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MOLECULAR AND CELLULAR CHARACTERIZATION OF MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT

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Molecular and Cellular Characterization of Midbrain Dopaminergic Neuron Development
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Dedicated to my parents.

ABSTRACT

Midbrain dopaminergic (mDA) development is a complex yet highly controlled mechanistic process that is conserved across species. The understanding of these molecular details can open windows to new avenues of therapeutic medicine. Parkinson's disease (PD) is a debilitating neurological disorder that to date has no cure or established cause. With various aspects of mDA development being revealed, the aim for a permanent treatment of PD itself is getting closer. Included within this thesis are four papers and two manuscripts covering diverse points of mDA neuron development and PD.

In **Paper I** we explore the role of transcription factor *Pbx1* to promote mDA differentiation through activation of *Pitx3* and repression of Onecut2. *Pbx1* is also involved in protection from oxidative stress through *Nfe2l1*, an important aspect of PD.

In **Paper II** we explore the cellular diversity of the ventral midbrain through the use of single-cell RNA-sequencing. The cellular transcriptional profiles aid in revealing the mDA neuron lineage and a cross-species comparison of mouse and human. To conclude, we use molecular tools to evaluate stem-cell derived mDA preparations for cell replacement therapy (CRT) in PD.

In **Paper III** we review the current knowledge of Wnt signaling related to mDA development and further investigate the human single-cell data set from **Paper II** for other possible Wnt components that have yet to be explored for their role in development.

In **Paper IV** we explore the composition of the mDA cellular environment using RNA-sequencing data. Here we apply a novel approach to gain insight to specific contributions from various cell types to the extracellular matrix, its modulators, and signaling ligands. We find a transcription factor network centered around *Arntl1* in radial glia type 1 cells, a putative progenitor to the neuronal lineage.

In **Paper V** we investigate the matricellular protein R-spondin 2. As a Wnt signaling activator, we show R-spondin 2 has a role in mDA differentiation when applied to embryonic stem cell differentiation protocols. This has direct translational impact in CRT for PD.

In **Paper VI** we explore the role of Wnt/planar cell polarity signaling in midbrain development. Specifically, we elucidate the roles of *Ror2* and *Vangl2* in mDA development and their participation in morphogenesis and neurogenesis.

In conclusion, this thesis encompasses research on midbrain development from molecular details at a single-cell level to cellular components affecting global developmental processes. Here I present findings to be included towards a greater understanding of midbrain development and novel ideas relevant to translational research in CRT for PD.

LIST OF SCIENTIFIC PAPERS

I. Villaescusa JC, Li B, Toledo EM, Rivetti di Val Cervo P, Yang S, Stott SR, Kaiser K, Islam S, **Gyllborg D**, Laguna-Goya R, Landreh M, Lönnerberg P, Falk A, Bergman T, Barker RA, Linnarsson S, Selleri L, Arenas E.
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The EMBO Journal. 2016 Sep 15;35(18):1963-78.

II. La Manno G*, **Gyllborg D***, Codeluppi S, Nishimura K, Salto C, Zeisel A, Borm LE, Stott SR, Toledo EM, Villaescusa JC, Lönnerberg P, Ryge J, Barker RA, Arenas E, Linnarsson S. (*Co-first authors)
Molecular Diversity of Midbrain Development in Mouse, Human and Stem Cells
Cell, 2016 Oct 6; 167(2):566-580

III. Toledo EM, **Gyllborg D**, Arenas E.
Translation of WNT developmental programs into stem cell replacement strategies for the treatment of Parkinson's disease
British Journal of Pharmacology, 2017 May 26; [Eprint] doi: 10.1111/bph.13871

IV. Toledo EM, La Manno G, Rivetti di Val Cervo P, **Gyllborg D**, Islam S, Villaescusa JC, Linnarsson S, Arenas E.
Molecular analysis of the midbrain dopaminergic niche during neurogenesis
Preprint at bioRxiv. 2017 Jun 26. doi: 10.1101/155846

V. **Gyllborg D**, Ahmed M, Toledo EM, Theofilopoulos S, Ffrench-Constant C, Arenas E.
The matricellular protein R-Spondin 2 (RSPO2) promotes midbrain dopaminergic neuron neurogenesis and differentiation
Manuscript

VI. **Gyllborg D**, Salašová A, Toledo EM, Gao B, Yang Y, Villaescusa JC, van Amerongen R, Arenas E.
Ror2 and Vangl2 control dopaminergic neurogenesis and multiple aspects of cell polarity in the midbrain floor plate
Manuscript

PUBLICATIONS NOT INCLUDED IN THESIS

- Zhang D, Yang S, Toledo EM, **Gyllborg D**, Saltó C, Villaescusa JC, Arenas E.
  Niche-derived laminin-511 promotes midbrain dopaminergic neuron survival and differentiation through YAP
  Science Signaling. Accepted Manuscript.

- Kaucka M, Zikmund T, Tesarova M, **Gyllborg D**, Hellander A, Jaros J, Kaiser J, Petersen J, Szarowska B, Newton PT, Dyachuk V, Li L, Qian H, Johansson AS, Mishina Y, Currie J, Tanaka EM, Erickson A, Dudley A, Brismar H, Southam P, Coen E, Chen M, Weinstein LS, Hampl A, Arenas E, Chagin AS, Fried K, Adameyko I.
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  Oligodendrocyte heterogeneity in the mouse juvenile and adult central nervous system
  Science. 2016 Jun 10;352(6291):1326-9.

CONTENTS

LIST OF ABBREVIATIONS

Ca^{2+}	Calcium
CE	Convergent extension
CRD	Cysteine-rich domain
CRT	Cell replacement therapy
DA	Dopaminergic
ECM	Extracellular matrix
E	Embryonic day
ES	Embryonic stem
FACS	Fluorescence-activated cell sorting
GWAS	Genome-wide association study
hPSCs	Human pluripotent stem cells
IsO	Isthmic organizer
IZ	Intermediate zone
iPS	Induced pluripotent stem
L-DOPA	Levodopa
mDA	Midbrain dopaminergic
MHB	Midbrain-hindbrain boundary
MZ	Marginal zone
P	Postnatal day
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	Parkinson's disease
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
RRF	Retrorubral field
scRNA-seq	Single-cell RNA-seq
SNc	*Substantia nigra pars compacta*
VM	Ventral midbrain
VTA	Ventral tegmental area
VZ	Ventricular zone

GENE LIST

Arntl / Bmal1	Aryl hydrocarbon receptor nuclear translocator-like
Apc	Adenomatosis polyposis coli
Axin1	Axin 1
Celsr(1-3)	Cadherin, EGF LAG seven-pass G-type receptor (1-3)
Ck1α/δ	Casein kinase 1 alpha/delta
Ddc / Aadc	Dopa decarboxylase
Dkk(1-4)	Dickkopf WNT signaling pathway inhibitor (1-4)
Dvl(1-3)	Dishevelled segment polarity protein (1-3)
En1/2	Engrailed 1/2
Fgf8	Fibroblast growth factor 8
Foxa1/2	Forkhead box A1/2
Fzd	Frizzled class receptor
Gsk3β	Glycogen synthase kinase 3 beta
Lgr(4-6)	Leucine rich repeat containing G protein coupled receptor (4-6)
Lmx1a/b	LIM homeobox transcription factor 1 alpha/beta
Lrp5/6	Low density lipoprotein receptor-related protein 5/6
Nr4a2 / Nurr1	Nuclear receptor subfamily 4, group A, member 2
Otx2	Orthodenticle homeobox 2
Pbx(1-4)	Pre B cell leukemia homeobox (1-4)

Pitx3	Paired-like homeodomain transcription factor 3
Ptk7	Protein tyrosine kinase 7
Rnf43	Ring finger protein 43
Ror1/2	Receptor tyrosine kinase-like orphan receptor 1/2
Rspo(1-4)	R-spondin (1-4)
Ryk	Receptor-like tyrosine kinase
Sfrp1/2	Secreted frizzled-related protein 1/2
Slc6a3 / Dat	Solute carrier family 6 (neurotransmitter dopamine transporter), member 3
Shh	Sonic hedgehog
Th	Tyrosine hydroxylase
Vangl2	Vang-like 2 (van gogh, *Drosophila*)
Wnt1	Wingless-type MMTV integration site family, member 1
Wnt5a	Wingless-type MMTV integration site family, member 5A
Znrf3	Zinc and ring finger 3

1 INTRODUCTION

1.1 THE SHAKING PALSY

"*Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.*"

-James Parkinson, 1817

Exactly 200 years ago, a general practitioner described, through his observations, six individual cases with similar symptoms and defined them as shaking palsy. James Parkinson published these observations in the *London Medical and Physical Journal* under the title *An Essay on the Shaking Palsy* and pioneered research into this disease as a unique malady to be distinguished from others. Many of his observations still hold true today and importantly, allowed for subsequent clinicians to supplement onto his findings. Although his proposed means of a remedy today would be considered erroneous, including induced skin blistering and bloodletting from the neck in order to reduce blood pressure to the brain, he carefully states how his observations should be considered and how to move forward.

"The inquiries made in the preceding pages yield, it is to be much regretted, but little more than evidence of inference: nothing direct and satisfactory has been obtained."

With his humility, and following the scientific method, we continue to question, observe, and analyze and build upon what those before us have discovered.

Even though he did not have a cure, he expressed hope of a treatment to stop the progress of the disease and advocated early intervention for success. Today, 200 years later, we continue to work in the realm of text written by James Parkinson in his essay about the shaking palsy.

"*…there appears to be sufficient reason for hoping that some remedial process may long ere discovered, by which, at least, the progress of the disease may be stopped.*"

1.1.1 History

It was not until 50 years after James Parkinson when Jean-Martin Charcot made more detailed observations and the term Parkinson's disease (PD) was first used. Charcot made a clear distinction in the symptoms, specifically between rigidity, weakness and bradykinesia. Today, by definition, the disease is manifested in the classical symptoms: resting tremor, rigidity, bradykinesia, and postural instability (Fahn, 2003). Starting in the early 1900's, more discoveries were made into the pathology of PD including observations made by Frederic Lewy in 1912, 100 years after Parkinson's initial observations. He described the discovery of

microscopic particles within cells of diseased patients which later were found to be mostly composed of α-synuclein and be termed Lewy body inclusions (Shults, 2006; Spillantini et al., 1998), a hallmark of PD. Later on, observations by Konstantin Tretiakoff and Rolf Hassler would confirm that the *substantia nigra pars compacta* (SNc) was the main cerebral structure affected. In the 1950s, a breakthrough in dopamine research came that would eventually earn Arvid Carlsson, along with Paul Greengard and Eric R. Kandel, the Nobel Prize in Physiology or Medicine in 2000. Arvid Carlsson discovered that dopamine was a neurotransmitter found in the basal ganglia region of the brain and that treating animals with reserpine caused PD like symptoms which could be reversed with the administration of L-DOPA (levodopa), a precursor in the dopamine synthesis process. Further research then translated to the first human trial with use of L-DOPA as a therapy under the research of Oleh Hornykiewicz in 1961. By 1967, L-DOPA was commonly used as a treatment for the movement symptoms of PD and is still being used today. Since the 1960's many discoveries surrounding the molecular processes of PD have been made due to the advancement in technology, methodologies, and overall understanding of the disease. The discovery by Spillantini, Trojanowski, Goedert (1997) that α-synuclein is the main component of Lewy bodies added significant understanding to the pathology of the disease (History of PD reviewed in Goetz, 2011).

As the culmination of research over the past decades continued, the true test would come with the introduction of cell transplants in human patients in the late 1980s and early 1990s. Today, these early trials are still considered as the proof of concept for a future stem cell-based cell replacement therapy (CRT) for PD that is now around the corner.

1.1.2 Current knowledge on PD

Today, PD affects about 2% of the world's population over the age of 65 and is the second most common neurodegenerative disorder after Alzheimer's disease. PD occurrence is highly correlated with age, but in some cases though, patients acquire early-onset forms of the disease (before 50 years of age) (de Lau and Breteler, 2006). Clinically, the loss of midbrain dopaminergic (mDA) neurons in the SNc results in the manifestation of the classical motor symptoms of the disease.

Although it is these symptoms that we associate with PD, many other cell types are affected such as noradrenergic neurons in the *locus coeruleus* (Hassler, 1938), peptidergic neurons (Agid et al., 1986), or serotonergic neurons (Chaudhuri et al., 2006) are responsible for non-motor symptoms. Some of the cell types impaired also give insight into how the disease progresses over time. One convincing idea, the Braak theory and staging system proposes the start of the disease in the lower part of the brainstem, the medulla oblongata as well as the olfactory bulb, where early Lewy bodies can be detected and then progresses upward in the brainstem and affects parts of the autonomic, limbic, and somatomotor systems (Braak et al., 2003, 2007). Furthermore, theories on origin of PD within the gut are also convincing, affecting cells of the enteric nervous system, consistent with early symptoms such as constipation, and then spreading of α-synuclein to the central nervous system (Klingelhoefer and Reichmann, 2015). Beyond these early affected regions, late stage PD symptoms in the form of cognitive

decline give indication to the spreading of the disease to the cortical regions of the brain (Schapira et al., 2017; Wolters and Francot, 1998).

Despite the extensive knowledge about the various pathological features of PD, the cause of the disease is still a mystery with some research reporting ties to environmental factors, and in less than 10% of cases some genetic links even though they are not fully penetrant. Some of these genetic hallmarks include mutations in genes such as α-synuclein (*SNCA*), leucine-rich repeat kinase 2 (*LRRK2*), *GBA*, *PARK2*, *PINK1*, and *VPS35* (Bonifati, 2014; Trinh and Farrer, 2013) among others. Dominantly inherited and late-onset forms of PD include missense mutations in *SNCA*, *LRRK2*, and *VPS35*. Recessively inherited missense mutations found in early onset forms of PD include the RBR E3 ubiquitin protein ligase, parkin (*PARK2*), the PTEN induced putative kinase 1 (*PINK1*), the protein deglycase DJ-1 *(PARK7*), and the ATPase type 13A2 (*ATP13A2*). Furthermore, a large-scale genome-wide association study has identified additional risk loci associated to PD (Nalls et al., 2014).

Mutations in these genes affect various molecular pathways leading to abnormal cellular processes that could lead to pathogenesis of PD. Many of these genes affect intracellular protein aggregation, protein trafficking and lysosomal-autophagy activity. The first major implicated gene in PD was α-synuclein. Under normal conditions, α-synuclein associates with synaptic vesicles and is broken down through lysosomes or proteasomal degradation. Under pathological conditions, α-synuclein accumulates and forms fibrils that are not degraded and disrupt processes such as intracellular trafficking, endosome-lysosome function and contribute to oxidative stress and mitochondrial dysfunction. α-synuclein can also be transmitted from one neuron to the next in a prion like manner, giving rise to a pathological spreading of disease. Some of the other genes mentioned above are also known to play various roles in these pathways and are thought to interact (for extensive review, see Abeliovich and Gitler, 2016).

90% of the cases of PD are currently thought to be idiopathic, that is without known cause. As in many other diseases, environmental factors such as diet, toxic substances and infectious agents have been suggested. However, evidence for a causative role has only been well documented in few patients exposed to the DA neurotoxin MPP+ (Langston et al., 1983). PD thus appears to be a multifactorial disorder where no single contributing factor is currently known or can explain the majority of the cases. At the same time, there is a certain degree of individual variation between PD patients with regard to onset, progression, clinical manifestations and response to treatment.

1.1.3 Current treatments

Without a clear understanding of origin of disease, current treatments do not address the underlying cause of disease and focus on symptomatic relief. In most cases, treatment of these patients involves administration of different types of drugs and in some cases more invasive surgical interventions.

Traditionally, replacing the loss of the neurotransmitter, dopamine, and its function has been the main target of pharmacological intervention. Today, the most common and the gold-

standard for the treatment of PD is L-DOPA, the precursor of dopamine and other catecholamines. L-DOPA, can be administered orally and is currently combined with carbidopa to prevent its metabolism and increase the proportion of L-DOPA that passes the blood-brain barrier. Once it has entered the central nervous system, it can be converted into dopamine by aromatic L-amino acidic decarboxylase (AADC; Dopa decarboxylase, DDC) and in turn increase available dopamine needed for neurotransmission. Long term use of L-DOPA results in decreased efficacy and adverse effects such as motor fluctuations and dyskinesias (Cenci, 2007; Huot, 2015; Whitfield et al., 2014).

