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A Wnt Signal Regulates Stem Cell Fate and Differentiation in vivo

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Abstract
Our knowledge about the normal generation of midbrain dopaminergic neurons in vivo is still rudimentary, despite many attempts to recapitulate the underlying events in vitro. Because the loss of these neurons is implicated in Parkinson's disease, this lack of information is one of the major drawbacks in the development of better therapies for this severe human neurological disorder. Recently, substantial advances have been made by demonstrating that the secreted molecule Wnt1 regulates a genetic network, including the transcription factors Otx2 and Nkx2-2, for the initial establishment of the dopaminergic progenitor domain in the mammalian ventral midbrain. In addition, Wnt1 appears to regulate the differentiation of the postmitotic progeny of these precursors by initiating the expression of midbrain dopaminergic-specific transcription factors. A genetic cascade controlled by the secreted molecule Sonic hedgehog, including the transcription factors Lmx1a, Msx1 and Nkx6-1, acts in parallel with the Wnt1-regulated network to establish the midbrain dopaminergic progenitor domain. The Sonic-hedgehog-controlled cascade may diverge from the Wnt1-regulated network at later stages of neural development through induction of proneural transcription factors required for the acquisition of generic neuronal properties by the midbrain dopaminergic progeny. Here we provide a brief overview of these regulatory gene networks.

Introduction

Neurons that synthesize the neurotransmitter dopamine are found at several locations in the mammalian brain (reviewed by Ang [1] and Prakash and Wurst [2]). The best studied population are the dopaminergic (DA) neurons located in two nuclei of the midbrain tegmentum: the substantia nigra (SN or A9 group) and the ventral tegmental area (or A10 group). Additional neurons are found in the retrorubral field (or A8 group). The mammalian midbrain dopaminergic (mDA) population plays a crucial role in several brain and body functions and behaviors. The mDA neurons have been in the focus of clinical interest for a long time because of their involvement in severe human neurological and psychiatric illnesses. Thus, the development of better treatments for these disorders has promoted their investigation. One potential therapy for Parkinson's disease, for example, would be the replenishment of the degenerating DA neurons in the human SN by healthy DA neurons that have

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been generated either in vitro or in vivo through the directed differentiation of stem cells [3]. To accomplish this, however, a full understanding of the genetic cues controlling the differentiation of a pluripotent, uncommitted neuroepithelial stem cell into a mature mDA neuron is required.

Generation of mDA Neurons Is Controlled by Two Signaling Centers in the Mouse Embryo

The embryonic mouse neural tube is patterned along its rostrocaudal and its dorsoventral axis by different signaling centers or secondary organizers (reviewed by Echevarria et al. [4]). The development of the mDA neuronal population is controlled by two of these signaling centers: the mid-/hindbrain boundary (MHB) or isthmic organizer acting along the rostrocaudal axis and the ventral midline of the neural tube or floor plate (FP) acting along its dorsoventral axis (reviewed by Prakash and Wurst [2, 5]). The secreted molecules Wnt1 and fibroblast growth factor (Fgf) 8 are expressed in the caudal midbrain or rostral hindbrain at the MHB, respectively, and the FP secretes the lipid-modified glycoprotein Sonic hedgehog (Shh). The mDA neurons arise at around day 10.5 of mouse embryonic development (E10.5) from the ventral midline [FP and basal plate (BP)] of the cephalic flexure close to the MHB (reviewed by Prakash and Wurst [2]), suggesting that these two signaling centers must play an important role in their generation. Indeed, we have shown that the position of the MHB during embryonic development determines the number and anteroposterior location of the mDA population and of the rostral hindbrain serotonergic (rh-5-HT) neurons, which are specified in the rostral hindbrain adjacent to the MHB [6]. Fgf8, secreted from the rostral hindbrain at the MHB, and Shh, secreted from the midbrain FP and BP, had previously been demonstrated to control the generation of ectopic mDA neurons in explant cultures or in vivo [7, 8]. More recent findings, however, have further refined our knowledge about the function of these two signaling centers, and of the molecules involved, in the generation of mDA neurons. These findings will be summarized in this review.

