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

Juan Pedro Martinez-Barbera1, Massimo Signore1, Pietro Pilo Boyl1, Eduardo Puelles1, Dario Acampora1,2, Robin Gogoi1, Frank Schubert1, Andrew Lumsden1 and Antonio Simeone1,2,*

1MRC Centre for Developmental Neurobiology, King’s College London, Guy’s Campus, New Hunt’s House, London SE1 1UL, UK
2International Institute of Genetics and Biophysics, CNR, Via G. Marconi 12, 80125, Naples, Italy

*Author for correspondence (e-mail: antonio.simeone@kcl.ac.uk)

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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 (hOtx1l1/hOtx12; 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, hOtx1l1/hOtx12; 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 hOtx1l1/hOtx12; 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 (Otx22–) in an Otx2 and Gbx2 null background (Otx22–/–; Gbx22–/–) recover both the headless phenotype exhibited by Otx22–/– embryos and forebrain- and midbrain-specific gene expression that is not observed in hOtx1l1/hOtx12; 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 so far been 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
morphealognic 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 En1, Pax2, Pax5, Pax8, Wnt1 and Fgf8; this loop, 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 GXB2 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 GXB2 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 due to the absence of OTX2 protein in the anterior neuroectoderm. The Otx2L allele was generated by inserting 300 bp of the λ phage DNA into the 3′ untranslated region (UTR) of the Otx2 locus (Pilo Boyle et al., 2001). In Otx2L mutants OTX2 protein level was drastically reduced in the epiblast and epiblast-derivatives. When the Otx2L was combined with an Otx2 null allele (Otx2L/–), OTX2 protein level in the anterior neuroectoderm was decreased by up to 20% of that seen in wild-type embryos and Otx2L/– embryos showed an almost complete head-less phenotype by 9 d.p.c. onwards (Pilo Boyle et al., 2001a).

Here we report that OTX2 and GXB2 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 GXB2 function is required for initiation of Fgf8 expression; (ii) Fgf8 is unable to repress Otx2 expression without the contribution of GXB2; (iii) GXB2 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 hOtx1L and Gbx2 single and double mutants is not dependent on Fgf8 expression and may be due to OTX2/GXB2 negative interactions.

**MATERIALS AND METHODS**

**Generation and genotyping of mice**

hOtx1L/hOtx1L; Gbx2–/– embryos were generated by intercrossing hOtx1L/– mice (Wassarman et al., 1997; Acampora et al., 1998); Otx1+/–: Otx2+–: males with Otx1+/–; Otx2+– females (Acampora et al., 1997); Otx2+–/–: Gbx2+–/– embryos were generated by crossing Otx2L/–; Gbx2L/– mice (Acampora et al., 1995; Wassarman et al., 1997; Pilo Boyle 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 Boyle 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, 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 hOtx1L/hOtx1L; Gbx2+–/– embryos**

hOtx1L/hOtx1L; Gbx2+–/– embryos were generated by crossing hOtx1L/– mice and Gbx2+–/– double heterozygous mice, which were viable and fertile. Parental hOtx1L/– and Gbx2+–/– were
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 hOtx1 2/hOtx1 2; Gbx2 2–/– 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 hOtx1 2/hOtx1 2; Gbx2 2–/– homozygous embryos were found (n=84). Therefore the hOtx1 2/hOtx1 2; Gbx2 2–/– mutant was embryonic lethal by 10 d.p.c.

At 9.75 d.p.c. the morphology of the anterior neural plate in hOtx1 2/hOtx1 2; Gbx2 2–/– mutants (Fig. 1D) was different from that of hOtx1 2/hOtx1 2 embryos (Fig. 1C). Indeed, in hOtx1 2/hOtx1 2; Gbx2 2–/– 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 hOtx1 2/hOtx1 2; Gbx2 2–/– by 10 d.p.c.

