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# A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo

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Midbrain neurons synthesizing the neurotransmitter dopamine play a central role in the modulation of different brain functions and are associated with major neurological and psychiatric disorders. Despite the importance of these cells, the molecular mechanisms controlling their development are still poorly understood. The secreted glycoprotein Wnt1 is expressed in close vicinity to developing midbrain dopaminergic neurons. Here, we show that Wnt1 regulates the genetic network, including Otx2 and Nkx2-2, that is required for the establishment of the midbrain dopaminergic progenitor domain during embryonic development. In addition, Wnt1 is required for the terminal differentiation of midbrain dopaminergic neurons at later stages of embryogenesis. These results identify Wnt1 as a key molecule in the development of midbrain dopaminergic neurons in vivo. They also suggest the Wnt1-controlled signaling pathway as a promising target for new therapeutic strategies in the treatment of Parkinson's disease.

**KEY WORDS: Dopaminergic neuron, Development, Midbrain, Progenitor domain, Cell fate specification, Wnt1, Otx2, Nkx2-2, Mouse**

## INTRODUCTION

Neurons synthesizing the neurotransmitter dopamine are found in different areas of the mammalian brain. The most prominent dopaminergic (DA) cell group is that of the ventral midbrain, comprising the substantia nigra (SN), the ventral tegmental area (VTA) and the retrorubral field (RrF) (Dahlstrom and Fuxe, 1964). Midbrain dopaminergic (mDA) neurons modulate a broad range of processes in the brain, including movement, cognition and reward. Thus, degeneration or dysfunction of mDA neurons in the human brain leads to severe neurological and psychiatric disorders, among them Parkinson's disease (PD) (Cooper et al., 2001; Lang and Lozano, 1998).

Understanding the development and maintenance of mDA neurons is of high clinical interest as replacement of this cell population in the diseased brain is considered to be one of the most promising therapeutic approaches for PD (Lindvall et al., 2004). Despite considerable advances made in recent years, the factors and steps controlling the development and maintenance of mDA neurons are far from being fully identified and understood. mDA neurons develop in the ventral midbrain in close vicinity to two important signaling centers of the embryonic neural tube, the floor plate (FP) and the mid-hindbrain boundary (MHB). The FP comprises specialized cells that secrete the glycoprotein sonic hedgehog (Shh). In turn, Shh regulates the expression of a variety of transcription factors, whose expression code confers the cellular identity along the dorsoventral axis of the neural tube (Jessell, 2000). One of these Shh-responsive genes is *Nkx2-2*, which encodes a type II homeodomain transcriptional regulator required for the specification of ventral cell populations in the hindbrain and spinal cord (Briscoe et al., 1999; Pattyn et al., 2003a; Pattyn et al., 2003b). The MHB is established at the expression border of two transcriptional repressors, Otx2 in the fore- and midbrain and Gbx2 in the hindbrain (Liu and Joyner, 2001a; Prakash and Wurst, 2004; Wurst and Bally-Cuif, 2001). In turn, transcription of the secreted proteins Wnt1 and fibroblast growth factor 8 (Fgf8) is initiated at the MHB, and transcription factors belonging to the engrailed (En) and Pax families are expressed across the MHB. Using explant cultures, Ye et al. (Ye et al., 1998) demonstrated that both Shh and Fgf8 are together required for induction of mDA neurons at ectopic locations, thus suggesting that the signals coming from the FP and the MHB play an important role in the development of these neurons. We have recently shown that the position of the MHB indeed controls the location and size of the mDA neuronal population (Brodski et al., 2003). Other factors implicated in the terminal differentiation and maintenance of mDA neurons are the LIM-homeodomain factor Lmx1b, transforming growth factors (TGFs) α and β, the En proteins, the orphan nuclear receptor Nr4a2 (Nurr1), and the mDA-specific paired-like homeodomain transcription factor Pitx3 (Alberi et al., 2004; Blum, 1998; Farkas et al., 2003; Hwang et al., 2003; Maxwell et al., 2005; Nunes et al., 2003; Simon et al., 2001; Smidt et al., 2000; Smidt et al., 2004; van den Munckhof et al., 2003; Zetterstrom et al., 1997). However, the precise mechanism that links early inductive signals to the molecular network regulating the differentiation and maintenance of mDA neurons still remains elusive.

Wnts are secreted palmitoylated glycoproteins involved in the control of cell proliferation, differentiation, polarity, migration and death (Baek et al., 2003; Hirabayashi et al., 2004; Megason and McMahon, 2002; Willert et al., 2003). Wnt1 is expressed in a ring encircling the neural tube at the MHB, in the dorsal midline (roof plate) of the midbrain, and in the ventral midline [FP and basal plate (BP)] of the cephalic flexure. The latter Wnt1 expression domain

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coincides with the region where mDA progenitors first arise at mouse embryonic day 9.5 (E9.5) (see Fig. S1 in the supplementary material) and mDA neurons later develop at E10.5-12.5. Previous in vitro studies indicated that Wnt5a, another member of the Wnt family (Parr et al., 1993), promotes mDA neuron differentiation, whereas Wnt1 predominantly enhances mDA progenitor proliferation (Castelo-Branco et al., 2003). In order to assess the role of Wnt1 in the generation of mDA neurons in vivo, we used different transgenic mouse lines as well as explant cultures. Here, we provide evidence for the first time that Wnt1 is indeed required for the generation of mDA neurons in vivo by controlling a molecular cascade that leads to the establishment of the mDA progenitor domain in early neural development and to the acquisition of the full mDA phenotype at later developmental stages.

## MATERIALS AND METHODS

### Transgenic mice

Generation and genotyping of $En1^{+/Wnt1}$ and $En1^{+/Otx2}$ knock-in, of conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$, and of $Nkx2\text{-}2^{-/-}$ and $Wnt1^{+/-}$ ($int1^{+/-}$) single mutant mice is described elsewhere (Broccoli et al., 1999; McMahon and Bradley, 1990; McMahon et al., 1992; Panhuysen et al., 2004; Puelles et al., 2004; Sussel et al., 1998; Thomas and Capecchi, 1990). Viable $En1^{+/Cre}$; $Otx2^{+/flox}$; $Nkx2\text{-}2^{+/-}$ triple heterozygous and $Nkx2\text{-}2^{+/-}$; $Otx2^{+/flox}$ double heterozygous mice were selected and intercrossed to generate compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutants.

### Radioactive in situ hybridization

Mouse embryos were fixed and processed for radioactive in situ hybridization as described elsewhere (Brodski et al., 2003). Probes used for in situ hybridization are as described previously (Brodski et al., 2003) and (Puelles et al., 2004).

### Immunohistochemistry

Immunohistochemistry was performed as previously reported (Castelo-Branco et al., 2003; Puelles et al., 2004). The rabbit antibodies were directed against Otx2 (1:3500), Shh (1:200; Santa Cruz Biotechnology), Th (1:125; Pel-Freez) and Pitx3 (1:100; kindly provided by P. Burbach); the mouse antibodies were directed against Nkx2-2 (1:100; Hybridoma Bank), Th (1:300; Chemicon) and 5HT (1:100; Chemicon).

### BrdU treatments

Injections of pregnant females with BrdU and immunochemical processing of the embryos was performed as described previously (Panhuysen et al., 2004).

### Explant cultures

Explant cultures of anterior neural plates of embryos derived from heterozygote $Wnt1^{+/-}$ intercrosses were essentially prepared as reported previously (Echevarria et al., 2001).