Wnt1 Secreted from the Ventral Midbrain Controls mDA Stem Cell Fate and Differentiation

Wnt1 expression is confined to a ring encircling the neural tube at the rostral border of the MHB in the caudal midbrain, the roof plate (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 first appear [9]. 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 in vivo [9]. The first hint came from the analysis of the $En1^{+/Wnt1}$ knock-in mouse, where *Wnt1* is expressed ectopically in the rostral hindbrain. Surprisingly, ectopic mDA progenitors and neurons develop in the rostral hindbrain FP of the $En1^{+/Wnt1}$ mutant [9]. These ectopic mDA neurons persist throughout development into adulthood [9]. The rostral hindbrain BP of the $En1^{+/Wnt1}$ mutants, however, normally generates the rh-5-HT neurons. The ectopic induction of mDA neurons coincided with an ectopic induction of *Otx2* in the FP and the repression of the most ventral *Nkx2-2* domain in the BP of the rostral hindbrain in $En1^{+/Wnt1}$ mutants [9]. These results already suggested that a regulatory network including *Wnt1*, *Otx2* and *Nkx2-2* is required for mDA neuron induction and specification (fig. 1). Indeed, the loss of *Otx2* in the FP and BP of the midbrain of conditional $En1^{+/Cre}$; $Otx2^{flox/flox}$ 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. At the same time, the *Wnt1* expression domain in the ventral midbrain is lost, and ectopic rh-5-HT neurons are generated instead of mDA neurons in this region of the $En1^{+/Cre}$; $Otx2^{flox/flox}$ mutant [9, 10]. 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 [9]. In addition to this 'early' patterning activity, Wnt1 is also required at later stages of neural development for the proper differentiation of mDA neurons in the mouse embryo. In $Wnt1^{-/-}$ knock-out mice, only few mDA precursors positive for tyrosine hydroxylase (Th), the rate-limiting enzyme in DA biosynthesis, are generated, and these cells fail to express the homeodomain (HD) transcription factor Pitx3 [9]. Furthermore, ectopic mDA neurons cannot be induced by Shh and Fgf8 in the absence of *Wnt1* in mouse embryo explant cultures [9]. In support of our findings, fate mapping of ventral *Wnt1*-expressing cells during mouse embryonic development revealed that a great extent of these cells will generate Th-expressing mDA neurons [11]. Previous data obtained

