Topical Review

Genetic networks controlling the development of midbrain dopaminergic neurons

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Recent data have substantially advanced our understanding of midbrain dopaminergic neuron development. Firstly, a Wnt1-regulated genetic network, including *Otx2* and *Nkx2-2*, and a Shh-controlled genetic cascade, including *Lmx1a*, *Msx1* and *Nkx6-1*, have been unravelled, acting in parallel or sequentially to establish a territory competent for midbrain dopaminergic precursor production at relatively early stages of neural development. Secondly, the same factors (Wnt1 and Lmx1a/Msx1) appear to regulate midbrain dopaminergic and/or neuronal fate specification in the postmitotic progeny of these precursors by controlling the expression of midbrain dopaminergic-specific and/or general proneural factors at later stages of neural development. For the first time, early inductive events have thus been linked to later differentiation processes in midbrain dopaminergic neuron development. Given the pivotal importance of this neuronal population for normal function of the human brain and its involvement in severe neurological and psychiatric disorders such as Parkinson's Disease, these advances open new prospects for potential stem cell-based therapies. We will summarize these new findings in the overall context of midbrain dopaminergic neuron development in this review.

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Neurons synthesizing the neurotransmitter dopamine (DA) are found at different positions in the vertebrate brain (reviewed by Prakash & Wurst, 2006). The best studied populations are the dopaminergic (DA) neurons located in two nuclei of the midbrain tegmentum in higher vertebrates: the substantia nigra (SN, also called the A9 group) and the ventral tegmental area (VTA, also called the A10 group). Additional neurons are found in the retrorubral field (RrF, also named the A8 group). The neurons of the SN and VTA project to the forebrain forming the nigrostriatal and mesocorticolimbic pathway, respectively (reviewed by Prakash & Wurst, 2006). The mammalian midbrain dopaminergic (mDA) population plays a fundamental role in several brain and body functions and behaviours. The mDA neurons have thus been the focus of clinical interest for a long time because of their involvement in severe human neurological and psychiatric illnesses. Besides a deeper understanding of their physiology, the development of better treatments for these disorders has promoted their investigation. One potential therapy for Parkinson's disease, for example, would be the restitution of the degenerating or lost DA neurons in the human SN by healthy DA neurons that have been generated either *in vitro* or *in vivo* through the directed differentiation of stem cells (Winkler *et al.* 2005). To accomplish this, a full understanding of the genetic cues controlling the differentiation of a pluripotent, uncommitted neuroepithelial stem cell into a mature mDA neuron is required. In this regard, substantial progress has been made in recent years through the analysis of the developmental programme governing the emergence of the mDA cell type during mammalian embryogenesis. This programme is inherited and can be subdivided into three distinct processes: (1) the induction of a progenitor field within the neuroepithelium competent to generate mDA precursors at early stages of neural development (approx. from embryonic day (E) 8.5 to E10.5 in the mouse); (2) the specification of a mDA neuronal fate in these precursors at intermediate stages (approx. from mouse E10.5 to E12.5); and (3) the acquisition of the mature phenotype or terminal differentiation of mDA neurons at relatively late stages of neural development (i.e. from E12.5 onwards).

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Cells that have been induced but not yet specified are still able to switch cell fate, but cells that have been specified are committed to a unique cell fate. This commitment includes the acquisition of a distinct phenotype by the cells, such as generic neuronal and special characteristics including neurotransmitter, electrophysiological and projection area identities. These processes may be envisioned as controlled by different genetic networks acting either sequentially or in parallel. We and others have recently reviewed the cell-biological and molecular aspects of mDA neuron development (Goridis & Rohrer, 2002; Smidt *et al.* 2003; Simeone, 2005; Prakash & Wurst, 2006). We will therefore restrict ourselves to a summary of the genetic cascades and networks controlling the development of this neuronal population in the mouse, emphasizing the most recent progress that has been made in the field.

Two important signalling centres of the mouse embryo control the generation of mDA neurons

