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Review

Wnt1-regulated genetic networks in midbrain dopaminergic neuron development

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Neurons synthesizing the neurotransmitter dopamine exert crucial functions in the mammalian brain. The biggest and most important population of dopamine-synthesizing neurons is located in the mammalian ventral midbrain (VM), and controls and modulates the execution of motor, cognitive, affective, motivational, and rewarding behaviours. Degeneration of these neurons leads to motor deficits that are characteristic of Parkinson's disease, while their dysfunction is involved in the pathogenesis of psychiatric disorders including schizophrenia and addiction. Because the aetiology and therapeutic prospects for these diseases include neurodevelopmental aspects, substantial scientific interest has been focused on deciphering the mechanistic pathways that control the generation and survival of these neurons during embryonic development. Researches during the last decade revealed the pivotal role of the secreted Wnt1 ligand and its signalling cascade in the generation of the dopamine-synthesizing neurons in the mammalian VM. Here, we summarize the initial and more recent findings that have unravelled several Wnt1-controlled genetic networks required for the proliferation and commitment of VM progenitors to the dopaminergic cell fate during midgestational embryonic stages, and for the correct differentiation of these progenitors into postmitotic dopamine-synthesizing neurons at late midgestational embryonic and foetal stages.

Keywords: Wnt1, dopamine, neuron, ventral midbrain, mouse

Introduction

Dopamine (DA)-synthesizing neurons arise in different regions of the mammalian forebrain and midbrain. However, the biggest population of dopaminergic (DA) neurons is found in two nuclei, the Substantia nigra pars compacta (SNc) or A9 group and the ventral tegmental area (VTA) or A10 group, and in the retrorubral field (RrF or A8 group) located in the ventral midbrain (VM) (Bjorklund and Dunnett, 2007). The SNc DA neurons are involved in the control and modulation of voluntary movements and habit learning; their loss in the human brain therefore leads to the typical motor symptoms of Parkinson's disease (PD), including the inability to initiate voluntary movements (akinesia or hypokinesia) and a general body stiffness (rigidity) (Dauer and Przedborski, 2003). The VTA DA neurons, by contrast, have been mostly implicated in the modulation of cognitive, motivational/rewarding, and emotional/affective behaviours; their improper function underlies several psychiatric disorders such as schizophrenia, addiction, attention deficit/hyperactivity disorders, and depression (Nieoullon, 2002; Hyman et al., 2006; Howes and Kapur, 2009). Therefore, the SNc and VTA DA neurons have received substantial clinical attention since their discovery. In particular, the prospects of regenerative treatments for neurodegenerative disorders such as PD have fuelled basic research aimed at understanding their generation, survival, and function in the mammalian brain.

It is meanwhile widely accepted that the SNc and VTA DA neurons arise from a progenitor population located in the ventral midline, the so-called floor plate (FP), of the midbrain (mesencephalon) and caudal forebrain (diencephalon) of the developing mammalian embryo (Ono et al., 2007; Bonilla et al., 2008; Joksimovic et al., 2009; Blaess et al., 2011). This progenitor population and their postmitotic progeny have therefore been subsumed under the name 'mesodiencephalic' dopaminergic (mdDA) progenitors/neurons (Smidt and Burbach, 2007), before they segregate into the distinctive SNc and VTA subpopulations around birth. The caudal limit of the mdDA neuronal population is defined by the boundary between the midbrain and the hindbrain, the so-called mid-/hindbrain boundary (MHB), where one of the most important secondary organizing centres of the mammalian embryo, the isthmic organizer (IsO), is located (Wurst and Bally-Cuif, 2001; Vieira et al., 2010). One of the earliest discoveries that laid the fundaments for the mdDA neurodevelopmental research field was the finding that two soluble factors, Sonic hedgehog (Shh) and Fibroblast growth factor 8 (Fgf8), secreted from the FP and caudal MHB, respectively, and their signalling pathways are both necessary and sufficient for the generation of mdDA neurons in the rodent brain (Hynes et al., 1995a, b, 1997; Ye et al., 1998).

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However, the pivotal importance of another secreted factor in this context, namely Wingless-related MMTV integration site 1 (Wnt1), was only recognized almost a decade later (Castelo-Branco et al., 2003; Prakash et al., 2006). This is even more surprising in view of the fact that *Wnt1* exhibits a very specific expression within the mdDA domain of the developing murine VM (Figure 1) (Parr et al., 1993; Brown et al., 2011). In the meantime, a considerable number of reports were published emphasizing the importance of Wnt1 in the development of mdDA neurons *in vivo* and *in vitro*, but also in their survival and neuroprotection in the adult brain. Here, we review the earliest to latest findings regarding the role of this secreted factor in mdDA neuron development, including its control of meanwhile well-established genetic networks that are required for the proper generation and survival of the mdDA neuronal population in the mammalian VM.

mdDA neurons are derived from Wnt1-expressing progenitors/precursors in the mouse VM

