

# Transcriptional control of midbrain dopaminergic neuron development

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Although loss of midbrain dopaminergic neurons is associated with one of the most common human neurological disorders, Parkinson's disease, little is known about the specification of this neuronal subtype. Hence, the recent identification of major transcriptional determinants regulating the development of these neurons has brought much excitement and encouragement to this field. These new findings will help to elucidate the genetic program that promotes the generation of midbrain dopaminergic neurons. Importantly, these discoveries will also significantly advance efforts to differentiate stem cells into midbrain dopaminergic neurons that can be used for therapeutic use in treating Parkinson's disease.

## Introduction

The midbrain dopaminergic (mDA) neurons (neurons that use dopamine as a neurotransmitter) constitute about 75% of dopaminergic neurons in the adult brain (Wallen and Perlmann, 2003). The midbrain dopamine system has been intensively studied because of its implication in diverse psychiatric and neurological disorders (Hornykiewicz, 1978; Carlsson, 2001). In addition, midbrain dopaminergic neurons play key roles in the generation of pleasure, and in the development of addictive behaviours such as drug abuse (Chao and Nestler, 2004).

Our current understanding of the location of dopaminergic (DA) neurons in the central nervous system (CNS) comes from immunohistochemical studies that determine the expression pattern of the enzyme tyrosine hydroxylase (Th), which is the rate-limiting enzyme of dopamine synthesis. Th<sup>+</sup> mDA neurons (historically called A8-A10) originate from the ventral part of a domain of the brain that extends rostrally to the ventral thalamus/hypothalamus border and caudally to the midbrain/hindbrain border (Fig. 1) (Bjorklund and Lindvall, 1984; Dahlstrom and Fuxe, 1964; Marin et al., 2005). These neurons can be anatomically divided into three main subgroups. The lateral A9 group corresponds to neurons of the substantia nigra pars compacta (SNpc), which mainly project into the dorsal striatum and form the nigro-striatal pathway. This group of neurons is mainly involved in the control of movement, as revealed by the resting tremor, rigidity, bradykinesia (abnormal slowing of voluntary movement) and gait disturbance that is seen in individuals with Parkinson's disease. These symptoms are due to the specific degeneration of the SNpc neurons (Lang and Lozano, 1998). The medial A10 and lateral A8 neuron groups define the ventral tegmental area (VTA) and retrorubal field of the midbrain, respectively. A10 and A8 groups project to the ventromedial striatum, as part of the mesocortical limbic system that is involved in emotional behaviour and mechanisms of reward (Dahlstrom and Fuxe, 1964; Tzschentke and Schmidt, 2000). Dysregulation of DA transmission in the limbic system has been linked to the

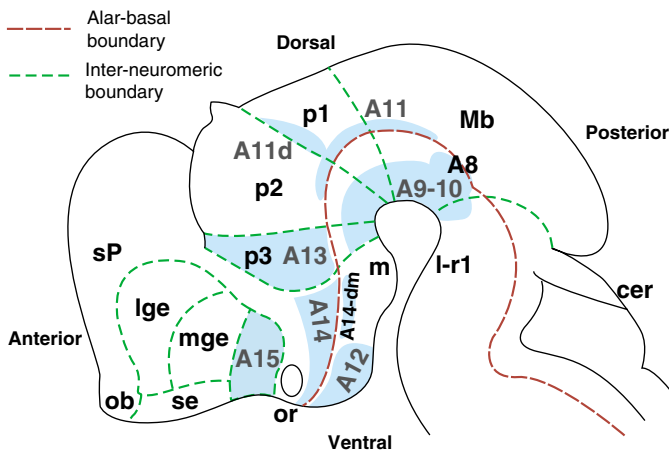
development of drug addiction (Kelley and Berridge, 2002; Wightman and Robinson, 2002) and depression (Dailly et al., 2004), and is thought to contribute to the psychotic symptoms of schizophrenia (Sesack and Carr, 2002).

As for all neurons, the generation of mDA neurons from a neural progenitor cell can be divided into distinct steps. At least three steps have been recognized based on the expression of molecular markers: (1) regional specification, (2) early and (3) late differentiation. Until recently, mDA progenitors were poorly defined and not easily distinguishable from other CNS progenitors. The recent discovery that certain transcription factors, specifically Otx2, Lmx1a and Lmx1b (Lmx1a/b), Engrailed 1 and Engrailed 2 (En1/2), Msx1 and Msx2 (Msx1/2), Neurogenin 2 (Ngn2) and Mash1, are all expressed in mDA progenitors has allowed these progenitors to be identified for the first time by a combinatorial transcription factor code (Simon et al., 2001; Puelles et al., 2003; Puelles et al., 2004; Andersson et al., 2006a; Andersson et al., 2006b; Kele et al., 2006). These new transcription factors provide additional markers with which to address the issue of heterogeneity among mDA progenitors, and are candidate regulators for promoting the specification and differentiation of progenitors into mDA neurons. Important insights into the role of these transcription factors in regulating the development of mDA neurons, which have arisen from loss- and gain-of-function experiments in chick and mice, will be discussed in this review. These new findings will underpin exciting research both in the stem cell and developmental biology fields during the next few years.

