From DEPARTMENT OF MEDICAL BIOCHEMISTRY AND BIOPHYSICS
Karolinska Institutet, Stockholm, Sweden

MECHANISMS CONTROLLING MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT

Shanzheng Yang

Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-print AB 2016

ISBN 978-91-7676-529-6

Mechanisms controlling midbrain dopaminergic neuron development

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Shanzheng Yang

Principal Supervisor:
Prof. Ernest Arenas
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Molecular Neurobiology

Co-supervisor(s):
Assist. Prof. J. Carlos Villaescusa
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Molecular Neurobiology

Prof. Katarina Le Blanc
Karolinska Institutet
Department of Laboratory Medicine (LABMED)
Division of Clinical Immunology

Opponent:
Assoc. Prof. Diogo S. Castro
Instituto Gulbenkian de Ciencia,
Oeiras, Portugal
Group of Molecular Neurobiology

Examination Board:
Prof. Finn Hallböök
Uppsala University
Department of Neuroscience

Assoc. Prof. Ana Teixeira
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Biomaterials and Regenerative Medicine

Prof. Lars Olson
Karolinska Institutet
Department of Neuroscience
Division of Genetic and molecular basis of nervous system disorders

To my family and my parents

ABSTRACT

The degeneration of midbrain dopaminergic (mDA) neurons accounts for some of the main motor symptoms of Parkinson's disease (PD). Efforts during the last decades have focused on understanding how mDA neurons are generated and maintained with the hope of developing novel stem cell-based replacement therapies. In this thesis, I address some of these questions in four papers.

Paper I: Wnt signaling controls multiple developmental processes in the embryo. Here we report that *Wnt1* deletion causes the loss of *Lmx1a* and *Ngn2* expression in the midbrain floor-plate resulting in the loss of mDA progenitor specification and neurogenesis in this region. Only a few ectopic LMX1A^{+}, NURR1^{+} and TH^{+} cells were transiently found in the basal plate. This phenotype and the morphogenesis defect found in *Wnt5a*$^{-/-}$ mice were worsened in *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice, indicating the existence of a previously unsuspected cooperation between Wnt1 and Wnt5a in mDA neuron development *in vivo*. Based on these results, we developed a combined Wnt protocol to promote the generation of mDA neurons from neural and embryonic stem cells *in vitro*. We conclude that coordinated Wnt actions promote mDA neuron development *in vivo* and in stem cells.

Paper II: In this study we report that the chemokine *Cxcl12* is expressed in the meninges, surrounding the ventral midbrain (VM) and that its cognate receptor CXCR4, is present and activated in NURR1^{+} mDA precursors and neurons. We found that VM meninges or CXCL12 promoted migration and neuritogenesis of TH^{+} cells in a CXCR4-dependent manner *in vitro*. Consistently, pharmacological blockade of CXCR4 or genetic deletion of *Cxcr4* resulted in an accumulation of TH^{+} cells in the lateral aspect of the intermediate zone in the VM. Moreover, the processes of TH^{+} cells in *Cxcr4*$^{-/-}$ mice were no longer radially distributed but disoriented. Thus our results indicate that CXCL12/CXCR4 regulate the radial migration of mDA neurons.

Paper III: We report that the homeodomain transcription factor ZEB2, is present at a high level in progenitor cells of the ventricular zone in the midbrain floor plate and that its expression diminishes in NURR1^{+} post-mitotic precursors. We found that ZEB2 upregulated *miR200c*, which in turn repressed *Zeb2*, to form a negative feedback loop. Overexpression of *Zeb2* reduced the levels of CXCR4 and NR4A2 in the developing VM *in vivo*, resulting in migration and mDA differentiation defects. This phenotype was phenocopied by *mir200c* knockdown, indicating that the Zeb2-miR200c loop prevents the premature differentiation of mDA progenitors into postmitotic cells and their migration.

Paper IV: We demonstrate that the extracellular matrix protein, laminin 511 (LM511), promotes midbrain dopaminergic neuron survival and differentiation via binding to integrin α3β1 and activation of the Yes-Associated protein, YAP. We found that LM511-YAP enhances mDA neuron survival by inducing the expression of miR-130a, which reduces the levels of PTEN, a negative regulator of the Akt/PKB pro-survival pathway, both *in vitro* and *in vivo*. Additionally, YAP up-regulates the expression of mDA differentiation genes such as *LMX1A*, *LMX1B* and *PITX3*, and prevents the loss of mDA neurons by oxidative stress. Thus our results

suggest the LM511-YAP pathway as a possible target for the development of novel therapies for PD.

In sum, the data presented in this thesis provide evidence that multiple factors control common aspects of mDA development such as neurogenesis, positively controlled by WNT1, WNT5A and *miR200c* and negatively by ZEB2. Similarly, the migration of mDA neurons is controlled by WNT5A, *miR200c* and CXCL12/CXCR4. Lastly, we found that the survival and differentiation of mDA neurons is not only controlled by neurotrophic factors, but also by the extracellular molecule, LM511, via YAP activation.

LIST OF SCIENTIFIC PAPERS

I. Andersson ER, Saltó C, Villaescusa JC, Cajanek L, **Yang S**, Bryjova L, Nagy II, Vainio SJ, Ramirez C, Bryja V, Arenas E.
Wnt5a cooperates with canonical Wnts to generate midbrain dopaminergic neurons in vivo and in stem cells.
Proc Natl Acad Sci U S A. 2013 Feb 12;110(7):E602-10.

II. **Yang S**, Edman LC, Sánchez-Alcañiz JA, Fritz N, Bonilla S, Hecht J, Uhlén P, Pleasure SJ, Villaescusa JC, Marín O, Arenas E.
Cxcl12/Cxcr4 signaling controls the migration and process orientation of A9-A10 dopaminergic neurons.
Development. 2013 Nov;140(22):4554-64.

III. **Yang S**, Toledo EM, Peng C, Uhlén P, Arenas E.
A Zeb2-miR200c loop controls midbrain dopaminergic neuron migration and differentiation.
Manuscript.

IV. Zhang D, **Yang S**, Toledo EM, Gyllborg D, Saltó C, Villaescusa JC, Arenas E.
Laminin-511 promotes midbrain dopaminergic neuron survival and differentiation via YAP.
Manuscript.

PUBLICATIONS NOT INCLUDED IN THE THESIS

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.
A PBX1 transcriptional network controls dopaminergic neuron development and is impaired in Parkinson's disease. *EMBO Journal.* 2016 Sep 15;35(18):1963-78

Theofilopoulos S, Griffiths WJ, Crick PJ, **Yang S**, Meljon A, Ogundare M, Kitambi SS, Lockhart A, Tuschl K, Clayton PT, Morris AA, Martinez A, Reddy MA, Martinuzzi A, Bassi MT, Honda A, Mizuochi T, Kimura A, Nittono H, De Michele G, Carbone R, Criscuolo C, Yau JL, Seckl JR, Schüle R, Schöls L, Sailer AW, Kuhle J, Fraidakis MJ, Gustafsson JÅ, Steffensen KR, Björkhem I, Ernfors P, Sjövall J, Arenas E, Wang Y.
Cholestenoic acids regulate motor neuron survival via liver X receptors. *J Clin Invest.* 2014 Nov 3;124(11):4829-42.

Ribeiro D, Laguna Goya R, Ravindran G, Vuono R, Parish CL, Foldi C, Piroth T, **Yang S**, Parmar M, Nikkhah G, Hjerling-Leffler J, Lindvall O, Barker RA, Arenas E.
Efficient expansion and dopaminergic differentiation of human fetal ventral midbrain neural stem cells by midbrain morphogens. *Neurobiol Dis.* 2013 Jan;49:118-27.

Toledo EM, **Yang S**, van Wijk KE, Gyllborg D, Sinha I, Varas-Godoy M, Islam1 S, Steffensen KR, Linnarsson S, Arenas E.
Liver X Receptors Induce Midbrain Dopaminergic Neurogenesis via a novel basic helix-loop-helix transcription factor cascade. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AADC	Aromatic L-amino acid decarboxylase
ALDH1A1	Aldehyde dehydrogenase-1a1
APC	Adenomatous polyposis coli
ApoER2	Apolipoprotein E receptor 2
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
BMPs	Bone morphogenetic proteins
BP	Basal plate
CamKII	Calcium-calmodulin-dependent kinase II
Cdkn1c	Cyclin-dependent kinase inhibitor 1C
CK	Casein kinase
CNS	Central nervous system
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
DA	Dopaminergic
DAB1	Disabled homolog 1
DAT	Dopamine transporter
DCX	Doublecortin
DLK1	Delta-like 1
DVL	Dishevelled
E	Embryonic day
ECM	Extracellular matrix
ES	Embryonic stem
FGF8	Fibroblast growth factor 8
FMRP	Fragile X mental retardation protein
FOXA2	Forkhead box A2
FP	Floor plate
Fz	Frizzled
GBX2	Gastrulation brain homeobox 2
GSK3β	Glycogen synthase kinase 3β

hFVM	human fetal ventral mesencephalic
ISH	*In situ* hybridization
IsO	Isthmic organizer
IZ	Intermediate zone
LEF	Lymphoid enhancer-binding factor
LIS1	Lissencephaly 1
LRP	Low-density lipoprotein receptor-related protein
MAP1B	Microtubule associated protein 1B
mDA	Midbrain dopaminergic
mFP	Midbrain floor plate
MHB	Midbrain-hindbrain boundary
MSX1	Muscle segment homeobox homolog 1
MZ	Marginal zone
NEUROG2	Neurogenin2
NFAT	Nuclear factor of activated T cells
NKX6-1	NK homeobox protein, family 6, member A
N-WASP	Neural Wiskott-Aldrich syndrome protein
OTX2	Orthodenticle homolog 2
PD	Parkinson's disease
PITX3	Pituitary homeobox 3; paired-like homeodomain transcription factor 3
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
RET	C-ret proto-oncogene
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1/2
RrF	Retrorubral field
SHH	Sonic hedgehog
SLC18A2	Solute carrier family-18 member-2
SLC6A3	Solute carrier family-6 member-3
SNc	Substantia Nigra pars compacta
SVZ	Subventricular zone

TCF	T cell factor
VLDLR	Very low density lipoprotein receptor
VMAT2	Vesicular monoamine transporter-2
VM	Ventral midbrain
VTA	Ventral tegmental area
VZ	Ventricular zone
WNT	Wingless-type MMTV integration site family
YAP	Yes-Associated Protein
ZEB1/2	Zinc finger E-box binding homeobox 1/2

1 INTRODUCTION

1.1 PARKINSON'S DISEASE AND CELL REPLACEMENT THERAPY

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting approximately 1% of the population over 65 years of age per year, with some variation depending on ethnicity and gender (Van Den Eeden et al., 2003; Wijeyekoon and Barker, 2009). The major symptoms of PD include bradykinesia, rigidity and a resting tremor (Kalia and Lang, 2015). At pathological level, the main features of PD include the loss of midbrain dopaminergic (mDA) neurons within substantia nigra pars compacta (SNc) and the presence of Lewy bodies, cellular aggregates formed by alpha-synuclein and other misfolded proteins. The cause of mDA neuron death is still not understood, but oxidative stress, alterations in autophagosome and proteasome systems, inflammation, and genetic factors have been shown to be involved (Eriksen et al., 2005; Miller et al., 2009; Tansey et al., 2007). Multiple genes and loci have been associated with PD, including causal mutations (*SNCA*, *PARK2*, *CHCHD2*, *LRRK2*, *VPS13C*, *VPS35*, *FBXO7*, *PLA2G6*, *PARK7*, *ATP13A2* and, *PINK1*) (Bonifati, 2014; Klein and Westenberger, 2012; Singleton and Hardy, 2016).

PD patients are currently treated with pharmacological tools (mainly L-DOPA and dopaminergic agonists) or surgery, such as deep brain stimulation. These treatments relieve the motor symptoms of PD (Hickey and Stacy, 2016; Smulders et al., 2016; Verhagen Metman et al., 2016), but they neither address the cause of disease nor do they replace the mDA neurons lost by disease. As a consequence, disease progresses and mDA neurons continue to degenerate, making therapies less effective. In advanced phases of disease, patients experience motor complications, as well as no-motor symptoms, including depression and dementia. Patients suffering PD end up experiencing progressive disability and reduced quality of life (Kalia and Lang, 2015). As PD progresses, the cost of the illness escalates, placing an economic burden on the healthcare system, society and patients themselves (Findley, 2007).

Proof of concept of cell replacement therapy (CRT) for PD has been provided by a series of studies in PD patients with intrastriatal grafts using human fetal ventral mesencephalic (hfVM) tissue (Barker et al., 2013; Barker et al., 2015; Lindvall and al., 1989; Lindvall et al., 1988). Grafted mDA neurons are known to innervate correct target cells in the host striatum (Kordower et al., 1995) and the patient's motor symptoms improve in some cases for as long as 16 years after transplantation (Politis et al., 2010). However logistic and ethical issues have hampered the wide use of hfVM in the clinic. In addition, variability in the quality of the tissue preparation causes variation in the number and types of cells within the preparations, leading to variable results and side effects. One such adverse effect is graft-induced dyskinesia, which is thought to be due to contamination of the midbrain tissue with serotonergic neurons from the hindbrain (Lindvall and Björklund, 2011; Politis et al., 2010). Recent advancements in stem cell and developmental biology have allowed to obtain high quality mDA neurons from human embryonic stem (hES) cells (Kirkeby et al., 2012; Kriks et al., 2011). These cell preparations have emerged as a promising cell source for transplantation as they can be readily generated

and characterized prior to clinical application (Arenas, 2002; Arenas, 2010; Arenas et al., 2015; Barker et al., 2015; Politis and Lindvall, 2012).

1.2 STEM CELLS

Stem cells are defined as cells that have the ability to continuously self-renew and give rise to differentiated progeny. Stem cells can be classified according to their origin (embryonic or adult) or their potential. Monopotent, such as adult hippocampal stem cells that can only give rise to neurons. Multipotent, cells that can give rise to all cell types in a tissue, such as long term neuroepithelial stem (lt-NES) cells derived from hES cells or fetal tissue, which are capable of giving rise to diverse types of neurons, astrocytes and oligodendrocytes (Falk et al., 2012; Koch et al., 2009; Tailor et al., 2013). Finally, pluripotent stem cells, such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, possess the ability to differentiate into all the cell types in an organism.

1.2.1 Embryonic stem (ES) cells

Mouse ES cells were the first to be derived from an organism, by cultivation of cells from the inner cell mass of blastocysts (Evans and Kaufman, 1981; Martin, 1981). These cells can be readily maintained and expanded as an undifferentiated pure cell population *in vitro*. Moreover, they can differentiate into every cell type in the body under appropriate conditions (Keller, 1995; Keller, 2005). Similar properties have been described for human ES cell lines, which were established almost two decades later (Thomson et al., 1998).

