Development/Plasticity/Repair

Otx2 Regulates Subtype Specification and Neurogenesis in the Midbrain

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The transcription factor Otx2 is required to determine mesencephalic versus metencephalic (cerebellum/pons) territory during embryogenesis. This function of *Otx2* primarily involves positioning and maintaining the mid-hindbrain organizer at the border between midbrain and anterior hindbrain. *Otx2* expression is maintained long after this organizer is established. We therefore generated conditional mutants of *Otx2* using the Cre/*loxP* system to study later roles during rostral brain development. For inactivation of *Otx2* in neuronal progenitor cells, we crossed *Otx2*$^{flox/flox}$ animals with *Nestin-Cre* transgenic animals. In *Nestin-Cre/+; Otx2*$^{flox/flox}$ embryos, Otx2 activity was lost from the ventral midbrain starting at embryonic day 10.5 (E10.5). In these mutant embryos, the mid-hindbrain organizer was properly positioned at E12.5, although Otx2 is absent from the midbrain. Hence, the *Nestin-Cre/+; Otx2*$^{flox/flox}$ animals represent a novel mouse model for studying the role of *Otx2* in the midbrain, independently of abnormal development of the mid-hindbrain organizer.

Our data demonstrate that *Otx2* controls the development of several neuronal populations in the midbrain by regulating progenitor identity and neurogenesis. Dorsal midbrain progenitors ectopically expressed *Math1* and generate an ectopic cerebellar-like structure. Similarly, *Nkx2.2* ectopic expression ventrally into tegmentum progenitors is responsible for the formation of serotonergic neurons and hypoplasia of the red nucleus in the midbrain. In addition, we discovered a novel role for *Otx2* in regulating neurogenesis of dopaminergic neurons. Altogether, these results demonstrate that *Otx2* is required from E10.5 onward to regulate neuronal subtype identity and neurogenesis in the midbrain.

Key words: **Otx2; midbrain; cerebellum; neuronal identity; dopaminergic neurons; serotonergic neurons; conditional inactivation**

Introduction

Specification of neuronal fates in the mesencephalic–metencephalic (mes-met) region begins with the acquisition of anterior–posterior (A-P) and dorsal–ventral (D-V) identities instructed by signals from underlying tissues and subsequently from organizing centers within the CNS (for review, see Lumsden and Krumlauf, 1996; Jessell, 2000). The mid-hindbrain (MHB) organizer, marked by the expression of *Fgf8* and *Wnt1* at the isthmic constriction, is crucial for patterning and growth of the mes-met region (for review, see Simeone, 2000; Liu and Joyner, 2001; Nakamura, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Echevarria et al., 2003; Raible and Brand, 2004). Mesencephalic and metencephalic progenitors are subsequently programmed by D-V signals from the roof plate and floor plate, including Bmps and Shh (Tanabe and Jessell, 1996). These cell–cell signaling events ultimately result in progenitors expressing distinct codes of transcription factors that endow them with the capacity to develop into distinct types of neurons.

Mes-met specification begins with the induction of gene expression that distinguishes the mesencephalon from the metencephalon. Loss of function of *Otx2* and *Gbx2* in mice indicates that these genes are required cell intrinsically for specification of the midbrain and cerebellum, respectively (Wassarman et al., 1997; Rhinn et al., 1998). In addition, *Otx2* and *Gbx2* act antagonistically in the mes-met region to position the MHB organizer (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). In

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contrast to *Otx2*, other transcription factors required for development of the mes-met region, such as the homeobox-containing genes *Pax2*, *Pax5*, *En1*, and *En2*, are required for the development of both the midbrain and cerebellum (Wurst et al., 1994; Hanks et al., 1995; Schwarz et al., 1997; Bouchard et al., 2000). Hence, among the critical mes-met determination genes, only *Otx2* is specifically required for the development of the mesencephalon.

To investigate later roles of *Otx2* in the mes-met region, we generated a conditional mouse mutant of *Otx2* using the Cre/*loxP* system and inactivated *Otx2* activity starting at embryonic day 10.5 (E10.5). Our studies revealed later roles for *Otx2* in regulating neuronal identity and neurogenesis that are distinct from its earlier role in A-P patterning and in positioning the MHB organizer. Strikingly, an ectopic cerebellar-like structure developed at the position of the colliculi that is preceded by changes in expression of genes involved in cerebellar development. Similar changes in cell fate occur in the ventral midbrain in which ectopic serotonergic neurons developed rostral to *Fgf8* expression, likely at the expense of Pou4f1$^+$ red nuclei (RN) neurons. In addition, hypoplasia of midbrain dopaminergic (DA) neurons is attributable to reduced *Mash1* and *Ngn2* and consequently decreased neurogenesis from the ventral midline. These results showed that some midbrain progenitors in *Otx2* conditional knock-out (*Otx2-CKO*) embryos adopt a hindbrain differentiation program (cerebellar granule and serotonergic neurons). Altogether, these data demonstrate novel roles for *Otx2* from E10.5 onward in regulating general neuronal and subtype differentiation program in the midbrain.

Materials and Methods

Generation and genotyping of mutants embryos and animals. Nestin-Cre transgenic and the *Otx2*$^{flox/flox}$ mouse strains were maintained in an outbred MF1 background. The *Nkx2.2* mutant strain was maintained in a mixed MF1-C57BL/6 background. To obtain conditional *Otx2* mutants, we crossed *Nestin-Cre* transgenic mice (Isaka et al., 1999) to animals homozygous for the *Otx2*^{flox} allele (Puelles et al., 2003). *Nestin-Cre/+*; *Otx2*$^{+/flox}$ male animals were then mated to *Otx2*$^{flox/flox}$ females. *Nestin-Cre/+*; *Otx2*$^{+/flox}$ males were also mated with *Nkx2.2*$^{+/-}$ females to generate *Nestin-Cre/+*; *Otx2*$^{+/flox}$; *Nkx2.2*$^{+/-}$ male animals. Simultaneously, *Otx2*$^{flox/flox}$ males were mated with *Nkx2.2*$^{+/-}$ females to generate *Otx2*$^{+/flox}$; *Nkx2.2*$^{+/-}$ female animals. *Nestin-Cre/+*; *Otx2*$^{flox/flox}$; *Nkx2.2*$^{-/-}$ mutants were obtained by crossing *Nestin-Cre/+*; *Otx2*$^{+/flox}$; *Nkx2.2*$^{+/-}$ males with *Otx2*$^{+/flox}$; *Nkx2.2*$^{+/-}$ or *Otx2*$^{flox/flox}$; *Nkx2.2*$^{+/-}$ females. The *Otx2*^{flox} allele was detected by PCR (Puelles et al., 2003), whereas the *Cre* transgene was detected by using a pair of primers (5′ ATC CGA AAA GAA AAC GTT GA 3′ and 5′ ATC CAG GTT ACG GAT ATA GT 3′) and PCR as described by Indra et al. (1999). A null mutation in the *Nkx2.2* gene was generated by eliminating the entire coding region (Sussel et al., 1998). For genotyping of *Nkx2.2* alleles, two sets of PCR primers were used. To detect the wild-type allele, a pair of primers (forward, 5′-CCC CCA GTA CCC GAC AGC ACA-3′; reverse, 5′-GGG GCC GGT TGG TCC TTT CTC-3′) and the PCR program includes an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min. To detect the mutant allele, a pair of primers (forward, 5′-AGA GGC TAT TCG GCT ATG ACT-3′; reverse, 5′-CCT GAT CGA CAA GAC CGG CTT-3′) and the PCR program includes an initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min. At all times, animals were handled according to the Society of Neuroscience Policy on the Use of Animals in Neuroscience Research, as well as the European Communities Council Directive.

Histological analysis. Postnatal brains were fixed in Bouin's fixative solution for 48 h at room temperature and then stored in 70% alcohol until embedding in paraffin. Paraffin sections (7 μm) were cut on a microtome and counterstained with hematoxylin–eosin or cresyl violet.

Whole-mount in situ *hybridization,* in situ *hybridization, and immunohistochemistry of brain sections.* Embryos or dissected brains were fixed for 2 h at 4°C in 4% paraformaldehyde in 0.1 M PBS and either stored in methanol at −20°C or cryoprotected with 30% sucrose in PBS, embedded in OCT compound (VWR International, Poole, UK), and cryosectioned on a cryostat (CM3050S; (Leica, Nussloch, Germany). Section and whole-mount *in situ* hybridization were performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993 and Conlon and Herrmann, 1993, respectively). The following mouse antisense RNA probes have been used: *Otx1* (Puelles et al., 2003), *Otx2*Δ (Puelles et al., 2003), *Otx2* (Rhinn et al., 1998), *Fgf8* (Crossley and Martin, 1995), *Wnt1* (Bally-Cuif et al., 1995), *Gbx2* (Bouillet et al., 1995), *Shh* (Echelard et al., 1993), *Patched1* (*Ptch1*) (Puelles et al., 2003), *Gli1* (Hui et al., 1994), *Pax3* (Goulding et al., 1991), *Gdf7* (Puelles et al., 2003), *Msx1* (Hill et al., 1989), *Math1* (Helms and Johnson, 1998), *Lmx1b* (Chen et al., 1998), *Nr4a2* [previously named *Nurr1* (Zetterstrom et al., 1997)], *Ptx3* (Puelles et al., 2003), *tyrosine hydroxylase* (*TH*) (Grima et al., 1985), *Pou4f1* [previously named *Brn3a* (Puelles et al., 2003)], *Ephrin-A5* (Zarbalis and Wurst, 2000), *Dll1* (Bettenhausen et al., 1995), *Hes5* (Akazawa et al., 1992), *Mash1* (Guillemot and Joyner, 1993), and *Ngn2* (Fode et al., 1998). For each probe, a minimum of three control and three mutant embryos were analyzed.