Another pharmacological treatment consists of the use of synthetic dopamine receptor agonists to stimulate the target neurons expressing dopamine receptors and regulate neural activity. Two of the more common medications are pramipexole and ropinirole. Although less potent than L-DOPA, dopamine receptor agonists can result in side-effects that vary patient to patient, such as hypersexuality and gambling behaviors, emphasizing the importance of case based recommended treatment.

Targeting dopamine degrading enzymes with inhibitors in order to supplement or maintain neurotransmitter production and reduce recycling is another method of medication. This includes MAO-B inhibitors to block an enzyme in the brain that catabolizes L-DOPA. COMT (catechol-O-methyl transferase) inhibitors can also be used, to affect the metabolism of L-DOPA and extending its biological half-life. Many other medications exist that help to alleviate specific symptoms of PD and can be prescribed based on patient circumstances.

Surgical treatments offer a different spectrum of treatments but are more invasive and reserved for more severe conditions. The most common surgical treatment is the implantation of electrodes in the subthalamic nucleus for deep brain stimulation. Deep brain stimulation has been approved as a PD treatment since 1997 and similar to the various prescribed medications, patient selection is key for positive outcome (Krack et al., 2017; Miocinovic et al., 2013).

Nevertheless, none of these treatments are a true cure as they do not change the progressive course of the disease and patients often require higher doses or increased stimulation over time. To this end, other methods need to be considered.

1.1.4 Cell replacement therapy

CRT has been developed over several years as a candidate alternative to dopamine neurotransmitter replacement therapy. Although several different cell types have been used for cell transplantation, it has become clear that mDA cells are required for efficient cell replacement. The first transplant study using human fetal ventral midbrain (VM) tissue was performed in Lund, Sweden, in 1989. This and subsequent trials demonstrated that CRT was possible, but that the cell preparations and transplantation had to fulfill certain criteria (reviewed in Lindvall and Kokaia, 2009). These pioneering clinical trials provided proof of concept for CRT. However, while some patients from these trials have shown positive benefits well over 15 years post-transplant (Kefalopoulou et al., 2014), others have shown little, none, or in some cases worsening of symptoms. To investigate further into possible explanations for

this, one more clinical trial is currently being conducted with fetal VM tissue to confirm the importance of variables such as cell preparation, transplantation, patient selection, immunosuppression and outcome measures for CRT (TRANSEURO) (Kirkeby et al., 2017a).

Nevertheless, we should remember that even though CRT can show long-term positive results, it still only aims at alleviating the motor symptoms of PD and it is now well understood that PD, as it progresses, affects more than just movement, and can give non-motor symptoms including depression, dementia, sleep disturbances, and gastro-intestinal complications, some of which arise even before the motor symptoms (Kalia and Lang, 2015; Park and Stacy, 2009; Schapira et al., 2017).

Although fetal tissue has been successfully used for CRT, this is not a viable solution for the treatment of PD in the future. The use of fetal tissue raises ethical issues that needs to be carefully taken into consideration. Fetal tissue is also not practical due to its limited access and the need of tissue pieces from several fetuses (up to 3-4) in order to get enough cells to treat just one brain hemisphere of a PD patient. This type of therapy also introduces a problem of standardization, as each cell preparation derives from various different donors and tissues are not in the same condition. For CRT to become a standardized method that could be implemented in a reproducible manner to multiple patients, it is essential to have an accessible and up-scalable cell source, standardize all the procedures and consistently obtain high quality cell preparations. It is currently thought that human pluripotent stem cells (hPSCs) are the most optimal starting population for CRT (Barker et al., 2015). hPSCs can be obtained from blastocysts (embryonic stem (ES) cells), or by reprogramming of somatic cells, such as skin fibroblasts, the so called induced pluripotent stem (iPS) cells. hPSCs have shown the capacity to differentiate into mDA neurons and provide functional recovery in animal models of PD (reviewed in Sundberg and Isacson, 2014).

A third novel strategy for CRT is the replacement of DA cells by transformation of existing non-neuronal cells in the brain. It has been shown that direct *in vivo* transformation of astrocytes to mDA neurons is possible by using viral vectors in a mouse model of PD (Rivetti di Val Cervo et al., 2017). These strategies for PD CRT are outlined in **Figure 1**.

hPSCs of various sources are currently used in clinical trials to treat a wide range of disorders including PD (Trounson and McDonald, 2015). hPSCs have the capacity to expand and to differentiate into all cell types of the body, therefore, treatment of PD and other disorders appears to favor this methodology. Although the general idea of the correct cell type required for CRT in PD is agreed upon (mDA neurons), there are many different protocols and the quest to produce bona fide SNc DA neurons (Arenas et al., 2015) is still ongoing. In addition, it is at present unclear what constitutes or defines a perfect human DA cell type for transplantation? and whether or not the cells produced in vitro resemble sufficiently their in vivo counterparts? and how much should they resemble with regard to gene expression and function to be considered as suitable for transplantation? To answer these questions, we need to: (1) gain a better definition of mDA neuron development in vivo and what is the composition of their environment, the VM; (2) Apply developmental knowledge to improve stem cell differentiation

protocols; (3) Establish molecular-functional correlates before and after transplantation with the goal to improve efficiency and safety of mDA cell preparations; and (4) Develop standard protocols to obtain cell preparations with reproducible quality and safety.

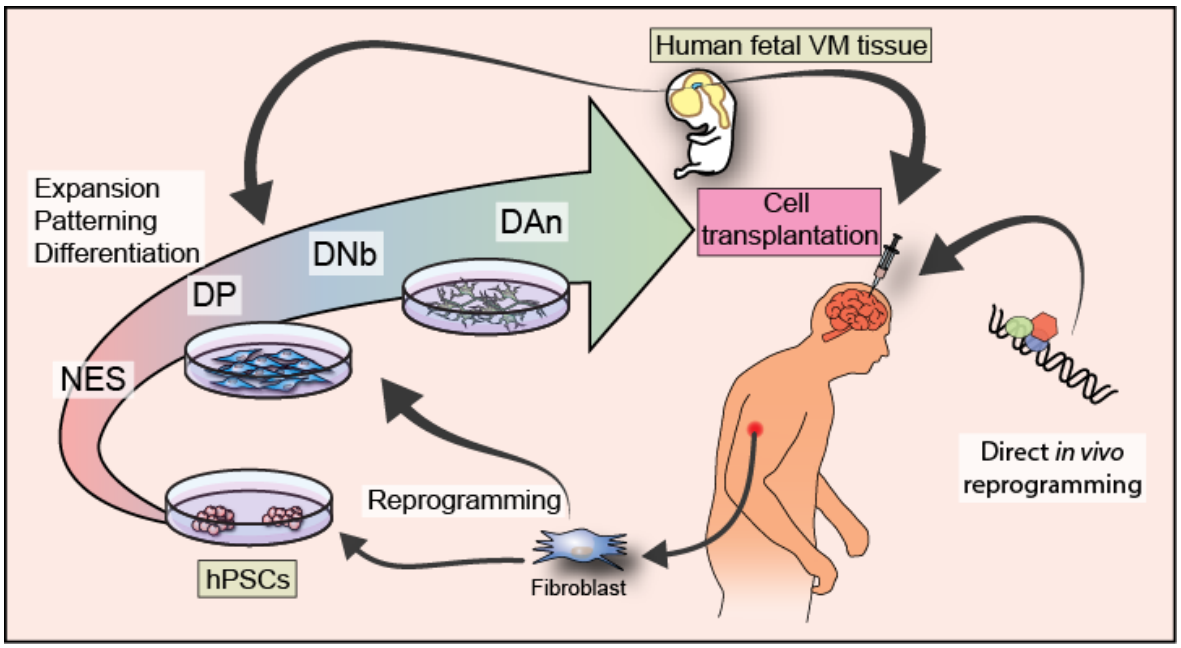

Figure 1: Cell replacement therapy strategies
Schematic of CRT approaches for PD. Different approaches include use of human fetal tissue for transplantation either directly or after expansion. hPSCs such as ES (derived from blastocysts) or iPS cell lines (derived from fibroblasts) could be differentiated into DA cells and used for transplantation. Fibroblasts could also be directly converted to mDA cells *in vitro* and then transplanted. Alternatively, somatic cells in the adult brain, such as astrocytes, could be reprogrammed *in vivo* into mDA neurons. NES: neuroepithelial stem cell; DP: dopaminergic progenitor; DNb: dopaminergic neuroblast; DAn: dopaminergic neuron.

As the scientific community in the field addresses and answers more of these questions, we are getting closer to the implementation of CRT for PD patients. Optimization and standardization of clinically safe cells for CRT is a whole endeavor in itself due to strict permissions and regulations on how to conduct these trials. Furthermore, making conclusions based on mouse pre-clinical data and translating that to human has shown to be difficult in several different clinical trials only to result in no efficacy for humans. This is in part related to the fact that the animal models do not capture all aspects of human disease, but also to the fact that many of the fundamental aspects of development applied to human stem cells come from studies in rodents and may differ from developmental processes in human, misguiding thus our efforts. One example of this are the species differences in Wnt/β-catenin signaling between mice and human; while Wnt/β-catenin activation was not required in mouse ES cell cultures for mDA differentiation, it has proven to be essential for the specification of hPSCs into midbrain floor plate and their correct differentiation into mDA neurons (Kirkeby et al., 2012; Kriks et al., 2011; Xi et al., 2012). More emphasis thus needs to be placed on comparative studies and in translating human developmental mechanisms to protocols to improve current stem cell preparations and make them suitable for human therapy. It is therefore essential that we gain a better understanding, in the case of PD, of the factors critical for VM development. Using

today's technologies to gain insight of *in vivo* developmental processes in human, such as single-cell RNA-sequencing (scRNA-seq) and CRISPR/Cas9 technologies, we are approaching a level of understanding of gene expression and function in human development, that is allowing us to improve the way we capture and recapitulate this process in a dish. We think this knowledge will translate in improved hPSC-derived mDA neuron preparations for CRT in PD.

One important aspect for translational research, in addition of cell intrinsic factors, such as transcription factors, and cell extrinsic factors, such as morphogens and growth factors, is the extracellular matrix (ECM). The physiological role of ECM components has been largely ignored in most differentiation protocols. The implementation of new ECM components, as well as new methodology to culture and differentiate stem cells in 3D environments may be important to replicate *in vivo* conditions and to improve CRT for PD in the future. Simple scaffold matrixes or manipulation of adhesion surface for cells to differentiate and migrate have shown to significantly impact cell maturation and survival (Ni et al., 2013; Wang et al., 2016; Zhang et al., 2017).

1.2 VENTRAL MIDBRAIN DEVELOPMENT

For the last few decades, numerous factors involved in midbrain development have been thoroughly studied (for comprehensive review see Arenas et al., 2015; Blaess and Ang, 2015; Bodea and Blaess, 2015). The following sections to come briefly describe the sequential steps taking place during VM and mDA neuron development: Specification, Proliferation, Neurogenesis, Differentiation, and Migration (**Figure 2**). The development of the neural tissue begins when the ectoderm gives rise to the neural plate at embryonic day (E) 7.5 in mouse, day 18 in human, followed by the formation of the neural folds and then the neural tube, which starts being formed by neurulation at E8 in mouse, day 20 in human, and is completely closed by E10.5 in mouse, day 29 in human (**Figure 2A**). The closing of the neural tube is a highly regulated process requiring Wnt/planar cell polarity (PCP), *Shh* (sonic hedgehog), and BMP (bone morphogenic protein) signaling. These signaling pathways and transcription factors such as *Zic2*, *Pax3*, *Cdx2* and *Grhl2/3* are in place to coordinate cell dynamics, including convergent extension (CE) movements, apical constriction and interkinetic nuclear migration required for proper neural tube formation (**Figure 2A**) (Nikolopoulou et al., 2017).

1.2.1 Specification

The neural tube is patterned by different types of secreted factors called morphogens that establish gradients in both the anterior-posterior and ventral-dorsal axis providing cells with regional identity. Two signaling centers are important for VM development: the floor plate, in the ventral neural tube, and the isthmic organizer (IsO), at the midbrain-hindbrain boundary (MHB). These structures provide the initial signals to define the VM. Extrinsic morphogens in early VM development include *Shh*, derived from the floor plate, and *Wnt1* (wingless-type

MMTV integration site family, member 1) as well as *Fgf8* (fibroblast growth factor 8), derived from the midbrain and hindbrain side of the IsO, respectively (**Figure 2B**).

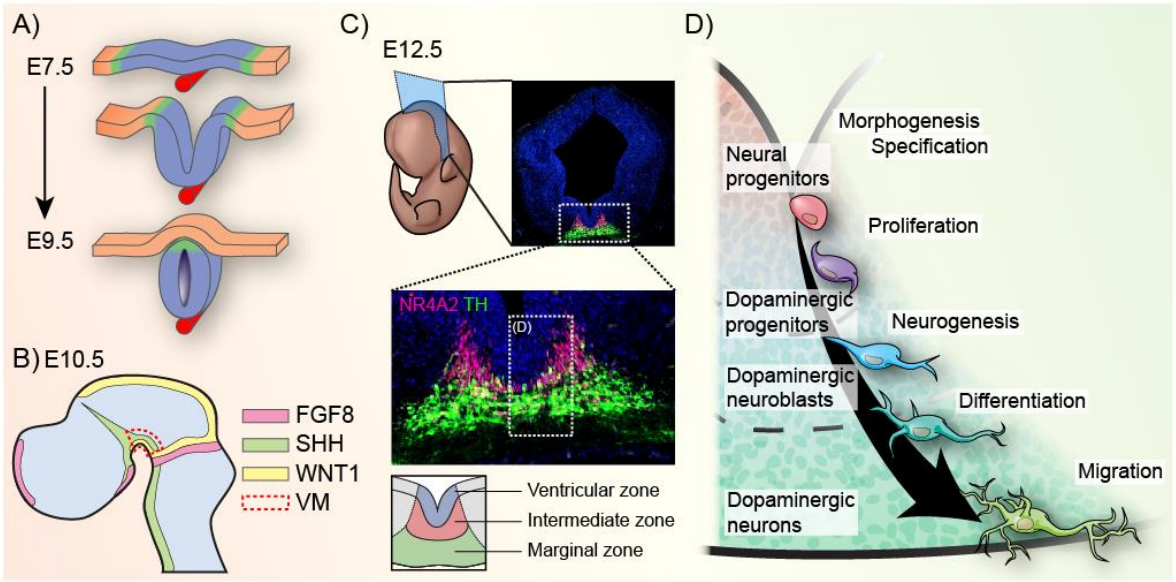

Figure 2: Ventral midbrain dopaminergic development
A) Formation of the neural tube at E8.5 in mouse starting from the neural plate (blue) and through CE movements, neural folds (green) join to form cord with SHH secreting notochord (red) positioned ventrally. B) Sagittal section of early morphogen established boundaries that set up the VM region with gradients of SHH and WNT1. C) Coronal section of mouse brain at E12.5 showing postmitotic cells of the mDA lineage. Three layers and developmental stages can be distinguished. Proliferating progenitors are found within the ventricular zone (VZ). Post-mitotic mDA neuroblasts ($NR4A2^+/TH^-$, red) are present in the intermediate zone (IZ). Differentiated mDA neurons become TH^+ (green) within the marginal zone (MZ). D) Schematic representation of the organization and steps during mDA neuron development.

The floor plate arises by the action of SHH, secreted initially by the notochord ventrally, induces the expression of *Foxa2* (forkhead box A2) in the ventral aspect of the neural tube, which defines the floor plate and in turn expresses *Shh* and initiates a SHH gradient that patterns the ventral-dorsal axis. More laterally, SHH regulates the expression of *Nkx6-1* and *Nkx2-2*, markers defining the basal plate (Prakash et al., 2009). On the other hand, the MHB is established by intrinsic signals, the transcription factors *Otx2* (orthodenticle homolog 2) and *Gbx2* (gastrulation brain homeobox 2). FGF8 secreted at the IsO allows for a gradient to form in the anterior-posterior axis and induces *Wnt1* at the anterior side of the MHB. At the same time, *Otx2* expression regulates the expression of *Lmx1a* and *Lmx1b* (LIM homeobox transcription factor 1 *alpha*/*beta*), which together with *Wnt1*/β-catenin form a critical auto-regulatory network that controls mDA neuron development (Chung et al., 2009). Loss of any of these three genes has drastic consequences for the proper development of mDA neurons (Andersson et al., 2006; Prakash et al., 2006; Smidt et al., 2000; Yan et al., 2011).

