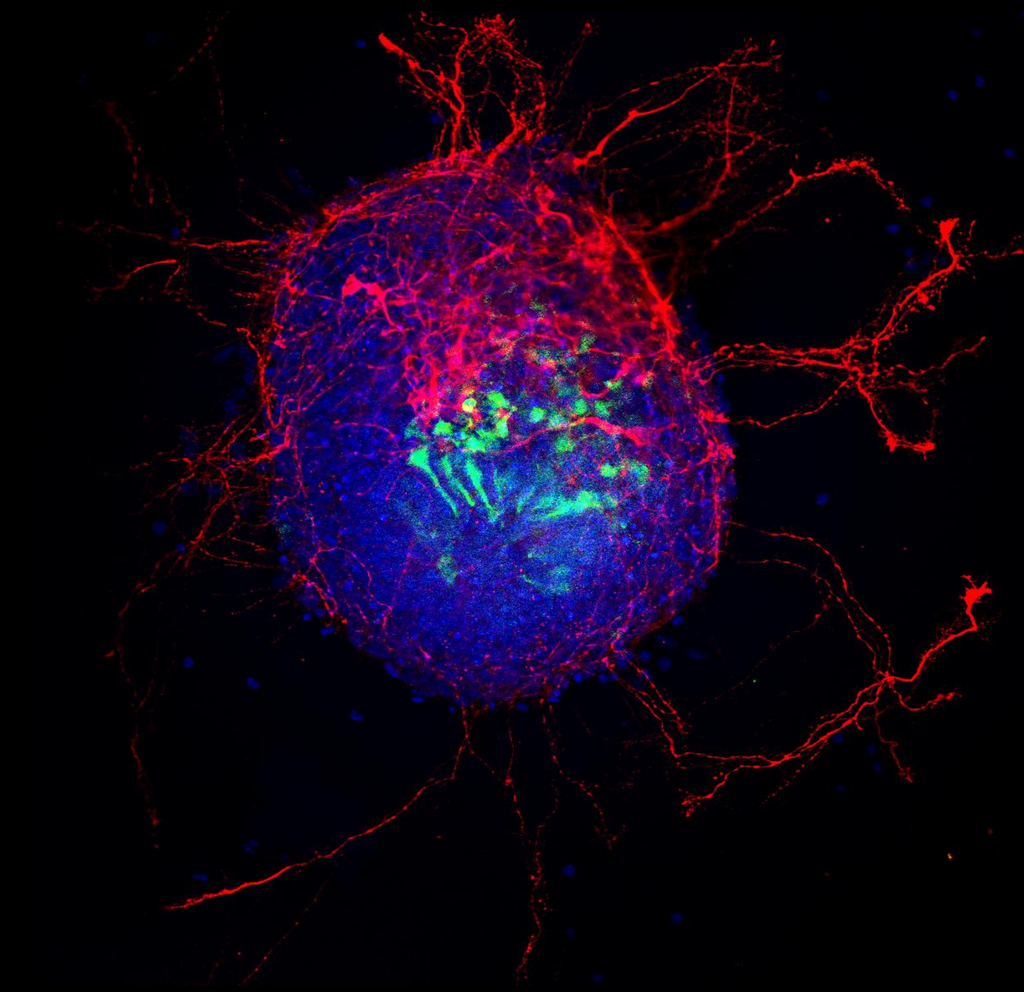
MOLECULAR CASCADES IN MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT: EMPHASIS ON WNTS



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MOLECULAR CASCADES IN MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT: EMPHASIS ON WNTS

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Cover illustration: On the cover is a confocal projection picture of a differentiated neurosphere derived from mouse fetal ventral midbrain cells transduced with Wnt1-GFP retrovirus. Detection of tyrosine hydroxylase with fluorescent antibodies (in red) reveals the presence of dopaminergic neurons. GFP-positive cells (in green) label Wnt1-secreting cells and DAPI staining (in blue) labels the cells nuclei. This thesis has, among other subjects, analyzed *Wnt1* gene regulation during the development of dopaminergic neurons.

"***Imagination is more important than knowledge***"

Albert Einstein

LIST OF PUBLICATIONS

The scientific content of the present thesis has been included in the publication of the following original articles:

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* denotes equal contribution

Para a minha mãe

Para o João

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LIST OF ABBREVIATIONS

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5HT	5-hydroxytryptamine
6-OHDA	6-hydroxydopamine
AADC	L-aromatic amino acid decarboxylase
A/P	Anteroposterior
APC	Adenomatous Polypus's Coli
AVE	Anterior visceral endoderm
BMP	Bone morphogenic protein
CAMK	Calmodulin-dependent kinase
CE	Convergent-extension
CNS	Central nervous system
CK1	Casein kinase 1
DA	Dopamine
DAergic	Dopaminergic
DAT	Dopamine transporter
DBS	Deep brain stimulation
Dkk	Dickkopf
DNA	Deoxyribonucleic acid
D/V	Dorsoventral
DVL	Dishevelled
E	Embryonic day
En	Engrailed
ES cell	Embryonic stem cell
F-DOPA	Fluor-DOPA
Fgf	Fibroblast growth factor
FoxA2	Forehead box A2
FP	Floorplate
Fez	Frizzled
GAP	GTPase-activating proteins
Gbx2	Gastrulation brain home box 2
GDI	Guanine nucleotide dissociation inhibitors
GEF	Guanine nucleotide exchange factor

GOI	Gene of interest
GSK3ß	Glycogen synthase kinase 3-ß
h	Human
HNF3ß	Hepatocyte nuclear factor 3-ß
ICM	Inner cell mass
IZ	Intermediate zone
iPD	Idiopathic Parkinson´s disease
iPS cell	Induced pluripotent stem cell
JNK	Jun-N-terminal kinase
L-DOPA	Levodopa (L-3,4-dioxyphenylalanine)
LEF	Lymphoid enhancer-binding protein
Lmx1a/b	LIM homeobox transcription factor 1 a/b
LOF	Loss of function
LRP	Low density-related lipoprotein receptors
m	Mouse
MEFs	Mouse embryonic fibroblasts
mes	Mesencephalic
MHB	Midbrain-hindbrain boundary
M/L	Mediolateral
Msx1	Muscle segment homeobox transcription factor 1
MZ	Marginal zone (or pial surface)
Ngn2	Neurogenin 2
Nurr1	Nuclear receptor related 1
Otx2	Orthodenticle homologue 2
PDL	Poly-D-lysine
PKC	Protein kinase C
PCP	Planar cell polarity
PD	Parkinson´s disease
PET	Positron emission tomography
Pitx3	Paired-like homeobox transcription factor-3
qPCR	Quantitative real-time PCR
RA	Retinoic acid
RNAi	RNA interference
Shh	Sonic hedgehog

SN	*Substantia nigra*
SNpc	*Substantia nigra pars compacta*
TCF	T-cell factor
TCL	Total cell lysate
TGFß	Transforming growth factor-ß
TH	Tyrosine hydroxylase
VM	Ventral midbrain/mesencephalon
VMAT2	Vesicular monoamine transporter
VTA	Ventral tegmental area
VZ	Ventricular zone
Wnt	Wingless-type MMTV integration site family

SUMMARY/RESUMO

SUMMARY

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects midbrain dopaminergic (DAergic) neurons in the *substantia nigra*, leading to a gradual depletion of dopamine (DA) in the *striatum*. Despite the increasing knowledge of the mechanisms underlying this degenerative process, some features of the disease remain elusive, with no cure for PD presently available. DAergic cell replacement therapy (CRT) has emerged in recent years as a possible approach to treat PD. The possibility of generating new DAergic neurons from stem cells *in vitro* and transplanting them into PD patients' brains represents a promising treatment that requires in depth knowledge of the molecular cascades regulating the generation of DAergic neurons during embryogenesis and their maintenance. However, this knowledge remains limited, hindering the use of correctly specified DAergic neurons for transplantation.

During the development of the ventral midbrain (VM), the assignment of regional identity and neuronal fate is controlled by sequential events requiring spatially and temporally coordinated interaction between organizing centers that emit inducing signals and responding cells that interpret these signals. One family of signaling proteins, which is crucial for midbrain development, is the Wnt family of proteins. The Wnt signaling pathway regulates neural patterning, cell fate determination, proliferation, differentiation, and neuronal maturation during vertebrate development. Wnt1 and Wnt5a are important morphogens, required for VM development, regulating progenitor proliferation and differentiation as well as specification and survival of DAergic neurons. Previous results have demonstrated a role for Wnt1 in promoting the proliferation of DAergic precursors and for Wnt5a in potentiating the final steps of differentiation in VM primary cultures. Nevertheless, a precise knowledge of the molecular cascades regulated by those morphogens during VM DAergic development is just beginning to emerge. This thesis identifies a novel player in the Wnt5a/ß-catenin-independent pathway during midbrain development, Tiam1, and investigates the gene regulation cascades by which a major ligand, Wnt1, regulates DAergic neuron development.

In this thesis, evidence is presented that Wnt1, which signals through the Wnt/ß-catenin pathway, is an important component of DAergic neuron development. We provide evidence that Wnt1 coordinates the expression of genes involved in the

specification and neurogenesis of midbrain DAergic neurons, such as *Shh, Lmx1a*, *Otx2 En1, Nurr1* and *Ngn2*. Moreover, we demonstrate for the first time that some of these genes (*Lmx1a*, *Otx2* and *Ngn2*) are regulated in a dose-dependent manner and in a positive or negative manner depending on the developmental time. Finally, we have observed that *Wnt1* levels in the progenitor cells in expansion correlate with the number of finally differentiated DAergic neurons, emphasizing the importance of Wnt1 in the early inductive events that link to later differentiation processes in midbrain DAergic neuron development.

In another study, we have identified Tiam1, a Rac1-guanine exchange factor, as a novel regulator of the Wnt5a/Dvl/Rac1 signaling pathway, a Wnt/ß-catenin-independent pathway, in midbrain DAergic cells. Wnt5a had been previously identified as DAergic pro-differentiation factor and this effect was show to be mediated by Rac1, a small GTPase. After knocking down Tiam1 in VM progenitor cultures, we have not only unraveled its functional requirement in Wnt5a-induced Rac1 activation, but also its role in Wnt5a-mediated induction of DAergic differentiation.

In summary, our data has broadened our knowledge on the gene regulation cascades regulated by Wnts during DAergic development and identifies a novel Wnt-signaling regulator of DAergic differentiation. We hope that this knowledge can contribute to the development of novel treatments for PD, including CRTs.

RESUMO

A doença de Parkinson é causada por uma degeneração progressiva dos neurónios dopaminérgicos da *substantia nigra*, levando a um desaparecimento gradual de dopamina no estriado. Apesar do conhecimento crescente sobre os mecanismos subjacentes ao processo degenerativo, algumas características da doença permanecem vagas, não havendo atualmente cura para a doença. A terapia celular surgiu nos últimos anos como uma abordagem possível para o tratamento do parkinsonismo. A possibilidade de gerar novos neurónios dopaminérgicos *in vitro* e transplantá-los nos cérebros dos doentes de Parkinson representa uma estratégia promissora de tratamento que requer um conhecimento aprofundado das cascatas moleculares que regulam a geração de neurónios dopaminérgicos durante a embriogénese. No entanto, esse conhecimento é ainda limitado, dificultando a geração de neurónios dopaminérgicos corretamente especificados para transplantação.

Durante o desenvolvimento do mesencéfalo ventral, a atribuição de identidade regional e destino neuronal é controlada por eventos sequenciais, que exigem uma interação coordenada a nível espacial e temporal entre centros de organização que emitem sinais indutores e células que interpretam esses sinais. Uma família de proteínas de sinalização que revelou ser crucial durante o desenvolvimento do mesencéfalo é a família de proteínas Wnt. A via de sinalização Wnt demonstrou regular a determinação do destino celular, proliferação, diferenciação e maturação neuronal durante o desenvolvimento dos vertebrados. Wnt1 e Wnt5a são dois morfogénios cruciais para o desenvolvimento do mesencéfalo ventral, regulando a proliferação, diferenciação e sobrevivência dos neurónios dopaminérgicos. Resultados anteriores demonstraram que o Wnt1 é requerido durante a proliferação de precursores dopaminérgicos, enquanto o Wnt5a é requerido durante as etapas finais da diferenciação em neurónios dopaminérgicos em culturas primárias de mesencéfalo ventral. No entanto, é necessário um conhecimento aprofundado acerca das cascatas moleculares intermediárias reguladas por esses morfogénios durante o desenvolvimento do mesencéfalo ventral, antes da sua potencial utilização a nível da terapia celular. Desta forma, esta tese identifica um novo componente na via Wnt5a/ß-catenina-independente durante o desenvolvimento do

mesencéfalo e analisa as cascatas de regulação genética pelo qual o Wnt1 regula o desenvolvimento dos neurónios dopaminérgicos.

Neste trabalho mostra-se que o Wnt1, que sinaliza através da via Wnt/ß-catenina-dependente, é um componente importante do desenvolvimento dos neurónios dopaminérgicos. Além disso, verifica-se que o Wnt1 coordena a expressão de genes envolvidos na especificação e neurogénese do mesencéfalo ventral, tais como *Shh, Lmx1a, En1 Otx2, Nurr1* e *Ngn2*. Por outro lado, demonstra-se, pela primeira vez, que alguns destes genes (*Otx2, Lmx1a* e *Ngn2*) são regulados num tempo específico, de uma forma dependente da concentração de *Wnt1*. Finalmente, observámos que os níveis de *Wnt1* nas células progenitoras em expansão se correlacionam com o número de neurónios dopaminérgicos diferenciados, dando ênfase à importância do Wnt1 nos primeiros eventos indutivos no mesencéfalo, nomeadamente na promoção da diferenciação dopaminérgica.

Adicionalmente, identificámos o Tiam1 (um fator de troca do nucleótido guanina (GEF) da Rac1) como um regulador da via de sinalização Wnt5a/Dvl/Rac1, uma via Wnt/ß-catenina-independente, em células dopaminérgicas do mesencéfalo ventral. O Wnt5a foi anteriormente identificado como um fator de pró-diferenciação dopaminérgica, por um processo mediado por Rac1, uma pequena GTPase. Após *knockdown* do Tiam1 em culturas de progenitores do mesencéfalo ventral verificámos a sua importância funcional não só na ativação da Rac1 induzida por Wnt5a, como também na indução da diferenciação dopaminérgica mediada por Wnt5a.

Em resumo, os nossos resultados permitem ampliar o conhecimento das cascatas moleculares reguladas por Wnts durante o desenvolvimento do mesencéfalo ventral e descrever um novo regulador da via de sinalização Wnt5a/Dvl/Rac1, crucial durante o processo de diferenciação dopaminérgica. Esperamos que este conhecimento possa contribuir para a otimização das atuais terapias celulares utilizadas no tratamento da doença de Parkinson.