For immunohistochemistry, sections were incubated overnight at 4°C with the appropriate primary antibody diluted in 0.1% Triton X-100 (TX-100) and 10% normal goat serum in PBS. Sections were then extensively washed in PBS plus 0.1% TX-100 and incubated 1 h at room temperature with a secondary antibody conjugated with a fluorochrome. Sections were then washed and mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). The following primary antibodies were used: rabbit anti-Otx (1:1000) (Baas et al., 2000), rabbit anti-serotonin (S5545, 1:5000; Sigma, St. Louis, MO), rabbit anti-Nkx2.2 (1:100) (Briscoe et al., 1999), rabbit anti-calbindin D-28k (CB38a, 1:1000; Swant, Bellinzona, Switzerland), rat anti-bromodeoxyuridine (BrdU) (OBT0030S, 1:10; Oxford Biotechnology, Kidlington, UK), rabbit anti-TH (AB152, 1:200; Chemicon, Temecula, CA), mouse anti-Pou4f1 (sc-8429, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-β-tubulin isotype III (SDL.3D10, 1:100; Sigma). All images were collected on a Zeiss (Oberkochen, Germany) LSM510 microscope or Leica TCS SP2 confocal microscope and processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA). TH$^+$ and Pou4f1$^+$ cell counting were done after immunohistochemistry using anti-TH or anti-Pou4f1 antibodies, respectively. Positive cells were numbered along the whole A-P axis of the midbrain every 50 μm. Altogether, 10 sections were analyzed at E12.5 and 14 sections at E15.5. Student's one-tailed *t* test was used to determine statistical significance.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling. Cryostat sections were washed once for 5 min in PBS–0.1% TX-100, permeabilized in ice-cold 0.01 M citrate buffer and 0.1% TX-100 for 2 min, and washed again in PBS–0.1% TX-100. The enzymatic reaction was then performed at 37°C according to the protocol of the manufacturer (1 684 795; Roche Diagnostics, Mannheim, Germany).

BrdU labeling. Pregnant females were injected intraperitoneally with a solution of BrdU (B-5002, at 10 mg/ml in physiological serum; Sigma) at 100 mg for 1 g of body weight and killed 1 h later. Proliferating cells were revealed by immunohistochemistry on frozen sections after *in situ* hybridization with an *Shh* antisense probe. BrdU$^+$ cells in the *Shh*$^+$ domain were counted on four adjacent sections per embryo at the level of DA and red nuclei neurons. Student's one-tailed *t* test was used to determine statistical significance.

Results

Nestin-Cre mediated inactivation of the *Otx2*^{flox} allele in the CNS

In *Nestin-Cre* transgenic animals, the *Cre* recombinase gene driven by the *Nestin* promoter and enhancer is specifically expressed in neural precursor cells (Isaka et al., 1999). To analyze Cre activity during embryogenesis, *Nestin-Cre/+; Otx2*$^{flox/+}$ mice were crossed with the *R26R* reporter mouse strain (Soriano,

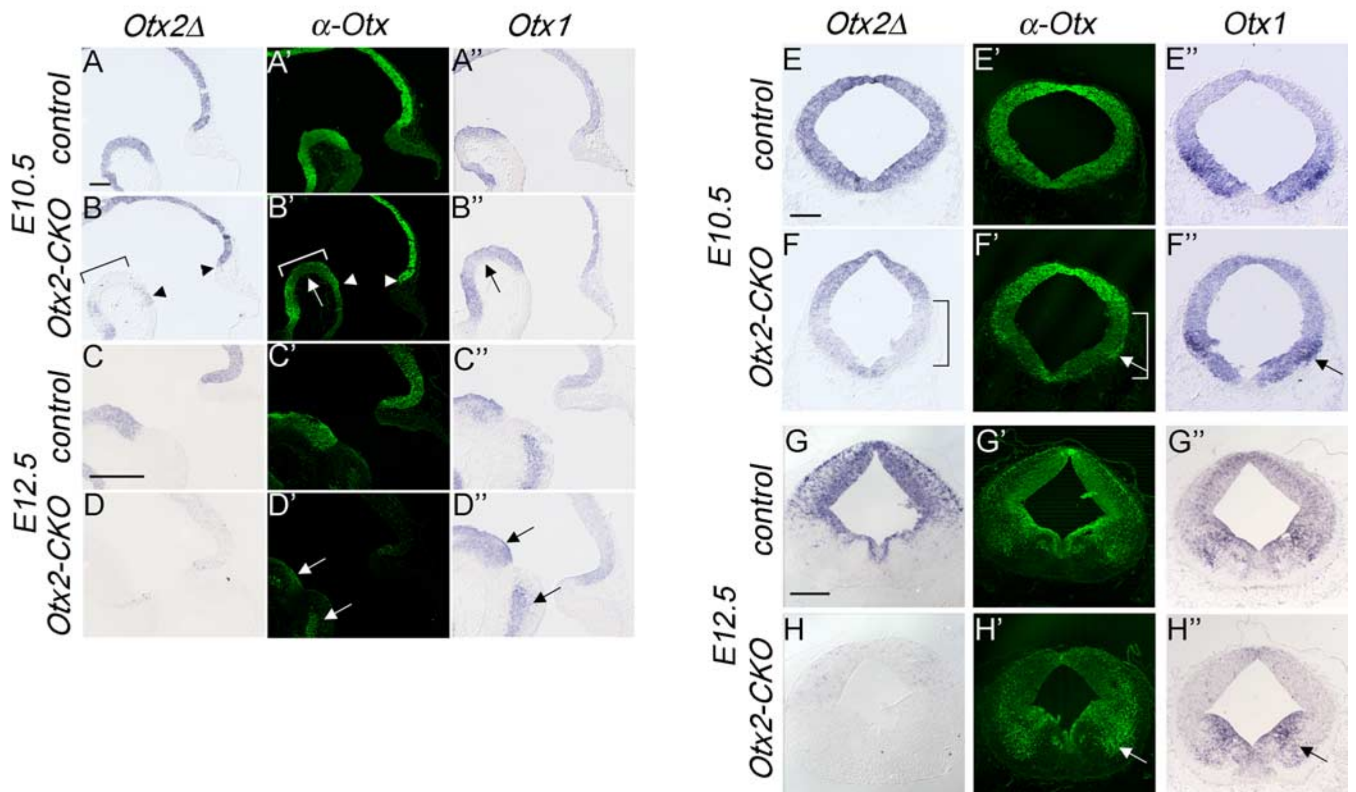

Figure 1. *Nestin-Cre*-mediated inactivation of *Otx2* in the midbrain. *In situ* hybridization (***A–H***, ***A″–H″***) and immunodetection of Otx proteins (***A′–H′***) on sagittal (***A″–D″***) or coronal (***E″–H″***) sections within the midbrain of control and *Otx2-CKO* embryos. Inactivation of *Otx2* starts in two lateral domains within the midbrain at E10.5 (brackets in ***B***, ***B′***, ***F***, ***F′***) but is excluded from the isthmus (arrowheads in ***B*** and ***B′***). The inactivation of *Otx2* progresses toward the dorsal part of the midbrain and the isthmus and is complete in the midbrain by E12.5 (***D***, ***D′***, ***H***, ***H′***). The domain of Otx $^{+}$ (green) cells corresponds ventrally to the expression of *Otx1* transcripts in the midbrain (arrows in ***B′***, ***B″***, ***D′***, ***D″***, ***F′***, ***F″***, ***H′***, ***H″***). At all stages studied, control embryos show expression of exon 2-containing transcripts of *Otx2* in all midbrain progenitors. Scale bars: (in ***A***) ***A***, ***A′***, ***A″***, ***B***, ***B′***, ***B″***, and (in ***E***) ***E***, ***E′***, ***E″***, ***F***, ***F′***, ***F″***, 100 μm; (in ***C***) ***C***, ***C′***, ***C″***, ***D***, ***D′***, ***D″***, and (in ***G***) ***G***, ***G′***, ***G″***, ***H***, ***H′***, ***H″***, 200 μm. Anterior is to the left for panels of sagittal sections.

1999). In *Nestin-Cre/+; R26R/+* embryos, the cells in which Cre is active constitutively expressed the β-galactosidase enzyme. Cre activity was first detected in the ventrolateral domain of the CNS at E10.5 and becomes progressively activated throughout the midbrain and most regions of the CNS by E12.5 (data not shown) in which *Otx2* is expressed.

To study the role of *Otx2* during neurogenesis, we generated *Nestin-Cre/+*; *Otx2$^{flox/flox}$* embryos (hereafter referred to as *Otx2-CKO* mutants). We analyzed *Otx2* inactivation using an anti-Otx antibody (Baas et al., 2000) or an RNA probe to detect exon 2, which is deleted on Cre recombination of the *Otx2^{flox}* allele (see Materials and Methods). The anti-Otx antiserum that we used likely recognizes both Otx1 and Otx2 proteins. The expression of Otx proteins observed in *Otx2-CKO* embryos at E10.5 and E12.5 corresponds to the remaining expression of *Otx1* (Fig. 1*B′*,*B″*,*D′*,*D″*,*F′*,*F″*,*H′*,*H″*).