Wnt1 transcription in the midgestational mouse embryo encompasses a ring-like domain in the caudal midbrain at the anterior (rostral) border of the MHB, the dorsal midline of the diencephalon, mesencephalon, caudal hindbrain (rhombencephalon), and spinal cord, and two parallel and wedge-shaped stripes that spare the medial FP along the VM and converge in the midline (the medial FP) of the caudal diencephalon (Parr et al., 1993; Brown et al., 2011). This unique expression pattern in the murine VM already suggested some functional implication of Wnt1 in the development of neuronal populations arising from this or nearby locations (Figure 1). Wnt1 is a hydrophobic, lipid-modified glycoprotein that is secreted from the cell surface in a highly regulated manner (Mikels and Nusse, 2006). It is therefore still unclear whether and how the secreted Wnt1 diffuses or is actively transported over longer distances in the mammalian VM and how far it can actually reach in this context (Mikels and Nusse, 2006), making it difficult to determine the extent and the identity of the VM cells that may receive a Wnt1 signal during embryonic development. Hence, a safe attempt is to determine the fate of Wnt1-expressing cells in the VM of the developing mouse embryo. Initial data indicated that at least a subset of the mdDA neurons is derived from progenitor cells expressing Wnt1 in the VM and at the MHB (Zervas et al., 2004). Using a transgenic mouse line expressing a fluorescent reporter protein under the control of *Wnt1* regulatory sequences and a so-called genetic inducible fate-mapping approach, a subsequent and more detailed analysis by the same group revealed that Wnt1-expressing progenitors in the murine VM give rise to the three mdDA neuronal groups (SNc, VTA, and RrF) throughout development (Brown et al., 2011). Interestingly, the Wnt1-derived progeny contributes predominantly to the mdDA neurons and to a clearly lesser and time-restricted extent to other neuronal populations found in the mammalian VM (Brown et al., 2011). Altogether, the initial and more recent findings confirm that postmitotic mdDA neurons belonging to all three mdDA neuronal subsets (SNc, VTA, and RrF) are indeed derived from Wnt1-expressing neural progenitors located in the murine VM.

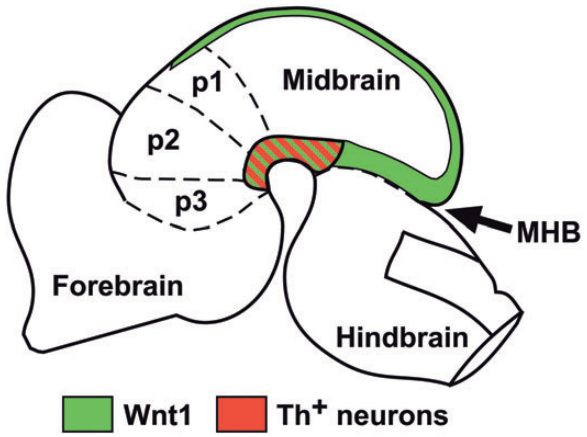

Figure 1 Expression of *Wnt1* in relation to the mdDA neuron domain in the mouse embryo. Schematic longitudinal (sagittal) overview of the neural tube in a late midgestational mouse embryo (embryonic day (E)10.5 to E12.5), depicting the region where *Wnt1* (green) is transcribed and where tyrosine hydroxylase (Th)-expressing mdDA neurons (red) arise. The $Wnt1^{+}$ domain encompasses a ring-like territory in the caudal midbrain (anterior border of the MHB), the dorsal midline of the diencephalon and mesencephalon, and a bilateral stripe in the lateral FP of the midbrain that converges in the medial FP of the prosomeres (p)1–3 in the diencephalon. Hatched areas indicate overlapping expression domains. Th^{+} neurons arise from the FP in the midbrain (mesencephalon) and caudal diencephalon (p1–3), and are therefore also called mdDA neurons.

Wnt1 is necessary and sufficient for the generation of mdDA neurons in the mouse

The crucial function of Wnt1 in the generation of mdDA neurons was revealed by a series of gain-of-function (GOF) and loss-of-function (LOF) experiments in the mouse embryo and *in vitro* cultured VM cells (Castelo-Branco et al., 2003; Prakash et al., 2006; Andersson et al., 2013). In transgenic $En1^{+/Wnt1}$ knock-in mice, in which the *Wnt1* cDNA was inserted into the murine Engrailed 1 (*En1*) locus, *Wnt1* expression is expanded across the MHB anteriorly into the caudal midbrain and posteriorly into the rostral hindbrain (Figure 2) (Panhuysen et al., 2004). The ectopic expression of *Wnt1* in the $En1^{+/Wnt1}$ mice results in the ectopic generation of mdDA precursors and neurons exclusively in the rostral hindbrain FP but not in the other *Wnt1*-expressing regions of the transgenic brain (Prakash et al., 2006). The exact reason for this specificity is not known at present, but it might be related to the ectopic induction of the homeodomain (HD) transcription factor (TF) orthodenticle homolog 2 (Otx2) (see below) and the presence of Shh in this region of the transgenic brain (Hynes et al., 1997; Prakash et al., 2006). Conversely, the lack of functional Wnt1 in the brain of $Wnt1^{-/-}$ knock-out (KO), *swaying* mutant, and *Wnt1* conditional KO (cKO) mice leads in nearly all cases (see Figure 2) to a strong reduction of mdDA progenitors and precursors, and later an almost complete loss of mdDA neurons in the mutant VM (Prakash et al., 2006; Ellisor et al., 2012; Andersson et al., 2013; Yang et al., 2013). This loss affects both the SNc and the VTA DA neurons that would normally arise from the midbrain FP, but some ectopically positioned mdDA neurons are found instead in the lateral midbrain (basal plate, BP) adjacent to the FP/mdDA domain of the *Wnt1* mutant (LOF) mice (Figure 2) (Ellisor et al., 2012; Andersson et al., 2013; Yang et al., 2013). The reason for this latter observation