### Bead implantations

Heparin-acrylic beads (Sigma) were soaked in 1 μg/μl recombinant mouse Fgf8b (R&D Systems) as described in (Echevarria et al., 2001). BSA (0.1% in PBS, Sigma)-coated beads were used as controls.

### Whole-mount in situ hybridization of explants

Explants were fixed and whole-mount in situ hybridization was carried out using standard procedures.

### RT-PCR of explants

Explant cultures were prepared and treated as described above. Total RNA from pooled explants was reverse-transcribed using random hexamers and the Advantage RT-for-PCR Kit (BD Biosciences Clontech). 4 μl each of 1:5 diluted single-stranded cDNA was amplified with primer pairs specific for *Th*, *Pitx3*, *Nr4a2*, *Aldh1a1*, *Wnt1* and *GAPD*. Primers and conditions are available upon request. cDNA from E12.5 CD1 mouse embryo heads was used as positive control. All gene-specific primer pairs except of *Nr4a2* were intron spanning.

## RESULTS

### Ectopic *Wnt1* induces *Otx2* only within the rostral hindbrain FP

First, we analyzed the expression pattern of different genes associated with the MHB in transgenic mice in which one *En1* allele was replaced by the *Wnt1* cDNA [$En1^{+/Wnt1}$ knock-in mice (Panhuysen et al., 2004)]. In these mutant mice, the *Wnt1* expression domain is expanded both anteriorly into the rostral midbrain and posteriorly into the rostral hindbrain (rostral third of rhombomere 1). Previous examination of these mice by means of whole-mount in situ hybridization revealed no alteration of the position and gene expression patterns at the MHB (Panhuysen et al., 2004). Most importantly, ectopic expression of *Wnt1* in the mid-hindbrain region of the $En1^{+/Wnt1}$ mice resulted only in an increase in size of the inferior colliculi (a caudodorsal midbrain derivative) without repatterning of this region. It thus had been argued that Wnt1 has no patterning activity on its own. It was therefore unexpected when a more thorough analysis of the $En1^{+/Wnt1}$ transgenic mice using radioactive in situ hybridization showed a caudal expansion of the *Otx2* domain but only within the FP of the rostral hindbrain (Fig. 1A). The caudal shift of *Otx2* expression was detected from E9.5 onwards, 24 hours after activation of the *En1* promoter (data not shown), and overlapped with the ectopic *Wnt1* expression in the rostral hindbrain FP albeit not to the same caudal extent (Fig. 1A). As in the wild type, *Fgf8* was not expressed within the FP of the rostral hindbrain in $En1^{+/Wnt1}$ embryos (Fig. 1A). Expression of other genes within the midbrain/rostral hindbrain FP such as *Wnt5a* and *Lmx1b*, was not changed in the $En1^{+/Wnt1}$ mutants (Fig. 1C and data not shown).

### Ectopic mDA neurons arise within the *Otx2*-positive hindbrain FP of $En1^{+/Wnt1}$ mutants

Concomitant with the ectopic induction of *Otx2* within the rostral hindbrain FP of $En1^{+/Wnt1}$ mice, a caudal shift of the aldehyde dehydrogenase 1 family member A1 (*Aldh1a1*, also known as *Raldh1*) expression domain was detected in the FP of these mutants (Fig. 1A). *Aldh1a1* is so far the only known marker for proliferating mDA progenitors and starts to be expressed at E9.5 within the cephalic flexure rostral to the MHB, overlapping with the *Wnt1* expression domain (Wallen et al., 1999) (see Fig. S1 in the supplementary material). At E12.5, the ectopic mDA progenitors had indeed developed into fully differentiated mDA neurons in the $En1^{+/Wnt1}$ mutants, as judged by the expression of tyrosine hydroxylase (*Th*), *Nr4a2* and *Pitx3* (Fig. 1B). At E15.5, the ectopic mDA neurons robustly expressed the dopamine transporter (*Slc6a3*), and they persisted into adulthood in $En1^{+/Wnt1}$ mice (see Fig. S2A,B in the supplementary material). The ectopic induction of *Aldh1a1* correlated with a repression of *Gbx2* in the same domain within the rostral hindbrain FP of $En1^{+/Wnt1}$ mutants (Fig. 1C), suggesting that *Gbx2* was repressed by the ectopic *Otx2* expression in this territory. Notably, no obvious increase in proliferating cells was detected in the FP of the $En1^{+/Wnt1}$ mutant midbrain and rostral hindbrain, as assessed by BrdU incorporation at E11.5 (Fig. 1D), indicating that the appearance of the ectopic mDA neurons is not due to an overproliferation of their progenitor pool. Given the tight link between the ectopic expression of *Wnt1* and *Otx2* in the rostral hindbrain FP of $En1^{+/Wnt1}$ mutants and the concomitant induction of ectopic mDA neurons in this region, we hypothesized that Wnt1 and Otx2 together are required for the generation of ectopic mDA neurons. Therefore, we analyzed *Wnt1* expression in the $En1^{+/Otx2}$ mouse mutant, which shows a caudal repositioning of

