Development 135, 3459-3470 (2008) doi:10.1242/dev.027003

Anterior-posterior graded response to Otx2 controls proliferation and differentiation of dopaminergic progenitors in the ventral mesencephalon

Daniela Omodei[1,*], Dario Acampora[1,2,*], Pietro Mancuso[1], Nilima Prakash[3], Luca Giovanni Di Giovannantonio[1], Wolfgang Wurst[3,†] and Antonio Simeone[1,2,†]

Meso-diencephalic dopaminergic (mdDA) neurons control voluntary movement, cognition and the reward response, and their degeneration is associated with Parkinson's disease (PD). Prospective cell transplantation therapies for PD require full knowledge of the developmental pathways that control mdDA neurogenesis. We have previously shown that Otx2 is required for the establishment of the mesencephalic field and molecular code of the entire ventral mesencephalon (VM). Here, we investigate whether Otx2 is a specific determinant of mesencephalic dopaminergic (mesDA) neurogenesis by studying mouse mutants that conditionally overexpress or lack *Otx2*. Our data show that Otx2 overexpression in the VM causes a dose-dependent and selective increase in both mesDA progenitors and neurons, which correlates with a remarkable and specific enhancement in the proliferating activity of mesDA progenitors. Consistently, lack of Otx2 in the VM specifically affects the proliferation of Sox2$^+$ mesDA progenitors and causes their premature post-mitotic transition. Analysis of the developmental pathway that controls the differentiation of mesDA neurons shows that, in the absence of Otx2, the expression of *Lmx1a* and *Msx1*, and the proneural genes *Ngn2* and *Mash1* is not activated in Sox2$^+$ mesDA progenitors, which largely fail to differentiate into Nurr1$^+$ mesDA precursors. Furthermore, proliferation and differentiation abnormalities exhibit increasing severity along the anterior-posterior (AP) axis of the VM. These findings demonstrate that Otx2, through an AP graded effect, is intrinsically required to control proliferation and differentiation of mesDA progenitors. Thus, our data provide new insights into the mechanism of mesDA neuron specification and suggest Otx2 as a potential target for cell replacement-based therapeutic approaches in PD.

KEY WORDS: Dopaminergic neuron, Cell proliferation, Cell differentiation, Otx2, Wnt1, Sox2, Lmx1a, Msx1, Ngn2, Parkinson's disease

INTRODUCTION

The dopaminergic (DA) neurons of the ventral tegmental area (VTA), substantia nigra (SN) and retrorubral field (RRF) represent a specialized population of neurons that originate in the floor-plate region of the mesencephalon and anterior prosomeres (P1-P3) (Hökfelt et al., 1984; Björklund and Lindvall, 1984; Smidt and Burbach, 2007; Smits et al., 2006; Marín et al., 2005). These neurons play a crucial role in the control of motor, sensorimotor and motivated behaviors (Björklund and Lindvall, 1984; Kelley and Berridge, 2002; Isacson, 1993). Degeneration of the DA neurons in the SN leads to the characteristic symptoms of Parkinson's disease (PD), whereas abnormal functioning of those in the VTA is involved in psychiatric and affective dysfunction (Jellinger, 2001; Egan and Weinberger, 1997; von Bohlen und Halbach et al., 2004). These disorders highlight the relevance of this population of neurons and the enormous effort to understand the molecular basis that controls their neurogenesis.

During development, neural cell-type specification is controlled by inducing signals and depends on the responsiveness of target progenitor cells (Lumsden and Krumlauf, 1996; Wurst and Bally-Cuif, 2001; Jessell, 2000; Briscoe and Ericson, 2001). Mesencephalic DA (mesDA) progenitors require the inducing properties of Fgf8 and Shh signals that are emitted at the isthmic organizer and floor-plate region, respectively, whereas competence in interpreting these inducing signals is provided by the molecular code that is defined by the expression of different transcription factors (Briscoe and Ericson, 2001; Ye et al., 1998; Simeone, 2005; Smidt and Burbach, 2007; Smits et al., 2006; Prakash and Wurst, 2006; Liu and Joyner, 2001). Several genes, including the transcription factors Pitx3, Lmx1a, Lmx1b, En, Msx1, Foxa2, Ngn2, Otx2, the orphan nuclear receptor Nurr1, the Wnt1 and Wnt5a members of the Wnt family, and retinoic acid play a relevant role in differentiation and survival of mesDA neurons (Saucedo-Cardenas et al., 1998; Semina et al., 1998; Smidt et al., 1997; Smidt et al., 2000; Smidt et al., 2004; Kele et al., 2006; Ferri et al., 2007; Andersson et al., 2006a; Andersson et al., 2006b; Ono et al., 2007; McCaffery and Dräger, 1994; van den Munckhof et al., 2003; Nunes et al., 2003; Simon et al., 2001; Zetterstrom et al., 1997; Castelo-Branco et al., 2003; Puelles et al., 2003; Puelles et al., 2004; Prakash et al., 2006). Among these, our attention has been focused on Otx2, a transcription factor containing a bicoid-like homeodomain that plays a crucial role in specification, regionalization and differentiation of forebrain and midbrain (Puelles et al., 2006; Acampora and Simeone, 1999; Simeone et al., 2002; Simeone, 2005). Accordingly, previous studies have shown that: (1) Otx2 together with Otx1 is required to control the positioning of *Shh* and *Fgf8* expression, and that failure in this control generates profound alteration in the identity code of progenitor domains in the ventral

[1]CEINGE Biotecnologie Avanzate, via Comunale Margherita 482, 80145 Naples, Italy and SEMM European School of Molecular Medicine - Naples site, Italy. [2]Institute of Genetics and Biophysics 'A. Buzzati-Traverso', CNR, Via P. Castellino 111, 80131 Naples, Italy. [3]Max-Planck-Insitute of Psychiatry, Molecular, Neurogenetics, Kraepelinstr. 2-16, 80804 Munich, Germany and Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Developmental Genetics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.

*These authors equally contributed to the work
†Authors for correspondence (e-mails: simeone@ceinge.unina.it; wurst@helmholtz-muenchen.de)

Accepted 2 September 2008

mesencephalon (VM), which exhibits a dorsal expansion of the mesDA compartment at the expense of the red nucleus (RN) domain (Puelles et al., 2003); (2) Otx2 is required in the VM to suppress the transcription factor Nkx2.2 and prevents the generation of 5-HT-containing neurons in place of RN and dorsalmost mesDA neurons (Puelles et al., 2004); and (3) Wnt1 and Otx2 may be engaged in a positive-feedback loop that, in turn, is required for proper development of mesDA neurons (Prakash et al., 2006). However, although these studies have indicated that Otx2 plays a relevant role in the establishment of the mesencephalic field and molecular code of the entire VM, they have not provided direct evidence on whether Otx2 is intrinsically required for mesDA neurogenesis. Here, we have investigated this crucial aspect by analyzing mutant mice that conditionally overexpress or lack Otx2 using *En1*-driven Cre recombinase. Our findings indicate that Otx2 controls selectively the proliferating activity of mesDA progenitors through a dose-dependent anterior-posterior (AP) graded effect and regulates the differentiation of mesDA neurons by inducing the expression of crucial determinants required to promote the transition of $Sox2^{+}$ mesDA progenitors into $Nurr1^{+}$ immature mesDA neurons. In general, these findings provide new insights into the regulatory mechanism that controls mesDA neurogenesis.

MATERIALS AND METHODS

Mouse mutants and ES cells

For the inducible overexpression of Otx2, we designed a molecule where the expression of the full-coding cDNA (49bp of 5′UTR-coding-204bp of 3′UTR), driven by the CMV enhancer-chicken βactin promoter, was blocked by a removable loxP-neo-triple polyA-loxP 'stop' cassette. In addition, an ires-GFP sequence was introduced downstream of the Otx2 cDNA to follow the transgene expression in vivo. The linearized construct was electroporated into E14Tg4a2 ES cells. DNAs extracted from 30 randomly integrated G418 resistant clones were digested with *Eco*RI and *Bam*HI restriction enzymes, and hybridized with two probes (*Neo* and *GFP*) localized across the two restriction sites (Fig. 1A). Only clones detecting single *Eco*RI and *Bam*HI bands longer than the minimum expected length were chosen for further analysis. Then, in order to detect potential disruption of any gene functions by the random integration of the construct, and for genotyping, we identified the chromosomal site and sequences flanking the transgene insertion in several ES clones by using the Vectorette System (Sigma). We chose, as the best candidate to generate a mouse line, a clone in which the transgenic and blocked Otx2 overexpressing ($tOtx2^{bov}$) construct was integrated in an intergenic region of chromosome 7 D2, 51 kb downstream of the *Isg20* gene and 111 kb upstream of the *Agc1* gene (Fig. 1A). To test the functionality and efficacy of the transgene expression, we re-transfected this clone to target the CreER-recombinase in the *Rosa26* (*R26*) locus.

The $tOtx2^{bov/+}$; $R26^{CreER/+}$ ES clone was tested for excision of the loxP-Neo-triple polyA-loxP cassette by using specific pairs of primers (black arrows in Fig. 1A and upper panel in Fig. 1B). The PCR products were 260 bp and 163 bp long for the excised and non-excised allele, respectively (Fig. 1B).

The $tOtx2^{bov/+}$; $R26^{CreER/+}$ ES clone was also tested to quantify the transgenic Otx2 and GFP gene products by western blot of total protein extracts of ES cells treated 24 hours with 100 nM of 4-hydroxytamoxifen (Sigma) (Fig. 1B). Based on these tests, the original $tOtx2^{bov/+}$ ES cell clone was injected into C57 blastocysts and chimeras were mated for germline transmission.

Litters were genotyped by using specific primers (open arrows in Fig. 1A). Primers sequences used in this study are available upon request. The $En1^{Cre/+}$; $Otx2^{flox/flox}$ mouse model to study Otx2 inactivation has been previously described (Puelles et al., 2004).

In situ hybridization and immunohistochemistry

In situ hybridization on sections and whole embryos was performed as described (Simeone, 1999; Puelles et al., 2006) with *GFP*, *Msx1*, *Lmx1a* probes, each corresponding to a PCR fragment of variable length between 300 bp and 500 bp or with *Wnt1*, *En1*, *Otx1*, *Gbx2* and *Fgf8* probes (Puelles et al., 2004). Immunohistochemistry was performed as previously reported (Puelles et al., 2004; Prakash et al., 2006). Details of primary antibodies can be provided on request.