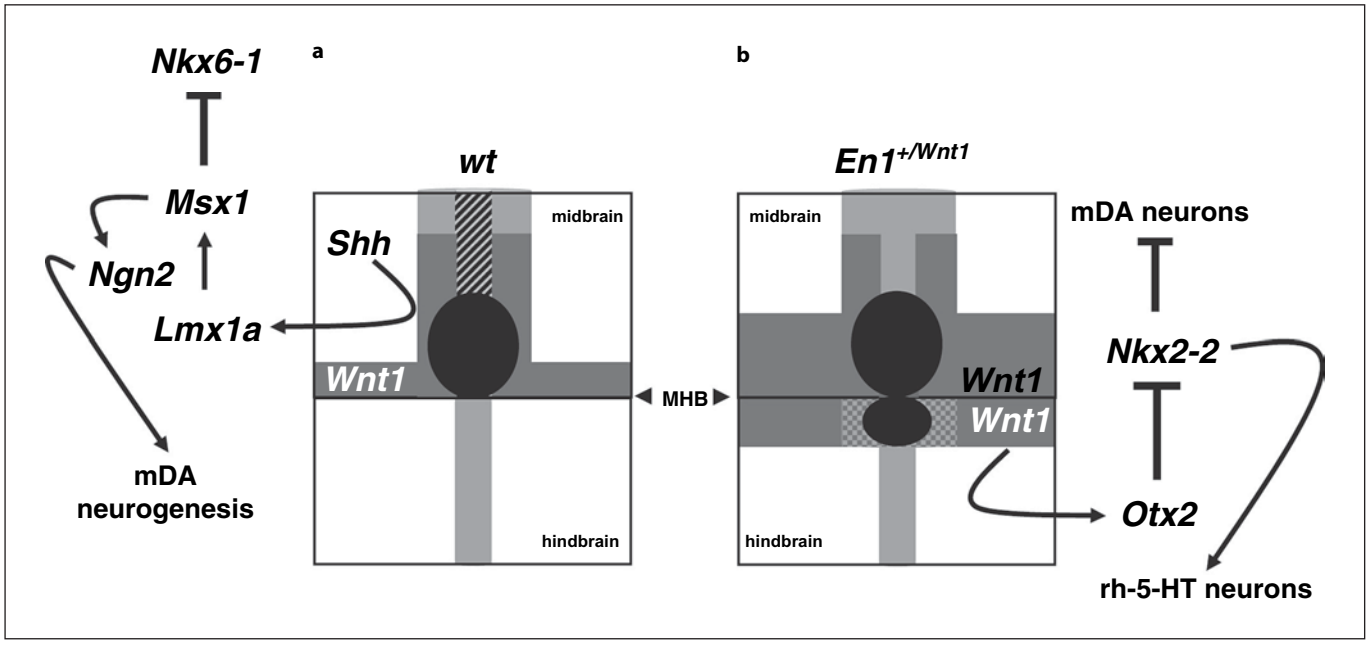

Fig. 1. A Wnt1- and a Shh-regulated genetic network act in parallel during the development of dopaminergic neurons in the ventral midbrain of the mouse embryo. **a** Top view on the ventral boundary between the midbrain and rostral hindbrain (the MHB) of an E10.5 wild-type *(wt)* mouse embryo depicting the region where mDA neurons (black) arise. *Wnt1* (dark grey) is expressed in a ring encircling the neural tube at the MHB and in two stripes adjacent to the *Shh*-positive FP (light grey). Induction of *Lmx1a* (hatched) within the midbrain FP at earlier stages requires Shh or a Shh-derived signal. Subsequently, *Msx1* (hatched) is induced by Lmx1a in the midbrain FP and represses *Nkx6-1* to establish the mDA progenitor domain in the ventral midline of the midbrain. The expression of the proneural factor *Ngn2* in this domain is then initiated by Msx1. Ngn2 is required for the acquisition of generic neuronal properties by the mDA neurons. **b** Top view on the ventral boundary between the midbrain and rostral hindbrain (the MHB) of an E10.5 $En1^{+/Wnt1}$ mouse embryo depicting the region where *Wnt1* (dark grey) is ectopically expressed and where ectopic mDA neurons (black) arise in the mutant. *Otx2* (chequered) is ectopically induced by Wnt1 in the *Shh*-positive FP of the mutant rostral hindbrain (light grey), which appears to be slightly broadened. Otx2 in turn represses the ventral *Nkx2-2* domain in the rostral hindbrain, thereby allowing the generation of ectopic mDA neurons in this region. Wnt1 may additionally control the acquisition of mDA-specific properties (not shown). Nkx2-2 is required for the normal generation of 5-HT neurons in the rostral hindbrain.

from in vitro experiments already suggested an important function of Wnt proteins in the generation of differentiated mDA neurons from cultured mDA precursors [12]. In these studies, however, it was proposed 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 [12]. Our own analysis of the $Wnt5a^{-/-}$ mutant mouse, however, does not confirm these findings in vivo [Minina et al., unpublished data]. The Wnt1-mediated signal transduction pathway (including receptors and intracellular effectors) regulating the generation of mDA neurons is unclear at present. Notably, the ectopic induction of mDA neurons in the $En1^{+/Wnt1}$ mutants occurred only within the rostral hindbrain FP and not in the dorsal neural tube, indicating that another signal from the FP must be present for mDA neuron induction.

Shh Secreted from the Midbrain FP Controls mDA Stem Cell Fate

As previously mentioned, Shh secreted from the ventral midbrain FP and BP together with exogenously applied Fgf8 are necessary and sufficient for the generation of ectopic mDA neurons in rat embryo explant cultures [8]. It was already shown before that ectopic expression of Shh and of its downstream effector molecule Gli1 in the

dorsal neural tube are able to induce ectopic mDA and 5-HT neurons in the dorsal mid-/hindbrain region, in areas where Wnt1 and Fgf8 are expressed normally [7]. More recent data have revealed that mDA neurons are not generated in *Shh*$^{-/-}$ null mutants and are considerably reduced by conditionally inactivating *Smoothened* (the essential positive mediator of Shh signaling) in the mouse embryo at E9.0 but remain unaffected when *Smoothened* is inactivated at later embryonic stages [13]. Therefore, Shh is necessary for induction of the mDA neuronal fate in ventral midbrain progenitors but appears to be dispensable for their later differentiation into mDA neurons [13].