The midbrain floor plate thus emerges under the unique influence of two morphogens, SHH and WNT1, and the transcription factor networks they control. Indeed, FOXA1/2, together with, LMX1A and LMX1B serve important functions in specification and differentiation of mDA neurons (Andersson et al., 2006; Ferri et al., 2007; Smidt et al., 2000), which originate

from progenitor cells in the ventricular zone (VZ) of the VM. These progenitors in addition to the transcription factors above, express *Corin* (Ono et al., 2007) as well as the redial glia marker *Glast/Slc1a3* (solute carrier family 1 (glial high affinity glutamate transporter), member 3) (Bonilla et al., 2008).

All these components are nowadays used to examine the quality of the differentiation of hPSCs into mDA neurons *in vitro*. Moreover, hPSCs are specified into midbrain floor plate cells by using SHH and activating Wnt/β-catenin signaling with FGF8 and/or GSK3β inhibitors early in the protocols.

1.2.2 Neurogenesis

Upon floorplate specification, mDA progenitors undergo neurogenesis, the process of by which cells divide to generate postmitotic daughter cells, which express key transcription factors such as the nuclear receptor *Nr4a2* (nuclear receptor subfamily 4 group A member 2, also known as *Nurr1*). Initial neurogenesis is controlled by the expression of proneural basic-helix-loop-helix genes, such as *Ascl1* (achaete-schute family bHLH transcription factor 1, also known as *Mash1*) and *Neurog2* (neurogenin 2, also known as *Ngn2*) (Kele et al., 2006), as well as the *Ferd3l* (Fer3-like (*Drosophila*)), which inhibits the proneural gene suppressor *Hes1* (hairy and enhancer of split 1 (*Drosophila*)) (Ono et al., 2010). These genes are influenced by the already established networks such as Shh-Foxa2 and Lmx1a/b-Wnt1, as well as Wnt5a, which also controls differentiation and cooperates with Wnt1 in mDA neurogenesis (Andersson et al., 2013; Castelo-Branco et al., 2003).

As a consequence of all these processes, the midbrain floor plate becomes a layered structure, with progenitors in the VZ, the layer of cells most proximal to the mesencephalic ventricle. As these cells undergo neurogenesis, postmitotic cells initially migrate in a radial fashion along radial glia, through the intermediate zone (IZ) and to the outer most layer, the marginal zone (MZ), where mDA neurons emerge (**Figure 2C-2D**). DA neurogenesis takes place between E10 and E14.5 in mouse and between 6 to 11 weeks post conception in humans (Almqvist et al., 1996; Nelander et al., 2009).

1.2.3 Differentiation

Commonly, mDA neurons are defined as such when they express the rate limiting enzyme for dopamine synthesis, *Th* (tyrosine hydroxylase), although maturation through electrical signaling properties and functional circuit integration come later. From neurogenesis, the cells subsequently differentiate into mature mDA neurons in the MZ (**Figure 2C-2D**). This process continues to build upon previous network of genes and is supported by additional players. Some transcription factors such as *Nr4a2*, *En1* (engrailed 1), and *Pitx3* (paired-like homeodomain transcription factor 3) have regulatory roles and control other genes such as neurotrophic factors and genes essential for proper functional integration and dopamine release. Trophic factors include *Bdnf* (brain-derived neurotrophic factor), *Gdnf* (glial cell line derived neurotrophic factor) and its receptor, the c-*Ret* proto oncogene. Other genes involved in neurotransmitter identity and function include *Slc18a2*/*Vmat2* (solute carrier family-18

member-2/vesicular monoamine transporter-2), *Slc6a3*/*Dat* (solute carrier family-6 member-3/dopamine transporter), *Drd2* (dopamine receptor D2), *Ddc*, and *Aldh1a1* (aldehyde dehydrogenase family 1, subfamily A1), all of which are part of the machinery essential for the production, release, and recycling of dopamine (reviewed in (Arenas et al., 2015; Hegarty et al., 2013; Rekaik et al., 2015)).

1.2.4 Migration

At around E14.5, no further progenitors are contributing to the mDA neuron pool. During neurogenesis, cells migrate in a radial fashion through the IZ to the MZ, in part due to chemokine signaling involving *Cxcr4*/*Cxcl12* (chemokine (C-X-C motif) receptor 4 / chemokine (C-X-C motif) ligand 12) as well as the ECM protein *Reln* (reelin) (Bodea et al., 2014; Yang et al., 2013b). From this point, mDA neurons migrate in a tangential direction until they reach their final positions where they will integrate into local circuitry postnatally, contributing to roles including, control of motor behavior, reward, decision making and learning. Several types of mDA neurons have been identified based on their anatomical position and where they project their axons. Briefly, these include the SNc DA neurons, which mainly project to the dorsal striatum, the VTA (ventral tegmental area) DA neurons that project to the prefrontal cortex and the limbic system, and the RRF (retrorubral field) DA neurons that project to the dorsal striatum, limbic system and prefrontal cortex (Bodea and Blaess, 2015; Roeper, 2013).

1.3 DOPAMINERGIC NEURONS

Understanding the different subtypes of mDA neurons in the VM is important not only from a developmental perspective, but also from a disease perspective, as SNc DA neurons are the most vulnerable to PD and their demise leads to the clinical manifestations that characterizes this disease (Damier et al., 1999; Javoy-Agid and Agid, 1980; McRitchie et al., 1996).

The developing VM is known to give rise to these three similar but spatially separated mDA neuron populations, the SNc, VTA, and RRF (Björklund and Dunnett, 2007). These distinct anatomically-defined mDA neurons have been more recently re-assessed and defined at a molecular level by examining the subsets of genes they express (Chung et al., 2005; Greene et al., 2005; Grimm et al., 2004). This type of work has led to the identification of markers such as *Slc6a3*/*Dat* and *Kcnj6* (potassium inwardly-rectifying channel, subfamily J, member 6, also known as *Girk2*), which mark the SNc and dorsolateral VTA, and *Calb1* (calbindin 1), expressed in the VTA and partially in the dorsal tier of the SNc (Fu et al., 2012; Thompson et al., 2005). More recently, *Sox6* (SRY (sex determining region Y)-box 6) has been shown to localize to a subset of SNc neurons and *Nolz1* (*Zfp503*, zinc finger protein 503) and *Otx2* mark subpopulations of VTA (Panman et al., 2014). These are only a few genes that have been explored but ongoing evaluation reveals new candidates for possible separation of the mDA neuron population, including studies on some cells being able to co-release GABA and classification of cells based upon expression of different voltage gated channels (Smits et al.,

2006, 2013; Tecuapetla et al., 2010). Other interesting studies have focused on birth dating neurons and tracing them to adulthood, which have shown that separate populations are born at different time points (Bayer et al., 1995; Bye et al., 2012).

The question is how do we define a cell type? If one would focus on just the neuronal diversity within the body, the list of criteria for classification causes inherent problems (Bota and Swanson, 2007; Fishell and Heintz, 2013; Nelson et al., 2016). Further classification of mDA cell types beyond the three anatomical types can be done using distinct parameters which are very likely to give further subdivisions and even different results (Clevers et al., 2017). For instance, defining a cell type based on anatomical features such as efferent projections vs. electrophysiological profiles will each give two sets of unique populations and up to 13 different subpopulations (Fu et al., 2012; Roeper, 2013). Thus, defining a subtype is dependent on the tools used to examine them and the interpretation of the results. One solution thus is to use a single level of information to exhaustively examine the cells and define their identity. For instance, the developmental programs required to acquire discrete mDA population identities has not yet been fully elucidated, in part due to the dynamic/continuous nature of the process (for review see Bodea and Blaess, 2015) and in part due to our incomplete knowledge of the genes and pathways involved. Although the definition of a cell type will be discussed for many years to come, new advanced technologies are aiding to cluster cells based on similar features, as no two cells will ever be identical.

1.3.1 Single-cell RNA-sequencing and defining cell types

Multicellular organisms have evolved by generating specialized cells that perform specific roles contributing to the overall function of the organism as a whole (Arendt, 2008). In the late 1800's when Santiago Ramón y Cajal illustrated the cell heterogeneity of the brain based solely on morphology, studies that would earn him the Nobel Prize in Physiology and Medicine 1906, cells were classified by morphology alone. Increasing levels of information in recent years makes it unavoidable to think how should we define a neuron today and at what point should we stop making distinctions between two cells (Bock, 2013). Whether classification occurs via morphology, as Ramón y Cajal did, or by connectivity and electrophysiological properties, it is no doubt that the complexity in the specialization and connectivity of neurons requires further investigation. This will aid in understanding what occurs under normal physiological function and how pathologies arise when a certain cell type malfunctions (Luo et al., 2008; Masland, 2004). Indeed, by being able to distinguish two similar cell types within the cellular diversity of an organism or tissue it could be possible to formulate new hypothesis, such as which cells can be targeted by specific diseases, should we know a disease mechanism; or the opposite, knowing cell types affected by disease, what may be the possible mechanisms.

Classical studies using transgenic mice and biochemical methods have been for many decades the tool of choice to study particular cells expressing specific genes. However, the recent development of advanced technologies have allowed to study developmental processes, with high scrutiny, at a global level, and are revolutionizing the way we study mechanisms of development (Poulin et al., 2016). A combination of three steps: single-cell capture methods,

nucleic acid amplification methods, and next-generation sequencing, provided proof of principle and allowed a very rapid development of scRNA-seq into a robust high-throughput method. This method has allowed for the generation of large data sets and gaining molecular insights into mechanisms of development and cell type definition (Linnarsson and Teichmann, 2016; Shapiro et al., 2013; Trapnell, 2015). The analysis of continuous process such as development, by capturing gene expression in cells at any given time point is like taking landscape pictures in time. With many pictures, a reconstruction of cell lineages can be obtained, contributing to understand the sequence of the underlying processes at a molecular level.

From a tissue piece to single cells and sequencing, a unique genetic fingerprint of the activity of individual cells can be taken (Islam et al., 2011, 2012). Not only does this method allow to identify individual cell types by their molecular signature, but it allows to count absolute RNA molecules and determine the transcriptional activation of individual genes (Islam et al., 2014; Kivioja et al., 2011). Applying this technology to the developing VM, or any other tissue, allows the discovery of unknown molecular players in development as well as novel cell types (Marques et al., 2016; Pollen et al., 2014; Treutlein et al., 2014; Zeisel et al., 2015). Moreover, gene expression levels can be used not only to examine the functional state of the cell, but also their identity.

A number of methods have been used to isolate single cells including manual picking, laser capture, FACS (Fluorescence-activated cell sorting), microfluidic devices, and microdroplets. Whichever method is used for single-cell isolation and capture, the following step require reverse transcription and nucleic acid amplification, which have just recently been modified to cover the genome for scRNA-seq (methods reviewed in Grün and van Oudenaarden, 2015; Kolodziejczyk et al., 2015). The method used in **Paper II**, STRT RNA-Seq, is a 5'-end amplification of cDNA method that allows for reduced technical variability and the incorporation of unique molecular identifiers for counting mRNA molecule and reducing amplification bias (Islam et al., 2012, 2014). Other methods allow for full-length coverage mRNAs (SMART-Seq) and others are 3'-end focused (CEL-Seq, Drop-Seq) (for methods review see Grün and van Oudenaarden, 2015; Kolodziejczyk et al., 2015). For every single-cell experiment, it is important to consider what the goal is and expected outcome. Whether the goal is to investigate a very heterogeneous tissue or a FACS sorted tissue to pre-select a specific cell type, it is important to consider both the number of cells to be sequenced and the sequencing depth (number of transcripts sequenced per cell). Sampling more cells or deeper sequencing will result in better resolution between clustered cell types but will eventually reach a point where no more information is gained. Ideally, one should sequence as many cells as possible to achieve the highest resolution, although this has not always been feasible because of the early, very high cost of such experiments. This is becoming less of a problem as the efficiency of these methods have improved and the cost has decreased.

The probability of finding exactly the same transcriptomic profiles in two cells using scRNA-seq methods is very low due to transcriptional dynamics, but grouping cells based on their

transcriptome similarity allows for the identification of similar cell types. Different clustering methods have their advantages and disadvantages (Bacher and Kendziorski, 2016; Stegle et al., 2015). In **Paper II**, BackSPIN is used for discovery and classification of cell types into groups (Zeisel et al., 2015). The high dimensionality of the data obtained from such methods has required very sophisticated bioinformatical tools in order to reduce this dimensionality into something comprehensible. The overall idea is to be able to group cells by reducing their dimensionality or the number of genes needed to distinguish cell type clusters. This is done by selecting genes that have high variance compared to that of the inherent technical noise. One such technique for visualizing this is t-distributed stochastic neighbor embedding (t-SNE) (Van der Maaten et al., 2008). Further clustering methods aid in the visualization of separation of groups and subgroups.

1.3.2 RNA-Sequencing and midbrain heterogeneity

More recently, Poulin et al., used a multiplexed single-cell qRT-PCR (quantitative real-time PCR) approach to resolve the heterogeneity of mDA neurons at postnatal day (P) 4 where five molecularly distinct mature mouse mDA neuron populations were identified based on a predetermined selection of genes (Poulin et al., 2014). However, a systematic and unbiased molecular definition of mDA neurons was not available and our knowledge of mouse, but particularly of human mDA neuron development was quite limited. As discussed in previous sections, elucidating how mDA cells diversify during development is of the outmost importance to be able to guide the generation of mDA neurons for PD CRT. Indeed, this information is very important as it may allow us to recapitulate mDA neuron development *in vitro* and make SNc DA neurons for PD CRT. Unbiased scRNA-seq currently offers the opportunity to interrogate the entire transcriptome of individual mDA neurons and determine their gene expression profiles in a systematic manner, allowing thus to reveal differences between cell types and transcriptional states. In **Paper II** (La Manno et al., 2016), we follow this approach to answer these questions.

Another recent study has used FACS sorted cells from *Lmx1a-EGFP* mice in order to delineate early mDA fates (Kee et al., 2017). Using a scRNA-seq approach, the authors were able to differentiate between two lineages, one being the mDA lineage and the other being that of closely related subthalamic nucleus neurons. This data has allowed to identify contaminating cell types in ES cell preparations containing mDA neurons and to improve mDA differentiation protocols for CRT in PD (Kirkeby et al., 2017b).

More recently, a single-cell resolution experiment has compared DA neurons from different regions of *Th-EGFP* mice at E15.5 and P7 (Hook et al., 2017). This study compared several DA populations in the brain in order to find specific traits of mDA cells. Although their resolution is lower than that in **Paper II** (identified only two mDA populations at E15.5), they did capture four populations at P7, including a SNc, a VTA, and surprisingly a postnatal progenitor population. They also took advantage of GWAS (genome-wide association study) data to identify the cell types expressing genes possibly involved in PD susceptibility.

1.4 WNT SIGNALING

As mentioned above, Wnt regulates multiple aspects of mDA neuron development by activating different Wnt signaling pathways (for full review see Arenas, 2014). Wnts are a family of secreted lipid-modified glycoproteins that consist of 19 different family members in mammals that function as morphogens. They bind to a family of G protein-coupled seven transmembrane receptors, Frizzled (Fzd), and several co-receptors (van Amerongen and Nusse, 2009). Wnt signaling plays a role in nearly all cells and tissues, where it controls multiple processes during embryogenesis, development, adulthood and disease. The functional diversity of this pathway is thought to be conveyed by the presence of multiple signaling components that are brought together to signaling complexes by different co-receptors (Willert and Nusse, 2012).

Structurally, Wnts are palmitoylated and this is fundamental for its secretion and interaction with receptors. Due to their high hydrophobicity, it has been difficult to crystallize and study the structure of Wnts. The crystal structure was finally resolved for *Xenopus* WNT8 connected to the extracellular cysteine-rich domain (CRD) of FZD8 and was shown to be in the shape of a 'hand' with the 'thumb' and 'index' finger creating the binding site to the Fzd receptor (Janda et al., 2012). The Fzd receptor has a hydrophobic pocket to mediate interaction with WNT and essential for protecting the lipid group on Wnt for crystal structure determination. The Wnt protein is divided into an N-terminal D1 domain and a C-terminal D2 domain with various hairpins and lipid-modification sites for binding. The structure has contributed to further understanding Wnt function and importantly, has implications for the design of new ways to target the signaling pathway and for drug discovery. However, the structural activation of the pathway, the Wnt-Fzd specificity, and the way Wnts interact with their co-receptors, still remains a mystery (Ke et al., 2013).