## Midbrain dopaminergic lineage

Molecular markers allow the distinction of three sequential cell populations in the mDA lineage: progenitors, immature neurons and mature neurons (see Fig. 2A,B). These populations are generated during successive developmental steps, which are called regional specification, early and late differentiation (see below). Until recently, the only molecule known to mark specifically DA progenitors in the midbrain was retinaldehyde dehydrogenase Aldh1a1 (Raldh1). Raldh1 catalyzes the oxidation of retinaldehyde into retinoic acid; however, the role of Raldh1 in mDA progenitors remains to be discovered. Remarkably, during the past two years several groups have shown that other transcription factors, such as Otx2, Lmx1a/b, En1/2, Msx1/2, Ngn2 and Mash1, are also expressed in these progenitors (Simon et al., 2001; Puelles et al., 2003; Puelles et al., 2004; Andersson et al., 2006a; Kele et al., 2006; Andersson et al., 2006b).

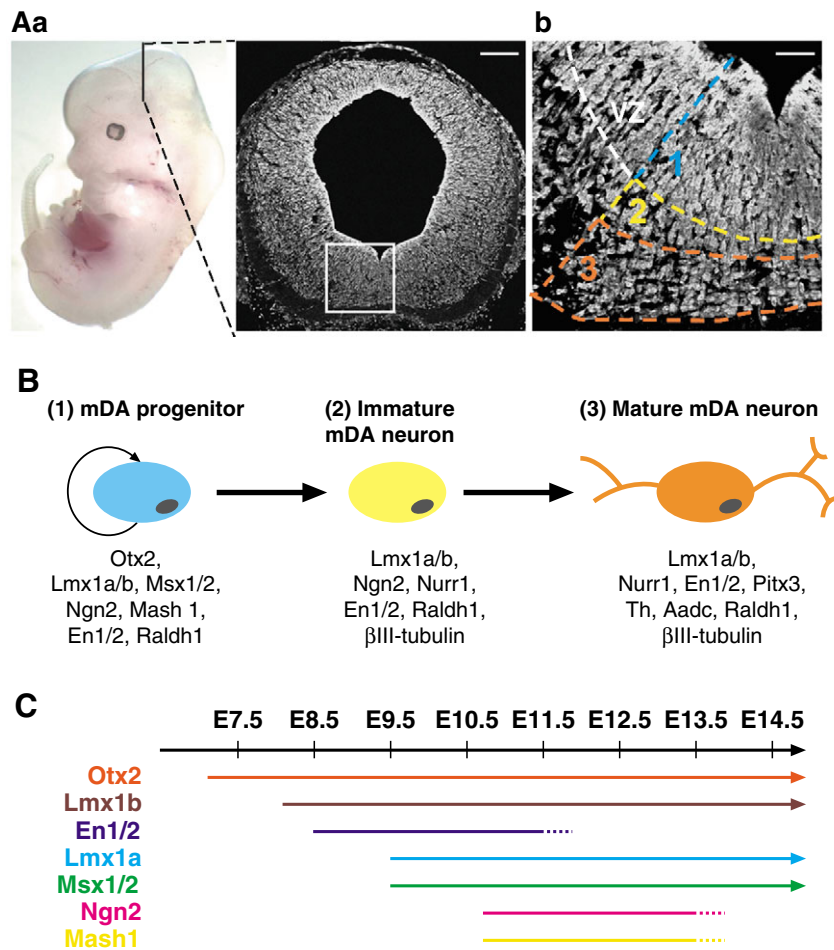
Despite this historical lack of information on molecular determinants of mDA progenitors, several key transcription factors have been identified and described that mark and regulate the development of postmitotic mDA neurons. For example, the orphan nuclear receptor Nurr1 was found to be required not for the generation, but for the maintenance of mDA neurons (Zetterstrom et al., 1997; Saucedo-Cadenas et al., 1998). It is also required for the expression of Th, vesicular monoamine transporter 2 (Vmat2),



**Fig. 1. Dopaminergic neurons in the CNS.** Schematic illustrating the location of Th<sup>+</sup> neurons, corresponding to A8-A15 groups, in mouse brains ranging from E12.5-E14.5. The DA groups (light blue areas) are depicted in relation to the neuromeric map [subdivision into transverse segments, see Puelles and Rubenstein (Puelles and Rubenstein, 2003)] and the alar (dorsal) and basal (ventral) plate subdivision. This figure is adapted, with permission, from Marin et al. (Marin et al., 2005). A16 and A17 groups are not shown. cer, cerebellum; d, dorsal; dm, dorsomedial; I-r1, isthmic region; lge, lateral ganglionic eminence; m, mamillary body; Mb, midbrain; mge, medial ganglionic eminence; ob, olfactory bulb; or; optic recess; p, prosomere; sP, secondary prosencephalon.

dopamine transporter (DAT) and Ret in mDA neurons (Wallen et al., 1999; Wallen et al., 2001; Smits et al., 2003). By contrast, En1/2 homeodomain proteins are required, in part, for the generation of mDA neurons and their survival (Simon et al., 2001). En1/2 proteins have also been shown *in vitro* to be required cell autonomously in mDA neurons for neuronal survival through their regulation of apoptosis (Alberi et al., 2004). Pitx3 is a paired homeodomain transcription factor that is required for the expression of Th in a subset of mDA neurons (Maxwell et al., 2005), and for the survival of primarily SNpc, but also VTA, neurons (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003).

