Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain

Eduardo Puelles[1], Alessandro Annino[1], Francesca Tuorto[2], Alessandro Usiello[3], Dario Acampora[1,2], Thomas Czerny[4], Claude Brodski[6], Siew-Lan Ang[5], Wolfgang Wurst[6,*] and Antonio Simeone[1,2,*]

[1]MRC Centre for Developmental Neurobiology, New Hunt's House, 4th Floor, King's College London, Guy's Campus, London Bridge, London SE1 1UL, UK
[2]Institute of Genetics and Biophysics 'A. Buzzati-Traverso', CNR, Via G. Marconi 12, 80125 Naples, Italy
[3]Department of Neuroscience, Retzius väg 8, Karolinska Institutet, S-17177 Stockholm, Sweden
[4]Institute of Animal Breeding and Genetics, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Wien, Austria
[5]Division of Developmental Neurobiology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, UK
[6]GSF-Research Center, Institute of Developmental Genetics, 85764 Munich/Neuherberg, Max-Planck-Institute of Psychiatry, Molecular Neurogenetics, Kraepelinstrasse 2-16, 80804 Munich, Germany
*Authors for correspondence (e-mail: antonio.simeone@kcl.ac.uk and wurst@gsf.de)

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Summary

The specification of distinct neuronal cell-types is controlled by inducing signals whose interpretation in distinct areas along the central nervous system provides neuronal progenitors with a precise and typical expression code of transcription factors.

To gain insights into this process, we investigated the role of Otx2 in the specification of identity and fate of neuronal progenitors in the ventral midbrain. To achieve this, *Otx2* was inactivated by Cre recombinase under the transcriptional control of *En1*. Lack of Otx2 in the ventrolateral and posterior midbrain results in a dorsal expansion of Shh expression and in a dorsal and anterior rotation of the midbrain-hindbrain boundary and *Fgf8* expression. Indeed, in this mutant correct positioning of the ventral site of midbrain-hindbrain boundary and *Fgf8* expression are efficiently controlled by Otx1 function, thus allowing the study of the identity and fate of neuronal progenitors of the ventral midbrain in the absence of Otx2. Our results suggest that Otx2 acts in two ways: by repressing Nkx2.2 in the ventral midbrain and maintaining the Nkx6.1-expressing domain through dorsal antagonism on Shh. Failure of this control affects the identity code and fate of midbrain progenitors, which exhibit features in common with neuronal precursors of the rostral hindbrain even though the midbrain retains its regional identity and these neuronal precursors are rostral to *Fgf8* expression. Dopaminergic neurons are greatly reduced in number, red nucleus precursors disappear from the ventral midbrain where a relevant number of serotonergic neurons are generated. These results indicate that Otx2 is an essential regulator of the identity, extent and fate of neuronal progenitor domains in the ventral midbrain and provide novel insights into the mechanisms by which neuronal diversity is generated in the central nervous system.

Key words: *Otx2*, Midbrain, Neuronal precursors, Dopaminergic neurons, Serotonergic neurons, Mouse

Introduction

During the development of the vertebrate central nervous system (CNS), the assignment of regional identity and neuronal fate is controlled by sequential events requiring spatially and temporally coordinated interaction between organising centres that emit inducing signals and responding tissues that interpret these signals (Jessell, 2000; Lumsden and Krumlauf, 1996; Rubenstein et al., 1998; Stern, 2001). The early anterior neuroectoderm is first regionalised into broad territories corresponding to the forebrain, midbrain and hindbrain and, subsequently, each of these areas is subdivided into smaller domains with distinct anteroposterior (AP) and dorsoventral (DV) character. Neuronal precursors within these domains exhibit a specific molecular identity and differentiate into specific neuronal cell types. These events require precise control to regulate the onset, maintenance and spread of organising signals (Jessell, 2000; Lumsden and Krumlauf, 1996; Rubenstein et al., 1998; Stern, 2001; Wolpert, 1969). Sonic hedgehog (*Shh*) and fibroblast growth factor *8* (*Fgf8*) genes encode two signaling molecules required to confer regional identity, to control growth and survival of neuronal precursors and to specify neuronal fate along the DV (Shh) and AP (Fgf8) axes of the neural tube (Agarwala et al., 2001; Briscoe and Ericson, 2001; Britto et al., 2002; Charrier, 2001; Crossley et al., 1996; Hynes and Rosenthal, 1999; Jessell, 2000; Litingtung and Chiang, 2000; Puelles et al., 2003; Shimamura and Rubenstein, 1997; Wurst and Bally-Cuif, 2001; Ye et al., 2001). In the spinal cord and hindbrain, the graded DV distribution of Shh is converted into specific progenitor cell identities by cross-repressive interactions between class I and class II homeoproteins that are, respectively, repressed or activated by *Shh* activity (Briscoe et

al., 1999; Briscoe et al., 2000; Briscoe and Ericson, 2001; Jessell, 2000; Pattyn et al., 2003; Vallstedt et al., 2001). Experiments performed in explant cultures have also indicated that Fgf8 and Shh signaling activities specify the identity and position of dopaminergic (DA) and serotonergic (Ser) neurons (Hynes and Rosenthal, 1999; Ye et al., 1998).

The homeoproteins encoded by *Otx1* and *Otx2* play crucial multiple roles in brain development, and during regionalisation they are required to control AP and DV patterning of the midbrain through a dose-dependent antagonism exerted on *Fgf8* and *Shh* expression, respectively (Acampora et al., 1997; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Puelles et al., 2003; Simeone et al., 2002). However, very little is known about the role of Otx genes in further decisions involving the allocation of midbrain neuronal fates under the influence of Fgf8 and Shh induction. To address this issue, we generated conditional mutants inactivating *Otx2* by Cre recombinase expressed under the transcriptional control of the *En1* gene (*En1*^{cre}) (Kimmel et al., 2000). *Otx2* inactivation in the ventral and posterior midbrain resulted in a dorsal expansion of *Shh* expression and in a dorsal and anterior rotation of the midbrain-hindbrain boundary (MHB). In *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos, the ventral territory rostral to the domain of *Fgf8* expression retained a midbrain identity but exhibited dramatic abnormalities in the identity and fate of neuronal precursors. DA neurons were greatly reduced in number and the precursor domain that normally generates red nucleus (RN) neurons gave rise to Ser neurons. This abnormality correlated with altered expression of Shh, Nkx2.2 and Nkx6.1, which define an expression code in the ventral midbrain similar to that normally observed in the rostral hindbrain.

These findings support an essential role for Otx2 in controlling the extent, identity and fate of neuronal progenitor domains of the ventral midbrain, thus providing novel insights into the molecular mechanisms that regulate neuronal diversity in the CNS.

Materials and methods

Production and genotyping of mutant embryos

The generation of *En1*$^{cre/+}$, *Otx2* $^{flox/+}$, *Otx2*$^{+/-}$, *Otx1*$^{cre/+}$ and *Otx1*$^{+/-}$ mutant mouse strains has been already reported (Acampora et al., 1995; Acampora et al., 1996; Kimmel et al., 2000; Puelles et al., 2003). *En1*$^{cre/+}$, *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$, *Otx1*$^{cre/+}$; *Otx2*$^{flox/-}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$; *Otx1*$^{-/-}$ mice and/or embryos were genotyped by allele specific PCR reactions. Genotyping was performed by using the following list of primers, specific to each allele: *En1*^{cre} allele (sense primer, 5′-AGAGAGCGAGATTTGCTCCACCAG-3′; antisense primer 5′-CAGGTATGCTCAGAAAACGCCTGG-3′); *En1* wild type allele (sense primer, 5′-CGAGCATGGAAGAACAGCAGCC-3′; antisense primer 5′-GACACCGGCACGCTGTCTCCATC-3′); *Otx2* wild type and flox allele (sense primer, 5′-ACTTGCCAGAA-TCCAGGGTGCAG-3′; antisense primer 5′-CCAGGCTAAAAGA-CCCTGGTTC-3′); *Otx1*^{cre} allele (sense primer, 5′-GGTGTG-CTTAGCAGACTTGGTAGA-3′; antisense primer 5′-CAGGTATGC-TCAGAAAACGCCTGG-3′); *Otx1* wild type allele (sense primer, 5′-CACTTGGGATTTTGCACCCTC-3′; antisense primer 5′-AGCA-GACACATGGAAACCTTC-3′).

