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THE ROLE OF LMX1A AND LMX1B IN REGULATING MESENCEPHALON DEVELOPMENT AND DOPAMINE NEURON SPECIFICATION

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We dance round in a ring and suppose, but the secret sits in the middle and knows.

Robert Frost
ABSTRACT
One of the most challenging questions in developmental biology is how neurons are specified, acquire their distinct characteristics and find their correct innervations to form functional circuits. The development of different subsets of neurons involves the expression of a program intrinsic to each cell type and the response to extrinsic environmental influences represented by soluble factors. Breakthroughs in the understanding of the genetic programs that controls the specification of ventral cell fates in the spinal cord and hindbrain, have provided useful tools for the study of similar genetic networks in the more complex rostral regions of the central nervous system, such as the mesencephalon (also called midbrain).

Midbrain dopamine (mDA) neurons are born in the ventral midline of the midbrain and regulate important functions in the brain, including motor control, cognition, emotions and learning. The degeneration of mDA neurons is the major hallmark of Parkinson’s disease (PD). The lack of knowledge regarding the factors involving in the early specification of mDA neurons has been one of the obstacles in applying embryonic stem cell (ESC)-based replacement therapy for PD. In paper I, we showed that Lmx1a and Msx1/2 are two key components in the development of mDA neurons. Lmx1a is necessary and sufficient for the acquisition of the proper mDA fate by activating the expression of downstream mDA neuron markers, while Msx1/2 synergizes with Lmx1a by suppressing alternative cell fates and promoting the progression of neurogenesis. Furthermore, we applied this knowledge to ESCs and showed that forced expression of Lmx1a could efficiently induce bona fide mDA neurons. In paper II, we continued to evaluate the role of Lmx1a in the mouse and compared the function of Lmx1a with its close homolog Lmx1b during mDA development. Surprisingly, loss of Lmx1a resulted in a moderate reduction of mDA neurons, which was partly due to the delayed conversion of floor plate into a neurogenic region at an early stage. Lmx1b could compensate to large extent for the loss of Lmx1a in mDA neuron generation as the compound genotype of the Lmx1 genes displayed a dose-dependent effect. Importantly, we showed that Lmx1a and Lmx1b have distinct roles in specifying two subgroups, i.e. medial and lateral mDA neurons. In addition, we revealed the function of Lmx1b in patterning other ventral cell types, i.e. oculomotor (OM) neurons and red nucleus (RN) cells. Loss of Lmx1b caused a dramatic reduction of OM neurons. By contrast, RN cells were born prematurely and were overproduced. Our current findings establish that Lmx1b influences the differentiation of multiple neuronal subtypes in the ventral
midbrain, while the activity of Lmx1a in the ventral midbrain appears devoted to the differentiation of mDA neurons.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:


* These authors contributed equally

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LIST OF ABBREVIATIONS

A-P Anterior-posterior
AVE Anterior visceral endoderm
ANR Anterior neural ridge
AADC Aromatic L-amino decarboxylase
bHLH basic Helix-loop-helix
BMP Bone morphogenetic protein
CNS Central nervous system
CLIM Cofactor of LIM domains
DA Dopamine
D-V Dorsal-ventral
DVE Distal visceral endoderm
DbH Dopamine β-hydroxylase
DAT Dopamine transporter
ESC Embryonic stem cell
eGFP Enhanced green fluorescence protein
FGF Fibroblast growth factor
FP Floor plate
GABA γ-aminobutyric acid
GDNF Glial cell line-derived neurotrophic factor
HD Homeodomain
ICM Inner cell mass
IsO Isthmic organizer
L-R Left-right
Lmx LIM homeobox gene
Ldb LIM-binding protein
mDA Midbrain dopamin neuron
MHB Mid-hindbrain boundary
Msx1 Msh-like homeobox gene 1
MN Motor neuron
NesE Nestin enhancer
OM Oculomotor
PD Parkinson’s disease
PS Primitive streak
RN Red nucleus
RA Retinoic acid
r1 Rhombomere 1
RP Roof plate
RRF Retrorubral field
Shh Sonic hedgehog
SNC Substantia nigra pars compacta
TGFβ Transforming growth factors beta superfamily
TH Tyrosine hydroxylase
vMB Ventral midbrain
VMAT2 Vesicular monoamine transporters 2
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-related MMTV integration site</td>
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<tr>
<td>ZLI</td>
<td>Zona Limitans intrathalamica</td>
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INTRODUCTION

Life begins with the fertilization of a single egg cell that gives rise to an entire organism through a complex series of processes including gastrulation, neurulation, regionalization and patterning. A longstanding interest of developmental biology is to understand the details of these events, especially the coming-into-being of the central nervous system (CNS) that is the most important controlling center of our bodies. The CNS consists of an extensive diversity of cell types that can be divided into neurons and glial cells. Glial cells constitute 90% of the brain. It has been increasingly appreciated that glial cells have more important functions in addition to provide support and protection for the neurons in the brain. However, neurons are still the most crucial components of the CNS. There are about 100 billion neurons in the brain. They process and transmit information through cell-cell networks to conduct the general functions of the brain.

Neurons are the progeny of neuroepithelial cells, which have a broad potential to give rise to all types of neurons in the CNS. However, this ability becomes restricted over time as a result of instructive positional cues. Once a neuron is born, its basic fate becomes fixed and its subsequent maturation proceeds according to the context that it resides in. Ultimately, specific neuronal subtypes become located in stereotypic regions of the brain to perform the corresponding functions.

With the exception of a few specific sites in the brain, such as hippocampus, the lateral subventricular zone and the olfactory bulb, neurons in adult brain cannot be regenerated to any larger extent after insult or injury. Consequently, the loss of particular neuronal subtypes results in corresponding disorders of the CNS, such as neurodegenerative diseases.

Parkinson’s disease (PD) is a neurodegenerative diseases caused by the progressive loss of dopamine (DA) neurons that are located in the substantia nigra in the ventral midbrain (vMB). It is the second most common neurodegenerative disorder of the CNS but so far there is no cure. However, transplantation of fetal ventral MB tissues has provided a proof-of-principle that cell replacement therapy may be a promising approach. The main focus of this thesis is to unravel the molecular and cellular mechanism of the specification of DA neurons in the vMB and to apply this knowledge to stem cells (e.g. ESCs) for future cell replacement therapy for PD. More specifically, we have studied in detail the transcription factors Lmx1a and Lmx1b that play important roles during the patterning and DA neuron generation in the vMB.
Gastrulation - formation of the three germ layers

Upon fertilization of an egg, the zygote undergoes serial rounds of cell cleavage to become a blastocyst. Before implantation, the blastocyst comprises of an outer cell layer called trophectoderm, surrounding a cluster of cells termed the inner cell mass (ICM), from which pluripotent ESCs are derived (Ralston and Rossant, 2005; Rossant and Tam, 2009). The trophectoderm gives rise to the placenta and portions of the pariental yolk sac whereas the ICM forms the embryo proper as well as the extraembryonic mesoderm. At the late blastocyst stage, a third lineage, the primitive endoderm cells are sorted out from the ICM (Niwa et al., 2005). Shortly after implantation into the uterus, the ICM goes through rapid proliferation and develops further into the primitive ectoderm, later called the epiblast as it differentiates to form a pseudostratified columnar epithelium lining the proamniotic cavity. Coinciding with the development of the epiblast, the primitive endoderm gives rise to the visceral endoderm—a tissue that surrounds the epiblast and pariental endoderm. Both are important sources of signals for future embryonic patterning (Arnold and Robertson, 2009; Gilbert, 2006; Rossant, 2004). See Box1A.

Gastrulation begins with the formation of the primitive streak (PS) at the border of the epiblast-extraembryonic interface. It is a process of the formation for the three germ layers which requires extensive movement of cells from the epiblast through the PS. Epiblast cells undergo an epithelial to mesenchymal transition, mobilize and transverse through the PS and then allocate between the epiblast and the visceral endoderm to become a new cell layer, i.e. the mesoderm. With the elongation of the PS towards the distal end of embryo, distinct mesodermal cell lineages become designated according to the time and site of ingestion through the PS. It is known that epiblast cells which enter through the posterior PS give rise to the extra-embryonic mesoderm as well as the visceral yolk sac mesoderm and blood islands. Lateral plate, paraxial, cranial and cardiac mesoderm appear slightly later from the intermediate and anterior levels of the PS. Finally, epiblast cells that migrate through the extreme anterior tip of the PS give rise to midline axial mesendoderm tissues that comprise the precordal plate, the notochord and the node, as well as the definitive endoderm cell lineage. As more definitive endoderm is incorporated into the pre-existing visceral endoderm layer, it progressively displaces the visceral endoderm anteriorly and proximally while covering the distal side of the embryo with a sheet of definitive endoderm. In contrast to
mesoderm and definitive endoderm, ectoderm is derived from the region of the epiblast that does not enter the PS. These three germ layers constitute the progenitor cells from which all fetal tissues will develop (Arnold and Robertson, 2009; Gadue et al., 2005; Gilbert, 2006; Tam and Loebel, 2007; Zernicka-Goetz et al., 2009). See Box1B.

**Box1:** (A) Epiblast and primitive endoderm lineages segregate from ICM in the blastocyst before implantation based on the gene expression of either GATA6 or Nanog. After implantation, the ICM, primitive endoderm and trophectoderm further proliferate and differentiate to form more defined structures. The blastocyst is transformed into the cup-like embryo cylinder. (B) Half a day after the emergence of the PS, cells continue to delineate from the epiblast and ingress into the PS to allocate between the epiblast layer and the visceral endoderm (hypoblast) along with the extension of the PS toward the distal tip of the embryo. Thus, three germ layers are formed and start to be specified into different tissues of the embryo in future. (Pictures are modified from Sebastian J. Arnold and Elisabeth J. Robertson 2009; Cindy C Lu et al., 2001; http://embryology.med.unsw.edu.au/Medicine/images/streak.jpg)

**Neural induction- making a commitment**

Neural induction constitutes the initial step in the generation of the vertebrate nervous system. Neural tissue is derived from the embryonic ectoderm, which also gives rise to
the epidermis. Therefore, each cell in the embryonic ectoderm undergoes a process of fate determination.