Presently, it is still unknown whether mDA progenitors represent a homogenous or a heterogeneous pool of progenitors. The specific vulnerability of SNpc DA neurons to toxic insults when compared with VTA DA neurons, as is evident in Parkinson's disease, indicates that these neurons are heterogeneous. The earliest molecular marker that distinguishes subpopulations of mDA neurons in mice is *Pitx3*. By using GFP to trace the lineage of *Pitx3*-expressing cells in *Pitx3* knock-in GFP mouse embryos, two partially overlapping groups of mDA neurons can be distinguished by their temporal profile of expression of Th and *Pitx3* (Maxwell et al., 2005). Ventrolateral mDA neurons express *Pitx3* prior to expressing Th, whereas the dorsomedial mDA neurons express Th ahead of *Pitx3* at E12.5. By



**Fig. 2. Molecularly distinct DA cell populations in the mouse ventral midbrain.** (A, part a) An E12.5 mouse embryo. The black line marks the plane of section shown in the image to the right. (A, part b) An enlarged view of the ventral midbrain (square box in A, part a), illustrating the position occupied by mitotic progenitors, immature and mature postmitotic neurons of the mDA lineage. (B) The sequential timing of transcription factor activation in mDA progenitors. The curved arrow indicates cycling cells. (C) The timing of expression of transcription factors in mDA progenitors (not in subsequent steps of the lineage) are shown. Dotted lines indicate that the precise timing of expression shutdown has not been determined. Images in A, part a, and A, part b are courtesy of the Ang Laboratory.

E13.5, all Th<sup>+</sup> cells express Pitx3. Although this study indicates that mDA neurons are a heterogeneous population transiently at E12.5, it remains to be determined whether their heterogeneous connectivity patterns and resistance to toxicity are already specified at the progenitor stage, or, alternatively, only later in postmitotic neurons.

### Regional specification of mDA progenitor

Studies using rat and chick neural plate explants have demonstrated that the floor plate-derived signal sonic hedgehog (Shh) and fibroblast growth factor 8 (Fgf8) from the mid-hindbrain boundary are required for the induction of DA neurons before E9.5 (Ye et al., 1998). In addition, Wnt1 (Prakash et al., 2006) and transforming growth factor- $\beta$  (Tgf $\beta$ ) (Farkas et al., 2003) probably also play a role in the patterning of midbrain progenitors. These signals specify anteroposterior (AP) and dorsoventral (DV) identity, and result in the activation of a combination of transcription factors, including Otx2, Lmx1a/b, En1/2, Msx1/2, Ngn2 and Mash1, in a temporal sequence (Fig. 2C). The expression of Otx2, Lmx1b and En1/2 genes is already initiated by E9.0 (Ang et al., 1994; Smidt et al., 2000). Subsequently, Lmx1a and Msx1/2 expression turn on around E9.5, and Ngn2 and Mash1 expression are not initiated until E10.75 (Andersson et al., 2006b). The molecular mechanisms leading to the sequential activation of these genes is not understood. Shh can induce Lmx1a and Msx1/2 expression in neural tube explants of chick embryos (Andersson et al., 2006b). However, these transcription factors are activated endogenously in mouse embryos one day later than the initiation of Shh expression (Echelard et al., 1993). These results suggest that Shh signalling induces another signal or factor that is required for the expression of Lmx1a and Msx1/2. Based on their ventral midline position, mDA progenitors are presumed to be derived from Shh<sup>+</sup> glial-like floor plate cells. Thus, another step in the specification of mDA progenitors requires the acquisition of neuronal potential by floor plate progenitors, a step that is likely to involve the activity of the proneural genes *Ngn2* and *Mash1* (Andersson et al., 2006a, Andersson et al., 2006b; Kele et al., 2006).

### Differentiating mDA neurons

#### Early differentiation: generation of immature mDA neurons

Birthdating studies using tritiated thymidine incorporation demonstrate that mDA progenitors exit the cell cycle and generate postmitotic immature mDA neurons between E9.5 and E13.5 in

mice (Bayer et al., 1995). Immature mDA neurons initiate Nurr1 expression (Zetterstrom et al., 1997) and En1/2 expression (Simon et al., 2001; Alberi et al., 2004) during this differentiation step (Fig. 2B). In addition, mDA progenitors, like many other CNS progenitors, downregulate Sox2 expression while initiating the expression of general neuronal markers, such as  $\beta$ III-tubulin, in postmitotic immature and mature mDA neurons (Kele et al., 2006).

#### Late differentiation: generation of mature mDA neurons

From E11.0 onwards, immature mDA neurons continue to migrate radially on radial glial fibres into the mantle zone, further differentiating into mDA neurons (Kawano et al., 1995). These neurons express Pitx3, Th and aromatic amino acid decarboxylase (Aadc), in addition to the earlier markers expressed in immature mDA neurons (Fig. 2B). Ngn2, however, is not expressed in mature mDA neurons. Aadc converts DOPA into dopamine. *Aadc* mRNA transcripts are thought to be expressed already in immature mDA neurons (Smidt et al., 2004).

#### Transcription factors required for mDA neuron development

The roles of transcription factors, such as Nurr1, En1/2 and Ptx3, acting during the late differentiation step of the mDA lineage (described briefly above, see Fig. 2B and Table 1) have been extensively reviewed elsewhere (Goridis and Rohrer, 2002; Riddle and Pollock, 2003; Wallen and Perlmann, 2003; Simeone, 2005; Smits et al., 2006; Prakash and Wurst, 2006). However, the roles of the transcription factors that govern the specification and early differentiation of mDA progenitors have only recently started to emerge during the past few years and will be the focus of this review (Table 1).