1.2.2 Differentiation of ES cells

As mentioned above, one important characteristic of ES cells is their capacity to generate differentiated cell types belonging to the three germinal layers both *in vivo* (Bradley et al., 1984) and *in vitro* (Keller, 1995; Smith, 2001). Protocols have been developed to differentiate ES cells to a broad spectrum of cell types including hematopoietic cells, cardiomyocytes, oligodendrocytes and pancreatic β cells (Keller, 1995; Murry and Keller, 2008). Knowledge obtained from understanding normal development has facilitated the optimization of specific differentiation protocols, taking cells from gastrulation to the induction of endoderm, mesoderm and ectoderm and to their downstream derivatives. Moreover, neuralized human ES cells have been differentiated into mDA neurons *in vitro* by using appropriate developmental factors such as SHH and activation of Wnt signaling (Kirkeby et al., 2012; Kriks et al., 2011).

1.3 ADULT MIDBRAIN DOPAMINERGIC NEURONS

Neurons capable of synthesizing the neurotransmitter dopamine, DA neurons, are distributed widely in the mammalian brain (Björklund and Dunnett, 2007). In the ventral midbrain (VM), three groups of mDA neurons, A8 (RrF), A9 (SNc) and A10 (VTA), compose the so-called mDA neurons. Of these, neurons in A9 (SNc) are the ones predominantly affected in PD patients.

Each mDA cell group is involved in the control and/or modulation of specific brain functions according to their distinct projection fields. DA neurons of the SNc innervate the striatum and form the nigrostriatal system that controls voluntary movement and body posture. The mesocortical and mesolimbic systems, originating in the A8 and A10 groups, are on the other hand involved in the modulation and control of cognitive and emotional/rewarding behaviors.

1.4 MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT

1.4.1 Neural induction and patterning

During gastrulation, a single cell layer called blastula gives rise to a three-layered structure formed by the ectoderm in the outside, the mesoderm in the middle and the endoderm in the inside. The notochord then induces the overlaying ectoderm into the neuroectoderm, which forms the neural plate, a flat sheet of neuroepithelial cells. The early neural plate undergoes neurulation, the process by which the neural plate rolls up to form the neural tube, where the prospective brain and spinal cord originate. Subsequently, gradients of developmental signals in the dorsoventral and anteroposterior axis pattern the neural tube, provide regional identity to different segments of the neural tube. The neural tube is partitioned along the dorsoventral axes under the influence of the underlying meso-endodermal tissue (ventral), as well as adjacent non-neural ectodermal structures (dorsal). Signals involved in this process include BMPs dorsally and SHH ventrally. These factors give rise from ventral to dorsal to the floor plate (FP), the basal plate (BP), the alar plate (AP) and the roof plate (RP) (Figure 1) (Wurst and Bally-Cuif, 2001).

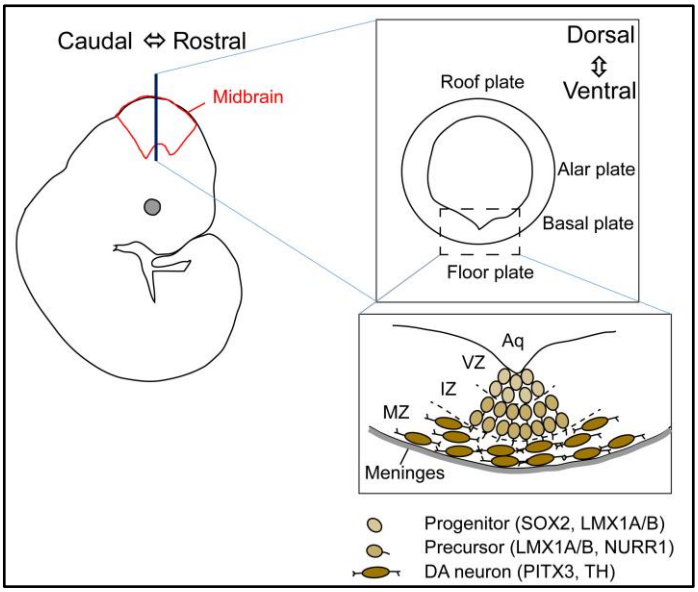

Figure 1. Schematic showing mouse midbrain at E11.5. Midbrain dopaminergic neurons reside in the floor plate, which can be divided into three layers: ventricular zone (VZ), intermediate zone (IZ) and marginal zone (MZ). mDA progenitors are in the VZ and express *Sox2, Lmx1a/b*. mDA precursors or neuroblasts are in IZ and express *Lmx1a/b, Nurrr1*. Mature mDA neurons can be found in MZ and express *Pitx3, Th*. Aq: Aqueduct.

Another important signaling center for the patterning of the neural tube, is the isthmic organizer (IsO), which defines the midbrain-hindbrain boundary (MHB) (Joyner et al., 2000; Rhinn et

al., 1998; Wassarman et al., 1997). At the end of gastrulation (E7.5 in mouse), the transcription factors, *Otx2* in the midbrain and *Gbx2* in the hindbrain, are expressed in a complementary pattern, to drive the formation of IsO in MHB (Broccoli et al., 1999; Millet et al., 1999; Wassarman et al., 1997). This center produces WNT1 in the midbrain side and FGF8 in the hindbrain side. During these and subsequent developmental stages, the combined action of transcription factors and morphogens from the IsO orchestrate multiple functions. These include providing regional identity of the VM, controlling the specification and proliferation of mDA progenitors, mDA neurogenesis, as well as the promoting the differentiation and survival of mDA neurons (Arenas et al., 2015; Dworkin and Jane, 2013).

1.4.2 Specification and differentiation of midbrain dopaminergic neurons

SHH, a morphogen initially secreted by the notochord at E8 and expressed in FP by E8.5 in mice, induces the expression of *Foxa2* in the FP (Ang et al., 1993; Sasaki et al., 1997). *Foxa2* plays a central role downstream of SHH as it is required for notochord and FP development as well as for ventral patterning (Ang et al., 1994; Weinstein et al., 1994). By contrast, in the BP, lower levels of SHH upregulate *Nkx6-1* and *Otx2*. FOXA2 also induces *Nkx6-1* in the BP (Nakatani et al., 2010) and suppresses *Nkx2-2* in the FP (Ferri et al., 2007). Moreover, FOXA2 directly represses *Gli1-3* and upregulates *Shh* expression (Metzakopian et al., 2012).

FOXA1/2 and OTX2 regulate the expression of two LIM homeobox transcription factors, *Lmx1b* and *Lmx1a* in mFP. Whereas *Lmx1b* is necessary for the differentiation of mDA progenitors (Smidt et al., 2000), *Lmx1a* is required for the specification of mDA neurons in the FP (Andersson et al., 2006a; Deng et al., 2011) and, via *Msx1*, suppresses the emergence of BP fates (Andersson et al., 2006a). Additionally, *Otx2* sustains the expression *Nkx6-1* in the BP and suppresses *Nkx2-2* in the FP (Puelles et al., 2004). Thus, the concerted action of the Shh-Foxa2 and the Otx2-Wnt1-Lmx1a/Msx1 networks is essential not only for the specification of the FP but also for the suppression of alternative neural fates.

In the FP, Wnt1/β-catenin (Ctnnb), Lmx1a and Lmx1b form a positive autoregulatory loop (Chung et al., 2009) required for mDA specification: on the one hand β-catenin, together with TCF/LEF, directly upregulates *Lmx1a* and *Otx2*, and, on the other hand, Lmx1a/b directly upregulate each other as well as *Wnt1, Msx1* and two key genes involved in mDA neuronal differentiation and survival, *Nurr1* (Nr4a2, nuclear receptor 4a2) and *Pitx3* (Pituitary homeobox 3). Combined, these results indicate that the specification of mDA neurons is controlled by the Lmx1a/b-Wnt1/Ctnnb autoregulatory loop together with Otx1/2 and Foxa1/2.

It has been recently shown that Foxa2 also interacts with components of the Wnt1-Lmx1a/b network. Indeed, FOXA2 directly promotes the expression of *Lmx1a/b* and *Msx1* and upregulates the expression of *Neurog2* (Metzakopian et al., 2012). *Neurog2* is a basic-loop-helix transcription factor required for mDA neurogenesis (Andersson et al., 2006b; Kele et al., 2006b), whereas *Ascl1* (Mash1, mouse achaete-schute homolog 1; another proneural gene of the basic-helix-loop-helix family) is capable of partially compensating for the loss of *Neurog2* (Kele et al., 2006b). These two proneural genes are directly or indirectly regulated by, and

integrate information from, the Shh-Foxa2 and Lmx1a/b-Wnt1-Otx2 networks (Arenas et al., 2015), as well as from nuclear receptors of the Liver X receptor family (Lxrα/Nr1h3 and Lxrβ/Nr1h2) (Sacchetti et al., 2009) and the morphogen Wnt5a (Andersson et al., 2008), which also control mDA neurogenesis.

1.4.3 MicroRNAs

MicroRNAs (miRNAs) are short (about 22 nucleotides (nt)) non-coding RNA molecules, which are produced in three steps: (1) Primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II; (2) Drosha, a nuclear-localized RNA endonuclease III, cleaves the pri-miRNA duplex transcripts into ~70 nt stem loops called precursor mRNAs (pre-miRNAs); (3) The pre-miRNAs are then exported to the cytoplasm where they are cleaved by the Dicer enzyme into ~22 nt mature miRNA double-stranded duplexes (Winter et al., 2009). Such duplexes are loaded into miRNA-induced silencing complexes where only one strand is left to serve as a guide for imperfect base pairing with its target mRNAs. Nucleotides 2~7 in the 5' terminus of the miRNA, called the "seed" sequence, are the most critical for recognition of the target mRNA (Pasquinelli, 2012).

MiRNAs act thus as post-transcriptional regulators of gene expression (Bartel and Chen, 2004; Ebert and Sharp, 2012). This layer of regulation is important because miRNAs are found in most organisms and regulate around 60% of the predicted protein-coding genes (Grün et al., 2005). The flexibility and reversibility of miRNA function enable a precise temporal and spatial gene regulation, which is crucial for the correct functioning of the nervous system (Davis et al., 2015). Indeed, miRNAs control multiple stages of neuronal development (Davis et al., 2015; Petri et al., 2014). In agreement with this, conditional knockout of Dicer in postmitotic mDA neurons has been shown to result in progressive apoptosis of mDA neurons, leading to behavioral defects and alterations in locomotion (Kim et al., 2007).

1.4.4 Wnt signaling

Wnts are a large family of lipid-modified proteins formed by 19 members that bind to and activate a common signaling module formed by Frizzled (Fz) receptors (10 family members), and the intracellular signaling component, dishevelled (Dvl; 3 members). In addition, Wnts interact with different types of co-receptors, which recruit additional signaling components and transduce the Wnt signal in different ways (Niehrs, 2012).

There are three main signaling pathways. The most well known and most well studied is the canonical or Wnt/Ctnnb1 signaling pathway. This pathway is activated by Wnts such as WNT1 or WNT3A and uses the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) as co-receptors. Upon activation, a destruction complex formed by adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α) is inhibited resulting in stabilization (lack of degradation) of CTNNB1, which then translocates to the nucleus and regulates, together with T cell factor (TCF) or lymphoid enhancer-binding factor (LEF), the transcription of Wnt target genes (Arenas, 2014). In the developing midbrain, this

pathway promotes key processes such as mDA neuron specification, proliferation and neurogenesis (Inestrosa and Arenas, 2010).

The second most well-known pathway is the Wnt/planar cell polarity (PCP) pathway. This pathway is activated by WNT5A in the developing midbrain and uses the receptor tyrosine kinase-like orphan receptor 1/2 (ROR1/2). This receptor has been found to interact with core PCP signaling components such as Van Gogh-like 2 (VANGL2) to transmit the morphogenetic gradients established by WNTs (Gao et al., 2011). Upon activation, this pathway leads to the activation of small GTPases, such as RAC1, by guanine exchange factors, such as TIAM1 (Čajánek et al., 2013). This pathway controls not only neural tube closure and morphogenesis, but also mDA neuron differentiation (Arenas, 2014).

The third main pathway is the Wnt/calcium pathway, which activates calcium sensitive proteins such as protein kinase C (PKC), calcium-calmodulin-dependent kinase II (CamKII) or calcineurin and regulate the nuclear factor of activated T cells (NFAT) (Arenas 2014). This pathway has not been linked to mDA neuron development, but can regulate processes such as proliferation and differentiation (Huang et al., 2011).

1.5 MIGRATION OF POSTMITOTIC DOPAMINERGIC NEURONS

1.5.1 Cell migration

Cell migration is a widespread process in the developing central nervous system (CNS). This process allows neural cells to move away from their place of origin in the germinal zones and reach their final positions. The migratory behavior of newborn cortical neurons is by far the best well studied. Three modes of migration have been described in the cortex: somal translocation and radial locomotion (both perpendicular to the ventricular surface), and tangential locomotion (Nadarajah and Parnavelas, 2002). Cortical pyramidal neurons are generated in the germinal ventricular zone (VZ) and begin their migration as they leave the VZ to populate the primordial plexiform layers. Somal translocation is adopted by early generated neurons once the tip of their process attaches to the pia surface (Miyata et al., 2001; Nadarajah and Parnavelas, 2002). As corticogenesis proceeds, the cortex becomes thicker and neurons start radial glia-dependent locomotion. In this process, newborn neurons attach to radial glia processes and migrate away from the VZ, towards superficial destinations. Cortical interneurons, which originate in the ventral telencephalon, instead migrate tangentially and invade the cortical plane (Evsyukova et al., 2013; Marín et al., 2010; Nadarajah and Parnavelas, 2002; Valiente and Marín, 2010).

1.5.2 Cellular mechanisms in neuronal migration

Locomotion is the standard way that neurons use for migration. At a cellular level, this process involves three steps: (1) extension of the leading process, to explore the immediate environment for attractive or repulsive cues; (2) movement of the nucleus into the leading process, also

called ‘nucleokinesis’; and (3) retraction of the trailing process/es (Evsyukova et al., 2013; Marín et al., 2010; Nadarajah and Parnavelas, 2002).

Newly born cortical pyramidal neurons have a single process when they leave VZ, but they transiently assume multipolar morphology within the lower intermediate zone (IZ) and SVZ (Evsyukova et al., 2013; Marín et al., 2010; Noctor et al., 2001). Multipolar neurons possess multiple thin processes that extend and retract in a dynamic but apparently random fashion. Once they attach to radial glia processes they become bipolar again, with a long leading process and a short trailing process. Multipolar-bipolar transition is critical for radial glia-based migration and depends on the function of filamin A, Lissencephaly 1 (LIS1), doublecortin (DCX), fragile X mental retardation protein (FMRP) and Wnts (Boitard et al., 2015; La Fata et al., 2014; LoTurco and Bai, 2006). The leading process acts as the compass of migrating neurons, selecting the direction of migration in response to chemotactic cues.

