ESTABLISHING DIVERSITY IN THE DOPAMINERGIC SYSTEM

Gabriela O. Bodea¹ and Sandra Blaess²*
¹Mater Research Institute - University of Queensland, Translational Research Institute, Woolloongabba QLD 4102; Queensland Brain Institute, University of Queensland, Brisbane QLD 4072, Australia
²Institute of Reconstructive Neurobiology, Life and Brain Center, University of Bonn, Bonn, Germany
*corresponding author: sblaess@uni-bonn.de

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ABSTRACT
Midbrain dopaminergic neurons (MbDNs) modulate cognitive processes, regulate voluntary movement, and encode reward prediction errors and aversive stimuli. While the degeneration of MbDNs underlies the motor defects in Parkinson's disease, imbalances in dopamine levels are associated with neuropsychiatric disorders such as depression, schizophrenia and substance abuse. In recent years, progress has been made in understanding how MbDNs, which constitute a relatively small neuronal population in the brain, can contribute to such diverse functions and dysfunctions. In particular, important insights have been gained regarding the distinct molecular, neurochemical and network properties of MbDNs. How this diversity of MbDNs is established during brain development is only starting to be unraveled. In this review, we summarize the current knowledge on the diversity in MbDN progenitors and differentiated MbDNs in the developing rodent brain. We discuss the signaling pathways, transcription factors and transmembrane receptors that contribute to setting up these diverse MbDN subpopulations. A better insight into the processes that establish diversity in MbDNs will ultimately improve the understanding of the architecture and function of the dopaminergic system in the adult brain.
1 DIVERSITY IN THE ADULT DOPAMINERGIC SYSTEM

MbDNs are organized anatomically into three major cell clusters: the substantia nigra pars compacta (SNC), the ventral tegmental area (VTA) and the retrorubral field (RRF) [1]. The SNC and VTA can be further divided into subnuclei (Figure 1). The rostral and caudal linear nucleus of the raphe (CLi and RLi) are sometimes considered as independent nuclei rather than as a part of the VTA [2,3].

Efferent projections to specific regions in the forebrain arise from essentially separate subpopulations of MbDNs and a lateral-to-medial topographic gradient roughly describes the relationship between the anatomical position of MBDN cell bodies and their projection targets in the forebrain. Laterally located MbDNs (SNC and dorsolateral VTA) establish connections to the dorsal striatum forming the mesostriatal pathway. More medially located MbDNs (dorsolateral VTA) project to the lateral regions of the nucleus accumbens. Medial MbDNs (ventromedial VTA) establish connections with the medial regions of the nucleus accumbens (NAc) and the prefrontal cortex (PFC) (mesocorticolimbic pathway) (Figure 1) [4-11]. MbDNs in the mesostriatal pathway are involved in the control of motor function and their degeneration results in the major symptoms of Parkinson's disease [12]. MbDNs in the mesocorticolimbic system regulate working memory, attention, decision making and reward-associated behavior. Dopamine imbalances in the mesocorticolimbic pathway have been implicated in drug abuse disorders, depression, attention deficit hyperactivity disorder and schizophrenia [13-18]. RRF neurons are thought to play a role in modulating the nigrostriatal and mesolimbic pathways [19]. MbDNs receive afferent connections from many different brain regions, and these are largely distinct for SNC and VTA neurons [10,11,20].

Molecular markers that unequivocally distinguish these anatomically defined subpopulations are not available, but the following restricted expression patterns have been widely employed to distinguish MbDNs on a molecular level and to define SNC or VTA fate at least approximately: KCNJ6 (Potassium inwardly-rectifying channel, subfamily J, member 6, also known as GIRK2) and SLC6A3 (Solute carrier family 6 member 3, also known as DAT) are expressed in MbDNs in the SNC and in the dorsolateral VTA. Calbindin1 (CALB1) is expressed in MbDNs in the VTA and in the dorsal tier of the SNC (Figure 1) [21-23]. However, there is increasing evidence for a high degree of molecular and neurochemical diversity in dopaminergic neurons [24-31]. For example it has been shown that MbDNs in the mesocortical pathway can co-release the neurotransmitter glutamate, while MbDNs in the SNC have the ability to co-release GABA (γ-aminobutyric acid) [32-35].

With the advance of optogenetic techniques and viral tracing systems, it has been possible to demonstrate that MbDNs that differ in their anatomical position and connectivity mediate distinct aspects of behavior [10,11,17,36-39]. Nevertheless, we are still lacking an overarching concept that integrates the above-mentioned characteristics to define what constitutes an MbDN subtype. The detailed knowledge of the developmental programs that generate discrete populations of MbDNs will offer an essential new perspective on understanding MbDN diversity. Studies in different model organisms and different areas of the CNS indicate that neuronal cell diversity is established by a genetic blueprint that specifies neuronal progenitors and that is later refined by experience-dependent mechanisms. The relative influence of the genetic- and experience-dependent processes on the final identity of neuronal cell types seems to vary in
different areas of the CNS [40-42]. In recent years, progress has been made in unraveling genetic mechanisms that contribute to the generation of MbDN subclasses. Still it is largely unclear how the subsequent interaction of differentiated MbDNs with their surrounding environment and their projection partners influence the ultimate profile of a particular MbDN neuronal subtype. Thus, we focus our review on the genetically determined processes that shape MbDN diversity in the embryonic rodent brain.

2 DEVELOPMENT OF DOPAMINERGIC NEURONS

MbDNs arise from a progenitor domain in the ventral midline (floor plate) of the midbrain. The secreted signaling molecules SHH (Sonic hedgehog), FGF8 (Fibroblast growth factor 8) and WNT1 (Wingless-related MMTV integration site 1) and the transcription factors OTX2 (Orthodenticle homolog 2), EN1/2 (Engrailed 1/2) and FOXA1/2 (Forkhead box A1/2) are critical for the induction of the ventral midbrain and for establishing the MbDN progenitor domain (reviewed in [43-45]). The induction of the floor plate by SHH signaling begins around embryonic day (E) 8.0 in the murine midbrain. About one day later, the MbDN progenitor domain emerges in the ventral midline with the onset of LMX1A (LIM homeodomain transcription factor 1A) expression [46]. These MbDN progenitors have characteristics of radial glia cells [47,48]. The beginning of MbDN neurogenesis around E10.5 is marked by the expression of the proneural gene Neurogenin 2 (NEUROG2) [46,49,50]. The first differentiated MbDNs appear around the same time and are characterized by the expression of the transcription factor NR4A2 (Nuclear receptor subfamily 4, group A, member 2; also known as NURR1). Expression of the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH) can be detected about half a day later (Figure 2) [51]. MbDN neurogenesis continues over several days, and ceases around E14.5 [52,53]. Simultaneously, MbDNs that leave the cell cycle mature further, migrate to their final positions and extend their axons towards their respective target areas. Each of these stages in MbDN development is defined by the expression of a set of transcription factors and other molecular markers. The signaling and transcriptional networks that control the specification of a general dopaminergic fate during development have been recently reviewed [43-45].

In the following sections we discuss the genes and developmental mechanism that are involved in generating MbDN diversity. We present evidence for a temporally shifting competence of MbDN progenitors, for the existence of molecularly distinct sets of MbDN progenitors and the ability of these subsets to develop into specific MbDN subclasses. We then summarize the existing knowledge on the diversity in MbDNs during their differentiation, migration and axonal pathfinding. Throughout, we use the SNc, dorsolateral VTA and ventromedial VTA terminology and the KCNJ6/SLC6A3/CALB1 molecular markers to describe subsets of MbDNs (Figure 1B).