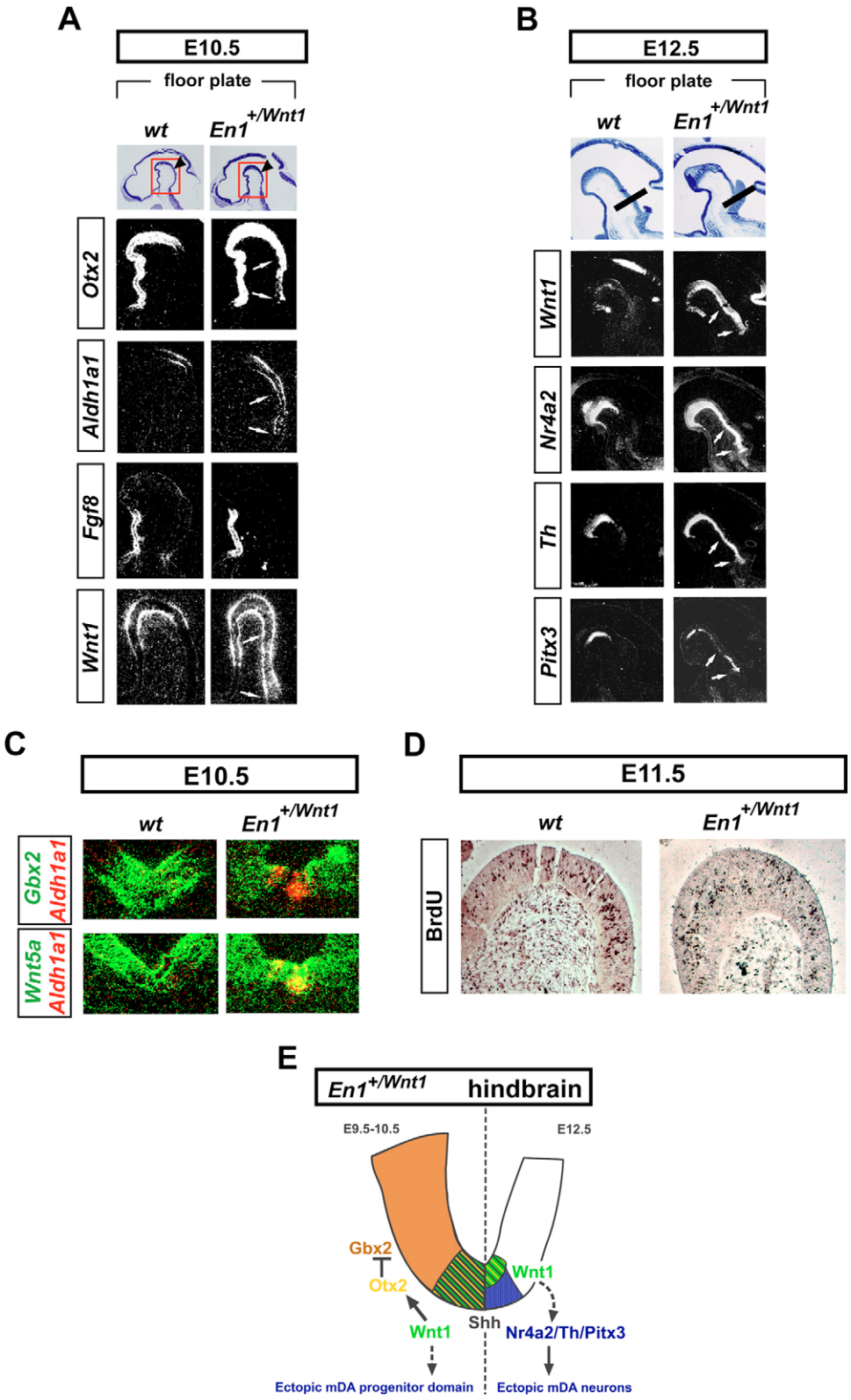


**Fig. 1. Ectopic expression of *Wnt1* in the rostral hindbrain leads to induction of *Otx2* and concomitant generation of ectopic mDA neurons only within the hindbrain FP.** (**A**) Midsagittal sections at the level of the FP of wild-type and *En1*$^{+/Wnt1}$ (*n*=6) embryos at E10.5 hybridized with probes for *Otx2*, *Aldh1a1*, *Fgf8* and *Wnt1*. Top row shows bright-field images; red rectangles depict the region of the dark-field images from consecutive sections shown below, except for those hybridized with *Wnt1*, which correspond to embryos of a distinct litter. Black arrowheads indicate the normal (wild-type) position of the MHB. White arrows indicate the ectopic expression domains. (**B**) Midsagittal sections of wild-type and *En1*$^{+/Wnt1}$ (*n*=4) embryos at E12.5 hybridized with probes for *Wnt1*, *Nr4a2*, *Th* and *Pitx3* (white arrows indicate ectopic expression). (**C**) Pseudocolored overlays of the corresponding dark-field images from coronal sections of E10.5 wild-type and *En1*$^{+/Wnt1}$ (*n*=4) embryos hybridized with probes for *Gbx2* (green), *Aldh1a1* (red) and *Wnt5a* (green). Overlapping domains appear yellow. (**D**) Immunodetection of BrdU in sagittal sections of E11.5 wild-type and *En1*$^{+/Wnt1}$ (*n*=2) embryos after 6 hours of cumulative labeling did not reveal in mutant embryos obvious abnormalities in proliferating activity along the FP region of the mid- and hindbrain. (**E**) Schematic drawing of a cross-section through the rostral hindbrain floor plate and basal plate of *En1*$^{+/Wnt1}$ mutants at two different time points of development, summarizing the events occurring in this region. The ectopic expression of *Wnt1* throughout the entire rostral hindbrain (rhombomere 1) of the *En1*$^{+/Wnt1}$ mutants has been omitted for clarity.

the posterior border of the *Otx2* expression domain and consequently of the MHB (Broccoli et al., 1999). In the *En1*$^{+/Otx2}$ transgenic mouse, additional mDA neurons are generated within the ectopic *Otx2* domain (Brodski et al., 2003). At E11.5, the *Wnt1* expression domain in the FP of *En1*$^{+/Otx2}$ embryos was enlarged caudally to the same degree as the ectopic mDA neurons (see Fig. S3 in the supplementary material). We thus concluded that a positive regulatory feedback loop controls ectopic expression of *Wnt1* and *Otx2* in the ventral midline (FP) of the hindbrain in these mutants, and that this regulatory network may be relevant for the subsequent ectopic generation of mDA neurons. We next asked for the downstream effectors of this

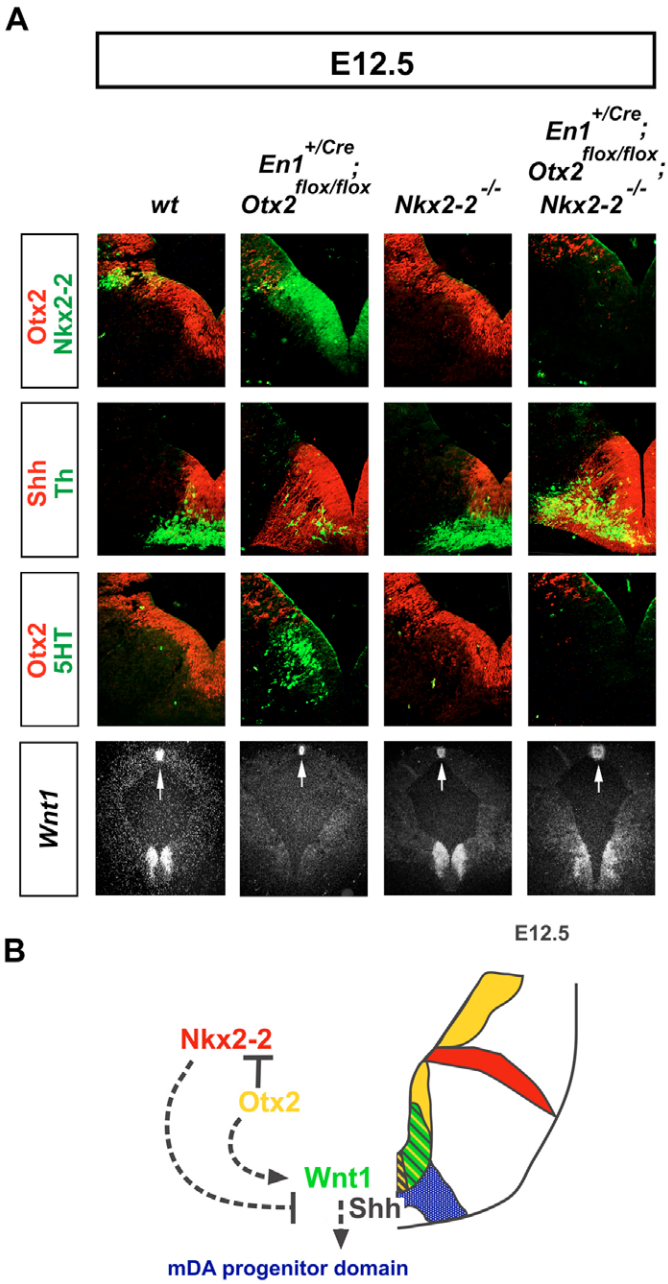


**Fig. 2. Ectopic Nkx2-2 is sufficient to repress the mDA neuronal fate and to induce rostral 5HT neurons in the ventral midbrain.** (**A**) Fluorescent immunostaining for Otx2 (red), Nkx2-2 (green), Shh (red), Th (green) and 5HT (green), or in situ hybridization with *Wnt1* probe on coronal sections of wild-type, conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$, $Nkx2\text{-}2^{-/-}$ single and compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutant mouse embryos at E12.5 ($n$=3 for each genotype). *Wnt1* expression in the dorsal midline (RP) of the midbrain remained unaffected in all mutants (white arrows). (**B**) Schematic drawing of a cross-section through the ventral midbrain of E12.5 wild-type mouse embryos summarizing our results.

regulatory feedback loop by analyzing another mouse mutant in which *Otx2* was conditionally inactivated in the mid/hindbrain region, including the ventral midline [$En1^{+/Cre}$; $Otx2^{flox/flox}$ mice (Puelles et al., 2004)].