1.4.1 History and background

The history of Wnts is relatively recent compared to other pathways. Just over 30 years ago, the gene *Int1* was originally identified as an oncogene as it was found to contribute to mammary carcinomas upon insertional activation by the Mouse Mammary Tumor Virus or MMTV (Nusse and Varmus, 1982, 2012). The genes of the *Drosophila wingless* (*Wg*) (Baker, 1987; Cabrera et al., 1987; Rijsewijk et al., 1987) and *mouse Int1* were later found to be homologous and by combining their two names, the word/gene '*Wnt1*' came out (Nusse and Varmus, 1992; Nusse et al., 1991). Over subsequent years, other Wnt family members and their downstream signaling components, including the receptor Fzd (Bhanot et al., 1996) and the co-receptor *Lrp5/6* (low-density lipoprotein receptor related protein 5/6) were discovered (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000).

Secretion of Wnts is performed with the aid of Porcupine (*Porcn*) in the endoplasmic reticulum and Wntless (*Wls*) within the Golgi network. *Porcn* is a palmitoyl transferase for lipid modification on the Wnt protein, which is necessary for binding to Fzd receptor, and *Porcn* loss leads to retention of WNT within the cell. *Wls*, is required for transport of WNT to the

plasma membrane and secretion. Beyond secretion, extracellular transport of Wnt is not clear as evidence suggests different mechanisms such as protein carriers, exosomes or secretory vesicles. Although Wnt secretion and transport appear simple, evidence is conflicting and seems to be cell context-dependent (Takada et al., 2017).

Wnt signaling has been classically divided into two main pathways. One being the β-catenin dependent signaling pathway, also referred to as the canonical pathway, and the other being β-catenin independent, referred to as the non-canonical pathways. Briefly, In the OFF state, a destruction complex leads to the phosphorylation and proteasomal degradation of β-catenin. In the ON state, activation of the WNT/β-catenin-dependent pathway by the ligand leads to a stabilization of β-catenin in the cytoplasm, which translocates to the nucleus and regulates gene transcription. There are two main WNT/β-catenin-independent pathways, the Wnt/PCP pathway, which induces cytoskeletal changes, and the Wnt/Calcium (Ca^{2+}) pathway, which induces calcium fluxes and is important for cell homeostasis (De, 2011). A schematic of the different Wnt signaling states and pathways is shown in **Figure 3** The presence and regulation of diverse Wnt signaling components, ligands, receptors and co-receptors, within a cell determines how the WNT signal is transduced. WNTs can thus signal in many different ways depending on the cell types, their position (exposure to different ligands and modulators) and time (varying signaling components). For reviews on the complexity of Wnt signaling, see for instance Komiya and Habas (2008) and Niehrs (2012).

1.4.2 Wnt/β-catenin signaling

In the OFF state, when Wnt is not present or signaling is being inhibited, the destruction complex residing in the cytoplasm binds to and phosphorylates β-catenin, marking it for degradation by the proteasome. The destruction complex consists of the scaffold protein AXIN (*Axin1/2*) interacting with β-catenin, adenomatosis polyposis coli (*Apc*), beta-transducin repeat containing E3 ubiquitin protein ligase (*Btrc*), and the serine-threonine kinases glycogen synthase kinase 3α/β (*Gsk3α/β*) and casein kinase 1 α/δ (*Ck1α/δ*). GSK3 and CK1 phosphorylate β-catenin which then allows the E3 ubiquitin ligase, β-TrCP, to ubiquitinate β-catenin, marking it for proteasomal degradation. Within the nucleus, target genes are not transcribed and transcription factors interacting with β-catenin, T cell factors (*Tcf*) and Lymphoid enhancer factor (*Lef*) are bound and repressed by *Groucho* (**Figure 3**, left) (Nusse and Clevers, 2017).

In the ON state, Wnt/β-catenin dependent signaling is initiated by Wnts binding to one of the ten G protein-coupled FZD receptors and dimerization with LRP5/6 occurs (**Figure 3**, middle). The binding of Wnt occurs between its lipid moiety in WNT and to the hydrophobic pocket in the CRD of FZD. The internal domain of LRP5/6 is phosphorylated and the scaffold protein AXIN is recruited to the membrane. DVL (dishevelled) is also recruited in the complex to the membrane and binds to the cytoplasmic part of the FZD receptor, and through its DIX domain mediates the complex of FZD-DVL-AXIN-LRP5/6. The destruction complex is then recruited to the membrane, subsequently β-catenin dissociates and its phosphorylation is then inhibited. Activated (dephosphorylated) free β-catenin accumulates in the cytosol and translocates to the

nucleus. Once in the nucleus, it acts as a co-activator to transcription factors of the TCF/LEF family to promote transcription of Wnt target genes. Target genes can be cell-type specific but some common targets genes include *C-myc*, *CyclinD1*, and *Axin2* (Nusse and Clevers, 2017).

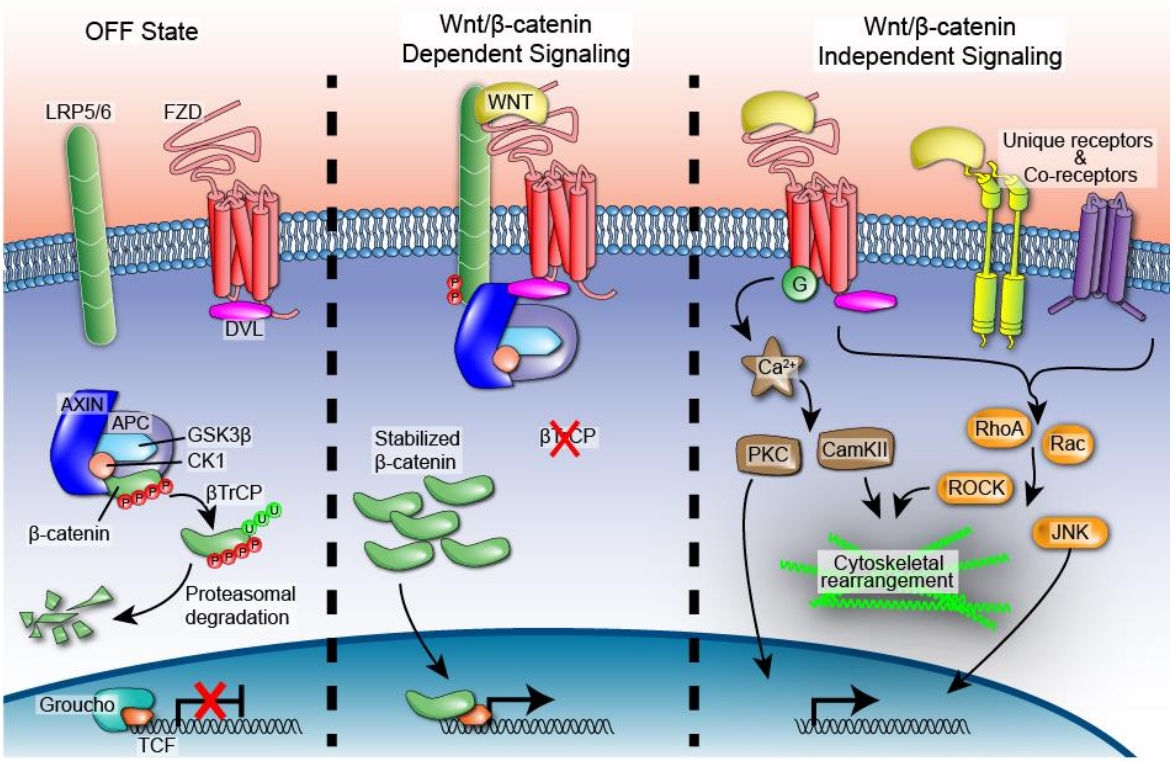

Figure 3: Wnt/β-catenin dependent and independent signaling
Overview of different states and pathways of Wnt signaling. Left: When WNT is not present or being inhibited, in the OFF state, β-catenin is phosphorylated by GSK3β and CK1 in the destruction complex allowing for the ubiquitination of β-catenin by βTrCP and destined from proteasomal degradation. Transcription is inhibited by transcriptional corepressor Groucho. Middle: Activation of Wnt/β-catenin dependent signaling is triggered by WNT binding to FZD-LRP5/6 receptors, DVL/AXIN is recruited to membrane along with the destruction complex. β-catenin is then neither phosphorylated nor targeted for degradation, allowing for the stabilization and accumulation of in the cytosol and its subsequent translocation to the nucleus to control transcription by binding to TCF/LEF. Right: Activation of Wnt/β-catenin independent signaling results from WNT binding to FZD or a number of other receptors coupled to alternative intracellular signaling components of the Ca^{2+} or PCP pathway.

Wnt signaling is highly regulated by various inhibitors and modulators. Dickkopf proteins (DKKs) are the best well characterized family of secreted Wnt/βcatenin signaling antagonists. This family is formed by 4 members (DKK1-4). While DKK1/2/4 bind to LRP5/6 to disrupt Wnt signaling, DKK3 does not bind LRP5/6 and it can either block or activate Wnt/βcatenin signaling. Another form of modulation is via the sFRPs (secreted FZD-related proteins) of which there are 5 members (*Sfrp1-5*). sFRPs contain a CRD that shows similarities to that of FZD, which allows them to bind to WNT directly. sFRPs can also bind to the Fzd receptor to prevent WNT binding. A similar mode of inhibition by binding directly to WNT can also occur with WIF1 (Wnt inhibitory factor-1). Due to the ability of both sFRPs and WIF1 to bind to WNT directly, these inhibitors can affect all the Wnt pathways. Other effectors of the Wnt pathway include *Wise*/*Sost*, *Igfbp-4*, *Shisa*, *Waif1a*, *Apcdd1*, and *Tiki1* (For comprehensive review see Cruciat and Niehrs, 2012; Malinauskas and Jones, 2014).

1.4.3 Wnt/planar cell polarity signaling

This pathway gets its name because it controls the polarization of cells within a plane. During development, the spatial and temporal distribution of cells is critical for the formation of the organism. WNT/PCP regulates the asymmetric distribution of proteins within the cells, the orientation of cilia and CE movements (cell migration and intercalation). In vertebrates, the process of CE is important first in anterior-posterior axis formation during gastrulation (Heisenberg et al., 2000; Tada and Smith, 2000)., Neurulation is also driven by CE movements (Ciruna et al., 2006; Tawk et al., 2007; Wallingford and Harland, 2002). Other developmental processes controlled by PCP signaling in mammals include, but are not limited to hair cell orientation (Guo et al., 2004), inner ear sensory hair cell orientation (Curtin et al., 2003; Montcouquiol et al., 2003), orientation of cilia (Song et al., 2010), axon guidance (Fenstermaker et al., 2010), and limb elongation (Gao et al., 2011). These cellular processes are highly conserved across species and essential for correct development and organogenesis.

Wnt/PCP was first discovered in *Drosophila* mutants exhibiting alterations in cuticular processes (Gubb and García-Bellido, 1982). Core PCP components in *Drosophila* include *frizzled*, *disheveled*, *prickle*, *van gogh/strabismus*, *flamingo*, and *diego*. Many of these core components are conserved in vertebrates and have expanded to form families of signaling components (Wansleeben and Meijlink, 2011), such as *Frizzled3/6*, *Dvl1/2/3*, *Prickle1/2* *Vangl1/2* (vang-like 1/2 (*van gogh,, Drosophila*)), *Celsr1/2/3* and *Ankrd6* (ankyrin repeat domain 6). Beyond the orthologs found in vertebrates and *Drosophila*, additional PCP related genes have been identified in vertebrates including *Ptk7* (protein tyrosine kinase 7), *Scribble* and *Ror1/2* (receptor tyrosine kinase-like orphan receptor 1/2) receptors. For comprehensive review on PCP signaling see (Gao, 2012; Singh and Mlodzik, 2012; Yang and Mlodzik, 2015).

Typically, Wnt/PCP signaling is activated by *Wnt5a* or *Wnt11* (Andre et al., 2015; Heisenberg et al., 2000; Yamaguchi et al., 1999) and involve the Fzd receptors *Fzd3* and *Fzd6* (Hua et al., 2014; MacDonald and He, 2012). However, this pathway can engage additional co-receptors and alternative receptors such as ROR2, RYK (receptor-like tyrosine kinase), and PTK7 (**Figure 3**, right). Upon binding of WNT to FZD and/or a co-receptor, FZD recruits DVL and complexes with DAAM1 (dishevelled associated activator of morphogenesis 1). Phosphorylation of DVL leads to downstream activation of the small GTPases RHOA and RAC, which leads to the activation of downstream effectors such as ROCK and JNK. This pathway induces modifications in actin and in the cytoskeleton, which is necessary for such processes as cell migration and polarity. As mentioned above, alternative receptor usage has also been described. One example is the Wnt5a-Ror-Dvl, which also controls morphogenesis (Ho et al., 2012). For review on Wnt/β-catenin-independent signaling see Sugimura and Li (2010), Gao (2012), and Yang and Mlodzik (2015).

Although the signaling pathway described above seems quite linear, the PCP pathway can affect the Wnt/β-catenin pathway at several levels in a receptor-dependent manner (van Amerongen et al., 2008). For example, *Ryk*, considered to signal independent from β-catenin was shown to regulate Wnt/β-catenin signaling and control neurite outgrowth (Lu et al., 2004).

This can also be seen in the inhibition of Wnt/β-catenin signaling by WNT5A in a dose dependent manner, where WNT5A binds to the ROR2 receptor. This does not affect β-catenin levels but instead inhibits transcriptional activation. In the same study, it was also shown that in some non-neural tissues WNT5A can bind to FZD4-LRP5 and activate Wnt/β-catenin signaling (Mikels and Nusse, 2006). These opposing effects on Wnt/β-catenin signaling has also been shown *in vivo* (Van Amerongen et al., 2012). Many more examples exist in which these pathways interact and control the same functions in cooperative or opposing manners. Evidence over the years has uncovered an unexpected level of complexity and interaction between these pathways. A more integrative and cell context-dependent view of Wnt signaling is thus emerging.

1.4.4 Wnt signaling and disease

Wnt signaling has been found to play a role in many diseases such as cancer and neurodegenerative disorders (MacDonald et al., 2009). Mutations in Wnt related components, such as APC, are associated to cancer and it also makes the Wnt pathway a target for therapeutic approaches (Serafino et al., 2016; Zimmerman et al., 2012). For review of Wnt signaling and cancer see Polakis (2012).

Within the nervous system, Wnt signaling plays important roles in maintaining proper homeostasis and diverse alterations have been found in neurodegenerative diseases, such as Alzheimer's or PD (Berwick and Harvey, 2012; Inestrosa and Arenas, 2010; Salašová et al., 2017; Toledo et al., 2008). In particular, Wnt dysfunction has been found to play a specific role in synaptic function (Purro et al., 2014). In agreement with this, blocking Wnt signaling with DKK1 induces synaptic disassembly and restoring Wnt reverses this effect (Galli et al., 2014; Marzo et al., 2016).

1.5 WNTS IN DOPAMINERGIC NEURON DEVELOPMENT

The two major Wnts during mDA neuron development are Wnt1 and Wnt5a, which activate the Wnt/β-catenin and Wnt/PCP pathways, respectively, but can have an effect on each other. While Wnt1 plays a general role in midbrain patterning, progenitor proliferation, DA specification and differentiation, Wnt5a has been shown to play a role in VM morphogenesis, decreasing DA progenitor proliferation and promoting mDA maturation (reviewed in Arenas, 2014; Inestrosa and Arenas, 2010).