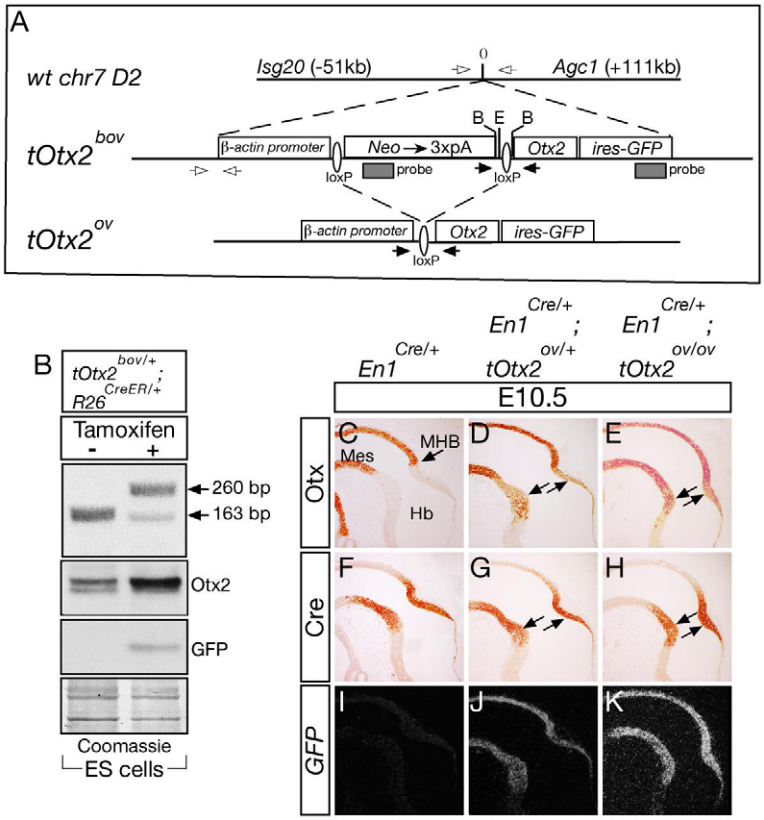

Fig. 1. Generation of the $tOtx2^{bov}$ mutant and Otx2 overexpression in ES cells and mutant embryos. (**A**) The genomic position at the chromosome 7 D2 region (upper line) where the $tOtx2^{bov}$ cassette (second line) is inserted allows the genotyping of mutant embryos by using two pairs of primers (open arrows in the upper line for the wild type and open arrows in the second line for the mutant). Cre-mediated removal of the Neo-triple polyA stop cassette generates the $tOtx2^{ov}$ allele (third line) and this event is monitored by the primers shown in the second and third line (black arrows). (**B**) The $R26^{CreER/+}$; $tOtx2^{bov/+}$ ES clone is treated with tamoxifen for 24 hours prior to monitoring the removal of the Neo-triple polyA cassette (upper panel), the Otx2 protein level (second panel) and the GFP activation (third panel). (**C-K**) Immunohistochemistry and in situ hybridization performed to detect the expression of Otx2 (C-E), *En1*-driven Cre recombinase (F-H) and *GFP* transcripts (I-K) in adjacent sagittal sections of E10.5 $En1^{Cre/+}$, $En1^{Cre/+}$; $tOtx2^{ov/+}$ and $En1^{Cre/+}$; $tOtx2^{ov/ov}$ embryos show that Otx2 is ectopically activated in the anterior hindbrain and cerebellum anlage (arrows in D,E) and, as revealed by *GFP* expression, overexpressed in the whole mesencephalon (J,K). Abbreviations: Mes, mesencephalon; Hb, hindbrain; MHB, midbrain-hindbrain border.

Dopaminergic cell counting

Cell counting of TH^+ neurons was performed on eight E18.5 dissected brains for each genotype (wild type, $En1^{Cre/+}$, $tOtx2^{bov/+}$, $En1^{Cre/+}$; $tOtx2^{ov/+}$ and $En1^{Cre/+}$; $tOtx2^{ov/ov}$). TH cell counting was also confirmed in three brains for each genotype by immunostaining with Pitx3 (data not shown). The procedure adopted for selection of sections and TH^+ cell-counting can be provided on request.

Cell proliferation experiments

To determine the labeling index (LI), pregnant females at E10.5 or E11.5 were intraperitoneally injected with 5′-bromo 2′-deoxyuridine (BrdU) at a concentration of 50 mg/kg of body weight and embryos collected 30 minutes after injection. To determine the Quit fraction (Qf), BrdU was administered as for LI experiments, but embryos were collected about 24 hours later. Further information on these experiments can be provided on request.

RESULTS

Generation of mouse mutants overexpressing Otx2

To assess the role of Otx2 in mesDA neurogenesis, we studied first the effect of Otx2 overexpression in the VM. To achieve this, we generated a transgenic mouse model that conditionally activates Otx2 by *En1*-driven Cre activity (see Materials and methods). The construct that conditionally overexpresses Otx2 (Fig. 1A) was transfected into ES cells to select the $tOtx2^{bov/+}$ clone that fulfils the following two requirements: containing a single copy of the construct inserted in a bona fide neutral genomic region; and overexpressing a moderate level of Otx2. The selected $tOtx2^{bov/+}$ ES cell clone contained a single insertion of the construct in an intergenic area of chromosome 7 at the chr7D2 position, 51 kb downstream of the *Isg20* gene and 111 kb upstream of the *Agc1* gene (Fig. 1A), and showed a moderate increase of about twofold of the Otx2 protein expressed in ES cells (Fig. 1B; see Materials and methods). In this assay, however, the level of GFP was lower than that expected on the basis of its mRNA (Fig. 1B; data not shown). This low level of GFP protein was also confirmed in vivo and not further investigated (data not shown). Based on these tests, the original $tOtx2^{bov/+}$ ES cell clone was used to generate the $tOtx2^{bov/+}$ strain. $tOtx2^{bov/bov}$ mice were healthy and fertile, and did not exhibit abnormalities. Next, we analyzed whether, at E10.5 in $En1^{Cre/+}$; $tOtx2^{ov/+}$ and $En1^{Cre/+}$; $tOtx2^{ov/ov}$ embryos, the $tOtx2^{bov}$ transgene was properly activated by the *En1*-driven Cre recombinase. As revealed by Otx2 immunostaining and *GFP* transcription, the transgenic Otx2 protein was detected in the mesencephalon and the rostral hindbrain (Fig. 1C-K). Indeed, the *GFP* mRNA and, consequently, the transgenic Otx2 were activated also in those territories such as the posterior ventral pretectum and anterior mesencephalon, where *En1*-driven Cre recombinase and the endogenous *En1* gene were not expressed at E10.5 (Fig. 1C-K; see Fig. S1A-F″ in the supplementary material). This indicated that the activation of the transgenic allele occurred earlier, when *En1* expression was broader than at E10.5. Indeed, at E9.2, as revealed by *GFP* transcripts, the Otx2 overexpressing allele was already activated in early progenitors expressing *En1* (see Fig. S1G-I″ in the supplementary material).

Next, we analyzed whether the Otx2 overexpression affected the integrity of the midbrain-hindbrain boundary (MHB) region by monitoring the expression of *Otx1*, *Fgf8* and *Gbx2*. Compared with E10.5 and E12.5 $tOtx2^{bov/+}$ embryos, the expression of these three

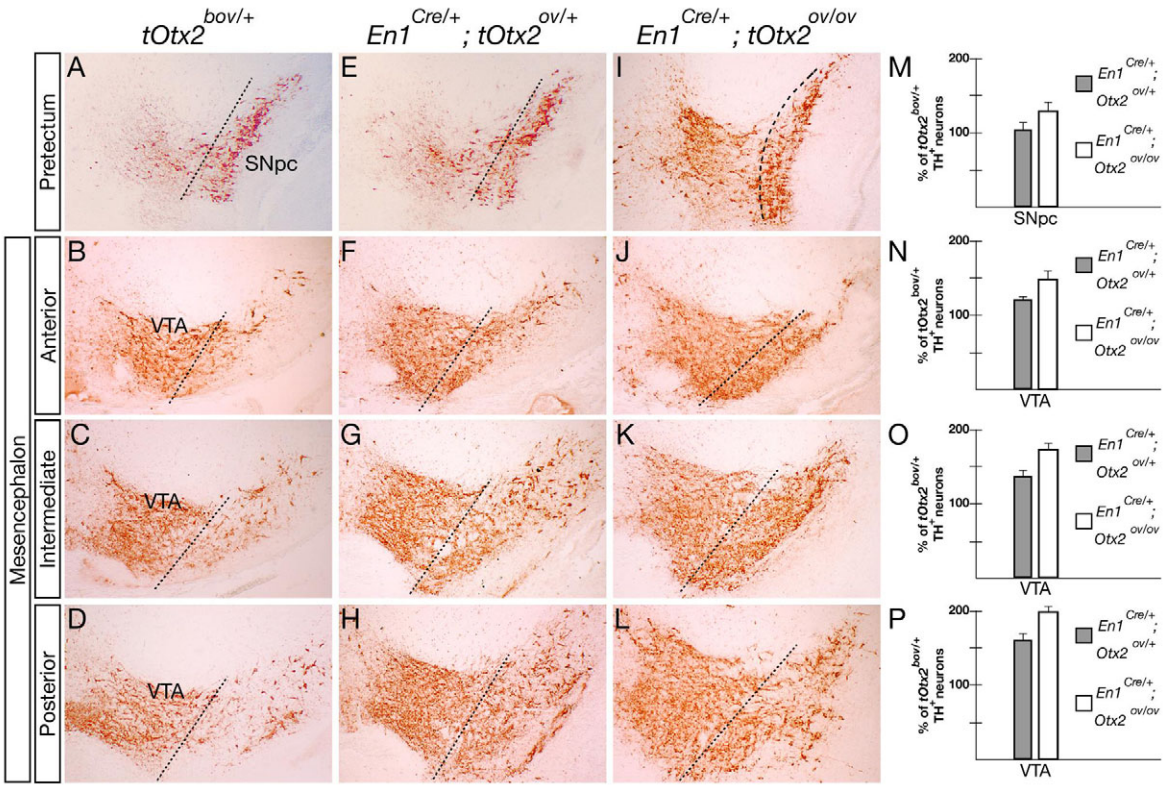

Fig. 2. AP differential response to Otx2 overexpression generates dose-dependent AP-graded increase of mesDA neurons. (**A-L**) TH immunohistochemistry performed at four anatomical levels corresponding to the posterior pretectum (A,E,I), anterior (B,F,J), intermediate (C,G,K) and posterior (D,H,L) mesencephalon. (**M-P**) Graphic representation showing the percentage of the $tOtx2^{bov/+}$ mesDA neurons detected in the two mutants at the anatomical levels corresponding to the histological sections shown. Abbreviations: SNpc, substantia nigra pars compacta; VTA, ventral tegmental area. The broken line demarcates the SNpc (A,E,I) or the VTA (B-D,F-H,J-L) territories analyzed for the cell-counting of TH^+ cells.