The embryonic mouse neural tube is patterned along its antero-posterior (A-P) and dorso-ventral (D-V) axis by the action of several important signalling centres. Patterning along the D-V axis is accomplished through the ventral (floor plate, FP) and dorsal (roof plate, RP) midline of the neural tube (reviewed by Chizhikov & Millen, 2004*b*; Placzek & Briscoe, 2005), whereas the most anterior edge of the presumptive forebrain (the anterior neural ridge, ANR), the boundary between the prospective dorsal and ventral thalamus in the diencephalon (the zona limitans intrathalamica, ZLI) and the mid-/hindbrain boundary (MHB) or isthmic organiser (IsO) together control the patterning of the anterior neural tube along the A-P axis (reviewed in Liu & Joyner, 2001; Wurst & Bally Cuif, 2001; Echevarria *et al.* 2003; Prakash & Wurst, 2004; Kiecker & Lumsden, 2005). These signalling centres are characterized by the expression of different secreted and transcription factors controlling the establishment of the adjacent neural territories and the specification of the distinct neuronal populations for each territory. A key feature of any signalling centre is that it must provide a diffusible signal that can be received by a group of cells, which in turn may then be 'primed' through transcription of cell-specific genes. The FP, for example, secretes the lipid-modified glycoprotein Sonic hedgehog (Shh), which is known for its pivotal role in the specification of the different ventral populations in the hindbrain and spinal cord by controlling the expression of different transcriptional regulators (reviewed by Placzek & Briscoe, 2005). The secreted molecules Wnt1 and fibroblast growth factor (Fgf) 8 are expressed in the caudal midbrain or rostral hindbrain at the MHB, respectively, and transcription factors belonging to the homeodomain (HD) and paired-box families such as Pax2/5 and Engrailed (En) 1 and 2 are expressed across this boundary in both the caudal midbrain and the rostral hindbrain. Expression of all these factors in the neural tube initiates at roughly the same time of mouse embryonic development, i.e. between E8.0 and E8.5 (see Prakash & Wurst, 2004). The mDA neurons arise at around E10.5 from the ventral midline (FP and basal plate, BP) of the cephalic flexure close to the MHB (reviewed by Prakash & Wurst, 2006), suggesting that these two signalling centres must play an important role in their development. Indeed, we were able to demonstrate that the position of the MHB during embryonic development also determines the number and location of the mDA neurons and of another cell population specified in the rostral hindbrain adjacent to the MHB, the rostral hindbrain serotonergic (rh-5HT) neurons (Brodski *et al.* 2003). The ectopic induction of mDA neurons in these transgenic animals occurred only in close proximity to the FP thus, supporting further the hypothesis that both signalling centres are necessary for mDA neuron induction.

Floor plate Shh controls mDA neuron induction

Shh is secreted from the ventral midbrain FP and BP and was identified as one important molecule acting on the neuroepithelial precursors of the mDA population to specify their neurotransmitter identity. It was indeed shown that Shh and Fgf8 together are necessary and sufficient for the generation of ectopic mDA neurons in rat embryo explant cultures (Ye *et al.* 1998). Ectopic expression of Shh as well as its downstream effector molecule Gli1 is able to induce ectopic mDA and 5-HT neurons in the dorsal mid-/hindbrain region (MHR), in areas where normally Wnt1 and Fgf8 are expressed (Hynes *et al.* 1997). In addition, in *Shh* null mutants mDA neurons are missing and by conditionally inactivating *Smoothened* (*Smo*) (a receptor of Shh signalling) at E9.0 mDA neurons are considerably reduced (Blaess *et al.* 2006). Thus, Shh is necessary for mDA progenitor induction. However, the question remains as to how two symmetric signals, Shh (which is expressed along almost the entire length of the neural tube) and Fgf8 (since the development of the rh-5HT neurons also depends on this molecule (Ye *et al.* 1998)) could provide sufficient information for conveying the mDA phenotype exclusively to cells residing in the ventral midbrain. The role of Fgf-signalling for mDA neuron development has been compromised by recent findings showing that the ablation of Fgf receptor (Fgfr) 1 in the MHR has no effect on the mDA neuronal population, but leads to a reduction of the most rostral hindbrain 5-HT neurons (Trokovic *et al.* 2003). Fgfr1 is the prominent receptor in the MHR, although its loss in the midbrain may be partly compensated by Fgfr2 (Blak *et al.* 2005). Furthermore, Fgf8 beads on *Wnt1* homozygous mutant forebrain explant cultures are unable to induce ectopic

mDA neurons in contrast to wild-type tissue (Prakash *et al.* 2006). Based on these observations we hypothesized that the secreted glycoprotein Wnt1 would be a good candidate to provide such an 'asymmetric' signal as *Wnt1* expression is confined to a ring encircling the neural tube at the rostral border of the MHB (caudal midbrain), the RP of the mes- and diencephalon, and two stripes adjacent to the FP of the midbrain. The latter expression domain overlaps with the region where mDA progenitors are first specified (Prakash *et al.* 2006).