INTRODUCTION

1. INTRODUCTION

Cell replacement therapy (CRT) have brought enthusiasm to the field because of the prospect of restoring Parkinson's disease (PD) brain damage (Björklund and Lindvall, 2000; Freed et al., 2001; Freed et al., 2001; Freed et al., 2001; Hagell and Brundin, 2001; Olanow et al., 2003; Olanow et al., 2003; Piccini et al., 1999; Piccini et al., 1999), but also disbelief because of the less than compelling results from clinical trials (Freed et al., 2001; Hagell and Brundin, 2001; Olanow et al., 2003; Piccini et al., 1999). However, clinical trials have been very useful because they have provided proof of principle for CRT and because they have pointed out what needs to be improved in order to develop a viable CRT for PD. Indeed, heterogeneity in cell composition and quality, inappropriate cell preparation, patient selection and insufficient immunosupression (Carlsson et al., 2007; Freed:2001ba, Hagell and Brundin, 2001; Piccini et al., 1999; Politis et al., 2011; Politis et al., 2010) are some of the obstacles that need to be overcome in order to implement CRT for PD.

In order to efficiently generate correctly specified DAergic neurons, the quality of transplantable cells should be standardized. Therefore, a detailed understanding of the molecular mechanisms and signaling pathways involved in their development is of the utmost importance. By identifying and understanding how different molecules interplay, it will be possible to mimic the *in vivo* conditions that regulate DAergic development, in *in vitro* differentiation protocols which will allow to expand and differentiate stem or progenitor cells as well as to standardize midbrain DAergic neuron cell preparations prior to transplantation.

Wnt signaling has been described as an essential pathway throughout development and in particular in VM development, promoting both proliferation of DAergic progenitors (Castelo-Branco et al., 2003; Castelo-Branco et al., 2003; McMahon and Bradley, 1990; Parish et al., 2008; Prakash et al., 2006; Thomas and Capecchi, 1990) and differentiation into DAergic neurons (Andersson et al., 2008; Parish et al., 2008). Nevertheless, it's role in the activation of DAergic downstream targets remains to be assessed.

This thesis focuses on the characterization of Wnt-mediated molecular mechanisms in the scope of mesencephalic (mes)DAergic neuron development, and how it can be used to improve CRT for PD.

1.1. *Parkinson's Disease*

PD was first described in 1817 by James Parkinson in his essay about the "Shaking palsy" (de Lau and Breteler, 2006; Parkinson, 2002). Almost 200 years later and there is still no cure. Nowadays, PD represents the movement disorder with the higher incidence and prevalence, and is the second most common neurodegenerative disease, after Alzheimer's disease, affecting between 0.3 to 1% of population in the range of 65 to 69 years old and being the estimated prevalence of 1 to 3% in people over 80 years old (de Lau and Breteler, 2006; Weintraub et al., 2008). The usual age of onset is the early 60s, although up to 10% of those affected are 45 years old or younger. About 40 000 cases of PD are diagnosed annually in the United States and there are currently up to 1 million with diagnosed PD (Covy and Giasson, 2011; Lesage and Brice, 2009; Vila and Przedborski, 2004; Weintraub et al., 2008; Westerlund et al., 2010).

PD is a chronic, progressive, neurodegenerative disease with a multifactorial etiology. About 90% of the cases are of sporadic origin (with no clear etiology) known as idiopathic PD (iPD), being the remaining 5 to 10% caused by inheritable genetic mutations. At least, 13 loci and 9 genes have been linked with both autosomal dominant and recessive forms of the disease (Covy and Giasson, 2011; de Lau and Breteler, 2006; Jenner and Olanow, 2006; Lesage and Brice, 2009; Olanow and McNaught, 2006; Tatton and Olanow, 1999; Vila and Przedborski, 2004; Westerlund et al., 2010).

The exact pathological mechanisms underneath iPD are not completely understood. It is believed that a combination of environmental (exogenous toxins and inflammation among others) and genetic factors leads to mitochondrial dysfunction with oxidative stress increase, decreased activity of the ubiquitin-proteasome system and activation of glial cells leading to neuronal death (de Lau and Breteler, 2006; Jenner and Olanow, 2006; McNaught et al., 2006; Olanow and McNaught, 2006; Pallone, 2007; Tatton and Olanow, 1999). Aging is indeed the only proven risk factor for iPD. Other environmental or lifestyle risk factors associated with development of PD are rural living, exposure to pesticides and herbicides, well-water drinking, and working with solvents (McNaught et al., 2006; Pallone, 2007). However, none of these factors has unequivocally been demonstrated to cause iPD.

Selective loss of mesDAergic neurons is one of the key pathological hallmarks of PD and is responsible for the motor features of PD. The degeneration of DAergic neurons in the SNpc, which project to the striatum (caudate nucleus and putamen) and

are involved in motor control, results in severe decrease of DA release in the striatum thereby playing a major role in PD´s cardinal symptoms – bradykinesia, resting tremor and rigidity. Current criteria also include gait disturbance with postural instability and freezing as main symptoms. Other neuronal systems also degenerate (eg. cholinergic, adrenergic and serotoninergic), particularly in the later stages of the disease, leading to the complex and variable non-motor features of PD, such as sleep disturbance, depression, cognitive deficits besides the autonomic dysfunction, gastrointestinal and genitourinary problems (Chaudhuri et al., 2006).

Another pathological feature of the disease is the presence of Lewy bodies, primarily localized to SN DAergic neurons. Lewy bodies are comprised of small cytoplasmic fibrillar inclusions that contain abnormal proteins including α-synuclein and ubiquitin, Parkin, and synphilin-1, in both central and peripheral autonomic neurons (Dawson and Dawson, 2003).

1.1.1. Current treatments

In the absence of a cure, the primary goal in managing PD is to preserve functionality and health-related quality of life. Modern neuropathology shows that many brain areas are affected in PD, but most prominent is the loss of DAergic neurons in the SN, in the VM. Outstandingly, the onset of PD symptoms is only observed when about 75% of DAergic neurons are lost. The diagnosis of PD remains a clinical one and is based on the recognition of symptoms and functional imaging such as F-DOPA positron emission tomography (PET), whereby fluoro-DOPA (an analogue of L-3,4-dioxyphenylalanine or L-DOPA) is taken up by the remaining DAergic cells (Jankovic, 2005).

The bastion of current pharmacological therapy in PD is based on replacement of the lost the neurotransmitter DA, providing symptomatic relief. This is achieved through administration of the precursor of DA, L-DOPA, that acts directly on the remaining DAergic neurons, replenishing the terminals and restoring the DA levels. However, beneficial effects tend to lessen with chronic use and severe motor/non-motor fluctuations (on-off phenomena) as well as abnormal, involuntary dyskinetic movements appear as debilitating drug-induced side effects. DA agonists have also been used alone

or in combination with L-DOPA (Turle-Lorenzo et al., 2006). Augmentation of the remaining DAergic neurotransmission can be achieved through inhibition of DA-degrading enzymes. Monoaminoxidase inhibitors, amantadine, catechol-O-methyl transferase inhibitors and anticholinergics, are some of the available drugs but older patients might be sensitive to them as they may cause symptoms like confusion, hallucinations, orthostatic hypotension and fatigue, making them inadequate in later PD stages.

Another current approach is surgical therapy. The first surgical procedures, based on surgical ablation of deep brain structures, such as thalamotomy or pallidothomy, are almost completely abandoned. Nowadays, Deep Brain Stimulation (DBS) of the subthalamic nucleus is the preferred procedure, but it is only used for patients whose symptoms cannot be adequately controlled with medication. DBS consists in surgically implanting an electrode to deliver electrical stimulation to target areas in the brain that control movement, blocking thus the abnormal nerve signals that cause tremor and PD symptoms.

Even though tremendous success has been achieved by these approaches, none of the currently available pharmacological or surgical therapies has been successful at halting the progress of PD symptoms or changing the course of the disease. Focus is thus now turning into discovering more effective therapeutic strategies where restoration of the degenerating DAergic system can be achieved.

1.1.2. Cell replacement therapy

Cell replacement therapy (CRT) has been considered one of the most promising therapeutic approaches for PD, in which DA-producing cells ectopically transplanted in the striatum could substitute for DA deficiency. The common consensus, based on animal studies, has been that a constant level of DA in the striatum is sufficient to restore motor behavior (Dunnett, 1991; Hagell and Brundin, 2001). However, transplantation in PD patients has indicated that the grafts mature and the symptoms improve after 6 months, a finding that has been interpreted as a result of synaptic integration of the cells in the target striatum (Piccini et al., 2000). It is therefore considered as the most practical solution, to transplant the grafts in the striatum, where

the grafted cells will release DA and exert its effects on the target cells, avoiding thus the need of reconstructing the nigrostriatal pathway.

In the past decade, different groups have managed to obtain *in vitro* cell populations enriched in DAergic cells that are derived from different sources, including neural tissue, primary cells, genetically engineered cell lines, as well as neural and embryonic stem (ES) cells. These cells have been used in PD models, but only fetal midbrain tissue has been used in clinical trials in an attempt to restore the DAergic system in the striatum.

For CRT to succeed, neurons must have the right identity and express genes that would allow them to survive, differentiate and integrate into local host circuits, establish new synapses, synthesize, release and take up DA in a similar manner to healthy host cells. In order to develop protocols to generate such cells, a precise understanding of the combination of transcription factors and soluble signals controlling mesDAergic development *in vivo* is of the utmost importance.

1.1.2.1. Fetal ventral midbrain

Transplantation of fetal VM tissue obtained from routine abortions in both rat models of PD (Bjorklund et al., 1980a; Bjorklund et al., 1980b) and, to a certain extent, in human patients (Freed et al., 1992a; Freed et al., 1992b; Lindvall et al., 1989) has shown to improve PD motor symptoms. Due to their origin, these cells are correctly specified towards a midbrain DAergic fate and could theoretically provide the required DA release in the striatum of PD patients.

Animal studies conducted in the late 70s and early 80s transplanted human fetal DAergic tissue to replace the lost of DA in PD rodent animal model assuming that it would induce long lasting clinical improvement (Bjorklund and Stenevi, 1971; Das and Altman, 1971; Dunnett, 1991; Lund and Hauschka, 1976). This data has demonstrated that intrastriatal grafted DA neurons, obtained from human fetal ventral mesencephalon, display many of the morphological and functional characteristics of normal DAergic neurons: they can reinnervate the dennervated striatum and form synaptic connections being spontaneously active and releasing DA. Successful reinnervation provided by the grafts was accompanied by a significant amelioration of Parkinson-derived symptoms in animal models (Dunnett, 1991; Goren et al., 2005).

These studies in experimental animals established a crucial developmental time window in which immature DAergic neurons could be harvested and were able to survive a subsequent grafting. Animal studies also revealed that the transplantation technique influenced the donor age window. These questions are essential since, even when using optimal donor age tissue, only around 5-20% of grafted dopaminergic neurons survive due to harvesting, dissection or transplantation procedures (Brundin et al., 2000a; Sortwell, 2003). Several attempts were done to enhance neuronal DAergic survival, including testing different grafting techniques, neurotrophic support, antioxidant therapies, increasing graft vasculature, promoting caspase inhibition, Bcl-2 overexpression along with others, whose main purpose was to reduce cell oxidative stress (Sortwell, 2003).

The first open label clinical trials using grafts of human fetal midbrain tissue started in the late 1980s and demonstrated the feasibility of performing this procedure as well as beneficial clinical effects on motor behavior (Lindvall et al., 1989; Lindvall et al., 1988). Since then, about 400 patients have been transplanted with fetal DAergic neurons in several clinical trials, which provided proof-of-principle that these cells can survive grafting (Brundin et al., 2000b; Hagell et al., 1999; Kordower et al., 1995; Lindvall et al., 1990; Mendez et al., 2005), restore DA release and ameliorate some PD motor features (Hagell and Brundin, 2001; Piccini et al., 1999).

However encouraging, the first open-label trials were unblinded, displaying variability in the processing protocols as well as on surgical methods. Moreover, there was a requirement for a more standardized, large and placebo controlled studies. For that purpose, two NIH-sponsored studies were performed in a double blind controlled-trial design (Freed et al., 2001; Olanow et al., 2003).

Freed and coauthors enrolled 40 PD patients in a study where they either received tissue from two embryos in each putamen or sham surgery and where the source of the human embryonic mesencephalic tissue consisted of aborted embryos seven to eight weeks after conception. The tissue was stored as solid tissue strands and maintained in culture for up to eight weeks prior to implantation. There was no postoperative immunosuppression given. PET scans with F-DOPA performed before and twelve months after the procedure showed no significant changes between the transplantation group and those submitted to sham surgery. Post-mortem examination on two deceased patients from unrelated causes revealed modest neuronal survival and fiber outgrowth (Freed et al., 2001). Despite modest clinical benefit held by younger

patients in this trial, there was a failure to reach statistic results. Several features were severely criticized and considered responsible for the lack of results, such as the absence of immunosupression, the insufficient amount of tissue, the storage method or the fact that the tissue was used as solid grafts, but also the rostro-caudal trajectory chosen instead of dorsoventral which was more frequently selected.

Olanow and colleagues, in a similar double-blind design, had thirty four PD patients randomized to receive bilateral grafting in the putamen of human embryonic mesencephalic tissue from one or four donor compared to sham surgery. The tissue contained DAergic neurons recovered from fragments of embryos aborted six to nine weeks after conception. All patients were treated with cyclosporin at a higher dose before the procedure and during six months after with a lower amount. From the results, one interesting feature was that four donor's transplanted patients demonstrated a trend to improve motor scores between six-nine months in comparison with what was reported in open-label trials, and worsened in the following months, showing in overall no significant differences. This deteriorating period matched the end of immunosupression which probably gave rise to graft rejection by the host's immune system (Olanow et al., 2003).

PET studies established considerable increases in striatal F-DOPA metabolism in grafted regions, with more pronounced changes in the four donor group. Post-mortem studies, from patients who died of unrelated causes, demonstrated robust graft survival with normal reinnervation of the striatum, once more with pronounced effects in the four donor group (Olanow et al., 2003). Taken together, the results from the two double-blind placebo-controlled trials failed to meet their primary end point despite increased striatal F-DOPA uptake on PET scans and post-mortem evidence of surviving transplanted neurons.

Methodological differences in these double-blinded studies are possible explanations to their lack of success, compared to their predecessors' open-label trials. Additional factors such as doctor/patient/caregiver bias in open label trials, cannot be excluded, but they cannot provide the only explanation as some patients have been out of medication and have continued to show improvement over decades (Piccini et al., 1999; Piccini et al., 2000). These double-blinded studies, are considered to be important as they may provide clues as to what are critical methodological and patient-derived factors contributing to successful neural grafts. Defining what is special about the successful cases would enable VM transplants to be used in some PD patients. Several

follow-up studies have allowed us to draw several conclusions relative to patient selection, graft tissue preparation and placement, the extent of immunosuppression and graft-induced dyskinesias. Different studies have demonstrated that the best results in the double-blind placebo-controlled trials were seen in patients with less severe disease, best preoperative response to L-DOPA (Björklund et al., 2003), and least loss of DA in the anterior putamen (Ma et al., 2010). Furthermore, other open-label studies have revealed that patients with DA loss that extends out of the dorsal striatum might do less well post grafting (Piccini et al., 2005). Therefore, choosing younger patients with less advanced disease and DA loss restricted to the dorsal and posterior putamen may produce better results with VM grafts. Regarding tissue preparation, the two NIH-funded and further successive studies have concluded that factors in the tissue preparation and prolonged storage time (Freed et al., 2001; Freeman, 2006; Olanow et al., 2003) adversely affect the survival of grafted DAergic neurons (Brundin et al., 2000b; Laguna Goya et al., 2008).