The early studies on neural induction were mostly performed in amphibian embryos. There it was discovered that transplantation of the most-dorsal lip of the amphibian blastopore (later called the Spemann organizer) to the ventral side resulted in the generation of a secondary CNS (Spemann, 1943; Spemann and Mangold, 2001). Since then, researchers had tried to find the “inductive signals” secreted from the organizer itself without success. This leads to the idea that the formation of neural tissue might be induced by the removal of some unknown inhibitory signals (Grunz and Tacke, 1989; Hemmati-Brivanlou and Melton, 1992; Sato and Sargent, 1989). Later, three BMP (Bone Morphogenetic Protein) antagonists, Noggin, Chordin and Follistatin were found to be expressed in the organizer and could neuralize animal cap explants of Xenopus (Hemmati-Brivanlou et al., 1994; Sasai et al., 1994; Smith and Harland, 1992; Smith et al., 1993). These findings led to the “default model” of neural induction proposing that cells within the ectoderm layer of the frog gastrula have an autonomous tendency to differentiate into neural tissue, which is otherwise inhibited by BMPs (Hemmati-Brivanlou and Melton, 1997; Stern, 2005).

However, with research carried further, the default model may be a little too simplistic and was challenged by some contradictory findings mostly in amniotes model systems such as chick and mouse. One study showed that some neural tissues could still be induced even when the node (i.e Spemann organizer in amphibian) was physically removed or genetically ablated (Ang and Rossant, 1994; Klingensmith et al., 1999). Another study demonstrated that the timing and expression pattern of BMP inhibitors did not correlate with the initiation of neural tissue (Wilson et al., 2000). Lastly and very importantly, BMP inhibitors are not sufficient to induce neural fate in epiblast explants (Streit et al., 2000; Streit et al., 1998), which means that BMP inhibitors might be required to provide a permissive environment for the induction of neural tissues but are not sufficient to do so alone.

Subsequently, it was revealed that FGFs ( Fibroblast Growth Factors) and WNTs (Wingless-related MMTV integration site) are involved in the neural induction process. Expression of dominant negative FGF receptors in animal cap cells in *Xenopus* blocks the ability of Noggin or Chordin to induce neural cells (Launay et al., 1996). FGF alone
can also directly induce neural fate in chick (Rodriguez-Gallardo et al., 1997) and in zebrafish (Kudoh et al., 2004) in the absence of other signals. One mechanism by which FGF signaling acts is via phosphorylation of a linker region in the BMP effector Smad1 which causes a downregulation of BMP signaling (De Robertis and Kuroda, 2004). However, it has been shown that BMP inhibitors induce anterior neural plate, while FGFs induce posterior neural plate (Furthauer et al., 2004; Rentzsch et al., 2004). The other mechanism of FGF signaling in neural induction is BMP independent and requires inhibition of the WNT pathway (Wilson et al., 2001). High level of WNT signaling can block the response of lateral epiblast cells to FGFs, and together with BMP signaling promotes an epidermal fate. When WNT signaling is attenuated, FGFs together with BMP inhibitors can transform the later epiblast cells to acquire neural fate (Wilson and Edlund, 2001; Wilson et al., 2001).

In reality, neural induction is a much more complicated process than what I have summarized here. It is a cascade of sequential events and requires cooperation of many different signaling pathways other than FGF, WNT and BMP. An increased understanding of the mechanisms of neural induction can be helpful in stem cell research in order to facilitate neural differentiation followed by producing certain neuronal subtypes that can be used to treat neurodegenerative disease, to understand the disease mechanisms and to screen for new drugs etc (Munoz-Sanjuan and Brivanlou, 2002). For example, treatment of human ESCs with Noggin greatly induces neural differentiation and prevents formation of extra-embryonic endoderm (Baharvand et al., 2007; Gerrard et al., 2005; Pera et al., 2004). In addition, FGF2 is used routinely in human ESCs cultures to promote the proliferation of neural progenitors and stabilize neural identity (Carpenter et al., 2001; Elkabetz et al., 2008; Okabe et al., 1996).

**Anterior-posterior patterning and regionalization of the neural tube**

Establishment of the body plan is a critical event during early embryonic development. Vertebrates have three body axes: anterior-posterior (A-P), dorsal-ventral (D-V), and left-right (L-R). The L-R axis is the last one to be determined, at least in the mouse. Recent studies have shown that L-R asymmetry is generated *de novo* by cilia at the embryonic node on the basis of pre-existing A-P and D-V information (Buceta et al., 2005; Okada et al., 2005). The cilia are tilted posteriorly and rotate in a clockwise direction which results in a leftward fluid flow (Nonaka et al., 2005).
unidirectional flow causes an uneven distribution of signaling factors such as Nodal, which in turn leads to asymmetric positioning of internal organs (Levin, 2005; Wright, 2001). Here, I will briefly summarize the regionalization of the neural tube along the A-P axis. The D-V patterning will be discussed in the next section.

The most prominent feature of the A-P patterning is the segmentation of the neural plate into forebrain, midbrain, hindbrain and spinal cord. The A-P axis is already determined prior to the gastrulation when Wnt3 expression is restricted to the posterior epiblast, where the PS will be induced. This process involves WNT and Nodal signaling (Morkel et al., 2003; Robertson et al., 2003). At the same time as the PS forms, a group of distal visceral endoderm (DVE) cells become specified as a signaling center and rapidly migrate towards the prospective anterior side of the embryo, thus establishing the anterior end of the embryo. The gross movement of the DVE creates the anterior visceral endoderm (AVE, see Box 1A). AVE is a head organizer and expresses secreted BMP antagonists such as noggin and follistatin, WNT antagonists including Dkk1, Frzb-1 and Crescent, as well as the Nodal antagonist activin and Lefty (Niehrs, 2004). It is an important neural-inducing tissue that promotes forebrain identity and protects anterior neural ectoderm from posteriorizing factors secreted from the PS. Besides, the AVE is also involved in the induction and spatial restriction of a number of anteriorly expressed genes such as Otx2, Lim1 and Hesx1 in order to consolidate the anterior neural properties (Acampora et al., 1995; Matsuo et al., 1995; Shawlot and Behringer, 1995).

The AVE is required for the formation of the forebrain. The question is then how the caudal region of neural plate is specified? The “activation-transformation model” was first proposed by Nieuwkoop. He suggested that nascent neural tissue adopts an anterior identity by default (a process that he referred to as “activation”), and that the posterior nervous system is subsequently generated through a process that he called “transformation” (Nieuwkoop, 1985; Nieuwkoop and Weijer, 1978; Rallu et al., 2002; Stern et al., 2006). The transformation signals are now known to be WNTs, FGFs and Retinoic acid (RA), that are derived from the node and the underlying mesoderm. Downstream of these posteriorizing signals, transcription factors such as members of the Hox gene family, are expressed from the rostral hindbrain to the tip of the tail. The combination of Hox gene expression is thought to be the most important instruction to
impose a posterior positional identity (Mallo et al., 2010; Young and Deschamps, 2009).

Subsequent to the A-P patterning, several distinct transverse domains are coarsely partitioned along this axis. The anterior neural tube balloons into three primary vesicles: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). Posteriorly, the long, uniformly narrow tube forms the spinal cord. These early morphological features of the neuroaxis accompanied by position-specific expression of developmental control genes dictate the overall plan of the CNS and predict its regional specializations (Lumsden and Krumlauf, 1996; Rallu et al., 2002).

The forebrain, midbrain and hindbrain are segmented by the transverse boundaries and have distinct histogenic competence based on gene expression patterns as well as morphological information (Rubenstein et al., 1994). These transverse zones have differential competence in responding to similar signaling cues such as Sonic hedgehog (Shh) that is expressed along the entire A-P axis. These zones are further specified by local signaling centers, i.e the anterior neural ridge (ANR) at the anterior end of the neural tube, the zona limitans intrathalamica (ZLI) in the middle of the diencephalon and the isthmic organizer (IsO) at the mid-hindbrain boundary (MHB). See Box2A. I will further describe these three signaling centers in the following part with the emphasis on the IsO, which participates in the DA neuron specification in the vMB.

**Important local signaling centers in CNS**

*Along the A-P axis*

A local signaling center is a group of cells emitting distinct morphogens responsible for the specific induction and/or patterning of neighboring tissues (Nieto, 1999). The ANR is the most anterior signaling center, first described in zebrafish (Houart et al., 1998). Fgf8 is expressed very early in ANR cells and is crucial for the specification of the anterior areas of the forebrain. Fgf8 is necessary for the induction and/or maintenance of FoxG1 (Bf1) expression, which in turn, is essential for forebrain precursor proliferation (Rubenstein and Beachy, 1998; Shimamura and Rubenstein, 1997). Furthermore, Fgf8 also regulates forebrain regional patterning through cooperation with WNT and Shh (Aboitiz and Montiel, 2007; Kuschel et al., 2003). The BMP antagonists
chordin and noggin also promote the inductive and trophic activities of the ANR in early development of the mammalian forebrain (Anderson et al., 2002).

The diencephalon develops from the caudal forebrain. It divides caudally to rostrally into three transverse domains called prosomere 1 to 3 (p1-3). The ZLI appears early on in the neural tube and locates between p2 and p3. The ZLI exhibits a unique pattern of molecular expression and is an important signaling center in diencephalic histogenesis and later on in thalamus patterning (Martinez and Puelles, 2000). WNT signaling is required for the initial positioning of the ZLI by induction of Irx3 and Six3 expression, which border the ZLI posteriorly and anteriorly, respectively (Braun et al., 2003; Lagutin et al., 2003; Lim and Golden, 2007). After the establishment of ZLI, the expression of Shh in ZLI activates the morphogenetic properties of this organizer, specifying in turn the compartmentalization and cell fate of the different diencephalic prosomeres through the control of specific gene expression (Kiecker and Lumsden, 2004; Lim and Golden, 2007). Gbx2 is expressed caudally to the ZLI and serves as a marker for the thalamus. Dlx2 and Nkx2.1 are expressed rostrally to the ZLI and are markers for the hypothalamus. Nkx2.2 and Fgf15 are expressed at both rostral and caudal sides. The combinatorial effects of these transcription factors contribute to the complexity of thalamic molecular regionalization (Bulfone et al., 1993; Chen et al., 2009; Kobayashi et al., 2002; Marin et al., 2002).