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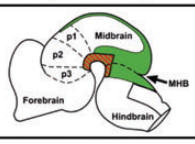
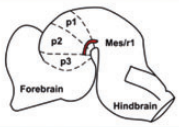
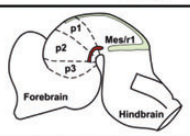
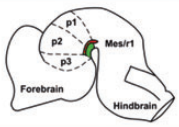
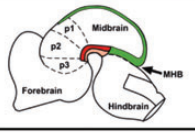
Wnt1 mouse mutant	Schematic *Wnt1* expression (E10.5–12.5)	*Wnt1* mRNA expression	mdDA phenotype	Reference(s)
***En1*$^{+/Wnt1}$** knock-in		Ectopic *Wnt1* expression in the *En1*$^{+}$ domain including the entire caudal midbrain and rostral hindbrain; *Wnt1* overexpression at the MHB and in the lateral FP of the caudal midbrain	Ectopic induction of Th^{+} mdDA neurons in the rostral hindbrain FP of transgenic mice	Panhuysen et al. (2004) and Prakash et al. (2006)
***Wnt1*$^{-/-}$** knock-out (KO)		Complete lack of *Wnt1* expression in the developing mouse embryo	Complete absence of Th^{+} mdDA neurons in the midbrain FP; ectopic Th^{+} neurons in the BP (lateral midbrain)	Prakash et al. (2006) and Andersson et al. (2013)
Swaying (*sw*, spontaneous *Wnt1* point mutation)		Residual *Wnt1* mRNA expression in the dorsal midline and MHB	Strong reduction of Th^{+} mdDA neurons, particularly in the VTA region	Thomas et al. (1991) and Ellisor et al. (2012)
***En1*$^{+/Cre}$;*Wnt1*$^{\Delta MHB/\Delta MHB}$** conditional knock-out (cKO)		Conditional ablation of *Wnt1* expression in the *En1*$^{+}$ domain at ~E9.0–9.5, including the dorsal midline, MHB and mesencephalic FP; residual *Wnt1* expression in the diencephalic FP	Severe depletion of Th^{+} mdDA neurons, only few remaining cells	Yang et al. (2013)
***Shh*$^{+/Cre:GFP}$;*Wnt1*$^{\Delta vMes/\Delta vMes}$** cKO		Conditional ablation of *Wnt1* expression in the *Shh*$^{+}$ domain at ~E9.0–9.5, i.e. in the mesodiencephalic FP	Strong reduction of medial Th^{+} mdDA neurons, expansion of lateral Th^{+} mdDA neurons	Yang et al. (2013)
***Wnt1-CreER*; *Wnt1*$^{\Delta MHB/\Delta MHB}$** cKO	*	Conditional ablation of *Wnt1* expression at E8.5 and E10.5 in the *Wnt1*$^{+}$ domain (dorsal midline, MHB, mesodiencephalic FP)	<u>*Wnt1* ablation at E8.5:</u> Depletion of caudal Th^{+} mdDA neurons, ectopic Th^{+} neurons in lateral domains (variable phenotype!) <u>*Wnt1* ablation at E10.5:</u> no mdDA phenotype	Yang et al. (2013)

Figure 2 Summary of *Wnt1* mRNA expression patterns and mdDA neuron phenotypes in *Wnt1* mutant mice. In the schematics, the *Wnt1*-expressing domain is depicted in green and the Th^{+} mdDA neuron domain is depicted in red; hatched areas indicate overlapping domains. Deletion of the mid-/hindbrain region in some of the mouse mutants is denoted by a missing MHB. The *Wnt1* expression spots in the dorsal rostral hindbrain (rhombomere 1) that were reported in *Wnt1 swaying* (*sw*/*sw*) (Ellisor et al., 2012) and *En1*$^{+/Cre}$;*Wnt1*$^{\Delta MHB/\Delta MHB}$ (Yang et al., 2013) embryos are omitted in the schematics. Asterisk (*): Ectopic expression of *Wnt1* from a *Wnt1-Cre* transgene was recently described in the *Wnt1-Cre* transgenic mouse line (Lewis et al., 2013) that was made using a very similar approach to the one used by Zervas et al. (2004) to generate the *Wnt1-CreER* transgenic mouse line. Yang et al. (2013) reported a variable or absent mdDA phenotype in the *Wnt1-CreER*;*Wnt1*$^{\Delta MHB/\Delta MHB}$ embryos that might in fact be due to an ectopic expression of *Wnt1* in these transgenic mice. Because the pattern of *Wnt1* expression in the *Wnt1-CreER*; *Wnt1*$^{\Delta MHB/\Delta MHB}$ embryos (Yang et al., 2013) is not known, we do not show any schematic overview for this mouse mutant.