## The mDA progenitor domain is established by Otx2-mediated repression of *Nkx2-2*

Previous analyses of the conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$ mouse mutant (Puelles et al., 2004) had shown that the loss of Otx2 within the midbrain FP and BP leads to a ventral expansion of the Nkx2-2 domain (Fig. 2A). In addition, the Shh domain was expanded dorsally in this conditional mutant (Fig. 2A). At the same time, the mDA neuronal population (marked by Th expression) was drastically reduced in the ventral midbrain and ectopic serotonergic (5HT) neurons were generated instead in this region (Fig. 2A). Most notably, *Wnt1* expression was lost only in the ventral midbrain (FP and BP) but left intact in the dorsal midline (roof plate, RP) of the midbrain of $En1^{+/Cre}$; $Otx2^{flox/flox}$ conditional mutants at E12.5 (Fig. 2A). This finding would be consistent with a positive feedback between *Otx2* and *Wnt1* that is also maintaining their expression in the ventral midbrain. The almost complete loss of mDA neurons in the $En1^{+/Cre}$; $Otx2^{flox/flox}$ mice could therefore be due to: (1) a specific and cell-autonomous requirement of Otx2 for the specification of mDA neurons; (2) the repression of the mDA neuronal fate by Nkx2-2 alone or in combination with Shh; and/or (3) a requirement of Wnt1 for the generation of mDA neurons. To address these possibilities we first investigated whether any alterations in the mDA and rostral 5HT cell populations could be detected in the $Nkx2\text{-}2^{-/-}$ single mutants. As had been published before (Briscoe et al., 1999), neither the mDA nor the rostral 5HT neuronal populations were affected in size or location in the $Nkx2\text{-}2^{-/-}$ single mutant embryos (Fig. 2A). Furthermore, expression of *Otx2*, *Shh* and *Wnt1* was not affected in the ventral midbrain of $Nkx2\text{-}2^{-/-}$ mice (Fig. 2A). Then we asked whether the ectopic expression of Nkx2-2 in the ventral midbrain of the conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$ mice was responsible for the loss of mDA neurons and the ectopic induction of 5HT neurons in this region. To resolve this issue, compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutant mice were generated.

The mDA neuronal population was rescued in the ventral midbrain of the compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutants compared with the $En1^{+/Cre}$; $Otx2^{flox/flox}$ mutant embryos (Fig. 2A). Furthermore, no ectopic 5HT neurons were detected in the ventral midbrain of the triple mutants (Fig. 2A). The rescue of mDA neurons occurred in the triple mutant, even though Otx2 was completely lost in the midbrain FP and BP from the earliest time point studied (E9.5) (Puelles et al., 2004) (Fig. 2A). Importantly, the ventral *Wnt1* expression domain was also rescued in the midbrain of the compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutants (Fig. 2A), although it had a somewhat 'fuzzy' appearance in the compound triple mutant. The reason for this is unclear at present. The rescue of the ventral *Wnt1* expression domain suggested that ectopic expression of Nkx2-2 in the ventral midbrain of conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$ mutant embryos also has a direct or indirect repressive effect on the transcription of *Wnt1* in this region. However, it also showed that Otx2 is not necessary for maintaining *Wnt1* expression in the ventral midbrain of compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutants. Furthermore, the comparison of the phenotypes observed in the conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$ mouse and the compound $En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutant suggested that Otx2 is required for the repression of *Nkx2-2* in the midbrain FP and BP, thereby establishing an Nkx2-2-negative territory from which mDA progenitors can develop (Fig. 2B).