The IsO is localized at the mid-hindbrain boundary and controls patterning of the midbrain and the anterior hindbrain. It also plays an important role to instruct the specification and normal development of neuronal subtypes such as DA neurons and serotonergic neurons in the vMB and hindbrain, respectively (Brodski et al., 2003). Initially the organizing activity of the IsO was shown by transplantation experiments where IsO grafts, after transplantation to the diencephalon, could transform the local fate of the diencephalon into MB optic tectum (Bally-Cuif et al., 1992; Bally-Cuif and Wassef, 1994; Martinez et al., 1991). Subsequently, Fgf8 was identified to be expressed by the IsO during early development and it was shown that this signaling molecule can mimic the isthmic activity (Crossley et al., 1996). Fgf8 is also required for cell survival around the IsO. Removal of Fgf8 in zebrafish and mice results in deletion of tectum and cerebellum regions (Brand et al., 1996; Meyers et al., 1998; Reifers et al., 1998). Fgf8 binds to its tyrosine kinase receptor, Fgfr1 and subsequently, the signal is relayed through the Ras-ERK signaling pathway (Sato and Nakamura, 2004). Furthermore, a
strong Fgf signal mediated by Fgf8b can induce cerebellar development and a lower level of signaling transduced by Fgf8a, Fgf17 and Fgf18 can induce MB development (Liu et al., 2003; Liu et al., 1999; Sato et al., 2001). Wnt1 is also secreted from the IsO region, but from a more anterior part than Fgf8. The expression of Wnt1 is initiated earlier than Fgf8 and is observed throughout the entire MB at the early somite stages. Wnt1 expression gradually becomes restricted to the posterior MB and dorsal midline of the CNS excluding rhombomere 1 (r1) of the hindbrain. Wnt1 mutant mice have a deletion of most of the MB and cerebellum and do not maintain Fgf8 expression (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). However, Wnt1 does not have isthmic-like activity as Fgf8 does.

Before the onset of Fgf8 and Wnt1 expression, the transcription factors Otx2 and Gbx2 are expressed in a complementary manner to position the IsO where Fgf8 and Wnt1 are going to be expressed later on. At an early developmental stage (around E7.5 in mouse), Otx2 and Gbx2 expression domains are still fuzzy and overlap slightly, but later (around E9 in mouse), the expression of these factors rapidly forms a sharp border that resides anterior to Fgf8 (Broccoli et al., 1999). Thus, Fgf8 overlaps with the anterior domain of Gbx2 expression. The shift of the Otx2-Gbx2 boundary causes a shift of the IsO and the expression of Fgf8 and Wnt1 correspondingly (Hidalgo-Sanchez et al., 2005). However, in either Otx2 and Gbx2 single or double mutant mice, Fgf8 and Wnt1 expressions are still induced and maintained (Martinez-Barbera et al., 2001). Actually, Lmx1b was shown to be essential for the initiation of Fgf8, as loss of Lmx1b leads to complete deletion of Fgf8 expression in the IsO (Guo et al., 2007). Interestingly, in chick, Lmx1b represses Fgf8 cell autonomously while induces Fgf8 non-cell autonomously through activating Wnt1 (Adams et al., 2000; Matsunaga et al., 2002). Transcription factors such as Pax2/5, En1/2 are also involved in the IsO gene cross-regulatory network. En1/2 and Pax2/5 are expressed in a gradient towards the MB and the r1, with a peak in the IsO. The expression of En1, Pax2 and Wnt1 is initiated prior to Fgf8, while En2 and Pax5 expressions are initiated after Fgf8 (Chalepakis et al., 1993; Rowitch and McMahon, 1995). En1 and En2, as well as Pax2 and Pax5 have redundant functions. Mice lacking both En1 and En2, or Pax2 and Pax5 display total loss of the MB and cerebellum (Hanks et al., 1995; Schwarz et al., 1997). Over-expression of En1/2 and Pax2/5 in chick showed that these proteins can regulate each other’s expression, as well as Fgf8 through forming a positive feedback loop (Sato et al., 2004). Meanwhile, several lines of evidence imply that En1/2 and Wnt1 are
involved in regulating each other’s expression although it is not clear whether this regulation is direct or indirect (Araki and Nakamura, 1999; Ristoratore et al., 1999). In addition to the positive regulatory loops that are present, there are also negative regulators such as Sprouty to Fgf8, Grg4 to En1/2 and Pax2/5, which exist to fine tune the IsO region (Minowada et al., 1999; Sugiyama et al., 2000). Interestingly, a recent study used a computational technique to predict the interplay of the various regulatory interactions in the IsO (Wittmann et al., 2009). The gene expression pattern around the IsO and the self-sustaining genetic network are summarized in Box2B.

**Box2:** (A) Schematic drawing of a mouse brain showing the subdivisions in side view. Local signaling centers: The ANR, ZLI and IsO are indicated in green, yellow and blue respectively. DA neurons in red are born anterior to the IsO in the vMB. (B) Gene expression patterns surrounding the IsO at E9.5 in the mouse. Otx2 and Gbx2 regulate each other negatively, leading to the establishment of the IsO. Gbx2 maintains Fgf8 expression, whereas Otx2 and Fgf8 repress each other. Meanwhile, the expression domains of Fgf8, Wnt1, En1/2 and Pax2/5 become interdependent and form a positive regulatory loop. Two negative feedback factors, Sprouty and Grg4 serve as brakes to restrict the expansion of the positive regulatory loop (the picture is modified from Wurst and Bally-cuif, 2001).

**Along D-V axis**

The D-V axis develops later than the A-P axis and is induced by the surrounding/underlying non-neural tissues such as the mesoderm. After the regression of the node, the notochord takes place as the axial mesoderm from the MB to the end of the neural plate. Anterior to the notochord, the axial mesoderm is called the prechordal plate. Both structures secrete Shh, which instructs the overlying neural plate to adopt a ventral fate. Consequently, a specialized structure in the neural plate, called the floor plate (FP) is induced and specified (Dodd et al., 1998; Placzek et al., 2000). Meanwhile, the neural plate rolls up to form the neural tube. When the two lateral tips
of the neural plate meet, the epidermis separates from the neural tube and secretes BMP and WNT to induce the roof plate (RP) formation at the tip of the underlying neural tube. The D-V axis together with the A-P axis creates the Cartesian coordinate to tell neurons where to reside and what to become.

The FP is a specialized glial structure located in the most ventral midline of the neural tube all the way from the MB into the tail region (Strahle et al., 2004). It controls neuronal subtype specification along the D-V axis through secretion of Shh, and axonal wiring through the secretion of the axon guidance cues Netrin-1 (Giger and Kolodkin, 2001; Jessell, 2000). The function of the FP as a ventral organizer of neural development is evolutionarily conserved from fish to mammals (Colamarino and Tessier-Lavigne, 1995; Tanabe and Jessell, 1996). Over the years, the mechanism of FP induction and the heterogeneity of the FP along the A-P axis have been extensively studied.

In the classic FP induction model, FP cells are considered to differentiate from neuroepithelial cells that occupy a ventral midline position, and are induced to a FP fate under the influence of Shh secreted from the underlying notochord cells (Placzek et al., 1990). Gain-of-function experiments in chick showed that Shh can induce the ectopic differentiation of FP cells in the neural plate in vitro (Marti et al., 1995; Roelink et al., 1994), while blockage of Shh in the notochord eliminates its ability to induce FP cells (Ericson et al., 1996). Furthermore, mutant mice lacking Shh or the components of the Shh signaling pathway, display defects in FP differentiation (Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998). Several recent studies have, however, challenged this model. Le Douarin and colleagues’ work indicates that the FP and the notochord may share common precursors. The analysis of chick-quail chimeras suggested that FP cells derive from pre-specified cells that intercalate from the node into the neural midline (Catala et al., 1996; Le Douarin and Halpern, 2000; Teillet et al., 1998). However, these discrepancies between different studies may be due to vertebrate species, embryonic stages and A-P levels chosen for the analyses.

The other challenge to the paradigm of notochord/Shh-mediated FP induction arose through observations in zebrafish embryos. Wild-type zebrafish embryos develop with two morphologically and molecularly distinct populations of FP cells: medial FP cells and lateral FP cells. Embryos mutant for either sonic you (syu) or you-too (yot), which
encode Shh and Gli2 respectively, lack lateral FP but have medial FP cells (Schauerte et al., 1998). Similar phenotypes are produced by several other mutations thought to disrupt components of the Hedgehog signaling pathways (Odenthal et al., 2000). Conversely, medial FP specification appears to require integration of Nodal and Notch signaling (Feldman et al., 1998; Gritsman et al., 1999). In addition, these studies indicate the heterogeneity in FP cells along the mediolateral axis.

Recent studies provide more and more evidence that FP cells also show distinctions along the A-P axis. Morphological and molecular variability can be found at different A-P levels (reviewed in (Placzek and Briscoe, 2005)). Interestingly, FP cells are ependymal-like cells, which were thought to be non-neurogenic and not to divide (Kingsbury, 1920). However, studies have revealed that FP cells in the vMB have neurogenic activity and generate dopamine neurons (Andersson et al., 2006b; Ono et al., 2007). The mechanisms underlying the control of neurogenic potential in the vMB FP cells begin to be revealed (Joksimovic et al., 2009; Ono et al., 2010) and will be further discussed in the “Results and Discussion” section.

The RP is a critical dorsal signaling center that occupies the dorsal midline of the developing CNS along its entire A-P axis. During neural tube development, the RP produces proteins of the BMP and WNT families controlling proliferation, specification, migration, and axon guidance of adjacent dorsal interneurons (Chizhikov and Millen, 2005). Downstream of these signaling factors, transcription factors such as Lmx1a, Lmx1b and Msx1/2 relay the information to consolidate the RP function (Chizhikov and Millen, 2004a, b, c). Interestingly, these genes are also involved in the vMB DA neuron developmental program, which I will come back to in “Results and Discussion” part.