remains unclear. Furthermore, mdDA neurons cannot be induced in explant cultures of the anterior neural plate in the absence of *Wnt1* (*Wnt1*$^{-/-}$ explants), even though Shh and Fgf8 are present in these explants (Ye et al., 1998; Prakash et al., 2006). Finally, the treatment of primary cell cultures from the rodent VM with partially purified Wnt1 protein increases the proliferation and subsequent differentiation of VM progenitor cells into DA neurons (Castelo-Branco et al., 2003; Tang et al., 2010). Altogether, these findings indicate that Wnt1 is both necessary and sufficient for the generation of endogenous and ectopic mdDA neurons in the developing mouse brain, independently of Fgf8 and Shh.

In the *En1*$^{+/Wnt1}$ knock-in mice, the induction of ectopic mdDA precursors expressing typical markers for these cells, such as the orphan nuclear receptor subfamily 4, group A, member 2 (Nr4a2, also known as Nurr1) and the retinoic acid (RA)-synthesizing enzyme Aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1, also known as Ahd2 or Raldh1) (Wallen et al., 1999), and their subsequent differentiation into postmitotic mdDA neurons expressing the mdDA-specific HD TF paired-like homeodomain transcription factor 3 (Pitx3) (Smidt et al., 1997) and the rate-limiting enzyme for DA synthesis, Tyrosine hydroxylase (Th), is delayed by ~1 day in the rostral hindbrain when compared with their endogenous counterparts in the VM (Prakash et al., 2006). This observation strongly suggested that a genetic network downstream of the ectopic Wnt1 had to be activated prior to the ectopic induction of mdDA progenitors and precursors in this region of the *En1*$^{+/Wnt1}$ mutant brain. It was therefore of upmost interest to unravel the signalling and genetic cascade downstream of Wnt1 that controls the generation of mdDA neurons at endogenous and ectopic sites of the mouse brain.

In this context, mouse mutants for the Wnt receptor Frizzled 3 (*Fzd3*$^{-/-}$ KO mice), which is prominently expressed in the murine VM (Fischer et al., 2007), and for the Wnt co-receptor Low density lipoprotein receptor-related protein 6 (*Lrp6*$^{-/-}$ KO mice), which is also expressed in the developing mouse VM (Castelo-Branco et al., 2010), both show a transient reduction of postmitotic mdDA neuron numbers during late midgestational stages (Castelo-Branco et al., 2010; Stuebner et al., 2010). The proliferation of mdDA progenitors and survival of VM cells, by contrast, are not affected in these

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mutant mice. Apart from revealing the presumably vast functional redundancy of Fzd/Lrp receptors/co-receptors in VM and mdDA neuron development, these findings suggest that extracellular Wnt1 (and any other Wnt expressed in the murine VM) ligands transduce their signal, at least in part, by the Fzd3 Wnt receptor and Lrp6 co-receptor to control the proper differentiation of mdDA precursors into mdDA neurons.

The establishment of the mdDA progenitor domain in the mouse VM is controlled by a cross-regulation between Wnt1 and Otx2