The issue of immunosuppressing patients receiving an intracerebral neural grafts has been also extensively debated, specially because no immunosupression was given in the Freed *et al.* study and in the Ollanow *et. al.* study, immunsuppression with cyclosporine was administered only during 6 months and clinical response deteriorated immediately after that period. Furthermore, immune reactivity was detected in and around the grafts in post-mortem tissue (Freed et al., 2001; Olanow et al., 2003; Olanow et al., 2009). Nevertheless, large VM grafts have shown to survive in patients where long-term immunosupression was suspended, without affecting graft survival or motor recovery induced by transplantation (Piccini et al., 2005). This provided evidence that lack or short-term immunosuppression can compromise the function and survival of the transplanted DAergic neurons and that immunosupression can be suspendend after long term treatment.

An additional concern to both NIH studies was the development of graft-induced dyskinesias. Freed and coworkers reported 5 out of 33 grafted patients with these involuntary movements in the range of 2-3 years after surgery, whereas Olanow and colleagues reported the same problem in about 56% of grafted patients. In both cases, however, dyskinesias persisted after therapeutic discontinuation and were considered a major drawback in the transplantation field. The present controversial but predominant hypothesis is that dyskinesias might be induced by a population of serotonergic or 5-hydroxytryptamine (5HT) neurons present in the graft (Carta et al., 2007; Ma et al., 2002; Politis et al., 2010). It has been shown that VM-grafted PD patients

have a 5HT hyperinnervation of the grafted striatum (Politis et al., 2010) and that exogenously supplied L-DOPA can be converted, stored, and released by the serotonergic terminals (Tanaka et al., 1999) but not inactivated because such cells lack DA transporters.

Twenty years have passed since the beginning of neuronal transplantation and many lessons have been learned from pre-clinical and clinical trials. It is believed that future developments in the field will undoubtedly depend on our capacity to identify novel cell sources and developmental processes critical to provide cells with the right identity, phenotype and purity, as well as sufficient numbers and quality needed for each procedure, which combined with optimized patient selection criteria and standard protocols should allow us to take cell replacement therapy to the next level.

1.1.2.2. Embryonic stem cells

One major limitation in the use of fetal embryonic tissue as a CRT is an ethical one. The need for several fetuses for each patient in order to obtain the sufficient numbers of DAergic neurons that could provide a motor improvement, has prompted the field of pluripotent cell research. Being able to expand pluripotent cells before differentiating them into DAergic neurons would potentially provide an endless supply of transplantable cells.

An alternative to fetal VM tissue has been the development of differentiation techniques for ES cells. These cells are derived from the inner cell mass (ICM) of the blastocyst and can theoretically proliferate indefinitely in a predictable manner. Being pluripotent, they can give rise to all kind of cells in an organism, including neural lineage. The first mouse ES cell lines were derived in 1981 (Evans and Kaufman, 1981; Martin, 1981) and the first human ES cell lines were generated in 1998 (Thomson et al., 1998).

Mouse ES (mES) cells have already demonstrated the ability to generate DAergic neurons in culture and to induce behavioral improvement in animal models of PD (Kawasaki et al., 2000; Kim et al., 2002; Lee et al., 2000). These DAergic neurons can survive transplantation, integrate into the host striatum, and reduce functional deficits in animal models of PD.

Despite the promising results from mES cells, the survival of human ES (hES) cell-derived DAergic neurons after transplantation has been very low and no functional benefit from these grafts has been reported for several years (Roy et al., 2006; Sonntag et al., 2007). Furthermore, the induction of DAergic cell fate in human hES varies between different cell lines (Perrier et al., 2004; Sato et al., 2003; Sonntag et al., 2007; Xu et al., 2005), as described previously in this section. One other major drawback for the potential clinical application has also been the risk of teratoma formation due to excessive proliferation and incomplete differentiation (Morizane et al., 2008). However, very recently, a report from the group of Lorenz Studer has provided proof of concept that human ES-derived DA neurons can engraft in rodent and non-human primates in the absence of outgrowths or teratomas and induce functional recovery in rodent models of PD (Kriks et al., 2011). This publication has been a milestone in the field and has opened the door to the development of hES cells as sources for cell replacement therapy for PD.

However, there are still many barriers to overcome before ES cell clinical application is possible: it must be assured that the stem cell source has an optimal differentiation potential with full integration and functional enhancement, bears measurable clinical benefits with minimum impact on host's immune system and does not lead to tumor formation.

1.1.2.3. Induced pluripotent stem cells

The most recent trend in the CTR field has been the generation of induced pluripotent stem (iPS) cells. The idea that patient-specific pluripotent cells could be derived from a patient, differentiated into DAergic neurons and re-engrafted into the same patient, would diminish the need for immunosuppression, while providing a cure for PD.

Ever since the cloning of Dolly, which established that adult cell nuclei could be reprogrammed into a previous undifferentiated stage under the action of molecules present in the oocyte (Wilmut et al., 1997), investigators proposed to search for the identity of such molecules so that similar reprogramming could be done without nuclear transfer. In 2006, Yamanaka was able to find four factors (Oct4; Sox2; c-Myc and Klf4)

sufficient for the reprogramming of mouse somatic cells into pluripotent cells (Takahashi and Yamanaka, 2006). Other investigators verified and replicated the same results in mouse cells (Okita et al., 2007; Park et al., 2008; Wernig et al., 2007) and rapidly there was a move on to human material (Takahashi et al., 2007). Different combination of genes have been tested in different cells with the same outcome: either by removing the tumorigenic c-Myc (Nakagawa et al., 2008; Wernig et al., 2008) or by adding Nanog to the Oct4 and Sox2 (Yu et al., 2007). Interesting for PD field, it has been also possible to derive iPS cells from the fibroblasts of PD patients (Park et al., 2008; Soldner et al., 2009) and the direct generation of neurons (Vierbuchen et al., 2010). More recently DAergic neurons have been directly generated from fibroblasts (Caiazzo et al., 2011; Pfisterer et al., 2011), avoiding thus not only the need for immunosuppression, but also the need of an intermediate pluripotent state and the risk of teratoma.

iPS cells are quite similar to hESC in terms of cell surface marker expression, karyotype, ability to differentiate into all the three primary germ layers *in vitro* as well as regarding teratoma formation, but they may retain epigenetic signals from the cell of origin. This means that the problems to use these cells in clinical trials are the same as for ES cells and that additional issues specific to iPS cells may arise as we learn more about this exciting cells. It is thus important to notice that iPS cell research can not replace ES cell investigation and that research with both cells types must be carried out in parallel in order to be able to exploit the tremendous potential of pluripotent cells and future clinical benefits of what it might be the true beginning of regenerative medicine.

For the time being and despite the enormous accomplishments that have been made in pursuit of the perfect cell for CRT, all efforts keep on bringing us back to the initial questions. In order to generate correctly specified DAergic neurons and in order to direct their efficient engraftment, it is crucial to know which extrinsic and intrinsic signals regulate their development *in vivo*.

1.2. *Midbrain Dopaminergic Neuron Development*

All the nine DAergic neuronal subtypes in the mammalian brain, classified from A8-A16, are TH positive (tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of DA) and able to synthesize and release DA, but only A8-A10 are localized in the

midbrain and of them, the A9 subtype from SN is the most severely affected in PD. How these particular cells derive from one-single-cell zygote into a highly organized organism through exquisitely orchestrated processes such as patterning, cell division, specification, migration and differentiation, is one of the most remarkable wonders of development. What makes A9 mesencephalic DAergic neurons unique, which extrinsic and intrinsic molecules are required for their specification and differentiation all the way through development, is the “Holy Grail” that many researchers have been looking for the past decades.

1.2.1. Early embryogenesis and gastrulation

After the fusion of the egg and sperm, the resulting fertilized zygote symmetrically proliferates until it reaches a compact 16-cell stage morula, at murine (m) embryonic day (E) 2. One day later, the first asymmetrical division occurs giving rise to the so-called blastocyst where cells with different size, polarity and expression of the transcription factors *Oct4* (or *Pou5f1*, POU domain class 5 transcription factor 1) and *Cdx2* (caudal-type homeobox protein 2), give rise either to the ICM within the sphere or the throphoblast, the outer shell that will give rise to the placenta, respectively (Niwa et al., 2005). By mE4.5, the ICM differentiates into two distinct layers: the epiblast, which will give rise to the embryo, and the hypoblast, which will become the yolk sac (Rossant and Tam, 2009; Zernicka-Goetz, 2002).

The process of gastrulation initiates then a morphological structuring of the developing embryo to form a multilayered embryo (gastrula). Through a series of convergent-extension (CE) movements and invaginations of the epiblast cells through the primitive streak, the intermediate mesoderm and the internal endoderm are formed. As the primitive streak extends rostrally, Hensen’s node is formed. Cells passing through the node will give rise to the prechordal plate and notochord, important organizing centers for the formation of the nervous system. Mesoderm, endoderm and ectoderm (the descendants of epiblast cells that do not pass through the primitive streak) constitute the three primary germ layers that contain progenitors of all tissues (Rossant and Tam, 2009; Tam and Loebel, 2007).

Throughout development, cell migration and specification seem to be coordinated by morphogen gradients along anteroposterior (A/P), dorsoventral (D/V) and/or mediolateral (M/L) axis. Gradients of morphogens lead to the activation of transcription of specific sets of genes, which then determine the spatial and temporal position of the cell within the tissue and the acquisition of specific cell fate. These processes are controlled by two signaling centers during gastrulation (the node and the anterior visceral endoderm, AVE) producing such morphogens (e.g. SHH, TGF-β, FGF, RA, and WNT) (De Robertis et al., 2000). The node seems to be responsible for the formation of the whole body, and the two signaling centers work together to form the anterior region of the embryo. The primitive streak and other posterior tissues are the sources of WNT and BMP proteins, whereas the node and its derivatives (such as the notochord) produce antagonists. *Fgf8* is expressed in the posterior tip of the gastrula (Figure 1).

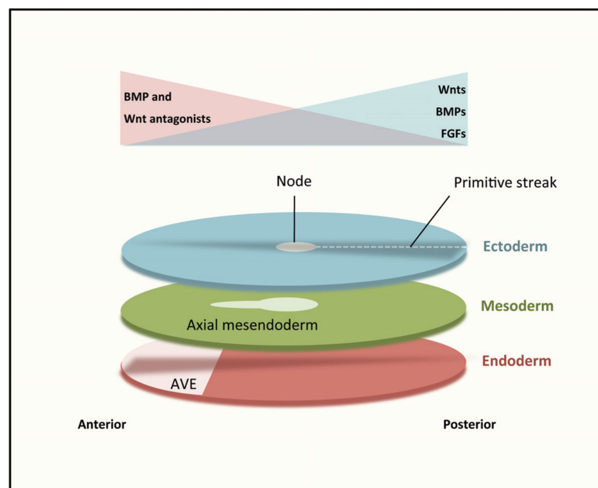

Figure 1 – Schematic view of the interacting signals for anterior-posterior patterning. The mouse embryo (depicted as a flattened disc) comprises of a stack of three germ layers and contains the primitive streak in the posterior region and the node in the central part of the embryo. The axial mesendoderm extends anteriorly from the node. The primitive streak and other posterior tissues are the sources of Wnt and BMP proteins, whereas the node, the axial mesendoderm and the AVE produce the antagonistic factors that modulate the level of signaling activity. Anterior patterning is therefore accomplished by diminishing the BMP and Wnt signals which promote differentiation and patterning of posterior tissues. Fgf8 is expressed in the posterior tip of the gastrula and continues to be made in the tail bud, creating a gradient across the posterior portion of the embryo. [adapted from (Robb and Tam, 2004)].

1.2.2. Neurulation

Neural induction is the process by which embryonic cells in the ectoderm make a decision to acquire a neural fate rather than to give rise to other structures such as epidermis. During late gastrulation, secretion of WNT and BMP antagonists (e.g. Noggin, Chordin and DKK) coming from the ventral endoderm and the axial mesendoderm (node and notochord) induce the neural plate in the overlying ectoderm (Hemmati-Brivanlou and Melton, 1997). By mE6-7, the neural plate has become longer and narrower due to CE movements. Furthermore, the lateral ends of the neural plate roll up and fuse to each other, forming the neural tube. This event marks the beginning of the development of the CNS (Beddington and Robertson, 1999). At this stage, ectoderm can be divided into three sets of cells: the internally positioned neural tube (which will form the brain and the spinal cord), the surface ectoderm or primarily epidermis, and the neural crest cells that eventually will migrate away and give rise to the future peripheral nervous system and melanocytes (Bronner-Fraser et al., 1991).

After neural tube closure, regionalization along a A/P axis starts by forming four neuromeres, that will establish the CNS main regions: the prosencephalon (forebrain), mesencephaplon (midbrain), rhombencephalon (hindbrain), and spinal cord (Lumsden and Krumlauf, 1996). Mesencephalon will give rise to the midbrain, where DAergic neurons are born. This segmented-type organization of the neural tube is achieved through the expression of a combination of soluble signals and transcription factors (Edlund and Jessell, 1999). Secretion of BMP from the roof of the neural tube (Lee and Jessell, 1999) and Sonic hedgehog (SHH) from the notochord and floor plate (Echelard et al., 1993) will promote the development of a D/V axis through the neural tube.

1.2.3. Midbrain patterning

The early midbrain is generated by the combined actions of two signaling centers, the isthmus at the midbrain-hindbrain boundary (MHB), and floorplate (FP), which promote the generation and position of DAergic neurons along the A/P and D/V axis, respectively. The major molecule involved in D/V signaling is SHH and the A/P pattern results from a more complex interaction of genes regulated by the isthmic organizer. The

formation of the isthmus, which produces fibroblast growth factor (FGF) 8, together with SHH signaling from the notochord and FP designates, at a specific region where these signals intersect each other, the region in which mesDAergic neurons will be borned.

1.2.3.1. Floor plate activity

1.2.3.1.1. Shh

The D/V polarity of the neural tube is induced by signals coming from its immediate environment: ventrally by the notochord and dorsally by the epidermis. SHH is secreted from the notochord and at E8.5 induces the ventral midline cells along the neural tube to become the FP, a secondary signaling center (Hynes et al., 1995a; Jessell et al., 1989; Yamada et al., 1993). These FP specialized cells will also secrete SHH, forming a gradient which is highest at the most ventral part of the neural tube (Briscoe et al., 1999; Hynes et al., 1995b). *Shh* is expressed in the ventral midbrain midline until E11.5 (Andersson et al., 2006b) and up to E13.5 in the notochord (Echelard et al., 1993). Moreover, the responsiveness window of the DAergic precursors to SHH is between E7.75 to E9 (Zervas et al., 2004).

SHH seems to be necessary and sufficient to induce mesDAergic neurons and this has been demonstrated by using recombinant SHH in FP-depleted midbrain explants (Hynes et al., 1995a), and FP explants in dorsal midbrain (Hynes et al., 1995b). On the other hand, SHH depletion compromises the development of the FP (Matise et al., 1998). Likewise, DAergic neuron development is impaired in midbrain explants when endogenous SHH is blocked by an antibody (Ye et al., 1998).