Nucleokinesis follows the extension of the leading process, following the direction of migration. Nucleokinesis is a subcellular process involving extensive and rapid cytoskeletal

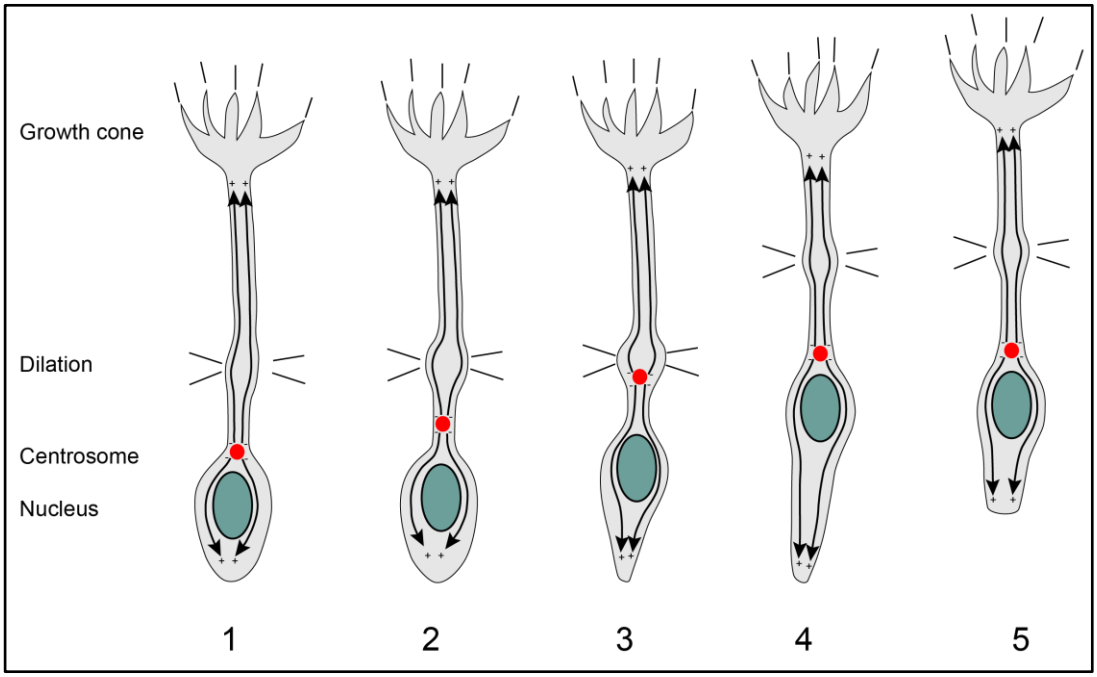

Figure 2. Coordinated events required for somal movement during saltatory migration. (1) The growth cone in the tip of the leading process extends toward the direction of the intended migration. (2) A swelling (dilation) forms at the base of the leading process where it adheres to the matrix substrate. The centrosome (red) moves towards the dilation. (3-4) The nucleus translocates into the dilation. This process is called nucleokinesis. (5) The trailing process retracts and a new dilation forms to start a new cycle of the process. +/-: plus/minus ends of microtubules. Modified from Cooper (2013).

rearrangements of both microtubules and actin, which results in the translocation of the nucleus into the leading process. During nucleokinesis, the centrosome and its accompanying organelles move into the swelling formed by the base of the leading process, followed by the

nucleus and the rest of the cell body (Figure 2) (Evsyukova et al., 2013; Valiente and Marín, 2010).

Extension of leading process and nucleokinesis are repeated to produce the typical saltatory movement of migrating neurons. When the leading process reaches the pial surface, migrating neurons detach form the radial glia process and move in a similarly way as during somal translocation to get to their final position.

1.5.3 Molecular cues regulating neuronal migration

A large numbers of secreted molecules and their membrane receptors have been shown to regulate neuronal migration, either chemoattractively or chemorepulsively. Most of those cues also regulate axon guidance (Evsyukova et al., 2013; Valiente and Marín, 2010), which also depend on the regulation of cytoskeletal elements such as microfilaments and microtubules (Dent et al., 2011; Evsyukova et al., 2013; Park et al., 2002; Valiente and Marín, 2010).

Reelin is a secreted glycoprotein and one of the most well-studied guidance cue molecules. Reelin regulates both radial and tangential migration and its mutation causes layering and migration defects in multiple brain regions, from the cerebral cortex to the VM and the spinal cord (Katsuyama and Terashima, 2009; Lambert de Rouvroit and Goffinet, 2001; Rice and Curran, 1999; Vaswani and Blaess, 2016). Reelin signaling involves binding to the ApoER2 and VLDLR receptors (Trommsdorff et al., 1998) and tyrosine phosphorylation of DAB1, which stabilizes microtubule through microtubule associated protein tau and MAP1B (Beffert, 2004; González-Billault et al., 2005; Hiesberger et al., 1999).

Another important signal is Slit, a secreted glycoprotein that binds to its cognate receptor ROBO (Roundabout), to induce repulsion and control neuronal tangential migration away from the midline (Wong et al., 2001; Ypsilanti et al., 2010). Binding of SLIT to ROBO increase the affinity of the intracellular CC3 motif of ROBO to SLIT-ROBO GTPase activation proteins (srGAPs) (Park et al., 2002; Wong et al., 2001). The srGAPs inactivate the small GTPases of the Rho family, mainly Cdc42 in SVZa cells, preventing it from activating an actin-nucleating factor, N-WASP, and thus abolishing actin polymerization associated with Arp2/3 protein activity and repelling cells from the slit-expressing SVZ region (Wong et al., 2001).

1.5.4 Migration of mDA neurons

When mDA precursors leave the VZ, they sequentially follow radial and tangential migration routes (Hanaway et al., 1971; Kawano et al., 1995). During radial migration mDA neurons attach to VIMENTIN^{+} radial glia fibers (Shults et al., 1990). After radial migration, when mDA neurons reach the marginal zone, mDA neurons destined to become SNc take a tangential pathway to migrate laterally and reach their final lateral destination. A panel of molecular cues have been reported to play a role in tangential migration of mDA neurons, such as neural L1 cell adhesion molecule (L1CAM) (Demyanenko et al., 2001; Ohyama et al., 1998) and Reelin (RELN) (Bodea et al., 2014; Kang et al., 2010; Nishikawa et al., 2003; Sharaf et al., 2015).

Netrin/Dcc signaling has also been shown to not only regulate mDA positioning but also cell death and the dopaminergic innervation of the striatum and cortex (Xu et al., 2010).

1.5.5 The CXCL12/CXCR4 signaling pathway

Chemokines (chemotactic cytokines) constitute a family of small protein ligands that are classified into four major groups (referred to as C, CC, CXC and CX3C), based on the location and organization of their cysteine residues. The CXC members, also called α-chemokine subfamily, have a single amino acid residue between the first two conserved cysteine residues (Zlotnik and Yoshie, 2012). CXCL12 (also known as stromal cell-derived factor 1, SDF1) is a member of the α-chemokine subfamily and, together with its cognate receptor, CXCR4, represent the best-known chemokine ligand/receptor pair.

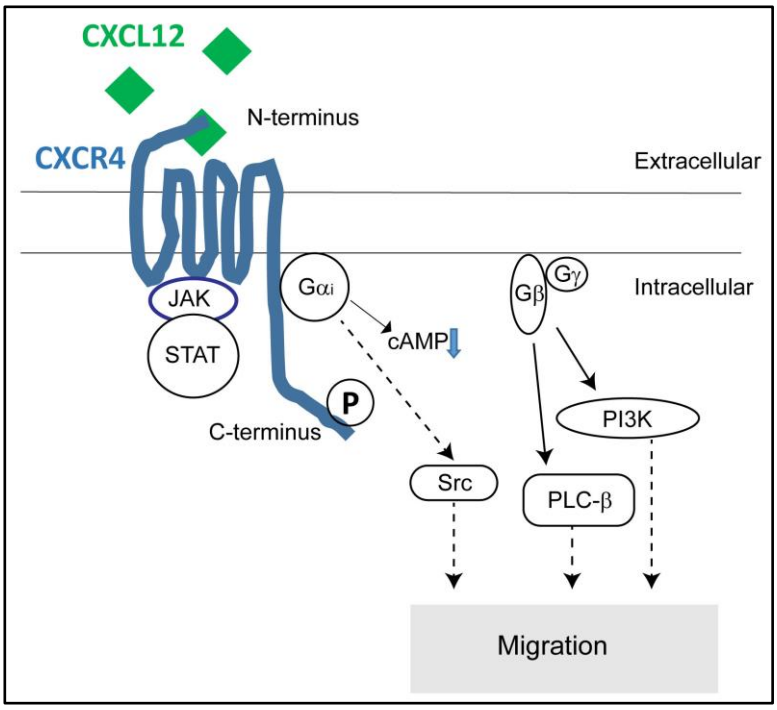

Figure 3. Components of the CXCR4 signaling pathway regulating cell migration. CXCL12 binding to CXCR4 leads to the activation of Gαi and liberation of Gβγ, which in turn activate phospholipase C-β (PLC-β) and phosphoinositide-3 kinase (PI3K), ultimately leading to the regulation of cell migration. Activated CXCR4 also recruits JAK/STAT and activates this pathway. CXCR4 is phosphorylated after its activation. Figure modified from Busillo and Benovic (2007).

The CXCR4 receptor is a member of the G protein coupled receptors (GPCRs) family. Activation of this receptor by CXCL12 leads to activation of G_i family of proteins. Activated G_i inhibit adenylyl cyclase and activate the Src family of tyrosine kinases, while liberated Gβγ activate phospholipase C-β (PLC-β) and phosphoinositde-3 kinase (PI3K) (Figure 3) (Busillo and Benovic, 2007).

Chemokines and their receptors are best known for their involvement in inflammatory and immune responses. Besides these roles, chemokines are expressed by all major cell types in the central nervous system (CNS). A growing body of evidence shows that chemokines and their receptors mediate cellular communication in the CNS (Mélik-Parsadaniantz and Rostène, 2008; Mithal et al., 2012; Zhu and Murakami, 2012). For example, projection neurons in the IZ/SVZ of the cortex express *Cxcl12* (Stumm and Höllt, 2007; Tiveron et al., 2006), and

regulate the tangential migration of CXCR4-expressing GABAergic interneurons generated in the ventral forebrain (López-Bendito et al., 2008; Tiveron et al., 2006). In addition, *Cxcl12* is persistently expressed in the leptomeninges (Paredes et al., 2006; Stumm et al., 2007), where it also regulates the tangential migration of GABAergic interneurons (López-Bendito et al., 2008) and hem-derived Cajal-Retzius cells throughout the MZ of the cortex (Borrell and Marín, 2006; Paredes et al., 2006). CXCL12/CXCR4 signaling is also responsible for the migration, assembly and positioning of cerebellar granule and Purkinje neurons (Ma et al., 1998), olfactory neurons (Miyasaka et al., 2007), as well as facial motoneurons (Sapède et al., 2005). Moreover, CXCL12/CXCR4 signaling is required for guiding the initial trajectory of ventral motoneurons (Lieberam et al., 2005) and for axon pathfinding of retinal ganglion cells and olfactory neurons (Li et al., 2005; Miyasaka et al., 2007). Similarly, the migration and final position of trigeminal and dorsal root ganglion cells, as well as their target innervation also require CXCL12/CXCR4 (Balabanian et al., 2005; Knaut et al., 2005; Odemis et al., 2005). Thus, CXCL12/CXCR4 signaling regulates the three key processes essential for the establishment of neural networks in different neuronal systems: neuronal migration, cell positioning and axon wiring.

1.5.6 Coordination of neurogenesis and migration

As mentioned above, postmitotic neurons start to migrate immediately after neurogenesis. Several lines of evidence indicate that neurogenesis and migration are coupled. Some of the most important genes regulating neurogenesis are basic helix-loop-helix (bHLH) genes, such as *Neurog1/2* and *Ascl1*, which initiate a cascade of bHLH gene activation events that eventually lead to the expression of terminal neuronal differentiation genes in central nervous system (Andersson et al., 2006b; Bertrand et al., 2002; Kele et al., 2006a; Ma et al., 1997). Once a progenitor is committed to neuronal fate by expressing proneural genes, Scrath1/2, an epithelial-mesenchymal transition (EMT) factor, is induced to initiate the migratory process (Itoh et al., 2013). Proneural bHLH factors down-regulate the RhoA small GTPase and up-regulate DCX and p35 expression, which modulate the actin and microtubule cytoskeleton assembly and enable newly generated neurons to migrate (Ge et al., 2006). Further analysis has shown that NEUROG2 induces the expression of small GTP-binding protein, Rnd2, to antagonize the activity of RhoA and promote multipolar-bipolar transition in the cortical IZ (Heng et al., 2008). Similarly, ASCL1 induce Rnd3 to inhibit RhoA activity via the Rho GTPase activating protein, p190RhoGap, in order to promote locomotion in the cortical plate (Pacary et al., 2011).

1.6 DIFFERENTIATION OF MIDBRAIN DOPAMINERGIC NEURONS

During migration from the IZ to the MZ, postmitotic precursors continue their differentiation, becoming mature mDA neurons. Early factors controlling mDA neurogenesis, such as *Otx2*, *Lmx1a/b*, *Foxa1/2* and *En1/2*, remain expressed and up-regulate later factors, such as *Nurr1/Nr4a2* and *Pitx3*. Nurr1 is required for mDA neuroblast survival and differentiation into

TH^+ mDA neurons (Zetterström et al., 1997). Accordingly, Nurr1/Nr4a2 has been found to regulate the expression of several genes that define a mature mDA neuron, including *Th*, *Slc18a2/Vmat2* (solute carrier family-18 member-2/vesicular monoamine transporter-2), *Slc6a3/Dat* (solute carrier family-6 member-3/dopamine transporter), *Ddc/Aadc*, and *Cdkn1c* (cyclin-dependent kinase inhibitor 1C) (Gil et al., 2007; Jankovic et al., 2005; Joseph et al., 2003; Saucedo-Cardenas et al., 1998; Smits et al., 2003; Wallen et al., 2001; Volpicelli et al., 2007; Zetterströmetal.,1997). In addition, Pitx3 directly regulates the *Aldh1a1* gene (aldehyde dehydrogenase-1a1), an enzyme in the synthesis of retinoid acid (Jacobs et al., 2007), which in turn regulate *Th, Dlk1* (Delta-like 1) and *D2R* (Jacobs et al., 2011). Moreover, PITX3 cooperates with NURR1 to activate transcription of *Aadc* (aromatic L-amino acid decarboxylase), *D2R, Dat* and *Vmat2* (Hwang et al., 2009; Jacobs et al., 2009). LMX1A/B directly regulates *Nurr1* and *Pitx3* (Chung et al., 2009). Moreover, FOXA1/2 are required for the expression of *Nurr1, En1* and *Aadc* in mDA neuroblasts and neurons, as well as for the expression of *Th* in mDA neurons (Ferri et al., 2007; Metzakopian et al., 2015; Stott et al., 2013).