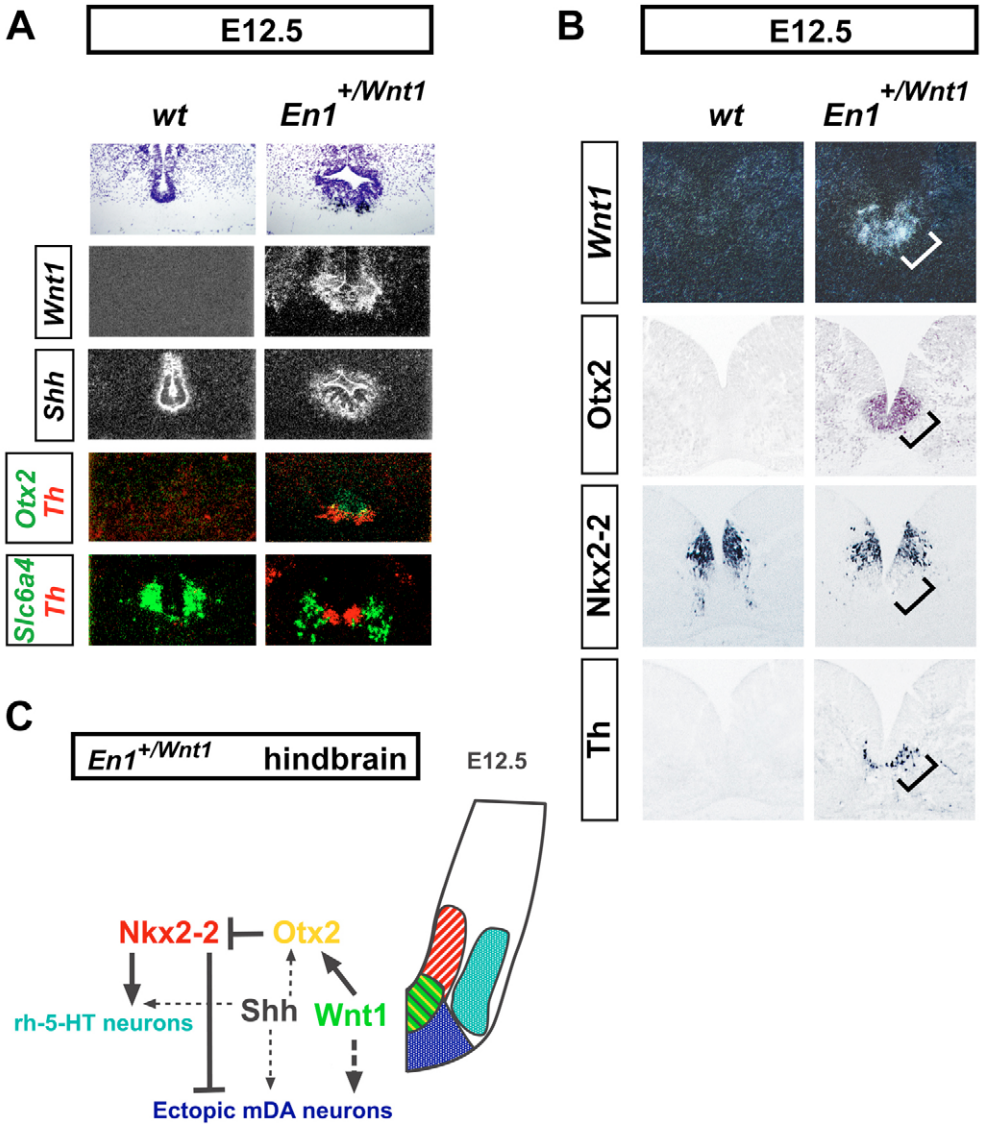


**Fig. 3. The ectopic mDA progenitor domain in *En1*$^{+/Wnt1}$ mutants is established through partial repression of *Nkx2-2* in the ventral hindbrain by Otx2.** (**A**,**B**) Coronal sections of E12.5 wild-type and *En1*$^{+/Wnt1}$ ($n$=4) embryos at the height of rhombomere 1-2 (black bars in Fig. 1B) hybridized with probes for *Wnt1*, *Shh*, *Otx2* (green), *Th* (red) and the serotonin transporter (*Slc6a4*, green), or immunostained for Otx2, Nkx2-2 and Th. Top row in A are bright-field pictures corresponding to the dark-field images. Pictures in A are partly pseudocolored overlays of the corresponding dark-field images. Brackets in B indicate the region where the most ventral part of the Nkx2-2 domain in the hindbrain was repressed by the ectopically induced Otx2. (**C**) Sketch of a cross-section through the *En1*$^{+/Wnt1}$ mutant rostral hindbrain at E12.5 summarizing these results. 5HT neurons were probably generated from the dorsolateral Nkx2-2-positive domain within the mutant BP in this region.

To confirm this hypothesis, we reinvestigated the *En1*$^{+/Wnt1}$ mutants, in which ectopic mDA neurons were generated within the *Otx2*-positive hindbrain FP. Coronal sections of E12.5 *En1*$^{+/Wnt1}$ embryos taken at the rostral hindbrain level revealed that the ectopic *Th*-expressing cells were located in the mantle zone of the ectopic *Otx2*-positive neuroepithelium within the *Shh* and *Wnt1* expressing FP of the mutant hindbrain (Fig. 3A). The ventral Nkx2-2 expression domain of the hindbrain, which extended ventrally towards, but did not overlap with, the FP in wild-type embryos (Fig. 3B), appeared to be repressed in its most ventral part overlapping with the region where the ectopic Otx2 was induced and the ectopic mDA neurons were generated in the *En1*$^{+/Wnt1}$ mutant (brackets in Fig. 3B). Changes of gene expression patterns in the hindbrain of *En1*$^{+/Wnt1}$ mutants were much more subtle. Thus, the tissue surrounding the ectopic mDA neurons retained its hindbrain identity as demonstrated by the presence of rostral hindbrain 5HT neurons, which were somewhat displaced but otherwise unaffected (Fig. 3A). Furthermore, expression of other marker genes, such as *Lmx1b*, *Wnt5a* and *Lhx1* was not changed in the rostral hindbrain tissue of *En1*$^{+/Wnt1}$ mutants surrounding the ectopic mDA neurons (data not shown). Other cell populations of the ventral mid- and hindbrain (such as motoneurons of the III and IV brain nuclei, and the red nucleus) also appeared not to be changed in *En1*$^{+/Wnt1}$ mice in regard of their location and size (data not shown). We thus concluded that the ectopic induction of mDA neurons in the hindbrain FP of *En1*$^{+/Wnt1}$ mutants is specific for this cell population, and is based on the ectopic induction of *Otx2,* which in turn represses the most ventral *Nkx2-2* expression domain (Fig. 3C). Thereby, an ectopic mDA progenitor domain is established in the hindbrain FP of the *En1*$^{+/Wnt1}$ mutants.

## mDA neurons do not differentiate properly in the absence of Wnt1

Our data support the conclusion that a *Wnt1*-controlled genetic network leads to the establishment of the mDA progenitor domain in the ventral midbrain during early stages of mouse neural development (i.e. between E9.5 and E12.5) by repressing *Nkx2-2* within this domain. The loss of *Wnt1* expression in the ventral midbrain of conditional *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mutants (which also lack mDA neurons) and the rescue of *Wnt1* expression in the same region of compound *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$ triple mutants (in which mDA neurons were also rescued) suggested that Wnt1 in addition plays a role in mDA cell fate specification. To clarify this point, a thorough analysis of the mDA domain was performed in

*Wnt1*$^{-/-}$ null mutant mice (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Based on the expression of *En1* and on the radial glial marker RC2, a residual ventral mid-hindbrain domain could still be detected in *Wnt1*$^{-/-}$ mice between E9.5 and E11.5 (Fig. 4A,B). This is in line with a previous report (McMahon et al., 1992). Within this residual *En1*-positive domain, cells expressing Nr4a2 and Th were detected between E10.5 and E12.5 (Fig. 4C; data not shown). The number of Th-expressing neurons, however, was strongly reduced in the *Wnt1*$^{-/-}$ null mutant at E11.5 when compared with the wild type (Fig. 4C). Remarkably, the Th-positive cells did not express other mDA neuron-specific marker genes, such as *Pitx3* or *Slc6a3*, at the time points analyzed in the *Wnt1*$^{-/-}$ mutants (Fig. 4C; data not shown). Furthermore, no *Aldh1a1*-expressing cells could be detected between E9.5 and E10.5 in the *Wnt1*$^{-/-}$ embryos (Fig. 4A), indicating a loss of proliferating mDA progenitors. To rule out the possibility that these Th-positive cells are noradrenergic neurons of the rostral hindbrain, expression of the enzyme dopamine-β-hydroxylase (*Dbh*, required for the synthesis of norepinephrine) was analysed in *Wnt1*$^{-/-}$ mutants at E11.5 and E12.5. However, no *Dbh* signal was detected in these cells (data not shown). In addition, *Otx2* was normally expressed and *Nkx2-2* was repressed in the region where the Th-positive cells arose (data not shown), suggesting that the mDA progenitor domain was still established in the *Wnt1*$^{-/-}$ embryos. This observation may be explained by the partially redundant action of other members of the Wnt family, in particular that of Wnt5a and Wnt7a, which are expressed in the same region (Castelo-Branco et al., 2003; McMahon et al., 1992; Parr et al., 1993). Therefore, we concluded that a DA precursor still develops in the remnants of the ventral midbrain in the *Wnt1*$^{-/-}$ mutant, but this precursor does not properly proliferate and differentiate into an mDA neuron in the absence of Wnt1 as judged by the lack of Pitx3 and *Slc6a3* expression (Fig. 4D). Even though the DA precursors in the *Wnt1*$^{-/-}$ knockout start to express Th, these cells were probably dying as they did not initiate