Ventral midbrain Wnt1 expression controls mDA neuron induction

Taking advantage of gain-of-function and loss-of-function *Wnt1* mutant mouse lines, we were able to demonstrate a crucial role of Wnt1 in the development of mDA neurons (Prakash *et al.* 2006). At early stages of mouse neural development (i.e. between E9.5 and E12.5), Wnt1 is required for the maintenance of *Otx2* expression in the region encompassing the FP and BP of the midbrain. Loss of *Otx2* in this region of $En1^{+/Cre}$; $Otx2^{flox/flox}$ mice leads to a ventral expansion of the midbrain Nkx2-2 expression domain, which is normally confined to a narrow stripe at the boundary between the BP and the alar plate of the mesencephalon. As a consequence, the *Wnt1* expression domain in the ventral midbrain is lost and ectopic rh-5HT neurons are generated instead of mDA neurons in this region of the mutant brain (Puelles *et al.* 2004; Prakash *et al.* 2006). The loss of *Wnt1* expression and of mDA neurons in the ventral midbrain of $En1^{+/Cre}$; $Otx2^{flox/flox}$ mice is probably a direct consequence of the repressive effect of Nkx2-2, as removal of this gene on an $En1^{+/Cre}$; $Otx2^{flox/flox}$ mutant background ($En1^{+/Cre}$; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ triple mutants) rescues the ventral *Wnt1* expression domain and the normal generation of mDA neurons (Prakash *et al.* 2006). In addition to this 'early' activity of Wnt1, it is also required at later stages of neural development (i.e. between E11.5 and E12.5) for the proper differentiation of mDA neurons in the mouse embryo (Prakash *et al.* 2006). In $Wnt1^{-/-}$ mice, few tyrosine hydroxylase (Th, the rate-limiting enzyme in DA biosynthesis)-expressing mDA precursors are still generated, but these cells fail to initiate expression of the HD transcription factor Pitx3. Furthermore, ectopic mDA neurons cannot be induced by Shh and Fgf8 in the absence of *Wnt1* in mouse embryo explant cultures (Prakash *et al.* 2006). In support of our findings, fate-mapping of ventral *Wnt1*-expressing cells during mouse embryonic development showed that a great extent of these cells will generate Th-expressing mDA neurons throughout the crucial stages (i.e. from E9.5 to E11.5) (Zervas *et al.* 2004). Previous data obtained from *in vitro* experiments already suggested an important function of Wnt proteins in the generation of differentiated mDA neurons from cultured mDA precursors (Castelo-Branco *et al.* 2003). In these studies, however, it was suggested that Wnt1 mostly controls the proliferation of mDA precursors and only to a minor extent their differentiation into Th-expressing mDA neurons, whereas Wnt5a was reported to be a more potent factor for the differentiation of these precursors into mDA neurons, with little effect on their proliferation (Castelo-Branco *et al.* 2003).

Wnt5a is another member of the mammalian Wnt family (comprising 19 proteins), which is expressed in the FP and BP of the neural tube including the ventral midbrain (Parr *et al.* 1993). Our own analysis of the $Wnt5a^{-/-}$ mutant mouse, however, does not confirm these findings *in vivo* (Minina *et al.* unpublished). The Wnt1-mediated signal transduction pathway (including receptors and intracellular effectors) regulating the generation of mDA neurons is unclear at present. However, based on *in vitro* data, it was suggested that the canonical Wnt/β-catenin pathway may be crucial in mediating the signal necessary for mDA development (Castelo-Branco *et al.* 2004). One can therefore envisage the distinct neuronal progenitor domains of the MHR being established within a cartesian grid system of diffusible signals along the A-P (Wnt1) and D-V (Shh) axes of the neural tube during early neural development (i.e. between E9.5 and E10.5).

Other secreted factors regulating mDA neuron development

The importance of other secreted factors and signalling pathways active in the FP and BP of the midbrain, such as transforming growth factors (Tgfs) α and β (Blum, 1998; Farkas *et al.* 2003), for mDA neuron development is less clear at present. They appear rather to provide trophic and/or mitogenic signals to the mDA progenitors, ensuring their proper survival and/or amplification. Although Tgfβ appears to be necessary for the induction of mDA neurons in the chicken embryo (Farkas *et al.* 2003), a similar requirement in the mouse embryo has not been reported so far. Nevertheless, both Tgfα and Tgfβ are required for the maintenance of mDA neurons in chicken and mice (Blum, 1998; Farkas *et al.* 2003).

But, how are the signals provided by these diffusible factors translated into a specific cell fate? Each of the secreted factors mentioned above has its specific receptors on the surface of the receiving cell. Binding of the corresponding secreted factor to its receptor activates the latter and elicits an intracellular signalling cascade that ultimately leads to protein modifications or changes in gene expression within the receiving cell (reviewed by Ciani & Salinas, 2005; Liu & Niswander, 2005; Thisse & Thisse, 2005; Huangfu & Anderson, 2006). Each of the aforementioned secreted factors can elicit different signalling cascades within the receiving cell, depending

on the type and complement of transducing (signalling) factors it will find inside the cell. The details of these signalling pathways involved in mDA neuron development are still totally unknown. However, target genes of these pathways are being unravelled, and it turns out that many of them are transcription factors activating and/or repressing a genetic programme that finally leads to the specification of the mDA neuronal phenotype in the receiving cell.