The amplification products are 301, 150, 197, 290, 235, 302 bp long, respectively. For the *Otx1*- and *Otx2*-null alleles, primers and conditions were previously reported (Acampora et al., 1995; Acampora et al., 1996)

In situ hybridisation, immunohistochemistry and apoptosis

In situ hybridisation and immunohistochemistry were performed as previously described (Acampora et al., 1998; Simeone, 1999). Probes for *Shh*, *Fgf8*, *Gbx2*, *Otx1*, *Grg4*, *Foxa2*, *Pet1*, *Pou4f1*, *Th*, *Isl1* have been already reported (Puelles et al., 2003) and correspond to PCR fragments ranging in length between 0.2 and 1 kb. The *Otx2Δ* probe corresponds to the *Otx2* exon 2 and has been previously described (Puelles et al., 2003); the Otx2-5′ probe is a PCR fragment including the last 150 bp of the exon containing the methionine; the probe for the *Otx1* null allele corresponds to a 700 bp *lacZ* DNA fragment (Acampora et al., 1996). Immunohistochemistry was performed as described (Puelles et al., 2003) with antibodies directed against Otx2 (1:2000), Nkx2.2 (1:100), Pou4f1 (α Brn3a) (1:100), Shh (1:200), Isl1 (1:100), Th (1:300), 5-HT (1:100) and Nkx6.1 (1:100) proteins. The αNkx2.2, α2H3 and αIsl1 are from Hybridoma Bank; the αShh and αPou4f1 from Santa Cruz Biotechnology; the αTh and α5-HT from Chemicon and the αNkx6.1 is a rabbit polyclonal serum kindly provided by J. Ericson. The α2H3 is a monoclonal antibody recognising the 165 kDa neurofilament subunit.

Apoptosis was detected according to the TUNEL method (Puelles et al., 2003).

Dopaminergic cell counting

The general procedure was essentially as previously reported (Acampora et al., 1999). Frontal sections through the midbrain of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ (n=4) and *En1*$^{cre/+}$ (n=3) adult mice were immunostained with Th antibody. For each brain a total of four sections (one every four consecutive sections) were selected along a comparable area and photographed at high magnification. Th-positive cell bodies were counted and the mean value for each genotype was calculated. The mean value of mutant brains was compared with that of control animals and the cell number reduction reported as a percentage.

Transfections, RNAse protection experiments and co-immunoprecipitation assays

A series of *Otx1* and *Otx2* cDNA molecules carrying non-overlapping deletions along the entire *Otx1*- or *Otx2*-coding region were generated by PCR. Wild-type and mutant versions of *Otx1* and *Otx2* were cloned in the pCT expression vector downstream of a CMV enhancer-promoter (Simeone et al., 1993; Thali et al., 1988). The *Grg4* expression plasmid has been previously reported (Eberhard et al., 2000). HeLa cells were electroporated with mouse *Otx1* and *Otx2* constructs, alone or in combination with the *Grg4* expressing plasmid. The amount of expression plasmids was equalised by addition of an empty CMV topping plasmid. Transactivation of a co-transfected multimerised *bts* or *np* target site was monitored by RNAse protection using as probe a fragment of the rabbit *β-globin* reporter gene (Simeone et al., 1993; Thali et al., 1988). Transactivations were normalised by monitoring the amount of the mRNA transcribed by the Otx and/or *Grg4* expression vectors (data not shown).

For co-immunoprecipitation assays, the *Grg4*-coding sequence was cloned in the pKW2T vector and its C terminus was fused to the Flag epitope by PCR. Transiently transfected HeLa cells were processed as described (Eberhard et al., 2000). Lysates were incubated for 30 minutes on ice, cleared from cellular debris and mixed with 10 μl of anti-Flag M2 affinity beads (Sigma) for 2 hours at 4°C under constant rotation. After extensive washing of the beads in buffer B (20 mM Tris-HCl pH 7.9, 200 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1% SDS), the precipitated proteins were analysed by SDS-PAGE and western blotting using polyclonal Flag, Otx2 and Grg4 antibodies. The input contains between 2% and 5% of the transfection.

Results

En1^{cre} mediated inactivation of the *Otx2*^{flox} allele

The generation of *Otx2*^{flox} and *En1*^{cre} mice has been reported previously (Kimmel et al., 2000; Puelles et al., 2003). As a first step, we monitored the inactivation of the *Otx2*^{flox} allele by comparing the expression of *En1*^{cre} mRNA with both *Otx2* mRNA and protein by using a specific *Otx2* probe (*Otx2Δ*) which detected only unfloxed *Otx2* transcripts, and an antibody against Otx2 (αOtx2) (Puelles et al., 2003). The *Otx2* inactivation was first detected at E9 (around 15 somites), but at this stage was still quite low (data not shown). From E9.5 (around 25 somites) onwards, a virtually complete inactivation of *Otx2* was detected in the ventral and caudal midbrain and functional *Otx2* transcripts were confined to the dorsolateral aspect of the anterior midbrain (Fig. 1).

Anatomical analysis of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants

Anatomical and histological inspection of the brain of conditional mutants revealed severe abnormalities of the caudal and ventral midbrain as expected by the lack of Otx2 in these areas. Indeed, these mutants lacked the inferior colliculus and exhibited a greatly expanded cerebellum showing supernumerary branches with apparently normal histology (Fig. 2A-C). Moreover, immunodetection of calbindin, parvalbumin, myelin binding protein and glial fibrillary acidic protein showed that number and position of Purkinje, basket and stellate cells, oligodendrocytes and Bergmann glia appeared unaffected (data not shown) (Mathis et al., 2003). Severe abnormalities were detected also in the ventral midbrain, such as lack of RN neurons, hypoplasia of the oculomotor nucleus (OM) (Fig. 2D,E), and extensive reduction and disorganisation of DA neurons (Fig. 2F). In particular, cell counting in four different mutant brains showed a 70±6% reduction of tyrosine hydroxylase (Th) positive midbrain neurons.

Abnormalities in the posterior midbrain of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants

To study the consequence of *Otx2* depletion on the development of the caudal midbrain and MHB, we examined the expression of *Otx2*, *Fgf8*, *Gbx2*, *Wnt1*, *Otx1*, *En1* and *Pax6*.

At E8.75 (around 10 somites), *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos showed a normal expression pattern of these markers according to a very mild inactivation of *Otx2* at this early stage (data not shown). At E9.5, the dorsal expression of *Fgf8* and *Gbx2* at the MHB was rostrally shifted and appeared expanded (Fig. 3B,C); the dorsolateral part of the ring of *Wnt1* expression was also shifted rostrally, while its expression in the roof plate and at ventral site of the MHB was unaltered (Fig. 3D); *Otx1* (Fig. 3E) was transcribed in the midbrain also in the area adjacent to the ventral site of the MHB and where *Otx2* was inactivated (compare Fig. 3E with 3A); the expression domain of *En1* (Fig. 3F) was also anteriorly shifted with an anterior border in close proximity with the posterior one of the functional *Otx2* (Fig. 3A); and *Pax6* was expressed in the pretectal domain (Fig. 3H). At E10.5 (around 35 somites), the *Fgf8* and *Gbx2* expression at the MHB (Fig. 3J,K,R,S) was sharpened ventrolaterally while along the dorsal edge of the neural tube remained rostrally expanded up to the caudal border of the functional *Otx2* domain (Fig. 3I). A corresponding rostral shift similar to that described at E9.5 was observed for *Wnt1* (Fig. 3L,T); *Otx1* transcription persisted at E10.5 in the Otx2-depleted region with a caudal and ventral border in close proximity of the *Fgf8* and *Gbx2* expression domains (Fig. 3M,U); the expression domain of *En1* included the ventral domain of *Fgf8* (arrow in Fig. 3N-V) and was adjacent to that of the functional *Otx2*; and *Pax6* demarcated the pretectal area (Fig. 3P), thus suggesting that the *Otx2Δ*-positive territory should also include the dorsolateral anterior midbrain. Therefore, a fairly normal positioning of both MHB and its molecular code were retained only ventrally in proximity of the strongest domain of *Otx1* expression while, dorsally, the MHB was rostrally shifted and the caudal and dorsal midbrain was repatterned into cerebellum