1.5.1 Wnt1

Wnt1 is essential for early development and specification of the floor plate in the VM and was one of the first genes to be targeted by knockout technology in mice (Thomas and Capecchi, 1990). *Wnt1* is one of the main activators of Wnt/β-catenin signaling and is expressed early in the developing midbrain floor plate, in the anterior part of the MHB, and in the roof plate. At later stages, when the first TH^+ neurons appear, *Wnt1* is detected as two bands on either side of

midbrain floor plate (Prakash et al., 2006). *Wnt1* null mice reveal a severe phenotype consisting on the loss of the MHB during development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). The midbrain shows a severe reduction in size, and few poorly mis-specified progenitors that give rise to severely reduced number of DA neurons that fail to mature (Andersson et al., 2013; Ellisor et al., 2012; Prakash et al., 2006). Other important functions of *Wnt1* include the maintenance of *En1* expression, which is required for the formation of the midbrain-hindbrain region (Danielian and McMahon, 1996), and of FGF8, which is required for maintaining *Wnt1* expression (Chi et al., 2003; Prakash et al., 2006; Ye et al., 1998). Furthermore, conditional deletion of *Wnt1* with $En1^{Cre}$ or Shh^{Cre}, result in a loss in specification as seen by the loss of LMX1A expression and reduced medial mDA neurons or reduced proliferation and neurogenesis, respectively (Yang et al., 2013a). Notably, *Wnt1* also antagonizes *Shh* signaling in the midbrain floor plate, a step necessary for midbrain neurogenesis (Joksimovic et al., 2009).

The extensive *in vivo* studies on the function of Wnt1 in midbrain development have had a profound impact on the development of *in vitro* protocols for the differentiation of pluripotent stem cells and neural progenitors into mDA neurons. Using either conditioned media or activation of the Wnt1 pathway with GSK3β inhibitors has allowed for the expansion and differentiation of VM cultures to mDA neurons (Castelo-Branco et al., 2003, 2004). Moreover, their subsequently implementation in hPSC differentiation protocols (Kirkeby et al., 2012; Kriks et al., 2011) has led to the achievement of correctly specified mDA neuron preparations that successfully engraft and rescue motor symptoms in animal models of PD. Together, *Wnt1* and activation of the Wnt/β-catenin pathway is critical for multiple steps of mDA neuron development from early specification to late survival, in mouse models *in vivo* and in human stem cells *in vitro*.

1.5.2 Wnt5a

Besides *Wnt1*, the other most well-studied Wnt in mDA development is *Wnt5a*, a Wnt that signals through the PCP pathway in the VM (Andersson et al., 2008). In particular, our group has provided evidence that Wnt5a controls morphogenesis, proliferation and differentiation of DA neurons (Andersson et al., 2008, 2013; Parish et al., 2008; Schulte et al., 2005). Wnt5a expression appears just after *Wnt1* in the VZ of the VM and progressively refines to the floor plate and the midline, extending also to the MZ (Andersson et al., 2008). $Wnt5a^{-/-}$ mice show increased progenitor proliferation and number of post-mitotic mDA neuroblasts that fail to fully differentiate into mDA neurons. $Wnt5a^{-/-}$ mice also showed morphogenesis defects such as a lateral expansion of the VM domain, shortening in the anterior-posterior axis and altered orientation of radial glia (Andersson et al., 2008). Furthermore, *in vitro* studies have shown that WNT5A protein decreases progenitor proliferation and promotes differentiation of neuroblasts towards mDA neurons in both primary cultures and stem cell preparations (Andersson et al., 2008, 2013; Bryja et al., 2007; Castelo-Branco et al., 2003; Parish et al., 2008; Schulte et al., 2005).

Wnt5a has also been shown to cooperate or oppose to the function of *Wnt1* in the VM. These types of interactions have been uncovered by the analysis of double *Wnt1* and *Wnt5a* null mice embryos (Andersson et al., 2013). This work showed that Wnt/β-catenin and Wnt/PCP pathways not only regulate distinct processes, but they also compete or synergize during mDA neuron development. Compound *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice showed a greater reduction in the number of post-mitotic NR4A2^{+};TH^{-} neuroblasts and TH^{+} mDA neurons compared to single null mutants, as well as a greater widening and shortening of the VM suggesting a cooperation of both Wnts in functions previously attributed to Wnt/β-catenin, such as neurogenesis, or typical Wnt/PCP functions, such as elongation of the anterior-posterior axis.

To date, the function of only a few possible WNT5A receptors have been examined in the VM, including *Fzd3*, *Fzd6*, *Celsr3*, and *Ryk* (Blakely et al., 2013; Fenstermaker et al., 2010; Stuebner et al., 2010). *In vitro* and *in vivo* analysis of these receptors have shown similar or stronger phenotypes compared to *Wnt5a*$^{-/-}$ mice. Indeed, *Celsr3* mutant mice showed abnormalities in anterior-posterior guidance of DA projections (Fenstermaker et al., 2010). The receptor *Ryk* regulated mDA differentiation and axon morphogenesis *in vitro*, but no effects on axonal growth and guidance were observed *in vivo* (Blakely et al., 2013). On the other hand, *Fzd3* and *Fzd6* were found to be required for midbrain morphogenesis, as shown in severe morphogenic defects in double knockout mice, including collapsed ventricle, but also show delay in mDA neuron development (Stuebner et al., 2010). These results point to a certain degree of receptor redundancy and to the possibility of compensations taking place *in vivo*.

To date, the function of other receptors such as *Ror2* and PCP components such as *Vangl2* in mediating WNT5A and Wnt/PCP signaling has not been examined in the VM. A previous report indicated that *Ror2* and *Vangl2* are required for *Wnt5a* signaling in limb development (Gao et al., 2011). In this system, a WNT5A gradient establishes PCP by inducing VANGL2 phosphorylation through ROR2 and CK1δ. The function of *Ror2* as a possible receptor for *Wnt5a* and of *Vangl2* in the VM is investigated in **Paper VI**.

1.5.3 Other Wnts and Wnt modulators

Other Wnts and Wnt pathway components have been examined in VM development. For example *Wnt2*, an activator of Wnt/β-catenin pathway was found to promote mDA progenitor proliferation both in primary VM cultures treated with WNT2 and in and *Wnt2*$^{-/-}$ mice (Sousa et al., 2010). This phenotype was similar to that of the *Lrp6* null mutant mice (Castelo-Branco et al., 2010), a co-receptor used by *Wnt2*. Other Wnts such as *Wnt8* are important in setting up the MHB in zebrafish (Rhinn et al., 2005, 2009), and *Wnt7a/b* has been found to regulate VM neurogenesis and DA axon growth and guidance (Fenstermaker et al., 2010; Fernando et al., 2014; Fougerousse et al., 2000).

Inhibitors of the Wnt pathway also have regulatory functions in mDA development. *Dkk1* deletion lacks significant head structures anterior to the midbrain during embryogenesis and analysis of the few surviving *Dkk1*$^{-/-}$ and *Dkk1*$^{+/-}$ embryos revealed that *Dkk1* is required for not only for midbrain morphogenesis, but also for mDA differentiation (Ribeiro et al., 2011).

Wnt modulators such as *Sfrp1* and *Sfrp2* are also expressed in the developing VM. Surprisingly, *Sfrp1*$^{-/-}$;*Sfrp2*$^{-/-}$ embryos display a similar phenotype to that of *Wnt5a*$^{-/-}$ embryos, indicating that *Sfrp1* and *Sfrp2* are required for Wnt/PCP signaling. Indeed, while low concentrations of SFRP1 and SFRP2 promoted mDA differentiation, high concentrations *in vitro* impaired their survival in VM primary cultures or in mouse ES cells differentiated into mDA neurons (Kele et al., 2012). Thus, SFRP1 and SFRP2 seem to play a dual role to activate or inhibit Wnt/PCP at low and high concentrations, respectively.

1.5.4 R-Spondins

R-Spondins (Rspo1-4) are a family of cysteine-rich secreted proteins containing a thrombospondin type I repeat (**Figure 4A**), initially discovered in 2002 (Chen et al., 2002). It has long been known that RSPOs are Wnt/β-catenin signaling activators (Kazanskaya et al., 2004) and they do so through their CRD (Kim et al., 2008). In addition, RSPO3 and RSPO4 can also have an effect on non-canonical signaling, by interacting through their thrombospondin domain with proteoglycans of the syndecan family (Astudillo et al., 2014; Ohkawara et al., 2011). In addition, RSPOs are not only considered as activators of Wnt signaling but rather as synergistic activators. Indeed, simultaneous exposure of cells to both RSPOs and WNT proteins result in a synergistic, robust activation of Wnt signaling (Kazanskaya et al., 2004; Kim et al., 2005).

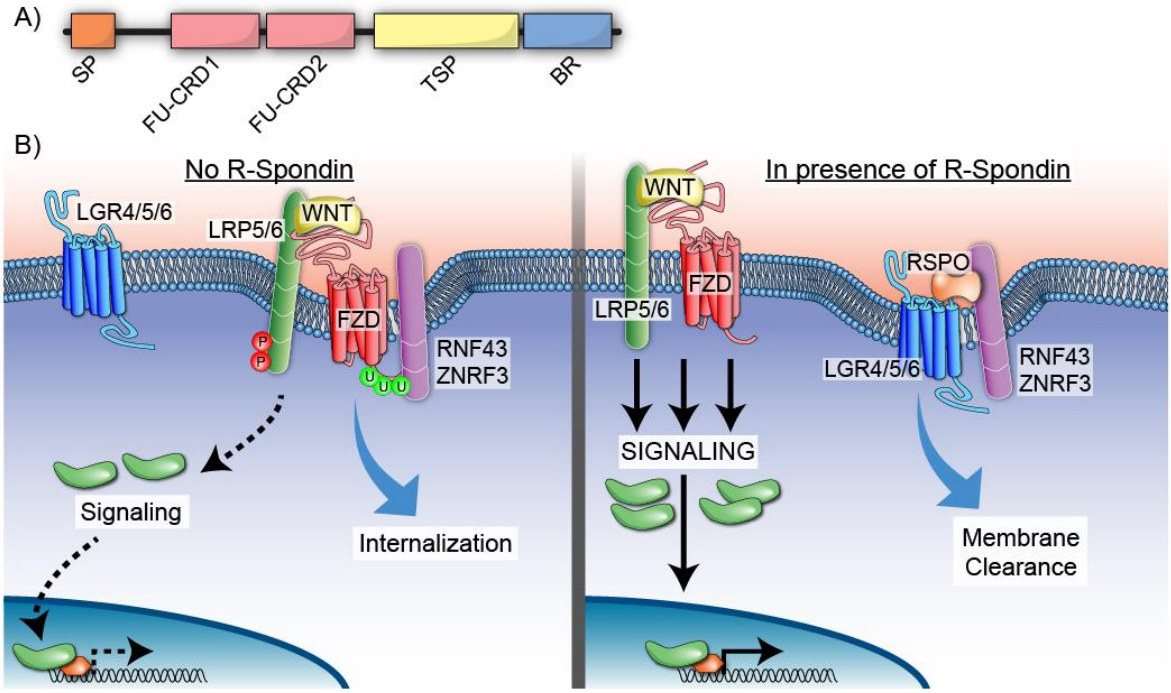

Figure 4: R-Spondin Signaling

A) Domain structure of R-Spondin. SP: signal peptide; FU-CRD: Furin-like cysteine-rich domain; TSP: Thrombospondin domain; BR: Basic amino acid rich domain. B) R-Spondins enhance Wnt signaling through sequestering away E3 ubiquitin ligase RNF43/ZNRF3. Left: When R-Spondin is not present, RNF43/ZNRF3 bind to and ubiquitinate the FZD receptor. This causes internalization of receptor complex and prevent further Wnt/β-catenin signaling from occurring. Right: In the presence of R-Spondin, RSPO binds to its receptor LGR4/5/6 and co-receptor RNF43/ZNRF3, thereby downregulating its activity at the membrane. This in turns allows for enhanced Wnt signaling.

The identity of RSPO receptors has been a matter of debate in the past. Initially it was reported that RSPOs bind to LRP5/6 receptors as Wnts do (Li et al., 2009; Nam et al., 2006; Wei et al., 2007), with some additional role of Fzds. Other studies (Binnerts et al., 2007) identified Kremen1 (*Krm1*), an LRP5/6 co-receptor, as a RSPO receptor. Subsequently, RSPOs were found to be the ligands of the leucine-rich repeat-containing G protein-coupled receptor (LGR) 4/5/6, but not LRP5/6 (Carmon et al., 2011; Glinka et al., 2011; de Lau et al., 2011). More recently, two additional WNT/RSPO signaling components: The transmembrane E3 ubiquitin ligases ring finger protein 43 (*Rnf43*) and the zinc and ring finger 3 (*Znrf3*) were identified as RSPO receptors (Hao et al., 2012; Koo et al., 2012). In the absence of RSPO, ZNRF3/RNF43 are thought to bind to the WNT/FZD complex and target the Fzd receptor for degradation by ubiquitination (**Figure 4B**, left). With RSPO present, RNF43/ZNRF3 form a complex that is cleared from the membrane leading to increased Wnt signaling (**Figure 4B**, right). Structural studies have shown a direct interaction of RSPOs with LGR receptors and RNF43/ZNRF3 (Chen et al., 2013; Wang et al., 2013; Zebisch et al., 2013). It is currently thought that RSPOs enhance Wnt signaling by contributing to the clearing RNF43/ZNRF3 from the membrane.

RSPO2 has been shown to play roles in morphogenesis *in vivo* in two different system such as the respiratory tract and limb development, two important areas of Wnt signaling (Bell et al., 2008). Recently, RSPO2 was found expressed in a subset of anterior mDA neurons and its expression levels were found to be reduced in *Lmx1a* mutants (Hoekstra et al., 2013). Moreover, the same study showed that deletion of *Rspo2* results in a modest decrease in TH and PITX3 levels. However, it is unknown whether loss of *Rspo2* results in reduced numbers of mDA neurons. It has also been recently reported that a *Lmx1b-miR135a2-Rspo2* regulatory circuit regulates Wnt signaling and the mDA progenitor pool (Anderegg et al., 2013). These two reports thus point to a role of RSPO2 in mDA neuron development, which so far remains to be fully elucidated and that we investigated in further detail in **Paper V**.

1.5.5 Ror receptors

The Ror-family of receptor tyrosine kinases are evolutionary conserved and known to participate in Wnt/PCP signaling. In vertebrates, this family consists of two members, Ror1/2. Structurally they are single pass transmembrane tyrosine kinases characterized by a kringle domain and a CRD domain similar to that of Fzd in the extracellular domain. The intracellular portion of the receptor includes a tyrosine kinase domain, two serine/threonine-rich domains, and a proline-rich domain (**Figure 5A**).

Wnt5a signaling can cause homo-dimerization and activation of the Ror2 signaling cascade (Liu et al., 2008) as well as hetero-dimerization between ROR1 and ROR2 (Paganoni et al., 2010) (**Figure 5B**). Signaling through Ror can be accomplished through several mechanisms depending on the cellular context, including other receptors and interactors of their cytoplasmic domains. To add to the complexity, ROR2 interacts with other Wnt receptors, including FZDs (Nishita et al., 2010; Randall et al., 2012). PCP signaling is usually mediated by a ROR-FZD

complex, but can also form complexes or directly interact with other signaling components such as the receptors PTK7 (Martinez et al., 2015) or RYK (Andre et al., 2012) and the secreted factor CTHRC1 (Yamamoto et al., 2008) (**Figure 5B**). Furthermore, ROR2 has been shown to phosphorylate and activate VANGL2 via CSNK1 (**Figure 5C**) (Gao et al., 2011). Other reported activities include antagonizing Wnt/β-catenin signaling and modulating BMP signaling (Stricker et al., 2017). For comprehensive review on Ror signaling, see Endo and Minami (2017); Petrova et al. (2014) and Stricker et al. (2017).