Transcription factors acting downstream of Wnt1 controlling mDA neuron development

In view of our result that *Pitx3* expression is not initiated in the absence of Wnt1, it remains to be shown whether *Pitx3* can be directly activated through canonical Wnt1-signalling. *Pitx3* seems to be regulated by Lmx1b, a member of the LIM-HD family. Lmx1b is expressed in the ventral midbrain and later confined to the postmitotic mDA progeny (Asbreuk *et al.* 2002). Lmx1b has previously been implicated in the development of mDA neurons in mice (Smidt *et al.* 2000). In the absence of *Lmx1b*, Th-positive mDA precursors fail to initiate expression of Pitx3 and later disappear, suggesting that Lmx1b is required for the induction of *Pitx3* in mDA neurons (Smidt *et al.* 2000). Interestingly, Lmx1b is able to induce ectopic expression of *Wnt1*, but not vice versa (Adams *et al.* 2000; Matsunaga *et al.* 2002). This would be in line with our findings showing no Pitx3 expression in mDA precursors in the absence of *Wnt1* (Prakash *et al.* 2006).

Another important finding in our studies was that the transcriptional repressor Otx2, which was previously thought of playing a crucial role in mDA neuron development (see Brodski *et al.* 2003; Puelles *et al.* 2003; Puelles *et al.* 2004; Vernay *et al.* 2005), does not appear to be required for the initial specification of mDA precursors and their differentiation into mDA neurons (Prakash *et al.* 2006). Deletion of *Otx2* from the ventral midbrain at E9.5 did not affect the normal generation of mDA neurons as long as *Nkx2-2* was also removed from the ventral midbrain, suggesting that Otx2 function in mDA neuron induction is to repress *Nkx2-2* (Prakash *et al.* 2006).

Transcription factors acting downstream of Shh control mDA neuron development

In a screen for HD transcription factors specifically expressed in the ventral midbrain and involved in mDA neuron development Lmx1a, like Lmx1b a LIM-HD family member, and Msx1, an orthologue of *Drosophila* muscle segment homeobox (msh) (reviewed by Ramos & Robert, 2005) have been identified (Andersson *et al.* 2006*b*). The factor Lmx1a (previously implicated only in dorsal neural tube development (Millonig *et al.* 2000; Chizhikov & Millen, 2004*a*), was shown to be expressed in the ventral midbrain in a spatio-temporal pattern correlating with the onset of mDA neurogenesis (Andersson *et al.* 2006*b*). The induction of ventral Lmx1a expression appeared to be Shh dependent. Most importantly, ectopic or over-expression of *Lmx1a* in the neural tube or in embryonic stem cells was sufficient to induce mDA neurons, albeit only in the ventral midbrain or in the presence of Shh (Andersson *et al.* 2006*b*). Lmx1a also seems to be required for the normal generation of mDA neurons, as RNA-interference (RNAi) experiments in the ventral midbrain of chicken embryos resulted in a drastic reduction of Nr4a2/Nurr1-positive cells in this region (Andersson *et al.* 2006*b*). A similar requirement of Lmx1a in the mouse has not been reported. Lmx1a appeared to exert its effects in part through the activation of another homeobox gene, *Msx1* (Andersson *et al.* 2006*b*). Its transcription in the ventral midbrain initiates somewhat later than *Lmx1a* (Andersson *et al.* 2006*b*). Expression of Msx1, in contrast to Lmx1a, is confined to proliferating precursors in the ventricular/subventricular zone (VZ/SVZ) of the ventral midline of the mesencephalon. Although *Msx1* was not sufficient to induce mDA neurons after ectopic expression in the chick midbrain and does not appear to be necessary for their generation in mice, it seems to be required for the repression of another homeobox gene, *Nkx6-1*, which is broadly expressed in the VZ/SVZ of the ventral neural tube (Andersson *et al.* 2006*b*). Repression of the most ventral *Nkx6-1* domain in the midbrain by Msx1 may therefore delimit the mDA progenitor domain from the more laterally located progenitors of motorneurons (Puelles *et al.* 2004; Andersson *et al.* 2006*b*). Whether *Lmx1a* and *Msx1* are regulated by Gli Zn-finger transcription factors, which are mediators of Shh signalling and control early mDA neuron development (Blaess *et al.* 2006) needs to be determined. Furthermore, Msx1 induced the expression of the proneural transcription factor Neurogenin2 (Ngn2) in the mesencephalic ventral midline of transgenic mice (Andersson *et al.* 2006*b*).

Proneural genes control mDA neuron differentiation

The proneural factor Ngn2 belongs to the family of basic helix-loophelix (bHLH) transcriptional regulators and confers mostly generic neuronal but also subtype-specific properties to differentiating neuroepithelial cells (reviewed by Bertrand *et al.* 2002). Indeed, Ngn2 has also been implicated in mDA neuron development (Andersson *et al.* 2006*a*; Kele *et al.* 2006). Ngn2 is expressed mostly in the VZ/SVZ of the ventral midbrain and in very few postmitotic Nr4a2/Nurr1-positive cells (Andersson *et al.* 2006*a*; Kele *et al.* 2006). In the absence of *Ngn2*, the mDA neuronal population was initially reduced to less than 20% and recovered postnatally to only about 50–60% of