**Table 1. Frequency of genotypes observed by intercrossing hOtx1 2/Otx2; Gbx2 2+/– mice**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Number of embryos</th>
<th>Expected frequency (%)</th>
<th>Observed frequency (%)</th>
</tr>
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<tbody>
<tr>
<td>hOtx1 2/hOtx1 2; Gbx2 2+/–</td>
<td>14</td>
<td>6.2</td>
<td>4.9</td>
</tr>
<tr>
<td>hOtx1 2/hOtx1 2; Gbx2 2+/–</td>
<td>24</td>
<td>12.5</td>
<td>8.4</td>
</tr>
<tr>
<td>hOtx1 2/Otx2; Gbx2 2+/–</td>
<td>30</td>
<td>12.5</td>
<td>10.5</td>
</tr>
<tr>
<td>hOtx1 2/Otx2; Gbx2 2+/–</td>
<td>74</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>hOtx1 2/hOtx1 2; Gbx2 2+/–</td>
<td>21</td>
<td>6.2</td>
<td>7.3</td>
</tr>
<tr>
<td>hOtx1 2/Otx2; Gbx2 2+/–</td>
<td>44</td>
<td>12.5</td>
<td>15.4</td>
</tr>
<tr>
<td>Otx2/Otx2; Gbx2 2+/–</td>
<td>14</td>
<td>6.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Otx2/Otx2; Gbx2 2+/–</td>
<td>42</td>
<td>12.5</td>
<td>14.7</td>
</tr>
<tr>
<td>Otx2/Otx2; Gbx2 2+/–</td>
<td>22</td>
<td>6.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Total</td>
<td>285</td>
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</table>
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 hOtx1<sup>2</sup>/hOtx1<sup>2</sup>; Gbx2<sup>–/–</sup> 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 hOtx1<sup>2</sup>/hOtx1<sup>2</sup>; Gbx2<sup>–/–</sup> double mutants. In hOtx1<sup>2</sup>/hOtx1<sup>2</sup>; Gbx2<sup>–/–</sup> embryos, Pax2, En1 and Wnt1 transcripts were broadly co-expressed with hOtx1, Gbx2 and Fgf8 along the anterior neural plate (compare Fig. 2V-X<sup>′</sup> with Y-α<sup>′</sup>). Notably, Pax2 was not transcribed along the lateral/dorsal edge of the neural plate (Fig. 2Y,Y<sup>′</sup>). At this stage, the forebrain marker Bf1 was expressed normally in Gbx2<sup>–/–</sup> mutants, but undetectable in hOtx1<sup>2</sup>/hOtx1<sup>2</sup> homozygous embryos (Fig. 2N,U). No neural expression of Bf1 was
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detectable in three \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) mutants analysed (Fig. 2β,β′).

Neural patterning in \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) embryos was also analysed at 9.7 d.p.c. Compared with wild-type littersmates (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 \( hOtx1^{2}/hOtx1^{2} \) 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 \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) 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 \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) mutants. At 9.7 d.p.c., \( Six3 \) and \( Bf1 \) expression was not detected in four \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) 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 \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) 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 \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) homozygous embryos (Fig. 3L) and posteriorly expanded in \( Gbx2^{+/-} \) mutants (Fig. 3R). Importantly, \( Atx \) was not expressed in \( hOtx1^{2}/hOtx1^{2}; Gbx2^{+/-} \) 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.
FGF8 could not repress hOtx1 expression without the contribution of GBX2.

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 Otx1 (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/Otx2 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/Otx2 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,
when this repatterning process takes place during embryonic development is largely unknown. In order to determine the precise onset of \textit{hOtx1} and \textit{Gbx2} abnormal expression and whether this process precedes the neuroectodermal activation of \textit{Fgf8} expression, a detailed expression analysis was performed at early stages in \textit{hOtx1} \textit{2}/\textit{hOtx1} \textit{2} and \textit{Gbx2} \textit{2/−} double mutants.

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

No abnormalities were observed in the expression patterns of \textit{Gbx2}, \textit{hOtx1}/\textit{Hoxb1} and \textit{Otx2}/\textit{Hoxb1} at late-streak and early headfold stages in \textit{hOtx1} \textit{2}/\textit{hOtx1} \textit{2}, \textit{Gbx2} \textit{2/−} and \textit{hOtx1} \textit{2}/\textit{hOtx1} \textit{2}; \textit{Gbx2} \textit{2/−} mutants when compared with wild-type littermates (Fig. 5A,\textit{A′′},B,\textit{B′},B′′ and L,M). However, at the late headfold and presomitic stages, \textit{hOtx1}, \textit{Otx2} and \textit{Gbx2} expression was clearly altered in these mutants. In \textit{hOtx1} \textit{2}/\textit{hOtx1} \textit{2} embryos, \textit{Gbx2} transcripts (Fig. 5D,\textit{D′′},F,\textit{F′′},H,\textit{H′′},J,\textit{J′′} and M,O) and \textit{Fgf8} (K,\textit{K′′}) probes at the stages indicated on the right side of the panel. The arrows indicate the anterior border of \textit{Hoxb1} expression in wild-type (A,\textit{C},E) and \textit{hOtx1} \textit{2}/\textit{hOtx1} \textit{2} (\textit{A′,E′}). In \textit{Gbx2} \textit{2/−} embryos at late headfold and presomitic stages this border could not be identified, being fused to the posterior expansion of \textit{Otx2} expression (\textit{C′,E′}).
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 presomatic 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<sup>l2</sup>/hOtx1<sup>l2</sup>; Gbx2<sup>–/–</sup> 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 prepattern 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 Atoh1. In this context, we decided to assess whether in a Gbx2<sup>–/–</sup> 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<sup>l</sup>) in an Otx2 and Gbx2 null background (Otx2<sup>2/2–/–</sup>; Gbx2<sup>–/–</sup>) were analysed.