**DA synthesis and clusters of DA neurons**

DA is a catecholamine neurotransmitter in the brain. Its function as a neurotransmitter was first discovered in 1957 by Arvid Carlsson (Carlsson et al., 1957; Carlsson et al., 1958). DA is synthesized in the body first by the hydroxylation of the amino acid L-tyrosine to L-DOPA via the enzyme tyrosine hydroxylase (TH). L-DOPA in turn is decarboxylated by aromatic L-amino acid decarboxylase (AADC). In some other catecholamine neurons, DA is further processed into norepinephrine by dopamine β-
hydroxylase (DBH). After synthesis, DA is packaged into vesicles by the vesicular monoamine transporter 2 (VMAT2), and DA is then released into the synapse in response to a presynaptic action potential and acts on its receptors D1-5 (D1R-D5R) depending on its location, e.g. D1R and D2R are highly expressed in the striatum. Re-uptake of DA into neurons by the dopamine transporter (DAT) and auto-regulation by D2R eliminate excess DA from the synaptic clefts and fine-tune release from dopamine terminals, respectively. DA is degraded into the inactive metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by catechol-o-methyl transferase (COMT) and monoamine oxidase (MAO). See Box3.

According to the nomenclature of Dahlstrom and Fuxe (Dahlstrom and Fuxe, 1964), there are a total of nine clusters of DA neurons, A8-A16 in the brain. A8-A10 is localized in the vMB, which accounts for 75% of total number of DA neurons. A11-A15 are found in the diencephalon and A16 is situated in the olfactory bulb (Bjorklund and Dunnett, 2007). DA as a neurotransmitters plays an important role in many aspects of brain functions including cognition, voluntary movement, motivation, reward, learning, mood and memory (Lang and Lozano, 1998a, b) (See Box4A).

**Midbrain DA neuron circuit and neurogenesis gradient**

The three clusters of DA neurons in the MB are the substantia nigra *pars compacta* (SNc:A9), the ventral tegmental area (VTA:A10) and the retrorubral field (RRF:A8) (Björklund A, 1984). DA neurons in the SNc degenerate in PD, while those in the VTA and RRF are less affected (Fearnley and Lees, 1991). Anatomically, the SNc is situated in a more rostral and lateral position of the vMB compared to the VTA, and the RRF is located dorsally and caudally to the SNc. Different models for cell genesis and
migration of the SNC, RRF and VTA in the MB have been described. The first model stated that DA precursors are generated in the neuroepithelium of the medial part of the MB and migrate ventrally along the process of radial glial cells and then laterally along the tangentially arranged nerve fibers. Around E15 in mice, these cells form a stream of an inverted Y shape and can be distinguished as the VTA and the SNC (Kawano et al., 1995). In the second model, the precursors of the SNC locate in the middle third of the basal plate, while, for VTA, the precursors locate in the medial one third of the basal plate. Subsequently, SNC and VTA neurons migrate in “parallel rows” radially (Hanaway et al., 1971). So far, there is no general consent although the study of the Pitx3-deficient aphakia mouse mutant supports the latter model (Smidt et al., 2004). Our study (see paper II) also indicates that the mDA progenitor domain has two different subpopulations that in turn give rise to medially or laterally located DA neurons. This is in line with the second model.

A9 DA neurons mainly innervate the dorsolateral striatum (sensorimotor related), forming the mesostriatal pathway. They play a pivotal role in regulating voluntary movement. A10 neurons are involved in the mesocortical and mesolimbic pathways that project to the frontal cortex and the medial ventral striatum. They regulate the process of reward, drug addiction, learning and memory (Bjorklund and Dunnett, 2007; Haber and Fudge, 1997; Van den Heuvel and Pasterkamp, 2008). A8 neurons project to the SNC and VTA and seem to be involved in interconnecting these two areas. They also project to the dorsal striatum via the nigrostriatal pathway, see Box4B (Arts et al., 1996; Ungerstedt, 1971; Wallen and Perlmann, 2003).

Box4: (A) Distribution of nine clusters of DA neurons in the developing rodent brain with A8-10 located in the vMB. (B) In adult rodent brain, these DA cell groups are positioned further apart. The specific projections of the different DA neuron groups to their targets are displayed by arrows. LGE: Lateral ganglionic eminence. (This picture is adapted from Björklund and Dunnet, Trends in Neuroscience, 2007)
The neurogenesis of DA neurons in the vMB extends from E10 to E14, with a peak at E11 and E12 in the mouse (Bayer et al., 1995b). A birth dating study showed that there is a neurogenic gradient within and between the SN and the VTA (Bayer et al., 1995a). In general, the SNc contains more early-generated neurons, while VTA contains more late-generated neurons. Furthermore, lateral parts of the SNc and VTA have a higher proportion of early generated neurons, while medial parts have a higher proportion of late-generated neurons. In addition, the anterior parts of the SNc and the VTA appear to have a higher percentage of early-born neurons than the posterior parts (Bayer et al., 1995a). Consequently, the neurogenic gradient appears to be composed of two axes, i.e. anterior to posterior and lateral to medial.

Summary of midbrain DA neuron development

Extrinsic signaling

Shh

The D-V axis of the MB is specified by the expression of Shh. Shh is expressed in the FP, and as such specifies a ventral identity by activating the downstream transcription factors Msx1/2 and Lmx1a in the vMB (paper I). Shh is necessary and required to induce mDA neurons (Hynes et al., 1995; Ye et al., 1998), but only until E10.5. After that, this function is instead relayed by Foxa1/2 (Ferri et al., 2007; Lin et al., 2009; Perez-Balaguer et al., 2009). Accordingly, we and others have found that Shh must be downregulated in the FP of the vMB to allow neurogenesis of mDA neurons (paper I, (Joksimovic et al., 2009; Ono et al., 2007)).

Fgf8

The A-P axis of the MB is specified by the expression of Fgf8. The duration as well as the strength of Fgf8 signaling is the key to patterning of the IsO region, which in turn, regulates the development of mDA neurons (Guo et al., 2010; Sato and Joyner, 2009). Fgf8 together with Shh is capable of inducing mDA neurons in a non-MB context, as shown by experiments using rostral forebrain explants (Wang et al., 1995; Ye et al., 1998). A combination of Shh and Fgf8 is now used as a standard method to differentiate ESCs into mDA neurons in vitro (Barberi et al., 2003; Kim et al., 2002). Inactivation of Fgf8 in the IsO results in the loss of tectum and cerebellum (Meyers et al., 1998; Reifers et al., 1998). In addition, Fgf8 is essential for the cell survival in the
MB and r1 (Chi et al., 2003) and directs the growth of axons from mDA neurons rostrally by inducing the repulsion factor *semaphorin 3F* (Yamauchi et al., 2009).

Wnts

The functions of Wnt signaling in mDA neuron generation have been studied extensively. Wnt1 is required to maintain the integrity of the MB (McMahon et al., 1992; Thomas and Capecchi, 1990) and the generation of mDA neurons (Panhuysen et al., 2004). Ectopic expression of Wnt1 in the rostral hindbrain results in the induction of ectopic mDA neurons through the activation of Otx2, which in turn represses Nkx2.2 expression and the generation of serotonergic neurons (Prakash et al., 2006). *In vitro*, Wnt1 acts to increase the proliferation and neurogenesis of mDA precursors and forms an autoregulatory loop with Lmx1a to control mDA neuron differentiation (Castelo-Branco et al., 2003; Chung et al., 2009). Recently it has been reported that β-catenin regulates mDA neurogenesis *in vivo*, providing evidence for the involvement of canonical Wnt signaling in DA neurogenesis (Tang et al., 2009). By contrast, Wnt5a acts through the non-canonical Wnt pathway. Wnt5a promotes vMB morphogenesis, reduces DA progenitor proliferation and neurogenesis in loss-of-function experiments *in vivo* (Andersson et al., 2008). Conversely, Wnt5a promotes the differentiation of mDA neurons and enhances their survival *in vitro* (Castelo-Branco et al., 2003; Parish et al., 2008).

Transforming growth factors beta (TGFβ)

The function of TGFβ on mDA neurons has been related to the regulation of the survival of young postmitotic cells. In this context, TGF-β2, TGF-β3 and BMP2/4/6 have been reported to exert survival-promoting effects in cultured rat mDA neurons and protect them against neurotoxins e.g 6-hydroxydopamine (Kriegstein et al., 1995a; Kriegstein et al., 1995b; Lin et al., 1993; Poulsen et al., 1994). Functional studies have shown that neutralization of TGFβ at a critical time window (E2-E3) in chick abolishes the induction of mDA neurons (Farkas et al., 2003). Unfortunately, we could not repeat these experiments. In addition, TGFβ2/3 double-knockout mouse embryos displayed only reduction TH+ cells in the vMB instead of the total loss, which further suggests that TGFβ has a role in promoting maturation and survival but not in the early induction of mDA neurons (Roussa et al., 2006).
Retinoic acid (RA)

The first indication that RA may be an additional early signal involved in DA cell differentiation, came from the fact that Aldh1 (previously named Ahd2), an aldehyde dehydrogenase, is expressed in the vMB already at E9.5 (Wallen et al., 1999). Aldh1 metabolizes retinaldehyde into RA (Lindahl and Evces, 1984). RA treatment can protect neurons from stress and increase cell survival in primary cultures (Friling et al., 2009b; Wallen-Mackenzie et al., 2003). Aldh1 expression is regulated by Pitx3 both in vivo and in vitro (Chung et al., 2005b; Jacobs et al., 2007). Maternal supplementation of RA can counteract part of the developmental defects caused by Pitx3 deficiency through rescuing an Aldh1-expressing mDA neuronal subpopulation (Jacobs et al., 2007).