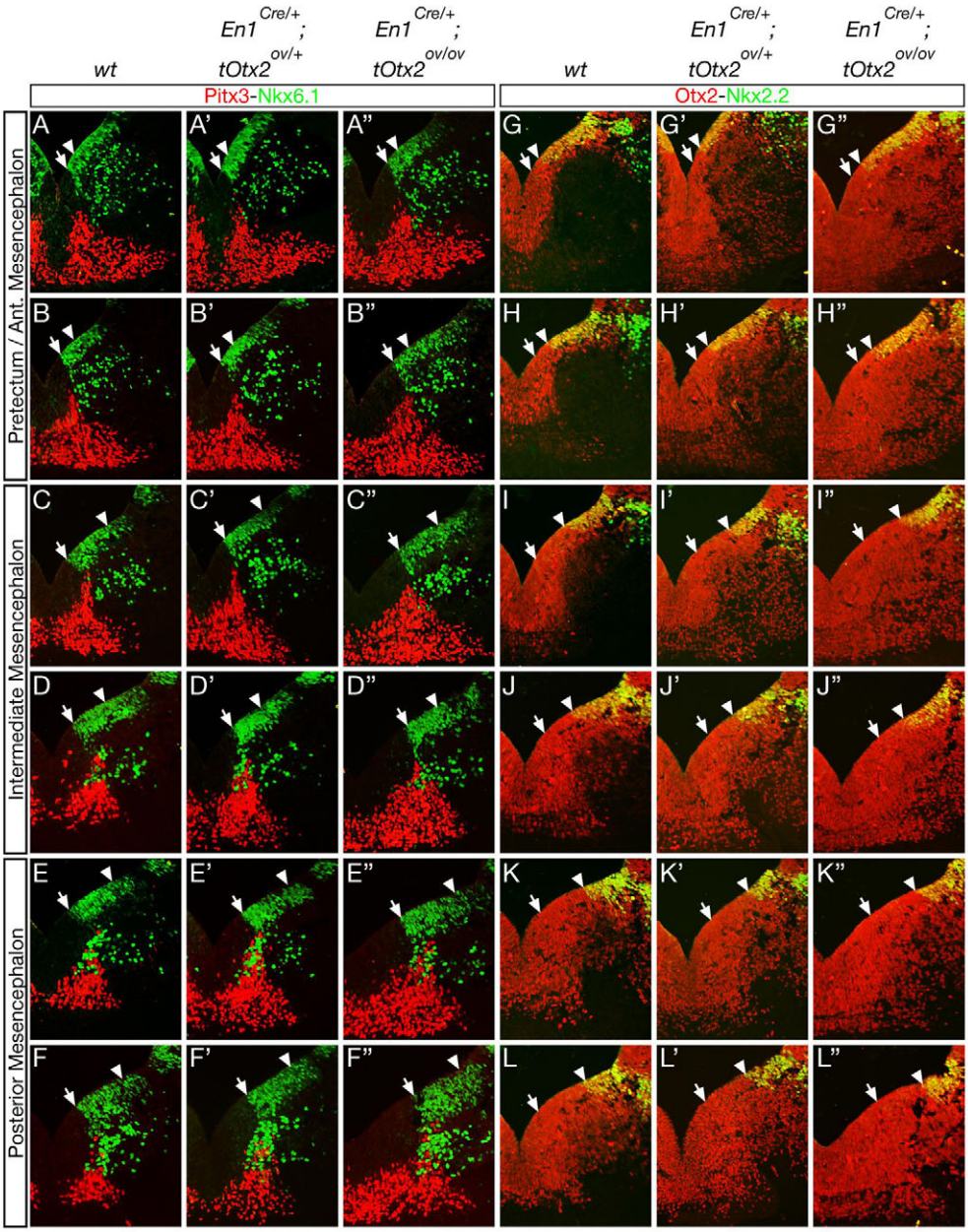

Fig. 3. Otx2 overexpression induces AP graded increase of mdDA neurons. (**A-L″**) Immunohistochemistry performed at six sequential anatomical levels corresponding to the posterior pretectum and anterior mesencephalon (A-B″,G-H″), the intermediate mesencephalon (C-D″,I-J″) and the posterior mesencephalon (E-F″,K-L″) of E12.5 wild-type, $En1^{Cre/+}$; $tOtx2^{ov/+}$ and $En1^{Cre/+}$; $tOtx2^{ov/ov}$ embryos with Pitx3 and Nkx6.1 (A-F″) and Otx2 and Nkx2.2 (G-L″) shows that in Otx2-overexpressing embryos the number of $Pitx3^+$ neurons gradually increases, moving from the anterior towards the posterior mesencephalon. This increase correlates with the copy number of the Otx2-overexpressing allele. Conversely, the Otx2 overexpression does not affect the extent of progenitor domains located dorsal to the mdDA domain; indeed, while the $Nkx6.1^-$-$Nkx2.2^-$ mdDA domain (ventral to the arrow) gradually expands, the $Nkx6.1^+$-$Nkx2.2^-$ (between arrow and arrowhead) and the $Nkx6.1^+$-$Nkx2.2^+$ (dorsal to the arrowhead) domains retain a similar distribution among the three genotypes at the different anatomical levels. Abbreviation: Ant, anterior.

genes was essentially unaffected in $En1^{Cre/+}$; $tOtx2^{ov/+}$ embryos (see Fig. S2A-J in the supplementary material), whereas in $En1^{Cre/+}$; $tOtx2^{ov/ov}$, the posterior border of the *Otx1* and *Fgf8* expression domains was moderately expanded in the anterior hindbrain at E10.5 (see Fig. S2K-M in the supplementary material). However, at E12.5 in $En1^{Cre/+}$; $tOtx2^{ov/ov}$ mutants, these MHB abnormalities appeared much less severe (see Fig. S2N,O in the supplementary material). Thus these data indicate that the $tOtx2^{bov}$ allele was properly activated by *En1*-driven Cre activity and that, probably owing to the moderate level of Otx2 activation, the integrity of the MHB was not remarkably affected. A more severe phenotype was instead observed in the cerebellum at E18.5 (data not shown).

Otx2 overexpression generates dosage-dependent and AP graded increase of mesDA neurons

Analysis of mesDA neurons was performed at four AP anatomical levels in E18.5 wild-type, $tOtx2^{bov/+}$, $En1^{Cre/+}$ and Otx2 overexpressing mice (n=8 per genotype). No difference in mesDA

neurons was detected among wild-type, *tOtx2*$^{bov/+}$ and *En1*$^{Cre/+}$ embryos (data not shown) (Sonnier et al., 2007), whereas in *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ embryos, the number of TH$^+$ neurons showed a graded AP increase (Fig. 2E-H,M-P). In particular, compared with control embryos, in *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ mutants, increases of about 20%, 35% and 50% of TH$^+$ neurons were detected, respectively, in the VTA of the anterior, intermediate and posterior mesencephalon. We then studied the phenotype of *En1*$^{Cre/+}$*; tOtx2*$^{ov/ov}$ mutants (n=8) to investigate whether, compared with *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ mutants, a more robust overexpression of Otx2 correlated with a higher number of TH$^+$ neurons. We found that, compared with control brains (Fig. 2A-D), *En1*$^{Cre/+}$*; tOtx2*$^{ov/ov}$ mutants generated up to about 80 and 100% more TH$^+$ neurons in the intermediate and posterior mesencephalon, respectively; and up to 25% and 40% more TH$^+$ neurons in the SNpc and anterior VTA, respectively (Fig. 2I-P). These findings were also confirmed by counting Pitx3$^+$ cells (data not shown).

Next, we analyzed whether this AP differential response to Otx2 overexpression was detected in meso-diencephalic DA (mdDA) progenitors and in early post-mitotic mdDA neurons by analyzing in wild type and Otx2 overexpressing embryos the expression of Foxa2, Nkx6.1, Otx2 and Nkx2.2 at E10.75 and that of Pitx3, Nkx6.1, Otx2 and Nkx2.2 at E12.5. At E10.75, we analyzed whether the relative extent of the mdDA domain (Foxa2$^+$-Nkx6.1$^-$) was gradually expanded along the AP axis and whether the dorsal Nkx6.1$^+$-Foxa2$^+$-Nkx2.2$^-$ and Foxa2$^+$-Nkx6.1$^+$-Nkx2.2$^+$ domains were affected by the Otx2 overexpression (Fig. 3; see Fig. S3 in the supplementary material). Compared with wild-type embryos, a selective and AP graded expansion of the Foxa2$^+$-Nkx6.1$^-$ mdDA domain was detected in Otx2-overexpressing mutants (Fig. S3A-F′; data not shown). In particular, this expansion was much less evident in *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$, where this expansion was detected in the posterior region of the intermediate mesencephalon and in the posterior mesencephalon (data not shown; Fig. 4). Instead, in *En1*$^{Cre/+}$*; tOtx2*$^{ov/ov}$ embryos, a mild expansion of the Foxa2$^+$-Nkx6.1$^-$-Nkx2.2$^-$ domain was detected in the pretectum and anterior mesencephalon (see Fig. S3A-B′ in the supplementary material); this expansion gradually increased, moving towards the posterior mesencephalon (see Fig. S3C-F′ in the supplementary material). Interestingly, the relative extent of the Nkx6.1$^+$-Foxa2$^+$-Nkx2.2$^-$ and Nkx6.1$^+$-Foxa2$^+$-Nkx2.2$^+$ progenitor domains appeared not affected by the Otx2 overexpression along the AP axis (see Fig. S3 in the supplementary material).

A similar experiment performed to detect early post-mitotic mdDA Pitx3$^+$ neurons showed an evident AP graded increase in the number of the Pitx3$^+$ neurons and in the extent of the Nkx6.1$^-$ mdDA domain (Fig. 3A-F″). Notably, the generation of Pitx3$^+$ neurons was apparently unaffected in the pretectum and anterior mesencephalon of *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ mutants and, similar to E10.75 embryos, the relative extents of the Nkx6.1$^+$-Nkx2.2$^-$ and Nkx6.1$^+$-Nkx2.2$^+$ domains were very similar in control and Otx2-overexpressing embryos (Fig. 3). Thus, these findings collectively suggest that mdDA progenitors exhibit an AP differential and dose-dependent sensitivity in their response to Otx2 overexpression through increased generation of mature mdDA neurons.