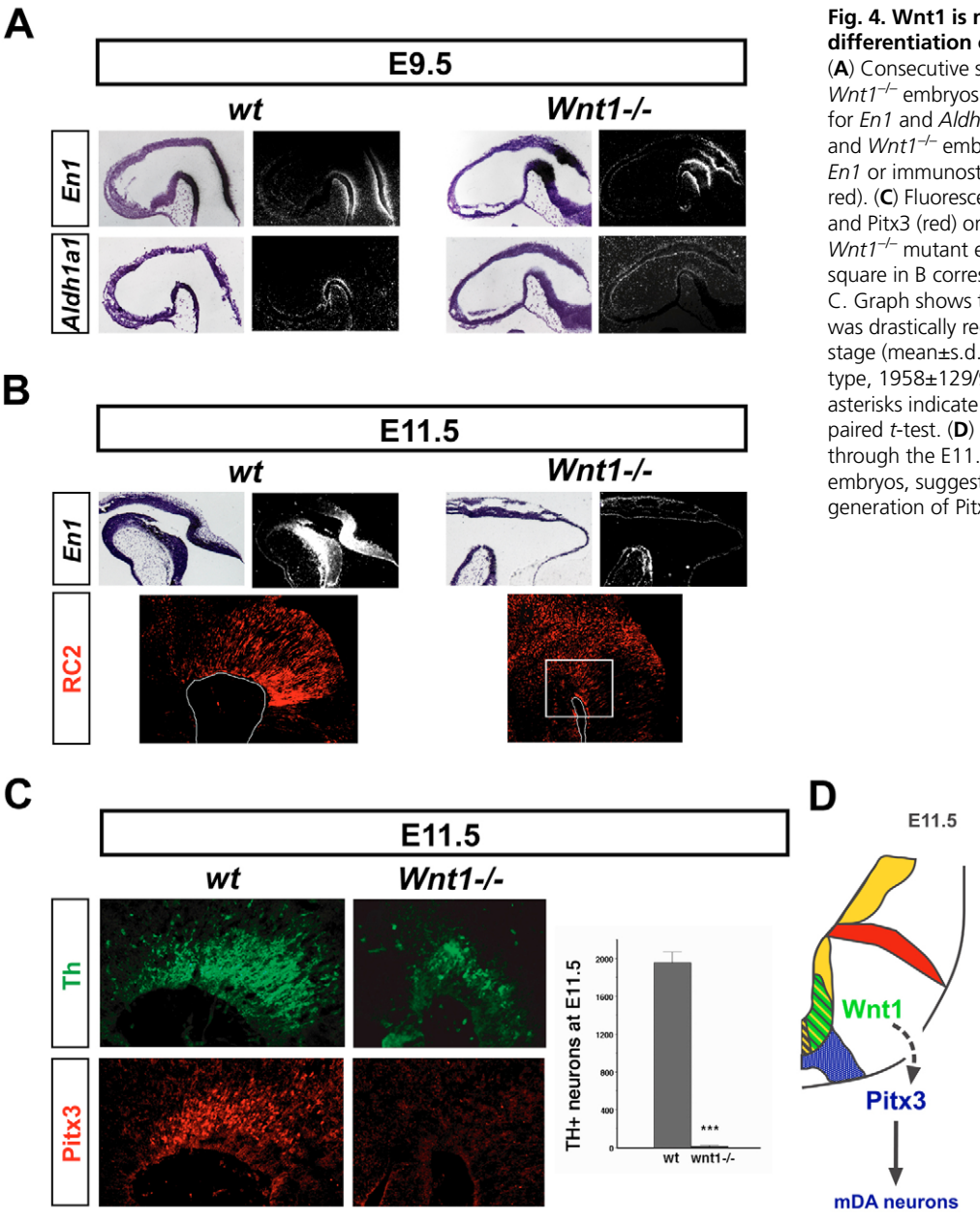


**Fig. 4. Wnt1 is necessary for terminal differentiation of Th-expressing mDA precursors.** (**A**) Consecutive sagittal sections of wild-type and *Wnt1*$^{-/-}$ embryos at E9.5 (*n*=8) hybridized with probes for *En1* and *Aldh1a1*. (**B**) Sagittal sections of wild-type and *Wnt1*$^{-/-}$ embryos at E11.5 (*n*=8) hybridized with *En1* or immunostained for RC2 (a radial glia marker, red). (**C**) Fluorescent immunodetection of Th (green) and Pitx3 (red) on sagittal sections of wild-type and *Wnt1*$^{-/-}$ mutant embryos at E11.5 (*n*=3). The white square in B corresponds to the region of the sections in C. Graph shows that the number of Th-positive cells was drastically reduced in the *Wnt1*$^{-/-}$ mutant at this stage (mean±s.d./s.e.m. from three sibling pairs: wild type, 1958±129/92 cells; *Wnt1*$^{-/-}$,14±11/8 cells). Triple asterisks indicate numbers that differ at *P*<0.0024, paired *t*-test. (**D**) Schematic drawing of a cross-section through the E11.5 ventral midbrain of wild-type mouse embryos, suggesting a possible role of Wnt1 in the generation of Pitx3-expressing mDA neurons.

their correct differentiation program. In agreement with this possibility, active caspase 3 staining was detected in the ventral midbrain of *Wnt1*$^{-/-}$ embryos at E11.5 (data not shown) and Th-positive cells were not detected in these embryos at stages later than E12.5, concomitant with the complete loss of the mid-hindbrain region (data not shown) (Chi et al., 2003).

### Wnt1 is necessary for ectopic induction of mDA neurons by Fgf8 and Shh

To further support the possibility that Wnt1 is indeed required for the generation of mDA neurons, in addition to other factors such as Fgf8 and Shh, we repeated the explant experiments performed by others (Ye et al., 1998) but using the whole anterior neural plate from *Wnt1*$^{-/-}$ mutant and wild-type (*Wnt1*$^{+/+}$ and *Wnt1*$^{+/-}$) mouse embryos at E8.0-8.5. It had previously been shown that ectopic mDA neurons were induced in forebrain explants only in the presence of both Shh and Fgf8 (Ye et al., 1998). We used whole anterior neural plate explants (comprising the presumptive fore-, mid- and hindbrain) for our experiments because the presumptive mid-hindbrain region will be eventually deleted in *Wnt1*$^{-/-}$ explants, and any negative result could therefore also be due to the loss of this tissue. Additional experiments have shown that *Wnt1* expression in the presumptive midbrain is maintained by Fgf8b-coated beads implanted in the presumptive ventral forebrain (Fig. 5B). Indeed, 6 days after implantation of an Fgf8b-coated bead close to the ventral midline of the presumptive forebrain, *Th*-expressing cells were detected at a distance from the bead in wild-type explants (Fig. 5A). By contrast, no *Th*-positive cells were detected in *Wnt1*$^{-/-}$ explants under the same conditions (Fig. 5A). The mDA identity of the induced *Th*-positive cells was further confirmed by RT-PCR for the specific marker *Pitx3*. After 6 days of culture in the presence of an Fgf8b-coated bead, expression of *Pitx3* was only detected in wild-type but not in *Wnt1*$^{-/-}$ explants (Fig. 5C). These results confirmed that, even in the presence of Fgf8 and Shh, no ectopic mDA neurons can be induced in the absence of *Wnt1*, indicating that Wnt1 is necessary for the generation of mDA neurons.

## DISCUSSION

We provide evidence that the secreted glycoprotein Wnt1 plays a crucial role in the generation of mDA neurons in vivo by exerting two different activities.