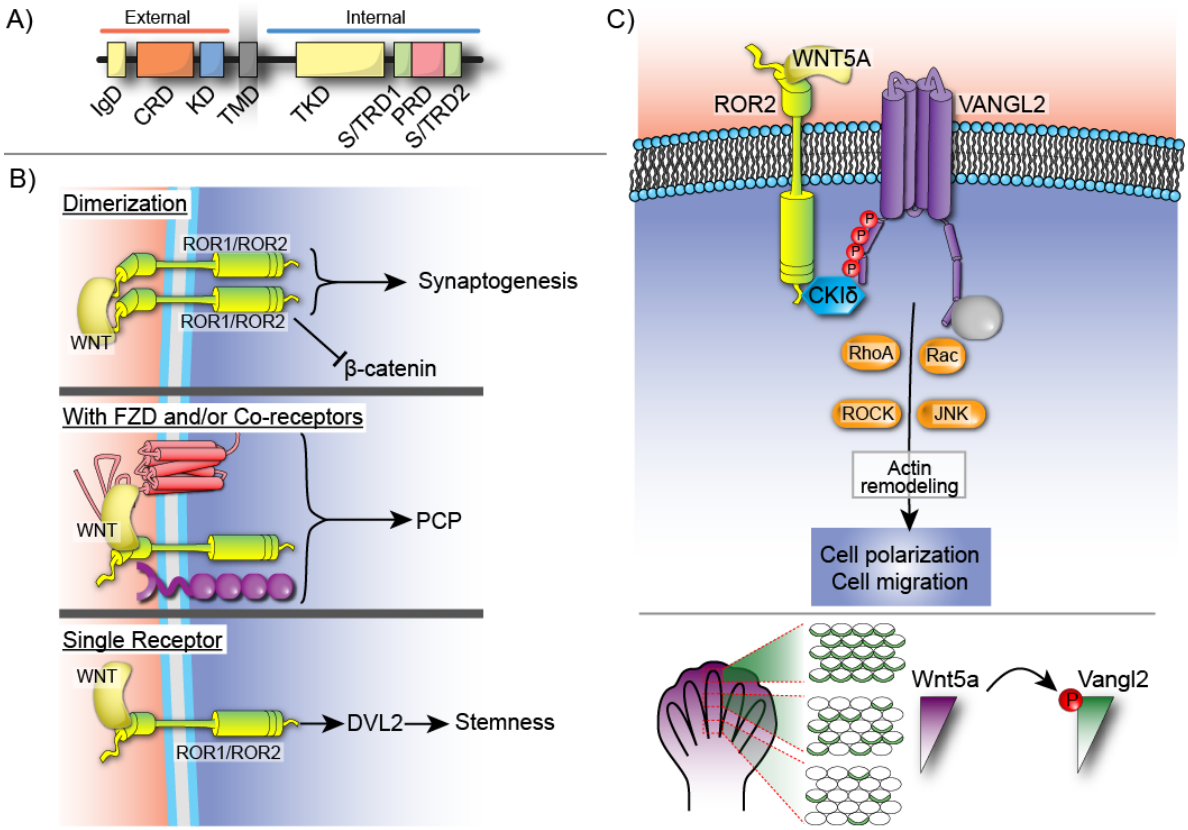

Figure 5: Ror and Vangl signaling
Signaling of ROR receptors and WNT5A-ROR2-VANGL2 signaling cascade. A) Schematic structure of the different domains of the ROR receptor. IgD: Immunoglobulin domain; CRD: cysteine rich domain; KD: kringle domain; TMD: transmembrane domain; TKD: tyrosine kinase domain; S/TRD1/2: serine/threonine rich domain; PRD: proline-rich domain. B) Possible signaling pathways and functions regulated by ROR receptor tyrosine kinases and other WNT receptors. Signaling is context dependent. Top: Shown to dimerize for synaptogenesis (Paganoni et al., 2010). Middle: In complex with FZD and/or possible other co-receptors such as PTK7, CTHRC1, and RYK. Part of largest signaling cascade leading to PCP pathway (Seifert and Mlodzik, 2007; Stricker et al., 2017). Bottom: Act as a sole receptor and signal through DVL2 to maintain neural progenitor cells (Endo et al., 2012; Ho et al., 2012). C) ROR2 can promote dose-dependent VANGL2 phosphorylation in response to a WNT5A gradient. This is accomplished through CKIδ. This signaling modality was found to control limb bud formation (Gao et al., 2011).

In the developing nervous system, Ror family members are expressed in the VZ of the dorsal telencephalon, where neural progenitor cells reside. Their expression decreases during development up till birth. In the postnatal nervous system, *Ror1* and *Ror2* are absent except for specific cell types in the cerebellum (Al-Shawi et al., 2001; Oishi et al., 1999). It has been suggested that *Ror1* and *Ror2* have redundant functions, but that some of their effects are not additive (Endo et al., 2012; Paganoni et al., 2010).

RORs control several cellular processes such as neurite extension, cell polarization, synaptogenesis, cell migration, neurogenesis and cell division. Null mutation of *Ror2* results in mice with developmental defects in heart and limb formation (Takeuchi et al., 2000). In humans, *ROR2* mutations lead to autosomal recessive Robinow syndrome and autosomal dominant brachydactyly type B (Afzal and Jeffery, 2003).

1.5.6 Vangl2

The Vangl family members, *Vangl1* and *Vangl2*, form a group of four-pass transmembrane proteins. They are known as a core PCP components for their capacity to regulate cell polarity and neuronal maturation (Torban et al., 2004).

Wnts, in a dose dependent manner, induce the phosphorylation of Vangl2 through the Wnt receptor Ror2 (Gao et al., 2011). As seen in limb development, WNT5A gradients induce correlative VANGL2 activity and cell polarity (**Figure 5C**). On the other hand, *Vangl2* deletions in mice are more severe and result in neural tube closure defects (Gao, 2012; Kibar et al., 2007, 2011; Lei et al., 2010). Studies in *Xenopus* have also shown the interaction between *Ror2*/*Vangl2* and their role in Wnt signaling (Martinez et al., 2015; Ossipova et al., 2015; Podleschny et al., 2015).

1.6 MIDBRAIN DEVELOPMENT: NEW TRANSCRIPTION FACTORS

As described in section 1.2, several transcription factors have been found to play key roles in midbrain development. Here I will focus on two additional families of genes relevant to this thesis.

1.6.1 Pbx1/3

The transcription factor family pre-B-cell leukemia homeobox (PBX) are composed of four members (PBX1-4) and are part of the larger TALE (three amino acid loop extension) homeodomain transcription factor family that includes PREP/PKNOX1/2, and MEIS1-3 proteins (Longobardi et al., 2014). The TALE proteins structurally consist of three main domains, all sharing a conserved helix-loop-helix homeodomain. The two other domains are family specific (PBC-A/B or MEIS-A/B) conserved regions. Null mutants for several of the TALE family members are early embryonic lethal as these proteins play roles in early vertebrate brain development in cell fate specification and differentiation (Schulte and Frank, 2014).

Previous studies have shown that *Pbx1* regulates regional identity in cortical progenitors and in newly generated neurons, and represses other neighboring fates (Golonzhka et al., 2015; Grebbin et al., 2016). Although PBX transcription factors are important in organogenesis, their role in midbrain development was poorly understood. Only the function of *Pbx1,* expressed in the VM, has been previously examined. *Pbx1*-deficient embryos show alterations in dopamine neuron axon guidance and in the regulation of netrin-1 receptor, *Dcc* (deleted in colorectal

carcinoma) (Sgadò et al., 2012). Other studies have identified *Pbx1* as a downstream target of LMX1A, up-regulated in *Lmx1a*-deficient mice (Hoekstra et al., 2013), and downstream of *Pitx3*, downregulated in *Pitx3*$^{-/-}$ embryos (Veenvliet et al., 2013). The latter result, however, is in contradiction with our analysis in **Paper I** where we show that PBX1 is upstream of PITX3 (Villaescusa et al., 2016). Indeed, our findings show: (1) that PBX1 protein is present in mDA neuroblasts and neurons, while the expression of PITX3 is only found in mDA neurons, (2) that *Pitx3* is a direct target of PBX1 as assessed ChIP-seq analysis; and (3) that the expression of *Pitx3* is lost in *Pbx1*$^{-/-}$;*Pbx3*$^{-/-}$ embryos.

1.6.2 Arntl

Arntl (aryl hydrocarbon receptor nuclear translocator like, previously known as *Bmal1*) is a member of the basic helix-loop-helix transcription factor gene family. ARNTL is a pioneer transcription factor that forms part of the circadian clock machinery in the cell (Menet et al., 2014; Stevens et al., 2008). Structurally it has a basic helix-loop-helix and two Per-Arnt-Sim (PAS) domains. ARNTL binds to CLOCK and form heterodimers that bind to E-boxes in the promoters of *Per1/2* and *Cry1/2* resulting in increasing protein levels, which repress the transcriptional activity of ARNTL:CLOCK heterodimers. The regulation of circadian rhythms involves additional genes such as *Rev-Erbα* and *Ror2*, which respective proteins repress or induce *Arnt1* and are induced by ARNTL:CLOCK (Buhr and Takahashi, 2013).

Mutations in *Arntl* have shown an accelerated ageing phenotype in mice (Kondratov et al., 2006). Interestingly, one of the common non-motor symptoms of PD patients is disturbances in sleep, a natural biological process controlled through your circadian rhythm. An abnormal circadian clock is shared among many neurodegenerative disorders including Alzheimer's, Huntington's disease and PD (Hood and Amir, 2017; Musiek and Holtzman, 2016). It has been shown that ARNTL, along with another clock protein PERIOD2, controls cell cycle entry and exit in during neurogenesis in the adult hippocampus (Bouchard-Cannon et al., 2013) and subventricular zone (Malik et al., 2015). Furthermore, it has been shown that ARNTL regulates the proneural transcription factor *Neurod1* and that *Arntl* gene silencing reduces the number of *Map2* positive cells (Kimiwada et al., 2009), suggesting a role in neurogenesis. The role of *Arntl* in mDA neuron development has not been previously studied and it now explored in **Paper IV**.

2 AIMS

This goal of this thesis is to gain knowledge of mDA development by better understanding the molecular components and machinery involved in establishing a cellular environment that allows for generation of a specific cell type. The ultimate goal is to use this information to control the mechanisms governing mDA differentiation in stem cells, which could then be directly used *in vivo*, for PD CRT, or *in vitro*, to model PD.

In this thesis, the following questions were addressed.

- What is the role of *Pbx1* in mDA development and how is it implicated in PD?
- How many cell types are present in the developing VM and in the DA lineage? How are they molecularly defined? How different is mouse and human VM development? How can we use scRNA-seq information to improve hPSC preparations for PD CRT?
- How can we extract data from scRNA-seq to better understand the role of new genes in mDA development? Have we overlooked additional Wnt signaling components present in the developing VM?
- Can we use a systems-based approach to reveal novel cell intrinsic and cell extrinsic candidates critical for mDA neuron development?
- What is the function of R-Spondins in mDA neuron development? Can they be used to promote mDA differentiation of hPSCs and advance stem cell-based CRT for PD?
- What are the roles of the Wnt/PCP genes *Wnt5a*, *Ror2*, and *Vangl2* in the development of the VM and mDA neurons?

To answer these questions, we have applied several different strategies. Our studies include the use of classical developmental methods such as analysis of mutant mice, analysis of PD postmortem samples, studies using primary cultures and embryonic stem cells, combined with analysis of cell signaling and advanced methods such as scRNA-seq and *in silico* analysis of gene expression.

3 RESULTS AND DISCUSSION

3.1 PAPER I: A PBX1 TRANSCRIPTIONAL NETWORK CONTROLS DOPAMINERGIC NEURON DEVELOPMENT AND IS IMPAIRED IN PARKINSON'S DISEASE

In this article, we explore the role *Pbx1* has in the development and survival of mDA neurons. The major findings of this paper (summarized in **Figure 6**) are: 1) PBX1A expression is first acquired in a subset of NR4A2^{+}/TH^{-} cells; 2) *Pbx1*, and in its absence *Pbx3*, are required for mDA neuron specification, differentiation and survival; 3) PBX1A represses *Onecut2* (one cut domain, family member 2) to inhibit lateral fates, induces *Pitx3* to promote mDA development, and induces *Nfe2l1* (nuclear factor, erythroid derived 2,-like 1) to protect against oxidative stress; 4) A significant reduction in PBX1A and NFE2L1 was detected in the nuclei of SNc mDA neurons in post-mortem samples of PD patients; 5) Overexpression of *Pbx1* in human neuroepithelial stem cells prevented the loss of DA neurons by oxidative stress. Together, our results show a function of PBX1 in mDA neuron development, suggest a role in PD and may have implications for the development of regenerative medicine and drug development for PD.

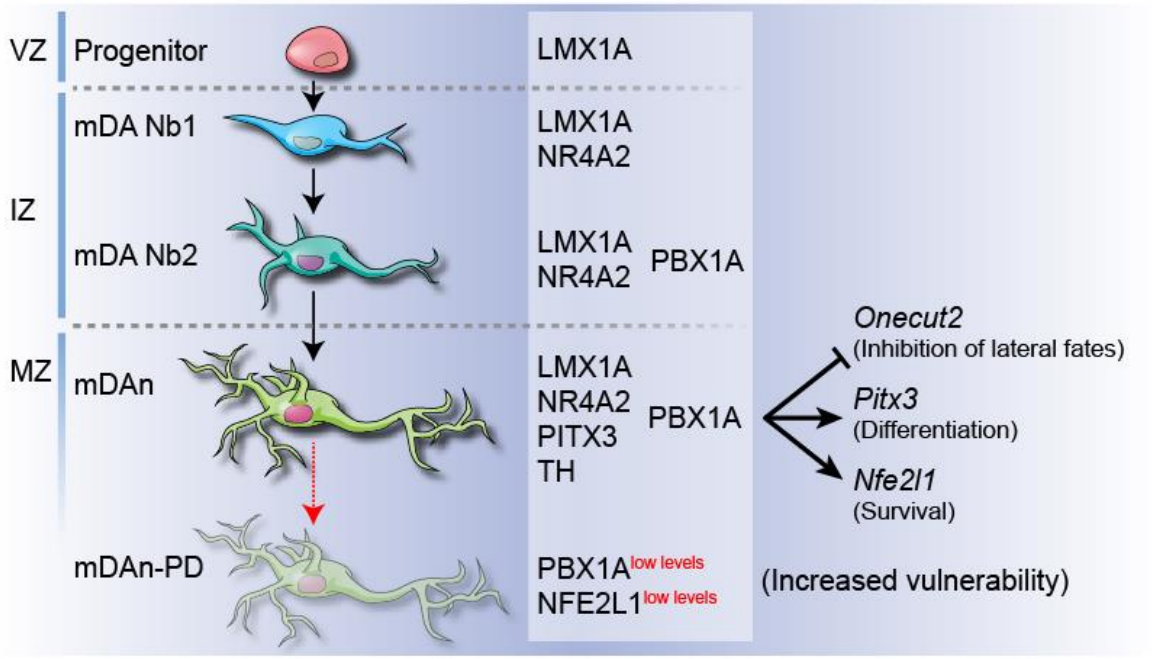

Figure 6: Role of PBX1 in ventral midbrain development and Parkinson's disease
Graphical summary of results showing stages of PBX1A expression, first appearing in mDA neuroblast 2 (Nb2) within the IZ prior to TH expression. PBX1A was found to target *Onecut2* to inhibit lateral fates, *Pitx3* to promote differentiation, and *Nfe2l1* for mDA survival. In PD patient samples, decreased levels of NFE2L1 and PBX1A were found in SNc DA neurons (Figure adapted from Villaescusa et al., 2016).

Previous studies on *Pbx1a* in VM development have either examined the expression or levels of this transcription factor in mouse embryonic and human fetal tissue or have focused on the axonal guidance phenotype in *Pbx1* null mice (Sgadò et al., 2012). Here we examined and defined the expression of the *Pbx1-3, Prep1-2* and *Meis1-2*. We found that PBX1A appears in mDA NR4A2^{+} neuroblasts, but prior to PITX3 in TH^{+} mDA neurons. Using knockout mice,

we observe an increase in the expression of *Pbx3* in *Pbx1* null mice, suggestive of a compensation. Conditional deletion of *Pbx1* in the context of *Pbx3*$^{-/-}$ mice, revealed a misspecification of mDA neurons and a decrease in mDA neuron numbers at later embryonic stages. Moreover, lentiviral mediated overexpression of *Pbx1* increased the number of TH^{+} cells. Lastly, direct clinical relevance was obtained by examining post mortem VM tissue from PD patients, where we found that the levels of PBX1 and NFE2L1 were reduced in the remaining SNc mDA neurons, but not in adjacent cell types.