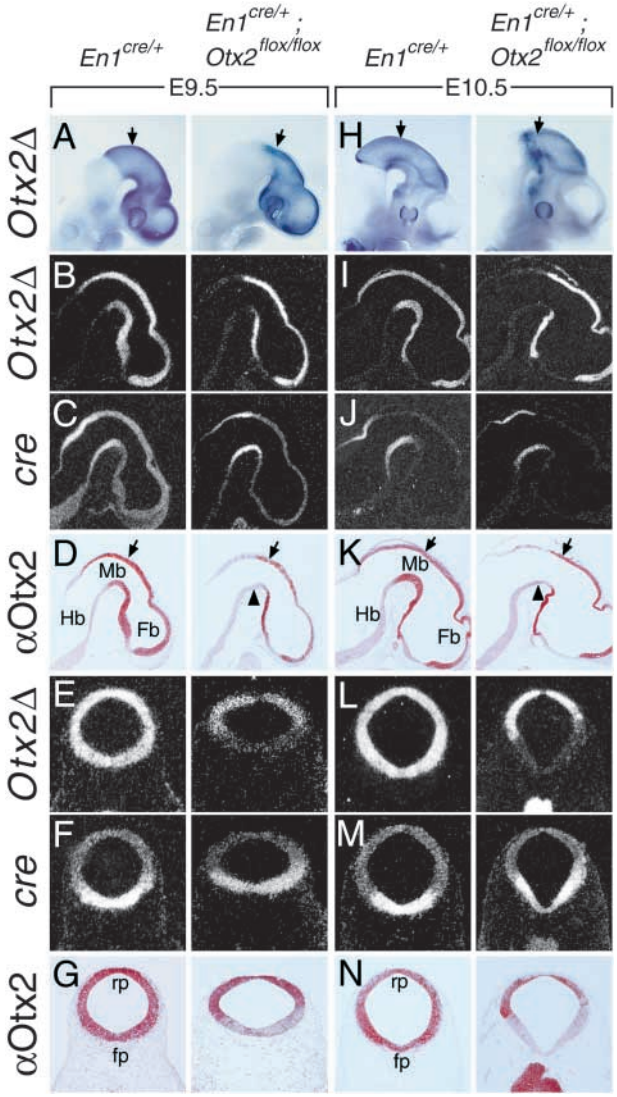

Fig. 1. *Otx2*^{flox} inactivation by *En1*-driven Cre recombinase. (A-N) *En1*$^{cre/+}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos are hybridised at E9.5 and E10.5 with *Otx2*Δ (A,B,E,H,I,L) and *Cre* (C,F,J,M) probes, or processed for Otx2 immunodetection (D,G,K,N). The arrows in A,D,H,K indicate the approximate position of frontal sections; the arrowheads in D,K indicate the ventral midbrain where Otx2 is lost. Fb, forebrain; Mb, midbrain; Hb, hindbrain; rp, roof plate; fp, floor plate.

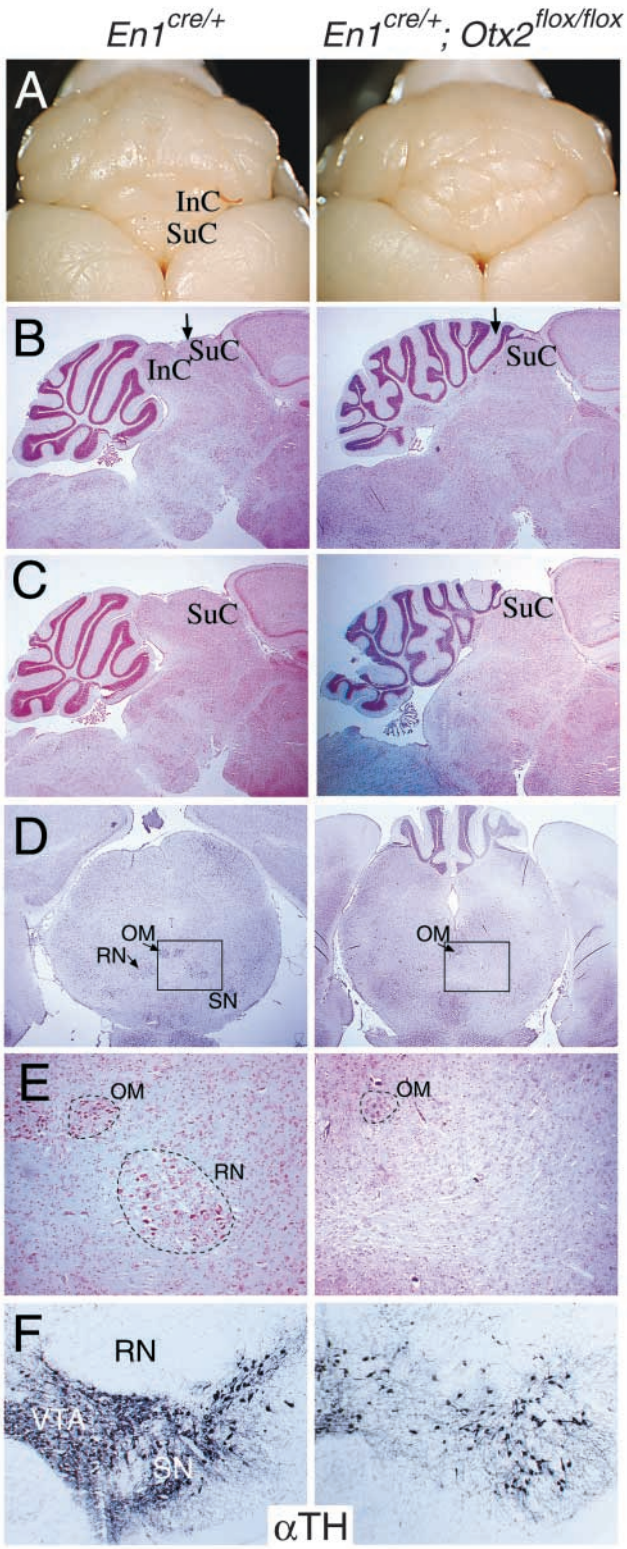

Fig. 2. Abnormalities in the adult brain of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants. (A-D) Midbrain and cerebellum of adult *En1*$^{cre/+}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mice are compared in dorsal view (A), or in sagittal (B,C) and frontal (D) Nissl-stained sections. (E) High magnification of the area demarcated by a square in (D). (F) Th immunostaining of the DA area in a frontal section close to (D). The arrows in B indicate the approximate position of frontal sections. InC, inferior colliculus; SuC, superior colliculus; OM oculomotor nucleus; RN, red nucleus; SN, substantia nigra; VTA, ventral tegmental area.

(Fig. 3X). This dorsal and rostral shift of the MHB can be visualised as a rotation of its DV axis describing an arc of about 45°. In this rotation the ventral site of the MHB represents the fixed point (Fig. 3X). To test the possibility that in the absence of Otx2, Otx1 may efficiently control the positioning of ventral MHB, we generated triple mutants in which *Otx2* inactivation by *En1*^{cre} was achieved in an *Otx1*-null background (Acampora et al., 1996). Compared with conditional mutants, *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$; *Otx1*$^{-/-}$ embryos revealed at E10.5 an anterior shift of the ventral domain of *Fgf8* expression (Fig. 4B) up to the border with the domain expressing functional *Otx2* transcripts in the ventral pretectum (Fig. 4A). Surprisingly, the ventral domain of *Gbx2* expression at the MHB did not move rostrally into the ventral midbrain and rather it was lost (Fig. 4C), raising the issue on the time-competence in responding to the lack of Otx proteins. Importantly, transcription of the *Otx1* null allele (*lacZ*) (Acampora et al., 1996) was retained along the presumptive ventral midbrain (Fig. 4D). The dorsal expression of *Fgf8* and *Gbx2* was very similar in triple and conditional mutants. Besides supporting a role for Otx1 in controlling at least the ventral positioning of *Fgf8* expression, these findings suggest that the rostral shift of the ventral domain of *Gbx2* expression at the MHB is sensitive to the lack of Otx gene products prior to E9-9.5. Next, to assess whether in conditional and triple mutants *Otx2* was transcribed in the Otx2-depleted territory, we analysed its expression with an *Otx2* probe (*Otx2*-5′) unaffected by Cre activity. In both mutants, robust transcription of *Otx2* was detected with this probe in the territory where *Otx2* was inactivated (Fig. 3G,O,W; Fig. 4E). Together these data indicate that in conditional mutants the Otx2-depleted territory rostral to *Fgf8* expression exhibits a midbrain regional identity and similarly, in triple mutants the Otx-depleted territory, although caudal to *Fgf8* expression, still retains relevant midbrain molecular features (transcription of *Otx1* and *Otx2* null alleles and absence of *Gbx2* expression).