In agreement with the analyses using *R26R* animals, expression of exon 2-containing transcripts and Otx2 protein were first lost in the ventrolateral midbrain at E10.5 but no activation occurred yet at the level of the floor plate and the isthmus (Fig. 1*B*,*F*). Subsequently, *Otx2* was progressively inactivated in the whole midbrain between E11.5 (data not shown) and E12.5. At E12.5, expression of Otx2 protein was almost completely missing in the midbrain of *Otx2-CKO* mutant embryos (Fig. 1*D*,*D′*,*H*,*H′*). The residual strong signal in the roof plate and ventral part of the midbrain (Fig. 1*H′*) correlated with the remaining sites of *Otx1* expression (Fig. 1*H″*). By comparison, the *Nestin:Cre/+; Otx2$^{flox/+}$* control (hereafter called control) embryos never showed any loss of Otx2 protein in the domain of Cre activity (Fig. 1*G*,*G′*) because one functional allele of *Otx2* remains.

In summary, the *Otx2-CKO* animals allow us to study any requirements of *Otx2* in the developing midbrain from E10.5 onward.

Otx2-CKO mice showed an ectopic cerebellar structure in the dorsal midbrain and abnormal development of tegmentum nuclei

Otx2-CKO mutants are found with the expected Mendelian ratio until birth, indicating that loss of Otx2 activity in the CNS from E10.5 onward does not result in embryonic lethality as in homozygous *Otx2* null mutants. (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Later, 30% of *Otx2-CKO* pups die during the first 3 weeks of their postnatal life, and the remaining 70% survive until adulthood. It remains unclear what is the cause of the postnatal lethality in *Otx2-CKO* mutants, but some postnatal animals that died showed growth defects. Histological analysis of pups at birth [postnatal day 0 (P0)], 1 week (P7), and 1 month (P28) revealed two major macroscopic defects in *Otx2-CKO* brain. First, an ectopic cerebellar-like structure formed at the dorsal midline (Fig. 2*A*,*A′*,*D*,*D′*,*E*,*E′*) but not in the lateral domain of the mutant midbrain at all stages analyzed (Fig. 2*B*,*B′*). Two dense layers of cell nuclei were observed in the ectopic structure, resembling the external granular cell layer (EGL)

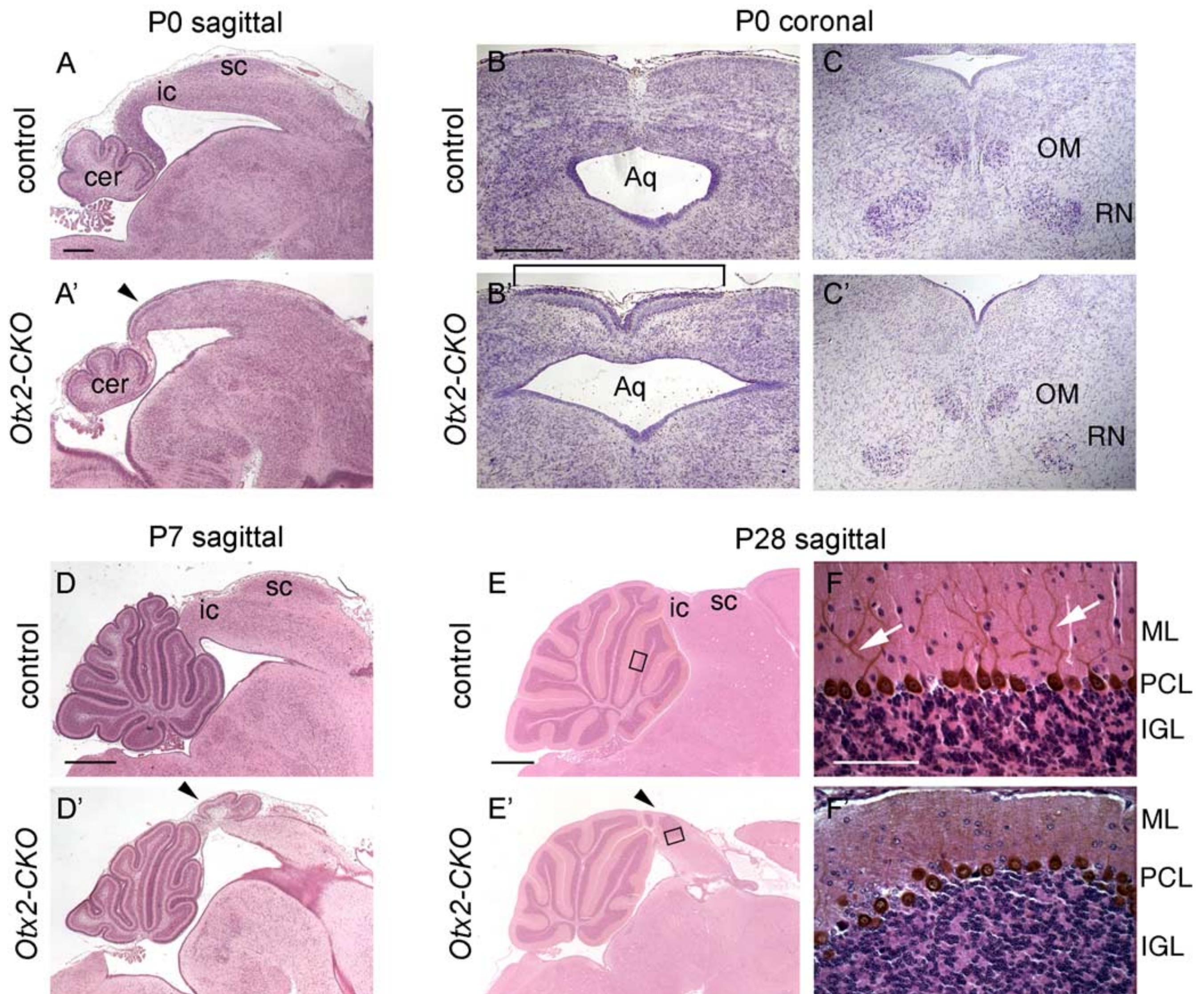

Figure 2. Anatomic abnormalities in the brain of postnatal *Otx2-CKO* mutants (***A′–F′***) compared with control (***A–F***) embryos. Sagittal sections counterstained with hematoxylin–eosin show the presence of an ectopic cerebellar-like structure in the dorsal midbrain (arrowheads) at P0 (***A′***), P7 (***D′***), and P28 (***E′***). Coronal sections at P0 (cresyl violet counterstained) show the restriction of the ectopic cerebellum to the midbrain dorsal midline (brackets in ***B′***). Cerebellar granule and Purkinje cells are identified in the ectopic cerebellar-like structure (***F*** and ***F′*** are higher-magnification images of the insets in ***E*** and ***E′***, respectively) either by morphological features (granule cells) or expression of calbindin detected by immunohistochemistry at P28 (Purkinje cells). White arrows indicate the Purkinje cell dendrites (***F***), which are not apparent in the ectopic cerebellum (***F′***). The ventral midbrain nuclei also show some defects: the red nuclei are hypoplastic (***C′***), whereas the OM nuclei develop normally (***C′***). aq, Aqueduct; cer, cerebellum; ic, inferior colliculus; IGL, internal granule cells layer; ML, molecular layer; PCL, Purkinje cells layer; sc, superior colliculus. Scale bars: (in ***A***) ***A***, ***A′***, and (in ***B***) ***B***, ***B′***, ***C***, ***C′***, 200 μm; (in ***D***) ***D***, ***D′***, and (in ***E***) ***E***, ***E′***, 500 μm; (in ***F***) ***F***, ***F′***, 100 μm. Anterior is to the right for sagittal sections.

and Purkinje cell layer in the normal cerebellum. Confirmation of the Purkinje cell layer was obtained by specific staining for calbindin protein at E17.5 (data not shown) and P28 (Fig. 2*F′*). However, in the ectopic cerebellar structure, the Purkinje cell layer was less organized than in the endogenous cerebellum (Fig. 2, compare *F*, *F′*). Second, compared with the control, the endogenous cerebellum in *Otx2-CKO* mutant embryos appeared truncated in its most posterior part in both vermis and hemispheres at all stages analyzed (Fig. 2 and data not shown). In addition, the remaining foliae showed an abnormal foliation pattern. The defects in the cerebellum will be the focus of another study. The formation of the ectopic cerebellum disrupted the dorsal midbrain structure, in particular the colliculi. Both superior and inferior colliculi could not be observed any more in adult *Otx2-CKO* animals (data not shown). Sagittal and coronal sections (Fig. 2*A′*,*B′*) of the brain of *Otx2-CKO* embryos also showed a severe reduction of these structures in the dorsal midbrain already by P0.

In the ventral midbrain, several groups of neurons are organized into nuclei that are visible by cresyl violet staining such as the RN and the oculomotor (OM) neurons. The OM neurons appeared normal, although there was a consistent reduction of the RN throughout the midbrain at birth (Fig. 2*C*,*C′*). In summary, the histological analyses revealed that *Otx2* is required for proper formation of neuronal subtypes in the midbrain.

Environmental signals along A-P and D-V axes are not modified in *Otx2-CKO* embryos

Previous genetic analyses in mice have shown that mouse mutants with rostral shifts of the MHB organizer develop an en-

larged cerebellum at the expense of the midbrain (Acampora et al., 1997; Suda et al., 1997). Hence, we examined whether the formation of an ectopic cerebellar-like structure in the midbrain may reflect an alteration of the position and/or function of this organizer. We therefore assessed the status of key regulatory molecules required for MHB organizer function in *Otx2-CKO* mutants. At E10.5 (data not shown) and E12.5, *Otx2* (Fig. 3*A*,*A′*), *Gbx2* (Fig. 3*D*,*D′*), *Wnt1* (Fig. 3*C*,*C′*), and *Fgf8* (Fig. 3*B*,*B′*) showed identical patterns of expression in mutant and control embryos, indicating that the MHB organizer was correctly positioned and maintained in the *Otx2-CKO* embryos.