On the other side of the neural tube, TGF-β proteins are secreted from the dorsal ectoderm and will also induce a secondary signaling center, the roof plate. The opposing gradients of these secreted factors will interact, thus instructing the synthesis of different transcription factors along the D/V axis of the neural tube.

The intersection between the FGF8-secreting isthmus with the SHH signalling from the notochord at the same time frame, designates the specific region in which mesDAergic neurons are born (Figure 2) (Hynes et al., 1995a; Ye et al., 1998).

1.2.3.1.2. *FoxA2/HNF3β*

In the midbrain, a subset of the SHH-secreting cells of the FP also express the transcription factor forkhead box A2 (*FoxA2*, also known as *HNF3β*) (Hynes et al., 1995b). *FoxA2* plays a crucial role in the early development of the endoderm and midline structures, including the notochord and FP. In the CNS, *FoxA2* is first detected in the presumptive node (Sasaki and Hogan, 1993). From the node, expression proceeds anteriorly in the head process, similar to *Shh*. At 2-3 somites-stage, around E8, *FoxA2* is already detected in the ventral midbrain and hindbrain. By the 5 somites-stage, expression in the midbrain broadens ventrolaterally and extends anteriorly into the forebrain and into the presumptive FP. Expression is maintained in the node and notochord up to 10 somites-stage (E8-9.25) and by 25-29 somites (E9-10.25), notochordal expression is restricted to the most extreme caudal regions. Expression in the FP is though stably retained until at least E11.5 (Ang et al., 1993; Sasaki and Hogan, 1994).

FoxA2 shows several similarities in expression to *Shh* (Sasaki and Hogan, 1993). *Shh* expressed from the notochord has been shown to directly induce *FoxA2* expression in the VM through Gli binding sites in the *FoxA2* gene (Sasaki et al., 1997). *FoxA2*, in turn, directly induces VM *Shh* expression through well-conserved *FoxA2* binding sites in the *Shh* gene (Echelard et al., 1993; Jeong and Epstein, 2003; Sasaki and Hogan, 1993). This strongly suggests that *FoxA2* is the main mediator of *Shh* signaling in mesDAergic development.

FoxA2 regulates midbrain DAergic patterning and differentiation by inhibiting an alternate fate (GABAergic and *Nkx2.2*-positive serotonergic neurons), inducing neurogenesis through *Ngn2*, and regulating *Nurr1*, *En1, TH* and *AADC* (Ferri et al., 2007; Lin et al., 2009; Sasaki et al., 1997) as well as regulating survival/maintenance of mesDAergic neurons (Arenas, 2008; Echelard et al., 1993; Jeong and Epstein, 2003; Kittappa et al., 2007). Futhermore, *FoxA1/2* cooperate with *Lmx1a/b* throughtout specification in a feedforward loop (Ferri et al., 2007; Lin et al., 2009; Lin et al., 2009; Nakatani et al., 2010). All the above mentioned factors will be further discussed in the next sections.

Ectopic expression of *FoxA2* has been reported to induce the formation of a dorsal FP, which in turn induces the expression of *Shh*, resulting in the formation of ectopic DAergic neurons (Arenas, 2008; Hynes et al., 1995b; Kittappa et al., 2007).

Additionally, two independent loss-of-function studies demonstrated lost expression of midbrain-specific developmental genes and reduced numbers of DAergic midbrain neurons in *Foxa2*-null mice (Ferri et al., 2007; Kittappa et al., 2007; Lin et al., 2009; Nakatani et al., 2010). Combined, these studies indicate that FoxA2 is both required and sufficient for midbrain DAergic neuron development.

1.2.3.2. Isthmic activity

1.2.3.2.1. Otx2/Gbx2

In the developing neural tube, the isthmic organizer demarcates the boundary between the midbrain and hindbrain around mE7.5. Long before the midbrain-hindbrain boundary (MHB) becomes perceptible, the expression of a set of genes is initiated in this region, which thus acts as an important organizing centre. The earliest anterior neuroectoderm marker gene is orthodenticle homologue 2 (*Otx2)*, a homeodomain transcription factor that is expressed in the AVE and in the epiblast prior to the onset of gastrulation (Acampora et al., 1995; Hynes et al., 1995b). During gastrulation, *Otx2* becomes progressively restricted to the anterior region of the mouse embryo in all three germ layers, including the prospective anterior neural plate. Gastrulation brain homeobox 2 (*Gbx2*), another homeobox-containing transcription factor, is on the other hand, expressed throughout all germ layers in the posterior part of the embryo (Ferri et al., 2007; Kittappa et al., 2007; Wassarman et al., 1997). Thus, the *Otx2* expression domain defines the prospective forebrain and midbrain territory, whereas the *Gbx2* expression field defines the prospective hindbrain region and spinal cord. The correct positioning of the isthmus is regulated by the interface between the regions expressing the two transcription factors at the MHB (Acampora et al., 1995; Liu and Joyner, 2001a; Prakash and Wurst, 2004; Simeone, 2000), even though they are not necessary for the induction of MHB genes (such as *Fgf8*) (Alavian et al., 2008; Wassarman et al., 1997). Loss of *Gbx2* function results in a caudal extension of *Otx2* expression (Liu and Joyner, 2001a; Millet et al., 1999; Prakash and Wurst, 2004; Simeone, 2000). Moreover, the midbrain and forebrain in *Otx2* mutant mice are absent, revealing the necessity of *Otx2* and *Gbx2* in early regionalizing events (Rhinn et al., 1999).

At the boundary between these two territories, signal molecules such as *Fgf8*, Paired box (*Pax*) 2, *Lmx1b*, and Wingless related 1 (*Wnt1)* are expressed across the MHB around mE8, and then their expression becomes restricted. Later on the expression Engrailed (*En*) 1/2, *Pax5*, and *Pax8* also overlaps in this region (Hynes and Rosenthal, 1999; Millet et al., 1999; Wurst and Bally-Cuif, 2001). Between mE9 and 10, the restricted expression of these genes defines an isthmic molecular code: *Wnt1* and *Otx2* identify the posterior mesencephalic side of the MHB and *Fgf8* and *Gbx2* identify the anterior hindbrain side (Figure 2).

1.2.3.2.2. Lmx1b

Expression of LIM homeobox gene *Lmx1b* is first detected in the anterior embryo at E7.5 and becomes then progressively restricted to the isthmus at E9 (Guo et al., 2007; Smidt et al., 2000). Its expression in the midbrain starts before neural tube closure and is found in a broad band, reaching from the ventral to the dorsal surface of the mesencephalon. *Lmx1b* is down-regulated around E11.5 when mesDAergic neurons are differentiating, being again detected later in the postmitotic neurons. From around E16 until adulthood, it is colocalized with *TH* and *Pitx3* in the VM (Andersson et al., 2006b; Prakash et al., 2006; Smidt et al., 2000).

Lmx1b seems to be necessary both as an initiator of the expression of *Fgf8* (Guo et al., 2007; Matsunaga et al., 2002) and in the maintenance and/or induction of the expression of *Wnt1* and *En1/2* in the MHB (Adams et al., 2000; Guo et al., 2007; Smidt et al., 2000). *Lmx1b* and *Wnt1* null mutants have very similar phenotypes in respect to midbrain DAergic neurons (Guo et al., 2007; Matsunaga et al., 2002; Smidt et al., 2000), a small residual population of TH-positive neurons in the ventral midbrain, which do not express *Pitx3* and totally disappear later during development. *Wnt1* is thought to act downstream of *Lmx1b* and thus mediate the effects of *Lmx1b* on *Pitx3*, in the independent pathway generating DAergic neurons (Smidt et al., 2000).

Loss-of-function studies demonstrate that *Lmx1b* regulates mid-hindbrain patterning, and consequently severe loss of mesDAergic neurons is primarily due to the early loss of most of the midbrain in Lmx1b$^{-/-}$ mouse embryos (Guo et al., 2007; Simon et al., 2003; Smidt et al., 2000). Deletion of *Lmx1b* results in the loss of isthmic and

cerebellar structures and subsequent loss of *Fgf8* and *Wnt1* expression (Adams et al., 2000; Guo et al., 2007; Matsunaga et al., 2002; O'Hara et al., 2005). On the other hand, misexpression of *Lmx1b* in the MHB of the chick embryo causes expansion of the tectum and cerebellum and induces *Fgf8* and *Wnt1* expression (Adams et al., 2000; Guo et al., 2007; Matsunaga et al., 2002). Furthermore, analysis of *Lmx1b* null animals has revealed reduced levels of *Pitx3* while other markers such as *Nurr1* and *TH* remain normal. *TH* expression is however lost after E16, suggesting that *Lmx1b* might be important for DAergic survival. Conversely, *Lmx1b* levels are unchanged in *Nurr1* null animals. Combined, these findings suggest that *Nurr1* and *Lmx1b* constitute two independent pathways, both of which are necessary for the generation of DA neurons (Guo et al., 2007; O'Hara et al., 2005; Smidt et al., 2000).

1.2.3.2.3. Fgf8

The soluble factor FGF8 is responsible for the A/P specification in the midbrain and starts to be secreted by the MHB between mE8 and mE8.5. By E9, *Fgf8* expression is restricted to a narrow ring encircling the isthmus on the *Gbx2*-expressing domain, in the rostral hindbrain (Crossley and Martin, 1995; Crossley et al., 1996; Rhinn et al., 1999) (Figure 2). *Fgf8* is considered as a crucial isthmic organizer molecule since it can mimic the properties of the isthmus and is required for the normal development of the midbrain and cerebellum. In fact, expression of *Fgf8* using FGF8-soaked beads can ectopically induce an isthmus, midbrain and cerebellum (Hynes and Rosenthal, 1999; Martinez et al., 1999; Wurst and Bally-Cuif, 2001). On the other hand, *Fgf8* conditional knockout animals, where *Fgf8* was specifically deleted from the MHB, the midbrain, cerebellum and isthmus were completely ablated (Chi et al., 2003; Crossley and Martin, 1995; Crossley et al., 1996). Partial deletion of *Fgf8* in an hypomorph, resulted in a deletion of midbrain and cerebellum (Martinez et al., 1999; Meyers et al., 1998).

1.2.3.2.4. Wnt1

Wnt1, a member of Wnt family (see chapter 1.3), is first detected at mE8, broadly expressed in the mesencephalon, but is not expressed in the FP region (Chi et al., 2003; McMahon et al., 1992; Parr et al., 1993). It becomes progressively restricted to the dorsal midline in the midbrain, a narrow ring immediately anterior to the *Fgf8* ring at the isthmus, and two stripes of expression flanking the ventral midline around E9-9.5 (Meyers et al., 1998; Prakash et al., 2006; Wilkinson et al., 1987) (Figure 2). Unlike *Fgf8*, *Wnt1* does not have induction activity but is essential for mid/hindbrain development (McMahon and Bradley, 1990; McMahon et al., 1992; Parr et al., 1993). *Wnt1* and *Fgf8* seem to cross-regulate each other (Chi et al., 2003; Lee et al., 1997; Liu and Joyner, 2001b; Prakash et al., 2006; Wilkinson et al., 1987). Interestingly, *Wnt1* is required for the induction of ectopic DAergic neurons by *Shh* and *Fgf8*, as these alone failed to do so in a *Wnt1*$^{-/-}$ background (McMahon and Bradley, 1990; McMahon et al., 1992; Prakash et al., 2006). Early *Wnt1* expression is largely restricted to the *Otx2*-positive territory and seems to be critical for the maintenance of *Otx2* expression in the midbrain and vice versa. *Wnt1* induces *Otx2* which represses *Nkx2.2* in the FP. *Nkx2.2* would otherwise repress the development of mesDAergic neurons and instead induce serotonergic neurons (Chi et al., 2003; Lee et al., 1997; Liu and Joyner, 2001b; Prakash et al., 2006).

Wnt1 is detected until E14.5 (Wilkinson et al., 1987) and is seen as a crucial factor in mesDAergic patterning. *Wnt1* mutant mice show an abnormal posterior midbrain, isthmus and rostral hindbrain, demonstrating its decisive role in the formation of the MHB. Futhermore, few DAergic neurons are born in *Wnt1*$^{-/-}$ mutants and those born do not manage to survive into later stages (McMahon and Bradley, 1990; Prakash et al., 2006; Thomas and Capecchi, 1990). This can be attributed to a loss of expression of its target gene *Engrailed*, as expression of *Engrailed1* under the *Wnt1* promoter rescues the loss of VM DAergic neurons in *Wnt1*$^{-/-}$ mutants (Danielian and McMahon, 1996). Moreover, expression of *Wnt1* under the *Engrailed1* promoter led to an expansion of the VM DAergic domain and increase in the number of DAergic neurons (McMahon and Bradley, 1990; Panhuysen et al., 2004; Prakash et al., 2006; Thomas and Capecchi, 1990). Additionally, partially purified WNT1 conditioned media increased the number of midbrain DAergic neurons obtained in mouse primary cultures, by increasing the proliferation of DAergic precursors in vitro (Castelo-Branco et al., 2003; Prakash et al., 2006).

Thus, Wnt1 is a major extrinsic molecule that plays a critical role in midbrain DAergic development, both in establishing the DAergic progenitor domain and promoting a correct differentiation of progenitors to mature DAergic neurons (Panhuysen et al., 2004; Prakash et al., 2006) (Figure 6). Nevertheless, a gene regulation assay enlightening which genes are transcriptionally regulated by *Wnt1* during VM development still has not been demonstrated.

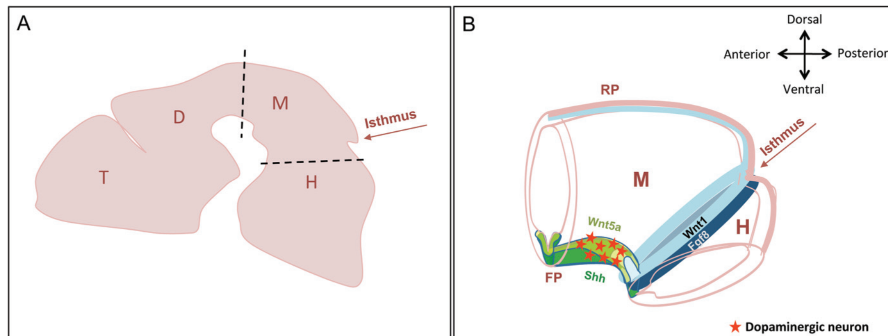

Figure 2 - Ventral midbrain patterning

A) A scheme of an E11.5 sagittal brain section, depicting the rostrocaudal, or A/P, axis. The midbrain (M) is situated anterior to the isthmus, between the diencephalon (D) and hindbrain (H). Dashed lines demonstrate which portion of the brain: midbrain, isthmus and R1, is depicted in B. **B)** Three-dimensional representation of the Shh-expressing floorplate (FP), and the Fgf8-expressing band around the isthmus. DAergic neurons (stars) arise in the intersection between Shh and Fgf8. A similar pattern of expression and subsequent intersection is formed by Wnt5a in the FP and Wnt1 in the isthmus. Wnt1 is also expressed in two stripes flanking the ventral midline, and in the roofplate (RP).

1.2.3.2.5. En1/En2

In the mouse, the patchy expression of two *engrailed* genes – *En1* and *En2* – at E8 in the anterior neuroectoderm, subsequently fuse to form a band of cells where the isthmic organizer emerges. Between E11 and E12, the two genes are detected in postmitotic DAergic neurons and are then continuously expressed throughout the entire life of these cells.

