ mutants were viable at 10.5 d.p.c. and displayed normal heart development (Fig. 6D).

In *Otx2*$^{\lambda}$*/–* embryos, lack of anterior neural tissue was evident at 8.7 d.p.c. when the *Otx2* expression domain was drastically reduced and confined to the anteriormost neural plate (Fig. 7F). At this stage, both *Gbx2* and *Fgf8* were transcribed more rostrally (Fig. 7G,H), and, in particular, *Fgf8* transcripts were detected within the *Otx2*$^{\lambda}$ expression domain (Fig. 7H). In *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ embryos, *Otx2* expression was wide-spread from the tip of the neural plate to the presumptive r3/r4 boundary (Fig. 7P) and both *Gbx2* and *Fgf8* expression domains were shifted posteriorly when compared with *Otx2*$^{\lambda}$*/–* single mutant embryos (Fig. 7Q,R). Nevertheless, compared with wild-type (Fig. 7B,C) or *Gbx2*$^{-/-}$ (Fig. 7L,M) embryos, *Fgf8* and *Gbx2* transcripts were still detected in an expanded domain within the presumptive midbrain and rostral hindbrain of *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ embryos (Fig. 7Q,R). Notably, *Fgf8* expression could also be identified in the presumptive position of the ANR (arrow in Fig. 7R).

Finally, in order to assess whether in *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$