We had also shown previously that the dosage of Otx proteins regulates the expression of *Shh*, a gene coding for a secreted molecule crucial for D-V patterning of the CNS (Puelles et al., 2003). Therefore, we examined D-V patterning in the midbrain by determining the expression of *Shh* and its ventral targets *Gli1* and *Ptch1*. At E12.5, the domain of *Shh* expression in *Otx2-CKO* embryos was identical to control embryos; however, the expression pattern appeared uniform, whereas in control embryos, *Shh* expression was slightly more intense at its dorsal limit (Fig. 3*E*,*E′*). This uniform expression pattern of *Shh* in the midbrain of *Otx2-CKO* embryos was similar to the expression pattern observed in the metencephalon of control embryos (see Fig. 5*B″*). We also observed an alteration in the morphology of the ventral midbrain. Specifically, the *Shh*-expressing cells showed a narrower V-shaped ventricular zone in *Otx2-CKO* compared with control embryos (Fig. 3*E*,*E′*), but the number of cells expressing *Shh* was similar in control and *Otx2-CKO* embryos. In addition, the alar-basal boundary of the midbrain, normally delimited by a sulcus, in control embryos was less apparent in *Otx2-CKO* embryos (arrows in Fig. 3*E–H* and *E′–H′* respectively). The expression domain of *Ptch1* (Fig. 3*F*,*F′*) and *Gli1* (Fig. 3*G*,*G′*) also appeared unchanged compared with control embryos, suggesting that the reception of Shh signaling is not modified in *Otx2-CKO* embryos. Consistent with the idea that Shh signaling is normal in these embryos, the expression pattern of *Pax3* (Fig. 3*H*,*H′*), a paired homeodomain-con-taining gene that is normally repressed by Shh signaling in ventral regions of the neural tube, was also not altered. Hence, we did not detect any evidence of abnormal *Shh* expression and signaling in these mutants.

Dorsally, the roof plate also functions as a signaling center secreting TGFβ-related superfamily molecules, including Gdf7 and Bmp6 that influence the differentiation of dorsal neurons. Expression of these molecules was identical in control and *Otx2-CKO* embryos (Fig. 3*I*,*I′* and data not shown). We also determined the response of dorsal midbrain cells to BMP signals in *Otx2-CKO* mutants. Expression of *Msx1*, a downstream target of BMP signaling, appeared slightly broader in the dorsal neural tube of *Otx2-CKO* mutants (Fig. 3*J′*) compared with its expression in control embryos (Fig. 3*J*).

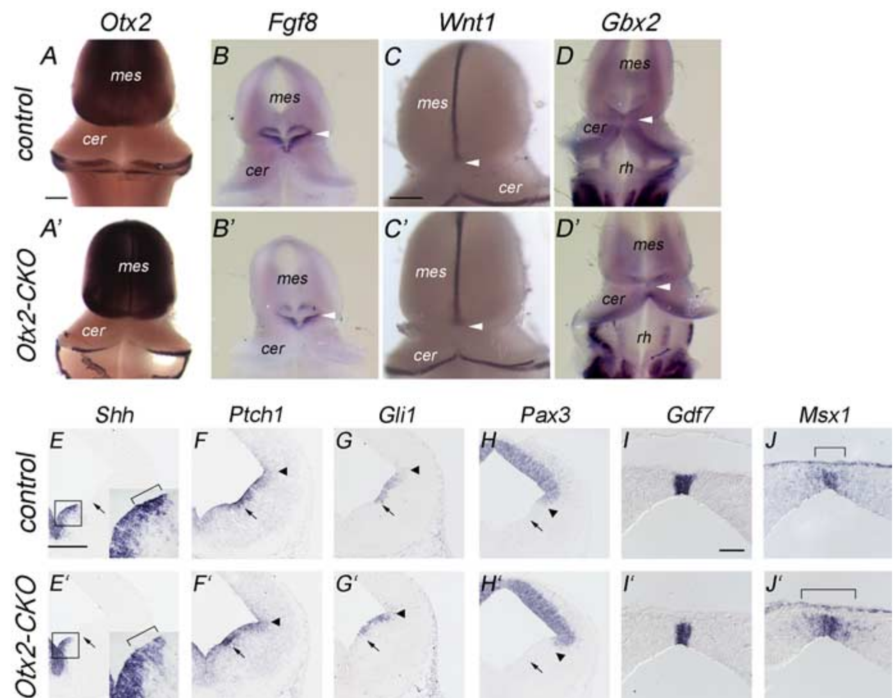

Figure 3. ***A***, Correct positioning and maintenance of the isthmic organizer. Whole-mount *in situ* hybridization of brains from control and *Otx2-CKO* embryos at E12.5 for *Otx2* (***A***, ***A′***), *Fgf8* (***B***, ***B′***), *Wnt1* (***C***, ***C′***), and *Gbx2* (***D***, ***D′***). All genes show identical patterns of expression in mutant and control embryos, indicating that the mid-hindbrain boundary organizer is correctly positioned and maintained in the *Otx2-CKO* mutants. Arrowheads mark the position of the isthmus. cer, Cerebellum; mes, mesencephalon; rh, rhombencephalon. Scale bars: (in ***A***) ***A***, ***A′***, ***B***, ***B′***, ***D***, ***D′***, and (in ***C***) ***C***, ***C′***, 200 μm. Anterior is to the top. ***B***, D-V patterning in the midbrain of *Otx2-CKO* embryos. *In situ* hybridization of coronal sections at the level of the midbrain DA field at E12.5. *Shh* expression is similar in control and *Otx2-CKO* mutant embryos (compare ***E***, ***E′***) with the exception of its most dorsal expression, in which the intensity of expression is decreased (brackets in insets in ***E*** and ***E′***). *Ptch1* (***F***, ***F′***) and *Gli1* (***G***, ***G′***) show no changes in their expression, and their dorsal limit of expression, marked by arrowheads, is identical in control and mutant embryos. Moreover, *Pax3* expression is not affected in *Otx2-CKO* compared with control embryos (arrowheads in ***H*** and ***H′*** indicate ventral boundary of expression of *Pax3*). Black arrows indicate the position of the alar-basal boundary (***E–H***, ***E′–H′***). *Gfd7* is also properly expressed in *Otx2-CKO* mutant embryos (***I***, ***I′***). In contrast, *Msx1* expression is slightly expanded in the roof plate of *Otx2-CKO* mutants (***J***, ***J′***). *Msx1* is also expressed in the surface ectoderm of *Otx2-CKO* mutants like in control embryos (Fig. 3 *J*, *J′*). Scale bars: (in ***E***) ***E***, ***E′***, ***F***, ***F′***, ***G***, ***G′***, ***H***, ***H′***, 200 μm; (in ***I***) ***I***, ***I′***, ***J***, ***J′***, 50 μm.

Altogether, these results demonstrate that that the expression of A-P and D-V patterning signals is primarily unaffected in *Otx2-CKO* mutants. However, the shape of the ventral midbrain was modified, and, in particular, the sulcus marking the alar-basal boundary was affected.

Midbrain progenitors express transcriptional codes similar to hindbrain progenitors in *Otx2-CKO* embryos

Next, we investigated whether *Otx2* might alter the fate of midbrain precursors by modifying progenitor code in the midbrain. The cerebellum is derived from the alar plate of the metencephalon (Wingate, 2001). The rhombic lip, located at the posterior dorsal edges, of the metencephalon gives rise to cerebellar granule cells, whereas the ventricular layer of the anterior metencephalon produces all other cerebellar cell types (for review, see Hatten and Heintz, 1995; Wang and Zoghbi, 2001). *Math1*, a basic helix-loop-helix (bHLH) transcription factor, is expressed in cerebellar granule progenitors in the rhombic lip and is required for the generation of cerebellar granule neurons (Ben-Arie et al., 1997). We found that *Math1* was ectopically expressed in the dorsal midbrain of E11.5, E12.5, and E15.5 *Otx2-CKO* mutants (Fig.