As mentioned above (see Introduction), embryos carrying the Otx2<sup>l</sup> hypomorphic allele in an Otx2 null background (Otx2<sup>2/2–/–</sup>) 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<sup>2/2–/–</sup>; Gbx2<sup>–/–</sup> double mutants exhibited an evident morphological rescue of the anterior defects observed in the Otx2<sup>2/2–/–</sup> mutants even though head and brain development still appeared compromised (Fig. 6D). Moreover, as compared with hOtx1<sup>l2</sup>/hOtx1<sup>l2</sup>; Gbx2<sup>–/–</sup> embryos (Fig. 1D), Otx2<sup>2/2–/–</sup>; Gbx2<sup>–/–</sup> mutants were viable at 10.5 d.p.c. and displayed normal heart development (Fig. 6D).

In Otx2<sup>2/2–/–</sup> 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<sup>2/2–/–</sup> expression domain (Fig. 7H). In Otx2<sup>2/2–/–</sup>; Gbx2<sup>–/–</sup> 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<sup>2/2–/–</sup> single mutant embryos (Fig. 7Q,R). Nevertheless, compared with wild-type (Fig. 7B,C) or Gbx2<sup>2/2–/–</sup> (Fig. 7L,M) embryos, Fgf8 and Gbx2 transcripts were still detected in an expanded domain within the presumptive midbrain and rostral hindbrain of Otx2<sup>2/2–/–</sup>; Gbx2<sup>2/2–/–</sup> 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<sup>2/2–/–</sup>; Gbx2<sup>–/–</sup>...
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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−/−; Gbx2−/− embryos. This was in marked contrast to the expression data obtained from the analysis of the Otx2−/−; Gbx2−/−; hOtx12/hOtx22; Gbx2−/− 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−/−; 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 hOtx12/hOtx22; Gbx2−/− single mutants is not transformed into a posterior (rostral hindbrain) fate as it is in the hOtx12/hOtx22 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 GXB2 functions and that in the absence of GXB2 protein, FGF8 is unable to repress hOtx1 (OtX1) expression. This conclusion is also supported by the observation that in hOtx1+/–; Otx2+/– and hOtx2+/–; Gbx2–/– mutant embryos, which contain significantly less OTX2 protein, Fgf8, Otx2 or Otx2+/– are co-expressed in a broad domain. Finally, we provide evidence indicating that induction of the forebrain- and midbrain-specific markers Bfl and Arx, respectively, depends on a dose-dependent balance between OTX2 and GXB2 functions. These findings provide evidence that competence and regionalisation of the anterior neuroectoderm is dependent on the mutual antagonism between OTX2 and GXB2.

**OTX2 and GXB2 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+/–; hOtx1+/– 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 presumptive-early somite stage (Fig. 5). At 8.5 d.p.c., hOtx1 transcripts are undetectable and IS0 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 IS0 positioning is under the control of OTX2 and GXB2 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+/–; hOtx1+/–; 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 GXB2 function. In this context, a similar conclusion has been previously deduced from the analysis of single mutants lacking only OTX2 (hOtx1+/–; hOtx1+/–) or GXB2 functions (Wassarman et al., 1997; Acampora et al., 1998). Nevertheless, before the analysis of double mutants (hOtx1+/–; hOtx1+/–; Gbx2–/–), the possibility was still open that only OTX2 or GXB2, would be sufficient for mediating Fgf8 activation. Therefore, rather than for activation, OTX2 and GXB2 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+/–; hOtx1+/–; Gbx2–/– embryos fails to activate forebrain- (Bfl) and midbrain- (Arx) specific gene expression, although Fgf8 appears to be expressed abundantly throughout the anterior neuroectoderm of these mutants. In contrast, Otx2+/–; Gbx2–/– embryos, which express reduced levels of OTX2 protein, recover more or less normal Bfl and Arx expression in the domains corresponding to the presumptive forebrain and midbrain, respectively. Together these data suggest that OTX2 and GXB2 may be crucial for conferring competence to the anterior neuroectoderm in responding to inducing activities required for fore-midbrain (OTX2) and rostral hindbrain (GXB2) 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 GXB2. Our results indicate that this antagonism is exerted by the relative amount of OTX2 and GXB2 gene products and may be reversible in appropriate genetic conditions. In the case of Otx2+/–; 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 GXB2; (ii) GXB2 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 hOtx1/2/hOtx1/2; 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+/− 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 hOtx1+/−/hOtx1−/−; Gbx2+/− or Otx2 expression in the Otx1+/−; Otx2+/− mutant embryos. Alternatively or in addition, a different and less potent FGF8 isoform (e.g. FGF8α) 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 hOtx1+/−/hOtx1−/− and hOtx1+/−/hOtx1+/−; 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 hOtx1+/−/hOtx1−/− 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 hOtx1+/−/hOtx1−/− 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 IS0 (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 hOtx1+/−/hOtx1−/− 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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