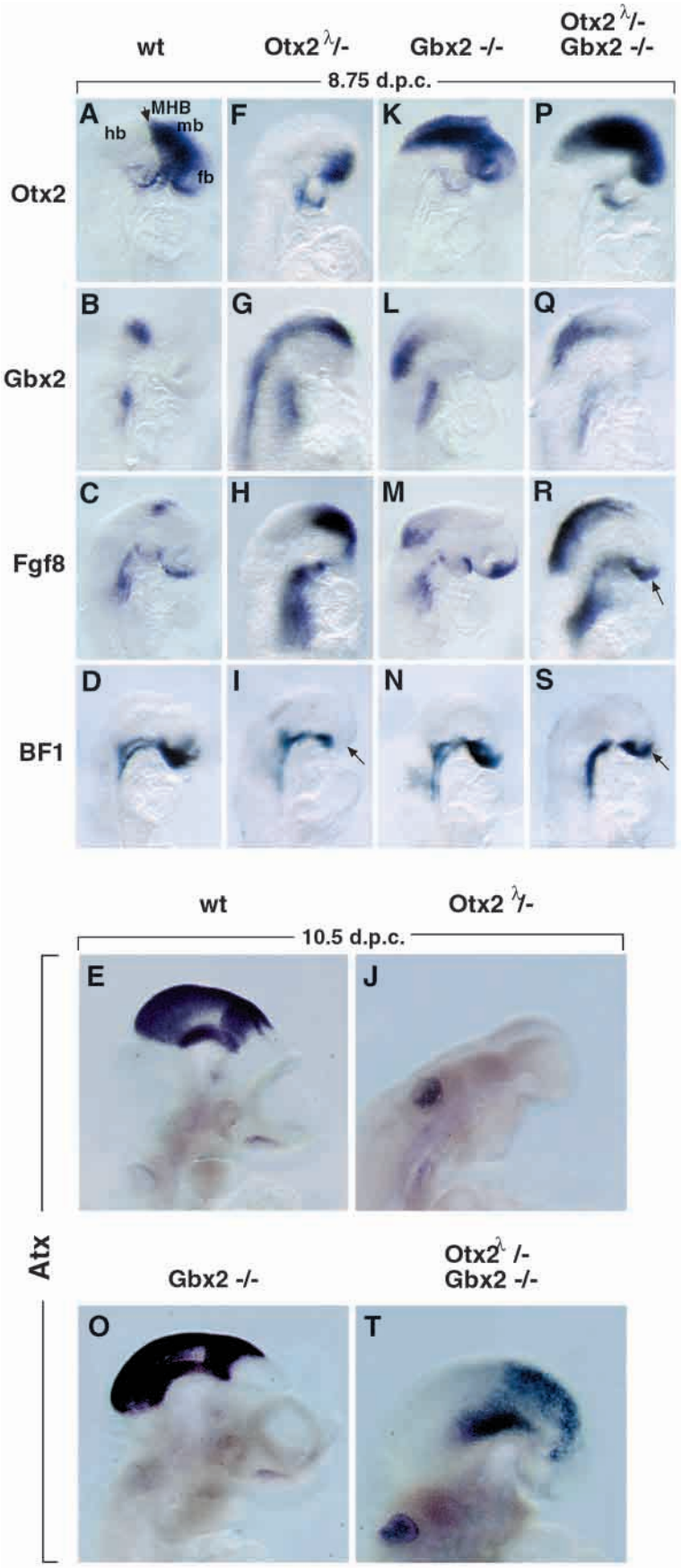

Fig. 7. A minimal amount of OTX2 protein suffices to displace *Fgf8* and *Gbx2* expression posteriorly and to induce forebrain- and midbrain-specific gene expression. (A-T) Whole-mount in situ hybridisation of wild-type (A-E), *Otx2*$^{\lambda}$/– (F-J), *Gbx2*$^{-/-}$ (K-O) and *Otx2*$^{\lambda}$/–; *Gbx2*$^{-/-}$ (P-T) embryos with *Otx2* (A,F,K,P,), *Gbx2* (B,G,L,Q), *Fgf8* (C,H,M,R), *Bf1* (D,I,N,S,) and *Atx* (E,J,O,T) probes shows that *Otx2*$^{\lambda}$/–; *Gbx2*$^{-/-}$ double mutants recover a wide-spread expression of *Otx2* (P), a posterior displacement of *Gbx2* (Q) and *Fgf8* (R) transcripts and the neuroectodermal expression of both the forebrain gene *Bf1* (compare arrow in I and S) and the midbrain gene *Atx* (T). The arrow in R indicates *Fgf8* expression in a region corresponding to the presumptive ANR; wild-type and *Gbx2*$^{-/-}$ embryos in A-C and K-M are the same as shown in Fig. 2. Abbreviations as in previous figures.

embryos fore- and midbrain development was at least in part recovered, *Bf1* and *Atx* expression was analysed at 8.75 and 10.5 d.p.c., respectively. Interestingly, *Bf1* (Fig. 7S) transcripts were detected in the most anterior neural plate and *Atx* (Fig. 7T) expression was observed more posteriorly in the presumptive midbrain region of the *Otx2*$^{\lambda}$/–; *Gbx2*$^{-/-}$ embryos. This was in marked contrast to the expression data obtained from the analysis of the *Otx2*$^{\lambda}$/– (Fig. 7I,J) single and *hOtx1*^{2}/*hOtx2*^{2}; *Gbx2*$^{-/-}$ (Fig. 2 and Fig. 3) double mutant embryos where no neural expression for *Bf1* and *Atx* was observed.

These findings emphasise the possibility that a relevant function of OTX2 is to provide competence to the neuroectoderm in responding to the fore- and midbrain inducing activities. Indeed, in a *Gbx2* null background, a low amount of OTX2 protein (*Otx2*$^{\lambda}$/–; *Gbx2*$^{-/-}$) makes the anterior neuroectoderm at least partially competent in activating forebrain- and midbrain-specific gene expression. However, direct experiments involving transplantation/recombination assays are necessary to assess whether tissue-competence and/or signalling pathway are affected. Nevertheless, together with previous data, these findings indicate that transformation of anterior identity to a more posterior value and vice versa depends on the dose-dependent antagonistic action of GBX2 and OTX2 functions.