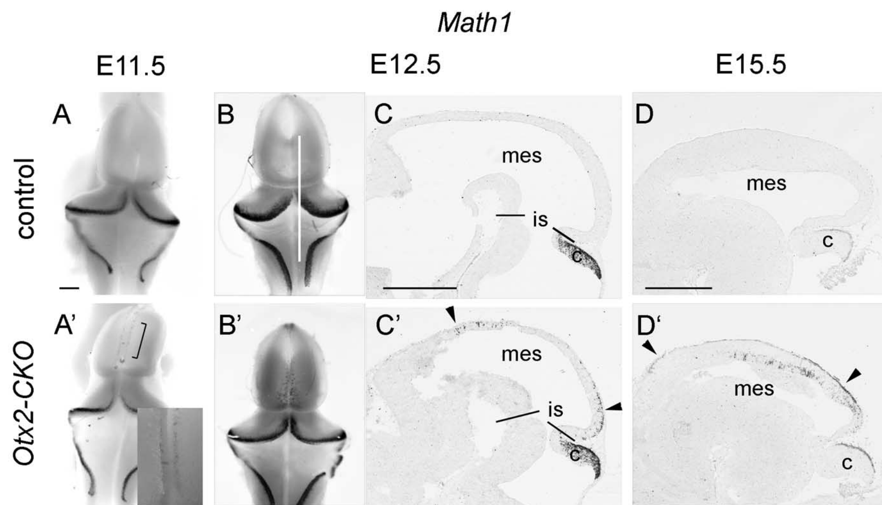

Figure 4. Dorsal midbrain cells ectopically express *Math1* in mutant embryos starting at E11.5 (***A–D***, ***A′–D′***). Whole-mount *in situ* hybridization showing *Math1* expression in the brain at E11.5 and E12.5 (***A–B′***). The first ectopic *Math1*$^{+}$ cells are observed in the posterior mutant midbrain at E11.5 (inset in ***A′***). *In situ* hybridization of sagittal sections of control and *Otx2-CKO* mutant embryos (***C–D′***). *Math1* expression marks granule cell progenitors in the cerebellum in control (***C***) and mutant embryos (***C′***) at E12.5, but ectopic expression of *Math1* is also detected in the dorsal midbrain both anteriorly and posteriorly (arrowhead in ***C′*** and ***D′***) in the mutant embryo. A white line in ***B*** indicates the plane of the section shown in ***B*** and ***B′***, and the black lines in ***C***, ***C′***, ***D***, and ***D′*** delineate the mid-hindbrain boundary. Abbreviations are the same as those for Figure 3. Scale bars: (in ***A***) ***A***, ***A′***, ***B***, ***B′***, 200 μm; (in ***C***) ***C***, ***C′***, and (in ***D***) ***D***, ***D′***, 500 μm. Anterior is to the left for sagittal sections. c, Cerebellum; is, isthmus; mes, mesencephalon.

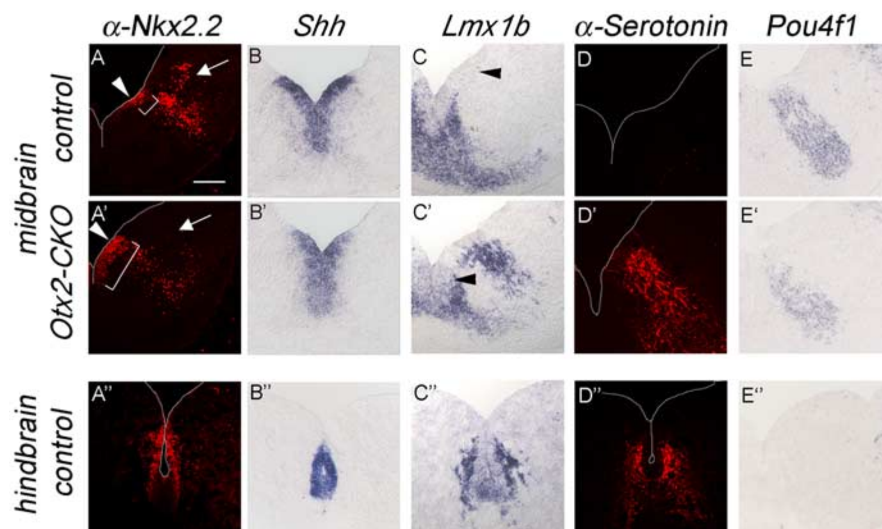

Figure 5. Ectopic serotonergic neurons in the ventral midbrain at E12.5. *In situ* hybridization (***B***, ***B′***, ***B″***, ***C***, ***C′***, ***C″***, ***E***, ***E′***, ***E″***) and immunohistochemistry (***A***, ***A′***, ***A″***, ***D***, ***D′***, ***D″***) of control and *Otx2-CKO* embryos. Coronal sections in the midbrain and transverse sections of rhombomere 1 in the hindbrain are shown. *Nkx2.2* is expressed in the *Shh*$^{+}$ domain in *Otx2-CKO* (***A′***, ***B′***) like in the control hindbrain (***A″***, ***B″***), whereas only limited or no coexpression is observed in the control midbrain (***A***, ***B***). Brackets in ***A*** and ***A′*** indicate the domain of *Nkx2.2*$^{+}$ expression in progenitors, and white arrowheads in the same panels indicate the dorsal limit of *Shh* expression as determined from data shown in ***B*** and ***B′***, respectively. The white line (***A–A″*** and ***D–D″***) shows the ventricular surface of the neuroepithelium. Above the alar-basal boundary, a patch of *Nkx2.2*$^{+}$ cells is missing in the *Otx2-CKO* embryo (arrows in ***A*** and ***A′***). Ectopic serotonergic neurons, marked by the expression of *Lmx1b* and serotonin, are observed at the level of *Shh* and *Nkx2.2* coexpression domain (***C′***, ***D′***) as seen in the hindbrain (***C″***, ***D″***). In the *Otx2-CKO* mutant, *Pou4f1* expression in the RN is reduced (***E***, ***E′***). In mutant embryos, *Nkx2.2* is now expressed in the most dorsal *Lmx1b*$^{+}$ DA progenitors. The black arrowheads in ***C*** and ***C′*** mark the ventral border of *Nkx2.2* domain as determined from data shown in ***A*** and ***A′***, respectively. Scale bar, 100 μm. All sections of control and *Otx2-CKO* embryos are adjacent sections, except for ***B***, ***B′***, and ***B″***.

4*A′–D′* and data not shown) compared with control embryos (Fig. 4*A–D*).

Mouse embryos with mutations in the netrin receptor *Unc5h3* and the transcription factor *Pax6* (Przyborski et al., 1998; Engelkamp et al., 1999; Goldowitz et al., 2000) also showed an ectopic *Math1*$^{+}$ EGL in the midbrain. However, their phenotype differed from *Otx2-CKO* embryos in three ways. First, the ectopic EGL in the former mutant embryos was observed when the endogenous EGL has covered the surface of the cerebellum after migrating out of the rostral rhombic lip between E13.0 and E16.0 in mouse. In *Otx2-CKO* mutants, the first ectopic *Math1*$^{+}$ cells in dorsal midbrain were detected at E11.5 (Fig. 4*A′*, inset) before the time when granule neuron progenitors are starting to migrate out of the rhombic lip at approximately E12.5–E13.0 (Miale and Sidman, 1961; Hatten and Heintz, 1995; Wang and Zoghbi, 2001). Second, two separate sites of ectopic granule cells appeared in the dorsal midbrain of *Otx2-CKO* mutants at E12.5 (Fig. 4*B*,*B′*), namely the posterior third of the midbrain and at the border with the diencephalon far away from the endogenous site of *Math1* expression. Third, we never observed any continuity between the endogenous EGL and the ectopic *Math1*$^{+}$ cells in histological sections of the midbrain (Fig. 2*A′* and data not shown). All of these data strongly suggest that ectopic *Math1*$^{+}$ cells are not coming from the cerebellum via migration through the isthmus but are likely induced in midbrain neuronal progenitors after *Otx2* inactivation.

We also observed that midbrain neuronal progenitors of *Otx2-CKO* embryos exhibited expanded ventral expression of the homeobox gene *Nkx2.2* into a region expressing *Shh* when compared with control embryos (Fig. 5*A′*,*B′*). These progenitors lie adjacent to ectopic *Lmx1b*$^{+}$ and 5-HT^{+} post-mitotic neurons (Fig. 5C′,*D′*), as observed in the hindbrain of control embryos (Fig. 5*C″*,*D″*). Normally, coexpression of *Shh* and *Nkx2.2* is only observed in hindbrain progenitors that generate 5-HT neurons starting at E10.5 (Puelles et al., 2004) (Fig. 5*A″*,*B″*). These results suggest that ectopic *Nkx2.2* may be responsible for the formation of 5-HT neurons abnormally in the midbrain. In agreement with this hypothesis, removal of *Nkx2.2* activity resulted in the disappearance of midbrain serotonergic neurons in *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos (Fig. 6*E*,*H*) at E12.5. Because the ectopic 5-HT neurons were found at the same D-V position as the endogenous Pou4f1-positive cells in the RN (Fig. 5*D′*,*E′*), hypoplasia of the RN (Table 1) may also be attributable to the abnormal expression of *Nkx2.2* by RN progenitors. This hypothesis is supported by the fact that the RN neurons are found in normal numbers in *Otx2-CKO; Nkx2.2*$^{-/-}$ (Fig. 6*G*, Table 1) compared with control (Fig. 6*A*, Table 1) embryos. Importantly, we also found that *Nkx2.2*$^{-/-}$ single mutants do not display

any defects in the development of OM and RN neurons at E12.5 (data not shown).

In summary, some midbrain progenitors exhibited a hindbrain-like progenitor code. This change in progenitor identity is responsible for the abnormal development of serotonergic neurons from the midbrain. It is noteworthy that, despite these fate transformations midbrain identity is not completely abolished because the undeleted portion of the *Otx2* gene (Fig. 3*A′*) and *EphrinA5* (data not shown) are still normally expressed in the midbrain of *Otx2-CKO* embryos.

Otx2 is required for the differentiation of ventral midline DA neurons

DA neurons that contribute to the substantia nigra and ventral tegmentum area are also generated from progenitors in the ventral midbrain (Zervas et al., 2004). To determine whether *Otx2* has a role in regulating the development of DA neurons, we examined the expression of genes that mark the DA lineage and are required for its development, such as *Lmx1b*, which is normally expressed in DA progenitors and DA neurons in the ventral midbrain (Smidt et al., 2000) (Fig. 5*C*). Expression of *Lmx1b* does not appear to be affected in DA progenitors but was reduced in the DA field of the ventral midbrain (Fig. 5*C′*), suggesting a reduction in the number of DA neurons. To confirm whether reduced *Lmx1b* expression in ventral region is attributable to a reduction in DA neurons, we also determined the expression *Nr4a2*, *Ptx3*, and *TH* (Smidt et al., 1997; Zetterstrom et al., 1997) in the midbrain of *Otx2-CKO* mutants at E12.5 and E15.5. Expression of all three genes was significantly reduced in the DA field at E12.5 and more severely reduced at E15.5 (Figs. 7, 8*A*,*B*,*C*,*C′*,*F*,*G*,*H*,*H′*).