The early onset of *Fgf8* is independent of the two transcription factors; however, its later expression in the isthmus seems to be under their direct regulatory control.

En1/2 expression from the isthmus is essential as it induces continuous production of *Fgf8*, allowing proper survival and neuronal development (Adams et al., 2000; Liu and Joyner, 2001b; Simon et al., 2001). *En1/2* expression is also dependent on proper *Otx2* expression, and necessary for the formation of the dorsal midbrain and cerebellum (Joyner, 1996; Rhinn and Brand, 2001; Smidt et al., 2000). In addition, *Wnt1* seems to directly or indirectly maintain *En1* expression across the MHB. Indeed, expression of *En1* driven by the *Wnt1* enhancer in *Wnt1*$^{-/-}$ mice rescues most of the *Wnt1*$^{-/-}$ mutant phenotype (Danielian and McMahon, 1996). While an early role for the *engrailed* genes has been shown, several reports have also documented an important role for this gene family in the survival of postmitotic DA neurons (Alberí et al., 2004; Joyner, 1996; Rhinn and Brand, 2001).

Neither of the two *engrailed* mutant strains, however, show a significant phenotype with respect to midbrain mesDAergic neurons. Studies in mice with null mutations for either *En1* or *En2* have shown a paired action in neuronal DAergic survival by compensating for the loss of one another. The requirement for the *engrailed* genes is apparent only in mutant mice deficient for both *En1* and *En2*, which exhibited complete loss of TH-positive DAergic neurons (Danielian and McMahon, 1996; Simon et al., 2001).

1.2.4. Ventral midbrain specification and differentiation

Around E9.5, the isthmus constriction fully separates midbrain from hindbrain and the neural tube is patterned longitudinally into roof plate, alar plate, basal plate and floor plate. The DAergic domain is then specified between E9.5 and E11.5. The ventral midbrain further subdivides into ventricular (VZ or neuroepithelium), intermediate (IZ) and marginal zones (MZ or pial surface). The mesDAergic progenitors generated in the neuroepithelium (VZ) of the VM will generate DAergic neuroblasts that exit the cycle and travel ventrally along the radial glial cells through the IZ (Bonilla et al., 2008; Kawano et al., 1995), and laterally to their final destinations near the pial surface (MZ), where they become fully differentiated mesDAergic neurons (Lumsden and Krumlauf, 1996; Simon et al., 2003) (Figure 3). This migration event is marked by the expression of aromatic amino acid decarboxylase (*AADC*), *Nurr1* (Bonilla et al., 2008; Kawano et al.,

1995; Zetterström et al., 1997), and *En1/2* (Lumsden and Krumlauf, 1996; Simon et al., 2001).

Twelve to twenty-four hours after becoming postmitotic, the first signs of the neurotransmitter phenotype appear, like the expression of the rate-limiting enzyme for dopamine synthesis, TH. Midbrain DAergic precursors/neuroblasts will eventually become fully differentiated DAergic neurons when they start expressing an array of genes that are essential for DA signaling. These key factors comprise the enzymes TH and AADC, which catalyze the conversion of L-tyrosine to L-DOPA and L-DOPA to dopamine, respectively; the vesicular monoamine transporter (VMAT2), which is required for vesicular storage and release of dopamine; and the dopamine transporter (DAT), involved in the reuptake of DA from the synaptic cleft. Several transcription factors such as *Lmx1b*, *Nurr1* and *Pitx3* are expressed postmitotically, which is essential for the differentiation and long-term survival of mesDAergic neurons (Jacobs et al., 2009; Saucedo-Cardenas et al., 1998; Smidt et al., 2000; Zetterström et al., 1997). DAergic neurons start then extending axonal outgrowths towards their target projection areas within the striatum and cortex.

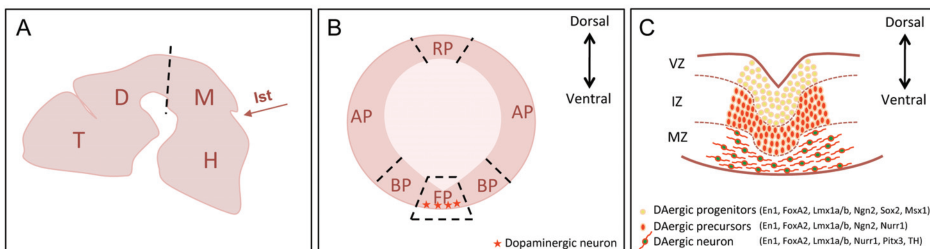

Figure 3 - Dopaminergic neuron differentiation

A) Segmentation of the neural tube into telencephalon (T), diencephalon (D), midbrain (M) and hindbrain (H) at E11.5. A dashed line shows the midbrain level represented for the coronal sections in B and C. Isthmus (Ist). **B)** The midbrain is dorso-ventrally divided into several compartments, from dorsal to ventral: roof plate (RP), alar plate (AP), basal plate (BP) and floor plate (FP). DAergic neurons are born and develop in the FP. **C.** DAergic progenitors in the ventricular zone (VZ) migrate through the intermediate zone (IZ) to the marginal zone (MZ), differentiating as they progress through these zones. Throughout this process, cells express a specific set of transcription factors.

1.2.4.1. Lmx1a

The first indications of the cellular phenotype of midbrain DAergic neurons are the appearance of *Lmx1a* and *Msx1* (muscle segment homeobox transcription factor 1) in the still proliferating progenitor cells. Andersson and collegues suggested that these genes function as midbrain DAergic neuron determinants (Andersson et al., 2006b). Both *Lmx1a* and *Msx1* are expressed in the midbrain when *Shh* signaling is present, early in the FP cells, suggesting they might be induced by *Shh*. Nevertheless, the analysis of conditional knockout mutants where *Shh* expression is lost in basal and floor plate progenitors already at E8.75 have shown that *Shh* is not necessary to maintain *Lmx1a* expression in the floor plate as there are no significant changes in Lmx1a+ cells (Lin et al., 2009). However, as discussed above, the Shh induces *FoxA1/2*, which is in turn known to cooperate with *Lmx1a/b* in DA specification via a feedforward loop (Lin et al., 2009; Nakatani et al., 2010).

Lmx1a is expressed before *Msx1* and is first identified at E9 in the DAergic progenitors in the VZ and persisting throughout life, both in postmitotic precursors functioning as a specific activator of downstream genes such as *Nurr1* and *Pitx3* (Andersson et al., 2006b) and in differentiated DAergic neurons. Thus, *Lmx1a* labels the whole midbrain DAergic lineage. Conversely, *Msx1* starts being expressed at E9.5 in the VZ progenitors and remains restricted to this domain. *Msx1* is induced and can cooperate with *Lmx1a* in inducing DAergic neurons but is not sufficient alone. It suppresses alternative cell fates (*Nkx6.1* gene) at the same time that restrains FP characteristics. It also induces panneural differentiation through the induction of proneural basic helix-loop-helix protein *Ngn2* triggering glial-to-neural switch (Andersson et al., 2006b; Lin et al., 2009).

Otx2, which is known to control A/P patterning of the neural plate, induces *Lmx1a* expression in FP cells, which, in turn, upregulates the proneural factor *Ngn2* to generate mesDAergic neurons (Andersson et al., 2006b; Nakatani et al., 2010; Ono et al., 2007). *Foxa2* also plays an important role in mesDAergic specification by cooperating with *Lmx1a/b* in FP cell differentiation in a autoregulatory loop trend (Andersson et al., 2006b; Nakatani et al., 2010) and is crucial for its maintenance (Lin et al., 2009; Ono et al., 2007). Moreover, they can induce ectopic DAergic neurons when co-expressed (Lin et al., 2009; Nakatani et al., 2010).

siRNA silencing of *Lmx1a* in chick embryos results in the loss of DAergic neurons in the midbrain, which failed to be rescued by *Lmx1b* (Andersson et al., 2006b; Lin et al., 2009), suggesting that *Lmx1b* cannot compensate for *Lmx1a* function in the development of these neurons. However, subsequent rodent studies have shown that *Lmx1a* and *Lmx1b* show similar roles in midbrain DAergic neuron development in gain of function studies both in mouse embryos (Lin et al., 2009; Nakatani et al., 2010) and in ES cells (Chung et al., 2009). However, in these experiments, *Lmx1b* induces *Lmx1a* expression in DAergic cells. Recent studies in Lmx1a and Lmx1b double mutant animals, have revealed a cooperative role for both genes in regulating the proliferation, neuronal commitment and differentiation of mesDAergic progenitors, as observed by the severe loss of mesDAergic neurons detected in these animals (Deng et al., 2011; Yan et al., 2011). An unique role for each gene has also been demonstrated as Lmx1a seems to be more important for neurogenesis in the medial DAergic domain, whereas Lmx1b is more important for the generation of lateral DAergic neurons (Deng et al., 2011).

1.2.4.2. Ngn2

Neurogenin 2 (*Ngn2*) is a proneural gene from the basic helix-loop-helix family involved in neuronal differentiation and subtype specification in various regions of the nervous system. *Ngn2* was described as the first gene expressed in the progenitors in the VZ (at E10.75) that is essential for proper differentiation, and whose loss of function causes impaired neurogenesis and almost complete loss of DAergic neurons in the midbrain (Andersson et al., 2006a; Kele et al., 2006).

At the onset of DAergic neurogenesis (E11.5), *Ngn2* expression is restricted to some proliferative cells within the VZ and also in Nurr1-positive cells in the IZ (Andersson et al., 2006a; Kele et al., 2006). Kele *et al.* reported that *Ngn2* is required for the differentiation of Sox2-positive VZ progenitors into Nurr1-positive postmitotic DAergic neuron precursors in the IZ, and that it may also be required for their subsequent differentiation into TH-positive DAergic neurons in the MZ (Kele et al., 2006). It has also been reported that *Ngn2* is expressed exclusively in the part of the VZ that gives rise to the migrating mesDAergic neuroblasts, but not in the differentiated DAergic neurons (Andersson et al., 2006a; Kele et al., 2006; Thompson et al., 2006).

Cells isolated by FACS from the developing VM of Ngn2-GFP knock-in mice were capable of generating mesDAergic neurons, both *in vitro* and after transplantation to the striatum of neonatal rats (Thompson et al., 2006). This group has also shown that surviving midbrain DAergic neurons in VM grafts were derived from early postmitotic, Nurr1-expressing precursors before they have acquired their fully differentiated neuronal phenotype. *Ngn2* also seems to be regulated by several genes such as *Foxa1/2*, as its expression is substantially reduced in Foxa1$^{-/-}$;Foxa2$^{-/-}$ mutants (Ferri et al., 2007), and a target of *Lmx1a* (most likely through *Msx1*) (Andersson et al., 2006b; Ono et al., 2007). Finally, as *Ngn2* does not promote DAergic neuron differentiation when ectopically expressed in dorsal and ventral midbrain E11.5 primary cultures (Andersson et al., 2006a; Ferri et al., 2007), it can be concluded that *Ngn2* is required but not sufficient for DAergic differentiation.

1.2.4.3. Nurr1

The nuclear receptor related 1 protein (*Nurr1* or *NR4A2)*, an orphan member of steroid/thyroid hormone receptor superfamily, is first expressed at E10.5 in postmitotic cells in the IZ and MZ of the VM, prior to the birth of DAergic neurons (Zetterström et al., 1997), persisting into adulthood.

Foxa2 (Ferri et al., 2007) and *Lmx1a* (Chung et al., 2009) have been found to regulate *Nurr1* expression, which in turn controls the expression of VMAT2 and DAT, that promotes the acquisition of the neurotransmitter phenotype (Ferri et al., 2007; Sacchetti et al., 2001; Smits et al., 2003). *Nurr1* has been used to induce a DAergic phenotype in ESCs (Chung et al., 2009; Chung et al., 2002; Kim et al., 2002) and neural progenitor cells (Grothe et al., 2004; Sacchetti et al., 2001; Wagner et al., 1999), even though their survival has been very low in all studies performed.

Nurr1$^{-/-}$ mice failed to produce midbrain TH-positive DAergic neurons, while other catecholaminergic cell groups appear unaffected (Saucedo-Cardenas et al., 1998; Zetterström et al., 1997). These mice displayed hypoactivity and died shortly after birth. Disturbances in cell migration, target innervation and cell death are also detected in Nurr1-null mice (Saucedo-Cardenas et al., 1998; Wallén et al., 1999; Zetterström et al., 1997). *Nurr1* is not required for the formation of midbrain DAergic progenitors since

En, *Lmx1b* and *Pitx3* are present at earlier stages in these animals (although later downregulated), but it is necessary for their differentiation and maintenance (Filippi et al., 2007; Grothe et al., 2004; Saucedo-Cardenas et al., 1998; Shim et al., 2007; Smidt et al., 2000). This data demonstrates that *Nurr1* is required for midbrain DAergic differentiation and survival but not necessary for the induction of all DAergic genes.

1.2.4.4. Pitx3

A second gene found to be important for final midbrain DAergic differentiation and survival is the paired-like homeobox transcription factor-3 (*Pitx3*), a member of the pituitary family of bicoid type homeobox transcription factors (Filippi et al., 2007; Saucedo-Cardenas et al., 1998; Semina et al., 1997; Smidt et al., 2000). In both rodent and human brain, *Pitx3* is strictly expressed in the mesDAergic neurons in the SN and VTA and its expression persists throughout adult life, being severely impaired in PD patients and completely absent from 6-OHDA lesioned rats (Saucedo-Cardenas et al., 1998; Smidt et al., 1997; Wallén et al., 1999).

It starts being expressed at E11 in the mouse midbrain matching the emergence of mesencephalic DAergic neurons, therefore with an almost complete overlap with *TH* expression (Semina et al., 1997; Smidt et al., 1997; Zhao et al., 2004). Besides, it has been demonstrated that *Pitx3* can activate the *TH* promoter *in vitro* (Cazorla et al., 2000; Lebel et al., 2001; Messmer et al., 2007; Smidt et al., 1997). *Pitx3* has also been shown to regulate the expression of *VMAT2* and *DAT* (Hwang et al., 2009; Smidt et al., 2004; Zhao et al., 2004), suggesting a possible cooperation between *Pitx3* and *Nurr1* in the DAergic neuron specification. The involvement of both transcription factors in the regulation of crucial mesDAegic genes suggests that these proteins might cooperate and participate in several processes during midbrain DAergic neuron development. Both factors are also expressed in mesDAergic neurons throughout adulthood, suggesting that *Pitx3* and *Nurr1* are important for the maintenance and normal physiology of mature midbrain DAergic neurons. Nevertheless, *Pitx3* was thought to be activated by *Lmx1b* (Lebel et al., 2001; Messmer et al., 2007; Smidt et al., 2000) in a pathway independent of *Nurr1* (Burbach et al., 2003; Smidt et al., 2000). Recently, Volpicelli and collegues have shown evidence that *Nurr1* directly regulates *Pitx3* by binding to a specific *Nurr1*-binding

region located in the *Pitx3* gene promoter (Volpicelli et al., 2012), thus shedding controversy to the field.