3 GENERATING DIVERSITY IN DOPAMINERGIC PROGENITORS

3.1 Temporal: Shifting competence of MbDN progenitors?

As discussed above, MbDNs differentiate over several days. Thus, one plausible mechanism for the generation of diverse populations of MbDNs is a changing competence of MbDN progenitors. Indeed,
birthdating studies provide some evidence that different subtypes of MbDNs might be generated at different embryonic time points. Bayer et al. administered radioactive H3-Thymidine at different embryonic stages to birthdate MbDNs. For each developmental stage, H3-Thymidine was given at two consecutive days (e.g. at E10.5 and E11.5). TH and H3-Thymidine double-labeled neurons were then assessed for their anatomical distribution in postnatal brains. The results show that MbDNs of the SNc and dorsolateral VTA are generated first, with a peak at E11.5, while ventromedial VTA-MbDNs are born later, with a peak at E12.5. Moreover, anterior MbDNs are in general born earlier than posterior MbDNs [53]. A more recent birthdating analysis used BrdU (Bromdesoxyuridine) injections at single time points to label proliferating MbDN progenitors, allowing for a more precise temporal dissection of their birthdates [54]. This study shows that the overall BrdU labeling of TH-expressing MbDNs decreases between E10.5 and E12.5. MbDNs born at E10.5 contribute equally to the SNc and VTA, while the majority of cells birth-dated at E11.5 give rise to VTA-MbDNs. This biased contribution to the VTA is even more pronounced at E12.5. Moreover, the few neurons labeled in the SNc at E12.5 are restricted to the medial parts of the SNc. Triple-labeling for KCNJ6/TH/BrdU and for CALB1/TH/BrdU confirmed the largely VTA-restricted contribution of later born MbDNs. The H3-Thymidine and BrdU study diverge on their conclusions on the time point of peak neurogenesis of SNc- and VTA-MbDNs, probably due to the different labeling methodologies. Nevertheless, they both suggest that MbDN progenitors might undergo a shift or progressive restriction in competence over time that biases them to give rise to more medial and more posterior MbDN subtypes at later time points of development. In such a model, the changing competence of progenitors might be based on – or at least reflected in – temporal changes in gene expression in MbDN progenitors. Interestingly, expression of a number of genes is temporally dynamic in MbDN progenitors (Figure 3). Whether these changing gene expression patterns are functionally important for a temporal shift in MbDN fate potential is not known.

3.2 Spatial: molecular codes defining distinct progenitor domains

An obvious prerequisite for studying whether MbDN progenitors consist of molecularly distinct subsets is to define what constitutes an MbDN progenitor. In 2006 Andersson et al demonstrated that Lmx1a is the key gene in determining MbDN progenitor identity in chick [46]. Based on this and a number of follow-up studies, it is generally assumed that LMX1A expression delineates the MbDN progenitor domain in the ventral midbrain of both mouse and chick [55-57]. In addition to LMX1A, several other transcription factors are expressed in the entire population of MbDN progenitors: LMX1B, FOXA1/2, ARX (Aristaless related homeobox), OTX2 and DMRTA2 (Doublesex and mab-3 related transcription factor like Family A2, also known as DMRT5). The expression patterns of these factors are however not confined to the LMX1A positive progenitor domain, since they extend further along the mediolateral or rostrocaudal axis of the neural tube (Figure 3). Except for ARX, all of these transcription factors have been shown to be of functional importance for the specification of murine MbDN progenitors [56-60].

In recent years, a number of genes have been identified that are differentially expressed within the LMX1A positive progenitor domain. We will separately describe the gene expression patterns along the
mediolateral and the anteroposterior axis of the MbDN progenitor domain, since most expression patterns have been characterized in detail in only one of the two axes. Furthermore, we will discuss the data available on the functional impact of these genes on MbDN diversity.

3.2.1 Diversity of MbDN progenitors along the mediolateral axis

3.2.1.1 Signaling pathways: SHH and WNT

The induction of MbDN progenitors depends on SHH signaling [61,62]. The response to SHH signaling can be monitored by the analysis of Gli1 (glioma-associated oncogene homolog 1) expression, a readout for the activated pathway [63]. In the ventral midbrain, Shh and Gli1 expression undergo dynamic changes. Starting around E7.5, Shh is expressed in the notochord and Gli1 expressing cells are located in the ventral midline of the presumptive midbrain. Between E8.0 and E8.5, ventral midline cells switch on Shh expression and Gli1 expression shifts to a domain laterally adjacent to the Shh-expressing cells. The Shh expressing domain expands further until E10.5; accordingly the adjacent Gli1 expressing domain moves further laterally. After E10.5, Shh is downregulated in ventral midline progenitors, but these medial progenitors remain unresponsive to SHH signaling and remain negative for Gli1 and other components of the canonical SHH signaling pathway [64-68]. The Shh expressing domain is eventually considerably broader than the MbDN progenitor domain (Figure 3B). Nevertheless, the early dynamic changes in Shh and Gli1 expression suggest that the medial and lateral MbDN progenitors are distinct based on the time when they express Shh and respond to SHH signaling (express Gli1). Genetic inducible fate mapping studies using reporter alleles in combination with ShhCreER or Gli1CreER knock-in lines confirmed that this is indeed the case: medial MbDN progenitors express Gli1 (respond to SHH) from E7.5 to E8.5 and express Shh from E8.5 to E10.5. In lateral MbDN progenitors the expression onset is delayed by about one day: Gli1 expression (SHH response) occurs around E8.5-E9.5, Shh expression around E9.5 [65,69,70] (Figure 3B). These studies also provide evidence that the medial and lateral MbDN progenitor domains give rise to different subsets of MbDNs in the prenatal or adult brain: medial progenitors (Shh-expressing at E8.5) develop predominantly into KCNJ6-expressing MbDNs in the SNc and dorsolateral VTA, while lateral progenitors (Shh-expressing at E11.5, Gli1-expressing at E9.5) generate preferentially CALB1-expressing MbDNs in the ventromedial VTA.

Inactivation of SHH signaling after E8.5 leads to a strong reduction in the size of the MbDN progenitor domain and in the number of mature MbDNs, but not to their complete absence. The number of mature MbDNs is severely decreased in these mutant mice [62,67,68,71,72]. Only complete inactivation of SHH signaling results in the loss of the entire MbDN progenitor population. A detailed analysis of one of the mutant mouse lines in which SHH signaling was inactivated after E8.5, shows that most of the remaining
MbDN progenitor domain has characteristics of medial MbDN progenitors (Corin positive) (Figure 3B). In these mutant mice, the decrease in the number of mature MbDNs is significantly more severe in CALB1 positive MbDNs than in the KCNJ6-expressing MbDNs. Importantly, VTA neurons that project to the PFC are essentially lost in these mutants, while projections to other target areas are at most only mildly affected [72]. These data strongly suggest that mesocortical MbDNs are derived from the lateral subset of MbDN progenitors that depend on SHH signaling after E8.5. Thus, these results demonstrate a functional role of SHH signaling in the establishment of MbDN diversity at the MbDN progenitor level.