the wild-type numbers (Andersson *et al.* 2006*a*; Kele *et al.* 2006). This recovery was probably due to the redundant activity of another proneural factor, Mash1, expressed in the same region as Ngn2, since removal of *Mash1* on an *Ngn2*$^{-/-}$ mutant background led to a further decrease in mDA neuron numbers, and the *Ngn2*$^{-/-}$ mutant phenotype could be partially rescued by overexpression of *Mash1* in *Ngn2*$^{Mash1/Mash1}$ knock-in mice (Kele *et al.* 2006). The remaining mDA neurons, however, differentiated normally into the SN and VTA subpopulations and established proper connections with their target fields in the forebrain in the absence of *Ngn2* (Andersson *et al.* 2006*a*; Kele *et al.* 2006). Overexpression of *Ngn2* in the dorsal midbrain or in cell cultures derived from it did not promote the generation of mDA neurons although it enhanced overall neurogenesis (Andersson *et al.* 2006*a*; Kele *et al.* 2006), indicating that it cannot specify a mDA neuronal fate in neural precursors. Neurogenesis appeared to be generally perturbed in the ventral midline of the *Ngn2*$^{-/-}$ midbrain as evidenced by a notorious lack of neuronal cell bodies and an increased expression of radial glia cell markers, concomitant with an aberrant expression of proneural and neurogenic genes like *Dll1* and *Hes5* in this region of the mutant midbrain (Andersson *et al.* 2006*a*; Kele *et al.* 2006). Since neighbouring cell populations such as the ventral mesencephalic motorneurons were not affected in the *Ngn2*$^{-/-}$ mutants (Andersson *et al.* 2006*a*), Ngn2 appears to be specifically required by the mDA precursors for the acquisition of generic neuronal properties, but not for their terminal differentiation.

Different transcription factors control distinct genetic networks required for the generation of mDA neurons

One of the first nuclear effectors recognized as being necessary for the proper development of mDA neurons is the orphan nuclear receptor Nr4a2/Nurr1 (reviewed by Perlmann & Wallen-Mackenzie, 2004; Prakash & Wurst, 2006). In the absence of *Nr4a2*/*Nurr1*, postmitotic mDA precursors are initially born but later disappear probably because they are lost by apoptosis (reviewed by Perlmann & Wallen-Mackenzie, 2004; Prakash & Wurst, 2006). Most importantly, the transcription of genes involved in DA biosynthesis and neurotransmission, such as *Th*, vesicular monoamine transporter 2 (*Vmat2*/*Slc18a2*) and the dopamine transporter (*Dat*/*Slc6a3*), is never initiated in these precursors in *Nr4a2*/*Nurr1* null mutant mice (reviewed in Perlmann & Wallen-Mackenzie, 2004). It was therefore suggested that Nr4a2/Nurr1 is required for the transcriptional activation of the genes encoding those proteins that confer DA neurotransmitter properties of mDA neurons (Sakurada *et al.* 1999). Another transcription factor playing a similar role in the differentiation of postmitotic mDA precursors into mDA neurons is the paired-like HD protein Pitx3. *Pitx3* was initially characterized as a bicoid-related homologue to *Pitx2* that is exclusively expressed in the mDA neuronal population (reviewed by Smidt *et al.* 2004; Prakash & Wurst, 2006). Pitx3 is required for the proper differentiation of a subset of mDA neurons, possibly by directly regulating *Th* expression in these cells (Maxwell *et al.* 2005). In the absence of *Pitx3*, the mDA neurons of the SN and around 50% of the VTA neurons are lost during development (Maxwell *et al.* 2005; reviewed by Smidt *et al.* 2004; Prakash & Wurst, 2006). Interestingly, the homologous gene *Pitx2* was shown to be directly activated through β-catenin/lymphoid-enhancer-factor (Lef) 1-mediated canonical Wnt-signalling during heart development (Kioussi *et al.* 2002). Finally, the En1 and En2 homeobox transcription factors, which are also under the transcriptional control of Wnt1, are required for the maintenance of the mDA neuronal population in later stages of embryonic development, but not for their initial specification (Simon *et al.* 2001).

Despite these considerable advances, many questions remain unanswered. The upstream regulators of *Nr4a2*/*Nurr1* and *Lmx1a* expression in the ventral midbrain, for example, are still unknown. Equally unknown are the targets conveying the mDA phenotype downstream of Lmx1a, although it is very likely that this pathway only operates in parallel with a pathway driven by Shh or another but yet unidentified ventralizing factor. The target genes of the HD factor Pitx3 in mDA neuron differentiation also await identification.