In addition to Shh, transforming growth factor (Tgf) β, which is another factor secreted from the ventral midbrain [14], appears to be required for the normal development of mDA neurons in mice [15]. In the absence of the two Tgf-β isoforms normally present in the ventral midbrain, Tgf-β2 and Tgf-β3, Th-positive mDA neurons are reduced by more than 30% compared to their wild-type numbers at midgestational stages [15].

Each of the aforementioned secreted factors can elicit different signaling cascades within the receiving cell, depending on the type and complement of transducing molecules (effectors) it will find inside the cell. The details of these signaling pathways involved in mDA neuron development are still totally unknown. However, target genes of these pathways are being unraveled, and it turns out that many of them are transcription factors activating and/or repressing a genetic program that leads to the specification of the mDA neuronal phenotype in the receiving cell.

Wnt1 May Activate Transcription Factors Required for mDA Neuron Differentiation

Our analysis of the *Wnt1* mutant mice indicated that expression of the paired-like HD protein Pitx3 is not initiated in the absence of Wnt1. Pitx3 is required for the proper differentiation of a subset of mDA neurons, possibly by directly regulating *Th* expression in these cells [16]. In the absence of *Pitx3*, the mDA neurons of the SN and around 50% of the ventral tegmental area neurons are lost during development [2, 16]. Transcription of the *Pitx3* gene was previously suggested to be regulated by the LIM-HD factor *Lmx1b* [17]. *Lmx1b* is expressed in the ventral midbrain and later confined to the postmitotic mDA progeny. In the absence of *Lmx1b*, Th-positive mDA precursors fail to initiate expression of Pitx3 and later disappear [17]. Notably, Lmx1b can induce ectopic expression of *Wnt1* [18], and *Pitx3* may therefore still be downstream of Wnt1 signaling.

Shh Signaling Targets Are Transcription Factors Required for mDA Neuron Development

More recently, a major breakthrough was achieved in identifying the possible targets of Shh signaling in mDA neuron development [19]. Both Lmx1a, like Lmx1b a LIM-HD family member, and Msx1, another HD protein, were identified in a screen for HD transcription factors expressed in mDA progenitors [19]. *Lmx1a* is transcribed in the ventral midbrain in a spatiotemporal pattern correlating with the onset of mDA neurogenesis, and initiation of ventral Lmx1a expression appeared to be Shh-dependent [19]. Notably, ectopic expression of *Lmx1a* in the chicken neural tube or overexpression of *Lmx1a* in mouse embryonic stem cells was sufficient to induce the mDA neuronal phenotype, although this was only possible in the chicken ventral midbrain or in the presence of Shh in embryonic stem cells [19]. Lmx1a also appeared to be necessary for the normal generation of mDA neurons, as RNA interference experiments in the ventral midbrain of chicken embryos resulted in a drastic reduction of cells positive for Nr4a2/Nurr1 (a marker for mDA progenitors/precursors) in this region [19]. A similar requirement of Lmx1a in the mouse has so far not been reported. Lmx1a appeared to exert its effects in part through the activation of another homeobox gene, *Msx1* [19]. Expression of Msx1 initiates somewhat later than Lmx1a and is confined to proliferating progenitors in the ventricular/subventricular zone (VZ/SVZ) of the ventral midline of the midbrain [19]. *Msx1* was not sufficient to induce mDA neurons after ectopic expression in the chick midbrain and does not appear to be essential for their generation in mice, as the numbers of mDA precursors and neurons were reduced only by 40% compared to their wild-type numbers in *Msx1* null mutants. Msx1, however, seems to be required for the repression of yet another HD gene, *Nkx6-1*, which is broadly expressed in the VZ/SVZ of the ventral neural tube [19]. Repression of the most ventral *Nkx6-1* expression domain in the midbrain by Msx1 may therefore delimit the mDA progenitor domain from the more laterally located progenitors of motorneurons [10, 19]. In addition to this repressive effect, Msx1 directly or indirectly induced the expression of the proneural gene *neurogenin 2* (*Ngn2*, see below) in the mesencephalic ventral midline of transgenic mice [19]. Taken together, the