Analysis of the molecular mechanism by which PBX1 regulates these functions was done with chromatin immunoprecipitation sequencing (ChIP-seq). ChIP-seq analysis identified binding motifs in proximity to genes related with signal transduction, RNA metabolic processes, transcription and response to stress as seen through Gene Ontology terms. After combining ChIP-seq data with TruSeq data, by Gene Set Enrichment Analysis (GSEA), target genes of *Pbx1* were identified to be differentially expressed in the VM. Producing a total of 29 genes that were either differentially upregulated of downregulated. Although a select few were validated, it still remains to be investigated the role of other target genes on the list and their possible role in mDA development. The dual function of PBX1 as an enhancer and repressor was briefly explored using bioinformatical analysis to show overrepresented transcription factor binding sites in which, for example, repressor loci were enriched on binding sites of NR4A2, hinting at the possibility that PBX1/NR4A2 repress common genes. On the other hand, the discovery of a *Pbx1-Nfe2l1* pathway that is affected in PD patients could open new developments into disease modeling and drug discovery. Together, we hereby describe a novel function of *Pbx1* in the specification and survival of mDA neurons and suggest that the *Pbx1-Nfe2l1* pathway is impaired in PD. More investigations into the mechanisms and possible therapeutic application of these findings are warranted.

3.2 PAPER II: MOLECULAR DIVERSITY OF MIDBRAIN DEVELOPMENT IN MOUSE, HUMAN AND STEM CELLS

In this article, we explore the cellular heterogeneity of the developing mouse and human VM at the single-cell level giving us data sets that can give insight into developmental programs that can be applied to CRT for PD. The major findings of this paper (some summarized in **Figure 7**) are: 1) A complete VM scRNA-seq data set that encompasses early mDA development in both mouse and human was obtained. 2) Novel cell types including three types of mouse and five types of human radial glia-like cells biased towards distinct fates were found. 3) Differences in cell types and gene expression between mouse and human mDA neuron development. 4) Single-cell analysis of mature mDA neurons showing five distinct clusters, emerging only postnatally. 5) A machine learning method to score mDA differentiation of stem cells was developed. Together, our data provides both the information and the methods required to assess the performance of DA differentiation protocols at a single-cell level. Our study describes mouse and human VM development *in vivo*, at a transcriptome level, which can be applied both to improve the mDA differentiation of hPSC and CRT protocols for PD, as well as the development of improved *in vitro* models of PD and its application in drug screening.

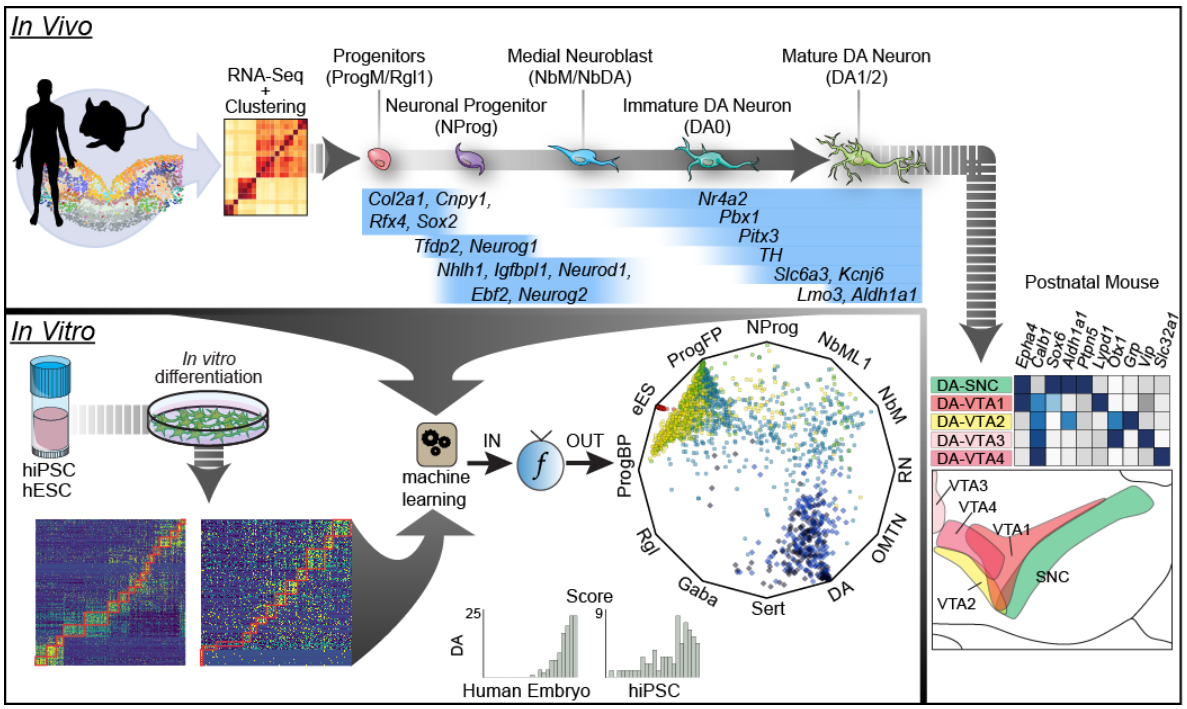

Figure 7: Single-cell analysis of developing ventral midbrain
Graphical scheme of single-cell analysis of mDA lineage *in vivo* and mDA differentiation of hPSCs *in vitro*. scRNA-seq was performed in embryonic mouse and fetal human VM tissue to perform a cross-species comparison. Several cell types in the mDA lineage were identified by the expression of distinct set of genes. Two embryonic mDA neurons were found to diversify into five cell types postnatally. Differentiated human ES and human iPS cells were compared to the cell types discovered in the *in vivo* human fetal data set. Using machine learning, we developed a method to score the quality of the *in vitro* differentiated cells.

In this study, we took advantage of cutting edge technologies such as scRNA-seq and new bioinformatical analysis methods to explore the cell types and molecular composition of the embryonic mouse and human fetal tissue. We generated a data set encompassing the critical time window when mDA neurogenesis takes place and generated a library of 1,977 human fetal cells, 1,907 mouse embryonic cells, 245 postnatal murine cells, and additionally, 2,052 cells hPSCs during their differentiation into mDA neurons *in vitro*. Using Back-SPIN clustering on the *in vivo* data sets we obtained 25 human and 26 mouse clusters that were largely conserved across species. By using unique molecular identifiers in our scRNA-seq analysis we were able to reveal the identity of cells based on their relative gene expression levels.

Focusing on the mDA lineage, we found from early neural progenitors, to neuroblasts and several post-mitotic embryonic DA cell types. Evaluating this sequential process, we discovered that each maturation step results from the acquisition of additional gene expression rather than their loss. To our surprise, the emergence of mature mDA neuron populations was not detected postnatally. mDA neurons have been previously classified by their electrical properties, anatomical position, and a handful of markers. With scRNA-seq technology, we are able to provide a complete unbiased evaluation of cell types at a molecular level. We discovered two mature fetal mDA neuron subtypes subdivide into five populations postnatally. This was validated with immunofluorescence where the five populations, such as one SNc and four VTA mDA neurons only emerged at P7. This is interesting due to the fact that PD specifically

impairs the function and survival of SNc neurons. In addition, finding the earliest decision a cell has to make to differentiate into a SNc mDA neuron may contribute to develop specific hPSC differentiation protocols for SNc neurons and improve thus CRT for PD.

Another interesting aspect of this paper was that we provide a tool to aid in the evaluation of hPSC-derived mDA neurons for CRT. All current evaluation techniques usually involve using a handful of gene markers to show the percentage of cells in the culture that are positive for these markers and then functional evaluations *in vitro* followed by *in vivo* assessment of their capacity to recover motor function after transplantation in PD mouse models. Although several studies claim a high percentage of cells in the mDA lineage, these preparations still contain multiple cell types belonging to other compartments and serving other functions. In addition, the critical question of what is the complete identity and functional capacity of cells unidentified as dopaminergic in these studies still remains to be answered. Using scRNA-seq, a global perspective and a very detailed level of information can be obtained at the same time, allowing to evaluate all the cell types present in the culture. As an example, in this paper, our evaluation of hPSC-derived mDA neurons revealed the presence of a near complete VM tissue containing unnecessary cell types for cell replacement such as GABAergic and red nucleus neurons, but also undesired cell types, such as hindbrain serotonin neurons that may cause graft-induced dyskinesias. Our study also raises the question of whether or not having a pure culture is important and whether or not some of additional cell types may be actually good to provide support to mDA neurons. Extensive testing would need to be done in the future. Either way, our machine learning tool can be used to examine the quality of hPSC-derived cells obtained by new protocols, which will be essential to improve cell preparations and to move forward the field of CRT for PD.

3.3 PAPER III: TRANSLATION OF WNT DEVELOPMENTAL PROGRAMS IN TO STEM CELL REPLACEMENT STRATEGIES FOR THE TREATMENT OF PARKINSON'S DISEASE

In this article, we review the role of *WNT* in the developing midbrain and extract new information from single-cell data acquired in **Paper II**. The major points of this review are: 1) We provide an overview of current knowledge on WNTs in mDA development and how they have been implemented into current hPSC differentiation protocols. 2) We reveal new WNT signaling components present in the developing human fetal VM development. 3) We discuss the possible importance of these new findings for mDA neuron development and for improving current hPSC mDA differentiation protocols and CRT for PD. A summary of this article is shown in **Figure 8**.

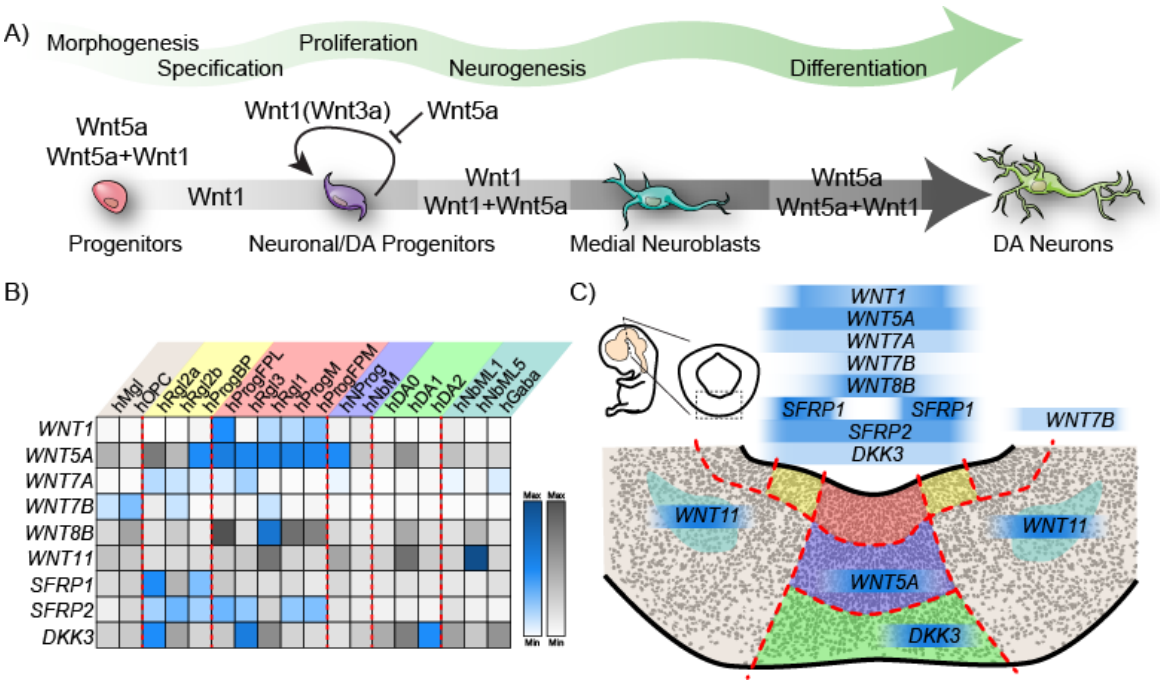

Figure 8: Expression of WNTs and related signaling components as well as their distribution in the human ventral midbrain

A) Cartoon summarizing the current knowledge of the functions of *Wnt1* and *Wnt5a* in the mDA lineage during embryonic development based on *in vitro* and *in vivo* mouse data. B) Heat map of *Wnt*s and Wnt signaling modulators found in the developing human VM in representative cell types revealed through scRNA-seq. C) Schematic representation of localization of *Wnts* and Wnt modulators found in Panel B (Figure adapted from Toledo et al., 2017).

Using the *in vivo* data set generated from human fetal tissue in **Paper II**, we were able to analyze distinct components of different signaling pathways. Here we focused on the ligands and modulators of the Wnt pathway expressed in the human dataset, including *WNT*s, *SFRP*s, and *DKK*s. As mentioned previously, the two critical ligands in VM development are WNT1 and WNT5A and beyond these, very little has been explored about the function of other possible players. Even less in known about human development, where in addition of the known expression of *WNT1* and *WNT5A*, we found the expression of *WNT7A*, *WNT7B*, *WNT8B* and *WNT11*, and Wnt signaling modulators (*SFRP1*, *SFRP2*, and *DKKs*) in various human VM cell types.

Moving forward, we think that careful manipulation of the Wnt pathway, especially the interplay between Wnt/β-catenin dependent and Wnt/PCP pathways, will be important in mDA specification for SNc type neurons. The location of Wnt signaling components and their influence on surrounding cell types could be critical due to the possibility that SNc neurons emerge from a distinct population compared to VTA in the developing VM. Interesting candidates for future studies include *WNT8B* due to its exclusive expression in radial glia-like 1 cell type (Rgl1), believed to be the neurogenic progenitor. However, it should be noted that nearly all the ligands and modulators of Wnt signaling found in our data sets are potentially interesting, regardless of the cell type of origin as the factors work both in an autocrine and

paracrine manners, affecting neighboring cells in the midbrain floor plate niche, where mDA neurons emerge.

Of interest for this thesis is also *Wnt5b*, a Wnt not included in this review since it was only found in the mouse data. We found that *Wnt5b* is expressed in progenitor cells, together with *Wnt5a*, suggesting the *Wnt5b* may be able to compensate for the loss of *Wnt5a* in the *Wnt5a*$^{-/-}$ mice, a possibility that remains to be examined.

In summary, the review presented here is only a small piece of what is possible to do with regard to the analysis of scRNA-seq data. Here, we only examined secreted factors of the Wnt signaling pathway. Other typical or non-typical Wnt signaling modulators such as *Tpbg*, *Apcdd1*, and *Trabd2a* showed interesting expression dynamics in the mDA lineage, but were not analyzed. Similarly, we did not consider receptors or tried to match cell types expressing receptor-ligand as the complexity of such networks grows exponentially. This type of analysis was performed in **Paper IV**. We conclude that *in silico* analysis can be used to systematically examine signaling components in candidate pathways that can then be then studied with experimental biological methods.

3.4 PAPER IV: MOLECULAR ANALYSIS OF MIDBRAIN DOPAMINERGIC NICHE DURING NEUROGENESIS

In this article, we explore the cell types and the signals that contribute to mDA neurogenesis. Using data from **Paper II,** additional VM bulk RNA-seq data and through bioinformatical analysis we define the concept of the mDA niche, that is the cell types and genes contributing to mDA neurogenesis. The main points of the study are: 1) The cell type contributing the most to mouse mDA neurogenesis was radial glia type 1 (Rgl1) identified in **Paper II**. 2) Analysis of cell extrinsic components identified participation of several cell types in the formation of the mDA niche, of which Rgl3 is the cell type expressing most ECM components and ligands for diverse signaling pathways for a specific VM niche. 3) Analysis of cell intrinsic factors identified different transcriptional networks in each of the three radial glia types and congruent with specific function for each radial glia. 4) Based on the analysis above we examine the function of the transcription factor *Arntl*, a central transcription factor in the pro-neurogenic network identified in Rgl1, and found a novel function of *Arntl* in controlling mDA neurogenesis. Results are summarized in **Figure 9**.

We started our study with the analysis of midbrain tissue and four adjacent brain regions from E11.5-E14.5, to define genes differentially expressed in the VM. The samples were also analyzed with weighted gene co-expression analysis where different gene modules were discovered and described different aspects of midbrain development as seen through gene ontology analysis. We further describe in detail a specific mDA module enriched in differentially expressed genes of the VM, which also is enriched in genes that describe different molecular processes, such as ECM formation. We then integrated the single-cell data from **Paper II** in order to identify the cell types contributing to the mDA module. Radial glia have

previously been reported to be part of the mDA lineage (Bonilla et al., 2008) and in our data represented a distinct cell type contributing the most to the ECM and its regulatory elements. We also develop a methodology to determine the main contributing cell types on a biological process. We analyzed both ligands and receptors for different developmental signaling pathways which included in addition to endothelial cells, pericytes and microglia, all the three radial glia types.