Summary

Recent advances have provided us for the first time with an insight into the genetic cascades active during the early and intermediate steps of mDA neuron development, including induction of the mDA progenitors and specification of the mDA neuronal fate. Firstly, a Wnt1-regulated network together with a Shh-controlled genetic cascade establishes the mDA progenitor domain at early stages of neural development. They do so by maintaining Otx2-expression in the ventral midbrain which in turn represses *Nkx2-2* in this domain, and by induction of Lmx1a and concomitantly of Msx1 in the mesencephalic ventral midline, which in turn re-presses *Nkx6-1* expression in this region. Thereby, a *Wnt1*$^{+}$, *Otx2*$^{+}$, *Lmx1a*$^{+}$, *Msx1*$^{+}$, *Shh*$^{+}$ but *Nkx2-2*$^{-}$, *Nkx6-1*$^{-}$ territory is established in the neuroepithelium of the ventral midbrain from which mDA precursors expressing the retinaldehyde dehydrogenase Aldh1a1 (reviewed by Prakash & Wurst, 2006) and Nr4a2/Nurr1 develop (Fig. 1). Failure to establish this transcriptional code results in a fate-switch of mDA progenitors into other identities such as rh-5HT neurons. It is not clear at present whether the

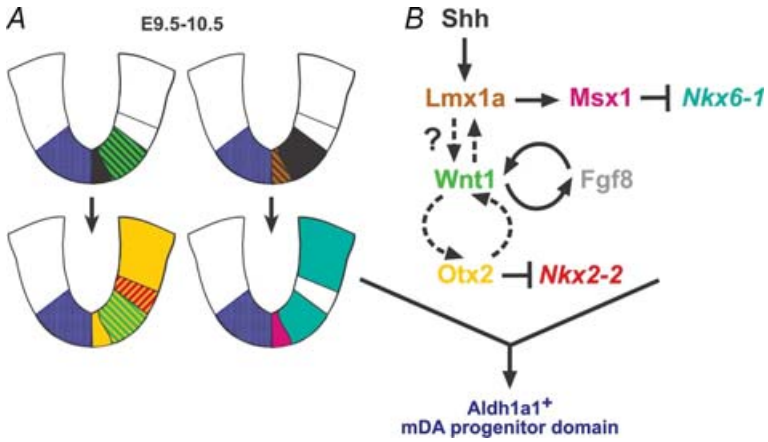

Figure 1. Establishment of the mDA progenitor domain during early stages of mouse neural development
A, cross-sections through the E9.5–10.5 mouse embryonic ventral midbrain depicting the expression domains of the secreted and transcription factors involved in the genetic networks required for the proper establishment of an Aldh1a1-positive (dark blue) mDA progenitor domain. *B*, the genetic networks required for the proper establishment of an Aldh1a1-positive mDA progenitor domain. Wnt1 (green) together with Shh (black) and Lmx1a (brown) are expressed in the ventral midline (FP) and/or in the adjacent BP of the mesencephalon. These factors positively control a genetic cascade including *Otx2* (yellow), required for repression of *Nkx2-2* (red) in the BP, and *Msx1* (pink), required for repression of *Nkx6-1* (turquoise) in the FP. Induction of *Lmx1a* in the ventral midbrain appears to require Shh. The possible interactions between Lmx1a and Wnt1 are not yet clarified (indicated by the broken arrows and question mark). Wnt1 and Otx2 mutually induce and/or maintain their expression in the ventral midbrain, although neither factor is necessary for it (indicated by the broken arrows). Wnt1 and Fgf8 (grey, not shown) are also engaged in a cross-regulation of their expression, but a strict requirement of Fgf8 signalling for the development of mDA neurons *in vivo* is uncertain. See text for details.