Otx antagonism on Shh expression

We have reported that maintenance of the *Shh* expression domain depends on dorsal antagonism exerted by Otx proteins on its expression in lateral midbrain, and proposed that this *Shh* antagonism might require functional interaction between Otx proteins and the co-repressor Grg4 (Puelles et al., 2003). We therefore studied whether *Otx2*-inactivation in ventral and lateral midbrain of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos was reflected in DV gene expression abnormalities. In conditional mutants at E10.5, *Shh* and *Foxa2* domains (Fig. 5D,E) were dorsally expanded within the Otx2-depleted neuroepithelium expressing *Otx1* and *Grg4* which, in turn, exhibited a weaker expression at their ventral domain (Fig. 5A-C); at E12.5, *Otx1* and *Grg4* domains (Fig. 5G,H) reset the presumptive alar-basal boundary (ABB) at the border of the *Shh* and *Foxa2* expanded domains (Fig. 5I,J). These data indicate that Otx2 is not required for maintenance of *Shh* expression in the ventral midbrain, rather it is important to antagonise the dorsal expansion of *Shh* and consequent increase in size of ventral midbrain. To support the possibility that Otx and Grg4

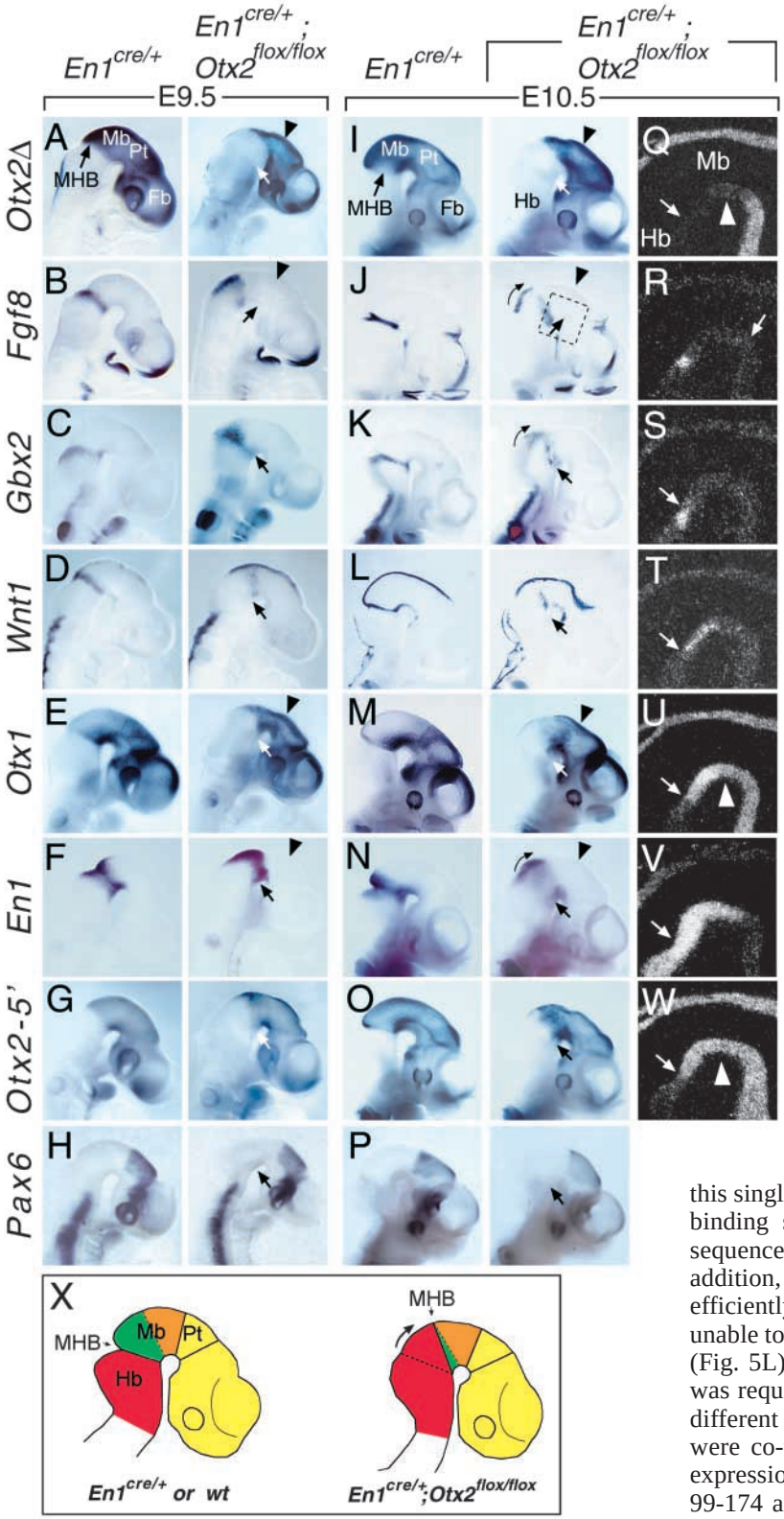

Fig. 3. MHB abnormalities in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos. (A-W) In situ hybridisation on E9.5 and E10.5 *En1*$^{cre/+}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos with *Otx2*Δ (A,I,Q), *Fgf8* (B,J,R), *Gbx2* (C,K,S), *Wnt1* (D,L,T), *Otx1* (E,M,U), *En1* (F,N,V), *Otx2*-5′ (G,O,W) and *Pax6* (H,P) probes. (X) Schematic representation summarising MHB abnormalities detected in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos. The parasagittal section in Q-W is focussed on the ventral midbrain of conditional mutants and approximately corresponds to the area included in the dotted square in J. In conditional mutant embryos, the arrows in A,C-H,I,K-Q,S-W indicate the corresponding position of the ventralmost expression of *Fgf8*, while the arrows in B,J,R indicate the corresponding position of the ventral and posterior border of the functional *Otx2* domain (*Otx2Δ*). The curved arrows in J,K,N,X indicates the dorsal and anterior rotation of the MHB whose ventral site remains in a fairly normal position; the arrowheads in A,B,E,F,I,J,M,N indicate the posterior border of *Pax6*; and the arrowheads in Q,U,W indicate the ventral midbrain area lacking functional *Otx2* transcripts (*Otx2Δ*) but still transcribing *Otx1* and *Otx2* (*Otx2-5′*). (X) Red, green, orange and yellow correspond to the hindbrain, posterior midbrain, anterior midbrain and forebrain, respectively. MHB, midbrain-hindbrain boundary; Pt, pretectum; Fb, forebrain; Mb, midbrain; Hb, hindbrain.

proteins may be responsible for the antagonism on *Shh/Foxa2* expression, we assessed in cell culture experiments whether Grg4 interacted with Otx proteins and modulate their transactivating ability. Otx1 and Otx2, like Bicoid and Goosecoid proteins (Desplan et al., 1988; Driever and Nusslein-Volhard, 1989; Hanes and Brent, 1991), bind to the *Bicoid target site* (*bts*) of the *Hunchback* promoter (Simeone et al., 1993). Strikingly, transactivating ability of Otx1 and Otx2 was strongly reduced by the addition of increasing amounts of the *Grg4* expressing plasmid (Fig. 5K). However, as the basal activity of the *bts* was undetectable, it could not be excluded that the repressive effect of Grg4 was contributed by a nonspecific negative effect on the *bts* reporter plasmid. To rule out this possibility, the lysine in position 50 of the Otx2 homeodomain was mutagenised to glutamine and this single amino acid substitution was sufficient to switch the binding specificity of Otx2 from the *bts* to the *np* target sequence (Desplan et al., 1988; Hanes and Brent, 1991). In addition, in this case the strong transactivation of Otx2 was efficiently suppressed by Grg4 and, moreover, Grg4 alone was unable to suppress the basal activity of the *np* reporter plasmid (Fig. 5L). To identify which part of Otx1 and Otx2 proteins was required for this cooperation, mutant molecules carrying different deletions of the *Otx1*- and *Otx2*-coding sequence were co-transfected alone or in combination with the *Grg4* expression plasmid. The *Otx1* molecule lacking amino acids 99-174 and the *Otx2* molecule lacking amino acids 102-208 did not respond to the Grg4 co-repressing activity (Fig. 5M,N). Detailed analysis of this region in Otx2 revealed that the sequence from amino acids 149 to 182 was required for cooperation with Grg4 (Fig. 5O). An almost identical sequence was identified also in the deleted region of the Otx1 mutant molecule not responding to Grg4 activity. To test whether Grg4 and Otx proteins may interact physically, Grg4-