Intrinsic factors

So far, many intrinsic factors (of which, most are transcription factors) have been identified to be involved in the proliferation/specification, differentiation, and maturation/maintenance of mDA neurons. The orphan nuclear receptor Nurr1 and the rate-limiting enzyme TH are two markers that were earliest described. Removal of TH results in a lack of DA production. Adult TH null mice are hypoactive as well as growth retarded, and eventually die at four weeks of age (Kim et al., 2000; Kobayashi et al., 1995; Zhou and Palmiter, 1995; Zhou et al., 1995). Nurr1 is required for the induction of TH and other DA phenotypic markers, i.e. VMAT2, DAT, AADC and c-Ret (Hermanson et al., 2003; Saucedo-Cardenas et al., 1998; Smits et al., 2003; Wallen et al., 1999; Wallen et al., 2001). In Nurr1 null mutants, mDA neurons are born but not maintained possibly as a consequence of the failure to acquire a proper phenotype (Kadkhodaei et al., 2009; Saucedo-Cardenas et al., 1998; Zetterstrom et al., 1997). Moreover, Nurr1 has been shown to physically interact with p57kip2, a CDK inhibitor, and to promote maturation (Joseph et al., 2003). In contrast to Nurr1, which is not a specific marker for mDA neurons, Pitx3 is expressed exclusively in mDA neurons in the brain. Interestingly, despite the ubiquitous expression in mDA cells, loss of Pitx3 leads to a selectively degeneration of DA neurons in SNc (Hwang et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003). This selective vulnerability could be due to the temporally distinct onset of TH expression in the SNc and VTA (Maxwell et al., 2005). More recently, microarray analyses have identified differential expressions of genes in the SNc and VTA (Chung et al., 2005a; Jacobs et al., 2006; Jacobs et al., 2009) and this information may shed more insight to solve the puzzle of the differential
vulnerability. **En1/2** is initially expressed all over the MB due to functions in the IsO (Millen et al., 1994). At later stages, the expression becomes restricted to postmitotic mDA neurons. En1/2 is required for the survival and maturation of mDA neurons but not for the induction or initial differentiation (Simon et al., 2001; Simon et al., 2004). En1/2 has anti-apoptotic functions (Alberi et al., 2004; Simon et al., 2001). **Lmx1b** was first identified as a crucial factor to control the onset of Pitx3 relative to TH and required for survival as all mDA neurons are lost after E16 in Lmx1b null mutants (Hwang et al., 2003; Jacobs et al., 2009; Maxwell et al., 2005; Smidt et al., 2000; Smidt et al., 2004; van den Munckhof et al., 2003). Lmx1b is also expressed in progenitors of mDA neurons, but its role during the early specification of mDA neurons is not fully understood. Our study begins to shed light on this topic (paper II).

The knowledge about early molecular players in mDA neuron development was for a long time quite limited, but has increased extensively in the last few years. **Otx2** controls the positioning of the IsO, which in turn, defines the mDA territory (Broccoli et al., 1999; Brodski et al., 2003; Millet et al., 1999). More direct links between Otx2 and the specification of mDA neurons have been revealed. Otx2 participates in the patterning of the MB, regulates proneural gene expression and activates downstream factors of mDA cell fate determinants, i.e Lmx1a and Msx1/2 (Omodei et al., 2008; Prakash et al., 2006; Puelles et al., 2003; Puelles et al., 2004; Vernay et al., 2005). Hence, Otx2 is in the high hierarchy in the mDA neuron developmental program. More interesting, Otx2 expression is maintained mostly in the VTA in the adult MB. Loss of Otx2 in adult shows reduced mesolimbic innervations but normal nigrostriatal innervations (Borgkvist et al., 2006; Chung et al., 2010). **Foxa1/2** has a broader expression domain than Shh in the vMB and is also maintained in postmitotic mDA neurons. Foxa1/2 acts in a gene dosage manner to regulate the differentiation and phenotypic maturation by controlling the expression of Nurr1, En1, TH and AADC (Ferri et al., 2007). Furthermore, Foxa1/2 is required for the maintenance of Lmx1a and Lmx1b expression and functions synergistically with these factors to induce ectopic mDA neurons (Lin et al., 2009; Nakatani et al., 2010). A long term study showed that Foxa2 heterozygous mice develop parkinsonian-like symptoms, which correlates with a selective loss of mDA neurons in the SNc (Kittappa et al., 2007). In addition, **Ngn2** is also a regulator in the mDA specification and neurogenesis. Its proneural function can be partially replaced by Mash1 (Andersson et al., 2006a; Kele et al., 2006). However, it is a key factor downstream of Lmx1a, Msx1/2 and Otx2 for the conversion of the glial-
like FP into a neurogenic region in the vMB (paper I+II, (Ono et al., 2007)). See Box5 for the representation of temporal sequence of gene induction in the developing mDA neurons.

Taken together, the elucidation of developmental pathways of mDA generation has facilitated the production of mDA neuron from stem cells *in vitro*. Our two studies presented below have further increased the understanding of the normal generation of mDA neurons during development and can contribute for the generation of *bona fide* mDA neurons from stem cells for cell replacement therapy or disease modeling for PD.

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**Box5: The temporal expression sequence of genes in the development of mDA neurons.** Among these, *Otx2*, *Foxa1/2*, *Lmx1b*, *Lmx1a* and *En1/2* are expressed in the progenitors and maintained until adult stage. *Msx1/2* is only expressed in the progenitors from E9.5 to E13.5. *Ngn2* is expressed in both progenitors and immature mDA neurons during the active period of mDA neuron generation (E10.5-E13.5). In contrast, the expression of *Nurr1*, *Pitx3* and *TH* is only initiated in the postmitotic mDA neurons with *Nurr1* being detected one day earlier than *Pitx3* and *TH*. 

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AIMS

The findings presented in this thesis aimed to acquire a further understanding of the transcriptional regulation for the specification of mDA neurons in particular. Specific questions addressed were:

- What are the early intrinsic factors to instruct the specification of vMB DA neurons? Can we use the knowledge gained from normal development of mDA neurons in vivo to steer ESC differentiation into bona fide mDA neurons in vitro? (Paper I)

- What are the common and different features of Lmx1a and Lmx1b genes during vMB patterning and the development of mDA neurons? (Paper II)
RESULTS AND DISCUSSION

Identification of intrinsic determinants of mDA neurons (Paper I)

Prior to this study, little was known regarding the molecular pathways involved in the early induction and specification of mDA neurons, while many factors which are important for the proper differentiation and maturation of mDA neurons had been identified. Considering the important roles that mDA neurons play in the normal brain function, and that little is known about the mechanism of PD, we think that a better understanding of the normal developmental pathways in the early specification of mDA neurons would contribute to this research field. More importantly, determinants identified in the early events of mDA neuron development can be exploited in a rational strategy to generate mDA neurons from stem cell in vitro, which in turn provide a source for cell replacement therapy for PD.

The rationale to search for early intrinsic factors involved in mDA neurons development came from models of patterning of ventral cell types in the spinal cord. Here, Shh is secreted from the FP and forms a ventral high and a dorsal low concentration gradient (Ericson et al., 1997a; Ericson et al., 1996; Ericson et al., 1997b; Roelink et al., 1994). Responding to different concentration threshold, a group of transcription factors, named the class II proteins are induced encompassing distinct ventral domains. These transcription factors typically contain a homeodomain (HD) in their DNA-binding motifs. Another set of HD proteins, the class I proteins are repressed by Shh directly or indirectly and therefore expressed in more dorsal regions. The class I and class II proteins pair up to form cross-repressive partners, which in turn results in combinatorial expression codes at different domains. Subsequently, five major ventral progenitor domains (p0-p3, pMN; MN: motor neuron) are established, which followed by the generation of different cell types (V0-V3, MN) (Briscoe and Ericson, 2001; Briscoe et al., 2000; Jessell, 2000; Muhr et al., 2001; Sander et al., 2000; Vallstedt et al., 2001). See Box6.

Based on the described patterning events in the spinal cord, we hypothesized that the unknown progenitor determinant(s) in the vMB most likely contains a HD DNA binding domain. Therefore, we designed strategies to look for HD proteins by using degenerate HD primers and RT-PCR to screen a cDNA library from dissected mouse
vMB tissue at embryonic day (E) 10.5. This approach, combined with a large-scale in situ hybridization screen, identified the Msh-like homeobox gene 1 (Msx1) and the LIM homeobox gene 1a (Lmx1a) that were specifically expressed in mDA progenitors. We provided evidence that both Lmx1a and Msx1 are induced downstream of Shh. Lmx1a is sufficient and required to induce mDA neurons while Msx1 potentiates neurogenesis in the FP of vMB and to represses alternative cell fates.

Box 6: Schematic drawing of morphogenetic activities of Shh secreted from the FP in the spinal cord and ventral patterning of the spinal cord. Shh regulates the expression of HD-containing (except Olig2, which is a bHLH protein) transcription factors in a concentration-dependent manner. Cross-repression between ClassII (in red and pink) and ClassI (in green) sets the border of 5 ventral domains. The combinatorial expression profiles of class II and class I patterning genes define the five progenitor identities and cell types.

The functions of Lmx1a
Lmx1a was discovered in the Dreher (dr) mouse, a spontaneously generated mutant with many developmental defects, including cerebellum and dorsal spinal cord (Millonig et al., 2000). Positional cloning of the gene responsible for the dr phenotype identified a 1.8kb cDNA clone located on chromosome 1, which shared significant identity throughout the coding region with the hamster Lmx1.1 (94% nucleotide identity, 98% amino-acid identity). This gene was termed Lmx1a, the mouse homolog of the hamster Lmx1.1 (Millonig et al., 2000). Follow-up studies showed that Lmx1a is widely expressed in the brain including RP, otic vesicles, vMB, hypothalamus and cortical hem etc (Costa et al., 2001; Failli et al., 2002). However, functional
characterization of Lmx1a was mostly done in the RP (Chizhikov and Millen, 2004a, b; Millen et al., 2004). Our study provided evidence that Lmx1a plays a crucial role during mDA neuron development.

**Lmx1a is sufficient and required for mDA neuron development in vivo**

Lmx1a is expressed both in progenitors and in postmitotic mDA neurons. Using chick in ovo electroporation, we assessed the function of Lmx1a by gain- and loss-of-function approaches. Forced overexpression of Lmx1a in the ventrolateral MB of chick embryos leads to a robust induction of ectopic Lmx1b+Nurr1+TH+ DA neurons at the expense of other neuronal subtypes (e.g. Lim1+ interneurons). However, the generation of ectopic mDA neurons is limited to the ventrolateral regions with the greatest frequency occurring ventrally. The fact that Lmx1a is not sufficient to induce ectopic DA neurons in the more lateral region of vMB indicates other factors are required to act in parallel to Lmx1a. One of the candidates is Foxa1/2, which was shown to cooperate with Lmx1a through a feedforward loop during the induction of mDA neurons (Lin et al., 2009). By contrast, in dorsal regions, Lmx1a participates in a different developmental program regulated by BMPs to specify a functional RP (Chizhikov and Millen, 2004b). Therefore, the activity of Lmx1a is context dependent.