DISCUSSION

In this study we have taken advantage of existing mouse models to investigate further the functions of *Otx2* and *Gbx2* in brain formation and IsO development. We present genetic evidence indicating that the absence of OTX2 and GBX2 leads to failure in regionalisation of the anterior neural plate, which is evident at the presomitic-early somite stage, and lack of competence of the anterior neuroectoderm in responding to forebrain and midbrain inducing properties. We show that the anterior neural plate (presumptive forebrain and midbrain) of the *hOtx1*^{2}/*hOtx1*^{2}; *Gbx2*$^{-/-}$ homozygous mutants is not transformed into a posterior (rostral hindbrain) fate as it is in the *hOtx1*^{2}/*hOtx1*^{2} single mutants, suggesting that GBX2 and OTX2 are key

factors mediating this repatterning process. Moreover, co-expression of *Fgf8, hOtx1* and *Gbx2* in the anterior neural plate of these double mutants suggests that activation and maintenance of *Fgf8* expression is independent of both OTX2 and GBX2 functions and that in the absence of GBX2 protein, FGF8 is unable to repress *hOtx1* (*Otx2)* expression. This conclusion is also supported by the observation that in *Otx1*$^{+/-}$*; Otx2*$^{+/-}$ and *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ mutant embryos, which contain significantly less OTX2 protein, *Fgf8, Otx2* or *Otx2*$^{\lambda}$ are co-expressed in a broad domain. Finally, we provide evidence indicating that induction of the forebrain- and midbrain-specific markers *Bf1* and *Atx*, respectively, depends on a dose-dependent balance between OTX2 and GBX2 functions. These findings provide evidence that competence and regionalisation of the anterior neuroectoderm is dependent on the mutual antagonism between OTX2 and GBX2.

OTX2 and GBX2 are required for competence and regionalisation of anterior neuroectoderm

Previously we have described that in mice carrying a single functional *Otx2* allele in an *Otx1* null background (*Otx1*$^{-/-}$*; Otx2*$^{+/-}$*)*, the posterior diencephalon and midbrain are transformed in an expanded cerebellum and pons. In this mutant, posterior repression of *Otx2* is paralleled by the co-ordinated anterior displacement of *Fgf8*, *Wnt1*, *Pax2* and *Gbx2* expression in a region anterior to the presumptive pretectal area (Acampora et al., 1997).

When the *Otx2* locus is replaced by the *hOtx1* cDNA, the gastrulation defects observed in *Otx2*$^{-/-}$ embryos (Acampora et al., 1995) are rescued because the *hOtx1* transcripts are efficiently translated in the VE. No hOTX1 protein is detected in the axial mesendoderm or anterior neuroectoderm of the *hOtx1*^{2}*/hOtx1*^{2} mutants, which results in absence of forebrain and midbrain regions by 8.5 d.p.c. Here we present evidence indicating that the lack of anterior structures is the consequence of an early transformation of the fore- and midbrain primordia into a metencephalic fate by the presomitic-early somite stage (Fig. 5). At 8.5 d.p.c., *hOtx1* transcripts are undetectable and IsO markers such as *Fgf8, Gbx2*, *Wnt1*, *En1* and *Pax2* are expressed at the rostral tip of the mutant embryos (Acampora et al., 1998). Conceptually similar, but obtained with a different transgenic approach, is the complementary transformation of the rostral hindbrain to a more anterior character that has been observed in mice ectopically expressing *Otx2* under *En1* transcriptional control (Broccoli et al., 1999).

An equally important role has been demonstrated for *Gbx2*. Mice lacking *Gbx2* exhibit abnormal patterning of the rostral hindbrain with early and permanent posterior expansion of *Otx2* (Wassarman et al., 1997; Millet et al., 1999) (Fig. 2 and Fig. 3). Embryos expressing *Gbx2* ectopically in the midbrain under *Wnt1* transcriptional control exhibit an opposite phenotype characterised by the midbrain-restricted repression of *Otx2* expression (Millet et al., 1999). In all the mentioned mouse models anterior or posterior displacement of either *Otx2* or *Gbx2* is paralleled by a similar displacement of FGF8 activity. The univocal interpretation of these findings is that IsO positioning is under the control of OTX2 and GBX2 transcription factors and occurs at the interface of their expression domains (Joyner et al., 2000; Simeone, 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Molecular analysis of *hOtx1*^{2}*/hOtx1*^{2}*; Gbx2*$^{-/-}$ embryos shows broad co-expression of the diagnostic markers *hOtx1, Gbx2, Pax2, Fgf8, Wnt1* and *En1* throughout the anterior neural plate in these mutants. This suggests a failure in regionalisation of the anterior neuroectoderm into forebrain, midbrain and rostral hindbrain territories. However, no differences in the expression of *Gbx2* and *hOtx1* is observed until the late streak/early headfold stages, suggesting that the initial induction of forebrain, midbrain and rostral hindbrain identities is normal in these embryos. Only later, at the presomitic-early somite stage, the early patterning is not maintained and *hOtx1* and *Gbx2* are co-expressed.