#### Otx2

Otx2 is a bicoid class homeodomain transcription factor that is widely expressed before gastrulation, but its expression becomes progressively restricted to the anterior third of the mouse embryo after E7.75 (Simeone et al., 1993; Ang et al., 1994). Within the nervous system, *Otx2* expression is restricted to the forebrain and midbrain between E8.5 and E12.5. In addition to these anterior brain regions, expression is also detected in the rhombencephalon from E12.5 onwards (Mallamaci et al., 1996). *Otx2* is required for the

**Table 1. A summary of the role of transcription factors in mDA neuron development**

Transcription factor	Expression in the mDA lineage	Function in mDA cells	References
Otx2	Progenitors	Required for regional and neuronal specification of mDA progenitors	Puelles et al., 2003; Puelles et al., 2004; Vernay et al., 2005
Msx1	Progenitors	Required for neuronal differentiation	Andersson et al., 2006b
Ngn2	Progenitors and immature neurons	Required for neuronal differentiation	Andersson et al., 2006a; Kele et al., 2006
Mash1	Progenitors	Not required for neuronal differentiation but can compensate for Ngn2 function	Kele et al., 2006
Lmx1b	Progenitors, immature and mature neurons	Required for maintenance of mature mDA neurons	Smidt et al., 2000
En1/2	Progenitors, immature and mature neurons	Required for the generation and survival of mature mDA neurons	Simon et al., 2001; Alberi et al., 2004
Nurr1	Immature and mature neurons	Required for the maintenance of mature mDA neurons and their expression of late differentiation markers	Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Wallen et al., 2001; Smits et al., 2003
Pitx3	Mature neurons	Required for Th-expression in a subset of mature mDA neurons and for the survival of primary SNpc, and also VTA, neurons	Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005

formation of the forebrain and midbrain as a result of its role in the anterior visceral endoderm, where it functions to restrict posterior fates (Mallamaci et al., 1996; Perea-Gomez et al., 2001) (reviewed by Simeone and Acampora, 2001). Subsequently, *Otx2* is also required for positioning the expression of *Wnt1* and *Fgf8* at the midbrain boundary (Brodski et al., 2003), and for limiting the dorsal extent of *Shh* expression in the ventral midbrain (Puelles et al., 2003).

*Otx2* also has additional roles in mDA progenitor specification and differentiation based on findings obtained from the phenotypical analyses of *Otx2* mutant mice. Several different conditional *Otx2* mouse mutants have been generated in an attempt to delete *Otx2* specifically at different developmental stages. For example, *Otx2* was deleted in the midbrain of *En1cre;Otx2<sup>flox/flox</sup>* embryos from E9.5 onwards (Puelles et al., 2004). In these *En1cre;Otx2<sup>flox/flox</sup>* mutant embryos, midbrain expression of *Shh* expands dorsally, whereas *Fgf8* expression, which is normally restricted to the anterior hindbrain, shifts anteriorly into the midbrain (Puelles et al., 2004). Despite these changes in AP and DV patterning molecules, a small domain of midbrain tissue develops normally. Within this domain, expression of the homeodomain protein *Nkx2.2* expands ventrally into presumptive DA progenitors around E9.5, indicating that *Otx2* is required for the repression of *Nkx2.2* in these progenitors (Prakash et al., 2006). Interestingly, serotonergic neurons are generated ectopically in these *Otx2* conditional mutants at the expense  $\text{Th}^+$  mDA neurons. Loss of mDA neurons is directly linked to the abnormal expression of *Nkx2.2* in mDA progenitors, as the presence of ectopic serotonergic neurons and the reduction in the number of mDA neurons are rescued in *En1cre;Otx2<sup>flox/flox</sup>;Nkx2.2<sup>-/-</sup>* embryos (Prakash et al., 2006). The ectopic expression of *Nkx2.2* in *En1cre;Otx2<sup>flox/flox</sup>* embryos is also associated with a loss of expression of *Wnt1*, which indicates that *Otx2* might regulate *Wnt1* expression indirectly via its repressive effects on *Nkx2.2* in mDA progenitors at E12.5. Accordingly, *Wnt1* expression is recovered in *En1cre;Otx2<sup>flox/flox</sup>;Nkx2.2<sup>-/-</sup>* embryos that lack both *Otx2* and *Nkx2.2*. It is noteworthy that *Wnt1* may also be required upstream of *Otx2*, as ectopic expression of *Wnt1* under the control of the *En1* promoter leads to an upregulation of *Otx2* in the floor plate of the rostral hindbrain of *En1<sup>Wnt1/+</sup>* embryos (Prakash et al., 2006).

A different role for *Otx2* in mDA progenitors was identified from studies of *Nestin-Cre;Otx2<sup>flox/flox</sup>* embryos (Vernay et al., 2005). In these conditional mutants, loss of *Otx2* protein from E10.5 onwards results in loss of expression of the proneural genes *Ngn2* and *Mash1* in ventral mDA progenitors. Subsequently, mDA neurons are missing at the ventral midline of the midbrain. In addition, *Nkx2.2* expression expands ventrally into the ventricular zone adjacent to the red nucleus neurons that are reduced in size in *Nestin-Cre;Otx2<sup>flox/flox</sup>* embryos (Vernay et al., 2005). Red nucleus neurons normally lie dorsal to mDA neurons, and are implicated in the control of locomotion (reviewed by Kennedy, 1990). This loss of red nucleus neurons is fully rescued in *Nestin-Cre;Otx2<sup>flox/flox</sup>;Nkx2.2<sup>-/-</sup>* embryos, but the loss of mDA neurons is not. Taken together, these results indicate that *Otx2*, presumably via regulating the expression of *Ngn2* and *Mash1* (see below), is also required for the generation of mDA neurons. A later role for *Otx2* in regulating neurogenesis in mDA progenitors seems contradictory to the observation that mDA neuronal development is recovered in *En1cre;Otx2<sup>flox/flox</sup>;Nkx2.2<sup>-/-</sup>* mutant embryos, despite the loss of *Otx2* in mDA progenitors prior to neuronal differentiation in these triple mutants at E9.5. In order to reconcile these two findings, I propose that the requirement for *Otx2* in regulating *Ngn2* expression in mDA progenitors may be bypassed by changes in *Shh* expression in the ventral midbrain of