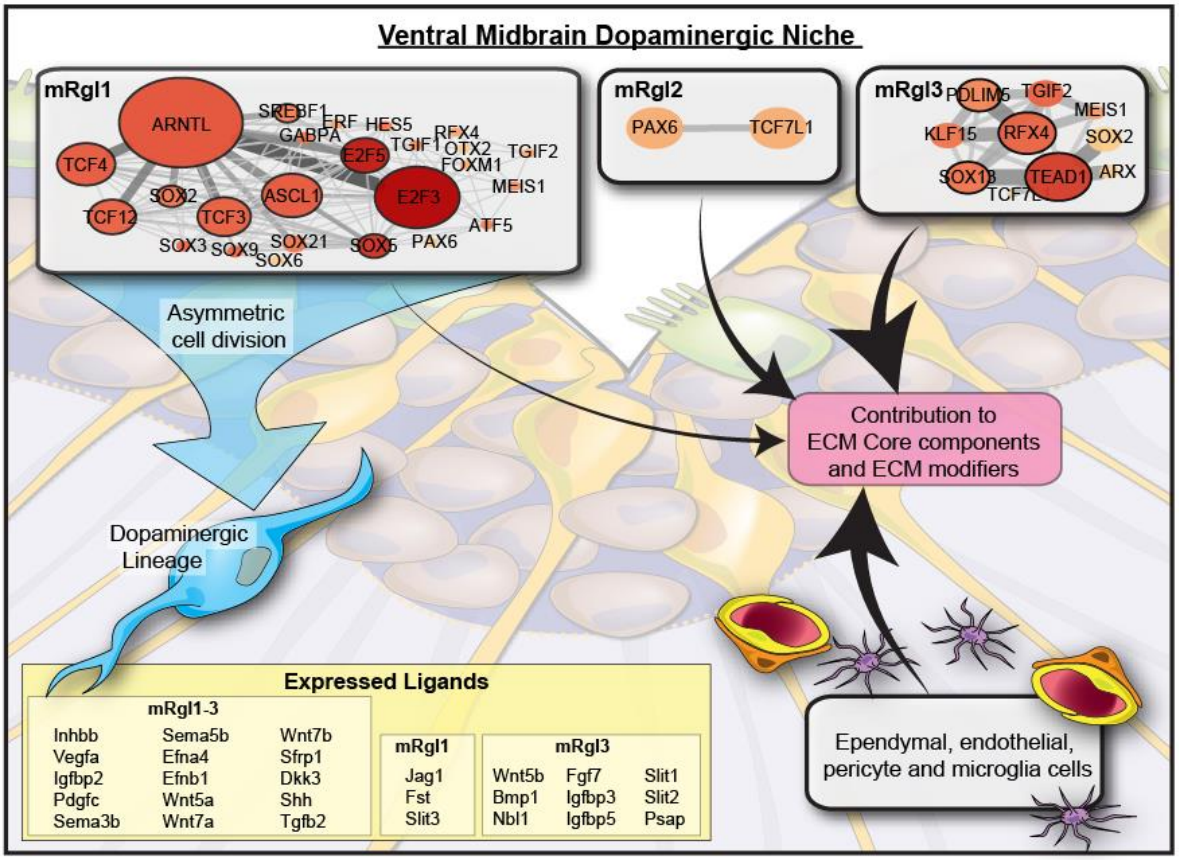

Figure 9: The ventral midbrain dopaminergic niche
Graphical abstract showing the transcriptional networks of the three types of radial glia (mRgl1-3) showing their contribution to the ECM core components, ECM modifiers, and ligands. Together, they contribute to a microenvironment in which mDA neurons emerge, the mDA niche. Furthermore, Rgl1, the putative radial glia in the mDA lineage, was characterized by a transcriptional network centered on the transcription factor *ARNTL*, which we found controls DA neurogenesis in human neuroepithelial stem cells.

Our approach suggests different functions for each of the radial glia (Rgl1-3) that contribute to mDA neurogenesis and the mDA niche and express known developmental signals and factors. Rgl1 expressing transcription factors required for mDA neurogenesis, Rgl2 expressing transcription factors for progenitor maintenance, and Rgl3 expressing transcription factors for formation of the mDA niche including ECM components. Together, we suggest a model in which Rgl1 serves as a neurogenic progenitor and reveal a transcriptional network centered around pioneering factor *ARNTL*, which controls cell cycle duration and here, progress into the mDA lineage. Using transcriptome data from the developing VM, we describe the mDA niche in which cells arise and the regulatory transcriptional networks involved in the setting up and maintenance of this environment.

3.5 PAPER V: THE MATRICELLULAR PROTEIN R-SPONDIN 2 (RSPO2) PROMOTES MIDBRAIN DOPAMINERGIC NEURON NEUROGENESIS AND DIFFERENTIATION

In this manuscript, we examine the role of a Wnt signaling regulator RSPOs. A lot of literature points to the fact that RSPOs potentiates Wnt signaling. We here investigate how RSPOs can be used in stem cell differentiation protocols in order to differentiate mDA neurons more effectively. Our findings include: 1) Exclusive expression of *Rspo2* during VM development. 2) RSPO2 has a significant effect on the differentiation of mDA neurons in mouse primary cultures as well as on the differentiation of mouse embryonic stem cells. 3) RSPO2 also has a significant effect on the differentiation of human ES cells into mDA neurons but was found to have an additional effect on mDA neurogenesis, which is of interest in order to improve cell preparations to be used in CRT for PD. Together, this data provides evidence that additional manipulations of the Wnt signaling pathway *in vitro* may contribute to improve current mDA differentiation protocols.

Previous reports have suggested a function of *Rspo2* in a sub-population of mDA neurons and a regulation by *Lmx1a* (Hoekstra et al., 2013). We hereby examine the expression and function of different spondins in the developing VM. We found that *Rspo2* is strongly expressed in the developing VM during neurogenesis and we thus decided to apply recombinant RSPO2 protein to determine its biological activity in mouse primary VM, mouse ES, and human ES cell cultures. All cultures treated with RSPO2 showed a robust significant increase in differentiation, as shown by an increase in the proportion of $NR4A2^+$ cells that become and TH^+. We found that this effect was exclusive for RSPO2, a protein that induced and additional increase in the number of the first postmitotic cell in the mDA lineage, the $NR4A2^+$ cells.

Previous studies have shown that the implementation of Wnt signaling in differentiation protocols is essential for proper specification of midbrain progenitors (Kirkeby et al., 2012; Kriks et al., 2011). Here, early activation of Wnt/β-catenin signaling was already used to improved mDA differentiation in hPSCs, as it is standard in the field. However, we found that a later manipulation of Wnt/β-catenin signaling with RSPO2 further improved neurogenesis and differentiation. It is important to note that currently protocols for hPSC-differentiation into mDA neurons use very high doses of GSK3β inhibitors, which have off target effects (discussed in **Paper III**). One possibility to improve such protocols would be to use RSPO2 at early stages to minimize these. Additionally, RSPOs have been critical in the setting up of organoids in other systems like the gut and liver and it would thus be of interest to examine whether RSPO2 could be also used to improve midbrain organoids. Furthermore, it remains to be determined whether RSPO2 can aid in the specification of SNc neurons, the cell type lost in PD.

3.6 PAPER VI: ROR2 AND VANGL2 CONTROL DOPAMINERGIC NEUROGENESIS AND MULTIPLE ASPECTS OF CELL POLARITY IN THE MIDBRAIN FLOOR PLATE

In this manuscript, we investigate the function of Wnt/PCP signaling components in VM development. Using mouse models, we find that the Wnt/PCP components, *Ror2* and *Vangl2* play essential roles in different aspects of mDA development that go beyond that of previous functions identified in *Wnt5a*$^{-/-}$ mice. These include: 1) *Wnt5a* overexpressing embryos partially phenocopy *Wnt5a*$^{-/-}$, affecting morphogenesis and mDA differentiation. 2) Double compound mutant mice for *Wnt5a* and its receptor *Ror2* reveal stronger morphogenesis defect than that of *Wnt5a*$^{-/-}$ embryos. 3) Double compound mutant embryos for the Wnt/PCP signaling components, *Ror2* and *Vangl2,* reveal novel alterations in mDA development including a very severe morphogenic defect with collapse of the ventricle and right-left asymmetry and reduction in mDA neurogenesis resulting in a significant reduction in the production of mDA neurons.

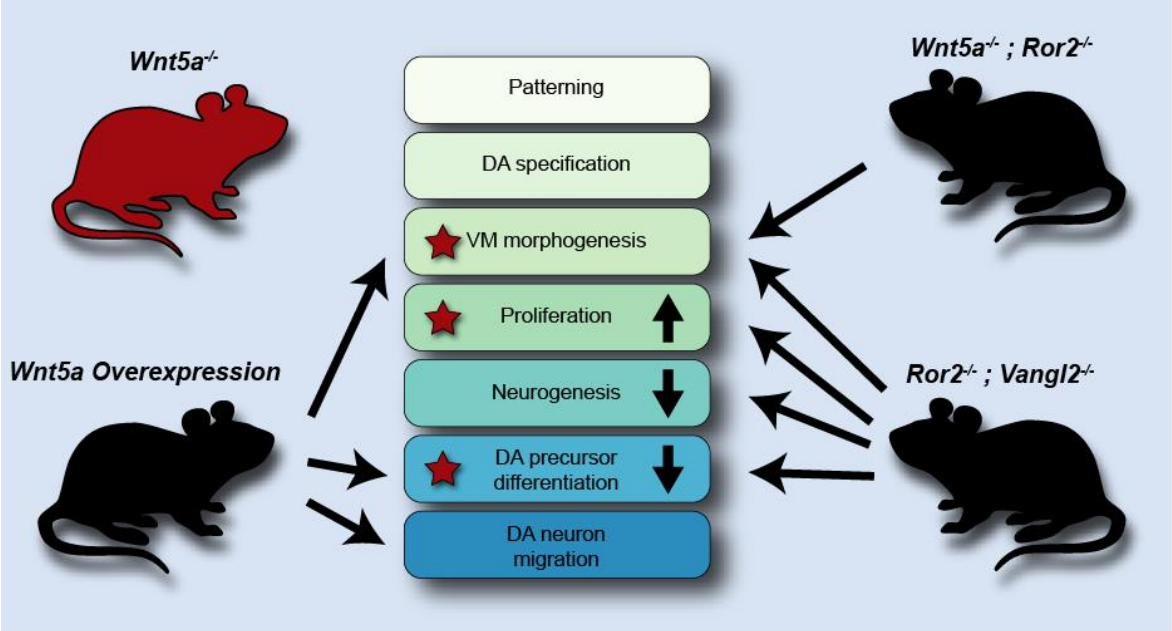

Figure 10: Function of *Wnt5a*, *Ror2*, and *Vangl2* in midbrain and dopaminergic neuron development
Graphical summary of conclusions obtained from analyzing mutant mice for the Wnt/PCP genes: *Wnt5a*, *Ror2*, and *Vangl2*. Previous analysis of *Wnt5a* mutant mice have shown impairments in VM morphogenesis, progenitor proliferation, and mDA differentiation in mice (red). Similarly, *Wnt5a overexpressing* mice showed clear morphogenic defects and alterations in mDA differentiation and mDA neuron migration. Morphogenesis was also impaired in *Wnt5a;Ror2* mice, to a larger extent than in *Wnt5a* deficient mice. *Ror2;Vangl2* double mutants showed an even more severe PCP morphogenesis phenotype and alterations in multiple aspects of mDA neurons development, including increase in proliferation and decreases in neurogenesis and differentiation

In this study, we first examined the expression of Ror and Vangl family member during development by various methods and found a very dynamic regulation of the expression of Vangl2 that correlated with mDA neurogenesis. We then took advantage of a novel transgenic mouse line where the expression of *Wnt5a* can be induced with doxycycline. Our results from the analysis of this mice revealed a surprising resemblance to the VM phenotype of the *Wnt5a*$^{-}$

$^{-/-}$ mice (Andersson et al., 2008) in that we see clear defects in morphogenesis but also early developmental delays that is compensated for at later time points. These findings support the hypothesis that the levels of Wnt5a are important for proper VM development. Next, since Wnt5a can signal via Ror2 we examined whether double mutant mice for these two genes worsened the *Wnt5a* phenotype. To our surprise, we found only a mild worsening of the morphogenesis phenotype of the *Wnt5a* null phenotype in *Ror2*$^{-/-}$*;Wnt5a*$^{-/-}$ mice embryos, although analysis of additional mutant mice is still necessary to complete this study. In agreement with previous results showing a cooperation between *Ror2* and *Vangl2* in limb development (Gao et al., 2011), analysis of *Ror2;Vangl2* mice revealed a strong Wnt/PCP phenotype in the VM, including a clear alteration of morphogenesis and mDA differentiation. *Ror2*$^{-/-}$*;Vangl2*$^{-/-}$ embryos also suffered from neural tube closure defects. In addition, we also found an alteration in left-right symmetry that altered the distribution of mDA progenitors, neuroblasts and neurons. The specification of the floor plate was not disrupted but we found an additional novel defect in mDA neurogenesis, as shown by a decrease in the number of postmitotic cells in the mDA lineage. Notably, severe morphogenic defects and decreased numbers of mDA neurons have not been previously observed in *Wnt5a* null embryos (Andersson et al., 2008). This finding suggests that the ROR2-VANGL2 complex is likely to transduce not only the signal from WNT5A, but also from additional Wnts. One likely candidate, based on our analysis in **Paper III** of the scRNA-seq data in **Paper II**, is that WNT5B, expressed in the same cell as WNT5A, may also signal through the same receptor complex. Future analysis of double *Wnt5a*$^{-/-}$;*Wnt5b*$^{-/-}$ mice would allow to answer this question. In addition it also remains to be determined whether additional Wnt/PCP receptors such as *Fzd3* and *Fzd6* (Stuebner et al., 2010) may also contribute to transduce the Wnt/PCP signal. In summary, this study finds novel roles of two Wnt/PCP signaling components in the developing VM and contributes to increase our understanding of the complex world of Wnt signaling.

4 CONCLUSION

This thesis is composed of four papers and two manuscripts covering various aspects of mDA neuron development. Our goal has been to gain a better understanding of the biological processes that govern this process. Based on the work within this thesis, I present the following conclusions:

- *Pbx1* and in its absence, *Pbx3* play a critical role in mDA differentiation and the survival of mDA neurons.
- Single-cell RNA-seq has unraveled a previously unsuspected cellular diversity and molecular complexity in the developing VM.
- Single-cell RNA-seq data allows to identify previously overlooked Wnt signaling components in the developing VM.
- *In silico* methods can be used to mine single-cell RNA-seq data and characterize cell intrinsic and extrinsic factors in the mDA neuron niche..
- R-spondin 2 robustly promotes the *in vitro* differentiation of endogenous mouse or human ES cell-derived mDA neurons.
- The Wnt/PCP membrane proteins *Ror2* and *Vangl2* control two novel functions in the developing VM: mDA neurogenesis and left-right symmetry.

And yet, here we are, 200 years after the publication of *An Essay on the Shaking Palsy*, still revealing critical molecular components and aiding in finding a cure for PD. Like James Parkinson, we look forward to a cure for this devastating disease. We just hope it will not take another 200 years!

5 ACKNOWLEDGEMENTS

Now to the page that is usually opened first by the majority of you. Even though I am not a person of many words, it is time to give gratitude for the people that have aided in the completion of what you have before you, whether it be scientific or just your presence. As I make this list, I can't help to notice how long I have been here and only a select few left from the first day I stepped into MolNeuro in 2010 when confocals, cryostats, and cell culture were all located in only our two main corridors.

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Enrique, where do I start? The walking encyclopedia of useless knowledge and the only person in the unit that not only knows what technique should be used to answer your scientific questions but also what the purpose of every reagent used is. At least I know I will always be able to fix your English.

As a person who likes to figure things out himself instead of going for help, there are a few things that I could not have done without you **Alessandra**. MolNeuro would not function without you, so thank you, especially these last few months!

Johnny, you left a hole in MolNeuro when you left and I think I can speak for everyone that MolNeuro is just different without you. Thank you so much for all the work you did in the lab even when you think it goes unnoticed sometime. The gratitude you deserve in all our projects is beyond fathomable, so wherever you are enjoying your free time, Thank YOU! And to **Ahmad**, for your continued support of entire MolNeuro when Johnny left, thank you.

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