WNT1 is an important regulator of proliferation and neurogenesis in MbDN progenitors and of Lmx1a and Otx2 expression (reviewed in [43-45]). Initially (until E8.5), Wnt1 is expressed throughout the presumptive midbrain. At later stages, Wnt1 expression is confined to the ventral midline, the roof plate and a ring of cells at the posterior midbrain [73,74]. From E9.5 to E11.5 Wnt1 is expressed in the lateral MbDN progenitor domain and with the onset of MbDN neurogenesis Wnt1 is also present in differentiated MbDNs. Wnt1 expression is downregulated in MbDN progenitors at E12.5, but maintained in differentiating MbDNs up to E14.5 [75-78] (Figure 3B). Brown et al. used genetic inducible fate mapping to investigate whether the Wnt1-expressing MbDN progenitors have a restricted fate potential. Using a Wnt1CreER transgenic mouse line to recombine reporter alleles, they labeled Wnt1-expressing progenitors at distinct 24-hour time points between E7.5 and E13.5 and analyzed whether their offspring contributes preferentially to any particular subset of MbDNs in postnatal mice. They did not uncover a biased contribution of descendants of Wnt1-expressing MbDNs to the SNc, VTA or RRF or to MbDN subtypes defined by the expression of KCNJ6, CALB1 or Calbindin 2 (CALB2 also known as Calretinin; expressed in the VTA and a small subset of SNc-MbDNs) [75]. These results appear to be in discrepancy with the above described birthdating experiments and fate-maps of Shh/Gli1 expressing MbDN progenitors that show that MbDNs with late birth dates and lateral MbDN progenitors preferentially give rise to VTA-MbDNs [53,54,65,69,70]. However, it is important to note that it has not been explored whether short-term fate mapping of Wnt1-expressing cells does indeed result in the marking of only lateral MbDN progenitors. It is also not clear whether the lateral Shh/Gli1 and Wnt1 expression domains are completely overlapping in MbDN progenitors at the critical developmental time points (E9.5-E11.5). Moreover, it should be taken into account that Wnt1 is expressed in MbDN progenitors and differentiated MbDNs [75,77]. Therefore, fate-mapping at time points after the onset of MbDN neurogenesis (E10.5) results not only in labeling of MbDNs originating from Wnt1-expressing MbDN progenitors but also in the direct marking of already differentiated, Wnt1-expressing MbDNs. Thus, the temporal restriction of fate potential in MbDN progenitors might be masked in this experimental approach.

While there is no evidence that Wnt1 expression delineates a MbDN progenitor domain with a particular fate potential, WNT signaling might play a role in determining diverse subsets of MbDN progenitors. MbDN progenitors have a temporally dynamic response to WNT signaling. Axin2, a readout and an intracellular negative regulator of WNT signaling, is expressed in only a few scattered MbDNs at E10.5, but is strongly expressed in the lateral MbDN progenitor domain at E11.5 [76,79]. Using a reporter mouse for canonical WNT-signaling (BAT-GAL transgenic mouse), Mesman et al showed that the
response to WNT is maintained in lateral progenitors at E12.5 [78]. Early inactivation of \textit{Wnt}1 (in \textit{Wnt}1 null mice or in mice in which \textit{Wnt}1 was inactivated in the mid/hindbrain around E8.5) leads to a severe reduction in the size of the MbDN progenitor domain. The few remaining MbDN progenitors generate a small amount of TH-positive neurons between E10.5 and E12.5, but these lack the ability to differentiate into mature MbDNs and are lost at later stages of embryogenesis [77,80,81]. Evidence that WNT1 signaling might have a biased effect on the development of VTA-MbDNs comes from the analysis of mutant mice homozygous for the \textit{Swaying} allele and of mice in which WNT signaling was inactivated in MbDN progenitors after E9.0. The \textit{Swaying} allele contains a point mutation in the \textit{Wnt}1 gene that results in a premature stop codon [82]. The phenotype of the \textit{Swaying} mutants is reminiscent of the \textit{Wnt}1 null phenotype, but it is less severe and approximately a fifth of the mutant mice survive to adulthood. In these mice, the caudal LMX1A positive MbDN progenitor domain is depleted. Both the KCNJ6/TH and CALB1/TH positive cells are severely decreased in the adult brain, but the reduction in the number of CALB1-expressing MbDNs is significantly more pronounced than of KCNJ6-expressing MbDNs. Since the mid/hindbrain boundary and the overall establishment of the midbrain are aberrant in the \textit{Swaying} mice, the changes in MbDNs might be indirect. In mice, in which \textit{Wnt}1 is inactivated in MbDN progenitors after E9.0, the percentage of medial MbDN progenitors that leave the cell cycle at E11.5 is decreased compared to wild-type, while the percentage of lateral MbDN progenitors exiting the cell cycle is increased. As a consequence, at E12.5, the number of medial MbDNs is reduced, while the number of lateral MbDNs is increased [77]. Later time points have not been investigated in these mice, thus it remains unclear whether the reduction in medial MbDN progenitors results in a reduced number of SNc-MbDNs (Figure 2). MbDN progenitor specific inactivation of \textit{\beta-catenin} (\textit{Ctnnb1}) abolishes all canonical WNT signaling, but elicits also structural changes in the MbDN progenitor domain due to the role of \textit{Ctnnb1} in adherens junctions [48,83]. In these mutants, MbDN neurogenesis is decreased and while not specifically analyzed, it appears that the reduction is particularly severe in the medial domain [48]. Both SNc and VTA MbDNs are reduced by approximately 50% in prenatal mutant brains compared to control brains. The converse experiment, in which WNT signaling is overactivated by forced expression of a constitutive-active form of \textit{Ctnnb1} in MbDN progenitors results in an expanded progenitor domain, reduced neurogenesis and a decrease in the numbers of MbDNs. SNc-MbDNs appear to be more severely reduced than VTA-MbDNs. Moreover, some MbDN progenitors lose their definitive MbDN progenitor identity and express markers of neighboring red nucleus progenitors [84,85]. In conclusion, these data suggest that WNT signaling is involved in the generation of MbDN diversity, possibly by regulating proliferation and neurogenesis differently in distinct MbDN progenitor subsets. However, further studies will be necessary (i.e. analysis of MbDN subsets in prenatal or postnatal \textit{Wnt}1 conditional knock-out mice, a genetic fate-map of Wnt-responding (\textit{Axin2}-expressing) cells) to fully understand the impact of WNT signaling on the development of MbDN subtypes and to dissect its role in MbDN progenitors and differentiated MbDNs.
3.2.1.2 Transcription factors

LMX1A and LMX1B are co-expressed in MbDN progenitors, but the LMX1B expression domain is slightly broader than the LMX1A domain (Figure 3). In mouse, LMX1A and LMX1B are partially redundant: only inactivation of both Lmx1a and Lmx1b results in the almost complete loss of MbDNs, while MbDNs are still generated in Lmx1a or Lmx1b single knock-outs, albeit in reduced numbers [55,86,87][28,56,57]. Surprisingly, the complete inactivation of Lmx1a or Lmx1b seems to have distinct effects on medial and lateral MbDN progenitors. In Lmx1a null mutants, a medial (Corin positive) and a lateral (Nk-6 homeobox 1 (NKX6-1)/Corin negative) MbDN progenitor domain is maintained but neurogenesis from the medial domain appears to be reduced [56] (but see also [57]) (Figure 3B). As a result, MbDNs in anterolateral areas, including the SNc and the lateral VTA are decreased in number, while MbDN numbers in the ventromedial VTA are less affected [28,56,57]. Complete inactivation of Lmx1b results in the loss of the lateral MbDN progenitor domain while the medial domain is established normally [56,57]. The effect in Lmx1b null mutants is likely indirect, since Lmx1b controls the expression of Wnt1 and Fgf8. In fact, when Lmx1b is inactivated in MbDN progenitors after E9.0, Wnt1 and Fgf8 expression is maintained, and the number of MbDNs is not reduced [57].