Shh-signaling cascade in mDA neuron development appears to include the activation of the transcription factors Lmx1a and Msx1 (fig. 1). Msx1 in turn represses the expression of *Nkx6-1* in the midbrain FP and directly or indirectly induces transcription of *Ngn2* in this region. Lmx1a seems to also activate other, still unknown genes necessary for mDA neuron generation, as Msx1 was unable to induce these cells at ectopic locations. It should be noted that, similar to our findings in the *Wnt1* mutants, neither transcription factor was able to exert its effects in the absence of Shh signaling, suggesting that additional signals or factors must be present in order to generate mature mDA neurons.

mDA Neurogenesis Requires Proneural Genes

The proneural factor Ngn2 belongs to the family of basic helix-loop-helix transcriptional regulators and confers mostly generic neuronal but also subtype-specific properties to differentiating neuroepithelial cells (reviewed by Bertrand et al. [20]). Ngn2 is expressed mostly in the VZ/SVZ of the ventral midbrain and in very few postmitotic Nr4a2/Nurr1-positive cells [21, 22]. In the absence of *Ngn2*, the mDA neuronal population was initially reduced to less than 20% and recovered postnatally to only 50–60% of the normal (wild-type) numbers [21, 22]. 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 resulted in a further decrease in mDA neuron numbers, and the *Ngn2*$^{-/-}$ mutant phenotype was partially rescued by overexpression of *Mash1* in *Ngn2*$^{Mash1/Mash1}$ knock-in mice [22]. The remaining mDA neurons, however, differentiated normally into the SN and ventral tegmental area subpopulations and established proper connections with their target fields in the forebrain in the absence of *Ngn2* [21]. Overexpression of *Ngn2* in the dorsal midbrain or in midbrain-derived cell cultures did not promote the generation of mDA neurons, although it enhanced overall neurogenesis [21, 22], indicating that Ngn2 cannot specify an mDA neuronal fate in neural precursors. Since an accumulation of radial glia-like precursor cells in the VZ/SVZ and a notorious lack of neuronal cell bodies were apparent in the ventral midline of the *Ngn2*$^{-/-}$ mutant midbrain, it was suggested that Ngn2 is required for normal neurogenesis in the ventral midbrain [21, 22]. Neighboring cell populations such as the ventral mesencephalic motorneurons, however, were not affected in the *Ngn2*$^{-/-}$ mutants [21]. Therefore, Ngn2 appears to be required specifically by the mDA precursors for the acquisition of generic neuronal properties but not for other aspects of their terminal differentiation.

Conclusions

Many details of the genetic networks controlling the early and intermediate steps of mDA neuron generation, including the establishment of the mDA progenitor domain and subsequent specification of the mDA neuronal fate in the offspring, have recently been revealed in several studies [9, 19, 21, 22]. Two networks appear to act in parallel during this process (fig. 1). (1) A Wnt1-regulated cascade is required for the maintenance of *Otx2* expression in the ventral midbrain, which in turn represses *Nkx2-2* in this domain. Failure to establish this transcriptional code results in a fate switch of mDA progenitors into other identities such as rh-5-HT neurons. Once the mDA progenitor domain is established, a Wnt1-mediated signal may directly or indirectly initiate or maintain the expression of the homeobox genes *Pitx3* and *En1/2* in the progeny. This signaling cascade acts together with the Nr4a2/Nurr1-regulated pathway conferring DA neurotransmitter identity (reviewed by Prakash and Wurst [2, 5]), thereby specifying the mDA phenotype in mDA precursors at intermediate stages of neural development. (2) A Shh-controlled network is required for the induction of Lmx1a and subsequently of Msx1 in the mesencephalic ventral midline. Msx1 in turn represses *Nkx6-1* expression in this region, thereby allowing the establishment of the mDA progenitor domain. At later stages, an Lmx1a/Msx1-regulated network probably acts independently of the Wnt1/Nr4a2/Nurr1-controlled genetic cascade to confer both generic neuronal (through activation of the proneural gene *Ngn2*) and mDA cell-type-specific (through activation of as yet unknown target genes) properties to the progeny. Nevertheless, neither the Wnt1-controlled cascade nor the Lmx1a-regulated network were able to induce ectopic mDA neurons in the dorsal neural tube, in contrast to previous results after overexpression of Shh or its effector Gli1 in this region of the neural tube [7]. This suggests that we are still far from knowing all signals and factors involved in the early steps of mDA neuron development. Many questions still remain open, and elucidation of their answers is currently the most important task in the field.

Acknowledgments

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