*En1cre;Otx2<sup>flox/flox</sup>;Nkx2.2<sup>-/-</sup>* mutant embryos (Prakash et al., 2006). This is because *Shh* can influence the expression of *Ngn2*, possibly via its effect on *Lmx1a* (see below). Alternatively, or in addition, the recovered *Wnt1* expression in *En1cre;Otx2<sup>flox/flox</sup>;Nkx2.2<sup>-/-</sup>* mutants is not completely normal and this may also affect *Ngn2* expression in these mutants (Prakash et al., 2006).

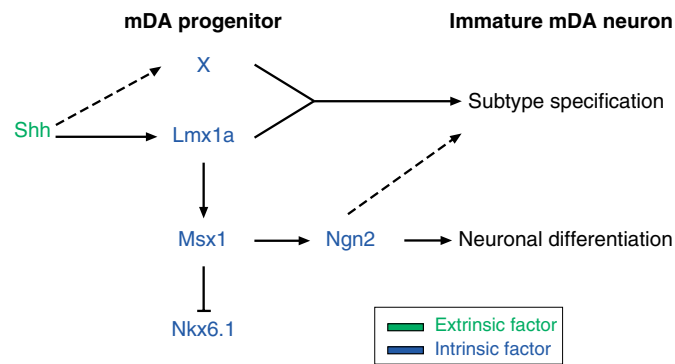
### Lmx1a and Lmx1b

*Lmx1a* and *Lmx1b*, members of the family of LIM homeodomain transcription factors, play important roles in the developing brain. *Lmx1b* is expressed in the midbrain from E8.0 onwards (Smidt et al., 2000), but this expression becomes restricted by E9.5 to the roof plate, the mid-hindbrain boundary and the ventral midbrain, including the floor plate. By contrast, *Lmx1a* expression begins at E9.5 in the ventral midbrain and then progressively expands dorsally (Andersson et al., 2006b). Double antibody labelling studies have revealed that at E9.5, *Lmx1b* expression encompasses more cells in the ventral midbrain than does *Lmx1a*, but that by E10.5 the expression domains of the two genes largely coincide. Because the expression of *Lmx1a* in progenitors directly overlies a region where  $\text{Th}^+$  neurons develop at E11.5, *Lmx1a* expression has been proposed to mark the dorsal boundary of mDA progenitors (Andersson et al., 2006b), whereas the initial expression of *Lmx1b* is believed to include progenitors for other cell types as well. This interpretation seems likely but awaits confirmation by lineage- and fate-mapping studies.

Loss-of-function studies have shown that *Lmx1b* is required for the maintenance of  $\text{Th}^+$  mDA neurons.  $\text{Th}^+$  neurons are found in *Lmx1b<sup>-/-</sup>* mutants up to E16.0, although they fail to express the paired homeodomain transcription factor *Pitx3* (Smidt et al., 2000). This differentiation defect results in the eventual loss of DA neurons in *Lmx1b<sup>-/-</sup>* embryos. Although this phenotype has been interpreted as demonstrating a role for *Lmx1b* in the maintenance of DA neurons, *Lmx1b* is initially broadly expressed in the midbrain; and earlier defects in patterning of the mid-hindbrain region may contribute to the loss of  $\text{Th}^+$  neurons at later stages. In addition, *Lmx1b.1* and *Lmx1b.2* genes are required for maintenance of the mid-hindbrain organizer and for the expression of *Wnt1*, *Wnt3a*, *Wnt10b*, *Pax8* and *Fgf8* in zebrafish embryos (O'Hara et al., 2005). Therefore, whether the mDA phenotype of *Lmx1b* mutant mouse embryos is due to an intrinsic role in the mDA lineage or to earlier patterning functions in the midbrain remains to be clarified. A detailed analysis of *Lmx1b* functions in mDA progenitors and neurons will be important to elucidate its roles at different developmental stages.

Positional cloning has identified *Lmx1a* as being the gene affected in the spontaneous mouse mutant *dreher* (Millonig et al., 2000). The point mutation in *Lmx1a* in *dreher* mice results in the loss of the caudal roof plate. In addition, overexpression studies have shown that *Lmx1a* can induce the expression of roof plate markers and of components of roof plate signalling (Chizhikov and Millen, 2004b), thus demonstrating that *Lmx1a* is both required and sufficient to induce roof plate formation.