An initial and notable observation was that the ectopic expression of Wnt1 and the ectopic induction of mdDA precursors in the rostral hindbrain FP of the *En1*$^{+/Wnt1}$ knock-in mice coincided with the ectopic expression of the HD TF Otx2 in this region of the mutant brain (Prakash et al., 2006). In line with this observation, the ectopic expression of Otx2 in the hindbrain FP is sufficient for the ectopic induction of mdDA neurons in this region (Ono et al., 2007). *Otx2* transcription is normally confined to neural tissues lying anterior (rostral) to the MHB. In fact, Otx2 is necessary for the development of anterior brain structures including the midbrain, and the mutual repression between Otx2 (expressed in the fore- and midbrain) and another HD TF, gastrulation brain homeobox 2 (Gbx2, expressed in the rostral hindbrain (rhombomere 1)), establishes an expression interface that determines the position of the MHB and IsO within the developing neural plate (Simeone, 2000; Vieira et al., 2010; Simeone et al., 2011). Consistent with this notion, the ectopic Otx2 represses the endogenous expression of *Gbx2* in the *En1*$^{+/Wnt1}$ rostral hindbrain FP. In the wild-type mouse, serotonin-producing (serotonergic, 5-HT) neurons arise from a bilateral domain adjacent to the rostral hindbrain FP that expresses the HD TF NK2 homeobox 2 (Nkx2-2) (Craven et al., 2004). In the *En1*$^{+/Wnt1}$ rostral hindbrain, the expression of the HD TF Nkx2-2 and the generation of 5-HT neurons are slightly affected (Prakash et al., 2006), suggesting that the ectopic Wnt1 activates a signalling cascade resulting in the ectopic transcription of *Otx2*, which in turn leads to the partial suppression of the normal genetic programme for the generation of 5-HT neurons and thus to the anteriorization of the *En1*$^{+/Wnt1}$ rostral hindbrain FP. It was therefore interesting to analyse the consequences of the deletion of *Otx2* in the murine VM. Mutant mice, in which the *Otx2* gene is conditionally inactivated across the MHB in the caudal midbrain and rostral hindbrain (*En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$ mice (Puelles et al., 2004)), show a drastic reduction of proliferating mdDA progenitors and postmitotic mdDA precursors and neurons, concomitant with the loss of *Wnt1* expression in the mutant VM (Puelles et al., 2004; Prakash et al., 2006; Omodei et al., 2008). Notably, the expression of Nkx2-2 is ectopically induced and ventrally expanded, and 5-HT neurons are generated instead of the mdDA neurons in the VM of the *En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$ mice and an analogous *Nestin-Cre*/+;*Otx2*$^{flox/flox}$ mouse (Puelles et al., 2004; Vernay et al., 2005; Prakash et al., 2006; Omodei et al., 2008). Because this phenotype is the exact opposite to the one in the *En1*$^{+/Wnt1}$ rostral hindbrain, these data indicate that the cross-activation of Wnt1 and Otx2 expression (which are both confined to neural tissues lying anterior to the MHB) is necessary and sufficient to induce the mdDA neuronal fate and repress an alternative rostral hindbrain serotonergic fate in neural progenitors of the murine VM.

The removal of the ectopic Nkx2-2 in compound *En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$;*Nkx2-2*$^{-/-}$ triple mutant mice rescues the expression of *Wnt1* and the generation of mdDA neurons that are lost in the *En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$ double mutant mice, and suppresses the generation of serotonergic neurons in the VM of these mice and *Nestin-Cre*/+;*Otx2*$^{flox/flox}$;*Nkx2-2*$^{-/-}$ mice (Vernay et al., 2005; Prakash et al., 2006). These results suggest a direct or indirect repressive effect of Nkx2-2 on the transcription of *Wnt1*, and indicate that Otx2 is not strictly required for the expression of *Wnt1* and the development of all mdDA neurons in the *En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$;*Nkx2-2*$^{-/-}$ VM from midgestational stages onwards. In fact, the cell-autonomous expression of Otx2 is most important for the development of an mdDA neuronal subpopulation arising from the intermediate and caudal midbrain (Omodei et al., 2008), which will generate a subset of VTA DA neurons expressing high levels of the calcium binding protein Calbindin 1 (Calb1) and Aldh1a1, but only very low or absent levels of the glycosylated (active) DA transporter (Dat, also known as Slc6a3) and the potassium inwardly rectifying channel, subfamily J, member 6 (Girk2, also known as Kcnj6) (Di Salvio et al., 2010a, b; Di Giovannantonio et al., 2013). Accordingly, the expression of *Wnt1* and its established target gene Cyclin D1 (*Ccnd1*) (Shtutman et al., 1999), as well as the proliferation and cell cycle-exit of mdDA progenitors and their differentiation into postmitotic mdDA neurons, is most affected in the posterior VM of the *En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$ mice (Omodei et al., 2008). Conversely, the over- and ectopic expression of Otx2 in the mid-/hindbrain region of *En1*$^{+/Cre}$;*tOtx*$^{ov/ov}$ mice induces the increased/ectopic transcription of *Wnt1* and *Ccnd1* and the increased proliferation of mdDA progenitors particularly in the posterior VM (Omodei et al., 2008). Moreover, the increased posterior mdDA progenitors/precursors in the *En1*$^{+/Cre}$;*tOtx*$^{ov/ov}$ mice preferentially differentiate into the Calb1- and Aldh1a1-expressing VTA DA subset (Di Giovannantonio et al., 2013).

In contrast to the apparent requirement of Otx2 for the correct expression of *Wnt1*, Wnt1 is not necessary for the transcription of *Otx2* in the murine VM (Ellisor et al., 2012; Yang et al., 2013; our unpublished data). However, as mentioned before, Wnt1 is sufficient for the ectopic induction of *Otx2* in the rostral hindbrain FP (Prakash et al., 2006). Altogether, the data summarized in this section demonstrate that the cross-regulation of Wnt1 and Otx2 is necessary and sufficient for the establishment of the mdDA progenitor domain in the midbrain FP of the midgestational mouse embryo (Figure 3A). Most importantly, they show that Otx2 is another important factor acting up- and downstream of Wnt1 in this context, and they corroborate the idea that mdDA neurons are not generated in the absence of *Wnt1*, highlighting the pivotal role of Wnt1 in the development of these neurons.