In *Aphakia* mice, characterized by a double deletion within the *Pitx3* gene (Burbach et al., 2003; Cazorla et al., 2000; Lebel et al., 2001; Semina et al., 2000; Simon et al., 2003), an almost complete ablation of DAergic neurons in the SN is observed, and a partial decrease in the VTA (Maxwell et al., 2005; Nunes et al., 2003; Volpicelli et al., 2012). The reason why SN neurons are more severely affected in SN than in VTA neurons is still unclear. Mice lacking *Pitx3* expression have shown to develop mesDAergic neurons in lower numbers and different location in the SN (Semina et al., 2000; Smidt et al., 2004), resembling PD. Thus, it seems that *Pitx3* is not required for inducing cell fate, but instead, is a key factor for terminal differentiation and final localization. Moreover, Pitx3 may be used to distinguishing A9 SN from A10 VTA DAergic neurons, since its expression is different in time in those two phenotypes (Hwang et al., 2003).

1.3. *Wnt signaling*

The Wnt family is a highly conserved group of secreted, cysteine-rich, lipid-modified glycoproteins that control cell proliferation, fate decisions, polarity, migration, and differentiation during development and homeostasis in the adult life (Cadigan and Nusse, 1997; Inestrosa and Arenas, 2010; Logan and Nusse, 2004; Maxwell et al., 2005; Nunes et al., 2003; Prakash and Wurst, 2007; van Amerongen and Nusse, 2009). Deregulation of these pathways is detected in several pathologies, such as colon, skin, and lung cancers (Clevers and Nusse, 2012; Giardiello et al., 1997; Smidt et al., 2004; Taipale and Beachy, 2001).

The name Wnt derives from the fusion of two words: *wingless* (wg), the *Drosophila* gene first identified for its function in wing and haltere formation, which is essential for segment polarity during fly development (Cadigan and Nusse, 1997; Logan and Nusse, 2004; Sharma and Chopra, 1976), and *int-1,* the homologue murine gene which was first identified as being able to induce breast cancer (Amoyel et al., 2005; Ciani and Salinas, 2005; Giardiello et al., 1997; Lundberg et al., 2008; McMahon and Bradley, 1990; Megason and McMahon, 2002; Nusse and Varmus, 1982; Taipale and Beachy, 2001;

Thomas and Capecchi, 1990). When both were identified as homologous, the term Wnt was given to designate this new signaling pathway (Nusse et al., 1991). This initial finding indicated that a pathway used for the development of an organism could participate in the process of oncogenesis.

Wnts can activate different pathways in a cell-specific and context-dependent manner. These pathways regulate multiple processes including proliferation, differentiation, migration, planar cell polarity (PCP), and convergent-extension (CE) movements. Initially, Wnts were divided into two groups, "canonical" or "non-canonical", based on their ability to transform C57MG mammary cells (Nusse and Varmus, 1982; Wong et al., 1994) or induce body axis duplication in *Xenopus* (Nusse et al., 1991; Sokol et al., 1991). Nowadays, this classification is becoming obsolete, as some Wnts previously considered non-canonical (unable to transform cells or induce axis duplication) have been discovered to either induce axis duplication (He et al., 1997; Wong et al., 1994) or activate ß-catenin (Mikels and Nusse, 2006b; Sokol et al., 1991) if a specific receptor is present, suggesting an extensive cross-talk between the two pathways. Nowadays, a more appropriate classification, based on the signaling pathway they activate, has emerged: the canonical pathway is now called Wnt/ß-catenin pathway (the transforming, dorsalizing, or axis-inducing class), whereas the non-canonical is refered to as ß-catenin-independent Wnt pathway (the non-transforming class), which is further subdivided into Wnt/PCP and Wnt/Ca^{2+} pathways depending on their downstream effectors (He et al., 1997; Veeman et al., 2003).

Studying Wnt function hasn't always been an easy task. Despite the successful generation of several knockout animals, many Wnt null mice are embryonically lethal and conditional knockouts are required in order to study their physiological contributions. As most extracellular proteins, Wnts contain cysteine residues that form disulfide bonds and prior to secretion they are glycosylated and palmityolated (Mikels and Nusse, 2006a; Willert et al., 2003). These modifications make Wnts highly hydrophobic, poorly soluble, and prone to localization close to cell membranes. The discovery that Wnts are palmitoylated helped develop a strategy for the first successful purification of Wnt proteins (Willert et al., 2003), which in turn has revolutionized the field of Wnt biology. However, not all Wnts have proven equally easy to purify. In fact, to date, other than Wnt3a, only Wnt5a (Mikels and Nusse, 2006b; Schulte et al., 2005) and Wnt2 (Sousa et al., 2010) have been successfully purified and revealed biological activity. This is why most studies regarding Wnt signaling use either Wnt3a or Wnt5a, the first two ligands

being purified. Difficulties in purifying other ligands most likely reflect differential lipid modification and/or glycosylation status of individual Wnt ligands.

In the last years, a horde of co-receptors, soluble antagonists, and other effectors of the Wnt signaling cascade have been identified, adding further complexity to understanding Wnt signals. In mammals there are 19 Wnt ligands, at least 15 receptors (10 Frizzleds, 2 Lrps, 1 Ryk and 2 Rors), 2 classes of inhibitors (Wnt-binding such as soluble Frizzled-related proteins (sFRPs), or receptor-binding such as Dickkopfs) and 2 classes of agonists (Norrin and R-spondins) (Castelo-Branco et al., 2003; Guo et al., 2007; Nusse, 2012; Veeman et al., 2003). Furthermore, considering that the intracellular signal transduction involves several proteins, phosphorylation events and feedback loops, it is easy to see the complex nature of this pathway. The following section of this thesis will discuss the separate Wnt pathways and the two Wnt ligands relevant to this thesis, Wnt1 and Wnt5a (Figure 6).

1.3.1. Wnt/β-catenin signaling pathway

The discovery that ectopic expression of the vertebrate *Wnt1* oncogene in *Xenopus* embryos resulted in axis duplication and two-headed frogs, solidified the role of Wnt as an important regulator of both embryonic development and oncogenesis (McMahon and Moon, 1989; Willert et al., 2003). In 1995, overexpression of certain domains of ß-catenin in *Xenopus* was also found to led to axis duplication, implying that ß-catenin had roles in signaling that were independent of its previously known role in cell adhesion (Funayama et al., 1995; Willert et al., 2003).

The most understood and documented Wnt pathway, the so-called canonical Wnt pathway, is transduced upon Wnt binding (such as Wnt1, Wnt3a and Wnt8) to members of two families of membrane receptors: Frizzleds (Fz) and low density-related lipoprotein receptors (LRP) 5/6. Upon binding, the ligand/receptor complex leads to a casein kinase 1 (CK1) γ-mediated phosphorilation of Dishevelled (Dvl) proteins (Davidson et al., 2005; Schulte et al., 2005; Zeng et al., 2005). Activated or phosphorylated Dvl, promotes disassembly of the destruction complex, consisting of Axin, Adenomatous Polyposis Coli (APC), CK1α and glycogen synthase kinase 3ß (GSK3ß) (Behrens et al., 1998; Itoh et al., 1998; Nusse, 2012). The activation and

membrane recruitment of Dvl recruits axin and the destruction complex to the plasma membrane, where axin directly binds to the cytoplasmic tail of LRP5/6, promoting its phosphorilation by GSK3ß. Destabilization of axin results in the release of ß-catenin from the destruction complex, making it active for signaling (McMahon and Moon, 1989; Tolwinski and Wieschaus, 2004). Dvl inhibition also prevents GSK3ß from phosphorylating ß-catenin, which would normally result in its ubiquitylation and targeting for degradation by the destruction complex.

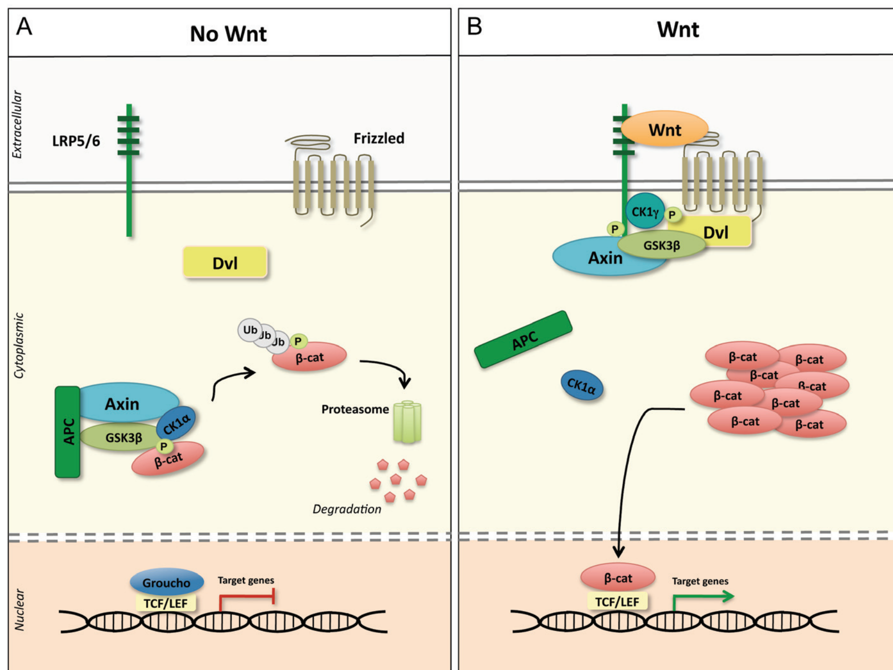

Figure 4 - Wnt/β-catenin signaling pathway

A) In the absence of Wnt, β-catenin is phosphorylated by a destruction complex composed of Axin, APC, GSK3β, and CK1α and subsequently degraded in the proteasome. **B)** Upon Wnt binding to LRP5/6 and Frizzled receptors, Dishevelled is recruited, interacting with Frizzled. This allows phosphorylation of LRP5/6 as well as inhibition of GSK3β and disassembling of the destruction complex. Dephosphorylated and activated β-catenin is no longer degraded and is stabilized in the cytoplasm. It translocates into the nucleus where it displaces repressors such as Groucho, binds to TCF/LEF transcription factors and promotes gene transcription.

As a consequence, free non-phosphorylated ß-catenin is stabilized and accumulates in the cytosol allowing its translocation into the nucleus, where it can bind to transcription factors of the lymphoid enhancer-binding protein (LEF)/ T-cell factor (TCF) family (Funayama et al., 1995; Hecht et al., 2000; Sun et al., 2000; Takemaru and Moon, 2000), thus activating the transcription of target genes. More than 100 direct target genes of Wnt/ß-catenin pathway have been identified, many being key regulators of cell cycle progression, cell fate specification, and differentiation (Davidson et al., 2005; Niehrs and Acebron, 2012; Nusse, 2012; Prakash and Wurst, 2007; Zeng et al., 2005).

In the absence of Wnt ligands, ß-catenin is recruited into the destruction complex, which facilitates the phosphorylation of ß-catenin by CK1α and then GSK3ß. This leads to the ubiquitylation and proteasomal degradation of ß-catenin (Aberle et al., 1997; Behrens et al., 1998; Itoh et al., 1998), maintaining the target genes in a repressed state in the nucleus by TCF/LEF transcription factors (which are rendered transcriptional repressors), associated with co-repressors of the Groucho family, and histone deacetylases (Cavallo et al., 1998; Chen and Struhl, 1999; O'Hara et al., 2005; Tolwinski and Wieschaus, 2004). When active, ß-catenin enters the nucleus and converts TCF/LEF to transcriptional activators in a coregulatory exchange that replaces Groucho with the histone acetylase CBP/p300 (cyclic AMP responsive element binding protein) (Adams et al., 2000; Hecht et al., 2000; Liu and Joyner, 2001a; Matsunaga et al., 2002; Simon et al., 2001; Sun et al., 2000; Takemaru and Moon, 2000)(Figure 4).

1.3.1.1. Wnt1: the ligand

Structurally, Wnt1 is a 41 kDA protein characterized by a cysteine-rich carboxy terminus, four potential glycosylation sites and a strongly hydrophobic amino terminus, which renders Wnt1 poorly soluble due to lipid modifications such as palmitoylation (Mikels and Nusse, 2006a; Willert et al., 2003), thus converting it into a membrane-anchored protein localized in specialized lipid rafts before secretion (Zhai et al., 2004). Wnt1 surpasses its insolubility in the aqueous space possibly by being transported by argosomes (extracellular vesicles that travel from cell to cell) (Greco et al., 2001; Katanaev et al., 2008; Kawano et al., 1995; Macdonald et al., 2009) and binding to lipoproteins (Macdonald et al., 2009; Mulligan et al., 2012; Panáková et al., 2005), which

allows short and long distance signaling. The developmental role of Wnt1 in the midbrain has been previously described in section 1.2.3.2.4 (Figure 6).

1.3.2. β-catenin-independent Wnt signaling pathways

As more Wnt isoforms were identified, it soon became clear that the Wnt/ß-catenin model was not sufficient to explain the actions of all identified Wnt isoforms. In early studies in *Xenopus,* overexpression of *Wnt5a* led to effects that were distinct from the axis duplication and neural malformations seen with *Wnt1, Wnt3* or *Wnt8* (Aberle et al., 1997; Du et al., 1995; Moon et al., 1993). In fact, in *Xenopus* this class of Wnts appeared to affect cell movements such as Planar Cell Polarity (PCP, the orientation of the cells within an epithelial plane), Convergence-Extension (CE, the narrowing and lengthening of a three-dimensional structure) and migration, rather than cell fate, which was different than what was observed Wnt/ß-catenin signaling. Moreover, these Wnts can even antagonize the ability of other Wnts to induce an ectopic axis (Cavallo et al., 1998; Chen and Struhl, 1999; Gieseler et al., 1999; Torres et al., 1996), pointing to an alternative Wnt-signaling pathway. Specific Wnts, such as *Wnt4*, *Wnt5a* and *Wnt11*, appear to activate "non-canonical" (ß-catenin-independent), rather than "canonical" pathways, although it has been argued that receptor expression patterns may, in fact, be more relevant than the ligand in the choice of downstream signaling (Beane et al., 2006; Habas et al., 2003; Kim and Han, 2005; Logan and Nusse, 2004; Schambony and Wedlich, 2007; Schlessinger et al., 2009; Tahinci and Symes, 2003).

ß-catenin-independent Wnt signaling pathways, as the name suggests, describes Wnt signals that are still driven by the binding of the Wnt ligand to Frizzled receptors, but whose downstream pathways are not mediated by GSK3ß or ß-catenin. ß-catenin-independent Wnt pathways are diverse and in many cases less characterized than the ß-catenin-dependent pathway. They are grouped into different categories for clarity and simplicity: Wnt/Ca^{2+} and Wnt/PCP pathways.

The term Wnt/Ca^{2+} signaling was coined after the observation that certain Wnts can lead to increases in intracellular calcium (Ca^{2+}) levels and activation of protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CamKII) in a ß-catenin-independent manner, upon binding to Fzd (Hecht et al., 2000; Kohn and Moon, 2005).