By using RNA interference (siRNA) to knock down Lmx1a in the chick vMB, we provided evidence that Lmx1a is required for the generation of mDA neurons. Analysis of siRNA-transfected chick vMB revealed a loss of postmitotic Lmx1b+Nurr1+ mDA neurons. Notably, although Msx1/2 expression was lost in progenitors, the expression of Lmx1b was maintained. Therefore, Lmx1a appears to be upstream of Msx1/2, but not of Lmx1b and Lmx1b is not able to compensate for the loss of Lmx1a in chick. Furthermore, the function of Lmx1a is specific for mDA neurons, since MNs are not affected by the loss of Lmx1a.

**Efficient derivation of DA neurons by forced Lmx1a expression in ESCs**

Over past few years, there has been an explosion of research focusing on the development of strategies to steer ESCs towards desired fates, with the hope for an ESC-based replacement therapy for diseases such as PD (Barberi et al., 2003; Kawasaki et al., 2000; Kawasaki et al., 2002; Lee et al., 2000; Okabe et al., 1996). One potential advantage of using ESC-derived mDA neurons compared with fetal MB tissue is the unlimited supply of cells to be used for transplantation, but the drawbacks
are the efficiency and purity. Previous ESC differentiation protocols have been relying on the addition of extrinsic factors, such as Shh, Fgf8 and Wnts or feeder cells. Many experiments have shown that high percentage of TH+/Tuj1+ neurons can be achieved from those culture conditions. However, it was not clear whether those TH+/Tuj1+ neurons are bona fide mDA neurons (Barberi et al., 2003; Buytaert-Hoefen et al., 2004). In addition, a mixture of cell types, including serotonin (5-HT) neurons and γ-aminobutyric acid (GABA) neurons is often present in the differentiating ESC culture (Reubinoff et al., 2001). It has been shown that 5-HT neurons mediate dyskinetic side effects in Parkinson’s patients with neural transplants (Politis et al., 2010). By contrast, GABAergic neurons send out long projections to their normal targets and affect behavioral improvement (Thompson et al., 2008). Considering the robust induction of mDA by Lmx1a in vivo, we wanted to examine whether this intrinsic determinant would be potent and efficient to induce mDA neurons in vitro. To this end, we transiently transfected mESCs with a construct in which the expression of Lmx1a was driven by a Nestin enhancer (NesE). This enhancer is only active in neuronal progenitor cells, but not in undifferentiated mESCs or in postmitotic neurons. A NesE-eGFP was used as control. The modified mESCs were differentiated as monolayer cultures in the presence of Shh and Fgf8 (Ying and Smith, 2003; Ying et al., 2003). In accordance with our in vivo data, we observed the induction of Msx1 and repression of Nkx6.1 at early time point in the differentiation cultures. A few days later, a battery of mDA markers, including Nurr1, En1/2, Pitx3, TH, Lmx1b and DAT were detected. Strikingly, over 80% of all Tuj1+ neurons were authentic mDA neurons and other cell fates such as GABA neurons were suppressed. We also noted that a very low concentration of Shh (1.7nM) was sufficient to drive the differentiation of NesE-Lmx1a ESCs. However, a higher concentration of Shh (15nM) was unable to coax NesE-eGFP ESCs to generate a significant number of mDA neurons. One explanation why Shh alone is unable to effectively induce mDA neurons could be a narrow “window of competence” for mDA neuron generation. The rapid induction of Lmx1a after NesE-Lmx1a transfection would synchronize the progenitors for the best production of mDA neurons. Frilling et.al followed up this study and established stably transfected Nes-Lmx1a mES cell line. They further showed that mDA neurons derived from the culture displayed electrophysiological profiles that were very similar to the properties of native mDA cells. Moreover, when transplanted into the striatum of 6-hydroxydopamine unilateral-lesioned neonatal rats, these cells expressed correct mDA postmitotic
markers and projected preferentially to dorso-lateral striatal regions (Friling et al., 2009a).

The present study provides another “proof-of-concept” of applying intrinsic factors in the generation of mDA neurons in vitro. Besides Lmx1a, others have used Nurr1, Ngn2, Foxa2 and Pitx3 alone or in combination and achieved a better yield of authentic mDA neurons (Andersson et al., 2007; Chung et al., 2005b; Chung et al., 2002; Kim et al., 2002; Lee et al., 2010; Martinat et al., 2006; Park et al., 2006). Notably, in contrast to Lmx1a, Lmx1b was not efficient in inducing mDA neurons. This suggests that Lmx1b is not a devoted mDA cell fate determinant as indicated by its early expression pattern. I will further discuss it in paper II.

The functions of Msx1/2

In addition to Msx1, we also found that its homolog Msx2 displayed an identical expression pattern in the vMB with Msx1 by using in situ hybridization. Since the biochemical properties of them are very similar (Catron et al., 1996), we focused our study only in Msx1 but the antibody we used recognized both Msx1 and Msx2.

Repression of alternative fates

Overexpression of Lmx1a in the vMB leads to ectopic induction of Msx1 in the progenitor zone, indicating that Lmx1a may act upstream of Msx1/2. However, Msx1 itself is unable to induce mDA neurons neither in vivo by chick in ovo electroporation nor in vitro by NesE-Msx1 ESCs differentiation. This suggests that the function of Lmx1a is not executed only through Msx1/2. The induction of mDA markers such as Nurr1 by Lmx1a is independent of Msx1/2. Previous studies have indicated that Msx1/2 can function as a transcriptional repressor that interacts with Groucho/TLE co-repressors (Catron et al., 1995; Zhang et al., 1996). Therefore, it is unlikely that Msx1/2 can induce a mDA fate directly, but instead may repress other factors in order to provide a permissive environment for the generation of mDA neurons. Indeed, we were able to confirm that Msx1 acts as a Groucho/TLE-dependent repressor in a reporter-gene assay, and that this activity is dependent on the putative Groucho/TLE binding eh1 domain. Accordingly, upon forced expression of Msx1, Nkx6.1 was promptly extinguished, resulting in a reduction of MN neurons, indicating that Nkx6.1 may be the direct downstream target of Msx1. However, Lmx1a is not able to repress Nkx6.1 itself without first inducing the expression of Msx1. Interestingly, we observed that
prior to the onset of Msx1 at E9.5, Nkx6.1 was expressed throughout the midline including in the mDA domain. Nkx6.1 expression was gradually retracted and eventually abutted the Msx1 domain. Furthermore, in Msx1 mutants, Nkx6.1 showed an increased expression level in the mDA domain. Thus, compared to Lmx1a, Msx1 does not directly induce mDA neuron fate, but instead suppresses other cell fates.

Do Msx1/2 and Nkx6.1 form a classical cross-repressive pair, as displayed in the spinal cord model? Unfortunately, we could not find any evidence of this. Overexpression of Nkx6.1 in the vMB in both chick and mouse did not alter either Msx1/2 expression or mDA neuron generation (our unpublished data). This data was confirmed by a recent study, which showed that Nkx6.1 failed to suppress mDA markers, by analysis of Nestin-Nkx6.1 transgenic mice (Nakatani et al., 2010). Instead, it was discovered that Sim1, which is expressed adjacent to the Msx1/2 domain, can disturb the further maturation of mDA neurons by blocking TH, En, and Pitx3 expression, but also cannot suppress Msx1/2 expression (Nakatani et al., 2010). However, another possibility for the demarcation of the Msx1/2 domain could be based on the dependence of Lmx1a or a high level of Shh signaling. A similar event has been noted in the ventral spinal cord, where Olig2 expression initially is present in the p3 domain, and then with the onset of Nkx2.2 expression, Olig2 becomes restricted to the pMN domain. In this case, the transient high level of Shh is required for the initiation of Nkx2.2 (Dessaud et al., 2007). Therefore, the factor directly repressing Msx1/2 remains unknown and refinement of Msx1/2 expression in the vMB could also be due to the dependence on the activation by other factor(s).

Promotion of neurogenesis

One unique feature of vMB DA neuron development is the origin from FP cells. As mentioned in the introduction, FP cells are characterized as glial-like non-neurogenic cells. Thus, the generation of mDA neurons must be preceded by a glial-to-neuronal conversion. Consistent with such transition, the proneural basic helix-loop-helix (bHLH) protein Ngn2 begins to be expressed in the DA domain in vMB around E10.75 and shortly thereafter, at E11.5, the expression of Shh is extinguished from the mDA domain. We observed that combined expression of Msx1 and Lmx1a, but not Lmx1a alone, resulted in premature generation of Nurr1+ DA neurons in the vMB during short-term transfection experiments. Msx1 therefore appears to influence the timing of mDA neuron induction and could mediate this conversion. To test this possibility, we
generated ShhE-Msx1 transgenic mice, in which Msx1 was activated at least 24h prior to its normal endogenous expression (Epstein et al., 1999). This premature induction of Msx1 resulted in an early retraction of Shh from the mDA domain and induction of Ngn2 followed by the premature generation of Nurr1+Pitx3+TH+ mDA neurons. Accordingly, in Msx1 mutant mice, there was an approximate 40% reduction in the number of Ngn2+ progenitor cells and Nurr1+ DA neurons. Considering the almost identical expression pattern of Msx1 and Msx2 in the vMB, this moderate phenotype could be due to the redundancy of Msx2. In future experiments, double mutants should be analyzed to see whether Msx1/2 is required for the generation of mDA neurons.

Following our study, the neurogenic character of the MB FP (mFP) has been analyzed in greater detail. Interestingly, it was shown that caudal FP (cFP) cells do not have the capacity to generate neurons when isolated and cultured in vitro (Ono et al., 2007). Furthermore, using ShhE-Mash1 transgenic mice, the cFP was converted to a neurogenic region, but fully differentiated DA neurons could still not be generated, indicating that a mDA identity is likely to be specified by mFP-selective factors. Indeed, ectopic expression of Otx2 in the cFP using ShhE-Otx2 transgenic mice induced a complete array of DA neurons markers including Lmx1a, Msx1/2, Ngn2, Nurr1 and TH. More recently, Nato3, which belongs to bHLH family, was identified to be also involved in the process of converting the mFP to a neurogenic region. Nato3 can repress Hes1, which in turn, derepresses Ngn2 through Hes1. However, Nato3 is downstream of Foxa1/2, not of Otx2 or Lmx1a (Ono et al., 2010). Therefore, more than one pathway is involved during the regulating the mFP neurogeneity.