MSX1 (Homeobox, msh-like 1) starts to be expressed in the ventral midline at E9.5, but it is excluded from the most lateral aspects of the LMX1A positive domain, where NKX6-1 is expressed. After E9.5, NKX6-1 is downregulated in lateral LMX1A positive progenitors but MSX1 remains restricted medially (Figure 3B). Premature expression of MSX1 in the floor plate results in the precocious induction of NEUROG2, while inactivation of Msx1 leads to reduced neurogenesis and a reduced number of NR4A2-expressing neurons at E11.5 [46]. A potential function of MSX1 in the generation of particular MbDN subtypes has not been explored, due to the embryonic lethality of Msx1 null mutants. A conditional gene inactivation approach - potentially in combination with inactivation of Msx2, which is also expressed in the ventral midline – will be necessary to explore whether the restricted expression of Msx1 has functional relevance for the generation of MbDN diversity.

FERD3L (Fer3-lik, also known as NATO3) is first expressed in the ventral midline at E8.5 and its expression overlaps with LMX1A positive progenitors at E9.5. At subsequent stages, FERD3L is downregulated in lateral MbDN progenitors, while expression is maintained medially (Figure 3). Similar to what has been described for MSX1, FERD3L misexpression induces premature neurogenesis, while inactivation of Ferd3l interferes with MbDN neurogenesis leading to a reduction in MbDN numbers. FERD3L is not required for the expression of midline specific markers such as MSX1 or Corin, but it maintains the proliferative state of ventral midline progenitors (Figure 3). The maturation of MbDNs or the specification of MbDN subtypes appears not be altered in adult Ferdl3 deficient mice. The authors report that MbDN numbers are reduced in both SNc and VTA and that the ratio of KCNJ6 to CALB1 positive cells is not significantly changed [88].

OTX2 is expressed in all MbDN progenitors [59,89], but expression appears to be higher in the lateral than in the medial progenitor domain, at least at E11.5 and E12.5 [30] (Figure 3). OTX2 is also expressed in differentiated MbDNs, in which it is largely restricted to CALB1 positive and KCNJ6 negative VTA.
neurons [22,30,90] (see below for further details). Fate-mapping of Otx2-expressing MbDN progenitors at E7.7, E8.5 or E9.5 shows that they give rise to Otx2-positive and Otx2-negative MbDNs in both the SNc and VTA [89]. Conditional inactivation of Otx2 in midbrain progenitors using the En1Cre line (En1-Otx2 cko) results in a severe reduction in the number of MbDNs, particularly in the VTA [91]. Virtually all of the remaining MbDNs have a ventral SNc or dorsolateral VTA fate (KCNJ6 positive, CALB1 negative) [89] (Figure 1). These neurons are also negative for Otx2, as assessed by monitoring GFP expression with an Otx2-GFP knock-in mouse. In contrast, forced expression of OTX2 in midbrain progenitors and their descendants (using the En1Cre line) results in an increased number of KCNJ6 negative VTA-MbDNs. These changes in OTX2 levels strongly affect MbDNs and MbDN progenitors at intermediate and posterior levels, but not at anterior levels [59]. Together, these data suggest a role for OTX2 in determining VTA fate in MbDN progenitors. However, whether this is a direct function of OTX2 in MbDN progenitors is difficult to determine, since both inactivation and forced expression of Otx2 result in additional severe defects [59,91]. Thus, OTX2 might not be involved in specifying a VTA-MbDN-generating MbDN progenitor subtype but might rather regulate the proliferation and neurogenesis of MbDN progenitors, possibly by regulating expression of Wnt1. Its effect on the expression of typical VTA markers could be primarily due to its later role in differentiated MbDNs [22] (see below).

Recently, it has been proposed that SOX6 (SRY box containing gene 10) and ZFP503 (zinc finger protein 503, also known as NOLZ1), together with OTX2, contribute to a transcriptional code that defines MbDN progenitors with distinct fate potentials [30]. The expression patterns of these two factors are consistent with this idea: At E11.5, SOX6 is expressed in medial progenitors. ZFP503 is restricted to lateral progenitors that constitute a subset of the MbDN progenitors expressing high levels of OTX2 (Figure 3). In differentiating and mature MbDNs, SOX6 is expressed in the SNc and dorsolateral VTA [30,31] while ZFP503 expression is confined to a subset of OTX2-expressing VTA neurons [30] (see below for further details). SOX6 expression is regulated by OTX2: In En1-Otx2 cko mice, SOX6 expression is expanded laterally in MbDN progenitors and SOX6 is widely expressed in the few differentiated MbDNs that are generated in these mutant mice. On the other hand, forced expression of OTX2 in differentiated MbDNs (but not in MbDN progenitors) leads to downregulation of SOX6. This regulation is nonreciprocal: Mice homozygous for a null allele of Sox6 maintain the normal expression pattern of OTX2 in MbDN progenitors. In Sox6−/− mice, the percentage of CALB1 positive/SLC6A3 negative cells is increased in the SNc. Whether these MbDNs are also KCNJ6-positive has not been examined. Consistent with a defect in SNc-MbDN specification, the dopaminergic innervation to the dorsal striatum, the main target area of SNc-MbDNs, is reduced in Sox6 null mutant mice and in mutant mice, in which Sox6 has been conditionally inactivated in differentiated MbDNs. The effect of the conditional gene inactivation on the percentage of CALB1 positive/SLC6A3 negative cells in the SNc has not been assessed. Thus, it remains to be resolved whether the apparent change in MbDN subset fate in Sox6 null mutants can be attributed to SOX6 function in MbDN progenitors or in differentiated MbDNs. The function of ZFP503 or the regulation of its expression through SOX6 or OTX2 has not yet been investigated. In conclusion, while the expression patterns of OTX2, SOX6 and ZFP503 suggest a potential role of these transcription factors in MbDN
progenitor diversification, further experiments will be necessary to dissect whether these transcription factors act primarily in MbDN progenitors or in differentiated MbDNs.

3.2.1.3 Transmembrane receptors and enzymes

The expression of several cell surface receptors is restricted to the medial MbDN progenitor domain: Corin, a transmembrane serine protease [55,92], Kitl (Kit ligand also known as SCF)[88] and Plxdc2 (Plexin domain containing 2, also known as Tem7r), a single-pass type I membrane protein. KITL promotes outgrowth of commissural axons in the spinal cord after they crossed the ventral midline [93]. PLXDC2, together with PLXDC1, can act as a receptor for PEDF (pigment epitheli um derived factor) [88,94], a neurotrophic and stem cell niche factor. It is not known whether the differential expression of Corin, Kitl and Pldc2 has any functional significance in MbDN progenitors, but interestingly all of these markers are expressed in the floorplate in the hindbrain and spinal cord. This suggests that the medial MbDN progenitor domain, despite its neurogenic potential, maintains most of the characteristics that define floor plate cells. Cadherin-11, a member of the type II subfamily of classical cadherins is expressed in the lateral MbDN progenitor domain and in adjacent lateral progenitors at E11.5 and E12.5 [95]. ALDH1A1 (aldehyde dehydrogenase family 1, subfamily A1, also known as Raldh1 or Ahd2), an enzyme in the retinol metabolism, is expressed in the entire MbDN progenitor domain at E9.5, but is restricted to the lateral MbDN progenitor domain at later developmental stages [51,85,96].