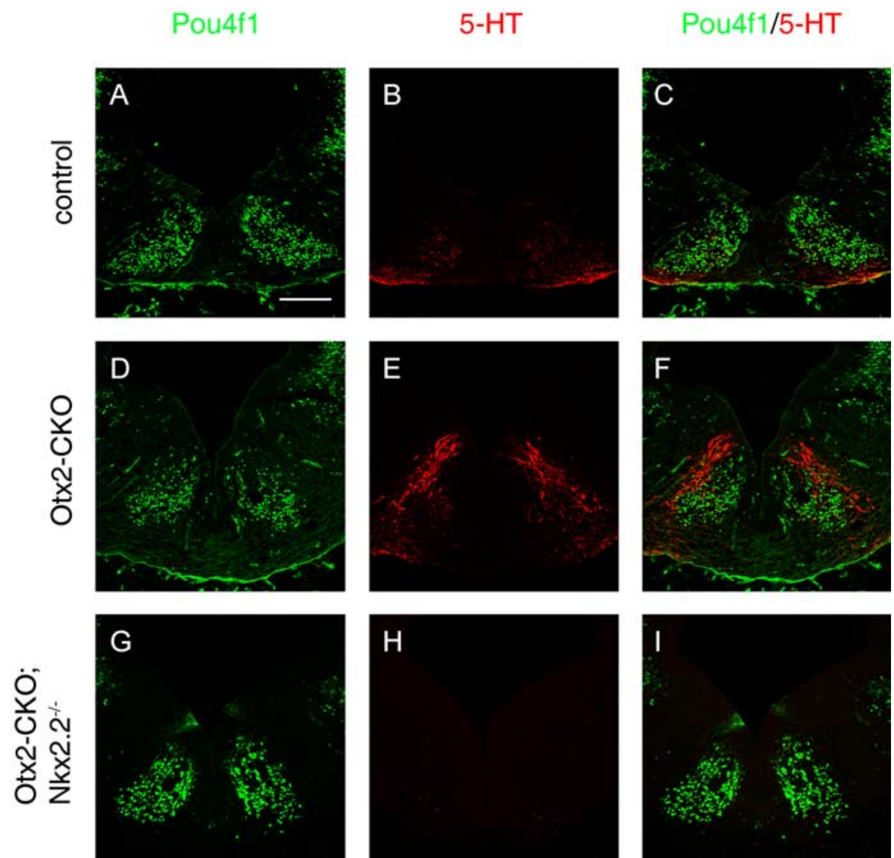

Figure 6. Ectopic serotonergic neurons are dependent on *Nkx2.2* expression in the midbrain. Immunohistochemistry (***A–I***) of coronal midbrain sections of control and *Otx2-CKO* embryos at E12.5. Pou4f1$^+$ neurons (RN) are reduced in number (***D***, ***F***), and ectopic serotonergic neurons are seen at the same level (***E***, ***F***). In *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos, rescue of the RN (***G***) and serotonergic (***E***) phenotypes are observed in the ventral midbrain. Counting of Pou4f1$^+$ neurons along the A-P axis of the midbrain shows a 30% reduction in number in *Otx2-CKO* compared with control and *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos (Table 1). Scale bar, 100 μm.

Table 1. Loss of RN neurons in *Otx2-CKO* ventral midbrain: RN (number of Pou4f1$^+$ cells)

	Control	*Otx2-CKO*^{a}	*Otx2-CKO; Nkx2.2*$^{-/-b}$
E12.5	1715 ± 47 (3)	1202 ± 120 (3)	1659 ± 54 (3)

Values represent the mean ± SD. Values in parentheses show the number of animals examined. *$p < 0.05$; **$p < 0.01$; *t* test. The percentage changes are obtained by comparing mutant results with control results (100% by default).

[a]The number of Pou4f1$^+$ neurons is reduced by 30% in Otx2-CKO embryos compared with control or *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos ($p < 0.05$).

[b]There is no significant difference between control and *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos.

Nkx2.2 expression expands ventrally into the DA neuron progenitor domain presumably marked by *Lmx1b* expression in *Otx2-CKO* embryos (Fig. 5*A′*,*C′*). The resulting change in progenitor code may affect the differentiation of *Lmx1b*$^+$ progenitors into DA neurons. To test this hypothesis and directly determine the contribution of ectopic *Nkx2.2* expression to the DA neuron deficit in *Otx2-CKO* mutants, we examined the expression of TH in DA neurons in *Otx2-CKO* mutants in an *Nkx2.2* null background. Reduction in the number of DA neurons was maintained in *Otx2-CKO*; *Nkx2.2*$^{-/-}$ embryos (Fig. 8*K*,*L*,*M*,*M′*) like in *Otx2-CKO* embryos (Fig. 8*F*,*G*,*H*,*H′*). We also determined the expression of TH in *Nkx2.2*$^{-/-}$ embryos and found normal numbers of TH$^+$ DA neurons (data not shown). This observation indicates that *Nkx2.2* ectopic ventral expression alone cannot explain the reduction in the number of DA neurons in *Otx2-CKO* embryos.

We next determined whether loss of DA neurons might be attributable to a defect in cell proliferation or cell death by BrdU labeling and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling analysis, respectively. No difference in the number of cells undergoing apoptosis was observed at E11.5 and E12.5 (data not shown). The percentage of BrdU$^+$ cells did not change at E11.5 but showed a 25% decrease in the mutants at E12.5 compared with controls (Table 2). Birth-dating analysis has shown that the first DA neurons are born at E10.5, with a peak of production at E11.5 and E12.5 in mouse (Bayer et al., 1995). The reduction in proliferation observed at E12.5 is therefore unlikely to be sufficient to explain the 40% reduction of TH$^+$ DA neurons observed at the same stage (Table 3). These results suggest that the loss of DA neurons at E12.5 is not primarily attributable to proliferation or apoptosis.

Because the expression of DA neuronal markers was almost completely missing beneath the ventral medial midbrain (Fig. 8*A′*–*D′*), this raised the possibility that neurons may be missing in this region. We therefore examined whether neurogenesis is

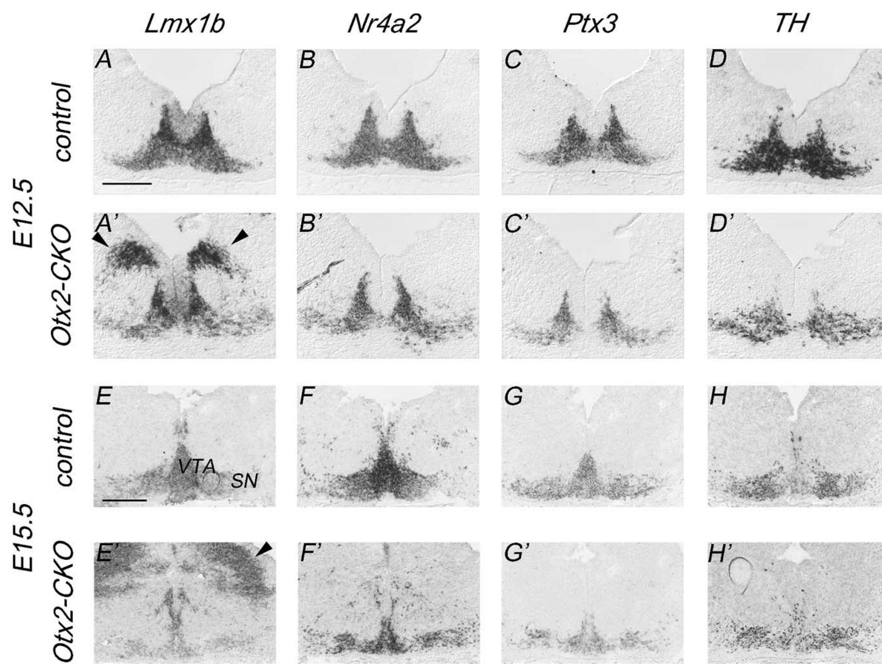

Figure 7. Loss of DA neurons in *Otx2-CKO* embryos. *In situ* hybridization of coronal sections at the level of the midbrain DA field at E12.5. The analyses of four midbrain DA neurons markers at E12.5 show a reduction in the size of the DA field (compare ***A–D***, ***A′–D′***). Later in development at E15.5 (***E–H***, ***E′–H′***), the reduction in the number of cells is more drastic in both the ventral tegmental area (VTA) and substantia nigra (SN). The arrowheads in ***A′*** point to the ectopic *Lmx1b*$^+$-expressing cells in the ventral midbrain. *Lmx1b* is also ectopically expressed in dorsal midbrain neurons (arrowhead in ***E′***). The significance of this expression remains to be determined. Scale bars: (in ***A***) ***A–D***, ***A′–D′***, 100 μm; (in ***E***) ***E–H***, ***E′–H′***, 200 μm.

affected in the ventral midline of the midbrain by analyzing the expression of a general neuronal marker, β-tubulin. β-Tubulin$^+$ neurons surrounded the ventricular surface including the floor plate in the midbrain of control embryos (Fig. 8*D*,*E*). In contrast, β-tubulin-labeled neuronal cell nuclei (detected by Toto-3 labeling) were missing from the ventral midline in the midbrain of mutants (Fig. 8*J*), although β-tubulin$^+$ fibers could still be detected (Fig. 8*I*). The observation of this acellular gap below the medial neural tube suggests a failure of neurogenesis in this region of *Otx2-CKO* mutants.