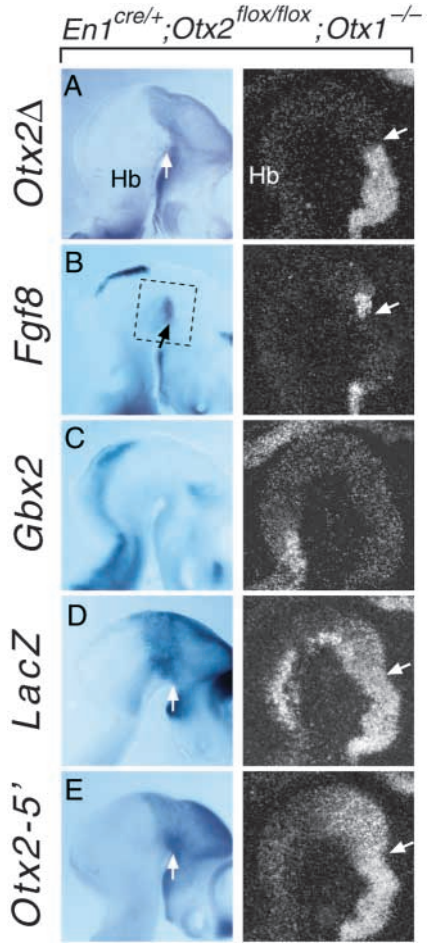

Fig. 4. *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$; *Otx1*$^{-/-}$ triple mutants show anterior shift of ventral expression of *Fgf8*. (A-E) In situ hybridisation on E10.5 *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$; *Otx1*$^{-/-}$ whole mount embryos and sections with *Otx2*Δ (A), *Fgf8* (B), *Gbx2* (C), *lacZ* (D) and *Otx2-5′* (E) probes. The sections are focussed on the ventral midbrain area approximately corresponding to the area included in the dotted square in B. The arrows in A,D,E indicate the anterior border of the *Fgf8* domain and the arrows in B indicate the ventral posterior border of the functional *Otx2* expression domain. Compared with *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos, the expression domains of *Fgf8* and functional *Otx2* are adjacent, while posterior to *Fgf8*, the presumptive midbrain yet exhibits transcription of *Otx1* (*lacZ*) and *Otx2* (*Otx2-5′*). Hb, hindbrain.

Otx co-immunoprecipitation assays were performed. In these experiments, Otx1 and Otx2 proteins were successfully co-immunoprecipitated by a Flag antibody specific for the Grg4-Flag protein (Fig. 5P) whereas the same antibody failed to co-immunoprecipitate Otx2 mutant proteins not responding to the Grg4 co-repressing activity (Fig. 5Q). Comparison between the Otx sequences sensitive to Grg4 and the Grg4 binding domain identified in insect and vertebrate transcription factors such as Six3, En, Pax, Nkx and Hairy-related proteins (Eberhard et al., 2000; Fisher et al., 1996; Fisher and Caudy, 1998; Muhr et al., 2001; Zhu et al., 2002) revealed significant homology (Fig. 5R). These results indicate that in cell culture experiments Grg4 may cooperate efficiently with Otx proteins to suppress their transactivating ability.

Otx2 controls the extent and identity of progenitor domains

To assess whether the lack of Otx2 affects the molecular code of progenitor domains in the ventral midbrain, we analysed the expression of Nkx6.1 and Nkx2.2. Compared with E10.5 and E12.5 control embryos, a drastic change of the Nkx expression domains was observed in conditional mutants. Indeed the ventral subventricular domain of Nkx6.1 was heavily reduced (Fig. 6B,E) and that of Nkx2.2 was ventrally enlarged (Fig. 6C,F) and overlapped the expanded domain of Shh (Fig. 6A,D). However, Nkx2.2 was excluded from the floorplate. The expression pattern described for Shh and Nkx genes in the ventral midbrain of conditional mutants showed an evident similarity with that exhibited in the rostral hindbrain of normal embryos (Fig. 6G-I). To assess whether the Nkx abnormal expression is due to the lack of Otx2 or to the overexpression of *Shh* or to both, we revisited the expression of Nkx2.2 and Nkx6.1 in conditional mutants inactivating *Otx2* by *Otx1*-driven Cre activity. In *Otx1*$^{cre/+}$; *Otx2*$^{flox/-}$ embryos, Otx2 was retained in the ventral midbrain (Puelles et al., 2003) and Shh expression was dorsally expanded similarly to what is observed in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ (Fig. 6J). Compared with control embryos and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants, in *Otx1*$^{cre/+}$; *Otx2*$^{flox/-}$ embryos the expression of Nkx6.1 was lost and that of Nkx2.2 was not ventrally expanded (Fig. 6K,L). These data indicate that in the ventral midbrain of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants, independent of the expression of *Shh*, the lack of Otx2 is reflected in ventral derepression of Nkx2.2, while Otx2-dependent expanded expression of Shh results in downregulation of Nkx6.1. Moreover, our findings are consistent with the possibility that Otx2 controls cell-autonomously Nkx2.2 and non-cell autonomously Nkx6.1.

Altered fate in the ventral midbrain of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants

To study whether neuronal fate was affected, we analysed the generation of RN, OM and DA neurons by specific markers such as *Pou4f1* (also known as *Brn3a*), *Isl1* and *Th*, respectively (Agarwala and Ragsdale, 2002; Puelles et al., 2003). Compared with sagittal view focussed on the ventral midbrain of E12.5 *En1*$^{cre/+}$ whole embryos, conditional mutants showed that rostral to the *Fgf8* expression at the ventral MHB (Fig. 7A), *Pou4f1*- and *Th*-positive cells were severely reduced in the midbrain and retained only rostrally in the presumptive ventral pretectum (Fig. 7B,C); conversely, the position, number and projection (2H3-positive axons) of *Isl1*-positive cells of the OM nucleus were not obviously affected (Fig. 7D,F). Frontal sections comparing the distribution of Pou4f1, Th and Isl1 proteins (Fig. 7I-K) to that of Nkx2.2 and Nkx6.1 (Fig. 7G,H) showed that in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos, the ventral neuroepithelium where Nkx2.2 was expanded, failed to generate Pou4f1-positive neurons and retained a fairly normal generation of Isl1-positive neurons (Fig. 7I,K), while DA neurons were confined to the residual floor plate region negative for Nkx2.2 (compare Fig. 7J with 7H). This suggests that the reduction of Th neurons should reflect a corresponding reduction in the extent of the DA progenitor domain because of the ventral expansion of the Nkx2.2 domain. As the expression code described in the ventral midbrain of conditional mutants for Shh, Nkx2.2 and Nkx6.1 (Fig. 6A-F) was similar to that normally exhibited by