Another interesting fact about the mFP is that a high level of Shh in the mDA domain is inversely correlated with proliferation and neurogeneisis. So what is the factor that restricts Shh from the mDA domain? Joksimovic et al. answered this question by showing that Wnt/β-catenin signaling for facilitation of mFP neurogenesis (Joksimovic, M, 2009). Early, but not late removal of Wnt genetically leads to maintenance of Shh expression in the mDA domain, which in turn hampered the neurogenesis (Joksimovic, M, 2009). Furthermore, Wnt1 can induce Otx2 and mDA neurons (Prakash N, 2006), but this ability is restricted rostrocaudally, with a caudal limit at the hindbrain level (Joksimovic, M, 2009). This further elucidates the heterogeneity of the FP and the influence of A-P expressed factors on the specification of cell fates.
Regulatory network between components in vMB DA neuron development

Previous and recent publications (Chung et al., 2009; Lin et al., 2009; Nakatani et al., 2010; Tang et al., 2010) have started to accomplish the “map” in which the genetic connections between different factors are linked directly or indirectly. It has been proposed that there are two main parallel pathways, i.e., Shh-FoxA2 and Wnt1/β-catenin-Lmx1a/Lmx1b, are active during mDA neuron development. These two pathways functionally interact with each other and synergistically induce postmitotic mDA markers through the cooperation between FoxA2 and Lmx1a (See Box7). These findings also indicate that Lmx1a and Lmx1b may have redundant effects, which is the main focus of paper II.

Box7: Regulatory network of two major signaling pathways in mDA neuron specification and differentiation. These genetic connections (directly or indirectly) between each component have been reported by loss- and/or gain-of-function (compliments of Ulrika Marklund).

Specific and redundant roles of Lmx1a and Lmx1b in vMB development and specification of dopamine neurons (Paper II)

As aforementioned, Dreher (dr) mice harbor a spontaneous mutation in the first LIM domain of the Lmx1a gene. This point mutation changes one amino acid from cysteine to tyrosine, which disturbs the function of the zinc finger and in turn disrupts the transactivity of Lmx1a (German et al., 1992; Johnson et al., 1997; Sanchez-Garcia and Rabbitts, 1994). dr/dr mice displayed a moderate reduction of DA neurons in vMB with around 30% less cells at E13.5 than control littermate (Ono et al., 2007). Since the expression of the altered Lmx1a polypeptide persists in DA progenitors and differentiating DA neurons in the MB of dr/dr mice, it remains unclear if the reduction
of mDA neurons observed in \( dr/dr \) embryos reflects a partial or complete loss of Lmx1a functional activity. Lmx1a is highly related to Lmx1b with 61% overall amino acid identity (100% identity in the HD and 67% and 83% in each LIM domain) (Hobert and Westphal, 2000). Although Lmx1b has is known to be essential for mDA neurons (Smidt et al., 2000), the MB patterning and specification of DA neurons in Lmx1b mutants are not fully understood. In this study, we have established two Lmx1a null mutant mouse strains and carefully compared the MB patterning and the initial specification of DA neurons as well as other ventral cell types in Lmx1a and Lmx1b null mutant embryos.

**Generation and analysis of Lmx1a null mutant mice**

We generated two new Lmx1a mutant mouse strains: termed Lmx1a\(^{eGFP}\) and Lmx1a\(^{cko}\), by homologous recombination in ESCs (see paperII Supplementary Figure 1 for details). A straight Lmx1a mutant strain (termed Lmx1a\(^{ckoΔGL}\)) was subsequently generated by crossing Lmx1a\(^{cko}\) mice with mice expressing the Cre-recombinase under the CMV promoter. Importantly, when compared to wild type and \( dr/dr \) mice, no Lmx1a protein was detected in the vMB in Lmx1a\(^{eGFP/eGFP}\) and Lmx1a\(^{ckoΔGL/ckoΔGL}\) embryos at E11.5. We also found that all three mutants displayed a similar reduction of mDA neurons at two developmental stages E11.5 and E13.5. Meanwhile, overexpression of Lmx1a protein with a \( dr \) mutation (Lmx1a\(^{dr}\)) in chick could not induce ectopic mDA neurons. These data provide evidence that Lmx1a function is not absolutely required for the specification of mDA neurons during mouse embryogenesis and that \( dr/dr \) is indeed a true knockout. This was also confirmed by a recent study in which different spontaneous Lmx1a mutations were compared, including truncations, missense, and frameshift mutations. Interestingly, all these different mutants displayed largely identical phenotypes in terms of cerebellar abnormalities (Chizhikov et al., 2006).

**Redundancy of Lmx1a and Lmx1b in the mouse**

The fact that Lmx1a is sufficient but not absolutely required for the specification of mDA neurons implies a certain degree of redundancy between Lmx1a and other proteins in mDA cell fate specification during mouse embryogenesis. We therefore compared the establishment of mDA progenitors and the specification of mDA neurons in wild type, Lmx1a\(^{eGFP/eGFP}\) null mutants and embryos homozygous for a previously established Lmx1b knockout allele (Lmx1b\(^{−/−}\)) (Chen et al., 1998).
First we asked why Lmx1a is not as important in mouse as it is in chick (Andersson et al., 2006b; Ono et al., 2007). We looked at the epistatic relation between Lmx1a and Lmx1b in two species. Studies in the RP in chick have shown that Lmx1b acts upstream of Lmx1a in the induction and specification of the functional RP (Chizhikov and Millen, 2004a). However, the RP function of Lmx1b is not conserved across vertebrates since Lmx1b is not expressed in the RP of the mouse spinal cord. However, Lmx1a and Lmx1b are expressed both in chick and in mouse vMB. In chick, by using \textit{in ovo} electroporation in the vMB, we performed gain- and loss-of-function studies for Lmx1b. Overexpression of Lmx1b could induce robust induction of Lmx1a expression in both progenitors and postmitotic cells which were also Nurr1⁺. Furthermore, downregulation of Lmx1b by siRNA led to a loss of Lmx1a expression both in progenitors and postmitotic cells (our unpublished data). However, in Lmx1b⁻/⁻ mice, we still detected Lmx1a expression, although the domain was smaller, which means Lmx1b is not absolutely required for the induction of Lmx1a expression in the mouse vMB. These results suggest that Lmx1b is upstream of Lmx1a in the mDA neuron developmental program, only in chick, but not in mouse, explaining why Lmx1a is required in chick but not in mouse for mDA neuron development.

The first evidence of overlapping function of Lmx1a and Lmx1b genes during embryonic CNS development came from the analysis of the RP in rb1, which differentiates into the epithelium of the choroid plexus, a structure with multiple physiological functions including the secretion of cerebrospinal fluid. This study showed that loss of Lmx1a completely abolishes RP induction in the spinal cord, but a residual RP still forms in rb1, where Lmx1a and Lmx1b are co-expressed. The double knockout of Lmx1a and Lmx1b displayed more severe phenotype than any of the single mutant (Mishima et al., 2009). We also examined Lmx1a<sup>eGFP</sup>/eGFP/Lmx1b compound mutants at E13.5. Interestingly, the number of null mutant for both genes at E13.5 was far below the Mendelian ratio and we were unsuccessful in getting enough material for statistical analysis. But we observed a dose-dependent effect when analyzing different combinations of Lmx1a and Lmx1b mutant genotypes at E13.5. Single heterozygotes of either Lmx1a or Lmx1b had comparable numbers of DA neurons to wild type littermates. However, embryos that were heterozygous for both Lmx1a and Lmx1b had a ~20-25% reduction in the number of Nurr1 and Pitx3.
expressing cells. With only one allele of Lmx1b left and no allele of Lmx1a, there was a ~70-75% loss of Nurr1 and Pitx3 expressing cells.

The reason why the number of null mutants for both genes is below the Mendelian ratio is still unclear. One possible explanation is that E13.5 is a late stage which may not reflect the direct effect of a loss of both Lmx1a and Lmx1b. It also implies early lethality and possible redundancy between these proteins in early developmental processes. In accordance with this, we observed that both Lmx1a and Lmx1b are expressed in the notochord (our unpublished data). As said before, the notochord is an important signaling source during early embryonic development. So the loss of Lmx1 genes may affect the function of the notochord, which in turn could cause abnormal embryonic development. The ideal situation would be to analyze the conditional double null mutants in order to make a conclusion. Therefore, we have already started to breed Lmx1a<sup>cko</sup> with Lmx1b<sup>cko</sup> mice (Zhao et al., 2006). By using different Cre transgenic mice line (e.g DAT-Cre), we will be able to dissect the specific functions of Lmx1a and Lmx1b during mDA neuron development.

**Lmx1a and Lmx1b have distinct functions**

*Lmx1a facilitates FP conversion in the vMB and is required for medial DA progenitors*

A unique feature of mDA neurons is that they are derived from ventral midline FP cells with a non-neurogenic character, while FP cells located more caudally do not generate any neurons (Andersson et al., 2006b; Ono et al., 2007). Thus, the generation of mDA neurons is associated with a transition of neuronal potential, in which FP cells must acquire the neuronal properties typical of DA progenitors. In Lmx1a<sup>eGFP/eGFP</sup> mice, we observed specific loss of mDA neurons in the most medial position, which was accompanied by a loss of Ngn2 expression and delayed retraction of Shh at E11.5. We therefore hypothesized that the specific reduction of mDA neurons at the ventral midline of Lmx1a<sup>eGFP/eGFP</sup> could reflect a role for Lmx1a in regulating the switch from a non-neurogenic FP character to neuronal mDA progenitors. We found that the expression of the Notch ligandDll1 as well as the bHLH proteins Hes5 and Tcf12 was lost specifically in the medial part of the mDA progenitor domain. Thus, Lmx1a appears to promote the expression of several genes implicated in Notch signaling pathway, presumably facilitating functional Notch signaling and regulating neurogenesis at the ventral midline.
**Lmx1b is required for lateral DA progenitors**

The induction of Ngn2 and the concomitant loss of Shh expression that occur in midline cells at around E10.5-11.5 are indicative of the conversion of FP cells into DA progenitors. In contrast to Lmx1a^eGFP/eGFP mice, Ngn2 was expressed and Shh was extinguished at the midline in Lmx1b^-/^- mice. In line with this, a significant number of Nurr1 and TH expressing neurons were detected ventral to the mDA progenitor zone in Lmx1b^-/^- at E11.5, including the ventral midline. However, the size of the mDA progenitor domain in Lmx1b^-/^- embryos was compromised and much narrower as compared to wild type and Lmx1a^eGFP/eGFP embryos. This raised the possibility that loss of Lmx1b function primarily affects lateral mDA progenitors. We searched for molecular markers that could distinguish between the medial and lateral mDA progenitor domains and found that Wnt1 and the Dopamine receptor D2 (D2R) were selective markers for the lateral DA progenitors at E11.5 and E13.5. Not surprisingly, both markers were lost in Lmx1b^-/^- mice. In conclusion, while different Lmx1a is required for the specification of mDA neurons in the medial progenitor domain, Lmx1b has a more pronounced role in establishing lateral mDA progenitors at early developmental stages. Furthermore, it is noteworthy that Lmx1b must also influence the differentiation of medially derived neurons, since the majority of Nurr1^+ neurons produced in Lmx1b^-/^- fail to initiate the expression of several markers of more mature mDA neurons e.g. TH and Pipt3 (Smidt et al., 2000). This could be due to a non cell-autonomous effect caused by the loss of Wnt signaling.