Otx2 overexpression induces a selective expansion of the mesDA progenitor domain

Based on previous findings, we analyzed in detail at E10.5 and E12.5 whether the identity and/or relative extent of VM progenitor domains was altered. At the anatomical level corresponding to the intermediate mesencephalon of E10.5 wild-type (data not shown) or *tOtx2*$^{bov/+}$ control embryos, Lmx1b was restricted to the mesDA domain (Fig. 4A); Shh expression included the Lmx1b$^+$ domain and the ventral half of the Nkx6.1$^+$ domain (Fig. 4B), and was adjacent to Nkx2.2 (Fig. 4C); the Nkx2.2$^+$ domain, in turn, partially overlapped with the dorsal region of both the Foxa2$^+$ and Nkx6.1$^+$ domains (Fig. 4D and compare Fig. 4B to C); and *Wnt1* was co-expressed in the mesDA domain with Lmx1b with the exception of the medialmost floor-plate region (Fig. 4E). In *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ and *En1*$^{Cre/+}$*; tOtx2*$^{ov/ov}$ embryos, no obvious abnormalities were identified in the boundary relationships between the *Nkx6.1*$^+$, *Nkx2.2*$^+$, *Shh*$^+$, *Lmx1b*$^+$, *Foxa2*$^+$ and *Wnt1*$^+$ domains or in the extent of the *Nkx6.1*$^+$ and *Nkx2.2*$^+$ domains (Fig. 4F-O), whereas a selective expansion of the *Lmx1b*$^+$ and *Wnt1*$^+$ domains was detected (Fig. 4F,K,J,O). In particular, this expansion was mild in *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ embryos and more pronounced in *En1*$^{Cre/+}$*; tOtx2*$^{ov/ov}$ mutants. In addition to the analysis performed with Pitx3, Nkx6.1 and Nkx2.2 (Fig. 3; see Fig. S3 in the supplementary

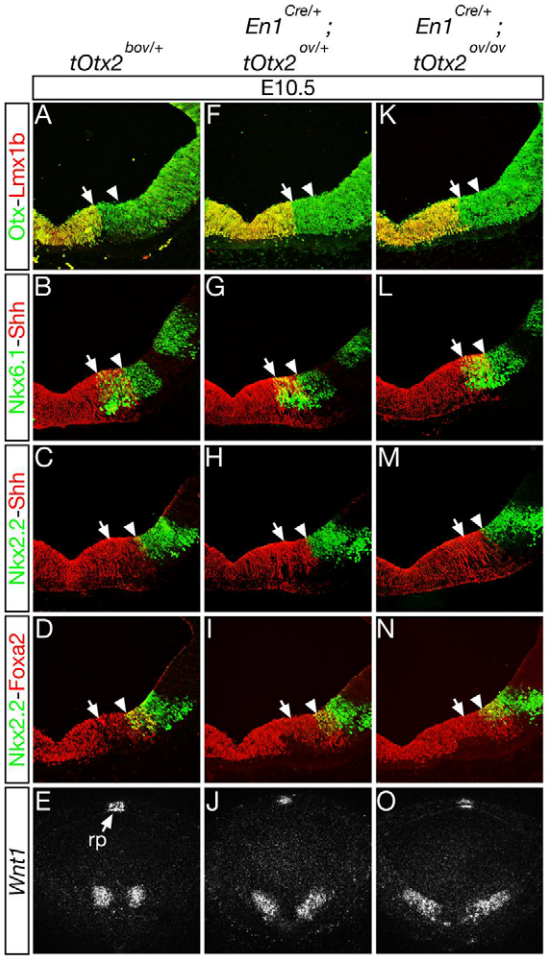

Fig. 4. Selective expansion of the mesDA domain in Otx2-overexpressing embryos. (**A-O**) Immunohistochemistry and in situ hybridization performed on adjacent sections through the intermediate mesencephalon of E10.5 *tOtx2*$^{bov/+}$, *En1*$^{Cre/+}$*; tOtx2*$^{ov/+}$ and *En1*$^{Cre/+}$*; tOtx2*$^{ov/ov}$ embryos with Otx2 and Lmx1b (A,F,K), Nkx6.1 and Shh (B,G,L), Nkx2.2 and Shh (C,H,M), Nkx2.2 and Foxa2 (D,I,N) antibodies, and *Wnt1* probe (E,J,O). The arrow indicates the dorsal border of Lmx1b expression, which is adjacent to the ventral border of Nkx6.1 and the arrowhead indicates the dorsal border of Shh, which is adjacent to or slightly overlapping the ventral border of Nkx2.2. Abbreviations: rp, roof plate.

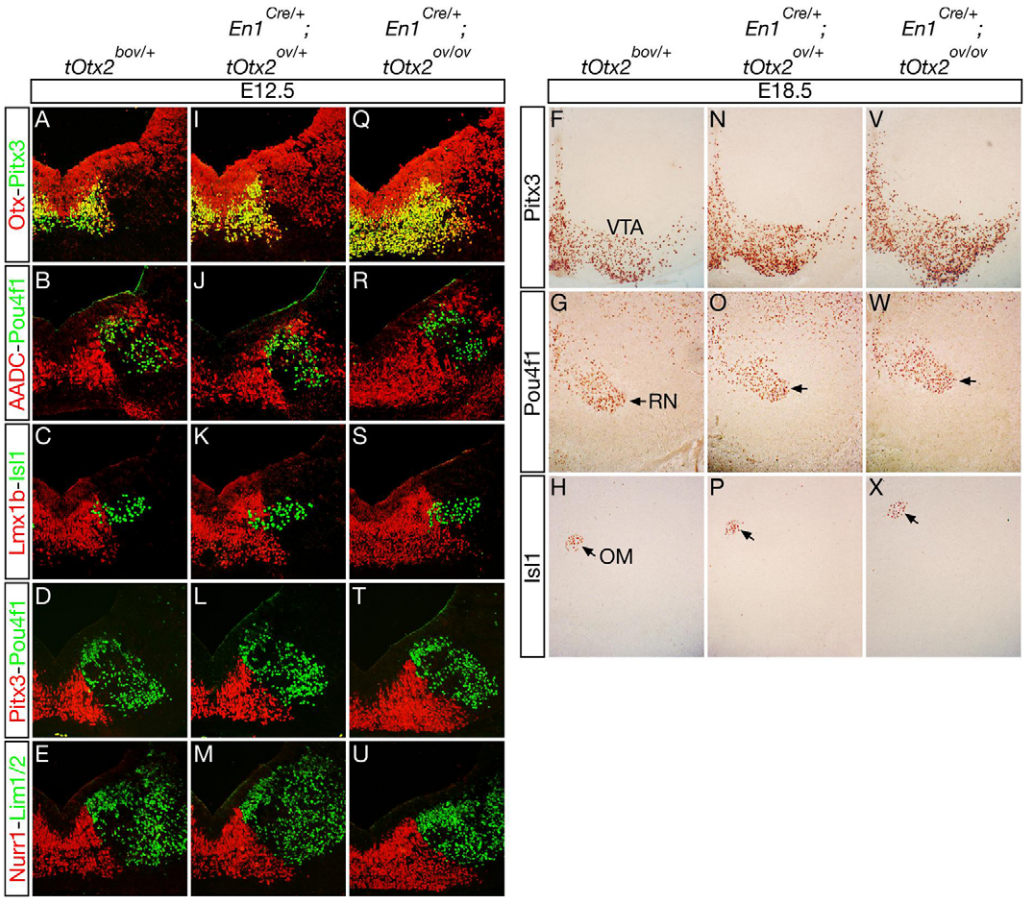

Fig. 5. Otx2 overexpression does not affect the generation and identity of RN and OM neurons. (**A-X**) Immunohistochemistry performed on the intermediate mesencephalon of E12.5 and E18.5 *tOtx2*$^{bov/+}$, *En1*$^{Cre/+}$; *tOtx2*$^{ov/+}$ and *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ with Otx2 and Pitx3 (A,I,Q), AADC and Pou4f1 (B,J,R), Lmx1b and Isl1 (C,K,S), Pitx3 and Pou4f1 (D,L,T), Nurr1 and Lim1/2 (E,M,U), Pitx3 (F,N,V), Pou4f1 (G,O,W) and Isl1 (H,P,X). Abbreviations: OM, oculomotor nucleus; RN, red nucleus; VTA, ventral tegmental area.

material), the phenotype described at E10.5 (Fig. 4) was confirmed at E12.5 by assessing also the combined expression of *Lmx1b*, *Nkx6.1*, *Nkx2.2*, *Foxa2* and *Shh* (Fig. S4A-C″).

Next, we studied whether Otx2 overexpression in the VM had a specific effect on the generation of mesDA neurons or also affected other neuronal populations. To achieve this, we first compared the expression of Isl1 and Pou4f1, two post-mitotic markers of the OM and RN neurons, with that of AADC, Lmx1b and Pitx3; and then the expression of Nurr1 with that of Lim1/2, which were, respectively, expressed in early post-mitotic mesDA and RN neurons. We found that, in contrast to the increase in the mesDA neurons, the number of Isl1$^+$ and Pou4f1$^+$ neurons, the size of the OM and RN and the identity of RN early post-mitotic precursors were similar in Otx2-overexpressing and control embryos (compare Fig. 5A-H with 5I-X). Collectively, these findings indicate that Otx2 overexpression induces a selective expansion of both mesDA progenitors and neurons, without affecting identity and size of adjacent progenitor domains or their post-mitotic progeny.

Otx2 is required to control proliferating activity of mesDA progenitors

Next, we studied whether Otx2 may control the extent of the mesDA domain by regulating selectively the proliferation of mesDA progenitors and/or their post-mitotic transition. To achieve this, we first determined the LI in the mesDA and Nkx6.1$^+$ domains by providing a short pulse of BrdU (30 minutes) and measuring the percentage of BrdU$^+$ cells over the total number of cells along the AP axis of the VM at E10.5 and E11.5 in control and mutant embryos. We found that the fraction of progenitors in S phase was remarkably increased in the mesDA domain of mutants at both stages analyzed, and that this increase was dose dependent and gradually more pronounced in the intermediate and posterior mesencephalon (Fig. 6A-H). Remarkably, almost no effect was detected in the proliferating activity of progenitors belonging to the Nkx6.1$^+$ domain (Fig. 6A-F,I,J).