1.7 SURVIVAL OF MIDBRAIN DOPAMINERGIC NEURONS

Understanding the factors that control the survival of mDA neurons, especially SNc neurons, has been an intense topic of research in the field. Genetic studies have shown transcription factors involved in the terminal differentiation of mDA neurons are also important for neuronal survival. Nurr1 regulates the expression of *Brain-derived neurotrophic factor (Bdnf)* and the glial cell-line derived neurotrophic factor (GDNF) receptor, *c-ret* (Barneda-Zahonero et al., 2012; Wallén A et al., 2001). Moreover, PITX3 has been found to be required for GDNF-induced *Bdnf* expression in SNc mDA neurons (Peng et al., 2011). These neurotrophic factors, BDNF (Barde et al., 1982; Leibrock et al., 1989) and GDNF (Lin et al., 1993) are the two most commonly used factors to maintain the survival and promote the differentiation of mDA neurons in stem cell biology. BDNF is known to promote the survival of fetal mDA neurons in primary cultures and to protect mDA neurons against neurotoxins both *in vitro* (Hyman et al., 1991) and *in vivo* (Frim et al., 1994). Similarly, GDNF was found to promote the survival and differentiation of mDA neurons *in vitro* (Lin et al., 1993) and to prevent their loss in animal models of PD (Beck et al., 1995; Tomac et al., 1995). Multiple other neurotrophic factors are currently known to promote the survival and/or differentiation of mDA neurons and have been suggested as therapeutic tools for PD (for review see Aron and Klein, 2011; Lindholm et al., 2016).

2 RESULTS AND DISCUSSION

2.1 Paper I

In this paper, we investigated whether the Wnt/β-catenin and Wnt/PCP signaling need to be in balance during mDA neuron development. Wnts are a family of secreted proteins that regulate multiple steps of neural development and stem cell differentiation. Two of them, Wnt1 and Wnt5a, activate distinct branches of Wnt signaling and individually regulate different aspects of mDA neuron development. However, several of their functions and interactions remain to be elucidated. Here we analyzed mDA neurogenesis in *Wnt1*$^{-/-}$ single and *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ compound knockout mice and developing an improved protocol for the differentiation of mouse ES cells into mDA neurons.

First, we found that *Wnt1* is required for mDA neurogenesis. Analysis of the *Wnt1*$^{-/-}$ mice revealed that the expression patterns of *Shh* and *Foxa2* were delayed, phenocopying the β-catenin$^{-/-}$ mice (Joksimovic et al., 2009). Moreover, *Lmx1a*, was found to be absent from the FP of Wnt1$^{-/-}$ mice at E11.5. Previous *in vitro* experiments have indicated that β-catenin regulates *Lmx1a* via an auto-regulatory loop (Chung et al., 2009) and that deletion of β-catenin results in ectopic expression of *Lmx1a in vivo* (Tang et al., 2009). Because Wnt1 activates Wnt/β-catenin signaling, our results indicate that it is Wnt1 that regulates the levels and position of Lmx1a *in vivo*. Interesting, *Lmx1a* was ectopically expressed in the BP of Wnt1$^{-/-}$ mice at E11.5, but the expression of other FP and BP markers, such as *Shh* and *Nkx6-1*, was conserved, indicating FP and BP patterning is not altered.

Then we asked whether general neurogenesis is affected in *Wnt1*$^{-/-}$ mice and examined the expression of two critical proneural transcription factors, *Neurog2* and *Mash1*, in the VM of *Wnt1*$^{-/-}$ mice at E11.5. We found that both *Neurog2* and *Mash1* were completely absent in the mFP, but *Neurog2* was expressed in the BP of Wnt1$^{-/-}$ mice at E11.5. In line with this, staining for TUJ1, a postmitotic pan-neuronal maker, and Topro3, a DNA dye to show nuclei, revealed no TUJ^{+}/Topro^{+} cell bodies in the FP, indicating that the FP region of *Wnt1*$^{-/-}$ mice contains no newborn neurons. Moreover, no NURR1^{+} mDA neuroblasts were detected in the FP. In fact, the only cell bodies found in the FP of *Wnt1*$^{-/-}$ mice were those of SOX2^{+} ventricular zone neuroepithelial cells and GLAST^{+} radial glia. Notably, a reduced number of NURR1^{+}/TH^{-} mDA neuroblasts and TH^{+} mDA neurons were found in an ectopic lateral position in the BP. Thus, our results indicate that *Wnt1* is required not only for the expression of *Lmx1a* and proneural genes (*Mash1* and *Ngn2*) in the midbrain FP but also for mDA specification and neurogenesis.

We then examined mDA neurogenesis in *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ compound knockout mice. The phenotype of these mice resembled the combination of the two single phenotypes and showed exacerbated caudal phenotypes, a shortened and truncated midbrain, and a flattening of the FP region. Previous studies have indicated that deletion of *Wnt1* reduces (Prakash et al., 2006) but deletion of *Wnt5a* increases (Andersson et al., 2008) progenitor proliferation in the midbrain FP. Although no difference in phospho-histone 3 (PH3) staining (for cells in M-phase) was

detected in the BP of *Wnt1*$^{-/-}$ or *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice, significant and specific changes were detected in the FP. Indeed, deletion of *Wnt5a* in *Wnt1*$^{-/-}$ mice rescued the loss of PH3^{+} cells in the FP. Moreover, Wnt5a served a stronger anti-proliferative role in the absence of endogenous Wnt1. Thus, our results indicate that *Wnt1* and *Wnt5a* interact in an antagonistic manner to regulate proliferation *in vivo*. Surprisingly, the partial rescue of progenitor proliferation by deletion of *Wnt5a* in *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice didn't lead to an increase of the number of Nurr1^{+} postmitotic precursors, but rather a further decrease in Nurr1^{+} cells from 57% in *Wnt1*$^{-/-}$ mice to 81% in *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice. Moreover, TH^{+} mDA neurons were nearly completely absent in VM of *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice at E12.5. These results indicated that Wnt1 and Wnt5a cooperate in the generation of postmitotic cells as well as in the maturation of mDA precursors.

Analysis of morphogenesis in compound *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mutants showed a *Wnt5a*$^{-/-}$-like phenotype, with a severe broadening of the ventricular cavity and flattening of the VM hinge point, while *Wnt1* single knockout leads to an elongation of the ventricular cavity and of the VM hinge point. Moreover, we found that the A-P distribution of the TH^{+} mDA domain, was further shortened from 55% in *Wnt1*$^{-/-}$ mice, to 85% in *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice. Additionally, TH^{+} cells occupied more lateral and dorsal positions in the *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice than in either of the single mutants. Thus, our results show that an unsuspected cooperation between Wnt1 and Wnt5a regulate VM morphogenesis *in vivo*.

These results indicate the existence of a broad cooperation of the Wnt/β-catenin and Wnt/PCP signaling pathways, which could be mediated by shared signaling components of the two pathways, such as Fz or Dvl. Indeed, we and others have reported previously that signaling components initially assigned to the Wnt/β-catenin pathway, such as the Lrp6 co-receptor, can contribute to convergent extension and Wnt/PCP signaling (Bryja et al., 2009; Tahinci et al., 2007). Thus, multiple mechanisms could account for the interaction between these two pathways.

We next examined whether activation of Wnt/β-catenin and Wnt/PCP signaling could be used to improve protocols for the differentiation of mouse neural and ES cells into mDA neurons. Because there is no commercially available active WNT1 protein (capable of activating the very sensitive Wnt/β-catenin reporter, TOPFLASH), we used WNT3A to activate Wnt/β-catenin signaling. We first examined neural stem cells and administered WNT3A for 3 days, to promote progenitor proliferation and specification, followed by 3 days without WNTs, and finally 3 days with WNT5A, to activate the Wnt/PCP/Rac1 pathway and promote progenitor cell-cycle exit, neurogenesis and NURR1^{+} precursor differentiation into TH^{+} DA neurons. Sequential administration of WNT3A and WNT5A increased the proportion of DA neurons per TUJ1-stained area by 2.5-fold compared with WNT3A, while WNT5A showed a small increase. This protocol was then adapted to mES cell cultures by shortening from 3 to 2 days each of the three steps (Wnt3a/no Wnt/Wnt5a) (Figure 4). These three steps were performed between days 7–13, after the neural-induction with Noggin (days 1–5) and the beginning of Fgf8/Shh-patterning (days 5–7) which continued until day 13. A final standard differentiation step with survival/differentiation factors such as BDNF and GDNF was included to enhance

the maturation of DA neurons on days 13–15/16. As observed with neural stem cells, treatment of mES cells with WNT3A had no effect on the number of TH^+ cells in the cultures. Sequential administration of Wnt3a followed by WNT5A led to a very significant (60%) increase in the percentage of TH^+ cells compared with control or Wnt3a alone. Additionally, sequential WNT3A and WNT5A treatment also increased by 80% the proportion of $TUJ1^+$ neurons that became TH^+. Finally, sequential Wnt treatment increased the proportion of TH^+ cells expressing *Foxa2* by 55%, *Lmx1a* by 29%, *Pitx3* by 31%, and *Nurr1* by 14%. Thus, our results indicate that, by improving our understanding of the basic mechanisms by which Wnts operate during normal midbrain development, it is possible to improve current protocols for the mDA differentiation of neural or ES cells.

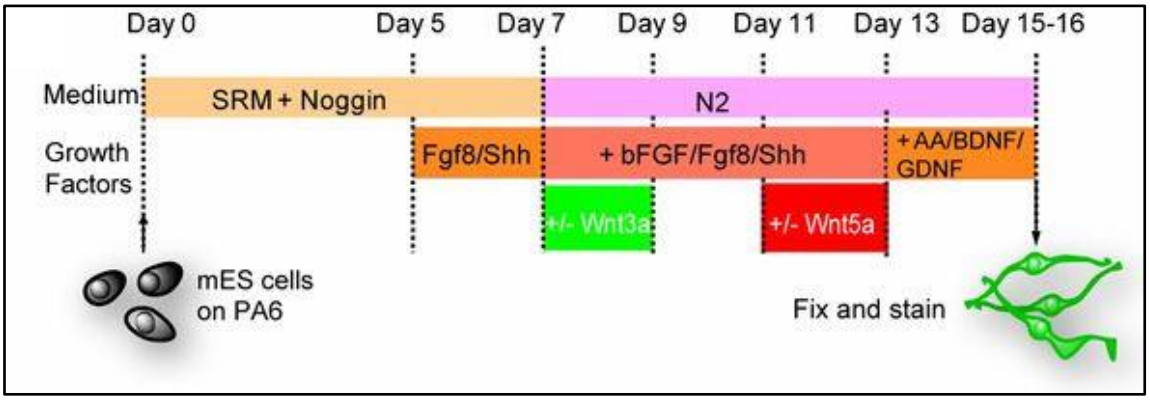

Figure 4. Scheme showing the protocol for mouse ES cells differentiation into dopaminergic neurons by incorporating sequential WNT3A and WNT5A treatment. From paper I (Andersson et al., 2013).

2.2 Paper II

In this paper, we investigated whether and how CXCL12/CXCR4 signaling is involved in migration and neuritogenesis of mDA neurons.

We first examined the expression of the chemokine *Cxcl12* and its receptor *Cxcr4* in developing VM in mice. *In situ* hybridization showed that *Cxcl12* is present in the meninges surrounding the VM during mDA neurogenesis and *Cxcr4* is expressed in the precursor domain, marked by *Nr4a2/Nurr1*. Immunostaining of CXCR4 and NURR1 confirmed that two proteins are co-localized in mDA precursors. The CXCR4 antibody (UMB-2) used in immunostaining is only able to recognize naive CXCR4 which has not been activated by phosphorylation (Sánchez-Alcañiz et al., 2011). Staining of CXCR4 in tissue sections pre-treated with protein phosphatase revealed the presence of additional signal corresponding to phosphorylated CXCR4. Comparison of consecutive sections with or without protein phosphatase treatment, revealed that phosphorylated active CXCR4 is present in both mDA precursors ($NURR1^+TH^-$) and TH^+ mDA neurons. This was also confirmed by immunostaining with antibodies against phosphorylated S324/325 and S338/339 of CXCR4. Notably, the

UMB-2 antibody recognizes non-phosphorylated S346/347 in human CXCR4, which is the main site for functional regulation of CXCR4 as it is required for subsequent phosphorylation of S324 and S338/339 (Mueller et al., 2013). These results revealed that CXCR4 begins its activation in $NURR1^+/TH^-$ precursors of the IZ and is fully activated in most $NURR1^+/TH^+$ mDA neurons of the MZ by E12.5, as they move closer to the meninges.

We then analyzed the function of meningeal CXCL12 on $CXCR4^+$ mDA neurons *in vitro*. First we did midbrain organotypic slice cultures. In slices where meninges were removed, more TH^+ cells were found in ectopic lateral positions in the IZ, compared with slices with intact meninges. The width of bundles containing TH^+ processes increased in slices without meninges. Importantly, CXCR4 immunoreactivity (UMB-2 antibody) was also higher without meninges, indicating less activation of the receptor. Migration assays were also performed to test whether the meninges are capable of promoting the migration of mDA neurons in a CXCR4-dependent way. Meninges were dissected out and when placed next to a piece of VM tissue, they induced the migration of TH^+ cells towards them and promoted neuritogenesis in TH^+ cells. Both effects were blocked by a CXCR4 antagonist AMD3100. These results clearly show that CXCL12/CXCR4 signaling may regulate mDA neuron migration and neuritogenesis.

To confirm these results we examined whether CXCL12/CXCR4 signaling is involved in mDA neuron migration *in vivo* and performed first gain of function experiments. When CXCL12 was overexpressed ectopically, lateral to the FP, TH^+ cells migrated towards *Cxcl12*-expressing GFP^+ cells. In agreement with the hypothesis that $CXCR4^+$ cells migrate along a CXCL12 gradient, when this gradient was disturbed by *Cxcl12* overexpression in the medial floor plate, TH^+ cells were retained in the IZ. Then we performed loss of function experiments and examined the phenotypes of mDA neurons when CXCR4 was either pharmacologically blocked by AMD3100 or genetically deleted in *Cxcr4* knockouts. At E11.5, TH^+ cells in $Cxcr4^{-/-}$ were retained in IZ and their processes were disoriented (not radially oriented as in wild type). The retention of TH^+ cells in the IZ was also found at later stages, in either $Cxcr4^{-/-}$ mice or in mice intraventricularly injected with AMD3100. We also found that the neurites of TH^+ cells reaching the lateral part of the MZ were more oriented towards the meninges in control $Cxcr4^{+/-}$ mice than in $Cxcr4^{-/-}$ embryos, a finding consistent with the fasciculation defects found in organotypic cultures without meninges.