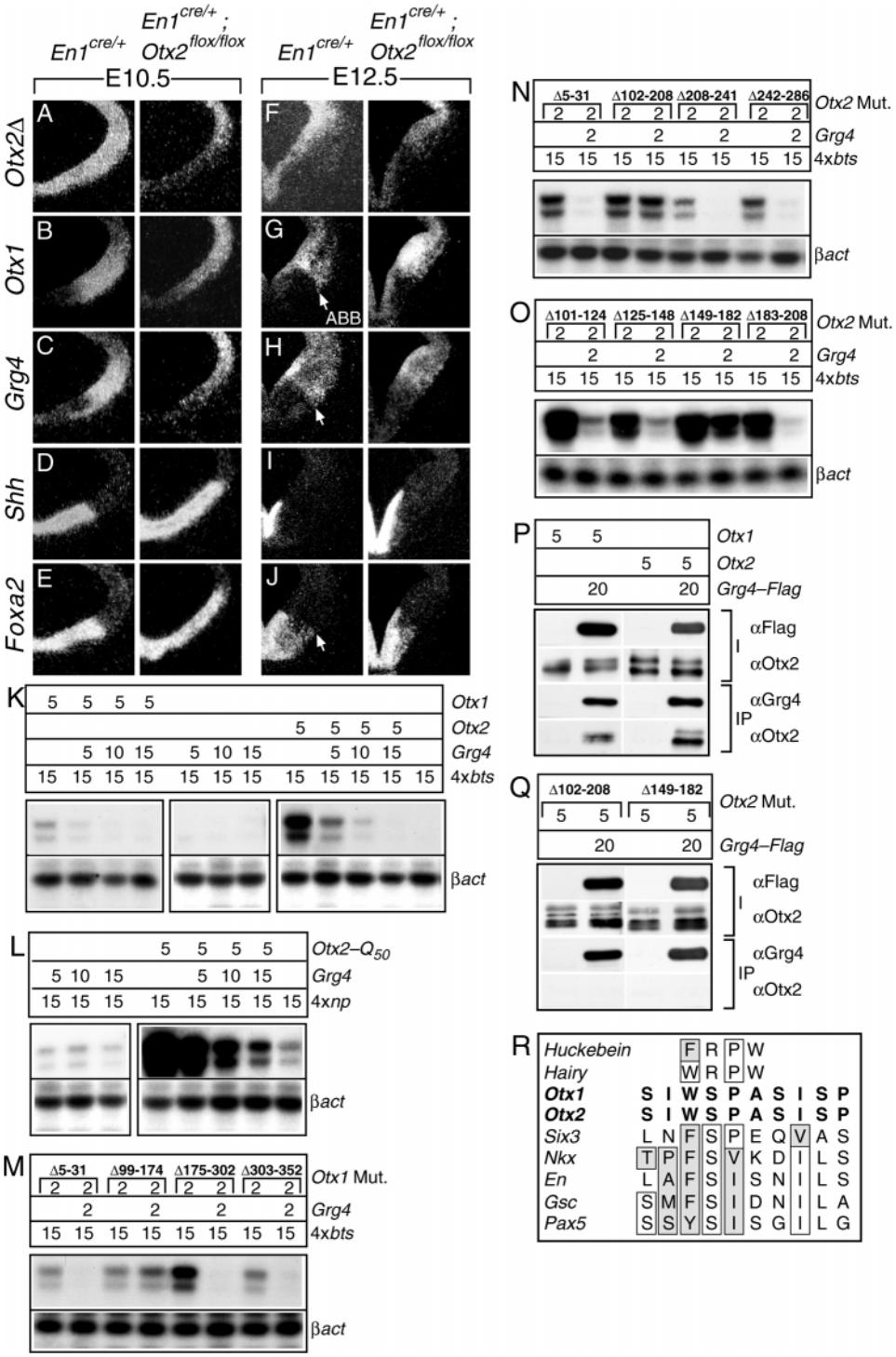

Fig. 5. Dorsal expansion of *Shh/Foxa2* expression and Grg4-mediated suppression of Otx trans-activating ability. (A-J) In situ hybridisation of E10.5 and E12.5 *En1*$^{cre/+}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos with *Otx2*Δ (A,F), *Otx1* (B,G), *Grg4* (C,H), *Shh* (D,I) and *Foxa2* (E,J) probes. (K,L) RNAse protection assays showing the trans-activating ability of Otx1, Otx2 and Otx2-Q_{50} alone or in combination with *Grg4* expressing plasmid on the multimerised (4×) *bts* (K) and *np* (L) target sequence. (M-O) *Otx1* (M) and *Otx2* (N,O) mutant molecules carrying the amino acid deletions (Δ) indicated are assayed alone or in combination with the *Grg4*-expressing plasmid. (P,Q) Co-immunoprecipitation assays between Grg4-Flag and Otx1 or Otx2 (P) and between Grg4-Flag and Otx2 mutant proteins not responding to Grg4-corepression (Q) show that, when co-transfected with the *Grg4-Flag* expressing vector, the Flag antibody co-immunoprecipitates Grg4-Flag and Otx1 or Otx2 (P), while it fails to co-immunoprecipitate Grg4-Flag and Otx2 mutant proteins (Q). (R) Comparison between the Otx1 and Otx2 amino acid domain (bold) responding to Grg4 and those known for other transcription factors reveals conservative substitutions (grey box) or identity (white box). The *β-act* indicates that a similar amount of total RNA is analysed for each transfection. Numbers indicate the DNA amount in micrograms transfected for each plasmid. ABB, alar-basal boundary; I, input; IP, immunoprecipitation.

the rostral hindbrain (Fig. 6G-I; Fig. 7M-R), we assayed whether Ser neurons were induced rostral to *Fgf8*. Strikingly, a relevant number of neurons expressing *Pet1*, an early serotonergic marker (Hendricks et al., 1999; Pfaar et al., 2002), or positive for 5-hydroxytrytamine (5-HT) was observed in this area (Fig. 7E,L) and, as in the rostral hindbrain (Fig. 7N,R), these Ser neurons differentiated from the neuroepithelium co-expressing Shh and Nkx2.2 (Fig. 6D,F, and bracket in Fig. 7H,L). Notably, their position and complementarity to OM neurons were very similar to those normally exhibited by RN neurons. To strengthen the finding that in conditional mutants, Ser neurons were generated in the area lacking functional *Otx2* transcripts and anterior to the *Fgf8* expression, we compared in horizontal sections the expression domains of *Otx2Δ*, *Fgf8*, *Th* and the 5-HT transporter (*Sert*; Slc6a4 – Mouse Genome Informatics), a third serotonergic marker (Fig. 7S-V). *Sert*-positive neurons (Fig. 7V) were detected anterior to *Fgf8* (Fig. 7T) and in the *Otx2Δ* negative territory (Fig. 7S), while *Th* neurons were remarkably reduced in number (Fig. 7U). This pattern of differentiation was stably retained at later stages except for a reduction of OM neurons (Fig. 8). Importantly, no staining for Pou4f1 and only a very few Th-positive neurons were detected in conditional mutants at E10.75 and E11.5 when, normally, RN and DA neurons began to be detectable (data not shown). Moreover, cell death

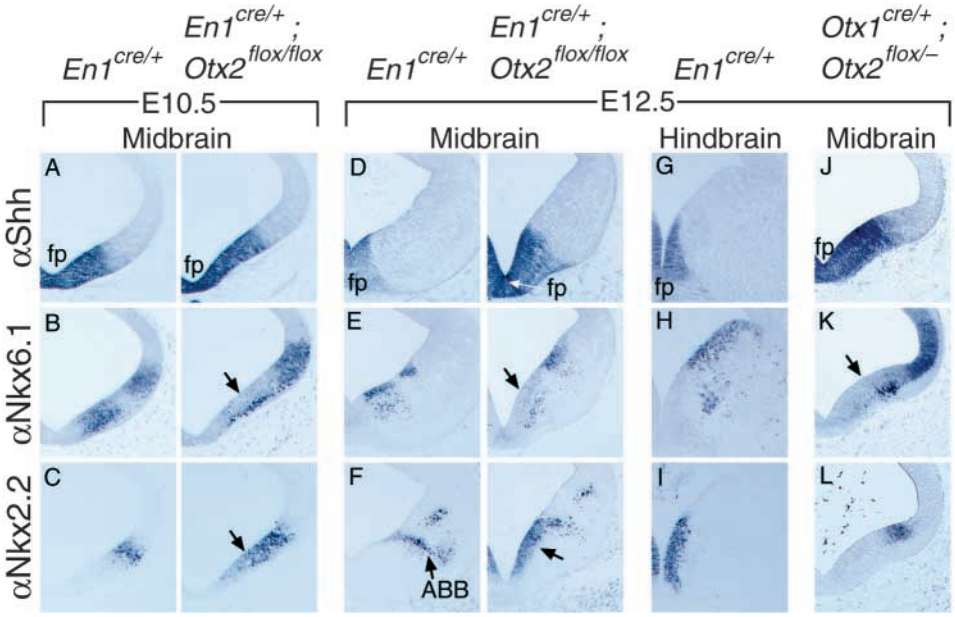

Fig. 6. Lack of Otx2 and increased expression of Shh affect the expression of Nkx6.1 and Nkx2.2. (A-L) Shh (A,D,G,J), Nkx6.1 (B,E,H,K) and Nkx2.2 (C,F,I,L) immunodetection in the ventral midbrain of E10.5 and E12.5 *En1*$^{cre/+}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos (A-F), in the rostral hindbrain of E12.5 *En1*$^{cre/+}$ embryos (G-I), and in the ventral midbrain of E12.5 *Otx1*$^{cre/+}$; *Otx2*$^{flox/–}$ embryos (J-L). The arrows in B,E,K indicate the ventral neuroepithelium where Nkx6.1 expression is lost, and the arrows in C,F indicate the ventral expansion of the Nkx2.2 domain. fp, floor plate; ABB, alar-basal boundary.

analysed at E10.5, E11.5 and E12.5 revealed no difference in TUNEL staining between control and conditional mutants (data not shown). Together these data strongly suggest that lack of RN and reduction of DA neurons depend on abnormal cell fate commitment of their progenitors rather than on cell survival. We have shown that triple mutants differ from *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos primarily in the position occupied by *Fgf8* with respect to the midbrain. Interestingly, in triple mutants, the Shh and Nkx expression patterns as well as the differentiation of ventral precursors were similar to those described in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos (Fig. 7W-β and data not shown) suggesting that, in the absence of Otx (after E9) and Gbx2 gene products, the differentiation program of the ventral midbrain is insensitive to the positioning of Fgf8 signaling.