In conclusion, the results discussed here indicate that the MbDN progenitor domain consists of at least two MbDN progenitor subgroups, namely medial and lateral MbDN progenitors, which are distinct in their gene expression, their responsiveness to signaling pathways and their fate potential.

3.2.2 Diversity of MbDN progenitors along the anteroposterior axis

Based on morphological, histological and gene expression characteristics of the MbDN containing region, anterior MbDNs appear to reside in the posterior diencephalon [97,98]. In addition, there is ample evidence for diversity of MbDN progenitors along the anteroposterior axis: a population of progenitors in the posterior diencephalon shares some core characteristics (En1 lineage, Foxa2, Otx2 and Lmx1a expression) with MbDN progenitors in the midbrain, but lacks others (En1/2 expression after E8.5) (Figure 3). Whether the diencephalic progenitors are the source of the diencephalic MbDNs has not been clarified, since fate-mapping studies have not yet been performed for this progenitor population.

3.2.2.1 Signaling pathways

FGF signaling, which is mainly mediated through FGF8 secreted from the mid/hindbrain boundary, is essential to establish and pattern the midbrain and anterior hindbrain territory [99,100]. After E8.5, FGF8 signaling is involved in setting up the anteroposterior identity of MbDN progenitors. In mice in which Fgf8 or the genes encoding FGF receptors are inactivated at E8.5, MbDN progenitors and TH-positive neurons are initially induced, but TH-positive neurons are lost by E15.5 [80,96,100,101]. Analyzing the anteroposterior characteristics of MbDN progenitors in the Fgf receptor conditional mutants in detail, Lahti
et al. show that FGF signaling is required for the specification of a posterior (midbrain) MbDN progenitor identity. In the mutant mice, MbDNs and their progenitors adopt the characteristics of diencephalic MbDNs/MbDN progenitors in the mutants. MbDN progenitors lack *En1/2* and *Aldh1a1* expression and differentiated MbDNs are positive for TH, *Nr4a2* and *Lmx1b*, but lack expression of *En1/2* and *PITX3* at E12.5 (Figure 2). By E15.5, TH positive cells can no longer be detected in the mutants [96]. Thus, FGF signaling appears to be required for the specification of a midbrain versus diencephalic fate in MbDN progenitors. Why the MbDNs with diencephalic characteristics do not acquire or retain MbDN identity in the mutant mice at later stages of development is not known. It is however clear that their disappearance is a consequence of the inactivation of FGF signaling in MbDN progenitors, since the inactivation of FGF receptors in differentiated MbDNs does not alter the number or the phenotype of MbDNs [96]. The fact that MbDN progenitors with a “diencephalic identity” are not able to produce mature MbDNs in the mutants and only display transient TH expression seem to suggest that posterior diencephalic progenitors might not have the capability to generate mature MbDNs during normal development.

After E10.5, *Wnt1, Wnt8b* and the *Wnt* target gene *Apcdd1* (Adenomatosis Polyposis Coli Down-Regulated 1 also known as *Drapc1*) have been described as being restricted to MbDN progenitors with midbrain identity [78,96]. At least for *Wnt1*, these data are inconclusive. Other studies report *Wnt1* expression along the entire anteroposterior extent of the LMX1A-expressing MbDN progenitor domain up to E12.5 [75,77]. In loss-of-function studies, the mosaic conditional inactivation of *Wnt1* after E8.5 (using *Wnt1-CreER* mice) results in the depletion of caudal MbDNs, whereas inactivation of *Wnt1* in MbDN progenitors after E9.5 (using *ShhCre* mice) appears to affect proliferation and neurogenesis of MbDN progenitors at all anteroposterior levels of the domain [75,77].

3.2.2.2 Transcription factors

*EN1/2* are essential for the establishment of MbDN progenitors and differentiated MbDNs [80,102-104], but they might also play a role in establishing MbDN diversity. As described above, *En1* is expressed in MbDN progenitors of both posterior and anterior identity before E9.5. Later both *En1* and *En2* are restricted to the posterior domain, before being downregulated in progenitors at E11.5 [96]. Complete inactivation of *En1* results in a severe reduction in the number of MbDNs in the anterolateral but not in the posteromedial MbDN domain at E13.5. Of several investigated MbDN markers only *Nr4a2* expression is maintained in both areas (Figure 2)[105]. The absence of most MbDN markers is reminiscent of the phenotype observed when FGF signaling is ablated in the midbrain, suggesting that EN1 might function downstream of FGF signaling in the specification of midbrain identity in MbDNs [96]. Conditional inactivation of *En1* using *En1Cre* results in a minor decrease in TH-positive neurons at E11.5 and a reduction in SNc-MbDNs in the adult mouse brain [80].

The transcription factors ETV4 (ets variant 4, also known as Pea3) and ETV5 (ets variant 5, also known as Erm) as well as DUSP6 (dual specificity protein phosphatase 6) have been identified as targets of FGF signaling. Their expression pattern is restricted to MbDN progenitors in the posterior midbrain at
E10.5, later their expression is downregulated in all MbDN progenitors [96]. Besides serving as readout for FGF signaling, the functional significance of this posteriorly restricted expression is unknown.

3.3 Integration of temporal and spatial domains

As discussed above there is increasing evidence that MbDN progenitors are diverse in their gene expression and their potential to give rise to specific MbDN subsets – both in space and in time. In the future, it will be important to integrate these distinct temporal and spatial processes to obtain a more complete picture on the early developmental mechanisms contributing to MbDN diversity. For example, the apparent restricted fate potential of lateral Shh-expressing MbDN progenitors observed in fate-mapping studies (these progenitors are marked at E11.5) could potentially be a consequence of a temporally restricted fate potential of E11.5 MbDN progenitors (as documented in birhtdating studies) rather than their identity as a lateral MbDN progenitor. Conversely, the restricted fate potential of late-born MbDN progenitors could be based on differences in the neurogenic potential of the medial and lateral domains: if medial progenitors cease neurogenesis before lateral progenitors, birth dating at later stages would only label lateral progenitors and thus result in a biased contribution of the birth-dated MbDNs to the VTA. Single cell lineage tracing of MbDN progenitors at the beginning and towards the end of their neurogenic might shed light on the general fate potential of MbDNs [106]. Moreover, a neuroanatomically unbiased RNA-seq approach of MbDN progenitors might give further insights into which subgroups of MbDN progenitors exist at different time points.

4 DIVERSITY IN DOPAMINERGIC NEURONS DURING DIFFERENTIATION AND MATURATION

4.1 Mechanisms influencing cell fate

4.1.1 Signaling pathways

Of the three signaling pathways that are potentially involved in establishing diversity in MbDN progenitors (SHH, FGF8 and WNT), the WNT pathway appears to be the only one in which canonical signaling might still play a role in differentiated MbDNs [67,68,75,77,78,96] (for non-canonical signaling see the section on axonal pathfinding below). Mesman et al. showed in their analysis of the BAT-GAL reporter mouse that canonical WNT signaling is activated primarily in medially located MbDNs [78]. Inactivation of Ctnnb1 using a TH-Cre line leads to reduced neurogenesis and a reduced number of MbDNs in both the SNc and VTA (MbDN subset markers were not analyzed) [48]. However, in these conditional knock-out mice a subset of MbDN progenitors are also recombined. Thus, the alterations in MbDNs observed in this mouse model cannot unequivocally be attributed to the loss of canonical Wnt signaling in differentiated MbDNs.