Our data indicate that initial induction of *Fgf8* in the neuroectoderm does not require OTX2 and GBX2 function. In this context, a similar conclusion has been previously deduced from the analysis of single mutants lacking only OTX2 (*hOtx1*^{2}*/hOtx1*^{2}) or GBX2 functions (Wassarman et al., 1997; Acampora et al., 1998). Nevertheless, before the analysis of double mutants (*hOtx1*^{2}*/hOtx1*^{2}*; Gbx2*$^{-/-}$), the possibility was still open that only OTX2 or GBX2, would be sufficient for mediating *Fgf8* activation. Therefore, rather than for activation, OTX2 and GBX2 are required for the refinement and restriction of *Fgf8* expression at the isthmus.

It is also apparent from this work that at 8.7 and 9.7 d.p.c. the anterior neural plate of *hOtx1*^{2}*/hOtx1*^{2}*; Gbx2*$^{-/-}$ embryos fails to activate forebrain- (*Bf1)* and midbrain- (*Atx)* specific gene expression, although *Fgf8* appears to be expressed abundantly throughout the anterior neuroectoderm of these mutants. In contrast, *Otx2*$^{\lambda}$*/–; Gbx2*$^{-/-}$ embryos, which express reduced levels of OTX2 protein, recover more or less normal *Bf1* and *Atx* expression in the domains corresponding to the presumptive forebrain and midbrain, respectively. Together these data suggest that OTX2 and GBX2 may be crucial for conferring competence to the anterior neuroectoderm in responding to inducing activities required for fore-midbrain (OTX2) and rostral hindbrain (GBX2) regional specification.

Alternatively, lack of forebrain- and midbrain-specific gene expression might be dependent on impairment (quantitative and qualitative) of signalling molecule(s) (FGF8) required for territorial specification. However, our findings are in close agreement with the idea that extension and patterning of fore-, mid- and rostral hindbrain territories depend on the antagonistic balance between OTX2 and GBX2. Our results indicate that this antagonism is exerted by the relative amount of OTX2 and GBX2 gene products and may be reversible in appropriate genetic conditions. In the case of *Otx2*$^{\lambda}$*/–* embryos, low levels of OTX2 protein in a *Gbx2*$^{+/+}$ background results in an almost head-less phenotype (posterior prevalence), whilst the same levels of OTX2 in a *Gbx2*$^{-/-}$ background lead to regionalisation of the anterior neural plate and maintenance of forebrain and midbrain identities (anterior prevalence).

Genetic interactions at the mid- and hindbrain regions

The analysis of the mouse models presented here has provided new insights into the molecular and genetic interactions required for proper development of the midbrain and hindbrain regions. Our findings suggest that: (i) FGF8 is unable to repress *Otx2* transcription without the contribution of GBX2; (ii) GBX2 is not required for ectopic expression of *Fgf8* and (iii) *Fgf8, Gbx2, En1* and *Pax2* have a differential sensitivity to the OTX2 repressive effect.

In *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ embryos, *Fgf8* is abundantly expressed throughout the anterior neuroectoderm overlapping with the *hOtx1* expression domain (Fig, 2). Since *hOtx1* is under the *Otx2* transcriptional control, this suggests that FGF8 is unable to repress *hOtx1* (*Otx2*) expression. Indeed, co-expression of *Fgf8* and *Otx2* transcripts is also observed in the *Otx1*$^{+/-}$; *Otx2*$^{+/-}$ double heterozygotes, which carry a wild-type copy of the *Otx2* locus. This is in marked contrast to the expression profile displayed by the *Otx2*$^{\lambda/-}$ and *Otx1*$^{-/-}$; *Otx2*$^{+/-}$ mutants, where there is a posterior repression of the *Otx2* expression domain coincident with the anterior expansion of *Gbx2* transcripts (Fig. 7) (Acampora et al., 1997).