In this paper, we thus identify that the CXCL12/CXCR4 signaling pathway controls mDA migration and neuritogenesis (Figure 5). CXCL12/CXCR4 has been shown to regulate the migration of multiple types of immature neurons and serve different purposes in different brain regions (see introduction). For instance, while meningeal CXCL12 serves as an anchoring factor to retain proliferating cerebellar precursors in the external granule layer (Reiss et al., 2002; Zhu et al., 2002) and in the dentate gyrus of hippocampus (Li et al., 2009), it works as chemoattractant to stimulate the migration of hem-derived Cajal-Retzius cells in the cerebral cortex (Borrell and Marín, 2006). In the developing midbrain, it has been known mDA neuroblasts migrate first ventrally and then tangentially towards their lateral destination

(Hanaway et al., 1971; Kawano et al., 1995). However little was known about the molecular players in the process, especially for radial migration. Here, we show that CXCL12/CXCR4 plays an important role in radial migration. In addition we found that that CXCL12/CXCR4 regulates neurite growth, a finding that is supported by previous studies showing that migration cues also regulate axon pathfinding (Marín et al., 2010; Ward and Rao, 2005). Finally, from an applicative point of view, we speculate that CXCL12 may be used in cell replacement therapies for PD, to improve neuroblast migration out of the graft and dopaminergic innervation of the host striatum, both of which may contribute to better functional integration of transplanted mDA neurons.

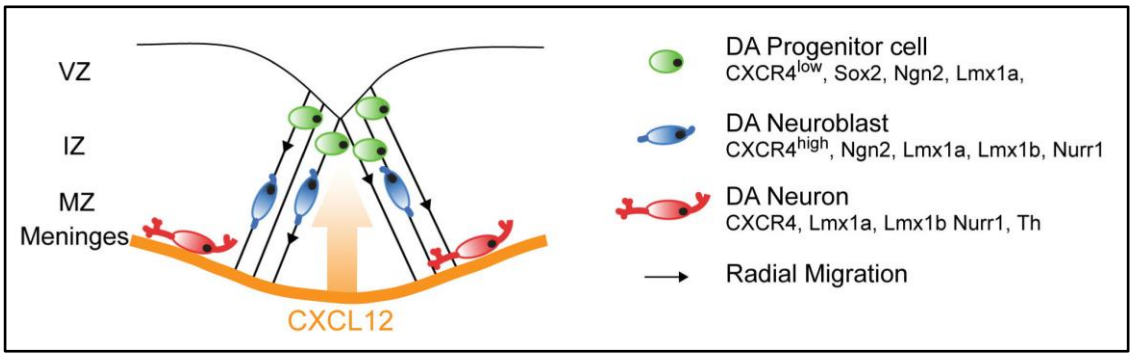

Figure 5. Migration of postmitotic DA neurons. Postmitotic DA neuroblasts (blue) migrate radially along the radial glia processes, from the IZ (intermediate zone) to MZ (marginal zone), following the gradient of CXCL12 secreted by the meninges (orange). VZ, ventricular zone. Modified from paper II (Yang et al., 2013).

2.3 Paper III

In this paper, we investigated how the homeodomain transcription factor, ZEB2, regulates mDA neurogenesis and migration.

First, we examined the expression of *Zeb2* in the VM during development. *In situ* hybridization showed that *Zeb2* is expressed in the VZ of the FP and BP, as well as in the IZ. Immunofluorescence confirmed the presence of ZEB2 in $SOX2^{+}$ VZ cells. The expression levels of ZEB2 in the IZ were much lower lever than in the VZ, and they were complementary to the levels of NR4A2/CXCR4, suggesting that ZEB2 is being downregulated in postmitotic mDA neuroblasts while they start migrating.

We then set out to investigate what are the target genes of ZEB2, and examined the DNA sequences bound by this transcription factor. For this purpose, we took the advantage of the ChiP-seq dataset from the ENCODE project. Analysis of the transcription factor binding-site motifs showed that ZEB2 and ZEB1 bind nearly identical sequences. Accordingly, these two proteins are known to serve similar functions (Bracken et al., 2008; Cong et al., 2013; Ellis et al., 2010; Park et al., 2008). ZEB transcription factors were found to interact with the promoter

of genes known to regulate mDA development, such as *NEUROG2*, *NR4A2*, *CXCR4* and *PITX3*. Moreover, overexpression of *Zeb2* in the substantia nigra dopaminergic cell line, SN4741, led to a down-regulation of *Cxcr4* mRNA, indicating that ZEB2 works as a transcription repressor.

To examine the function of ZEB2 *in vivo*, we overexpressed Zeb2 in the midbrain FP by *in utero* electroporation. We found that CXCR4 and NR4A2 (NURR1) immunoreactivity was lower in *Zeb2* overexpressing cells compared with *Gfp* cells, indicating that ZEB2 represses the expression of these two ZEB2 target genes. As expected, down-regulation of CXCR4 in migratory mDA precursors reduced their migration and impaired their multipolar-to-bipolar transition, which is required for migration.

Zeb2 has also been reported to represses the expression of *miR-200* family members in cancer cells (Bracken et al., 2008). The *miR-200* family is present in the VZ of the developing midbrain (Peng et al., 2012). We found that primary *miR-200*c (pri-miR-200c) is expressed in the VZ, as assessed by ISH, confirming the previous report. Moreover, we found that *pri-miR-200c* is also expressed in the IZ of the FP, in $CXCR4^+$ cells where ZEB2 immunoreactivity is much lower than that in the VZ. Interestingly, overexpression of ZEB2 up-regulated *pri-miR-200c* at E12.5, 1 day after electroporation; and conversely, overexpression of *pMiR-200c* down-regulated ZEB2 immunoreactivity in the FP *in vivo*. These results suggested a negative feedback loop formed by ZEB2 and miR-200c in the VM.

Finally we examined the function of ZEB2-miR-200c loop in mDA neuron development by overexpressing a *miR-200* sponge vector (*pCAG-Sponge*) to inhibit members of the miR-200 family (Peng et al., 2012). We found that ZEB2 immunoreactivity was elevated when *miR-200s* were knocked-down by overexpression of *pCAG-Sponge* in primary VM cell cultures. Overexpression of *pCAG-Sponge* resulted in the reduced migration and less $NR4A2^+/GFP^+$ out of the total GFP^+ cells. These results indicated that a ZEB2-miR-200c loop controls the neuronal migration and differentiation of mDA neurons.

In this study we thus describe a new regulatory loop involving the transcription factor ZEB2 and the microRNA miR-200c in developing VM that controls the differentiation and migration of mDA neurons. The unilateral negative feedback nature of the loop indicates that the level of ZEB2 protein is critical for its function during FP development. These two factors, ZEB2 and miR-200c, seem to reach a balance as indicated by their co-expression in progenitor cells in VZ, while in the IZ, ZEB2 protein diminished in spite of the presence of both *Zeb2* mRNA, indicating an ongoing inhibition by miR-200c. We thus suggest that ZEB2 may function to prevent premature mDA differentiation by repressing genes, such as *Neurog2*, *Nr4a2*, *Cxcr4* and *Pitx3* in progenitors. Interestingly, we show here that ZEB2 promotes the expression of *miR-200c*, which is not the case in other cell types (Bracken et al., 2008; Park et al., 2008), reflecting the fact that ZEB2 can act as a transcriptional repressor or activator, depending on co-factor recruitment (Sánchez-Tilló et al., 2011). Notably, members of the miR-200 family can target a large number of proteins, two of which, SOX2 and E2F3 have been shown to regulate cell cycle exit in midbrain progenitors during neurogenesis (Peng et al., 2012). We

thus conclude that a complex regulatory network involving microRNAs of the miR-200 family and ZEB2 regulates not only mDA migration, but also neurogenesis.

2.4 Paper IV

In this paper, we investigated the impact of the extracellular matrix (ECM) on mDA neuron development in mouse embryos and in human long-term neuroepithelial stem (lt-NES) cells in culture.

We first examined the composition of the ECM in the midbrain FP by RNA-sequencing. We focused on laminins, which are trimeric proteins consisting of α, β and γ subchains (Lama, Lamb and Lamc, respectively). Analysis of the mouse VM tissue revealed that Laminin subchains *Lama1*, *4* and *5*, *Lamb1* and *2*, and *Lamc1* are abundant in the VM at E12.5. Interestingly, *Lama*5 was differentially expressed compared to the dorsal midbrain or ventral diencephalon or hindbrain. LAMA5 was detected in VM by immunofluorescence at E10.5 and E12.5 and was found to increase from low levels in the VZ to high levels in the MZ.

Our sequencing data suggested that six possible trimeric laminins can be formed in the VM. Five of them (LM111, LM411, LM421, LM511, LM521) are currently available as protein and were used to test their functionality in primary mDA cultures. LM511 increased the percentage of $LMX1A^+$ and TH^+ cells, while LM211 and LM332 decreased that compared to LM111. Notably, LM521, which contains only a different β chain compared with LM511 (β2 instead of β1), did not support mDA cells as LM511 did, indicating a highly specific role of the β1 laminin chain. Indeed, LM511 reduced cell death in primary mDA neurons with or without oxidative stress challenge (induced by 6-hydroxydopamine). We also found that LM511 represses the level of phosphatase and tensin homolog (PTEN) in the substantia nigra dopaminergic cell line, SN4741. PTEN, which is present in the developing VM, has been previously shown to antagonize PI3K activity and promote mDA neuron death (Stiles et al., 2004). Moreover, we also found that LM511, but no other laminin, was able to upregulate YAP signaling in mDA neurons. YAP is a key transcriptional co-activator involved in the transduction of extracellular mechanical information (Dupont et al., 2011) as well as proliferation, differentiation and survival (Pan, 2010), but has not been linked to mDA neuron development.

In order to dissect out the pathway by which LM511 may regulate YAP and PTEN, we first looked for the receptor mediating LM511 signaling. We thus performed a screen with blocking antibodies against the laminin receptors, integrins, and used YAP target genes *CTGF* and *ANKRD1* as a readout. We found that Integrin α3 (ITGA3) blocking antibodies partially decreased the transcription of YAP target genes. Moreover, knock-down of Integrin α3, but not Integrin α6 (another receptor for LM511) (Hynes, 2002), induced YAP phosphorylation and upregulated PTEN levels. Interestingly, RNA-seq analysis showed *Itga3* and *Itgb1* are the two most abundant integrin transcripts in developing VM. Since Integrin α3β1 (ITGA3B1) is

the only α3 containing integrin among the 24 integrin heterodimers (Hynes, 2002), we conclude that ITGA3B1 mediates LM511-YAP signaling in mDA neurons.

Immunostaining revealed that YAP is expressed in TH^+ cells in the midbrain FP. We then set out to examine the function of YAP in mDA neurons. First, *Yap* overexpression was found to downregulate PTEN levels and knockdown of YAP increased PTEN levels in SN4741 cells. As expected from the pro-apoptotic role of PTEN, we found that *Yap* knockdown increased and overexpression reduced cell death and active Caspase 3 immunoreactivity in primary mDA neurons treated with the neurotoxin 6-hydroxydopamine, compared to shRNA control. The importance of YAP in mDA survival was confirmed *in vivo* by *in utero* electroporation of *Yap-shRNA* in the midbrain FP, where more active Caspase 3^+ cells were found compared with control. These results strongly indicate that YAP is required for mDA neuron survival *in vitro* and *in vivo*. We next sought to identify the factors mediating the effects of YAP and the inhibition of PTEN. Analysis of microRNA binding sites in the regulatory regions of *Pten* revealed several potential candidates. Of them, miR-130a and miR-107 were enriched in the midbrain tissue, but only miR-130a was up-regulated by LM511. Moreover, overexpression or knockdown of *Yap* respectively increased or decreased miR130a expression in SN4741 cells. Furthermore, LM511 induced the expression of miR130a, an effect that was partially blocked by *Yap* knockdown. Finally, overexpression of miR130a in the midbrain FP at E13.5, by *in utero* electroporation, knocked-down PTEN. Thus, our data show that LM511 up-regulates miR-130a via YAP, which in turn reduces the levels of PTEN in dopaminergic cells.

In addition to the pro-survival role of YAP in mDA neurons, we also examined whether YAP promotes mDA neuron differentiation. We then overexpressed Yap in human lt-NES cells (Falk et al., 2012; Koch et al., 2009), which were subsequently differentiated towards mDA neurons. A set of mDA differentiation specific genes, such as *LMX1A*, *LMX1B* and *PITX3*, were up-regulated by *Yap* overexpression, compared with *Gfp* overexpression. Moreover, immunostaining revealed that LMX1A, LMX1B, PITX3, Aldehyde dehydrogenase1A1 (ALDH1A1) and dopamine transporter (SLC6A3) proteins were increased in TH^+ neurons derived from Yap-lt-NES cells compared to Gfp-lt-NES cells. Finally, overexpression of *YAP* in the developing VM also induced ectopic expression of PITX3, without affecting proliferation, suggesting an additional pro-differentiation effect of YAP.

In this study we identify a novel pathway activated by LM511 in the ECM that controls mDA neuron survival and differentiation (Figure 6). Previous studies have identified that G protein-coupled receptors (Yu et al., 2012; Zhou et al., 2014) and integrins (Elbediwy et al., 2016; Martin et al., 2016; Wong et al., 2016) transduce signals from the ECM to the cells via YAP signaling. However, the molecular identity of the ECM signals remained unknown. Our study identifies LM511 as the first molecular entity in the ECM capable of activating YAP. Notably, YAP signaling is low in the brain because of the low stiffness of the tissue and the abundance of cell-to-cell contacts (Dupont et al., 2011; Varelas et al., 2012; Zhao et al., 2007). Our results suggest that the presence of LM511 in restricted sites of the ECM, such as the VM FP, may thus provide a mechanism to active YAP and regulate the survival and differentiation of

specific cell types such as mDA neurons. These novel functions of YAP, combined with its canonical role in transducing ECM stiffness (Dupont et al., 2011), indicate that the LM511-YAP pathway may serve as an integrator of diverse ECM properties and as a coordinator of cellular responses to niche-derived signals.

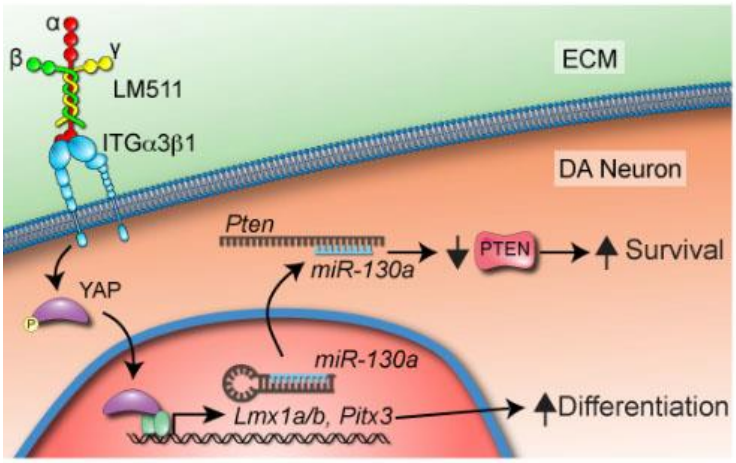

Figure 6. Schematic figure showing that the LM511-ITGA3B1-YAP axis enhances mDA neuron survival via suppression of PTEN by *miR-130a* and promotes mDA neuron differentiation via up-regulation of mDA neuron transcription factors such as *Lmx1a/b* and *Pitx3*. From paper IV (manuscript).