The retinoic acid (RA) signaling pathway has been linked to the differentiation of SNc-MbDNs [107]. ALDH1A1 is involved in the generation of RA from retinol. As described above, ALDH1A1 is expressed in MbDN progenitors. In differentiated MbDNs at E14.5, Aldh1a1 appears to be confined to anterolateral MbDNs at E14.5 while its expression is largely restricted to the SNc and the ventromedial parts of the VTA in the adult brain [107,108]. The transcription factor PITX3 (Paired-like homeodomain transcription factor
3) directly regulates *Aldha1* expression in MbDNs and administration of RA can partially rescue the subpopulation of MbDNs that are lost in *Pitx3* deficient mice (see below) [107,109]. Maxwell et al. report that SNc-MbDNs are not lost but merely downregulate their TH expression in E14.5 *Pitx3* null mutants [110]. Thus, it appears that RA treatment might restore the identity of *Pitx3* deficient MbDNs, enabling their differentiation. It remains to be investigated whether RA signaling has a role independent of *Pitx3* (i.e. by analyzing whether the phenotypes of *Aldh1a1* and *Pitx3* loss-of-function mutants are similar).

### 4.1.2 Transcription factors

*PITX3* is generally assumed to be expressed in all differentiated MbDNs (but see: [110,111]). Maxwell et al. report that they can identify subgroups of MbDNs between E12.5 and E14.5 based on the temporal sequence of *PITX3* and TH expression: MbDNs in dorsomedial positions turn on TH prior to *PITX3*, while ventrolateral MbDNs express *PITX3* before TH [110]. Mice homozygous for the *Pitx3* null allele *aphakia*, show a progressive reduction in MbDNs in lateral positions starting at E12.5. In prenatal and adult *aphakia* mice, the great majority of SNc-MbDNs is lost, while VTA-MbDNs are reduced by about half [110,112,113]. These data suggest that SNc-MbDNs are more sensitive to the loss of *Pitx3* function than VTA-MbDNs. Several genes that are regulated by *PITX3* in MbDNs have been identified: *Th*, *Slc18a2* (vesicular monoamine transporter), member 2, also known as VMAT2), *Slc6a3*, *Drd2* and *Aldh1a1* are positively regulated by *PITX3*; *Dlk* (mitogen-activated protein kinase kinase 12, also known asa MAP3K12), *Cck* (Cholecystokinin) and *En1/2* are negatively regulated by *PITX3*. Some of these genes are expressed in either anterior (*Aldh1a1*) or posterior (*Cck*) MbDNs. In anterior MbDNs, *PITX3* regulates *Th*, *Drd2* and *Dlk* expression via ALDH1A1 mediated RA signaling, but downregulates *Cck* independently of RA signaling. In posterior MbDNs, the control of *Slc6a3*, and *Slc18a2* expression through *PITX3* is also independent of RA signaling [109].

The expression patterns of SOX6, OTX2 and ZFP503 in differentiated MbDNs have briefly been described in the section on MbDN progenitors; here we present a more detailed account: In MbDNs migrating away from their progenitor domain at E13.5, OTX2 is expressed at high levels in the lateral migratory stream. The most lateral subset of these migrating MbDNs expresses ZFP503. SOX6 is expressed medially and in a small number of lateral MbDNs that appear to be OTX2 negative. MbDNs that have reached the mantle layer and start to move laterally also express SOX6 (see Figure 2A for the migratory routes of MbDNs). At E18.5, when MbDNs have reached their final position in the SNc and VTA, SOX6 is mainly expressed in the SNc and in a few cells in the dorsolateral VTA, where its expression overlaps with SLC6A3 and KCNJ6. OTX2 is primarily expressed in the VTA (CALB1 positive, KCNJ6 negative cells); a subset of these cells co-express ZFP503 in the dorsolateral aspects of the VTA [22,30,89,90]. As summarized above, SOX6 inactivation in differentiated MbDNs leads to decreased innervation of the dorsal striatum. The function of ZFP503 in MbDNs remains to be investigated [30]. The inactivation of *Otx2* in differentiated MbDNs results in an increased percentage of KCNJ6/SLC6A3 positive MbDNs within the MbDN population that would normally express OTX2. In the converse experiment – overexpression of OTX2 in differentiated MbDNs – the percentage of SLC6A3- and OTX2-doublepositive MbDNs and of KCNJ6 positive VTA-MbDNs is decreased as compared to wildtype littermates.
Semiquantitative reverse transcription PCR and RNA *in situ* hybridization experiments indicate that OTX2 controls the expression of SLC6A3 through negatively regulating *Slc6a3* mRNA levels. In summary, these results imply that OTX2 regulates the subtype identity of MbDNs by its antagonistic effect on SLC6A3 and KCNJ6 positive MbDNs in the dorsoateral VTA [22].

Developing MbDNs transiently express early B cell factors (EBF1-3). Each EBF family member plays a distinct role in controlling differentiation or migration of MbDN subpopulations [114-116]. *Ebf1* is expressed in immature MbDNs at E11.5 and in SNc-MbDNs at E15.5. At later stages, *Ebf1* expression is downregulated. *Ebf1* deficient mice have severe defects in the formation of the SNc that are already apparent at E15.5. The total number of MbDNs is not changed in these mutants and the number of MbDNs in the VTA is slightly increased at birth. The distribution of MbDN subset markers (CALB1, KCNJ6, SLC6A3) has not been analyzed in these mutant mice [115]. *Ebf2* is transiently expressed in immature MbDNs (E12.5 to E14.5). Inactivation of *Ebf2* does not affect SN- or VTA-MbDNs, but results in reduced numbers of TH positive cells in the periaqueductal grey. The periaqueductal grey surrounds the cerebral aqueduct and contains a small number of MbDNs in its ventrolateral quadrant [117]. TH-positive projections to a small area within the olfactory tubercle appear to be specifically reduced in these *Ebf2* null mice (Yang:2015ko). Since neither cell death nor proliferation of MbDN progenitors are altered in *Ebf1* or *Ebf2* mutant mice, both EBF1 and EBF2 likely influence differentiation or migration of MbDN subsets [115,116]. *Ebf3* appears to be expressed in all developing MbDNs at E11.5, but by E18.5 there is little overlap between *Ebf3*- and *Th* expressing cells in the ventral midbrain [114]. EBF3 can promote the generation of TH-positive neurons from murine embryonic stem cells [118], but the function of EBF3 in the development of MbDN subtypes has not yet been investigated.

**4.2 Migratory routes of MbDNs and their differential response to stimuli**

MbDNs giving rise to the medial VTA or the SNc follow different migratory paths to reach their final target: neurons giving rise to the VTA migrate radially from the progenitor domain towards the pial surface. MbDNs that form the SNc undergo a three-step migration process: 1) migrate radially from the progenitor domain towards the pial surface, 2) migrate a short distance tangentially resulting in the segregation of SNc and VTA neurons, 3) undergo long distance tangential migration to reach the final position in the ventral-lateral midbrain. A likely prerequisite for the ability of SNc and VTA neurons to follow different migratory routes and reach their different positions is their differential response to migratory stimuli and
guidance factors. The extracellular matrix molecule Reelin plays an important role in migratory processes in several brain areas [120]. In mutants with inactivated Reelin signaling, the SNc is disorganized; SNc neurons do not reach their final lateral position and are clustered close to the VTA. The VTA shows no major defect in these mutants [121-124]. Consistent with a specific role of Reelin signaling in the migration of SNc-MbDNs, the expression of the intracellular effector of Reelin signaling, Dab1 (Disabled1), is restricted to laterally migrating MbDNs [124].