First, Wnt1 is involved in the establishment of the mDA progenitor domain by maintaining Otx2 expression in the ventral midbrain, which in turn is required for the repression of *Nkx2-2* in this territory (Fig. 6A). Failure to repress *Nkx2-2* in the midbrain FP and BP leads to the generation of 5HT instead of mDA neurons. The function of Wnt1 in this early developmental context may be similar to the role of Fgf8 in the maintenance of *Gbx2* expression within the rostral hindbrain (Chi et al., 2003; Liu and Joyner, 2001b; Liu et al., 1999; Sato et al., 2001). As Fgf8 can induce or maintain *Wnt1* expression non-cell-autonomously (Chi et al., 2003; Liu and Joyner, 2001b), and *Wnt1* is required for maintaining *Fgf8* expression in the rostral hindbrain (Lee et al., 1997), this early activity of Wnt1 may be part of an integrated regulatory network controlling the maintenance of the MHB and consequently the establishment of the distinct progenitor domains in the ventral mid- and hindbrain. It should be noted, however, that this Wnt1 activity appears to be restricted to the ventral neural tube, as the overexpression of *Wnt1* in the dorsal neural tube leads only to an enhanced proliferation of dorsal neural precursors (Panhuysen et al., 2004). Our results also revealed a so far underestimated plasticity of the ventral mid-and hindbrain neural precursors, in the sense that these precursors are able to generate both mDA and rostral 5HT neurons, depending on the genetic program and the environmental cues acting on these cells. Furthermore, the specification of rostral hindbrain 5HT neurons appears to depend in part on the same signals and factors required for the development of the more-caudal 5HT neuronal populations in the hindbrain and spinal cord, namely Shh and its downstream effectors of the Nkx family (Briscoe et al., 1999; Pattyn et al., 2003a). This finding is in line with a recent report on a different conditional *Otx2* mouse mutant (Vernay et al., 2005). Given the particular expression pattern of Nkx2-2 in the ventral midbrain, a repressive effect of this transcription factor on the specification of the mDA neuronal fate would not be detected in the *Nkx2-2*$^{-/-}$ single mutant. The normal appearance of the rostral 5HT population in the *Nkx2-2*$^{-/-}$ mouse, however, indicates that the loss of Nkx2-2 in the rostral hindbrain

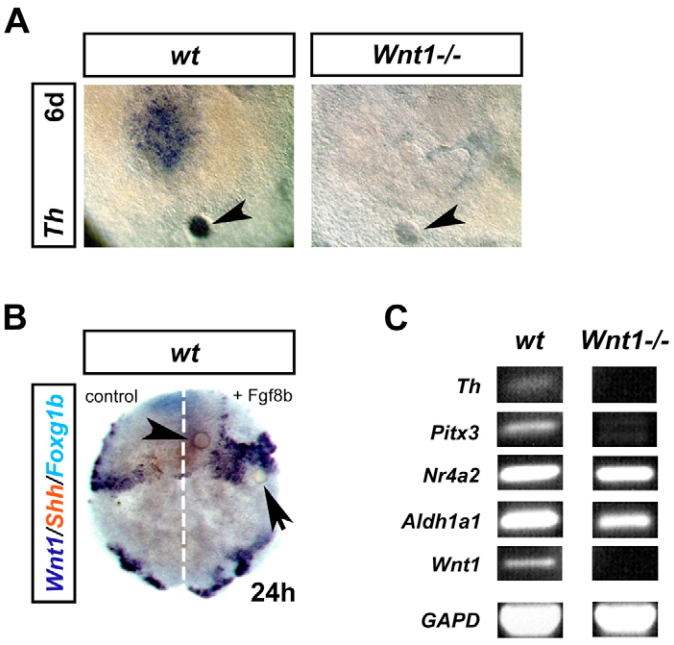


**Fig. 5. *Wnt1* is required for ectopic induction of mDA neurons by Fgf8 and Shh.** (**A**) In situ hybridization with *Th* probe (dark blue) on E8.0-8.5 wild-type (*Wnt1*$^{+/+}$ and $^{+/-}$; *n*=20/28) and *Wnt1*$^{-/-}$ (*n*=8/9) mouse anterior neural plate explants 6 days after implantation of Fgf8b-coated beads (arrowheads) close to the ventral midline of the presumptive forebrain. No induction of *Th*-positive cells was seen after implantation of BSA-coated (control) beads (data not shown; *n*=8/9 for wild-type and *n*=4/5 for mutant explants). (**B**) In situ hybridization of wild-type E8.0-8.5 mouse anterior neural plate explants 24 hours after implantation of Fgf8b-coated beads with *Foxg1b* (light blue) and *Shh* (red) to confirm the forebrain and ventral identity of the tissue where the bead was implanted (arrowhead). No *Wnt1* expression (dark blue) was detected in the tissue surrounding the bead located outside the endogenous *Wnt1* domain (arrowhead). However, expression of *Wnt1* was maintained at a distance from the bead when compared with the contralateral control side of the explant (broken line coincides with the ventral midline). Repression of endogenous *Wnt1* around another Fgf8b-coated bead located in the presumptive dorsal midbrain is apparent (arrow). (**C**) RT-PCR for *Th*, *Pitx3*, *Nr4a2*, *Aldh1a1* and *Wnt1* on total RNA from pooled wild-type and *Wnt1*$^{-/-}$ explants cultured under the same conditions. Detection of ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) was used as control. Expression of *Nr4a2* and *Aldh1a1* is not restricted to mDA neurons and was therefore detected in wild-type as well as in *Wnt1*$^{-/-}$ explants.

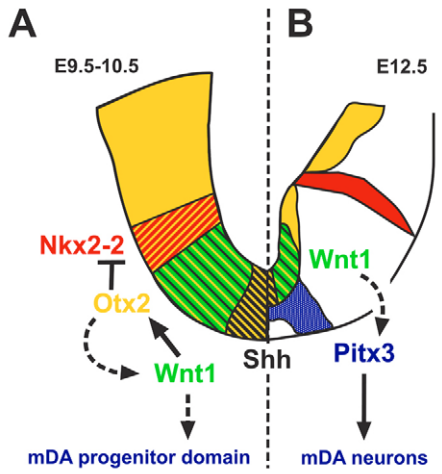


**Fig. 6. Wnt1 may control two different steps in the generation of mDA neurons in vivo.** (**A**) Schematic drawing of a cross section through the mouse ventral midbrain close to the MHB at E9.5-10.5 showing the overlapping expression of Shh (black) and Wnt1 (green) in the midbrain FP and BP. Wnt1 is spared from the ventral midline (medial FP), where only Shh is expressed. Otx2 (yellow) is expressed throughout the midbrain neuroepithelium at this stage, overlapping with a narrow stripe corresponding to the Nkx2-2 expression domain (red). Both Wnt1 and Otx2 are engaged in a positive feedback loop controlling their expression within the ventral midbrain such that secreted Wnt1 protein induces and/or maintains *Otx2* expression in the FP and BP of the midbrain and vice versa. Otx2 protein is in turn required for repression of *Nkx2-2* within this territory of the neural tube. The mDA progenitor domain is thus established by this Wnt1-controlled regulatory network during early neural development. (**B**) Schematic drawing of a cross-section through the mouse ventral midbrain close to the MHB at E12.5. The Shh (black) and Wnt1 (green) expression domains have refined to a narrow area corresponding to the ventricular and subventricular zone and partly overlapping with the Otx2-positive (yellow) proliferative neuroepithelium of the midbrain FP and BP. The Nkx2-2 (red) domain is now restricted to a wedge-shaped transversal stripe at the alar-basal boundary corresponding to the region of lowest Otx2 protein levels. At this stage, proliferating mDA progenitors within the midbrain FP/BP have already generated Th-expressing mDA precursors. Wnt1 may be required for terminal differentiation of these cells by inducing expression of Pitx3 (blue). The as yet hypothetical nature of a direct role of Wnt1 in the generation of mDA neurons is indicated by broken lines.

must be compensated by yet another factor, probably the related Nkx2-9 transcriptional regulator showing a similar expression pattern as Nkx2-2 (Briscoe et al., 1999; Pattyn et al., 2003a).