We then investigated whether the increase of mesDA neurons may also be contributed by a decreased number of early mesDA progenitors exiting the cell cycle. The percentage of cycling progenitors quitting the cell cycle within 24 hours of BrdU administration at E10.5 was calculated by measuring the fraction of BrdU$^+$ cells that were Ki67$^-$ (Qf). Our data showed that the Qfs measured in the mesDA domain, but not those in the Nkx6.1$^+$ domain, were significantly reduced and, also in this case, correlated with the level of Otx2 overexpression (see Fig. S5 in the supplementary material). These findings thus suggest that the expansion of the mesDA domain may be caused by a selective enhancement in the proliferating activity of mesDA progenitors coupled to a decrease in the percentage of mesDA progenitors quitting the cell cycle. Based on these results, we studied the proliferation in the VM of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ embryos (Puelles et al., 2004). Previously, we reported that, in this mutant, the lack of Otx2 generates ventral de-repression of Nkx2.2, loss of Nkx6.1 expression in progenitors, dorsal expansion of Shh and lack of *Wnt1* expression. All these events resulted in the generation of 5-HT-containing neurons from RN and dorsalmost mesDA progenitors, and heavy reduction of mesDA neurons (Puelles et al., 2004; Prakash et al., 2006). Interestingly, these abnormalities mildly affected the pretectum and anterior mesencephalon (see Fig. S4D-I′ in the supplementary material), with increasing severity in the intermediate and posterior mesencephalon (about 80% less TH$^+$ neurons) (see Fig. S4J-L′ in the supplementary material) (Puelles et al., 2004).

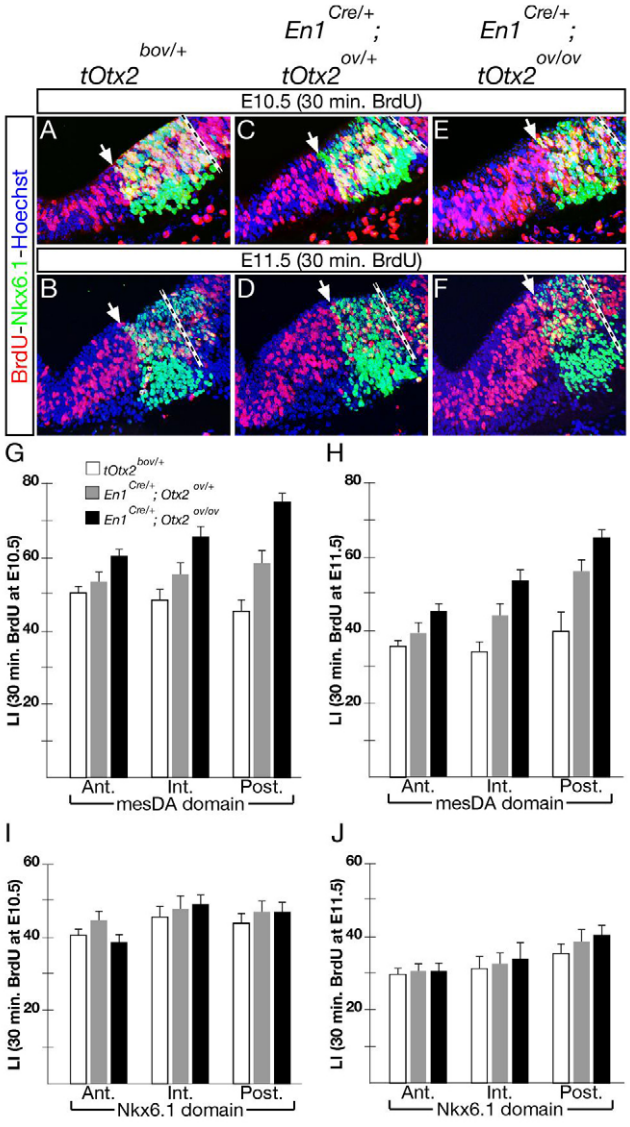

Fig. 6. The proliferating activity of mesDA progenitors is enhanced in Otx2 overexpressing mutants. (**A-F**) Representative adjacent sections through the intermediate mesencephalon of *tOtx2*$^{bov/+}$ (A,B), *En1*$^{Cre/+}$; *tOtx2*$^{ov/+}$ (C,D) and *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ (E,F) embryos pulsed with BrdU for 30 minutes at E10.5 or E11.5, are immunostained with BrdU and Nkx6.1, and stained with Hoechst to determine the LI of progenitors in the mesDA and Nkx6.1 domains. The arrow and the broken line indicate approximately the Nkx6.1$^+$ domain analyzed. (**G-J**) Graphic representation of the LI detected along the mesDA (G,H) and the Nkx6.1$^+$ (I,J) domains shows a selective dose-dependent increase in the proliferating activity of mesDA progenitors developing in the intermediate and posterior mesencephalon. Abbreviations: Ant., anterior; Int., intermediate; Post., posterior.

As previous studies have not addressed which process(es) was affected in mesDA neurogenesis of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants, we investigated whether the heavy reduction in mesDA neurons may be caused by abnormality in proliferation and/or premature post-mitotic transition and/or differentiation of their progenitors. First, we studied proliferation in the mesDA (Nkx2.2$^-$) and Nkx2.2$^+$ domains. Proliferating activity determined at E10.5 and E11.5 showed that the LI was heavily reduced in the mesDA domain of mutant embryos (Fig. 7A-J). By contrast, the LI detected in the Nkx6.1$^+$ domain of control embryos and in the Nkx2.2$^+$ domain of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants did not differ significantly (Fig. 7A-H,K,L). Next, we analyzed whether in these mutants mesDA progenitors quit the cell cycle prematurely. Compared with wild-type embryos, the Qfs for the intermediate and posterior (but not for the anterior) mesencephalon were dramatically increased in mutants (Fig. 7M-Q) and, remarkably, the expression of Ki67 was switched off in a relevant fraction of mesDA progenitors of the intermediate and posterior VM (Fig. 7M,N). As for the LI, the Qfs determined in the Nkx2.2$^+$ domain also showed only a mild reduction when compared with those of the Nkx6.1$^+$ domain of control embryos (Fig. 7M-P,R). Next, as Ki67 expression was heavily affected, we determined whether mesDA progenitors lacking Otx2 retained the expression of Sox2, which is normally transcribed by most of cycling CNS progenitors (Ki67$^+$) and downregulated when they exit the cell cycle and differentiate (Graham et al., 2003; Kele et al., 2006). We found that, despite the severe lack of both Ki67$^+$ and BrdU$^+$ cells (Figs 7, 8), the expression of Sox2 was retained (Fig. 8G,H), indicating that the Otx2-dependent impairment in proliferation did not affect Sox2 expression. Because this and previous studies showed that *Wnt1* expression was lost in the intermediate and posterior mesencephalon of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants (Prakash et al., 2006) (Fig. 8D,E) and expanded in Otx2-overexpressing embryos (Fig. 8F), we studied whether in the absence of *Wnt1*, Sox2$^+$-Otx2$^-$ progenitors expressed Cyclin D1 (CycD1), a direct target of the Wnt canonical pathway (Shtutman et al., 1999; Tetsu and McCormick, 1999). A remarkable loss of CycD1 expression (Fig. 8K) was detected in the Sox2$^+$-Nkx2.2$^-$ (Fig. 8B,H) domain of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ embryos. Conversely, p27^{kip1}, which is normally expressed at high level in quiescent post-mitotic neuronal cells (Lee et al., 1996), was strongly activated in the Sox2$^+$-Nkx2.2$^-$ cells (Fig. 8H). Compared with *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ embryos, in mutants overexpressing Otx2 an essentially opposite phenotype was detected. Indeed, *Wnt1* was expanded (Fig. 8F), BrdU$^+$ (Fig. 8C,L) and CycD1$^+$ (Fig. 8L) cells remarkably increased in number, and a high level of p27^{kip1} was detected only in Sox2$^-$ post-mitotic neurons (Fig. 8I).

We then investigated whether abnormalities in *Wnt1* expression correlated with the mesencephalic territory primarily affected in cell proliferation by Otx2 (overexpression or inactivation). We found that, although in the caudal mesencephalic area the expression of *Wnt1* was expanded in Otx2 overexpressing mutants and lost in embryos lacking Otx2 (see Fig. S6C-D″ in the supplementary material), in the rostral mesencephalon of both *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ and *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants, the expression of *Wnt1* was slightly affected (see Fig. S6A-A″ in the supplementary material), thus suggesting a close correlation between the territory requiring Otx2 for proliferation of mesDA progenitors and abnormalities in *Wnt1* expression. Collectively, these findings indicate that Otx2 selectively controls the proliferating activity of intermediate and posterior mesDA progenitors and prevents their premature post-mitotic transition, possibly, through activation/maintenance of the Wnt canonical pathway.

MesDA differentiation in *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ and *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants

Relevant studies have shown that Shh-dependent induction of Lmx1a is required for Msx1 expression, and that Msx1 is necessary for Ngn2 activation, which, in turn, promotes the differentiation of Sox2$^+$ mesDA progenitors into Nurr1$^+$ post-mitotic DA precursors (Kele et al., 2006; Andersson et al., 2006a; Andersson et al., 2006b; Ono et al.,