Proneural bHLH genes have been implicated in regulating neurogenesis in the CNS and PNS (Bertrand et al., 2002). Both *Mash1* (Fig. 8*N*) and *Ngn2* (Fig. 8*O*,*P*) proneural genes are expressed in the ventral midbrain of wild-type embryos at E11.5. In *Otx2-CKO* embryos, *Mash1* expression was extinguished (Fig. 8*S*), whereas *Ngn2* expression was severely reduced in proliferating ventricular zone progenitors (Fig. 8*T*,*U*) of *Otx2-CKO* embryos. We then examined two components of the Notch signaling pathway, *Dll1* and *Hes5*, whose expression are dependent on proneural gene activity in the neuroepithelium. *Dll1*, the earliest known marker of cell cycle exit in the neuroepithelium (Henrique et al., 1995), is a ligand of Notch and is probably a direct transcriptional target of proneural proteins (Bertrand et al., 2002), whereas *Hes5*, an effector of notch signaling, is induced when newborn neurons activate Notch in neighboring cells (Kageyama and Ohtsuka, 1999). *Dll1*, normally expressed in a salt and pepper pattern throughout the ventricular zone (Henrique et al., 1995) (Fig. 8*R*), was no longer detectable at the ventral midline (Fig. 8*W*), and *Hes5* expression was also abolished at the same level in *Otx2-CKO* embryos (Fig. 8*V*). Altogether, these results indicate that loss of *Otx2* leads to a specific loss of proneural genes in the medial floor plate. This results in a failure of neurogenesis at the ventral midline that correlates well spatially and precedes the absence of the medial DA neurons, suggesting a causal relationship between these events. Additional loss of TH-positive neurons between E12.5 [40% reduction (Table 3)] and E15.5 [50% reduction (Table 3)] determined by immunohistochemistry on tissue sections could be contributed by the decrease in proliferation of ventricular zone cells that is observed in *Otx2-CKO* mutants at E12.5.

Discussion

Transient requirement for *Otx2* in regulating the position of the mid-hindbrain organizer and *Shh* expression

Otx2 has been shown previously to coordinate A-P and D-V patterning by regulating the expression of key morphogenetic signals such as *Fgf8* and *Shh*, respectively. Inactivation of *Otx2* before E10.5 in *Otx1*$^{cre/+}$; *Otx2*$^{flox/-}$ (Puelles et al., 2003) and *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ (Puelles et al., 2004) embryos led to shifts in the anterior boundary of *Fgf8* and an expanded dorsal domain of *Shh*. However, complete inactivation of *Otx2* by E12.5 in *Otx2-CKO* embryos did not affect the expression of *Fgf8* and *Shh* in the MHB region. Although *Otx1* is still expressed at the MHB boundary (Fig. 1*D*″), it is unlikely that *Otx1* is compensating for *Otx2* in maintaining the MHB boundary in its dorsal domain, because *Otx1* cannot compensate for *Otx2* in maintaining the MHB boundary dorsally when *Otx2* is inactivated in *En1*$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos at E9.5 (Puelles et al., 2004). Altogether, these results demonstrate that *Otx2* is no longer required to maintain the MHB organizer and the dorsal extent of *Shh* expression at E12.5.

Otx2 inhibits the expression of transcription factors regulating hindbrain fate

In this paper, we demonstrated a later role for *Otx2* from E10.5 onward in regulating midbrain identity. In the midbrain of *Otx2-CKO* mutants, *Nkx2.2* and *Shh* are abnormally coexpressed in some progenitors as in serotonergic hindbrain precursors, and ectopic 5-HT neurons are formed adjacent to these progenitors. Rescue of 5-HT neurons in *Otx2-CKO* mutants in an *Nkx2.2* mutant background indicate that *Nkx2.2* is required for the generation of the 5-HT neurons. These results therefore strongly suggest that *Otx2* normally prevents serotonergic development in the midbrain by limiting the ventral expression of *Nkx2.2*. How does *Otx2* regulate *Nkx2.2* in the midbrain? *Otx2* may directly or indirectly regulate the activity of the *Nkx2.2* promoter. Whatever the mechanism, *Ox2* must cooperate with different spatially restricted cofactors in regulating D-V region-specific gene expression. *Shh* has been shown previously to regulate *Nkx2.2* expression (Briscoe et al., 1999). *Shh* signaling does not appear to be modified in *Otx2-CKO* embryos and hence is probably not responsible for the enlarged domain of *Nkx2.2* expression. Stronger evidence that the ectopic expression of *Nkx2.2* is independent of *Shh* signaling at E10.5 comes from previous work on *Otx1*-$^{cre/+}$; *Otx2*$^{flox/flox}$ embryos. In these conditional mutants, *Shh* signaling

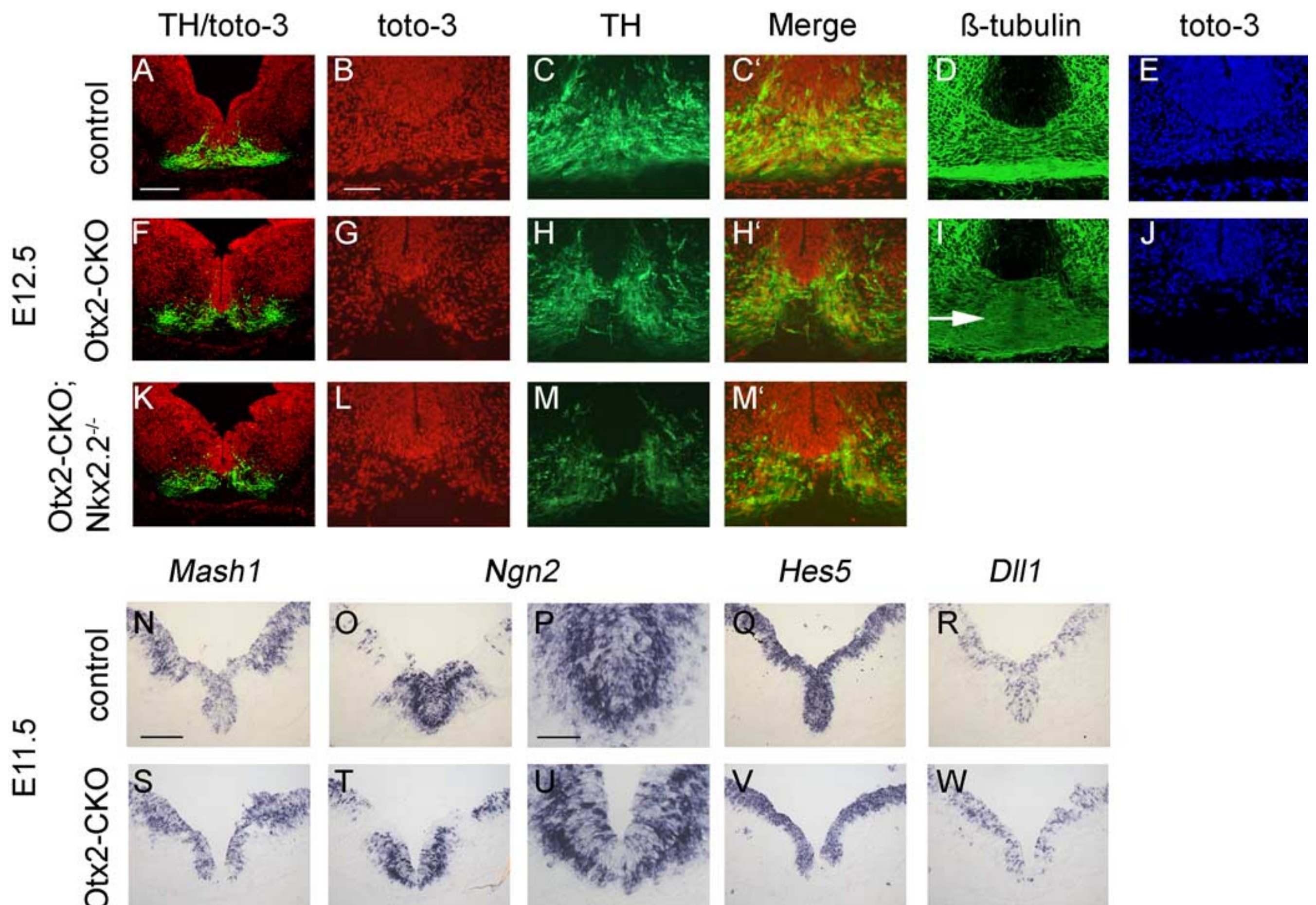

Figure 8. Partial loss of DA neurons is a consequence of the lack of neurogenesis of ventral DA progenitors of the midbrain. *In situ* hybridization (***N–W***) and immunohistochemistry (***A–M′***) of control and *Otx2-CKO* embryos are shown. Ventral midline DA neurons are lost in *Otx2-CKO* (***F***) compared with control (***A***) embryos. The resulting acellular gap, shown by the absence of cell nuclei (***G***, ***J***) is filled by fibers (arrows in ***I***). *Nkx2.2* ectopic expression in DA progenitors is not responsible for the reduction in the number of midbrain DA neurons in *Otx2-CKO* embryos (***F***), because the DA phenotype is not rescued in *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos (***K***). At E11.5, *Mash1* (***N***) and *Ngn2* (***O***, ***P***) are expressed in midbrain DA progenitors. In *Otx2-CKO* embryos, *Mash1* expression is not detected in ventral midline progenitors (***S***), and *Ngn2* expression is strongly reduced in the same progenitors but not in the adjacent postmitotic precursors in the mantle zone (***T***, ***U***). Expression of *Hes5* and *Dll1* is also switched off where *Mash1* expression is lost in *Otx2-CKO* embryos (***V***, ***W***). ***N–R*** and ***S–W*** are adjacent sections. ***P*** and ***U*** are higher-magnification images of ***O*** and ***T***, respectively. Scale bars: (in ***A***) ***A***, ***F***, ***K***, and (in ***B***) ***B***, ***C***, ***C′***, ***D***, ***E***, ***G***, ***H***, ***H′***, ***I***, ***J***, ***L***, ***M***, ***M′***, 25 μm; (in ***N***) ***N***, ***O***, ***Q–T***, ***V***, ***W***, 100 μm; (in ***P***) ***P***, ***U***, 50 μm.