In the Wnt/PCP pathway, activated Dvl signals through small Rho GTPases such as Rho, Rac and Cdc42 (Choi and Han, 2002; Habas et al., 2003; Habas et al., 2001; Nusse and Varmus, 1982) and also through Jun-N-terminal kinase (JNK) (Boutros et al., 1998; Du et al., 1995; Moon et al., 1993; Yamanaka et al., 2002), leading to cytoskeletal modifications. Small GTPases have different intracellular targets and appear to constitute separate pathways (Beane et al., 2006; Gieseler et al., 1999; Habas et al., 2003; Kim and Han, 2005; Schambony and Wedlich, 2007; Schlessinger et al., 2009; Tahinci and Symes, 2003; Torres et al., 1996). Therefore, this thesis will describe Wnt/Rho and Wnt/Rac signaling pathways into more detail. Nevertheless, such classifications are not rigid as these pathways overlap with or intersect one another and are constantly evolving.

1.3.2.1. Wnt/PCP pathway

One of the most fascinating features of cells undergoing morphogenesis is their ability to communicate with each other within the epithelial or mesenchymal tissue, so that the cells orient and/or move in the same direction, in a coordinated way as a whole population. Achievement of this coordinated movement requires the integration of polarity of individual cells into the plane of a tissue. This process, referred to as planar cell polarity (PCP), was initially studied by genetic tools and described in the adult wing of *Drosophila M.* (Choi and Han, 2002; Gubb and García-Bellido, 1982; Habas et al., 2001; Habas et al., 2003).

In vertebrates, the molecular and cellular mechanisms underlying PCP-dependent processes remain largely obscure compared to those in *Drosophila*. These pathways are known to be involved in several diverged processes that require the coordination of cell polarity and cell cohesion within a cluster of cells in vertebrates. These processes include CE during gastrulation and neurulation, ear hair cell orientation, ciliogenesis, neural tube closure, and hindbrain neuron migration (Boutros et al., 1998; Darken et al., 2002; Goto and Keller, 2002; Shariatmadari et al., 2005; Yamanaka et al., 2002). During the development of the neural tube, groups of cells move in the same direction in a coordinated manner and these movements are regulated by PCP signaling. Although the final output is different in these developmental processes, a similar set of signaling components is required, which includes Frizzled (Fz), Dishevelled, (Dvl) and a set of

PCP-specific genes (Prickle, Strabismus/Vangl2, Flamingo/Celsr1, and Diego) (Nakatani et al., 2010; Seifert and Mlodzik, 2007). While Fz and Dvl are important intermediaries for its initial transduction in the PCP pathway, activation of Dvl (likely via phosphorylation) does not lead to the stabilization of ß-catenin or changes in calcium concentrations, such as in ß-catenin-dependent and Wnt/Ca^{2+} pathways, respectively. Instead, the PCP pathway functions via the activation of small GTPases from the Rho family, the heterotrimeric G proteins, and c-Jun N-terminal kinase (cJNK) (Choi and Han, 2002; Habas et al., 2003; Tahinci and Symes, 2003).

Small Rho GTPase proteins are molecular switches common to all eukaryotic cells that use the energy from GTP hydrolysis to modulate and control numerous aspects of actin filament dynamics and cytoskeleton structure. They cycle between an inactive (GDP-bound) and an active (GTP-bound) conformation, where they interact with effector proteins, and this transition is under the tight control of guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Kranewitter et al., 2001; Moon and Zheng, 2003; Olofsson, 1999). GTP-binding also influences the cellular localization of the Rho GTPases, with the GDP-bound state existing solely in the cytoplasm due to their association with GDIs. Cellular signals (e.g. growth factors) influence the activity of GEFs at the plasma membrane, which in turn, catalyze the exchange of GDP for GTP on Rho GTPases, thus promoting their activation.

The three best studied members of this family, RhoA, Rac1, and Cdc42, influence diverse processes such as adhesion, cell migration, morphogenesis and axon guidance. Moreover, they are also involved in cell polarity, endocytosis and cell cycle (Etienne-Manneville and Hall, 2002; Govek et al., 2005; Jaffe and Hall, 2005; Ng et al., 2002; Schlessinger et al., 2009). Rho GTPases seem to integrate Wnt-induced signals spatially and temporally to promote morphological and transcriptional changes affecting cell behavior (Witze et al., 2008). Indeed, they have shown to be able to compensate for PCP phenotype caused by loss of Dvl and Fz in *Drosophila* (Fanto et al., 2000; Strutt et al., 1997). Nevertheless, RhoA and Rac1 seem to regulate separate pathways as it has been shown that, in RhoA loss-of-function studies, CE defects cannot be rescued by Rac1, and conversely, Rac1 depletion cannot be rescued by RhoA (Habas et al., 2003; Kohn and Moon, 2005; Tahinci and Symes, 2003). RhoA is known to play a role in regulating the assembly of actin-myosin filaments to generate contractile forces, while Rac1 and Cdc42 promote actin polymerization at the cell periphery to generate

protrusive forces (Gubb and García-Bellido, 1982; Jaffe and Hall, 2005). The specific mechanical and chemical cues that modulate their activity to each of the above mentioned processes is likely to vary between different cell contexts, cell or tissue types, receptors, or stimuli. Although Rho GTPases integrate many signaling events by their interaction with multiple components, they may act more as permissive factors for cytoskeleton reorganization rather than as direct mediators (Song and Poo, 2001).

1.3.2.1.1. Wnt/Dvl/RhoA signaling

In vertebrates, certain Wnt ligands, such as Wnt5a and Wnt11, are capable of activating the small GTPase RhoA (Darken et al., 2002; Endo et al., 2005; Goto and Keller, 2002; Shariatmadari et al., 2005; Zhu et al., 2006). RhoA GTPase activity is primarily associated with the formation of stress fibers and contractile bundles. Its activity influences myosin light chain kinase, which in turn, causes increased myosin activity and contraction.

Upon binding of Wnt ligand to Fz receptor, the signal is transduced via Dvl to activate RhoA (Habas et al., 2001; Logan and Nusse, 2004; Schlessinger et al., 2009). It has been shown that RhoA can be activated upon Dvl overexpression (Habas et al., 2001) and Daam1 has been identified as critical regulator of this pathway, by mediating the interaction between Dvl and RhoA (Choi and Han, 2002; Habas et al., 2001; Habas et al., 2003). RhoA then activates Rho kinase (ROCK) (Jaffe and Hall, 2005; Leung et al., 1996; Leung et al., 1995), leading to the assembly of actin-myosin filaments and focal adhesion complexes (Endo et al., 2005; Ridley et al., 1992; Zhu et al., 2006). Indeed, RhoA has been shown to be required for CE movements in *Xenopus* and zebrafish (Habas et al., 2001; Schlessinger et al., 2009; Tahinci and Symes, 2003; Zhu et al., 2006), and can rescue the CE defects seen in *Wnt5a* and *Wnt11* zebrafish mutants (Habas et al., 2001; Zhu et al., 2006). Moreover, Daam1 is able, to some extent, to rescue the CE movement defects caused by Dvl loss of function during *Xenopus* embryogenesis (Leung et al., 1995; Leung et al., 1996; Liu et al., 2008a) (Figure 5).

1.3.2.1.2. Wnt/Dvl/Rac signaling

Rac GTPase activity is primarily associated with actin dynamics and actin-based structures in the lamellipodium, sheet-like membrane protrusions found at the leading edge of motile cells such as endothelial cells, neurons, immune cells and epithelial cells (Del Pozo et al., 2002; Woo and Gomez, 2006).

Rac role in PCP first emerged through experiments in which a dominant-negative protein was expressed in the *Drosophila* eye (Fanto et al., 2000). These studies concluded that Rac and Rho likely mediate distinct pathways downstream from Fz/Dvl (Habas et al., 2003; Ridley et al., 1992) and that Rac signals to the nucleus through the JNK/p38 MAP kinase cascades and the Fos and cJun transcription factors (Weber et al., 2008), though they likely promote cytoskeletal changes as well. Nonetheless, RhoA and Cdc42 have also shown to regulate JNK in the context of Wnt/PCP pathway. While activation of JNK by Wnt5a in *Xenopus* does not require Rac1, and instead requires Cdc42 (Habas et al., 2003; Hikasa et al., 2002; Schambony and Wedlich, 2007), Wnt5a activation of JNK in mammalian cells has been shown to require Rac1 activity, but not RhoA or Cdc42 (Andersson et al., 2008; Bryja et al., 2008; Habas et al., 2003). Therefore, evidence for direct target gene regulation via this signaling branch is unclear (Schambony and Wedlich, 2007; Yamamoto et al., 2009), if compared to numerous genes regulated by the Wnt/ß-catenin pathway (Boutros et al., 1998; Habas et al., 2003; Nishita et al., 2010; Nusse, 2012; Rosso et al., 2005).

To add even more complexity to this pathway, Ror receptors have been described to be involved in Wnt-mediated Rac1 activation in mouse cells, together with Frizzled receptors (Nishita et al., 2010; Nishita et al., 2006; Oishi et al., 2003; Sato et al., 2010), explaining how Wnt signal diversity is achieved. Both Ror2 and Wnt5a exhibit overlapping expression patterns, phenocopying one another when deleted. Wnt5a is thought to bind to the orphan tyrosine kinase receptor Ror2, activating JNK signaling (He et al., 2008; Liu et al., 2008b), and this has been shown to require Rac1 activation, but not RhoA or Cdc42 (Habas et al., 2003). On the other hand, Ror2 has been demonstrated to be involved in the transduction of Wnt5a-mediated Cdc42 activation in *Xenopus* (Schambony and Wedlich, 2007). As JNK has also been described to directly regulate cytoskeleton rearrangements (Bishop and Hall, 2000; Kim and Han, 2005; Schambony and Wedlich, 2007; Xia and Karin, 2004), the exact contribution of nuclear JNK signaling to the Wnt/PCP pathway remains unclear (Figure 5).

1.3.2.1.2.1. Tiam1: a Rac GEF

Little is known about the immediate upstream regulators of RhoA and Rac1 - the GEFs and GAPs - during PCP. All GTPases have at least one GEF that regulates the exchange of GDP for GTP, promoting a great diversity in this group of proteins. One of the most studied GEFs that regulates the small GTPases of the Rho family is T-cell lymphoma invasion and metastasis 1 (Tiam1). Tiam1, a GDP–GTP exchange factor for the small GTPase Rac, was originally implicated in tumor invasion and metastasis in T-lymphoma cells. Interestingly, it is also expressed in the developing CNS, and has shown to increase cellular migration in fibroblasts and to promote neuronal cell migration (Ehler et al., 1997; Leeuwen et al., 1997; Michiels et al., 1995). Indeed, Tiam1 is expressed at low levels in most tissues but at markedly higher levels in brain and testis (Ehler et al., 1997; Habets et al., 1995). It seems that Tiam1 is required in cytoskeletal reorganization during cell migration and neurite extension in defined neuronal populations (Ehler et al., 1997). Furthermore, in neuroblastoma cells, overexpression of Tiam1 results in cell spreading and adhesion, but also affects neurite outgrowth (Leeuwen et al., 1997). These effects are dependent on Rac and can be abrogated by co-expression of constitutively active Rho (Leeuwen et al., 1997). Tiam1 overexpression caused membrane ruffling and cytoskeletal changes in a Rac1-dependent manner (Michiels et al., 1995), demonstrating a specific nucleotide exchange ability for Rac *in vivo*, although it has also demonstrated GEF activity *in vitro* for Cdc42 and RhoA (Michiels et al., 1995). However, the possible involvement of Tiam1 in Wnt/PCP signaling has not been addressed prior to this work.

1.3.2.1.3. Wnt/Dvl/Cdc42 signaling

During vertebrate CE movements, cells elongate and intercalate at the midline. Activation of Fz promotes Rho, Rac, and Cdc42 activation, required for several different outcomes: Rac and Cdc42 are required for polarized protrusive activity and cell elongation. A dominant-negative form of *Drosophila* Cdc42 expressed in the wing resulted in the failure of cells to elongate in the apical-basal direction, in part because of misorganization of the actin cytoskeleton (Eaton et al., 1995). Cdc42 was also required for proper wing hair formation, specifically for actin polymerization in the wing hair

(Eaton et al., 1996). Dominant-negative Cdc42 was also able to rescue the impairment of cell–cell adhesion induced by overexpression of Wnt5a (Choi and Han, 2002). The observations that Cdc42 can rescue Wnt11 and Wnt5a-induced phenotypes through a Dvl-independent route involving heterotrimeric G proteins and PKC, suggest that both Wnt ligands may influence cell–cell adhesions during CE through Cdc42. In addition, Cdc42 activation in *Xenopus* has been recently shown to be under the control of Wnt5a and Ror2, thus regulating the CE movements via activation of JNK (Schambony and Wedlich, 2007) (Figure 5).

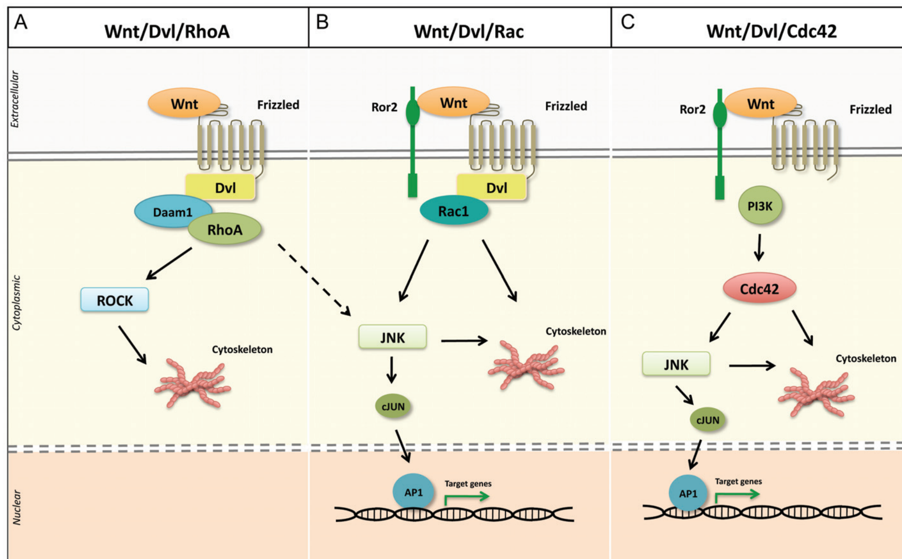

Figure 5 - Wnt/PCP signaling pathways

Wnt/Dvl/RhoA (A), Wnt/Dvl/Rac (B) and Wnt/Dvl/Cdc42 (C) signaling pathways are depicted in the picture. **A)** Wnt/Dvl/RhoA pathway: Wnt binds to Frizzled and via the Dvl/Daam1 complex induces activation of RhoA which regulates cytoskeleton via its effectors. Dashed arrow indicates a possible activation of JNK (see main text). **B)** Wnt/Dvl/Rac pathway: Wnt binds to Frizzled and Ror2. Subsequently, the signal from the receptor complex is transduced via Dvl to activate Rac1. Rac1 induces changes in cytoskeleton via set of effectors (not depicted) and/or via activation of JNK. JNK can also phosphorylate transcription factor cJUN, which enters the cell nucleus and together with transcription factor cFos forms the AP1 complex, which regulates gene expression. **C)** Wnt/Cdc42 pathway: Wnt binds to Ror2 and Cdc42 is activated in a PI3K-dependent manner. It can regulate either cytoskeleton or gene expression via activation of the JNK/cJUN cascade (AP1 – complex of cJUN and cFos).