Multiple questions arise from these results, such as: How is the LM511 signal transduced from ITGA3B1 to YAP? Or what is the identity of the transcription factors interacting with YAP and contributing to provide cell type specificity in mDA neurons? Or what are the target genes of YAP in mDA neurons other than the ones identified here?

From an applicative point of view, LM511 may be used to improve *in vitro* differentiation of mDA neurons derived from various types of stem cells. In the future it will be also interesting to investigate whether and how the ECM plays role in the pathogenesis of Parkinson's disease, since some evidence indicates that ECM degradation and alteration of ECM enzymes takes place in PD (Lorenzl et al., 2002; Yang et al., 2015).

3 CONCLUSIONS

In this thesis, I present four projects, covering diverse aspects of midbrain development such as the specification, neurogenesis, migration, differentiation and survival of mDA neurons.

The main conclusions of our studies are the following:

1 *Wnt1* and *Wnt5a* genetically interact and cooperate to control multiple aspects of mDA neuron development.
2 Sequential application of Wnt3a and Wnt5a improves the mDA neuron differentiation of mouse ES Cells .
3 CXCL12/CXCR4 regulates the radial migration and neuritogenesis of mDA neurons during development both *in vitro* and *in vivo*.
4 ZEB2 activates the expression of *miR-200c*, which in turn represses *Zeb2* expression, forming a unilateral negative feedback loop in VM floor-plate progenitors.
5 ZEB2 represses the expression of *Cxcr4* and *Nr4a2 (Nurr1)*, to prevent premature migration and differentiation of mDA progenitors.
6 LM511 activate YAP pathway throug Intergrin $\alpha 3\beta 1$ in mDA neurons.
7 LM511-YAP promotes the differentiation of mDA neurons.
8 LM511-YAP induces miR-130a, which represses PTEN, resulting in increased survival of mDA neurons.

The incorporation of the knowledge from developmental biology to regenerative medicine, particularly to improve the differentiation of pluripotent cells into mDA neurons, has been proven to be a successful strategy and I think it can further contribute in the future. The results we obtained from the four projects in my thesis have expanded our understating of mDA specification, neurogenesis, migration, differentiation and survival. My hope is that these studies will also contribute to the future development of novel therapeutic strategies for PD, such as cell replacement or neuroprotective therapies.

4 ACKNOWLEDGEMENTS

First, I would like to thank my Supervisors: Ernest Arenas, Carlos Villaescusa and Katarina Le Blanc.

I would like to thank Ernest for giving me the opportunity to explore developmental biology as a PhD student. Thank you for sharing your knowledge, enthusiasm and always seeing the bright side of things and fit them in a big picture. Thanks for your trust, support and allowing me to freely explore new ideas. I am especially grateful for your help with the thesis until the very last moment.

I would like to thank you, Carlos, for all your help and discussions about my projects, for your knowledge and high standard for science, and for your advices for my future career.

External Collaborators

I would like to thank Juan Antonio Sánchez-Alcañiz, Nicolas Fritz, Changgeng Peng, Per Uhlén and Oscar Marín for their contribution to the articles. It has been a pleasure to work with all of you.

Internal Collaborators

I would like to thank former Arenas lab members: Emma Andersson, Linda Edman, Spyros Theofilopoulos, Diogo Ribeiro, Lukas Cajanek, and Vitezslav Bryja, as well as to current lab members Carmen, Dawei, Enrique and Daniel for their help, discussions and contribution. It has been great to work and learn from all of you!

Opponent and examination board

I would like to thank Dr. Diogo S. Castro and members of examination board Drs. Finn Hallböök, Ana Teixeira and Lars Olson for accepting the invitation and for their participation in my PhD thesis defense.

Past and present members of Arenas lab

I would like to thank all present and past lab members: Carmen, Enrique, Dawei, Alca, Linda, Daniel, Karol, Kaneyasu, Chika, Pia, Geeta, Mark, Fabia, Willy, Catarina, Isabel, Kim and Linda Adlerz, for all the help, reagents and discussions; Lottie for your help in ordering and tips for living in Sweden.

MolNeuro

I would like to thank Patrik Ernfors, Per Uhlén, Sten Linnarsson, Gonçalo Castelo-Branco, Jens Hjerling-Leffler and Ulrika Marklund for making MolNeuro an excellent and unique place for doing science; Alessandra for all your help, especially the one with accommodation even before I came here; Johnny for always keeping the lab running smoothly.

I would like to thank all the past and present members at MolNeuro who I got the opportunity to work with, for your effort to make the division such a vibrant environment. Especially I would like to thank: Songbai for your help since the first day I came here and for your expertise and dedication; Changgeng for your advices and vision in science; Satish for your suggestions, advices, all the talks and collaboration; Shigeaki for your expertise and many helps; Amit for your advice for Postdoc and for your hard working attitude and productivity; Erik for your help with calcium imaging and I hope something will come out; Ana M. and Carolina for help with in situ hybridization; Sueli for help with MAC sorting; Alessandro for your help with antibodies, your excellent PhD work and your advice for Postdocs; Boris for your optimistic attitude; Hind for being officemates long time ago; Per, Göran and Connla for running the CLICK facility; David for teaching me how to make sandwiches in a professional way; Gioele, Lars and Hannah for scientific lunch talks; Ana F. for discussion on the idea that ideas are cheap; Ivar for your interest in Eastern culture; Lili, Daohua, Yiwen, Dawei, Jianren, Yiqiao, Mingdong, Xiuzhe, Xiangyu, Zhengwen and Xiao Fei for creating a Chinese community here; Carmen for proofreading of the thesis. I would like to thank all the people that I have met over last years for your patience with my English.

I would like to thank my previous supervisor Prof. Feng-yan Sun for supporting me to conduct PhD study at KI and for your training me in neuroscience, for your knowledge and expertise.

Last but not least, I would like to thank my parents for your endless support; my wife Jie and my lovely son Yichen, for your support and love.

5 REFERENCES

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J. (2006a). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* **124**, 393–405.

Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F. and Björklund, a (2006b). Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development* **133**, 507–16.

Andersson, E. R., Prakash, N., Cajanek, L., Minina, E., Bryja, V., Bryjova, L., Yamaguchi, T. P., Hall, A. C., Wurst, W. and Arenas, E. (2008). Wnt5a regulates ventral midbrain morphogenesis and the development of A9-A10 dopaminergic cells in vivo. *PLoS One* **3**, e3517.

Andersson, E. R., Saltó, C., Villaescusa, J. C., Cajanek, L., Yang, S., Bryjova, L., Nagy, I. I., Vainio, S. J., Ramirez, C., Bryja, V., et al. (2013). Wnt5a cooperates with canonical Wnts to generate midbrain dopaminergic neurons in vivo and in stem cells. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E602-10.

Ang, S. L., Wierda, a, Wong, D., Stevens, K. a, Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development* **119**, 1301–1315.

Ang, S. L., Rossant, J., Ang, S. L., Rossant, J., Ang, S.-L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J., et al. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78**, 561–74.

Arenas, E. (2002). Stem cells in the treatment of Parkinson's disease. *Brain Res. Bull.* **57**, 795–808.

Arenas, E. (2010). Towards stem cell replacement therapies for Parkinson's disease. *Biochem. Biophys. Res. Commun.* **396**, 152–6.

Arenas, E. (2014). Wnt signaling in midbrain dopaminergic neuron development and regenerative medicine for Parkinson's disease. *J. Mol. Cell Biol.* **6**, 42–53.

Arenas, E., Denham, M. and Villaescusa, J. C. (2015). How to make a midbrain dopaminergic neuron. *Development* **142**, 1918–36.

Aron, L. and Klein, R. (2011). Repairing the parkinsonian brain with neurotrophic factors. *Trends Neurosci.* **34**, 88–100.

Balabanian, K., Lagane, B., Infantino, S., Chow, K. Y. C., Harriague, J., Moepps, B., Arenzana-Seisdedos, F., Thelen, M. and Bachelerie, F. (2005). The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J. Biol. Chem.* **280**, 35760–6.

Barde, Y. A., Edgar, D. and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1**, 549–53.

Barker, R. A., Barrett, J., Mason, S. L., Björklund, A., Thompson, W., Björklund, A., Stenevi, U., Björklund, A., Dunnett, S., Stenevi, U., et al. (2013). Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. *Lancet. Neurol.* **12**, 84–91.

Barker, R. A., Drouin-Ouellet, J. and Parmar, M. (2015). Cell-based therapies for Parkinson disease—past insights and future potential. *Nat. Rev. Neurol.* **11**, 492–503.

Barneda-Zahonero, B., Servitja, J. M., Badiola, N., Miñano-Molina, A. J., Fadó, R., Saura, C. A. and Rodríguez-Alvarez, J. (2012). Nurr1 protein is required for N-Methyl-D-aspartic Acid (NMDA) receptor-mediated neuronal survival. *J. Biol. Chem.* **287**, 11351–11362.

Bartel, D. P. and Chen, C.-Z. (2004). Opinion: Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat. Rev. Genet.* **5**, 396–400.

Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A. and Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* **373**, 339–341.

Beffert, U. (2004). Reelin and Cyclin-Dependent Kinase 5-Dependent Signals Cooperate in Regulating Neuronal Migration and Synaptic Transmission. *J. Neurosci.* **24**, 1897–1906.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517–30.

Björklund, A. and Dunnett, S. B. (2007). Dopamine neuron systems in the brain: an update. *Trends Neurosci.* **30**, 194–202.

Bodea, G. O., Spille, J., Abe, P., Andersson, A. S., Acker-Palmer, A., Stumm, R., Kubitscheck, U. and Blaess, S. (2014). Reelin and CXCL12 regulate distinct migratory behaviors during the development of the dopaminergic system. *Development* **141**, 661–73.

Boitard, M., Bocchi, R., Egervari, K., Petrenko, V., Viale, B., Gremaud, S., Zgraggen, E., Salmon, P. and Kiss, J. Z. (2015). Wnt Signaling Regulates Multipolar-to-Bipolar Transition of Migrating Neurons in the Cerebral Cortex. *Cell Rep.* **10**, 1349–1361.

Bonifati, V. (2014). Genetics of Parkinson's disease â€“ state of the art, 2013. *Park. Realt. Disord.* **20**, S23–S28.

Borrell, V. and Marín, O. (2006). Meninges control tangential migration of hem-derived Cajal-Retzius cells via CXCL12/CXCR4 signaling. *Nat. Neurosci.* **9**, 1284–93.

Bracken, C. P., Gregory, P. A., Kolesnikoff, N., Bert, A. G., Wang, J., Shannon, M. F. and Goodall, G. J. (2008). A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res.* **68**, 7846–7854.

Bradley, A., Evans, M., Kaufman, M. H. and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255–6.

Broccoli, V., Boncinelli, E. and Wurst, W. (1999). The caudal limit of Otx2 expression positions the isthmic organizer. *Nature* **401**, 164–168.

Bryja, V., Andersson, E. R., Schambony, A., Esner, M., Bryjová, L., Biris, K. K., Hall, A. C., Kraft, B., Cajanek, L., Yamaguchi, T. P., et al. (2009). The extracellular domain of Lrp5/6 inhibits noncanonical Wnt signaling in vivo. *Mol. Biol. Cell* **20**, 924–36.

Busillo, J. M. and Benovic, J. L. (2007). Regulation of CXCR4 signaling. *Biochim.*

Biophys. Acta **1768**, 952–63.

Čajánek, L., Ganji, R. S., Henriques-Oliveira, C., Theofilopoulos, S., Koník, P., Bryja, V. and Arenas, E. (2013). Tiam1 regulates the Wnt/Dvl/Rac1 signaling pathway and the differentiation of midbrain dopaminergic neurons. *Mol. Cell. Biol.* **33**, 59–70.

Chung, S., Leung, A., Han, B. S., Chang, M. Y., Moon, J. Il, Kim, C. H., Hong, S., Pruszak, J., Isacson, O. and Kim, K. S. (2009). Wnt1-lmx1a Forms a Novel Autoregulatory Loop and Controls Midbrain Dopaminergic Differentiation Synergistically with the SHH-FoxA2 Pathway. *Cell Stem Cell* **5**, 646–658.

Cong, N., Du, P., Zhang, A., Shen, F., Su, J., Pu, P., Wang, T., Zjang, J., Kang, C. and Zhang, Q. (2013). Downregulated microRNA-200a promotes EMT and tumor growth through the Wnt/??-catenin pathway by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma. *Oncol. Rep.* **29**, 1579–1587.

Cooper, J. A. (2013). Mechanisms of cell migration in the nervous system. *J. Cell Biol.* **202**, 725–734.

Davis, G. M., Haas, M. A. and Pocock, R. (2015). MicroRNAs: Not "Fine-Tuners" but key regulators of neuronal development and function. *Front. Neurol.* **6**, 1–12.

Demyanenko, G. P., Shibata, Y. and Maness, P. F. (2001). Altered distribution of dopaminergic neurons in the brain of L1 null mice. *Brain Res. Dev. Brain Res.* **126**, 21–30.

Deng, Q., Andersson, E., Hedlund, E., Alekseenko, Z., Coppola, E., Panman, L., Millonig, J. H., Brunet, J.-F., Ericson, J. and Perlmann, T. (2011). Specific and integrated roles of Lmx1a, Lmx1b and Phox2a in ventral midbrain development. *Development* **138**, 3399–408.

Dent, E. W., Gupton, S. L. and Gertler, F. B. (2011). The Growth Cone Cytoskeleton in Axon outgrowth and guidance.pdf. *Cold Spring Harb. Perspect. Biol.*

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S., et al. (2011). Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179–183.

Dworkin, S. and Jane, S. M. (2013). Novel mechanisms that pattern and shape the midbrain-hindbrain boundary. *Cell. Mol. Life Sci.* **70**, 3365–3374.

Ebert, M. S. and Sharp, P. A. (2012). Roles for MicroRNAs in conferring robustness to biological processes. *Cell* **149**, 505–524.

Elbediwy, A., Vincent-Mistiaen, Z. I., Spencer-Dene, B., Stone, R. K., Boeing, S., Wculek, S. K., Cordero, J., Tan, E. H., Ridgway, R., Brunton, V. G., et al. (2016). Integrin signalling regulates YAP and TAZ to control skin homeostasis. *Development* **143**,.