**Lmx1b controls the generation of oculomotor neurons and red nucleus cells**

Studies of Lmx1b in the MB have primarily focused on DA neurons. However, compared to Lmx1a, Lmx1b is broadly expressed in the vMB at early developmental stages (Andersson et al., 2006b). This implies that Lmx1b may have other function in the vMB. We therefore examined the generation of cell types situated laterally to DA neurons in the vMB of Lmx1b mutant mice.

In the vMB, two other neuron subtypes are located dorsally to mDA domain, i.e. OM neurons and RN. OM neurons are born around E9 and control eye movement and vestibulo-ocular reflexes. RN cells are located in close vicinity to OM neurons, sharing the same progenitor domain. The RN contains both excitatory glutamatergic neurons and inhibitory GABAergic neurons, which project to the cerebellum, brainstem and
spinal cord and play fundamental roles in the control of limb movement (Evinger, 1988; Keifer and Houk, 1994). RN neurons are born one day later than OM neurons, around E10. The paired-like homeobox gene Phox2a, which is expressed in OM progenitors, is a crucial fate determinant for OM precursors (Coppola et al., 2005; Pattyn et al., 1997). Single-minded homolog 1 (Sim1) and Nkx6.1 have been reported to be the intrinsic determinants for the RN cells (Nakatani et al., 2010; Prakash et al., 2009).

Three distinct progenitor domains could also be defined in the ventral-most MB region in Lmx1b-/- embryos, as determined by Lmx1a, Sim1/Phox2a and Nkx2.2 expression. Interestingly, however, we observed a dramatic reduction of Phox2a expression, and a loss of Isl1/2+ OM neurons, within the presumptive OM/RN progenitor domain between E9.5 to E11.5 in Lmx1b-/- mutants (while no change was observed in Lmx1a+GFP/eGFP mice). Moreover, the loss of OM neurons was accompanied by a premature induction and overproduction of RN cells, as indicated by increased numbers of Brn3a+ and Lim1/2+ neurons within the OM progenitor domain. In addition, a significant number of Brn3a+ and Lim1/2+ cells in Lmx1b-/- mutants was found to be intermingled with Nurr1+ cells within the presumptive Lmx1a+ mDA progenitor domain. This finding indicates that the impaired specification of DA neurons observed in Lmx1b-/- mutants reflects, at least in part, that a subpopulation of prospective mDA progenitors adopts a RN fate instead of mDA neuron differentiation program in the absence of Lmx1b function.

In this study, we used conventional Lmx1b-/- mutant mice. One concern of these mutant mice is that the IsO is also affected. As said before, Lmx1b is expressed and involved in a gene regulatory loop in the IsO. Removal of Lmx1b results in a loss of Wnt1 expression, which further affects Fgf8, En1/2 and Pax2/5 expressions (Adams et al., 2000; Guo et al., 2007; Matsunaga et al., 2002). Are the phenotypes we observed in the Lmx1b-/- mutant mice secondary defects due to dysfunction of the IsO? We cannot exclude this possibility, although we still do not think a dysregulation of the IsO could explain the whole phenotype. First of all, it has been shown that Otx2 and Pax6 expressions are not affected in Lmx1b-/- mice (Guo et al., 2007). Although Gbx2 is not maintained in Lmx1b-/- mice, Otx2 does not extend into the anterior hindbrain as expected, indicating that other unknown factor(s) repress Otx2. Meanwhile, Pax6 expression, which is antagonized by En1/2 and defines the border between the
diencephalon and the MB, remains unchanged. Therefore, the MB still exists and can be analyzed. Secondly, other mutant strains with IsO defects do not show the same phenotype as Lmx1b<sup>−/−</sup> mice. We examined Wnt1<sup>−/−</sup> mice since Wnt1 is downstream of Lmx1b and essential for the IsO function. As previously reported, mDA neurons are greatly lost in Wnt1<sup>−/−</sup> mice (Panhuysen et al., 2004; Prakash et al., 2006). However, our unpublished data showed that OM neurons and RN cells are also equally affected, which suggested that loss of Wnt1 results in general agenesis of cells. This data does not completely resemble the Lmx1b<sup>−/−</sup> mutant phenotype and subsequently indicates that Lmx1b has specific functions other than establishing the IsO.

The LIM-HD family and its co-factors

Lmx1a and Lmx1b belong to the LIM-HD family, which contains two tandem cysteine-rich LIM motifs and DNA-binding HD motif. The HD is thought to form a helix-turn-helix structure that binds regulatory DNA sequences of target genes (Gehring et al., 1994). The LIM domains may interact with the HD to prevent its binding to DNA in the absence of a LIM binding partner. Binding with another protein relieves this restraint and simultaneously provides the opportunity for the two domains to act cooperatively in the assembly of a regulated transcription complex (Curtiss and Heilig, 1998). Proteins reported to bind LIM domain are cofactor of LIM domains (Clim) /LIM-binding protein (Ldb) (Agulnick et al., 1996). In mice, there are two highly conserved genes in the Clim family: Clim2 (Ldb1) and Clim1 (Ldb2). The LIM binding portion of Clim has been localized to the carboxy-terminus, while the amino-terminal region is involved in homodimer formation (Agulnick et al., 1996; Jurata and Gill, 1997; Matthews and Visvader, 2003). Through these interaction properties, Clim may act as specific protein-binding adapters, facilitating assembly of large complexes of the nuclear LIM proteins, as well as of other classes of transcription factors such as Pitx1, GATA, bHLH DNA-binding proteins (Bach, 2000; Dawid et al., 1998; Lee and Pfaff, 2003; Ma et al., 2008). It has elegantly been shown that activation of the somatic MN gene HB9 is dependent on Clim2-mediated assembly of heterodimers between two LIM-HD proteins, Lim3 and Isl1 (Thaler et al., 2002). Later, it was found that Ngn2 but not Mash1 actively participated with Lim3 and Isl1 in this complex to synergize the transcriptional activation of HB9, providing a link between neurogenesis and neuronal subtype specification (Bach, 2000; Dawid et al., 1998; Lee and Pfaff, 2003; Ma et al., 2008). Hence, by virtue of their LIM domains, it would be possible that Lmx1a and Lmx1b perform distinct functions through assembly of different transcriptional
complexes via Clim. It would be interesting in the future to find out whether there are any physical interactions between Lmx1a, Lmx1b with Clim, what kind of transcriptional complexes are formed by Lmx1a and Lmx1b respectively, and what downstream genes are activated by them.
SUMMARY AND FUTURE PERSPECTIVES

PAPER I: In this study, we revealed the important functions of two transcription factors, Lmx1a and Msx1/2, in the early specification of mDA neurons. Lmx1a is sufficient and required to activate downstream mDA neuronal markers. Msx1/2 acts in parallel to suppress alternative cell fates and promote neurogenesis by converting the FP into a neurogenic region. Following our study, several publications have continued to revealed the network regulating mDA neuron identity, in which Lmx1a has been placed downstream of Otx2 and Foxa1/2 and upstream of Nurr1 and Pitx3. It would be interesting to identify novel direct/indirect downstream factors of Lmx1a to further expand the knowledge regarding the process of the differentiation and maturation of mDA neurons. For this purpose, enriched mDA neurons derived from NesE-Lmx1a ESCs provide sufficient material for downstream applications, such as ChIP-chip and ChIP-Seq. In addition, these mDA neurons, generated in vitro, are suitable for studying detailed mechanisms of mDA neuron degeneration in response to neurotoxin or environmental insults, and for performing a large scale of drug screens with the aim to identify compound with a neuroprotective function and the potential to block the progression of PD.

PAPER II: In this study, we further investigated the function of Lmx1a in mouse and compared the phenotype of Lmx1a with its homolog Lmx1b. In contrast to the chick, Lmx1a is not absolutely required for mDA development in the mouse, which is partly due to the redundant function of Lmx1b. However, subgroups of mDA neurons show different requirement for Lmx1a and Lmx1b. Medial mDA progenitors require Lmx1a for neurogenesis at an early stage during the conversion of the FP to a region with neurogenic potential, while Lmx1b is essential for lateral mDA progenitors. More and more studies have shown that mDA neurons are not a homogeneous population. They display differential vulnerability to the loss of different factors, such as Pitx3 and Otx2, at a late developmental stage. Our data suggest that this heterogeneity could originate from the early specification period, wherein only medially located mDA progenitors are derived from bona fide FP cells. Lateral mDA precursors would instead be born with a neurogenic potential, which is similar to neurons generated in the caudal neural tube. Interestingly, both Lmx1a and Lmx1b continue to be expressed in mDA neurons in the adult. The future plan is to delineate the functions of Lmx1a and Lmx1b in
maintenance and target-finding of different subpopulations of mDA neurons in the adult brain by using conditional Lmx1a and Lmx1b mutants.

Compared to the spinal cord and hindbrain, little is known about the patterning events of the ventral cell types in the MB. We show that Lmx1b plays a role in the specification of multiple ventral cell types, including OM neurons and RN cells. Lmx1b may act upstream of Phox2a, which is an intrinsic determinant of OM neurons genesis. Although the exact ontogenesis of RN cells is not well-known, our data indicated that these cells may share progenitor domain with OM neurons and are generated subsequent to OM neurons. The sequential generation of visceral MNs and 5-HT neurons from the same progenitor domain has been elegantly shown in the hindbrain. It would be extremely interesting to further investigate whether such a mechanism can apply here.
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