Second, Wnt1 has a later specifying activity required for the proper differentiation of mDA precursors into mature mDA neurons (Fig. 6B). In the absence of *Wnt1*, no *Aldh1a1* expression was detected at E9.5 and E10.5, and less than 1% of Th-positive cells were born within a residual *En1*-positive ventral mid-hindbrain domain. Moreover, these cells did not express *Pitx3* and *Slc6a3*. It is possible that for the generation of the few Th-positive cells in the $Wnt1^{-/-}$ mutants, other members of the Wnt family, such as Wnt5a and Wnt7a may have in part compensated for the loss of *Wnt1*, as they are expressed in this region and Wnt5a exhibits partially overlapping activities in vitro (Castelo-Branco et al., 2003; McMahon et al., 1992; Parr et al., 1993). Another possibility is that the remaining Th-expressing cells in the $Wnt1^{-/-}$ embryos are generated independently of a Wnt signal and may directly derive from the medial FP, similar to the few Th-positive neurons in the conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$ mutants. Previous studies of mDA neuron development have suggested that two independent pathways are active during terminal differentiation of these cells: a Nr4a2-controlled pathway required for initiation of *Th* and *Slc6a3* transcription (Kim et al., 2003; Saucedo-Cardenas et al., 1998; Smits et al., 2003; Zetterstrom et al., 1997); and an Lmx1b-controlled pathway necessary for *Pitx3* expression (Smidt et al., 2000). As *Wnt1* is induced and/or maintained by Lmx1b, but not vice versa (Adams et al., 2000; Matsunaga et al., 2002), and as we could not detect any changes of *Lmx1b* expression patterns in our mutant mice, it is very likely that *Wnt1* is acting downstream of *Lmx1b* in the regulation of *Pitx3* expression. Pitx3 is a homeobox transcription factor uniquely expressed in all mDA neurons (Smidt et al., 2004; Smidt et al., 1997; Zhao et al., 2004), and loss-of-function experiments have shown that *Pitx3* is required for the terminal differentiation and survival of a subset of mDA neurons, mainly those of the SN (Hwang et al., 2003; Maxwell et al., 2005; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). Notably, we did not detect *Slc6a3* expression in the $Wnt1^{-/-}$ mutant at the time points analysed. As Slc6a3 expression is found in the remaining Th-positive cells of the *Pitx3* mutant (Hwang et al., 2003; Smidt et al., 2004) but not in the $Nr4a2^{-/-}$ (Smits et al., 2003) or $Wnt1^{-/-}$ embryos, it could be that transcription of the *Slc6a3* gene is controlled by both Wnt1 and Nr4a2. However, it is not known whether activation of the *Slc6a3* gene is controlled directly or indirectly by Wnt1. The ability of Wnts to promote the acquisition of a mDA phenotype was recently demonstrated in vitro using ventral midbrain precursor cultures (Castelo-Branco et al., 2003). In this study, Wnt1 had a predominantly proliferative effect on these neural precursors, whereas Wnt5a, another member of the Wnt family expressed throughout the FP of the mid- and hindbrain (Parr et al., 1993), promoted the differentiation of ventral midbrain precursors into mDA neurons without affecting their proliferation in vitro. However, in contrast to the in vitro studies, our present findings show that Wnt1 alone could control the specification of the mDA neuronal fate in vivo. The drastically reduced number of Th-positive cells in the $Wnt1^{-/-}$ mouse, however, suggests that Wnt1 in addition controls the proliferation of mDA progenitors in vivo. Importantly, the appearance of ectopic mDA neurons in the hindbrain of $En1^{+/Wnt1}$ mice and in wild-type but not in $Wnt1^{-/-}$ explants together with the severe loss of DA neurons in the $Wnt1^{-/-}$ mutants indicate that Wnt1 has a crucial role in the development of mDA neurons. One reason for the discrepancy with the in vitro results could be that cultured ventral midbrain precursors lose domain-specific positional information and do not keep the regulatory network normally controlling their differentiation into mDA neurons in vivo. It is not yet clear how the Wnt1-signal is transduced in mDA progenitors. However, recent in vitro data have shown that activation of the canonical Wnt-pathway in ventral midbrain precursor cultures enhances the differentiation of DA neurons in these cultures (Castelo-Branco et al., 2004), suggesting that this pathway may as well participate in mDA cell differentiation in vivo.

Our data also indicate that in the absence of Wnt1, both Shh and Fgf8 are not sufficient for ectopic induction of mDA neurons. The activity of these two secreted factors in mDA fate specification has recently been investigated in vivo. First, a dorsal expansion of the ventral midbrain Shh territory does not always correlate with an ectopic induction or increase of the mDA population. The ventral *Shh* domain is dorsally expanded in conditional $Otx1^{+/Cre}$; $Otx2^{-/flox}$ mutant embryos, in which *Otx2* is inactivated in the lateral midbrain but left intact in the midbrain FP and BP (Puelles et al., 2003). In

these conditional mutants, the number of mDA neurons is remarkably enlarged owing to an expansion of the mDA progenitor domain that is positive for *Shh* and to an increased proliferation of their precursors (Puelles et al., 2003). A similar dorsal expansion of the Shh domain is seen in the conditional *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mutants, but in these mice, the mDA phenotype is almost completely absent (Puelles et al., 2003; Puelles et al., 2004). This indicates that Shh may rather act as a mitogen on mDA precursors, thus expanding their progenitor pool during development, but cannot compensate on its own for the loss of the mDA fate in the conditional *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mutant. Second, the position and extent of the *Fgf8* expression domain in the ventral hindbrain is not affected in *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ mice (Puelles et al., 2004), indicating that Fgf8 alone or together with the dorsally enlarged Shh domain is also not able to rescue the mDA cell population in these mutants. Notably, the different phenotypes of the two conditional mouse mutants regarding the mDA neuronal population can be explained by the differences in *Otx2*, *Nkx2-2* and *Wnt1* expression between them. Expression of Otx2 in the midbrain FP and BP is unaffected in the *Otx1*$^{+/Cre}$; *Otx2*$^{-/flox}$ embryos and, as a consequence, *Nkx2-2* is still repressed and mDA neurons are present in this region of the mutant midbrain (Puelles et al., 2003). However, loss of *Otx2* in the midbrain FP and BP of *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ embryos leads to a ventral expansion of the midbrain Nkx2-2 domain and to the loss of mDA neurons [this work and that of Puelles et al. (Puelles et al., 2004)]. Most importantly, the specification of the mDA neuronal fate strongly correlates with the expression of *Wnt1* in the ventral midbrain or hindbrain of all mutant mouse lines analysed. Thus, the *Wnt1* domain and mDA neurons are unaffected in the ventral midbrain of conditional *Otx1*$^{+/Cre}$; *Otx2*$^{-/flox}$, of *Nkx2-2*$^{-/-}$ single and of compound *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$; *Nkx2-2*$^{-/-}$ triple mutants [this work and that Puelles et al. (Puelles et al., 2003)]. By contrast, *Wnt1* expression is lost and mDA neurons are miss-specified in the ventral midbrain of conditional *En1*$^{+/Cre}$; *Otx2*$^{flox/flox}$ and *Wnt1*$^{-/-}$ embryos. Finally, ectopic expression of *Wnt1* together with *Otx2* in the rostral hindbrain FP of *En1*$^{+/Wnt1}$ and *En1*$^{+/Otx2}$ transgenic mice is sufficient to induce ectopic mDA neurons in this region of the mutant hindbrain. Our results therefore indicate that Shh and Fgf8 act in concert with a Wnt1-controlled regulatory network (including Otx2 and Nkx2-2), leading to the establishment of the mDA progenitor domain in the ventral midbrain and the subsequent terminal differentiation of mDA neurons. Furthermore, our present data suggest the Wnt1-controlled signaling pathway as a promising target in the treatment of diseases affecting mDA neurons, including PD.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/24/89/DC1

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