Discussion

Otx and Shh/Fgf8 functional intersections coordinate regional and neuronal patterning of the midbrain

In this study we focussed our attention on the role of Otx2 in the generation of neuronal cell types of the ventral midbrain. This important process is the final step of a complex series of morphogenetic events requiring sequential intersections between Otx and Shh/Fgf8 functions. These functional intersections coordinate the extent, identity and fate of midbrain subregions (e.g. dorsal versus ventral and rostral versus caudal midbrain) and, within them, those of ventral progenitor domains. Although in conditional mutants a rostral shift of the MHB was expected, we have observed only dorsally an anterior rotation of its DV axis. This MHB rotation describes a dorsal arc of about 45° along the caudal midbrain. The dorsolateral midbrain posterior to this shift is respecified into cerebellum. Moreover, the analysis of the triple mutant has indicated that, at least after E9.5, Otx1 alone is sufficient to maintain the positioning of the ventral site of the MHB and antagonise the anterior shift of *Fgf8* expression. Surprisingly, in triple mutants the ventral expression of *Gbx2* did not extend rostrally in the midbrain, unlike the expression of *Fgf8*. Possibly as a consequence of this, the ventral midbrain, although now caudal to the *Fgf8* source, retained expression of *Otx1* and *Otx2* null alleles. These findings provide novel functional information on the control exerted by Otx genes on MHB positioning and *Fgf8* expression after E9.5. Indeed this control is not uniform along the DV axis of the MHB and, owing to the cooperative effect of Otx1 and Otx2, it appears more efficient lateroventrally. This suggests that at E9.5, the identity of dorsal midbrain may be still flexible. Our analysis also suggests that *Gbx2* expression in the ventral midbrain is prevented after E9.5 by an Otx2-independent negative mechanism. Indeed our data suggest that failed anteriorisation of ventral expression of *Gbx2* is insensitive to the lack of Otx1 and Otx2 proteins after E9.5. However, this is not in contrast with previous data showing rostral shift of the ventral domain of *Gbx2* and in all these cases Otx proteins are lost before E9.5 (Simeone et al., 2002). Interestingly, in the conditional mutant inactivating *Gbx2* by *En1*-driven Cre recombinase, *Otx2* expression was slightly expanded and only on the dorsal side of rhombomere 1, even though Gbx2 was ablated from the entire rhombomere (Li et al., 2002). One possible explanation, as suggested by the authors, is the existence of a Gbx2-independent negative and late (after 8 somite stage) regulation of *Otx2* expression in the rostral hindbrain. Therefore, our findings and those previously reported (Li et al., 2002) strongly suggest that the identity of ventral midbrain and rostral hindbrain is maintained after E9-E9.5 through an Otx- and Gbx2-independent negative control of *Gbx2* and *Otx2* expression, respectively.

A second relevant feature of *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ mutants is represented by the dorsal expansion of *Shh* expression in response to the *Otx2* inactivation in lateral and ventral midbrain. A similar expansion of the Shh domain has been reported recently in embryos with reduced level of Otx1 and Otx2 in the lateral midbrain (Puelles et al., 2003). Apart from confirming these previous findings, the data reported here

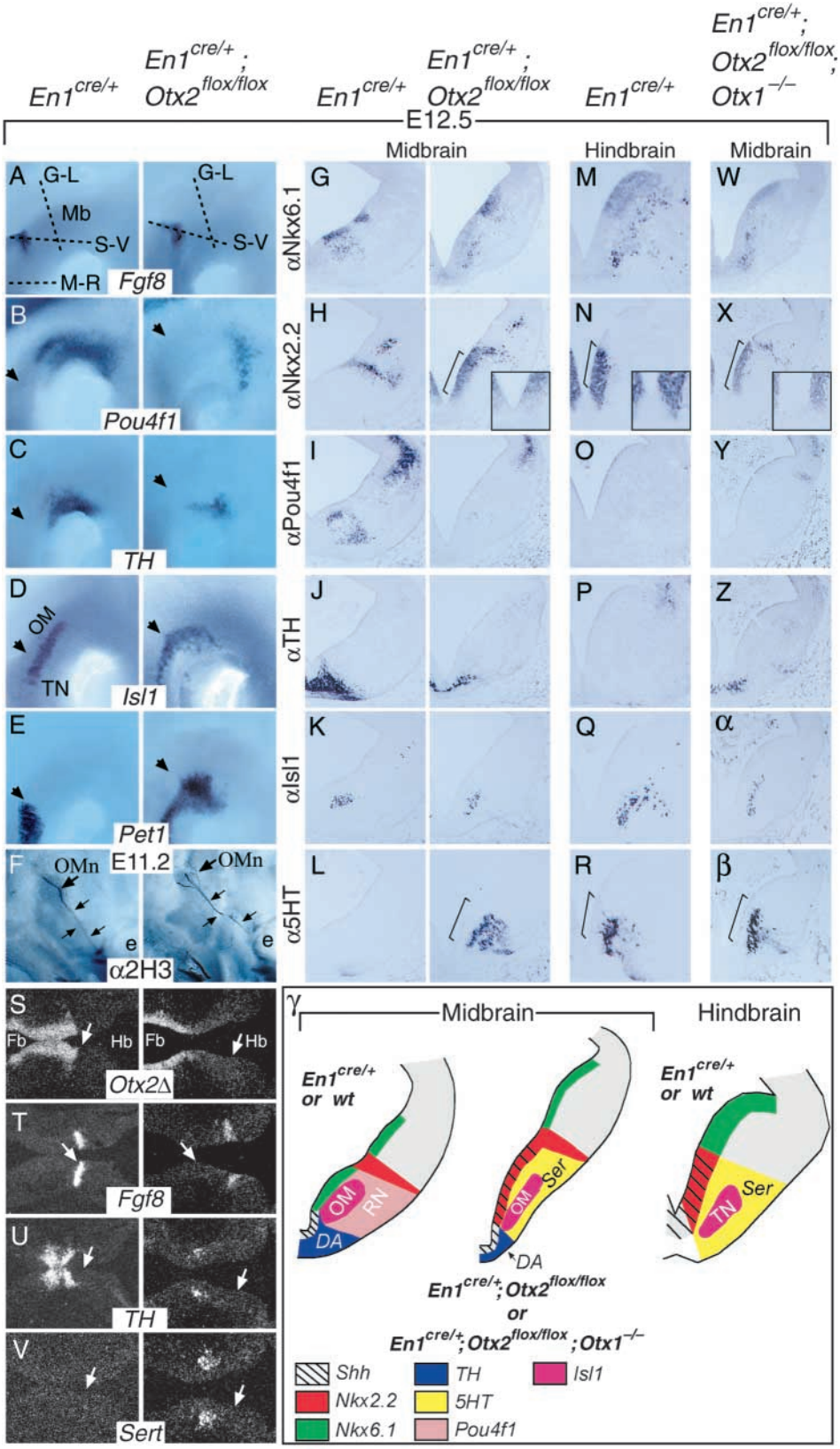

Fig. 7. Abnormalities in extent, identity and fate of progenitor domains. (A-F) In situ hybridisation (A-E) and immunohistochemistry (F) on whole-mount embryos focussing on sagittal view of the ventral midbrain of *En1*$^{cre/+}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos at E12.5 and E11.2 with *Fgf8* (A), *Pou4f1* (B), *Th* (C), *Isl1* (D), *Pet1* (E) probes and 2H3 (F) antibody. (G-β) Immunohistochemistry (G-R,W-β) and in situ hybridisation (S-V) at E12.5 in the midbrain of *En1*$^{cre/+}$, *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$; *Otx1*$^{-/-}$ embryos and in the rostral hindbrain (M-R) of *En1*$^{cre/+}$ embryos with Nkx6.1 (G,M,W), Nkx2.2 (H,N,X), Pou4f1 (I,O,Y), Th (J,P,Z), Isl1 (K,Q,α) and 5-HT (L,R,β) antibodies and with *Otx2Δ* (S), *Fgf8* (T), *Th* (U) and *Sert* (V) probes. (γ) Schematic representation summarising the expression pattern of Shh, Nkx6.1 and Nkx2.2 and neuronal cell types detected in control and mutant embryos. The broken lines in A indicate the approximate position of frontal (G-L) and horizontal (S-V, M-R) sections; the position of frontal sections of the triple mutant (W-β) are at a level similar to that of the conditional mutant, but in the triple mutant this position is posterior to *Fgf8* expression; the arrowheads in B-E and the arrows in S,U,V indicate the corresponding position of *Fgf8* expression and the arrows in T indicate the posterior border of functional *Otx2* domain; the inset in H,N,X highlights the ventral expression of Nkx2.2; the bracket demarcates the neuroepithelium expressing Nkx2.2 and generating Ser neurons in the midbrain of mutant embryos (H,L,X,β) and in the rostral hindbrain of control embryos (N,R). OM oculomotor nucleus; RN, red nucleus; Fb, forebrain; Mb, midbrain; Hb, hindbrain; TN, trochlear nucleus; OMn, oculomotor nerve; e, eye; DA, dopaminergic cells; Ser, serotonergic cells.