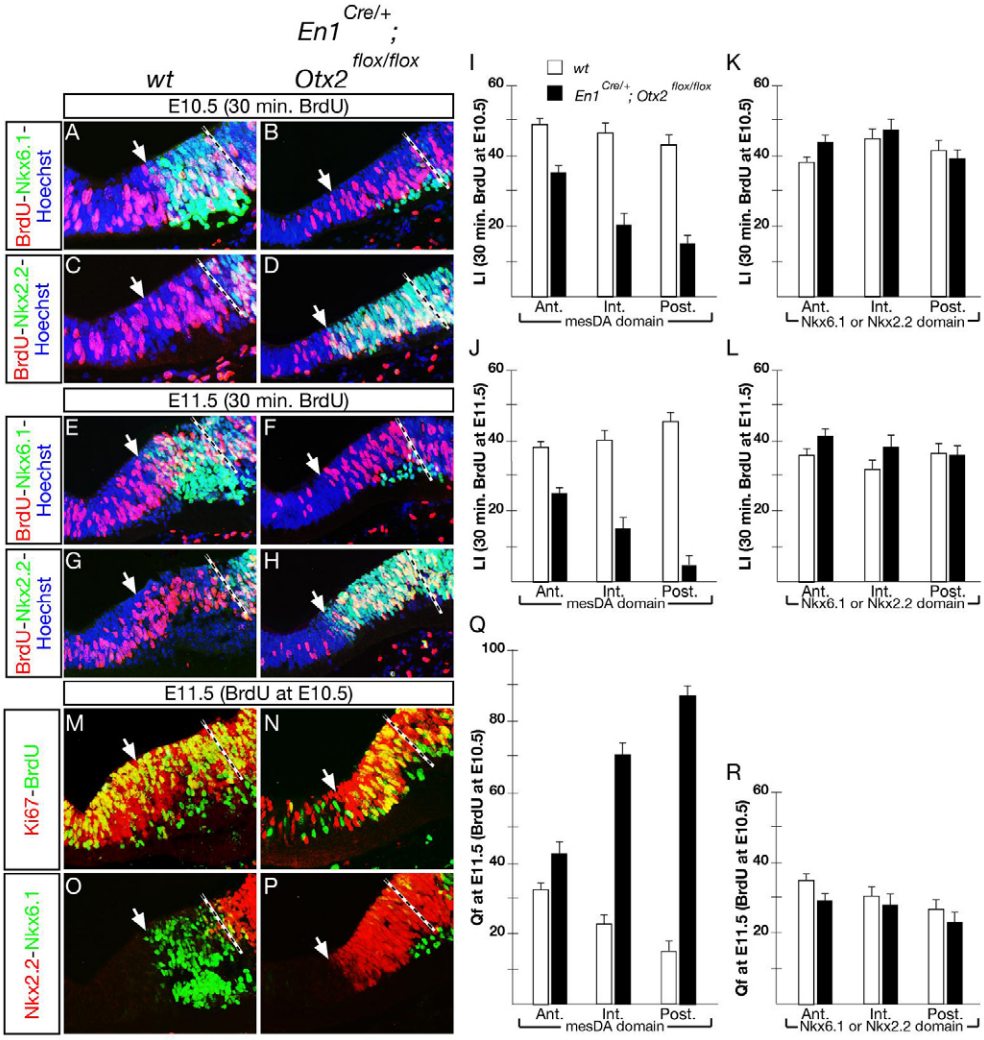

Fig. 7. Otx2 controls proliferation and post-mitotic transition of mesDA progenitors. (**A-H**) Representative adjacent sections through the intermediate mesencephalon of E10.5 (A-D) and E11.5 (E-H) wild-type and *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ embryos immunostained with Nkx6.1 and BrdU (A,B,E,F), Nkx2.2 and BrdU (C,D,G,H), and then stained with Hoechst show that the number of BrdU$^+$ cells is dramatically and selectively reduced in the mesDA domain of mutants. (**I-L**) Graphic representation showing the AP graded reduction of the LI in the mesDA domain of mutants (I,J), whereas the LI measured in the Nkx2.2$^+$ domain of mutant embryos is similar to that detected in the Nkx6.1$^+$ domain of control embryos (K,L). (**M-P**) Representative adjacent sections through the intermediate mesencephalon of E11.5 wild-type and *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ mutants immunostained with Ki67 and BrdU (M,N), and Nkx2.2 and Nkx6.1 (O,P) show that, in mutant embryos, Ki67 is switched off in the majority of mesDA progenitors and that most of the mesDA BrdU$^+$ cells (labeled at E10.5) become post-mitotic (Ki67$^-$) at E11.5. Conversely, in the Nkx2.2$^+$ domain, most of the BrdU$^+$ progenitors retain Ki67 expression as in the Nkx6.1$^+$ domain of control embryos. (**Q**,**R**) Graphic representation of the Qfs in the mesDA (Q), Nkx6.1$^+$ (in wild type) and Nkx2.2$^+$ (in *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ embryos) domains (R) shows a selective increase in the number of mesDA progenitors quitting the cell-cycle in mutant embryos, whereas a mild reduction is detected in the Nkx2.2$^+$ domain. The arrow and the broken line in (A-H,M-P) indicate the Nkx6.1$^+$ or the Nkx2.2$^+$ territories analyzed. Abbreviations: Ant., anterior; Int., intermediate; Post., posterior.

2007). On this basis, we first analyzed the expression of Otx2 in relation to several markers at E11.5 and E12.5 in wild-type embryos. This analysis showed that in the Nkx6.1$^-$Lmx1b$^+$ mesDA domain (see Fig. S7A,A′ in the supplementary material), Otx2 was co-expressed in progenitors with *Sox2*, *Mash1*, *Ngn2*, *Lmx1a*, *Msx1* and *Lmx1b* (see Fig. S7B-D′ in the supplementary material; Fig. 9C,D,E,F,O,P), in early post-mitotic precursors with Ngn2, Nurr1 and Pitx3 (see Fig. S7D-F′ in the supplementary material), and in more mature mesDA neurons with a large subset of Pitx3$^+$ cells (see Fig. S7F,F′ in the supplementary material). We then investigated whether the expression of these genes and, consequently, the differentiation of mesDA progenitor subsets was altered in response to increased level or lack of Otx2. These experiments showed that, compared with wild-type embryos, in Otx2 overexpressing mutants *Lmx1a* and *Msx1* expression was expanded and the number of Ngn2$^+$-Sox2$^+$ and Mash1$^+$-Sox2$^+$ progenitors was increased, whereas, in *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ embryos, *Lmx1a* and *Msx1* transcription was silenced and the expression of Ngn2 and Mash1 was confined to sporadic mesDA Sox2$^+$ progenitors (Fig. 9C-J″). In contrast to these genes, the expression of Lmx1b was retained in *En1*$^{Cre/+}$*; Otx2*$^{flox/flox}$ mutants, where it was also expanded dorsally within the Nkx2.2$^+$ domain from which 5-HT$^+$ neurons were generated (Fig. 9O-P″) (Puelles et al., 2004). Finally, we analyzed the generation of Ngn2$^+$-Nurr1$^+$ and Nurr1$^+$-Pitx3$^+$ subpopulations of post-mitotic mesDA neurons, which,

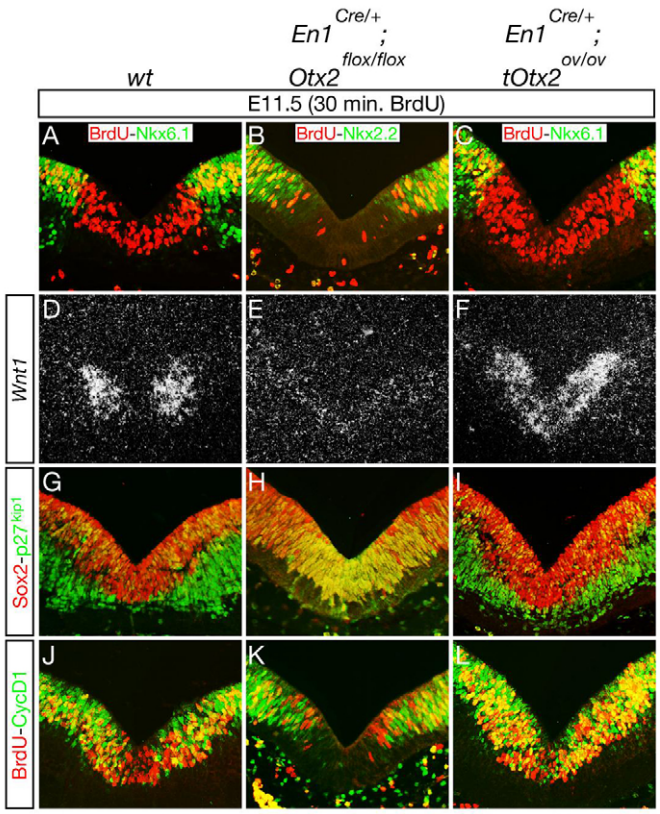

Fig. 8. *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ and *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ embryos exhibit complementary expression abnormalities in cell-cycle components. (**A-L**) Adjacent sections through the intermediate mesencephalon of E11.5 wild type, *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ and *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ embryos pulsed with BrdU for 30 minutes and immunostained with BrdU and Nkx6.1 (A,C), Nkx2.2 and BrdU (B), Sox2 and p27^{kip1} (G-I) and BrdU and CycD1 (J-L) or hybridized with the *Wnt1* probe (D-F).

as expected, were remarkably expanded in *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ mutants and virtually absent at E11.5 or heavily reduced at E12.5 in *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ embryos (Fig. 9K-N″). These findings show that overexpression of Otx2 induces increased generation of mesDA neurons that correlates with a corresponding expansion of the subpopulations of differentiating progenitors, while lack of Otx2 results in the general failure of the mesDA differentiation program.

DISCUSSION

Previous studies have indicated that the specification of mesDA neurons is characterized by sequential steps that determine, first, the mesDA field and, subsequently, the progressive maturation of mesDA progenitors into post-mitotic immature precursors and, then, mature neurons expressing the TH neurotransmitter gene (reviewed by Smidt and Burbach, 2007; Smits et al., 2006; Prakash and Wurst, 2006). In this process, Otx2 is early required to control the positioning of *Fgf8* and *Shh* expression, to regulate *Wnt1* expression and to maintain the integrity of progenitor domains in the whole VM (Puelles et al., 2003; Puelles et al., 2004; Simeone et al., 2002; Prakash et al., 2006; Liu and Joyner, 2001; Smidt and Burbach, 2007). In this study, we have investigated for the first time the potential role of Otx2 as an intrinsic determinant of the developmental program of mesDA neurogenesis, and have discovered that Otx2 controls selectively the proliferating activity of mesDA progenitors and is required to promote their differentiation by directly or indirectly activating the Lmx1a-Msx1-Ngn2 genetic cascade (Kele et al., 2006; Andersson et al., 2006a; Andersson et al., 2006b; Ono et al., 2007).

Otx2 controls selectively the generation of mesDA neurons through a dose-dependent AP graded effect on the proliferation of mesDA progenitors

This study shows that Otx2 overexpression causes increased generation of mesDA neurons. This increase correlated with the level of overexpressed Otx2 protein and was more dramatic in the posterior and intermediate mesencephalon than in the anterior mesencephalon and pretectum. Consistently, embryos that lack Otx2 exhibit heavy reduction of mesDA neurons in the intermediate and posterior mesencephalon, whereas in the anterior mesencephalon and pretectum their generation is less affected (Puelles et al., 2004) (see Fig. S4 in the supplementary material). These data, therefore, indicate that, depending on the position occupied along the AP axis of VM, mesDA progenitors exhibit a differential response to Otx2. A second finding of this study is that although Otx2 is overexpressed even in the VM progenitor domains adjacent to the mesDA compartment, in these domains, neurons generation is not increased. Indeed, boundary relationships among Nkx6.1, Nkx2.2, Shh and Foxa2, as well as the number and identity of OM and RN neurons are unaffected by Otx2 overexpression. This suggests that Otx2 may exert a selective control on the generation of mesDA neurons by modulating their number along the AP axis.