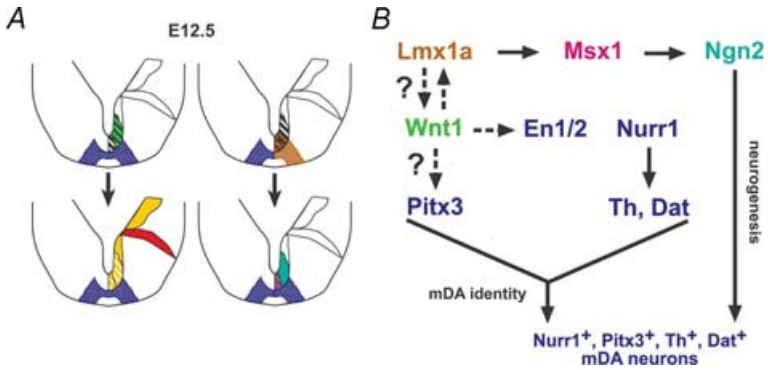

Figure 2. Determination of the mDA-specific and generic neuronal cell fate in mDA precursors at intermediate stages of mouse neural development
A, cross-sections through the E12.5 mouse embryonic ventral midbrain depicting the expression domains of the secreted and transcription factors involved in the genetic networks required for the differentiation of mDA precursors into mature mDA neurons (dark blue). *B*, the genetic networks required for the differentiation of mDA precursors into mature mDA neurons. Wnt1 (green) and Lmx1a (brown) continue to be expressed in the ventral midline (FP) and/or in the adjacent BP of the mesencephalon, but Wnt1 expression is now restricted to the VZ/SVZ of the neuroepithelium, whereas Lmx1a is also expressed in the mantle zone containing the postmitotic progeny. At this stage, these factors positively control a genetic cascade including *Msx1* (pink), required for expression of *Ngn2* (turquoise) in the VZ/SVZ of the ventral midbrain, and probably also *Pitx3* (dark blue), although this remains to be shown (indicated by the broken arrow and question mark). Wnt1 may also maintain the expression of *En1/2* (dark blue) in postmitotic mDA neurons at these stages. A complementary pathway controlled by Nurr1/Nr4a2 induces the expression of DA biosynthetic enzymes or transporters (*Th* and *Dat/Slc6a3*, dark blue) in these cells. Expression of Nurr1/Nr4a2, Pitx3, Th, Dat/Slc6a3, and En1/2 together define the mDA-specific identity of the postmitotic neurons, whereas their generic neuronal properties are regulated by the expression of the proneural factor Ngn2. The possible interactions between Lmx1a and Wnt1 at this stage are unknown (indicated by the broken arrows and question mark). Neither Otx2 (yellow stripes) nor Shh (black stripes) are required for the generation of mature mDA neurons at this developmental stage. Red: *Nkx2-2*. See text for details.

Wnt1- and the Lmx1a-regulated cascades do act in parallel or also act sequentially, as an induction of *Lmx1a* by Wnt1 has not been reported so far. It should be noted, however, that Lmx1a is able to induce *Wnt1* expression in the chicken dorsal spinal cord (Chizhikov & Millen, 2004*a*). The same appears to apply for Msx1, which was reported to induce *Wnt1* transcription after ectopic expression in the chicken neural tube, although this could rather be an indirect effect (reviewed by Ramos & Robert, 2005). It is almost certain, however, that both pathways require the presence of Shh or a Shh-regulated factor, as neither *Wnt1* nor *Lmx1a* was able to induce ectopic mDA neurons in dorsal domains of the neural tube (hindbrain or midbrain, respectively), although both factors were present (Andersson *et al.* 2006*b*; Prakash *et al.* 2006). Secondly, Wnt1-mediated signalling may directly or indirectly initiate or maintain expression of the homeobox genes *Pitx3* and *En1/2* in the progeny. This signalling cascade acts together with the Nr4a2/Nurr1-regulated pathway conferring DA neurotransmitter identity (initiation of *Th* and *Dat*/*Slc6a3* expression), thereby specifying the mDA phenotype in mDA precursors at intermediate stages of neural development (Fig. 2). At this stage, an Lmx1a/Msx1-controlled genetic cascade probably acts independently of the Wnt1/Nr4a2/Nurr1-regulated network to confer both generic neuronal (through activation of the proneural *Ngn2* gene) and mDA cell type-specific (through activation of as yet unknown target genes) properties to these precursors. Again, the interactions between the Wnt1-, and the Lmx1a-regulated networks remain unknown, but a requirement of Shh or a Shh-mediated signalling cascade for mDA neuron development at this stage may be excluded based or more recent evidence (Andersson *et al.* 2006*b*; Blaess *et al.* 2006; Prakash *et al.* 2006). Nevertheless, as pointed out before, many questions still remain open, and elucidation of their answers is currently the most important task in the field.

References

Adams KA, Maida JM, Golden JA & Riddle RD (2000). The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer. *Development* **127**, 1857–1867.

Andersson E, Jensen JB, Parmar M, Guillemot F & Bjorklund A (2006*a*). 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 & Ericson J (2006*b*). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* **124**, 393–405.

Asbreuk CHJ, Vogelaar CF, Hellemons A, Smidt MP & Burbach JPH (2002). CNS expression pattern of *Lmx1b* and coexpression with *Ptx* genes suggest functional cooperativity in the development of forebrain motor control systems. *Mol Cell Neurosci* **21**, 410–420.

Bertrand N, Castro DS & Guillemot F (2002). Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* **3**, 517–530.

Blaess S, Corrales JD & Joyner AL (2006). Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. *Development* **133**, 1799–1809.

Blak AA, Naserke T, Vogt Weisenhorn DM, Prakash N, Partanen J & Wurst W (2005). Expression of Fgf receptors 1, 2, and 3 in the developing mid- and hindbrain of the mouse. *Dev Dyn* **233**, 1023–1030.

Blum M (1998). A null mutation in TGF-α leads to a reduction in midbrain dopaminergic neurons in the substantia nigra. *Nat Neurosci* **1**, 374–377.

Brodski C, Weisenhorn DMV, Signore M, Sillaber I, Oesterheld M, Broccoli V, Acampora D, Simeone A & Wurst W (2003). Location and size of dopaminergic and serotonergic cell populations are controlled by the position of the midbrain-hindbrain organizer. *J Neurosci* **23**, 4199–4207.