MbDNs undergoing radial migration express the chemokine receptor CXCR4 (C-X-C chemokine receptor type 4) between E11.5 and E17.5, while CXCR4 appears to be downregulated once the cells reach the pial surface and switch to tangential migration [124,125]. CXCR4 expressing MbDNs respond to the chemokine CXCL12 (chemokine (C-X-C motif) ligand 12, also known as SDF1), a factor acting as an attractant in the migration of several neuronal populations [126-128]. In the midbrain, CXCL12 is expressed in the meninges of the pial surface. Loss of CXCL12 signaling results in altered orientation of MbDN processes at E11.5, followed by a transient ectopic accumulation of a small number of MbDN neurons close to the progenitor zone during development [124,125].

Netrin 1 (NTN1), another known guidance molecule for migrating neurons, is expressed in the VTA and medial SNcs during embryogenesis [129-131]. Its receptor, DCC (Deleted in Colorectal Cancer) appears to be expressed in all MbDNs during migration [132]. Mice deficient for Dcc or Ntn1 have ectopic MbDNs in a dorsolateral position between the SNc and the VTA, suggesting a role of NTN1-DCC signaling in regulating MbDN migration [131,133]. However, the expression of CALB1/KCNJ6/SLC6A3 has not been assessed in these mice and thus it remains unclear whether NTN1-DCC signaling affects a particular subpopulation of MbDNs. Future studies combining precise labeling of MbDN subpopulations with time-lapse imaging and gene inactivation are required to fully understand the requirement of above mentioned molecules in regulating the distinct migratory pathways of different MbDN subsets.

4.3 Response to axonal pathfinding cues

The functional diversity of MbDNs has its roots in a complex circuitry. A precise regulation of MbDN axonal growth along the anteroposterior and dorsoventral axes during development is likely required to form these distinct circuits. The profile of cell surface receptors that allows MbDNs to respond to different guidance factors and segregate their projections should differ in distinct MbDN subpopulations. However, there are only a few detailed studies on the expression and function of known axonal guidance molecules in developing MbDNs. Netrin, Semaphorins, WNTs and SHH all have been directly implicated in establishing distinct MbDN connectivity and will be briefly discussed below. SLIT and Ephrin signaling might however also play a role in establishing diverse connections in the dopaminergic system, based on subset restricted expression patterns of ligands and receptors [30,135-141].

4.3.1 Netrins

In addition to regulating neuronal migration, Netrins are bifunctional axon guidance cues that act as attractants via the receptor DCC or as repellents through the UNC5 (unc-5 homolog) receptors (often co-
expressed with DCC) [130]. Both VTA and SNc axons are attracted to NTN1 and to the striatum in a DCC-dependent manner. However, the two subpopulations show a preference for different NTN1 concentrations. SNc-MbDNs show significant neurite outgrowth at low NTN1 concentrations, whereas higher NTN1 concentrations are required for an outgrowth response of VTA-MbDNs in explant cultures. This differential preference of SNc and VTA axons is consistent with data showing that Ntn1 is expressed at low levels in the dorsal striatum, the target area of SNc axons and at higher levels in the ventral striatum, the target area for VTA axons [131]. NTN1 is also expressed in the PFC [142]. Inactivation of Ntn1 or its receptor Dcc leads to midline crossing defects of MbDN axons, but the phenotypes in the terminal target area of MbDN projections differ in the two mutants: Ntn1 but not Dcc null mutants have a reduced innervation of the dorsal striatum. In Dcc null mutants, the innervation of the ventral striatum appears to be shifted dorsally and innervation of the PFC is reduced [133]. Mice heterozygous for Dcc show increased MbDN innervation in the PFC [142]. Whether Ntn1 null mutants have defects in mesocortical projections has not been investigated. Finally, MbDN-lateral habenula projections require NTN1 as a short-range attractant to innervate the lateral habenula. In absence of Ntn1 or Dcc, MbDN axons accumulate at the border of the lateral habenula [143].

4.3.2 Semaphorins

Semaphorins (SEMA) are secreted or membrane bound proteins, which are known to play a role in axonal guidance by interacting with their receptors plexins and neuropilins (NRP) [144]. Plexins A1 and A3 are expressed in MbDNs at E13.5 (in the rat). Nrp2 is expressed in an anterior subset of MbDNs in the medial VTA between E12.5 and E14.5, while NRP1 is diffusely expressed throughout the medial VTA. NRP1 and NRP2 are both expressed by MbDN axons either in combination or alone [145-148]. SEMA3A, 3C and 3F are expressed in the PFC at E14.5 [147], suggesting that SEMA/NRP-mediated guidance might regulate axon pathfinding of mesocortical MbDN projections. In addition, Sema3F is expressed in the dorsal midbrain and diencephalon during MbDN axonal outgrowth and in the mid/hindbrain boundary [146,147] indicating that it might be important for channeling MbDN axons toward the forebrain. MbDN neurite outgrowth is inhibited by SEMA3F in ventral midbrain explants [146,148]. Kolk et al. propose that MbDNs in the anterior medial VTA change their response to SEMA3F from repulsion to attraction as they develop: NRP1 initially mediate a repellent response necessary for axon fasciculation, subsequently they are required for an attractive response of mesocortical axons to SEMA3F [147]. However, Torre et al. did not detect MbDN axonal defasciculation in Nrp2 null mice, but they demonstrate that the projections of MbDNs to the lateral habenula are essentially lost in these mutants [148].

4.3.3 WNT and SHH

WNT proteins act through several different signaling pathways. While the canonical β-catenin dependent pathway regulates neuronal proliferation and differentiation, the WNT/planar cell polarity (PCP) pathway plays a role in the axonal organization of MbDNs [149, 150]. At E14.5, Wnt5a is expressed in a medial MbDN subset with highest levels posteriorly, whereas Wnt7b is expressed in all MbDNs with the
highest expression anteriorly. WNT5A repels SNc and VTA axons, whereas WNT7A attracts them in E12.5 explants cultures. Wnt5a null mice show only a transient defect in MbDN axonal projection, probably because of a compensatory effect of other WNTs. The PCP components Frizzled3 (FZD3), Celsr3 (cadherin, EGF LAG seven-pass G-type receptor 3, flamingo homolog) and Vangl2 (VANGL planar cell polarity protein 2) are expressed in all MbDNs from E11.5 onwards. Fzd3 null mice have a large subset of laterally located MbDN axons that are aberrantly oriented into posterior or dorsal directions or towards the midline. MbDN axons in these mutants do not respond to WNT5A or WNT7B and do not innervate the striatum or the PFC at E14.5 and E17.5 [150,151]. In Celsr3 null mice, a medial subset of MbDN axons reaches the striatum. In contrast, in mice deficient for Vangl2, MbDN axons innervate the striatum. One explanation for these differences in phenotypes is the potential redundancy of PCP components [150].