indicate that Otx2 is not required to regulate *Shh* expression in the floor-plate region ventral to the ABB. Moreover, we provide evidence that the Grg4-co-repressor may interact with Otx proteins to attenuate their transactivating ability. Together with expression data, these findings strengthen the possibility that dorsal antagonism on *Shh/Foxa2* expression at the ABB may require direct or indirect interaction with the Otx-Grg4 repressing complex. Grg4 is able to interact with different classes of transcription factors including Pax and Nkx homeodomain proteins (Muhr et al., 2001; Ye et al., 2001). Thus, our findings provide further support for the general idea that a combinatorial series of interactions between co-repressor molecules and transcription factors belonging to different gene families define a sophisticated regulatory network controlling

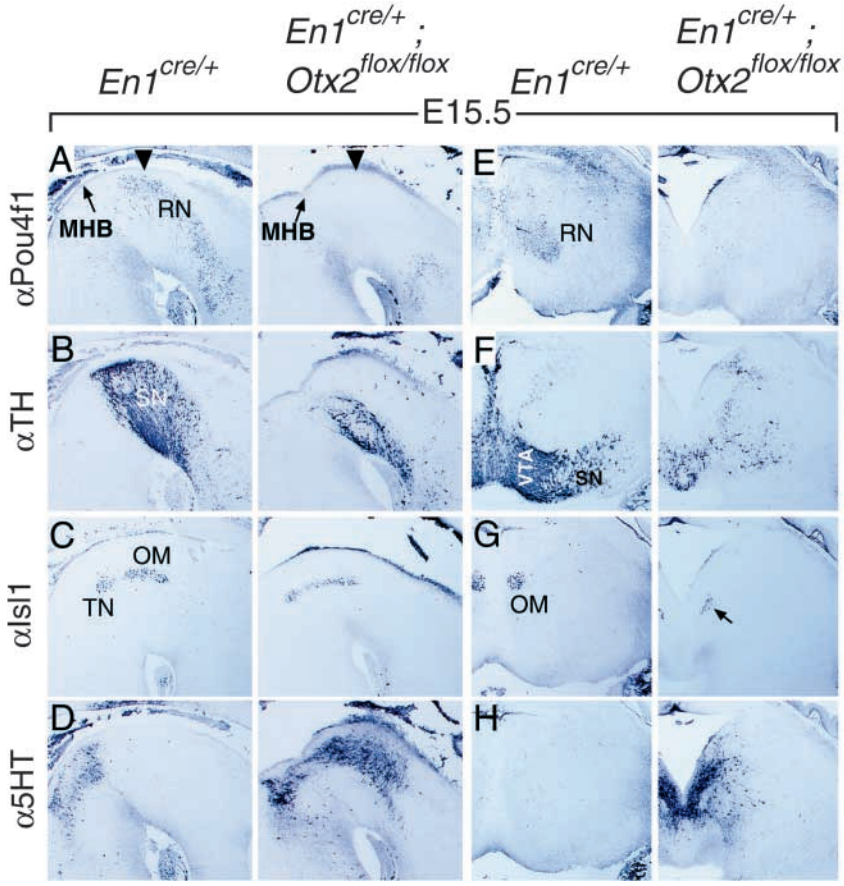

Fig. 8. Abnormal differentiation is maintained at later stages in the ventral midbrain of conditional mutants. (A-H) Immunohistochemistry in sagittal (A-D) and frontal (E-H) sections of E15.5 control and conditional mutant embryos with Pou4f1 (A,E), Th (B,F), Isl1 (C,G) and 5-HT (D,H) antibodies. The arrowheads in A indicate the position of frontal sections and the arrow in G indicates the residual Isl1-positive neurons. RN, red nucleus; SN, substantia nigra; VTA, ventral tegmental area; MHB, midbrain-hindbrain boundary; TN, trochlear nucleus; OM, oculomotor nucleus.

the transcription of signaling molecules and cellular determinants.

The role of Otx2 in formation and maintenance of ventral progenitor domains

Studies on Shh and Fgf8 have provided crucial information for understanding molecular events controlling the sequential steps of neuronal development (Agarwala et al., 2001; Briscoe and Ericson, 2001; Hynes and Rosenthal, 1999; Jessell, 2000).

In the spinal cord and hindbrain, graded distribution of Shh activity is interpreted by class II Nkx factors, which, in turn, are crucial intermediaries in the assignment of the identity and fate of neuronal progenitor domains (Briscoe et al., 1999; Briscoe et al., 2000; Sander et al., 2000). Interestingly, in the spinal cord, rostral hindbrain and midbrain the molecular code defined by Shh and Nkx expression patterns is not uniform but exhibits a characteristic, regionally restricted profile. In particular, in the rostral hindbrain Nkx2.2 is co-expressed with Shh and is ventral to the Nkx6.1 domain, while in the ventral midbrain the Nkx6.1 domain is located between that expressing Shh and that positive for Nkx2.2 (Fig. 7γ). Adjacent to the MHB, Shh and Fgf8 signaling activities induce and position the Ser and DA neuronal populations in the rostral hindbrain and in the midbrain, respectively (Hynes and Rosenthal, 1999; Ye et al., 1998). Ser neurons originate from the progenitor domain expressing Shh and Nkx2.2, while most if not all DA neurons arise from the ventralmost neuroepithelium positive only for Shh. This suggests that the different expression code of these two progenitor domains may be relevant in the establishment of the Ser and DA neuronal phenotype. Indeed, it has been shown that Nkx2.2 is essential for the coordinated generation of hindbrain Ser neurons (Briscoe et al., 1999; Pattyn et al., 2003).

Therefore, a crucial issue was to elucidate the regulatory mechanism(s) and factor(s) controlling the identity code of midbrain and hindbrain progenitor domains. Our study provides in vivo evidence that Otx2 is a major genetic determinant of this process in the ventral midbrain. Indeed, lack of Otx2 from E9.5 produces relevant abnormalities in the expression pattern of Shh, Nkx6.1 and Nkx2.2. This event generates a major change in the identity and fate of DA and RN progenitors, which, in turn, exhibit a molecular code similar to that observed in the rostral hindbrain (Fig. 7γ). This strongly suggests that Otx2 is required to provide midbrain neuronal precursors with a specific differentiation code suppressing that of the anterior hindbrain. To perform this role, Otx2 exerts a dual control; that is, repression of Nkx2.2 in the ventral midbrain and maintenance of the Nkx6.1 expression domain through dorsal antagonism on Shh expression. Failure of this dual control in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos affects identity and fate of dorsal DA and RN neuronal precursors which, as in the hindbrain, co-express Shh and Nkx2.2 and generate Ser neurons (Fig. 7γ). However, in *Otx1*$^{cre/+}$; *Otx2*$^{flox/–}$ embryos, failed antagonism on Shh expression and consequent lack of Nkx6.1 generates a remarkable increase of DA neurons (Puelles et al., 2003) because in this case presumptive RN precursors (normally positive for Nkx6.1 and negative for Shh and Nkx2.2) acquire the identity and fate of presumptive DA progenitors (positive for Shh and negative for Nkx6.1 and Nkx2.2). Therefore, in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos where Otx2 is inactivated in ventral and lateral midbrain, progenitor domains undergo an anterior into posterior change of identity and fate, while in *Otx1*$^{cre/+}$; *Otx2*$^{flox/-}$ mutants (Puelles et al., 2003), where Otx2 is inactivated only in the lateral midbrain, they undergo a dorsal into ventral transformation.

In *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos, OM neurons are not severely affected and the DA cell type is never completely abolished. For OM neurons, a likely explanation is based on the fact that, as revealed by BrdU experiments and Isl1 immunodetection (data not shown), this neuronal cell type is generated quite early (between E9.5 and E10) and, therefore should not be severely affected by the Otx2 inactivation. For

midbrain DA, our data suggest that the ventralmost fraction of DA precursors is excluded from the Nkx2.2 ventralisation, and thereby retains its proper identity and fate. Why these neuronal precursors are not permissive to express Nkx2.2 remains to be determined. Complete suppression of the DA phenotype is observed only in Otx mutants exhibiting full transformation of midbrain into rostral hindbrain and coordinated anterior shift of both MHB and expression of *Fgf8* and *Gbx2* at early somite stage (Acampora et al., 1997; Brodski et al., 2003). Finally, in conditional and triple mutants, the presumptive ventral midbrain showed a similar and abnormal differentiation program, regardless of the site of *Fgf8* expression. Together, these data indicate that Fgf8- and Shh-inducing signals require Otx2 in the ventral midbrain to be properly interpreted. This suggests that Otx2 should play a crucial role in the establishment of the cellular competence to respond to these inducing signals. This supports the idea that midbrain-polarised activity of Shh and Fgf8 depends on the molecular identity of the responding tissue and might not represent an intrinsic property of these inducing molecules.

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