The finding that in our mutants, FGF8 is unable to repress *Otx2* without the contribution of GBX2 is apparently in conflict with recent evidence indicating that FGF8 can repress *Otx2* independently of GBX2 (Liu and Joyner, 2001).

Reasons for this discrepancy might lie in the different approach used by Liu and Joyner (Liu and Joyner, 2001), where explants taken from the anterior midbrain of *Gbx2*$^{-/-}$ mutants were cultured with FGF8b-soaked beads. In our genetic study, the level of FGF8 protein might be not sufficient to repress *hOtx1* expression in the *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ or *Otx2* expression in the *Otx1*$^{+/-}$; *Otx2*$^{+/-}$ mutant embryos. Alternatively or in addition, a different and less potent FGF8 isoform (e.g. FGF8a) might be expressed in response to a low level of OTX2 in our mouse models compared with the strong FGF8b isoform used in the in vitro studies. Conversely, the fact that GBX2 is required for *Otx2* repression is in agreement with recent finding indicating that GBX2 may down-regulate *Otx2* expression (Garda et al., 2001).

Our results suggest that GBX2 is not required for *Fgf8* ectopic expression. This conclusion is supported by the *Gbx2* expression profile observed in the *hOtx1²/hOtx1²*single and *hOtx1²/hOtx1²; Gbx2*$^{-/-}$ double mutants. In these embryos a consistent and significant anteriorisation of *Gbx2* expression is detectable at the presomitic and early somite stage, a few hours before the onset of *Fgf8* expression at the MHB (Fig. 5). Moreover, *Fgf8* expression was induced at the rostral tip of the *hOtx1²/hOtx1²*mutants at the 5-6 somite stage, possibly at the rostral limit of the ectopic *Gbx2* expression domain (Fig. 5). These data strongly suggest that *Gbx2* anteriorisation in the *hOtx1²/hOtx1²* mutants is not dependent on *Fgf8* and that positioning of *Fgf8* expression is predated by the rostral limit of *Gbx2* and the posterior one of *Otx2*. This finding is, however, not in contrast with the result that FGF8 may play a role in stabilising and up-regulating *Gbx2* expression for maintenance of *Otx2/Gbx2* limit at the IsO (Garda et al., 2001), rather it may highlight different interactions between OTX2 and GBX2 necessary for the initial setting of the MHB or for its maintenance. Our data (Fig. 5) suggest that the repatterning observed in the *hOtx1²/hOtx1²* and *Gbx2*$^{-/-}$ embryos depends on mutual negative interactions between GBX2 and OTX2. This implies that *Gbx2* expression might be normally repressed by OTX2 in the forebrain and midbrain and conversely, *Otx2* might be silenced by GBX2 in the rostral hindbrain. Whether this antagonism is direct or indirect remains unknown.

Finally, our analysis has provided evidence for a differential transcriptional response of *Fgf8, Gbx2, Pax2* and *En1* to different levels of OTX proteins. In *Otx1*$^{+/-}$; *Otx2*$^{+/-}$ embryos, *Fgf8, Pax2* and *En1*, but not *Gbx2* expression domains are expanded along the dorsal midbrain. Only a further reduction of OTX proteins (in the *Otx1*$^{-/-}$; *Otx2*$^{+/-}$ mutants), or specifically in OTX2 protein levels (Acampora et al., 1998; Pilo Boyl et al., 2001), results in a marked rostral expansion of *Gbx2* transcripts. This supports the idea that adequate levels of OTX proteins are required for repression of Gbx2 transcription in the anterior neural plate. Further studies will be necessary to understand the nature of this differential repressive effect. This implies that, directly or indirectly, the sensitivity to the OTX2 repressive effect may play an important modulatory role in the establishment of the molecular interactions operating at the MHB.

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