Ellis, A. L., Wang, Z., Yu, X. and Mertz, J. E. (2010). Either ZEB1 or ZEB2/SIP1 can play a central role in regulating the Epstein-Barr virus latent-lytic switch in a cell-type-specific manner. *J. Virol.* **84**, 6139–52.

Eriksen, J. L., Wszolek, Z. and Petrucelli, L. (2005). Molecular pathogenesis of Parkinson disease. *Arch. Neurol.* **62**, 353–7.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.

Evsyukova, I., Plestant, C. and Anton, E. S. (2013). Integrative mechanisms of oriented neuronal migration in the developing brain. *Annu. Rev. Cell Dev. Biol.* **29**, 299–353.

Falk, A., Koch, P., Kesavan, J., Takashima, Y., Ladewig, J., Alexander, M., Wiskow, O., Tailor, J., Trotter, M., Pollard, S., et al. (2012). Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. *PLoS One* **7**, e29597.

Ferri, A. L. M., Lin, W., Mavromatakis, Y. E., Wang, J. C., Sasaki, H., Whitsett, J. a and Ang, S.-L. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. *Development* **134**, 2761–9.

Findley, L. J. (2007). The economic impact of Parkinson's disease. *Park. Relat. Disord.* **13**, 8–12.

Frim, D. M., Uhler, T. a, Galpern, W. R., Beal, M. F., Breakefield, X. O. and Isacson, O. (1994). Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5104–5108.

Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M. A., Andre, P., Robinson, J., Sood, R., Minami, Y., et al. (2011). Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. *Dev. Cell* **20**, 163–76.

Ge, W., He, F., Kim, K. J., Blanchi, B., Coskun, V., Nguyen, L., Wu, X., Zhao, J., Heng, J. I.-T., Martinowich, K., et al. (2006). Coupling of cell migration with neurogenesis by proneural bHLH factors. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1319–1324.

González-Billault, C., Del Río, J. A., Ureña, J. M., Jiménez-Mateos, E. M., Barallobre, M. J., Pascual, M., Pujadas, L., Simó, S., La Torre, A., Gavin, R., et al. (2005). A role of MAP1B in reelin-dependent neuronal migration. *Cereb. Cortex* **15**, 1134–1145.

Grün, D., Wang, Y.-L., Langenberger, D., Gunsalus, K. C. and Rajewsky, N. (2005). microRNA target predictions across seven Drosophila species and comparison to mammalian targets. *PLoS Comput. Biol.* **1**, e13.

Hanaway, J., McConnell, J. A. and Netsky, M. G. (1971). Histogenesis of the substantia nigra, ventral tegmental area of Tsai and interpeduncular nucleus: an autoradiographic study of the mesencephalon in the rat. *J. Comp. Neurol.* **142**, 59–73.

Heng, J. I.-T., Nguyen, L., Castro, D. S., Zimmer, C., Wildner, H., Armant, O., Skowronska-Krawczyk, D., Bedogni, F., Matter, J.-M., Hevner, R., et al. (2008). Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. *Nature* **455**, 114–118.

Hickey, P. and Stacy, M. (2016). Deep brain stimulation: A paradigm shifting approach to treat Parkinson's disease. *Front. Neurosci.* **10**, 1–11.

Hiesberger, T., Trommsdorff, M., Howell, B. W., Goffinet, A., Mumby, M. C., Cooper, J. A. and Herz, J. (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of Disabled-1 and modulates tau

phosphorylation. *Neuron* **24**, 481–489.

Huang, T., Xie, Z., Wang, J., Li, M., Jing, N. and Li, L. (2011). Nuclear factor of activated T cells (NFAT) proteins repress canonical Wnt signaling via its interaction with dishevelled (Dvl) protein and participate in regulating neural progenitor cell proliferation and differentiation. *J. Biol. Chem.* **286**, 37399–37405.

Hwang, D.-Y., Hong, S., Jeong, J.-W., Choi, S., Kim, H., Kim, J. and Kim, K.-S. (2009). Vesicular monoamine transporter 2 and dopamine transporter are molecular targets of Pitx3 in the ventral midbrain dopamine neurons. *J. Neurochem.* **111**, 1202–12.

Hyman, C., Hofer, M., Barde, Y.-A., Juhasz, M., Yancopoulos, G. D., Squinto, S. P. and Lindsay, R. M. (1991). BDNF is a neurotrophic factor of dopaminergic neurons of the substantia nigra. *Nature* **350**, 230–232.

Hynes, R. O. (2002). Integrins: Bidirectional, allosteric signaling machines. *Cell* **110**, 673–687.

Inestrosa, N. C. and Arenas, E. (2010). Emerging roles of Wnts in the adult nervous system. *Nat. Rev. Neurosci.* **11**, 77–86.

Itoh, Y., Moriyama, Y., Hasegawa, T., Endo, T. a, Toyoda, T. and Gotoh, Y. (2013). Scratch regulates neuronal migration onset via an epithelial-mesenchymal transition-like mechanism. *Nat. Neurosci.* **16**, 416–25.

Jacobs, F. M. J., Smits, S. M., Noorlander, C. W., von Oerthel, L., van der Linden, A. J. a, Burbach, J. P. H. and Smidt, M. P. (2007). Retinoic acid counteracts developmental defects in the substantia nigra caused by Pitx3 deficiency. *Development* **134**, 2673–2684.

Jacobs, F. M. J., van Erp, S., van der Linden, A. J. a, von Oerthel, L., Burbach, J. P. H. and Smidt, M. P. (2009). Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. *Development* **136**, 531–540.

Jacobs, F. M. J., Veenvliet, J. V, Almirza, W. H., Hoekstra, E. J., von Oerthel, L., van der Linden, A. J. a, Neijts, R., Koerkamp, M. G., van Leenen, D., Holstege, F. C. P., et al. (2011). Retinoic acid-dependent and -independent gene-regulatory pathways of Pitx3 in meso-diencephalic dopaminergic neurons. *Development* **138**, 5213–22.

Joksimovic, M., Yun, B. a, Kittappa, R., Anderegg, A. M., Chang, W. W., Taketo, M. M., McKay, R. D. G. and Awatramani, R. B. (2009). Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. *Nat. Neurosci.* **12**, 125–31.

Joyner, A. L., Liu, A. and Millet, S. (2000). Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr. Opin. Cell Biol.* **12**, 736–741.

Kalia, L. V. and Lang, A. E. (2015). Parkinson's disease. *Lancet* **386**, 896–912.

Kang, W.-Y., Kim, S.-S., Cho, S.-K., Kim, S., Suh-Kim, H. and Lee, Y.-D. (2010). Migratory defect of mesencephalic dopaminergic neurons in developing reeler mice. *Anat. Cell Biol.* **43**, 241–51.

Katsuyama, Y. and Terashima, T. (2009). Developmental anatomy of reeler mutant mouse. *Dev. Growth Differ.* **51**, 271–286.

Kawano, H., Ohyama, K., Kawamura, K. and Nagatsu, I. (1995). Migration of dopaminergic neurons in the embryonic mesencephalon of mice. *Brain Res. Dev. Brain Res.* **86**, 101–13.

Kele, J., Simplicio, N., Ferri, A. L. M., Mira, H., Guillemot, F., Arenas, E. and Ang, S.-L. (2006a). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495–505.

Kele, J., Simplicio, N., Ferri, A. L. M., Mira, H., Guillemot, F., Arenas, E. and Ang, S.-L. (2006b). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495–505.

Keller, G. M. (1995). In vitro differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* **7**, 862–9.

Keller, G. (2005). Embryonic stem cell differentiation : emergence of a new era in biology and medicine. 1129–1155.

Kim, J., Inoue, K., Ishii, J., Vanti, W. B., Voronov, S. V, Murchison, E., Hannon, G. and Abeliovich, A. (2007). A MicroRNA feedback circuit in midbrain dopamine neurons. *Science* **317**, 1220–4.

Kirkeby, A., Grealish, S., Wolf, D. a., Nelander, J., Wood, J., Lundblad, M., Lindvall, O. and Parmar, M. (2012). Generation of Regionally Specified Neural Progenitors and Functional Neurons from Human Embryonic Stem Cells under Defined Conditions. *Cell Rep.* **1**, 703–714.

Klein, C. and Westenberger, A. (2012). Genetics of Parkinson ' s Disease.

Knaut, H., Blader, P., Strähle, U. and Schier, A. F. (2005). Assembly of trigeminal sensory ganglia by chemokine signaling. *Neuron* **47**, 653–66.

Koch, P., Opitz, T., Steinbeck, J. a, Ladewig, J. and Brüstle, O. (2009). A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 3225–30.

Kordower, J. H., Freeman, T. B., Snow, B. J., Vingerhoets, F. J., Mufson, E. J., Sanberg, P. R., Hauser, R. a, Smith, D. a, Nauert, G. M. and Perl, D. P. (1995). Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *N. Engl. J. Med.* **332**, 1118–1124.

Kriks, S., Shim, J.-W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* **480**, 547–51.

La Fata, G., Gärtner, A., Domínguez-Iturza, N., Dresselaers, T., Dawitz, J., Poorthuis, R. B., Averna, M., Himmelreich, U., Meredith, R. M., Achsel, T., et al. (2014). FMRP regulates multipolar to bipolar transition affecting neuronal migration and cortical circuitry. *Nat. Neurosci.* **17**, 1693–700.

Lambert de Rouvroit, C. and Goffinet, A. M. (2001). Neuronal migration. *Mech. Dev.* **105**, 47–56.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P.,

Thoenen, H. and Barde, Y.-A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149–152.

Li, Q., Shirabe, K., Thisse, C., Thisse, B., Okamoto, H., Masai, I. and Kuwada, J. Y. (2005). Chemokine signaling guides axons within the retina in zebrafish. *J. Neurosci.* **25**, 1711–7.

Li, G., Kataoka, H., Coughlin, S. R. and Pleasure, S. J. (2009). Identification of a transient subpial neurogenic zone in the developing dentate gyrus and its regulation by Cxcl12 and reelin signaling. *Development* **136**, 327–35.

Lieberam, I., Agalliu, D., Nagasawa, T., Ericson, J. and Jessell, T. M. (2005). A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. *Neuron* **47**, 667–79.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–2.

Lindholm, D., Mäkelä, J., Di Liberto, V., Mudò, G., Belluardo, N., Eriksson, O. and Saarma, M. (2016). Current disease modifying approaches to treat Parkinson's disease. *Cell. Mol. Life Sci.* **73**, 1365–1379.

Lindvall, O. and al., et (1989). Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. *Arch. Neurol.* **46**, 615–631.

Lindvall, O. and Björklund, A. (2011). Cell Therapeutics in Parkinson's Disease. *Neurotherapeutics* **8**, 539–548.

Lindvall, O., Rehncrona, S., Gustavii, B., Brundin, P., Astedt, B., Widner, H., Lindholm, T., Björklund, A., Leenders, K. L., Rothwell, J. C., et al. (1988). Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. *Lancet (London, England)* **2**, 1483–4.

López-Bendito, G., Sánchez-Alcañiz, J. A., Pla, R., Borrell, V., Picó, E., Valdeolmillos, M. and Marín, O. (2008). Chemokine signaling controls intracortical migration and final distribution of GABAergic interneurons. *J. Neurosci.* **28**, 1613–24.

Lorenzl, S., Albers, D. S., Narr, S., Chirichigno, J. and Beal, M. F. (2002). Expression of MMP-2, MMP-9, and MMP-1 and Their Endogenous Counterregulators TIMP-1 and TIMP-2 in Postmortem Brain Tissue of Parkinson's Disease. *Exp. Neurol.* **178**, 13–20.

LoTurco, J. J. and Bai, J. (2006). The multipolar stage and disruptions in neuronal migration. *Trends Neurosci.* **29**, 407–413.

Ma, Q., Sommer, L., Cserjesi, P. and Anderson, D. J. (1997). Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. *J. Neurosci.* **17**, 3644–3652.

Ma, Q., Jones, D., Borghesani, P. R., Segal, R. a, Nagasawa, T., Kishimoto, T., Bronson, R. T. and Springer, T. a (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9448–53.

Marín, O., Valiente, M., Ge, X. and Tsai, L.-H. (2010). Guiding neuronal cell migrations.

Cold Spring Harb. Perspect. Biol. **2**, a001834.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78**, 7634–7638.

Martin, K., Pritchett, J., Llewellyn, J., Mullan, A. F., Athwal, V. S., Dobie, R., Harvey, E., Zeef, L., Farrow, S., Streuli, C., et al. (2016). PAK proteins and YAP-1 signalling downstream of integrin beta-1 in myofibroblasts promote liver fibrosis. *Nat. Commun.* **7**, 12502.

Mélik-Parsadaniantz, S. and Rostène, W. (2008). Chemokines and neuromodulation. *J. Neuroimmunol.* **198**, 62–8.

Metzakopian, E., Lin, W., Salmon-Divon, M., Dvinge, H., Andersson, E., Ericson, J., Perlmann, T., Whitsett, J. a, Bertone, P. and Ang, S.-L. (2012). Genome-wide characterization of Foxa2 targets reveals upregulation of floor plate genes and repression of ventrolateral genes in midbrain dopaminergic progenitors. *Development* **139**, 2625–34.

Metzakopian, E., Bouhali, K., Alvarez-Saavedra, M., Whitsett, J. a., Picketts, D. J. and Ang, S.-L. (2015). Genome-wide characterisation of Foxa1 binding sites reveals several mechanisms for regulating neuronal differentiation in midbrain dopamine cells. *Development* **142**, 1315–1324.

Miller, R. L., James-Kracke, M., Sun, G. Y. and Sun, A. Y. (2009). Oxidative and inflammatory pathways in Parkinson's disease. *Neurochem. Res.* **34**, 55–65.

Millet, S., Campbell, K., Epstein, D. J., Losos, K., Harris, E. and Joyner, a L. (1999). A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. *Nature* **401**, 161–164.

Mithal, D. S., Banisadr, G. and Miller, R. J. (2012). CXCL12 signaling in the development of the nervous system. *J. Neuroimmune Pharmacol.* **7**, 820–34.

Miyasaka, N., Knaut, H. and Yoshihara, Y. (2007). Cxcl12/Cxcr4 chemokine signaling is required for placode assembly and sensory axon pathfinding in the zebrafish olfactory system. *Development* **134**, 2459–68.

Miyata, T., Kawaguchi, A., Okano, H. and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31**, 727–741.

Mueller, W., Schütz, D., Nagel, F., Schulz, S. and Stumm, R. (2013). Hierarchical organization of multi-site phosphorylation at the CXCR4 C terminus. *PLoS One* **8**, e64975.

Murry, C. E. and Keller, G. (2008). Differentiation of Embryonic Stem Cells to??Clinically Relevant Populations: Lessons from Embryonic Development. *Cell* **132**, 661–680.

Nadarajah, B. and Parnavelas, J. G. (2002). Modes of neuronal migration in the developing cerebral cortex. *Nat. Rev. Neurosci.* **3**, 423–32.

