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

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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 despite 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; 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; Puellés 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 (En1cre (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 En1cre/+; Otx2fl/fl 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 of Shh, Nkx2.2 and Nkx6.1, which define an expression code...
Results

En1\textsuperscript{cre} mediated inactivation of the Otx2\textsuperscript{flox} allele

The generation of Otx2\textsuperscript{flox} and En1\textsuperscript{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\textsuperscript{flox} allele by comparing the expression of En1\textsuperscript{cre} mRNA with both Otx2 mRNA and protein by using a specific Otx2 probe (Otx2\textsubscript{D}) which detected only unfloxed Otx2 transcripts, and an antibody against Otx2 (\alpha Otx2) (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\textsuperscript{cre}; Otx2\textsuperscript{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\textsuperscript{cre}+; Otx2\textsuperscript{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\textsuperscript{cre}+; Otx2\textsuperscript{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. 3G). At E10.5 (around 35 somites), the Fgf8 and Gbx2 expression at the MHB (Fig. 3I,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 Otx2\textsubscript{D}-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...
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 En1cre was achieved in an Otx1-null background (Acampora et al., 1996). Compared with conditional mutants, En1cre/+; Otx2flox/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 (Puõelles et al., 2003). We therefore studied whether Otx2-inactivation in ventral and lateral midbrain of En1cre/+; Otx2flox/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. 5L,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...
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. To test whether Grg4 and Otx proteins may interact physically, Grg4-
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 Otx1cre+/−; Otx2flox/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 En1cre/+; Otx2flox/flox (Fig. 6J). Compared with control embryos and En1cre+/−; Otx2flox/flox mutants, in Otx1cre+/−; Otx2flox/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 En1cre+/−; Otx2flox/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 En1cre+/−; Otx2flox/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 En1cre+/− 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 En1cre+/−; Otx2flox/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. 7L). 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
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-hydroxytryptamine (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 Otx2D, 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

The SI specifies conditions that simulate the 'time-evolving' nature of the system, facilitating the exploration of the model's dynamic responses. The SI offers a comprehensive survey of the system's configurations and outcomes under varied conditions, enabling a deeper understanding of its underlying mechanisms. The SI is instrumental in elucidating the model's behavior across different scenarios, allowing for a more nuanced and refined analysis of the system's properties and potential applications.
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<sup>cre/+;</sup> Otx2<sup>flox/flox</sup> 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<sup>cre/+;</sup> Otx2<sup>flox/flox</sup> 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<sup>cre/+;</sup> Otx2<sup>flox/flox</sup> 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
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...
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. 7). 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 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 En1cre/+; Otx2flox/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. 7). However, in Otx1cre/+; Otx2flox−/− 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 En1cre/+; Otx2flox/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 Otx1cre/+; Otx2flox−/− mutants (Puelles et al., 2003), where Otx2 is inactivated only in the lateral midbrain, they undergo a dorsal into ventral transformation.

In En1cre/+; Otx2flox/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

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**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.
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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