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

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

Chizhikov VV & Millen KJ (2004*a*). Control of roof plate formation by Lmx1a in the developing spinal cord. *Development* **131**, 2693–2705.

Chizhikov VV & Millen KJ (2004*b*). Mechanisms of roof plate formation in the vertebrate CNS. *Nat Rev Neurosci* **5**, 808–812.

Ciani L & Salinas PC (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat Rev Neurosci* **6**, 351–362.

Echevarria D, Vieira C, Gimeno L & Martinez S (2003). Neuroepithelial secondary organizers and cell fate specification in the developing brain. *Brain Res Brain Res Rev* **43**, 179–191.

Farkas LM, Dunker N, Roussa E, Unsicker K & Krieglstein K (2003). Transforming growth factor-βs are essential for the development of midbrain dopaminergic neurons *in vitro* and *in vivo*. *J Neurosci* **23**, 5178–5186.

Goridis C & Rohrer H (2002). Specification of catecholaminergic and serotonergic neurons. *Nat Rev Neurosci* **3**, 531–541.

Huangfu D & Anderson KV (2006). Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from *Drosophila* to vertebrates. *Development* **133**, 3–14.

Hynes M, Stone DM, Dowd M, Pitts-Meek S, Goddard A, Gurney A & Rosenthal A (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. *Neuron* **19**, 15–26.

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

Kiecker C & Lumsden A (2005). Compartments and their boundaries in vertebrate brain development. *Nat Rev Neurosci* **6**, 553–564.

Kioussi C, Briata P, Baek SH, Rose DW, Hamblet NS, Herman T, Ohgi KA, Lin C, Gleiberman A & Wang J (2002). Identification of a Wnt/Dvl/β-catenin $\rightarrow$ Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell* **111**, 673–685.

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

Liu A & Niswander LA (2005). Bone morphogenetic protein signalling and vertebrate nervous system development. *Nat Rev Neurosci* **6**, 945–954.

Matsunaga E, Katahira T & Nakamura H (2002). Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development. *Development* **129**, 5269–5277.

Maxwell SL, Ho H-Y, Kuehner E, Zhao S & Li M (2005). Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Dev Biol* **282**, 467–479.

Millonig JH, Millen KJ & Hatten ME (2000). The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. *Nature* **403**, 764–769.

Parr BA, Shea MJ, Vassileva G & McMahon AP (1993). Mouse *Wnt* genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247–261.

Perlmann T & Wallen-Mackenzie A (2004). Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. *Cell Tissue Res* **318**, 45–52.

Placzek M & Briscoe J (2005). The floor plate: multiple cells, multiple signals. *Nat Rev Neurosci* **6**, 230–240.

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

Prakash N & Wurst W (2004). Specification of midbrain territory. *Cell Tissue Res* **318**, 5–14.

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

Puelles E, Acampora D, Lacroix E, Signore M, Annino A, Tuorto F, Filosa S, Corte G, Wurst W, Ang SL & Simeone A (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 SL, Wurst W & Simeone A (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037–2048.

Ramos C & Robert B (2005). *msh*/*Msx* gene family in neural development. *Trends Genet* **21**, 624–632.

Sakurada K, Ohshima-Sakurada M, Palmer TD & Gage FH (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* **126**, 4017–4026.

Simeone A (2005). Genetic control of dopaminergic neuron differentiation. *Trends Neurosci* **28**, 62–65.

Simon HH, Saueressig H, Wurst W, Goulding MD & O'Leary DD (2001). Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J Neurosci* **21**, 3126–3134.

Smidt MP, Asbreuk CH, Cox JJ, Chen H, Johnson RL & Burbach JP (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat Neurosci* **3**, 337–341.

Smidt MP, Smits SM & Burbach JPH (2003). Molecular mechanisms underlying midbrain dopamine neuron development and function. *Eur J Pharmacol* **480**, 75–88.

Smidt MP, Smits SM & Burbach JPH (2004). Homeobox gene Pitx3 and its role in the development of dopamine neurons of the substantia nigra. *Cell Tissue Res* **318**, 35–43.

Thisse B & Thisse C (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev Biol* **287**, 390–402.

Trokovic R, Trokovic N, Hernesniemi S, Pirvola U, Vogt Weisenhorn DM, Rossant J, McMahon AP, Wurst W & Partanen J (2003). FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals. *EMBO J* **22**, 1811–1823.

Vernay B, Koch M, Vaccarino F, Briscoe J, Simeone A, Kageyama R & Ang S-L (2005). Otx2 regulates subtype specification and neurogenesis in the midbrain. *J Neurosci* **25**, 4856–4867.

Winkler C, Kirik D & Bjorklund A (2005). Cell transplantation in Parkinson's disease: how can we make it work? *Trends Neurosci* **28**, 86–92.

Wurst W & 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 JL, Hynes MA & Rosenthal A (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755–766.

Zervas M, Millet S, Ahn S & Joyner AL (2004). Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. *Neuron* **43**, 345–357.

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