SHH is a known guidance cue for commissural axons in the spinal cord and for axons of retinal ganglion cells. SHH signals through a non-canonical pathway that includes the SHHco-receptor BOC (biregional cell adhesion molecule-related/down-regulated by oncopgenes (Cdon) binding protein), Src-family kinases, protein kinasce C and integrin linked kinase [152]. Shh expression in the ventral midline extends from the midbrain into the hypothalamus, where it acts as a guidance cue for a subset of MbDNs. Inactivation of the gene encoding for the SHH receptor Smoothened (SMO) at a time when SHH signaling does no longer play a role in MbDN progenitors (after E10.5) results in subtle defects in the guidance of ventrally coursing MbDN axons. In explant cultures, only MbDN axons in explants from the medial ventral midbrain are attracted to a source of SHH, indicating a differential sensitivity of medial and lateral MbDNs to SHH [153]. How this differential sensitivity is established remains unclear, since SMO is expressed in all MbDNs at E12.5. Whether non-canonical components of the SHH pathway are expressed in the medial subset of MbDNs has not been investigated.

In conclusion, it is still not well understood how a range of axon guidance molecules and the combinatorial expression patterns of their receptors establish the highly diverse connections of the dopaminergic system. Studying these different pathways in isolation is likely not sufficient to understand their impact on MbDN connectivity, especially in light of evidence that receptors for different guidance molecules have to interact to elicit a full downstream response [154].

5 CONCLUSION AND FUTURE DIRECTIONS

The systematic application of developmental genetic tools has been pivotal for advancing our understanding of neuronal diversity in several regions of the central nervous system, most notably in the cortex and the spinal cord. Thus, the combination of developmental and functional research approaches that are beginning to be applied to the dopaminergic system will deliver essential insights into what constitutes a particular MbDN subclass and how it is established during development. In the long-term, a comprehensive understanding of developmental mechanisms and gene expression patterns in combination with optogenetic tools for circuit analyses of genetically defined neurons will make it possible
to launch a systematic genetic dissection of the dopaminergic system in the mouse and to further increase our understanding of its development, organization and function. In addition, it will be of great interest to explore the role of epigenetic mechanisms in the generation of MbDN subtypes. Advances in single cell genomics and improved methods for the isolation of specific neuronal subtypes will open new doors to explore how epigenetic modifications add additional levels of complexity to neuronal diversity in the dopaminergic system.

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FIGURE LEGENDS

Figure 1. Midbrain dopaminergic neuron (MbDN) diversity in the adult mouse brain. (A) Schematic of MbDN nuclei projecting to their target areas in the forebrain; SNc: substantia nigra pars compacta; VTA: ventral tegmental area; RRF: retrorubral field; SNCD: SNc dorsal tier; SNCL: SNc lateral tier; SNCM: SNc medial tier; SNCV: SNc ventral tier; PBP: parabrachial pigmented nucleus; PN: paranigral nucleus; PIF: paraintersicular nucleus; IF: interorsicicular nucleus; dStr: dorsal striatum; vStr: ventral striatum; PFC: prefrontal cortex. (B) Anatomical organization of SNc and VTA at three anteroposterior levels (coronal view). Differently colored dots show the location of MbDN cell bodies with distinct projection targets (see C). Areas that contain KCNJ6/SLC6A3-expressing MbDNs have a light grey background; areas that contain CALB1-expressing MbDNs have a dark grey background. KCNJ6/SLC6A3: also known as GIRK2/DAT; CALB1: Calbindin 1; RLi: rostral linear nucleus; CLI: caudal linear nucleus of the raphe; NAc: nucleus accumbens; mShell: medial shell; iShell: lateral shell. (C) Schematics of MbDN projection target areas at three anteroposterior levels (coronal view). Schematics are based on [5,6,21-23].

Figure 2. MbDN development. (A) Timeline of MbDN development. MbDN progenitors are induced in the ventral midline of the midbrain. Subsequently, MbDNs differentiate, migrate, form projections and establish synaptic contacts with neurons in their forebrain target areas. SNc-MbDNs: dark green, VTA-MbDNs: light green, RRF-MbDNs: turquois. Coronal views illustrate mediolateral location of progenitors and MbDNs during development. Note that SNc-MbDNs are preferentially derived from medial progenitors, VTA-MbDNs from lateral progenitors. The arrows below the E10.5 coronal section indicate the migratory paths for SNc- or VTA-MbDNs. (B) Key signaling molecules and transcription factors involved in the different steps of MbDN development from progenitors to immature and to mature MbDNs. DDC: dopa decarboxylase. Other abbreviations are explained in the main text.

Figure 3. Key factors expressed in subsets of MbDN progenitors. (A) Coronal and sagittal schematics of the MbDN progenitor domain. The posterior diencephalon (pD) domain is hatched, since it has not been clarified whether these progenitors can give rise to mature MbDNs. L: lateral; M: medial; DV: dorsoventral axis; aMb: anterior midbrain; pMb: posterior midbrain; AP: anteroposterior axis. (B) Drawings illustrate dynamic and/or subset specific expression patterns of various factors expressed in MbDN progenitors between E9.5 and E11.5. The expressions patterns of the factors are organized from broad to restricted expression. Abbreviations are explained in the main text.
REFERENCES


90 Di Salvio M, Di Giovannantonio LG, Omodei D, Acampora D & Simeone A (2010) Otx2 expression is restricted to dopaminergic neurons of the ventral tegmental area in the adult brain. Int J Dev Biol 54,
939–945.
108 McCaffery P & Dräger UC (1994) High levels of a retinoic acid-generating dehydrogenase in the meso-felencephalic dopaminergic system. Proc Natl Acad Sci USA 91, 7772-7776


Figure 1

Projection diversity with corresponding target areas

- **SNC**: SNCL + SNCD + SNCM + SNCV
- **VTA**: dorsolateral VTA: PBP
- ventromedial VTA: PN+ PIF+IF
- **RRF**

**B**

- **dStr**
- **NAc Core**
- **NAc mShell**
- **NAc lShell**
- **Olfactory tubercle (Tu)**
- **prefrontal cortex (PFC)**
- **hippocampus**
- **basolateral amygdala (Bla)**

**C**

- **KCNJ6, SLC6A3**
- **CALB1**
**Figure 2**

The figure illustrates the process of spinal cord development, highlighting the transition from embryonic to postnatal stages. It focuses on the expression of various transcription factors and genes at different developmental stages (E10.5, E14.5, and adult).

**A**
- **Embryonic Development**
  - E10.5
  - E14.5
  - Adult

**B**
- **MbDN Progenitors**
  - SHH
  - WNT1
  - FGF8
  - ARX
  - FOXA1/2
  - LMX1A/B
  - OTX2

- **Immature MbDNs**
  - ALDH1A1
  - EN1/2
  - FOXA1/2
  - LMX1A/B
  - NR4A2
  - OTX2

- **Mature MbDNs**
  - DDC
  - EN1/2
  - FOXA1/2
  - LMX1B
  - NR4A2
  - PITX3
  - SLC18A2
  - TH

The diagram shows the induction, differentiation, migration, axonal pathfinding, maturation, and synaptogenesis processes during spinal cord development.
**A**

Coronal view

Sagittal view

*pD* or *pMb* domain, at low levels/in few cells

*M* or *aMb* domain

*L* or *pMb* domain, at low levels/in few cells

*pD* domain

outside of MbDN progenitor domain

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**B**

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<tr>
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