The selective effect on the generation of mesDA neurons has been investigated by analyzing whether Otx2 is required to regulate the proliferating activity and post-mitotic transition of mesDA progenitors. This analysis has shown that Otx2 plays a major role in controlling proliferation of mesDA progenitors. Indeed, in Otx2 overexpressing embryos, the LI is significantly increased and the Qf is reduced. Conversely, in mutants that lack Otx2, the LI exhibits a drastic reduction and the Qf a dramatic increase. Furthermore, in *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ embryos, a large fraction of Sox2^{+} progenitors in the intermediate and posterior mesencephalon switch off Ki67 and induce high level of p27^{kip1}, suggesting that they represent a type of ‘frozen’ progenitor that prematurely exits the cell cycle. Remarkably, these abnormalities are restricted to mesDA progenitors, as, in mutants that overexpress or lacking Otx2, the adjacent Nkx6.1^{+} or Nkx2.2^{+} domains are apparently unaffected or exhibit mild impairments in LI, Qf and in expression of Ki67 and p27^{kip1}. Thus, our data provide the first evidence that (1) Otx2 may regulate selectively the generation of mesDA neurons by controlling the proliferating activity of their progenitors; and (2) this control exhibits an AP graded effect. This and previous studies have shown that *Wnt1* expression is lost in the VM of *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mice and that Wnt molecules are differentially required, being indeed involved in promoting proliferation (Wnt1) or mesDA differentiation (Wnt5a) (Panhuysen et al., 2004; Castelo-Branco et al., 2003; Castelo-Branco et al., 2004). Our data suggest that Otx2 may control mesDA proliferation through the maintenance and/or activation of the Wnt/β-catenin pathway. In this context, active Wnt pathway through the TCF/LEF/β-catenin nuclear complex may modulate the transcription of a broad range of target genes, including cyclins and, in particular, Cyclin D1, a major regulator of the cell cycle progression (Fodde and Brabletz, 2007; Shtutman et al., 1999; Tetsu and McCormick, 1999; Lin et al., 2000; Arber et al., 1997). We show that CycD1 expression is suppressed specifically in the

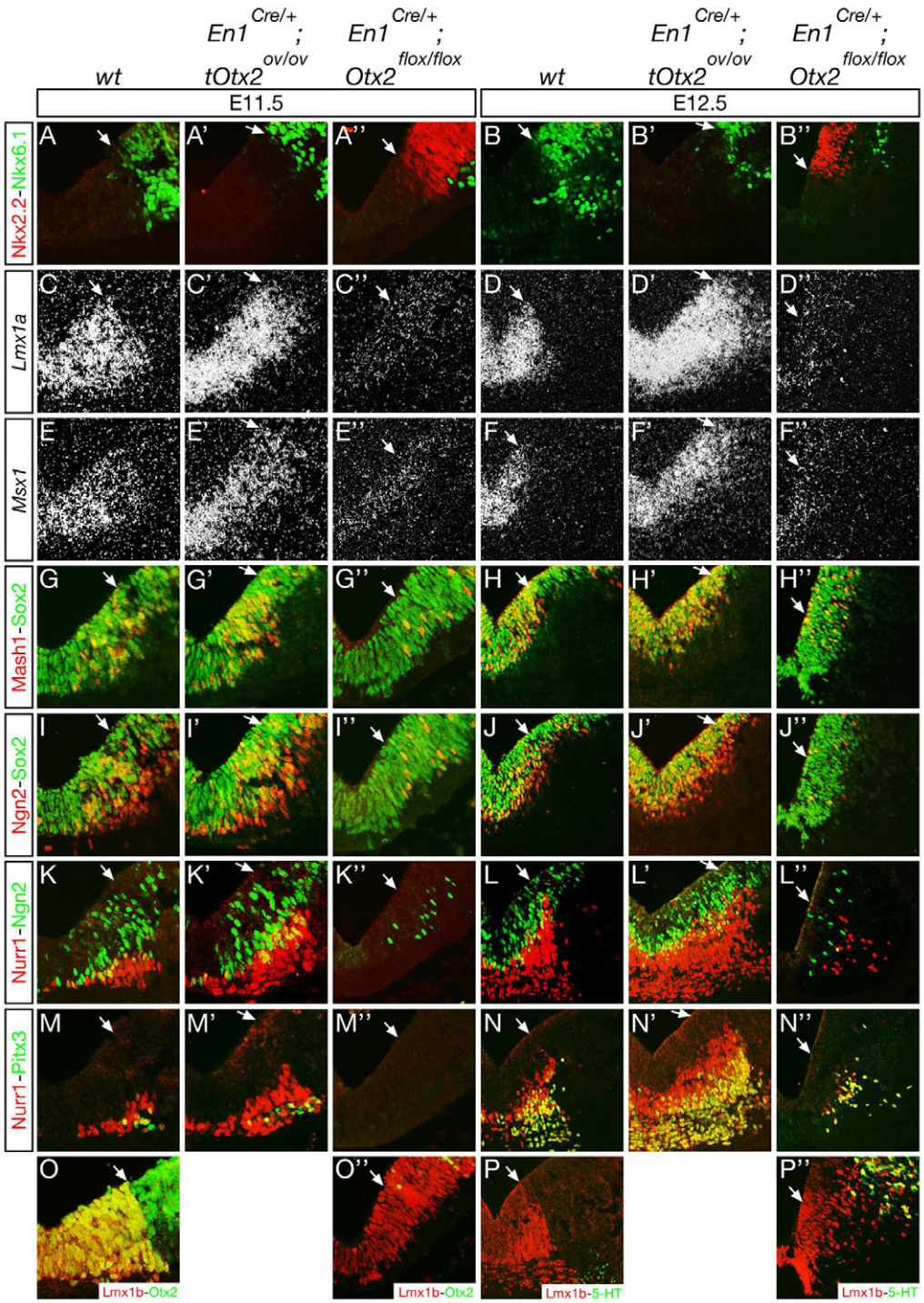

Fig. 9. Differentiation of mesDA neurons requires Otx2. (**A-P″**) Adjacent sections through the intermediate mesencephalon of E11.5 and E12.5 wild type, *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ and *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ embryos immunostained with Nkx6.1 and Nkx2.2 (A-B″), Mash1 and Sox2 (G-H″), Ngn2 and Sox2 (I-J″), Nurr1 and Ngn2 (K-L″), Nurr1 and Pitx3 (M-N″), Lmx1b and Otx2 (O,O″), Lmx1b and 5-HT (P,P″) or hybridized with *Lmx1a* (C-D″) or *Msx1* (E-F″) probes. In *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants, Lmx1b is dorsally expanded within the Nkx2.2^{+} domain that generates 5-HT^{+} neurons, and is abundant at E12.5 in the Sox2^{+} mesDA progenitors (O″,P″). The arrow indicates the ventral border of Nkx6.1^{+} domain in wild-type and *En1*$^{Cre/+}$; *tOtx2*$^{ov/ov}$ embryos and the ventral border of the Nkx2.2^{+} domain in *En1*$^{Cre/+}$; *Otx2*$^{flox/flox}$ mutants.

mesDA domain of mutants that lack *Wnt1* expression, whereas in embryos overexpressing Otx2, the expanded expression of *Wnt1* correlates with a higher number of CycD1^{+} cells. Therefore, these findings strongly support the possibility that Otx2 may control the proliferation of mesDA progenitors through the maintenance/activation of the Wnt canonical pathway by regulating the expression of *Wnt1*. In view of the selective effect on mesDA progenitors, our data indicate propagation of the Wnt1 proliferative signal over a very short range.

Otx2 is required for mesDA differentiation

Recent studies have indicated that the differentiation of mesDA progenitors and their transition to post-mitotic mesDA neurons require an intricate pathway of transcription factors (reviewed by Smidt and Burbach, 2007). Indeed, experiments performed in chick and analysis of mouse mutants collectively suggest that early in mesDA differentiation Shh-dependent expression of Lmx1a activates Msx1, which induces Ngn2; Ngn2, in turn, is required for the differentiation of Sox2^{+} progenitors into Nurr1^{+} post-mitotic young mesDA neurons

(Andersson et al., 2006a; Andersson et al., 2006b; Kele et al., 2006; Ono et al., 2007). In this context, however, although in chick embryos the silencing of Lmx1a abolishes Nurr1 expression, mouse *Lmx1a*-null mutants exhibit only 30% fewer TH neurons and, similarly, *Msx1*-null embryos lack 40% of Ngn2$^+$ progenitors and Nurr1$^+$ mesDA neurons (Ono et al., 2007; Andersson et al., 2006b). As reported, this might suggest the existence of compensatory functions and/or, in addition to their sequential requirement, an Lmx1a-Msx1 synergistic action (Andersson et al., 2006b). Our data show that, compared with control embryos, in Otx2-overexpressing embryos, the expression of *Lmx1a*, *Lmx1b*, *Msx1*, *Ngn2* and *Mash1* is expanded in a higher number of mesDA progenitors, which generate more Ngn2$^+$-Nurr1$^+$ immature precursors and Pitx3$^+$ mature mesDA neurons; conversely, in embryos lacking Otx2, the expression of *Lmx1a*, *Msx1*, *Ngn2* and *Mash1* is lost or severely affected in Sox2$^+$ mesDA progenitors, which are greatly impaired in their ability to generate Nurr1$^+$ post-mitotic mesDA neurons.

Thus, these findings indicate that Otx2 is required to activate the genetic pathway leading to mesDA neuron generation. In this context, Otx2 might directly control the activation of *Lmx1a* and, consequently, the subsequent steps of mesDA differentiation. Supporting this possibility is the finding that ectopic expression of Otx2 in the ventral hindbrain is sufficient to induce Lmx1a, Msx1 and proneural gene expression, and the generation of TH$^+$-Pitx3$^+$ DA neurons (Ono et al., 2007). A second possibility is that Otx2 is indirectly required for *Lmx1a* activation by providing early mesDA progenitors with competence to respond to Shh-mediated induction of *Lmx1a*. In molecular terms, Otx2 might be necessary to repress a Shh-independent repressor of *Lmx1a*. A third possibility is that Shh might activate in mesDA progenitors an Otx2 co-activator required for Otx2-mediated induction of *Lmx1a*. The analysis of the mesDA genetic cascade in conditional mutants lacking Otx2 or Shh or both functions might shed light on this important aspect in the future. However, although our data suggest that Otx2-dependent expression of *Wnt1* is more likely to be required to control proliferation of mesDA progenitors and prevent their premature exit from cell cycle, they do not exclude a priori that Wnt1 signaling may be necessary for one or more steps of mesDA differentiation, including competence of mesDA progenitors to Shh-inducing activity. A final finding of this study is that in the mesDA domain of *En1$^{Cre/+}$; Otx2$^{flox/flox}$* mutants most of the Sox2$^+$ progenitors are Ki67$^-$p27^{kip1+} and that these 'post-mitotic' progenitors do not activate any of the genes required for post-mitotic maturation of mesDA neurons (e.g. Nurr1). This suggests that, at least in the *En1$^{Cre/+}$; Otx2$^{flox/flox}$* mouse model, Sox2 expression does not require cell proliferation activity and that post-mitotic maturation of mesDA progenitors is not activated only by a block in proliferating activity and/or cell cycle exit, but, rather, depends on the correct progression of the differentiation process culminating with the transition of Sox2$^+$-Ngn2$^+$ progenitors into Sox2$^-$Ngn2$^+$-Nurr1$^+$ post-mitotic immature mesDA neurons. Interestingly, it has been recently shown that Otx2 and Sox2 physically interact to activate *Rax1* expression in the retina (Danno et al., 2008). Whether Otx2 and Sox2 may interact also in mesDA progenitors and regulate the expression of target gene(s) remains to be investigated.