Table 2. Cell proliferation is reduced in *Otx2-CKO* ventral midbrain at E12.5: mitotic index (number of BrdU$^+$ cells/total number of cells)

	Control	*Otx2-CKO*	% Change
E11.5	38.46 ± 0.35 (3)	37.40 ± 1.66 (3)	0%
E12.5	22.50 ± 1.65 (3)	15.88 ± 1(4)**	−25%

Values represent the mean ± SD. Values in parentheses show the number of animals examined. *$p < 0.05$; **$p < 0.01$; *t* test. The percentage changes are obtained by comparing mutant results with control results (100% by default).

Table 3. Loss of DA neurons in *Otx2-CKO* ventral midbrain: midbrain DA neurons (number of TH$^+$ cells)

	Control	*Otx2-CKO*	% Change
E12.5	2505 ± 90 (3)	1503 ± 180(3)**	−40%
E15.5	3250 ± 94 (3)	1625 ± 86(3)**	−50%

Values represent the mean ± SD. Values in parentheses show the number of animals examined. *$p < 0.05$; **$p < 0.01$; *t* test. The percentage changes are obtained by comparing mutant results with control results (100% by default).

is enhanced, leading to a reduction of *Nkx6.1* expression, but no changes in *Nkx2.2* expression was observed. Given these results, we favor the hypothesis that *Otx2* is directly involved in the regulation of *Nkx2.2*. However, we cannot formally rule out a role for Shh in contributing to changes in gene expression in ventral midbrain progenitors in *Otx2-CKO* mutants.

The concomitant rescue of the serotonergic neurons and hypoplasia of the RN suggest a causal relationship between these two events. The progenitors adjacent to Pou4f1$^+$ red nuclei neurons ectopically express Nkx2.2, and loss of Nkx2.2 activity in *Otx2-CKO; Nkx2.2*$^{-/-}$ embryos results in normal development of the red nuclei. These results strongly suggest a role for Nkx2.2 in modifying the identity of red nuclei progenitors.

Dorsally, midbrain progenitors also ectopically express a hindbrain progenitor determinant *Math1*. Ectopic expression of *Math1* as early as E11.5 anticipates and is likely involved in the formation of the ectopic cerebellar granule cells in the midbrain of *Otx2-CKO* embryos. Altogether, the transformation of some dorsal and ventral midbrain progenitors into more caudal hindbrain-like progenitors in *Otx2-CKO* embryos suggest that *Otx2* maintains mesencephalic identity in part by repressing alternative hindbrain fates. It would be interesting to determine whether the molecular mechanism involved in the repression of *Math1* expression in midbrain progenitors is similar or distinct from that involved in the repression of Nkx2.2 expression.

Otx2 regulates neurogenesis of DA progenitors in the ventral midbrain

Although *Otx2* negatively regulates serotonergic differentiation via repression of *Nkx2.2* expression, it positively regulates the development of ventral midline DA neurons. Loss of DA neurons in the ventral midbrain of *Otx2-CKO* embryos is also not rescued in an *Nkx2.2* mutant background, indicating that the ectopic *Nkx2.2* expression in progenitors is not responsible for the partial loss of DA neurons. This result is somewhat expected because the ectopic expression of *Nkx2.2* occurred only in a small subset of dorsal $Lmx1b^+$ DA progenitors and is therefore unlikely to lead to the loss of the most ventral DA neurons. Instead, we suggest that loss of DA neurons is attributable to the absence of neurogenesis in ventral midline progenitors in the midbrain of *Otx2-CKO* embryos for the following reasons. (1) Ventral midline progenitors normally express *Mash1* and *Ngn2*, which are sufficient and necessary to drive neurogenesis in other regions of the CNS (Bertrand et al., 2002). (2) These progenitors likely differentiate into the DA neurons that coexpress β-tubulin and TH directly underneath them. (3) In *Otx2-CKO* mutants, loss of *Mash1* and *Ngn2* expression in ventral midline progenitors precedes and correlates well spatially with the region in which DA neurons are lost. (4) *Ngn2* is required in these progenitors for the differentiation of DA neurons because $Ngn2^{-/-}$ mutants lack almost all DA neurons (our unpublished results). Together, these facts suggest that hypoplasia of DA neurons is attributable to loss of neurogenesis in ventral midline progenitors of *Otx2-CKO* embryos. However, this may not be the only reason for the loss of DA neurons in *Otx2-CKO* embryos, because, more dorsally, $Lmx1b^+$ progenitors can also generate DA neurons. It is noteworthy that ventral midline cells of the hindbrain normally do not undergo neurogenesis (Pattyn et al., 2004). Given our hypothesis that *Otx2* regulates neurogenesis in the midline progenitors of the midbrain, it will be interesting in the future to determine whether *Otx2* is able to positively activate neurogenesis when ectopically expressed in the ventral midline of the hindbrain.

Otx2 is expressed in all $Lmx1b^+$ DA progenitors, yet in *Otx2-CKO* embryos, neurogenesis is only affected in the most medial progenitors. Lack of neurogenesis defects in more dorsal DA progenitors may be attributable to functional compensation by Otx1 protein and/or the late timing of inactivation of Otx2 protein in these cells. Neurogenesis of DA progenitors initiates at E10.5 (Bayer et al., 1995), so neurogenesis at the earliest stages is likely not affected by loss of *Otx2* expression in the conditional mutants. We also found that proliferation is reduced in *Otx2-CKO* embryos at E12.5 in the region of the ventral midbrain in which DA neurons originate. The reduction in cell proliferation likely also contributes to an additional reduction of DA neurons occurring between E12.5 and E15.5.

Comparison between *Nestin-Cre/+;Otx2* $^{flox/flox}$ and *En1*$^{Cre/+}$*; Otx* $^{flox/flox}$ embryos

While this work was in progress, we reported on the phenotype of *En1*$^{Cre/+}$*;Otx2*$^{flox/flox}$ embryos (Puelles et al., 2004). The phenotype in the RN and DA neurons in these two conditional mutants is similar except that *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ embryos exhibit a much stronger phenotype, likely attributable to the earlier inactivation of the Otx2 protein in these embryos at E9.5. In *En1*$^{Cre/+}$*;Otx2*$^{flox/flox}$ embryos, the change of identity observed in the ventral midbrain is more severe because the ventral expansion of *Nkx2.2* is likely earlier and more pronounced, whereas in *Nestin-Cre/+;Otx2* $^{flox/flox}$ mutants, the DA area is only slightly affected by the *Nkx2.2* misexpression. Consistent with this finding, the correct number of DA neurons was not recovered in the *Nkx2.2* null background. Rather, in *Nestin-Cre/+;Otx2* $^{flox/flox}$ embryos, loss of *Otx2* revealed a specific and novel role for this gene in controlling the number of DA neurons by regulating proneural gene expression in their progenitors, as well as their proliferation.

Otx2-CKO mutants as a new tool for understanding cell fate specification in the cerebellum

We have so far identified two cerebellar cell types in the ectopic cerebellar-like structure located at the dorsal midline of the midbrain of *Otx2-CKO* mutants: granule neurons and Purkinje cells. The origin of the cerebellar granule neurons is relatively well understood, and several studies have identified the precursors (for review, see Wingate, 2001) as well some of the signals required for their formation (Alder et al., 1999). In contrast, molecular mechanisms controlling the generation of all other cerebellar cell types are poorly understood. Indeed, although their origin (ventricular zone), their time generation (from E10 to postnatal stages), their sequential generation (the deep nuclei, the Purkinje cells, and other classes of cerebellar neurons), and their migration pattern have been elucidated, the signals and transcription factors that regulate the induction and specification of these cerebellar cell types remain to be deciphered (Altman and Bayer, 1997). The formation of an ectopic cerebellum in the midbrain of *Otx2-CKO* embryos could provide a unique model for identifying genetic pathways involved in cell fate specification in the cerebellum. For example, comparison of gene expression profiles in the dorsal midbrain of wild-type and *Otx2-CKO* mutants may identify candidate genes involved in cerebellar development. In addition, although ectopic granule neurons are observed along the whole A-P axis of the midbrain, Purkinje cells are only formed ectopically in the caudal midbrain. This result suggests that the signal(s) required for Purkinje cell development may be localized near the MHB organizer. *Otx2-CKO* embryos could also be helpful to unravel interactions between granule and Purkinje neurons.

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