Nakatani, T., Kumai, M., Mizuhara, E., Minaki, Y. and Ono, Y. (2010). Lmx1a and Lmx1b cooperate with Foxa2 to coordinate the specification of dopaminergic neurons and control of floor plate cell differentiation in the developing mesencephalon. *Dev. Biol.* **339**, 101–13.

Niehrs, C. (2012). The complex world of WNT receptor signalling. *Nat. Rev. Mol. Cell Biol.* **13**, 767–779.

Nishikawa, S., Goto, S., Yamada, K., Hamasaki, T. and Ushio, Y. (2003). Lack of Reelin causes malpositioning of nigral dopaminergic neurons: evidence from comparison of normal and Reln(rl) mutant mice. *J. Comp. Neurol.* **461**, 166–73.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714–720.

Odemis, V., Lamp, E., Pezeshki, G., Moepps, B., Schilling, K., Gierschik, P., Littman, D. R. and Engele, J. (2005). Mice deficient in the chemokine receptor CXCR4 exhibit impaired limb innervation and myogenesis. *Mol. Cell. Neurosci.* **30**, 494–505.

Ohyama, K., Kawano, H., Asou, H., Fukuda, T., Oohira, a, Uyemura, K. and Kawamura, K. (1998). Coordinate expression of L1 and 6B4 proteoglycan/phosphacan is correlated with the migration of mesencephalic dopaminergic neurons in mice. *Brain Res. Dev. Brain Res.* **107**, 219–26.

Pacary, E., Heng, J., Azzarelli, R., Riou, P., Castro, D., Lebel-Potter, M., Parras, C., Bell, D. M., Ridley, A. J., Parsons, M., et al. (2011). Proneural transcription factors regulate different steps of cortical neuron migration through rnd-mediated inhibition of RhoA signaling. *Neuron* **69**, 1069–1084.

Pan, D. (2010). The hippo signaling pathway in development and cancer. *Dev. Cell* **19**, 491–505.

Paredes, M. F., Li, G., Berger, O., Baraban, S. C. and Pleasure, S. J. (2006). Stromal-derived factor-1 (CXCL12) regulates laminar position of Cajal-Retzius cells in normal and dysplastic brains. *J. Neurosci.* **26**, 9404–12.

Park, H. T., Wu, J. and Rao, Y. (2002). Molecular control of neuronal migration. *Bioessays* **24**, 821–7.

Park, S. M., Gaur, A. B., Lengyel, E. and Peter, M. E. (2008). The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.* **22**, 894–907.

Pasquinelli, A. E. (2012). MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat. Rev. Genet.* **13**, 271–82.

Peng, C., Aron, L., Klein, R., Li, M., Wurst, W., Prakash, N. and Le, W. (2011). Pitx3 is a critical mediator of GDNF-induced BDNF expression in nigrostriatal dopaminergic neurons. *J. Neurosci.* **31**, 12802–15.

Peng, C., Li, N., Ng, Y.-K., Zhang, J., Meier, F., Theis, F. J., Merkenschlager, M., Chen, W., Wurst, W. and Prakash, N. (2012). A unilateral negative feedback loop between miR-200 microRNAs and Sox2/E2F3 controls neural progenitor cell-cycle exit and differentiation. *J. Neurosci.* **32**, 13292–308.

Petri, R., Malmevik, J., Fasching, L., Åkerblom, M. and Jakobsson, J. (2014). miRNAs in brain development. *Exp. Cell Res.* **321**, 84–89.

Politis, M. and Lindvall, O. (2012). Clinical application of stem cell therapy in Parkinson's disease. *BMC Med.* **10**, 1.

Politis, M., Wu, K., Loane, C., Quinn, N. P., Brooks, D. J., Rehncrona, S., Bjorklund, A., Lindvall, O. and Piccini, P. (2010). Serotonergic neurons mediate dyskinesia side effects in Parkinson's patients with neural transplants. *Sci. Transl. Med.* **2**, 38ra46.

Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M. V., et al. (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development* **133**, 89–98.

Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S.-L. L., Wurst, W. and Simeone, A. (2004). Otx2 regulates the extent, identity and fate of neuronal progenitor domains in the ventral midbrain. *Development* **131**, 2037–48.

Reiss, K., Mentlein, R., Sievers, J. and Hartmann, D. (2002). Stromal cell-derived factor 1 is secreted by meningeal cells and acts as chemotactic factor on neuronal stem cells of the cerebellar external granular layer. *Neuroscience* **115**, 295–305.

Rhinn, M., Dierich, a, Shawlot, W., Behringer, R. R., Le Meur, M. and Ang, S. L. (1998). Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125**, 845–856.

Rice, D. S. and Curran, T. (1999). Mutant mice with scrambled brains: Understanding the signaling pathways that control cell positioning in the CNS. *Genes Dev.* **13**, 2758–2773.

Sacchetti, P., Sousa, K. M., Hall, A. C., Liste, I., Steffensen, K. R., Theofilopoulos, S., Parish, C. L., Hazenberg, C., Richter, L. A., Hovatta, O., et al. (2009). Liver X receptors and oxysterols promote ventral midbrain neurogenesis in vivo and in human embryonic stem cells. *Cell Stem Cell* **5**, 409–19.

Sánchez-Alcañiz, J. A., Haege, S., Mueller, W., Pla, R., Mackay, F., Schulz, S., López-Bendito, G., Stumm, R. and Marín, O. (2011). Cxcr7 controls neuronal migration by regulating chemokine responsiveness. *Neuron* **69**, 77–90.

Sánchez-Tilló, E., Siles, L., de Barrios, O., Cuatrecasas, M., Vaquero, E. C., Castells, A. and Postigo, A. (2011). Expanding roles of ZEB factors in tumorigenesis and tumor progression. *Am. J. Cancer Res.* **1**, 897–912.

Sapède, D., Rossel, M., Dambly-Chaudière, C. and Ghysen, A. (2005). Role of SDF1 chemokine in the development of lateral line efferent and facial motor neurons. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 1714–8.

Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313–22.

Sharaf, A., Rahhal, B., Spittau, B. and Roussa, E. (2015). Localization of reelin signaling pathway components in murine midbrain and striatum. *Cell Tissue Res.* **359**, 393–407.

Shults, C. W., Hashimoto, R., Brady, R. M. and Gage, F. H. (1990). Dopaminergic cells align along radial glia in the developing mesencephalon of the rat. *Neuroscience* **38**, 427–36.

Singleton, A. and Hardy, J. (2016). The Evolution of Genetics: Alzheimer's and Parkinson's Diseases. *Neuron* **90**, 1154–1163.

Smidt, M. P., Asbreuk, C. H., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat. Neurosci.* **3**, 337–41.

Smith, A. G. (2001). Embryo-derived stem cells: of mice and men. *Annu. Rev. Cell Dev. Biol.* **17**, 435–62.

Smulders, K., Dale, M., Carlson-Kutha, P., Nutt, J. and Horak FB (2016). Pharmacological treatment in Parkinson's disease: Effects on gait. - PubMed - NCBI. *Park. Relat. Disord.* **16**, 30263–2.

Stiles, B., Groszer, M., Wang, S., Jiao, J. and Wu, H. (2004). PTENless means more. *Dev. Biol.* **273**, 175–184.

Stott, S. R. W., Metzakopian, E., Lin, W., Kaestner, K. H., Hen, R. and Ang, S.-L. (2013). Foxa1 and foxa2 are required for the maintenance of dopaminergic properties in ventral midbrain neurons at late embryonic stages. *J. Neurosci.* **33**, 8022–34.

Stumm, R. and Höllt, V. (2007). CXC chemokine receptor 4 regulates neuronal migration and axonal pathfinding in the developing nervous system: implications for neuronal regeneration in the adult brain. *J. Mol. Endocrinol.* **38**, 377–82.

Stumm, R., Kolodziej, A., Schulz, S., Kohtz, J. D. and Höllt, V. (2007). Patterns of SDF-1alpha and SDF-1gamma mRNAs, migration pathways, and phenotypes of CXCR4-expressing neurons in the developing rat telencephalon. *J. Comp. Neurol.* **502**, 382–99.

Tahinci, E., Thorne, C. A., Franklin, J. L., Salic, A., Christian, K. M., Lee, L. A., Coffey, R. J. and Lee, E. (2007). Lrp6 is required for convergent extension during Xenopus gastrulation. *Development* **134**,.

Tailor, J., Kittappa, R., Leto, K., Gates, M., Borel, M., Paulsen, O., Spitzer, S., Karadottir, R. T., Rossi, F., Falk, A., et al. (2013). Stem cells expanded from the human embryonic hindbrain stably retain regional specification and high neurogenic potency. *J. Neurosci.* **33**, 12407–22.

Tang, M., Miyamoto, Y. and Huang, E. J. (2009). Multiple roles of beta-catenin in controlling the neurogenic niche for midbrain dopamine neurons. *Development* **136**, 2027–38.

Tansey, M. G., McCoy, M. K. and Frank-Cannon, T. C. (2007). Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. *Exp. Neurol.* **208**, 1–25.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–7.

Tiveron, M.-C., Rossel, M., Moepps, B., Zhang, Y. L., Seidenfaden, R., Favor, J., König, N. and Cremer, H. (2006). Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/intermediate zone. *J. Neurosci.* **26**, 13273–8.

Tomac, a, Lindqvist, E., Lin, L. F., Ogren, S. O., Young, D., Hoffer, B. J. and Olson, L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature* **373**, 335–339.

Trommsdorff, M., Borg, J. P., Margolis, B. and Herz, J. (1998). Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J. Biol. Chem.* **273**, 33556–60.

Valiente, M. and Marín, O. (2010). Neuronal migration mechanisms in development and disease. *Curr. Opin. Neurobiol.* **20**, 68–78.

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. a and Nelson, L. M. (2003). Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. *Am. J. Epidemiol.* **157**, 1015–1022.

Varelas, X., Wrana, J. L., Ashe, H. L., Briscoe, J., Cockburn, K., Rossant, J., Mayor, R., Carmona-Fontaine, C., Harvey, K. F., al., et, et al. (2012). Coordinating developmental signaling: novel roles for the Hippo pathway. *Trends Cell Biol.* **22**, 88–96.

Vaswani, A. R. and Blaess, S. (2016). Reelin Signaling in the Migration of Ventral Brain Stem and Spinal Cord Neurons. *Front. Cell. Neurosci.* **10**, 62.

Verhagen Metman, L., Pal, G. and Slavin, K. (2016). Surgical Treatment of Parkinson's Disease. *Curr. Treat. Options Neurol.* **18**, 49.

Wallén A, a, Castro, D. S., Zetterström, R. H., Karlén, M., Olson, L., Ericson, J. and Perlmann, T. (2001). Orphan nuclear receptor Nurr1 is essential for Ret expression in midbrain dopamine neurons and in the brain stem. *Mol. Cell. Neurosci.* **18**, 649–663.

Ward, M. E. and Rao, Y. (2005). Investigations of neuronal migration in the central nervous system. *Methods Mol. Biol.* **294**, 137–56.

Wassarman, K. M., Lewandoski, M., Campbell, K., Joyner, A. L., Rubenstein, J. L., Martinez, S., Martin, G. R., Abbott, L., Jacobowitz, D., Acampora, D., et al. (1997). Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. *Development* **124**, 2923–34.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M., Darnell, J. E., Ang, S. L., Rossant, J., Ang, S. L., et al. (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78**, 575–88.

Wijeyekoon, R. and Barker, R. a (2009). Cell replacement therapy for Parkinson's disease. *Biochim. Biophys. Acta* **1792**, 688–702.

Winter, J., Jung, S., Keller, S., Gregory, R. I. and Diederichs, S. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* **11**, 228–234.

Wong, K., Ren, X. R., Huang, Y. Z., Xie, Y., Liu, G., Saito, H., Tang, H., Wen, L., Brady-Kalnay, S. M., Mei, L., et al. (2001). Signal transduction in neuronal migration: Roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. *Cell* **107**, 209–221.

Wong, K.-F., Liu, A. M., Hong, W., Xu, Z. and Luk, J. M. (2016). Integrin α2β1 inhibits MST1 kinase phosphorylation and activates Yes-associated protein oncogenic signaling in hepatocellular carcinoma. *Oncotarget* **5**,.

Wurst, W. and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of

the isthmic organizer. *Nat Rev Neurosci* **2**, 99–108.

Xu, B., Goldman, J. S., Rymar, V. V., Forget, C., Lo, P. S., Bull, S. J., Vereker, E., Barker, P. a., Trudeau, L. E., Sadikot, a. F., et al. (2010). Critical Roles for the Netrin Receptor Deleted in Colorectal Cancer in Dopaminergic Neuronal Precursor Migration, Axon Guidance, and Axon Arborization. *Neuroscience* **169**, 932–949.

Yang, S., Edman, L. C., Sánchez-Alcañiz, J. A., Fritz, N., Bonilla, S., Hecht, J., Uhlén, P., Pleasure, S. J., Villaescusa, J. C., Marín, O., et al. (2013). Cxcl12/Cxcr4 signaling controls the migration and process orientation of A9-A10 dopaminergic neurons. *Development* **140**, 4554–64.

Yang, P., Pavlovic, D., Waldvogel, H., Dragunow, M., Synek, B., Turner, C., Faull, R. and Guan, J. (2015). String Vessel Formation is Increased in the Brain of Parkinson Disease. *J. Parkinsons. Dis.* **5**, 821–836.

Ypsilanti, A. R., Zagar, Y. and Chédotal, A. (2010). Moving away from the midline: new developments for Slit and Robo. *Development* **137**, 1939–52.

Yu, F.-X., Zhao, B., Panupinthu, N., Jewell, J. L., Lian, I., Wang, L. H., Zhao, J., Yuan, H., Tumaneng, K., Li, H., et al. (2012). Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell* **150**, 780–91.

Zetterström, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248–50.

Zhao, B., Zhao, B., Wei, X., Wei, X., Li, W., Li, W., Udan, R. S., Udan, R. S., Yang, Q., Yang, Q., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* **21**, 2747–2761.

Zhou, X., Wang, Z., Huang, W. and Lei, Q. Y. (2014). G protein-coupled receptors: Bridging the gap from the extracellular signals to the Hippo pathway. *Acta Biochim. Biophys. Sin. (Shanghai).* **47**, 10–15.

Zhu, Y. and Murakami, F. (2012). Chemokine CXCL12 and its receptors in the developing central nervous system: emerging themes and future perspectives. *Dev. Neurobiol.* **72**, 1349–62.

Zhu, Y., Yu, T., Zhang, X.-C., Nagasawa, T., Wu, J. Y. and Rao, Y. (2002). Role of the chemokine SDF-1 as the meningeal attractant for embryonic cerebellar neurons. *Nat. Neurosci.* **5**, 719–20.

Zlotnik, A. and Yoshie, O. (2012). Review The Chemokine Superfamily Revisited. *Immunity* **36**, 705–716.