Wnt1 regulates a transcriptional network including Lmx1a and Pitx3 required for the proper differentiation and survival of mdDA neurons

Apart from the early function of Wnt1 in the establishment of the mdDA progenitor domain summarized in the previous section, Wnt1 also plays a role in the later differentiation of these progenitors into mdDA neurons. In fact, the lost (in the *Wnt1*$^{-/-}$ and *En1*$^{+/Cre}$;*Otx2*$^{flox/flox}$ VM) or ectopic/increased (in the *En1*$^{+/Wnt1}$ rostral hindbrain FP and *En1*$^{+/Cre}$;*tOtx*$^{ov/ov}$ VM) expression of Wnt1 also correlates with the impaired generation of mdDA neurons and

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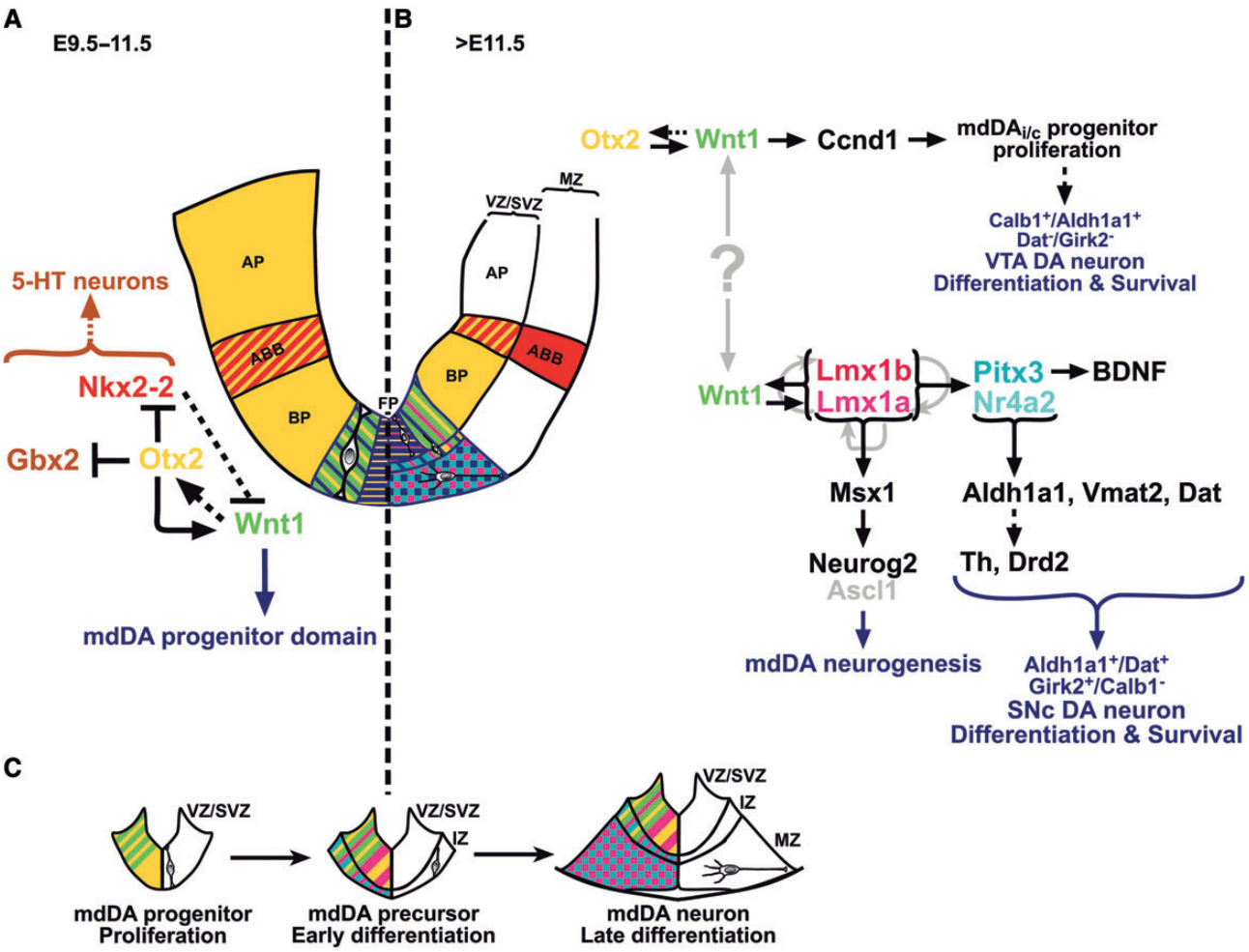