A recent breakthrough study by the groups of Ericson and Perlmann has identified *Lmx1a* as a crucial determinant of mDA neuron fate development (Andersson et al., 2006b). In this study, these researchers showed that the overexpression of *Lmx1a* in the ventral midbrain of chick embryos promoted the generation of DA neurons over that of other neuronal subtypes. The induction of DA neurons in chick embryos was preceded by a re-specification of progenitor cells, as indicated by the activation of *Msx1* and the repression of *Nkx6.1* expression. Importantly, the activity of *Lmx1a*



**Fig. 3. Model of mDA neuron specification.** Shh induces *Lmx1a* and X (an unknown transcription factor) in mDA progenitors. Based on the timing of induction of endogenous *Lmx1a* expression compared with Shh expression, the induction of *Lmx1a* may be indirect. *Lmx1a* and X then act cooperatively to specify immature mDA neurons. *Lmx1a* in turn activates *Msx1*, which induces *Ngn2*. *Ngn2* promotes neuronal differentiation and, perhaps, also the subtype specification of immature mDA neurons. In addition, *Msx1* is required and is sufficient for the suppression of *Nkx6.1* expression in DA progenitors. Dotted arrows indicate hypothetical functions that remain to be proven. This model is modified, with permission, from Andersson et al. (Andersson et al., 2006b).

is context dependent, as *Lmx1a* can only induce ectopic DA neurons in ventral, and not in dorsal, midbrain cells. This context dependence suggests a role for additional factors that are specifically expressed in ventral midbrain cells. Alternatively, *Lmx1a* might not be able to inhibit dorsal differentiation programs and thus cannot convert dorsal midbrain cells to a more ventral fate. It is noteworthy that in this context, the overexpression of *Foxa2* and *Gli* in transgenic mice results in the generation of ectopic Th<sup>+</sup> neurons in the dorsal midbrain (Sasaki and Hogan, 1994; Hynes et al., 1997). In these transgenic embryos, ectopic DA neurons were found adjacent to dorsal sites of *Shh* expression. These results indicate that some dorsal midbrain progenitors are competent to acquire a mDA fate in mouse embryos at developmental stages equivalent to those of the chick experiments discussed above (Sasaki and Hogan, 1994; Hynes et al., 1997). Taken together, these results favour the hypothesis that *Lmx1a* alone is not sufficient to induce mDA neurons, and that it functions cooperatively with ventral factors induced by the Shh pathway (Fig. 3).

Additional support for cooperative interactions between *Shh* and *Lmx1a* has come from studies using the differentiation of embryonic stem (ES) cells. Mouse ES cells transfected with *Lmx1a* differentiate into DA neurons in the presence, but not in the absence, of Shh (Andersson et al., 2006b). Impressively, *Lmx1a* promotes Th expression in up to 80% of βIII-tubulin<sup>+</sup> neurons that were derived from ES cells. Importantly, these neurons also expressed other mDA neuron markers, such as Pitx3, *Lmx1b*, En1/2 and DAT. By contrast, mock-transfected ES cells produced Th<sup>+</sup> neurons that were mostly GABA<sup>+</sup> and Pitx3<sup>-</sup>.

A complementary loss-of-function study by RNA interference also supports a role for *Lmx1a* in the generation of DA neurons in chick embryos (Andersson et al., 2006b). In these experiments, *Lmx1a* knockdown by siRNA electroporation resulted in a loss of DA neurons, which was not compensated for by unperturbed expression of *Lmx1b*. This result was surprising, as studies in mice have demonstrated a role for *Lmx1b* in the development of mDA neurons (Smidt et al., 2000); *Lmx1b* can also partially rescue roof

plate formation in *dreher* mice (Chizhikov and Millen, 2004a). One way to reconcile these results is to propose that the two genes have overlapping roles in mDA development, with *Lmx1a* being perhaps more efficient at the specification step, while *Lmx1b* is required for later differentiation events in the DA lineage. This hypothesis is consistent with the observation that *Lmx1b* is much less efficient than *Lmx1a* at promoting mDA neuron differentiation in ES cells (Andersson et al., 2006b). It is also possible that the requirements for *Lmx1a* and *Lmx1b* genes are different for the two species. Whether *Lmx1a* and *Lmx1b* have unique and/or redundant roles in the development of mDA neurons in mice awaits further studies of *Lmx1a*, *Lmx1a*;*Lmx1b* double mutant embryos and of *Lmx1b* conditional mutants.

### Msx1 and Msx2

The mouse *Msx* genes, *Msx1*, *Msx2* and *Msx3*, encode homeodomain transcription factors that share 98% homology in the homeodomain and function as transcriptional repressors (reviewed by Ramos and Robert, 2005). *Msx1* and *Msx2* are expressed in DA progenitors in the ventral midbrain (Andersson et al., 2006b), in addition to in the roof plate and adjacent cells in the dorsal neural tube and neural crest, as well as in many other sites where epithelial-mesenchymal inductive interactions occur, such as in the limbs and tooth buds, heart, branchial arches and in craniofacial processes. *Msx3*, by contrast, is expressed exclusively in the dorsal aspect of the neural tube in the mouse, caudal to the mid-hindbrain boundary (Shimeld et al., 1996; Wang et al., 1996).

*Msx1*<sup>-/-</sup> embryos exhibit a 40% reduction in the normal number of mDA neurons, probably as a result of the downregulation of *Ngn2* expression (Andersson et al., 2006b). This partial reduction of mDA neurons in *Msx1* mutants may be due to compensation by *Msx2*, a possibility that remains to be addressed by the analysis of *Msx1*;*Msx2* double mutants. In addition, *Msx1* is able, and is required, to repress *Nkx6.1* expression in ventral midbrain progenitors (Andersson et al., 2006b) (Fig. 3). Premature expression of *Msx1* in the midbrain in transgenic mice also leads to the precocious expression of *Ngn2* and *Nurr1*, and to the downregulation of *Shh* in the floor plate, indicating that *Msx1* sets the timing of mDA neuron generation possibly by inducing *Ngn2* expression in ventral midbrain progenitors (Andersson et al., 2006b). Given that *Msx* genes normally function as repressors, *Msx1* may regulate the activity of a repressor of *Ngn2* in mDA progenitors.