Concluding remarks

This and previous studies show that Otx2 is required for multiple steps of mesDA neuron development. Indeed Otx2 controls early specification of VM by both positioning Shh and Fgf8 signals, and maintaining the identity of progenitors domains (Puelles et al., 2003; Puelles et al., 2004; Prakash et al., 2006). Here, we have provided evidence that Otx2 exerts a crucial influence over mesDA neurogenesis by regulating the proliferating activity and differentiation of mesDA progenitors. Collectively, these and previous findings suggest that Otx2 represents a potentially relevant genetic determinant in future ES cell- or mesDA progenitor-based studies that are focused on improving the generation of authentic mesDA neurons and providing potential tools for the treatment of Parkinson's disease.

We thank D. Grieco, C. Missero and T. Russo for helpful discussions and criticisms on the manuscript, and the staff of the CEINGE animal house for excellent animal care. We are also indebted to G. Corte for the Sox2 and Otx2 antibodies, and to D. J. Anderson for the Ngn2 antibody. This work was supported by the Italian Association for Cancer Research (AIRC), the FP6 for the EuTRACC Integrate Project (LSHG-CT-2007-037445) and the 'Fondazione Cassa di Risparmio' of Rome to A.S., and by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung and the European Communion to W.W.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/20/3459/DC1

References

Acampora, D. and Simeone, A. (1999). The *TINS* lecture. Understanding the roles of Otx1 and Otx2 in the control of brain morphogenesis. *Trends Neurosci* **22**, 116-122.

Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F. and Björklund, A. (2006a). Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* **133**, 507-516.

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J. (2006b). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* **124**, 393-405.

Arber, N., Doki, Y., Han, E. K., Sgambato, A., Zhou, P., Kim, N. H., Delohery, T., Klein, M. G., Holt, P. R. and Weinstein, I. B. (1997). Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. *Cancer Res.* **57**, 1569-1574.

Björklund, A. and Lindvall, O. (1984). Dopamine-containing systems in the CNS. In *Handbook of Chemical Neuroanatomy: Classical Transmitters in the CNS* (ed. A. Biörklund and T. Hökfelt), Vol. 2, pp. 55-121. Amsterdam, The Netherlands: Elsevier.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin Neurobiol* **11**, 43-49.

Castelo-Branco, G., Wagner, J., Rodriguez, F. J., Kele, J., Sousa, K., Rawal, N., Pasolli, H. A., Fuchs, E., Kitajewski, J. and Arenas, E. (2003). Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. *Proc. Natl. Acad. Sci. USA* **100**, 12747-12752.

Castelo-Branco, G., Rawal, N. and Arenas, E. (2004). GSK-3beta inhibition/beta-catenin stabilization in ventral midbrain precursors increases differentiation into dopamine neurons. *J. Cell Sci.* **117**, 5731-5737.

Danno, H., Michiue, T., Hitachi, K., Yukita, A., Ishiura, S. and Asashima, M. (2008). Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. *Proc. Natl. Acad. Sci. USA* **105**, 5408-5413.

Egan, M. F. and Weinberger, D. R. (1997). Neurobiology of schizophrenia. *Curr. Opin Neurobiol* **7**, 701-707.

Ferri, A. L., Lin, W., Mavromatakis, Y. E., Wang, J. C., Sasaki, H., Whitsett, J. A. and Ang, S. L. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. *Development* **134**, 2761-2769.

Fodde, R. and Brabletz, T. (2007). Wnt/beta-catenin signaling in cancer stemness and malignant behavior. *Curr. Opin Cell Biol.* **19**, 150-158.

Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.

Hökfelt, T., Matensson, A., Björklund, S., Kleinau, S. and Goldstein, M. (1984). Distributional maps of tyrosine hydroxylase-immunoreactive neurons in the rat brain. In *Handbook of Chemical Neuroanatomy: Classical Transmitters in the CNS* (ed. A. Biörklund and T. Hökfelt), Vol. 2, pp. 227-379. Amsterdam, The Netherlands: Elsevier.

Isacson, O. (1993). On neuronal health. *Trends Neurosci.* **16**, 306-308.

Jellinger, K. A. (2001). The pathology of Parkinson's disease. *Adv. Neurol.* **86**, 55-72.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

Kele, J., Simplicio, N., Ferri, A. L., Mira, H., Guillemot, F., Arenas, E. and Ang, S. L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495-505.

Kelley, A. E. and Berridge, K. C. (2002). The neuroscience of natural rewards: relevance to addictive drugs. *J. Neurosci.* **22**, 3306-3311.

Lee, M. H., Nikolic, M., Baptista, C. A., Lai, E., Tsai, L. H. and Massagué, J. (1996). The brain-specific activator p35 allows Cdk5 to escape inhibition by $p27^{Kip1}$ in neurons. *Proc. Natl. Acad. Sci. USA* **93**, 3259-3263.

Lin, S. Y., Xia, W., Wang, J. C., Kwong, K. Y., Spohn, B., Wen, Y., Pestell, R. G. and Hung, M. C. (2000). Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. USA* **97**, 4262-4266.

Liu, A. and Joyner, A. (2001). Early anterior/posterior patterning of the midbrain and cerebellum. *Annu. Rev. Neurosci.* **24**, 869-896.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Marín, F., Herrero, M. T., Vyas, S. and Puelles, L. (2005). Ontogeny of tyrosine hydroxylase mRNA expression in mid- and forebrain: neuromeric pattern and novel positive regions. *Dev. Dyn.* **234**, 709-717.

McCaffery, P. and Dräger, U. C. (1994). High levels of a retinoic acid-generating dehydrogenase in the meso-telencephalic dopamine system. *Proc. Natl Acad. Sci. USA* **91**, 7772-7776.

Nunes, I., Tovmasian, L. T., Silva, R. M., Burke, R. E. and Goff, S. P. (2003). Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc. Natl. Acad. Sci. USA* **100**, 4245-4250.

Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H. et al. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development* **134**, 3213-3225.

Panhuysen, M., Vogt Weisenhorn, D. M., Blanquet, V., Brodski, C., Heinzmann, U., Beisker, W. and Wurst, W. (2004). Effects of Wnt1 signaling on proliferation in the developing mid-/hindbrain region. *Mol. Cell. Neurosci.* **26**, 101-111.

Prakash, N. and Wurst, W. (2006). Development of dopaminergic neurons in the mammalian brain. *Cell Mol. Life Sci.* **63**, 187-206.

Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M. et al. (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* **133**, 89-98.

Puelles, E., Acampora, D., Lacroix, E., Signore, M., Annino, A., Tuorto, F., Filosa, S., Corte, G., Wurst, W., Ang, S. L. et al. (2003). Otx dose-dependent integrated control of antero-posterior and dorso-ventral patterning of midbrain. *Nat. Neurosci* **6**, 453-460.

Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037-2048.

Puelles, E., Acampora, D., Gogoi, R., Tuorto, F., Papalia, A., Guillemot, F., Ang, S. L. and Simeone, A. (2006). Otx2 controls identity and fate of glutamatergic progenitors of the thalamus by repressing GABAergic differentiation. *J. Neurosci.* **26**, 5955-5964.

Saucedo-Cardenas, O., Quintana-Hau, J. D., Le, W. D., Smidt, M. P., Cox, J. J., De Mayo, F., Burbach, J. P. and Conneely, O. M. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc. Natl. Acad. Sci. USA* **95**, 4013-4018.

Semina, E. V., Ferrell, R. E., Mintz-Hittner, H. A., Bitoun, P., Alward, W. L., Reiter, R. S., Funkhauser, C., Daack-Hirsch, S. and Murray, J. C. (1998). A novel homeobox gene PITX3 is mutated in families with autosomal-dominant cataracts and ASMD. *Nat. Genet.* **19**, 167-170.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA* **96**, 5522-5527.

Simeone, A. (1999). Detection of mRNA in tissue sections with radiolabelled riboprobes. In *In Situ Hybridization. A Practical Approach*, 2nd edn (ed. D. G. Wilkinson), pp. 69-86. Oxford: IRL, Oxford University Press.

Simeone, A. (2005). Genetic control of dopaminergic neuron differentiation. *Trends Neurosci.* **28**, 62-66.

Simeone, A., Puelles, E. and Acampora, D. (2002). The Otx family. *Curr. Opin. Genet. Dev.* **12**, 409-415.

Simon, H. H., Saueressig, H., Wurst, W., Goulding, M. D. and O'Leary, D. D. (2001). Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J. Neurosci.* **21**, 3126-3134.

Smidt, M. P. and Burbach, J. P. (2007). How to make a mesodiencephalic dopaminergic neuron. *Nat. Rev Neurosci.* **8**, 21-32.

Smidt, M. P., van Schaick, H. S., Lanctôt, C., Tremblay, J. J., Cox, J. J., van der Kleij, A. A., Wolterink, G., Drouin, J. and Burbach, J. P. (1997). A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc. Natl. Acad. Sci. USA* **94**, 13305-13310.

Smidt, M. P., Asbreuk, C. H., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat. Neurosci* **3**, 337-341.

Smidt, M. P., Smits, S. M., Bouwmeester, H., Hamers, F. P., van der Linden, A. J., Hellemons, A. J., Graw, J. and Burbach, J. P. (2004). Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. *Development* **131**, 1145-1155.

Smits, S. M., Burbach, J. P. and Smidt, M. P. (2006). Developmental origin and fate of meso-diencephalic dopamine neurons. *Prog. Neurobiol.* **78**, 1-16.

Sonnier, L., Le Pen, G., Hartmann, A., Bizot, J. C., Trovero, F., Krebs, M. O. and Prochiantz, A. (2007). Progressive loss of dopaminergic neurons in the ventral midbrain of adult mice heterozygote for Engrailed1. *J. Neurosci.* **27**, 1063-1071.

Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-426.

van den Munckhof, P., Luk, K. C., Ste-Marie, L., Montgomery, J., Blanchet, P. J., Sadikot, A. F. and Drouin, J. (2003). Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. *Development* **130**, 2535-2542.

von Bohlen und Halbach, O., Schober, A. and Krieglstein, K. (2004). Genes, proteins, and neurotoxins involved in Parkinson's disease. *Prog. Neurobiol.* **73**, 151-177.

Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. *Nat. Rev Neurosci* **2**, 99-108.

Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.

Zetterström, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248-250.