Figure 3 Wnt1-regulated genetic networks ensure the correct establishment of the mdDA progenitor domain and the proper neurogenesis, acquisition of DA metabolic functions, and survival of the emerging postmitotic mdDA precursors and neurons in the mammalian VM. (**A**) Schematic view of a hemisection through the VM of a midgestational mouse embryo (E9.5 to E11.5), depicting the region where the mdDA progenitor domain (dark blue hatched area) is established by a Wnt1-driven genetic network including Otx2. See text for details. (**B**) Schematic view of a hemisection through the VM of a late midgestational mouse embryo (after E11.5), depicting the region where proliferating mdDA progenitors (in the ventricular zone, VZ/subventricular zone, SVZ) and postmitotic mdDA precursors (in the intermediate zone, IZ) and neurons (in the mantle zone, MZ) (dark blue hatched areas) emerge under the control of two Wnt1-driven genetic networks. Note that these two genetic networks might intersect at the level of Wnt1 (grey double arrow with question mark). See text for details. Solid arrows/lines indicate genetic interactions that are necessary and sufficient; broken arrows/lines indicate genetic interactions that are only sufficient for the activation/repression of the corresponding gene; grey arrows indicate cross- or auto-regulatory interactions between the two Lmx1 orthologues; and grey letters indicate not yet experimentally validated target genes. (**C**) Schematics of the transition from a proliferating mdDA progenitor to a postmitotic early differentiating mdDA precursor to a late differentiating (mature) mdDA neuron and the corresponding domains in the murine VM according to the time-scale of the sketch depicted above. The proliferating mdDA progenitors are confined to the VZ/SVZ; the postmitotic mdDA precursors and differentiating mdDA neurons migrate radially out of the IZ into the MZ, and later orient tangentially to reach their final destinations (SNc, VTA, and RrF) in the mouse VM. ABB, alar-basal boundary; AP, alar plate.

the reduced/abolished or ectopic/increased expression, respectively, of a number of genes in the mutant VM. Among these genes are the HD TFs LIM homeobox transcription factor 1 alpha (*Lmx1a*), *Pitx3,* and msh homeobox 1 (*Msx1*), and the proneural basic helix-loop-helix (bHLH) TFs neurogenin 2 (*Neurog2*, also known as *Ngn2*) and achaete-scute complex homolog 1 (*Ascl1*, also known as *Mash1*) (Prakash et al., 2006; Omodei et al., 2008; Andersson et al., 2013; Yang et al., 2013; our unpublished data), suggesting that they are direct or indirect targets of the Wnt1 signalling pathway in the murine VM.

In support of this notion, the overexpression of Wnt1 in differentiating mouse pluripotent (embryonic) stem cells strongly induces the transcription of *Lmx1a* and, to a lesser extent, *Otx2* and *Pitx3* (Chung et al., 2009). Using chromatin-immunoprecipitation (ChIP) and quantitative PCR (qPCR) assays on VM and differentiating pluripotent stem cell extracts, Chung et al. (2009) showed that the *Lmx1a* and *Otx2* promoter regions are bound by Wnt1-activated β-catenin, the nuclear effector that is stabilized in the presence of a Wnt signal (MacDonald et al., 2009), thus confirming that these are direct target genes of the Wnt1/β-catenin signalling pathway in murine

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cells. Similar ChIP/qPCR assays and GOF and LOF analyses performed by the same group showed that the Lmx1a TF and its orthologue LIM homeobox transcription factor 1 beta (Lmx1b) bind to the *Wnt1*, *Pitx3*, *Nr4a2,* and *Msx1* promoter regions, thus corroborating these genes as direct targets of the Wnt1-induced Lmx1a and Wnt1-independent Lmx1b TFs and revealing a positive feedback regulation between Wnt1 and Lmx1a (Chung et al., 2009). This study also disclosed a cross-activation of the *Lmx1a* and *Lmx1b* promoters by Lmx1b and Lmx1a, respectively, and the self-activation of the *Lmx1a* promoter, thereby substantiating the functional redundancy between the two orthologue Lmx1 TFs (Chung et al., 2009).

The aforementioned direct and indirect Wnt1 target genes are in turn required for the proper neurogenesis and differentiation of mdDA neurons (Smidt and Burbach, 2007; Hegarty et al., 2013). Lmx1a and Msx1 are sufficient for the ectopic induction of mdDA neurons in the chicken and mouse embryo, respectively, and necessary for the generation of a subset comprising ~30%–40% of all mdDA neurons in the murine VM (Andersson et al., 2006b; Ono et al., 2007; Nakatani et al., 2010; Deng et al., 2011; Yan et al., 2011). In the latter context, Lmx1a acts in a redundant manner with Lmx1b (Deng et al., 2011; Yan et al., 2011). Notably, the expression of *Wnt1*, *Ccnd1*, *Msx1*, *Neurog2*, and *Ascl1* is reduced in a dose-dependent manner in the midbrain FP of *Lmx1a*/*Lmx1b* single and double mutant (KO) mice (Deng et al., 2011; Yan et al., 2011), confirming that these are direct and indirect target genes of the Lmx1a/b TFs. Pitx3 is particularly important for the generation and survival of rostrolateral mdDA precursors giving rise to the SNc DA neuron subset (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005). In this context, Pitx3 induces the transcription of brain-derived neurotrophic factor (*Bdnf*) and cooperates with the nuclear receptor Nr4a2 (Nurr1) for the activation of a number of target genes (Jacobs et al., 2007, 2009a, b; Peng et al., 2011). These include the RA-synthesizing enzyme *Aldh1a1* and enzymes, transporters, and receptors involved in DA metabolism, such as *Th*, the vesicular monoamine transporter 2 (*Vmat2*, also known as *Slc18a2*, required for the synaptic vesicle packaging of DA), *Dat* (*Slc6a3*, required for the re-uptake of extracellular DA), and the DA receptor D2 (*Drd2*, also known as *D2R*) (Jacobs et al., 2007, 2009a). The induction of *Aldh1a1* as a Pitx3 target gene and the concomitant production of RA and activation of the RA signalling pathway appear to be important for the transcription of some of the Pitx3 targets, such as *Th* and *Drd2* (Jacobs et al., 2011). The supplementation of *Pitx3*$^{-/-}$ pregnant mice with RA or the treatment of *Pitx3*$^{-/-}$ VM primary cell cultures with BDNF rescues the loss of mdDA neurons in these mice or cultures, respectively (Jacobs et al., 2007; Peng et al., 2011), indicating that Aldh1a1 and BDNF mediate some of the pro-survival functions of Pitx3 in the SNc DA neuronal subset. The two proneural TFs Neurog2 and Ascl1 are required for the proper neurogenesis of mdDA precursors/neurons, but not for the specification of their cell fate (Andersson et al., 2006a; Kele et al., 2006). *Neurog2* appears to be activated by Msx1 in the midbrain FP (Andersson et al., 2006b), although this has not been established conclusively so far.