### Ngn2 and Mash1

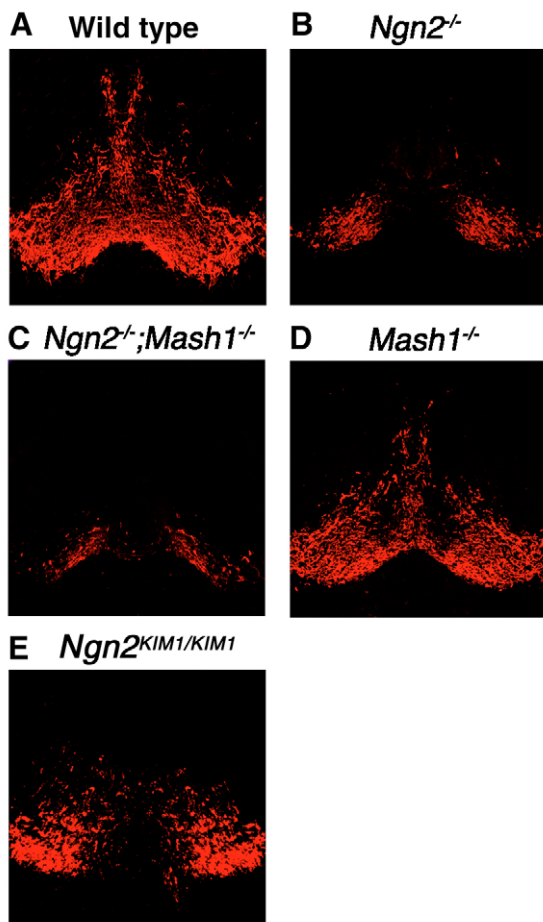
Proneural basic helix-loop-helix genes are crucial regulators of neurogenesis and of subtype specification in many areas of the nervous system. In the ventral midbrain, the proneural genes *Mash1*, *Ngn2* and *Ngn1* show an intricate pattern of expression. *Ngn2* and *Mash1* are expressed in mDA progenitors, whereas *Ngn1*, *Ngn2* and *Mash1* are co-localized in the ventricular zone more dorsally (Kele et al., 2006). *Ngn2* is also expressed in newly born postmitotic immature mDA neurons immediately adjacent to the ventricular zone at the ventral midline. *Ngn2* is required for the generation of *Nurr1*<sup>+</sup> immature mDA neurons, and probably also for their subsequent differentiation into Th<sup>+</sup> mature mDA neurons (Andersson et al., 2006a; Kele et al., 2006). Although *Mash1* by itself is not required for mDA neuron development, the loss of both *Mash1* and *Ngn2* in *Mash1*;*Ngn2* double mutant mouse embryos leads to a greater loss of mDA neurons than occurs in *Ngn2* single mutants (Fig. 4), suggesting that *Mash1* can partially compensate for the loss of *Ngn2* function in mDA progenitors. Accordingly, this

results in a further rescue of Th<sup>+</sup> neurons (Fig. 4) in *Ngn2<sup>KIMash1/Mash1</sup>* embryos that express *Mash1* under the control of the *Ngn2* promoter (Kele et al., 2006).

*Ngn2* has a role in regulating generic neuronal, as well as subtype-specific, differentiation programs in other parts of the CNS (reviewed by Bertrand et al., 2002). The reduced number of Th<sup>+</sup> neurons in *Ngn2* mutants is due to a failure in neuronal production, as suggested by the loss of  $\beta$ III-tubulin<sup>+</sup> neurons, which lie directly beneath mDA progenitors (Kele et al., 2006). Moreover, the loss of two markers of proneural activity, *Dll1*, a Notch ligand, and *Hes5*, an effector of Notch signalling, in the ventral midbrain is consistent with a role for *Ngn2* in regulating the generic aspect of neurogenesis (Kele et al., 2006). In other parts of the CNS, the role of *Ngn2* in subtype specification has been demonstrated by the inability of other classes of proneural genes to compensate for *Ngn2* activity (reviewed by Bertrand et al., 2002). *Mash1* is able to compensate partially for *Ngn2* function, as 60% of the normal number of mDA neurons are generated in *Ngn2<sup>KIMash1/KIMash1</sup>* embryos. This partial

compensation suggests some unique role for *Ngn2* in specification of the mDA neuronal subtype. In addition, the expression of *Ngn2*, but not *Mash1*, in postmitotic DA neurons is consistent with an additional and unique role for *Ngn2* in regulating later differentiation steps in immature mDA neurons. Complementary gain-of-function studies performed by electroporating *Ngn2* in the dorsal midbrain of mouse embryos have demonstrated a role for *Ngn2* in promoting the migration of newborn neurons from the ventricular zone to the mantle zone and in inducing expression of the general neuronal marker  $\beta$ III-tubulin. However, these studies show that *Ngn2* alone is insufficient to promote the ectopic expression of DA neuron markers and the generation of ectopic DA neurons (Kele et al., 2006). This finding is not surprising because *Ngn2* is known to function in other parts of the CNS in a context-dependent manner, working cooperatively with other transcription factors (Bertrand et al., 2002). Further insights into the function of *Ngn2* in mDA neuron development will come from the identification of its transcriptional targets and cofactors in both mDA progenitors and immature mDA neurons.

In summary, the studies described in this section have identified new transcription factors that not only better define mDA progenitors and neurons, but also regulate their specification and differentiation. In light of recent studies on the development of other DA groups in the CNS, a brief comparison of the genetic programs that govern the development of DA neurons is discussed below.