The findings summarized in this section altogether indicate the existence of complex and prominently Wnt1-controlled genetic networks, including a partly self-reinforcing, positive feedback regulation between its members, which are essential for the development of mdDA neurons in the mammalian VM (Figure 3B). In these networks, Otx2 and the Lmx1a/b TFs lie upstream of *Wnt1* because *Wnt1* expression is not activated in their absence, whereas Wnt1/β-catenin signalling induces the transcription of *Otx2* in certain contexts (at ectopic sites) and activates the transcription of *Lmx1a* directly and *Pitx3* directly or indirectly, because the expression of these TFs is lost in the absence of *Wnt1*. The Lmx1a/b TFs in turn directly activate the transcription of *Pitx3*, *Nr4a2,* and *Msx1*, and mutually cross-regulate or even self-activate (in the case of Lmx1a) their expression. Further downstream, the HD TF Pitx3 and the Nr4a2 nuclear receptor (co-)activate the expression of genes encoding the most important enzymes, transporters, and receptors involved in DA/RA synthesis, DA transport, and DA reception, as well as genes encoding neurotrophic factors and their receptors (Jacobs et al., 2009b), and the HD TF Msx1 potentially induces the expression of the proneural bHLH TFs Neurog2 and Ascl1. Activation of these downstream targets of the Wnt1-regulated genetic pathways thereby ensures the proper neurogenesis, acquisition of DA metabolic functions, and survival of the emerging postmitotic mdDA precursors and neurons in the mammalian VM (Figure 3C).

Future directions

Although considerable advances have been made regarding the details of the Wnt1/β-catenin genetic pathways that are active during the development of the mdDA neurons, several aspects remain to be clarified in this context. For example, it is still unknown whether the Wnt1-expressing mdDA progenitors are identical to the cells receiving the Wnt1 signal (Wnt1-responsive cells) in the mammalian VM, or whether the number and the identity of the Wnt1-responsive mdDA progenitors, precursors, and/or neurons are currently being under- or overestimated. Another unclear issue is that on the one hand, the Wnt1/β-catenin genetic cascade appears to control in an unbiased manner the establishment of the mdDA progenitor domain and the generation of mdDA neurons in the rodent VM. On the other hand, the cross-regulatory interaction of Otx2 and Wnt1 in the intermediate and posterior VM during late midgestation appears to control preferentially the generation of the VTA DA neuronal subpopulation (Omodei et al., 2008; Di Giovannantonio et al., 2013), whereas Wnt1/β-catenin signalling is also involved directly or indirectly in the control of *Pitx3* expression (Prakash et al., 2006; Chung et al., 2009), a HD TF that is required in particular for the proper differentiation and survival of the SNc DA neuronal subset (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005; Jacobs et al., 2007; Peng et al., 2011). It therefore remains to be clarified whether the Wnt1-controlled genetic pathways play a general and/or a specific function in the generation of the three (SNc, VTA, and RrF) mdDA neuronal subsets. Finally, the mechanistic details of the Wnt1-activated signal transduction pathway(s), including the receptor(s) and nuclear effector(s) involved in these processes and the identity of all Wnt1 target genes in these contexts, are still not understood. Elucidation of these unknown aspects is particularly important for devising novel cellular (*in vitro*) disease models of PD, schizophrenia, and addiction, for therapeutic drug screenings using these *in vitro* models, and for regenerative approaches to the treatment of PD. The latter include the generation of mdDA neurons or

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more specifically the SNc DA neuronal subset either *in vitro* in the culture dish by the directed differentiation of pluripotent stem cells, or *in situ* by the direct conversion (reprogramming) of somatic cells into these neurons (Qiang et al., 2013).

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