**Fig. 4. *Ngn2* is required for Th<sup>+</sup> DA neuron generation, and *Mash1* partially compensates for *Ngn2* function in the ventral midbrain.** Coronal sections showing the expression of Th in DA neurons in the ventral midbrain of (A) wild-type and (B–D) mutant E14.5 mouse embryos. A severe reduction in Th<sup>+</sup> DA neuron number in (B) *Ngn2<sup>-/-</sup>* and in (C) *Ngn2<sup>-/-</sup>;Mash1<sup>-/-</sup>* mutant embryos is observed, which is not evident in (D) *Mash1<sup>-/-</sup>* embryos, which have wild-type numbers of Th<sup>+</sup> DA neurons. (E) Partial rescue in the number of Th<sup>+</sup> DA neurons in *Ngn2<sup>KIMash1/KIMash1</sup>* (*Ngn2<sup>KIM1/KIM1</sup>*) embryos. Modified, with permission, from Kele et al. (Kele et al., 2006).

### DA neuron development via distinct transcription factor networks

Besides the neurons in the A8 (retrosubstantia nigra), A9 (SNpc) and A10 (VTA) areas of the midbrain discussed so far, some catecholaminergic neurons in the forebrain are also dopaminergic. They are found in areas A11 to A15 (diencephalic and hypothalamic groups), A16 (periglomerular cells in the olfactory bulb), and A17 (interplexiform cells in the retina; see Fig. 1). Very little is known about the specification of the DA fate in these groups, except for the A13 and A16 groups. The A13 DA neurons reside in the alar plate of neuromere segment p3. Recent studies demonstrate that A13 progenitors express homeodomain transcription factors *Dlx1/2* and *Pax6*, and differentiate into *Pax6<sup>+</sup>/Islet<sup>+</sup>/Th<sup>+</sup>* neurons (Andrews et al., 2003; Mastick and Andrews, 2001). In *Dlx1/Dlx2* double mutant mice, neurons generated by A13 progenitors fail to express *Pax6*, *Islet1* and *Th*. These results thus identify a role for *Dlx1/2* in the specification of A13 DA neurons. *Pax6*, however, is not required for A13 progenitors to differentiate into Th<sup>+</sup> neurons. By contrast, *Pax6* is a molecular determinant of A16 progenitors that is required for the neurogenesis and subtype determination of olfactory bulb periglomerular A16 DA neurons. These programs also differ from the one that promotes mDA differentiation, indicating that distinct programs are involved in specifying DA differentiation at different CNS positions. In support of this hypothesis, lateral tuberal hypothalamic DA neurons in the forebrain of chick embryos express *Nkx2.1* and *Msx1* homeodomain proteins, and have been shown to depend on the expression of the homeodomain transcription factor *Six3* in progenitors for their development (Ohyama et al., 2005). By contrast, *Six3* is not expressed in A13 and mDA progenitors.

The distinct differentiation programs for CNS DA neurons highlight the importance of identifying a DA neuron by its developmental history in addition to its neurotransmitter phenotype. Crucial parameters affecting the success of a transplantation therapy for the treatment of Parkinson's disease include good graft

integration and functional reinnervation (reviewed by Bjorklund and Isacson, 2002; Lindvall and Bjorklund, 2004; Snyder and Olanow, 2005). Recent findings indicate that grafted SNpc and VTA DA neurons differ in their axon projection patterns in the DA-denervated forebrain of adult mice, suggesting that mDA neuronal subtypes display distinct responses to axon guidance cues and target recognition mechanisms regulating reinnervation in the forebrain (Thompson et al., 2005). These and earlier studies (Hudson et al., 1994; Zuddas et al., 1991) indicate that the success of transplantation therapies will be strongly influenced by the type of DA neurons used in these procedures. Therefore, elucidating the molecular determinants that regulate mDA neuron differentiation from CNS progenitor cells in vivo should facilitate the generation of specific populations of DA neurons from stem cells that will be useful for transplantation therapies.

## Conclusions

Although some of the key molecular players required in specifying neural progenitors towards a mDA differentiation program are now known, their precise functions remain to be deciphered. Gain-of-function studies suggest that the major determinants, such as *Otx2*, *Lmx1a/b*, *Msx1/2*, *Ngn2* and *Mash1*, are likely to act in a combinatorial manner to promote the mDA fate, as the ectopic expression of some of these genes individually fails to promote the differentiation of dorsal midbrain progenitors into DA neurons (Kele et al., 2006; Andersson et al., 2006b). Future work will shed light on the specific combinatorial interactions of transcription factors that govern the differentiation of midbrain progenitors into mDA neurons, and will also lead to the discovery of downstream components of these regulatory networks. Besides these transcription factors, members of the forkhead/winged helix transcription factor family, *Foxa1* and *Foxa2*, are also expressed in mDA progenitors (Puelles et al., 2003), and their role in the development of mDA neurons is currently being investigated in our laboratory. In addition, the molecules responsible for the generation of SNpc versus VTA DA neurons remain unknown. SNpc and VTA DA neurons settle in different positions along the mediolateral axis of the midbrain and have distinct axon projection patterns. A major challenge for the future is to discover the molecules that are responsible for these distinct migratory and axon growth behaviours. The ability to generate DA neurons from neural progenitor/stem cells of the SNpc subtype and to purify them based on a specific set of molecular markers will lead to significant progress in stem cell-based therapies for Parkinson's disease.

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