1.3.2.2. Wnt5a: the ligand

Wnt5a is a highly conserved diffusible protein, initially attributed to the ß-catenin-independent class of Wnts due to lack of axis duplicating and transforming ability (Nusse, 2012; Torres et al., 1996; Wong et al., 1994). However, it is known that the classification of Wnts depending on their transforming activity does not necessary reflect their specific signaling pathways and downstream events. It is now believed that Wnt5a signal is transduced by Fz receptors and/or co-receptors including the atypical tyrosine kinases Ryk and Ror2 (discussed in section 1.3.2.1.2). Dependent on the receptor and cell type, Wnt5a has been shown to activate three signaling pathways: the Wnt/ß-catenin pathway, the Wnt/Ca^{2+} pathway, and the Wnt/PCP pathway (Mikels and Nusse, 2006b; Qian et al., 2007; Witze et al., 2008).

To illustrate this, Wnt5a overexpression in *Xenopus* embryos, induced defective morphogenic movements (Castelo-Branco et al., 2003; Moon et al., 1993), such as incomplete closure of the anterior tube and a shortened anterior–posterior axis, which are distinct from those achieved by Wnt1, Wnt3a and Wnt8. Moreover, *Wnt5a* mRNA injection in zebrafish and *Xenopus* embryos revealed that expression of Wnt5a stimulates intracellular Ca^{2+} by activating Ca^{2+}-dependent effector molecules such as CamKII, NFAT, and PKC (Malbon and Karoor, 1998; Schambony and Wedlich, 2007; Yamamoto et al., 2009). On the other hand, Wnt5a has also been shown to inhibit Wnt/ß-catenin signaling (Moon et al., 1993). Co-expression of Wnt5a with Wnt8 (a well described ß-catenin-dependent Wnt) in *Xenopus* abolishes the ability of Wnt8 to induce secondary axis formation (Bishop and Hall, 2000; Torres et al., 1996; Xia and Karin, 2004). Conversely, Wnt5a has been shown to activate TCF when co-expressed with Fzd4 and Lrp5 (Mikels and Nusse, 2006b), to activate ß-catenin when fused to the Lrp5/6-binding domain of Dkk2 (Liu et al., 2005) and to induce axis duplication in the presence of Fzd5 (He et al., 1997), indicating that Wnt5a-mediated inhibition or activation of the ß-catenin pathway might be dependent on the receptor context.

Despite the cumulative knowledge, little is known about which of these pathways and downstream signaling components mediate Wnt5a's influence on axon growth and guidance. Because the ß-catenin-independent pathway includes multiple signaling cascades in addition to the PCP and Ca^{2+} pathway, Wnt5a regulates a variety of cellular functions, such as proliferation, differentiation, migration, adhesion and polarity.

First detection of Wnt5a is observed during gastrulation. Wnt5a$^{-/-}$ mice suffer perinatal lethality by asphyxia due to the severe anterior-posterior extension defects (Yamaguchi et al., 1999). In these mice, VM morphology and DAergic population distribution are altered, indicating that Wnt5a mediates several functions in the VM and DAergic neuron development (Andersson et al., 2008). In the midbrain, Wnt5a is detected as early as E8.75 (Yamaguchi et al., 1999), continuing throughout DAergic development (Andersson et al., 2008; Castelo-Branco et al., 2003). Wnt5a was therefore uncovered as a critical regulator of DAergic differentiation (Inestrosa and Arenas, 2010) (Figure 6).

Purification of Wnt5a (Schulte et al., 2005) highlighted its potential to induce DAergic differentiation of Nurr1-positive precursors into TH-positive DAergic neurons in primary mesencephalic neuronal cultures (Andersson et al., 2008; Castelo-Branco et al., 2003), in VM-derived neurospheres (Parish et al., 2008), in a DAergic cell line (Bryja et al., 2007b; Schulte et al., 2005) and in ES cells (Lonardo et al., 2010; Sanchez-Pernaute et al., 2008), through activation of Dvl (Bryja et al., 2007b; Schulte et al., 2005) and Rac1 (Andersson et al., 2008). This differentiation occurs without increasing the total number of precursors, suggesting that its effects are specific to the maturation process. Moreover, Wnt5a induced increase in *Otx2* and *En1* mRNA levels (Castelo-Branco et al., 2003). Interestingly, VM glia has shown to secrete Wnt5a (Castelo-Branco et al., 2003), suggesting a role for glia-derived Wnts in the regulation of DAergic neuron development by embryonic glia (Hall et al., 2003; Wagner et al., 1999).

On the other hand, a transient increase in the number of proliferating progenitors and Nurr1-positive precursors is detected in Wnt5a-/- mice, plus an impairment in the DAergic differentiation at E12.5, which is later recovered (Andersson et al., 2008). Finally, Wnt5a-transfected VM neurospheres grafted into 6-OHDA-lesioned rats, have shown an improvement in behavior compared to control-transfected grafts (Parish et al., 2008). Similar results were achieved with Wnt5a-treated parthenogenetic primate ES cells, which also revealed improved DAergic neuron differentiation, graft survival and behavioral recovery in 6-OHDA-lesioned rats (Sanchez-Pernaute et al., 2008). While these reports support a role for Wnt5a in developing DAergic precursors both *in vitro* and *in vivo* (Figure 6), it remains to be determined how this differentiation is achieved, and through which pathways is it regulated.

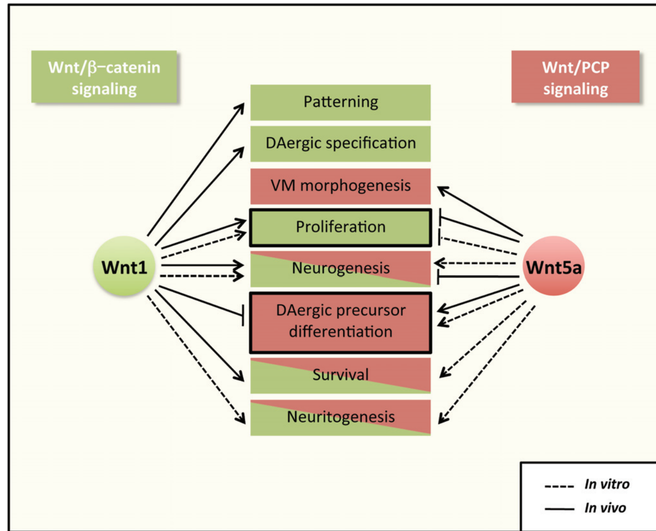

Figure 6 - Function of Wnt1 and Wnt5a in midbrain dopaminergic neuron development

Midbrain development depends on the sequential regulation of multiple functions by cell-intrinsic and cell-extrinsic factors, such as Wnts. The delicate balance between activation of distinct Wnt signalling pathways by ligands such as Wnt1 (which signals through the Wnt/β-catenin pathway) and Wnt5a (which signals through the Wnt–planar cell polarity pathway) controls midbrain dopaminergic neuron development. Loss- and gain-of-function experiments in vivo (continuous arrows) and in vitro (discontinuous arrows) have contributed to our understanding of this balance. Arrowheads indicate activation; blunt-ended arrows indicate inhibition. Green shading indicates functions controlled by Wnt1; red shading indicates functions controlled by Wnt5a. The black bold box indicates a positive by regulation by one Wnt and opposing action of the other Wnt. VM, ventral midbrain. [adapted from (Inestrosa and Arenas, 2010)].

AIMS

2. AIMS

Understanding the molecular mechanisms underlying midbrain dopaminergic development is of the utmost importance for the successful implementation of CRT in PD. Wnt signaling has been shown to play critical roles throughout ventral midbrain dopaminergic development. In the broader sense, this thesis examines the function of two key signaling players, Wnt1 and Wnt5a in midbrain dopaminergic neuron development. Our work comprised the following objectives:

Study I:

- To identify genes that are regulated by Wnt1 signaling in midbrain dopaminergic progenitors in culture.
- To evaluate the role of Wnt1 overexpression on ventral midbrain DAergic neuron differentiation.

Study II:

- To identify novel components and regulators of the Wnt5a/PCP/Rac1 pathway.
- To investigate the function of the Rac1 GEF, Tiam1, in the differentiation of midbrain DAergic precursors into DAergic neurons by Wnt5a.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. *Ventral Midbrain Precursor Cultures*

Ventral midbrain (VM) precursor cultures were grown as neurospheres, as previously described (Parish et al., 2008), with slight variations according to the experimental design. For Wnt1 gene regulation studies, VMs from embryos at stage E10.5 were collected from CD-1 mice. Embryonic brains were dissected in ice-cold PBS supplemented with 0.2% glucose and mechanically dissociated with a fire-polished Pasteur glass pipette to single-cell . Cells were plated at a final density of 1 – 1.5 x 10^5 cells/cm^2 on uncoated wells for a suspension culture and kept in an incubator at 37°C with 5% CO2. The medium for neurosphere culture consisted of 1:1 mixture of Minimum Essential Medium and Ham's F12, which is supplemented with N2 supplement, 15 mM HEPES, 1 mM Glutamine, 3 mg/ml AlbuMAX (all from Gibco) and 6 mg/ml Glucose. To induce proliferation cultures were supplemented with basic fibroblast growth factor (FGF2) 10 ng/ml, brain-derived neurotrophic factor (BDNF) 20 ng/ml and B27 supplement (1:50, Gibco) during the first 5 days *in vitro* (DIV). Fresh media was changed every second day. After 5 DIV, neurospheres were collected by centrifugation at 500 rpm for 3 minutes, resuspended in N2 medium without factors and plated for differentiation at a final density of approximately 50 spheres/cm^2 in poly-D-lysine (PDL)-coated wells (10 μg/ml, Sigma). No patterning factors were added to the media in these experiments to avoid interference in the Wnt1 gene regulation. Differentiated cells were collected for analysis 3 DIV later.

In Tiam1 siRNA experiments, VMs were dissected of E10.5 mouse embryos, enzymatically dissociated to single-cell with collagenase/dispase for 30min at 37°C in a rocking platform, followed by mechanical dissociation. Next, cells were plated at a density of 100000 cells/cm^2 in 1 ml of N2 media (supplemented with 250 ng/ml Shh, 25 ng/ml FGF8, and 20 ng/ml FGF2) and grown as neurospheres for 7 DIV. 0.5 ml of fresh media was added every second day. The factors Shh and FGF8 were added to the media in order to promote specification of VM DAergic neurons. At day 7, spheres were collected and dissociated into small cell clusters (collagenase/dispase treatment plus mechanical trituration), which were then seeded at a density of 100000 cells/cm^2 on

PDL/laminin pre-coated plates in N2 media (no growth factors) and transfected with appropriate siRNA (50-100 nM) using Lipofectamine 2000. Fresh N2 media was added 24h after transfection (supplemented with BDNF and GDNF to final concentration 20 ng/ml of each, to promote cell survival). Cells were harvested 3 days after the transfection.

For Tiam1 shRNA lentiviral knockdown studies, the VM tissue was dissected and plated as described above for Tiam1 siRNA studies. The cells were transduced immediately after dissection (DIV 0) in N2 media containing Shh (250 ng/ml), FGF8 (25 ng/ml) and FGF2 (20 ng/ml) with 1×10^7 to 3×10^7 transducing units per mL, as detailed in section 3.4. 48 hours after dissection, the media was replaced with fresh one, removing all viral particles. Cells were grown as neurospheres for 5 DIV, with addition of fresh media every 2—3 days. At day 5, cells were collected and seeded at an approximate density of 50 spheres/cm^2 on PDL/laminin pre-coated plates in N2 media (with BDNF and GDNF, 20 ng/ml each) and differentiated for 3 DIV.

CD1 mice (Charles River Breeding Laboratories) were housed, bred, treated and sacrificed according to Karolinska Institute guidance for animal experiments, and to the guidelines of the European Communities Council (directive 86 609 EEC). All experiments were approved by the local ethical committee (ethical permit number N145/09 from the *Stockholms Norra Djurförsöketiska Nämnd*, Sweden).

3.2. *Cell Culture, Transfection, and Treatments*

The mouse SN4741 DAergic cell line (Son et al., 1999) was propagated as previously described (Bryja et al., 2007a; Schulte et al., 2005). For the purpose of transient gene overexpression, cells were transfected using Superfect (Qiagen) according manufacturer's instructions. Following constructs were used: MYC-Dvl2 (Lee et al., 1999; Torres et al., 1996; Wong et al., 1994), FLAG-Dvl3, Dvl2-EGFP (Chen et al., 2003; Malbon and Karoor, 1998), HA-Dvl2, FLAG-Tiam1 (Martin et al., 2006; Tolias et al., 2005), MYC-Rac1 (Ridley et al., 1992; Swift et al., 2001). For experiments involving siRNA, the SN4741 cells were transfected with 50-100 nM siGENOME Tiam1 siRNA (SMARTpool M-047-08-01) or non-targeting siRNA (both from Dharmacon) using Lipofectamine 2000 (Invitrogen) according manufacturer's recommendations. The

amount of siRNA was determined through function of the target and the efficiency of the silencing was assessed by western blotting or quantitative real-time PCR (qPCR). For immunocytochemistry, SN4741 cells (10,000-20,000 cells/cm^2) were grown overnight on glass coverslips and transfected with the indicated plasmids. 24 hours post-transfection, cells were fixed in 4% paraformaldehyde. For analysis of cellular signaling, SN4741 cells were stimulated with 100 ng/ml of mouse Wnt5a (R&D Systems) for 2.5 h. Control stimulations were done with vehicle (0.1% BSA, 0.05 % CHAPS in PBS).

For viral production, the following cells lines were used: HEK293T and human Phoenix gag-pol packaging cell line (www.stanford.edu/group/nolan/retroviral_systems/phx.html) (Swift et al., 2001; Torres et al., 1996). These and the HEK293A cell line were grown in DMEM containing 10% FCS, 2mM L-glutamine, 50 units/ml penicillin, and 50 units/ml streptomycin. Transient transfections of HEK293A were carried out with PEI (Polyethylimin) with ratio 1 μg DNA per 3 μl of PEI in 100ul serum free media, incubating the mixture for 10 minutes at room temperature, and adding the mixture drop wise to HEK293A cells in their culture media.

3.3. *Retroviral vectors construction, production and transduction*

The Wnt1 cDNA fragment was isolated from pCA-Wnt1-HA vector by PCR using the following primers containing Not1 and Bgl2 restriction sites, respectively: 5'-TTT GCG GCC GCA TGG GGC TCT GGG CGC T-3' and 5'- TCT GCA CGA GTG TCT ATC CAG ATC TTT T -3'. The resulting PCR fragment was then inserted into the pPRIG-IRES-eGFP retroviral vector, kindly provided by Dr. Philippe Pognonec (http://www.unice.fr/FRE3094/PRIG/PRIG_home.html) (Martin et al., 2006; Parish et al., 2008). These bi-cistronic retroviruses encode an internal ribosomal entry sequence (IRES2, Clontech) and enhanced green fluorescent protein (eGFP, Clontech). Control retrovirus contained no additional cDNA (IRES2-eGFP). Cloning procedures such as restriction, digestion, dephosphorylation of DNA fragments, ligations, and transformation of newly generated vectors in *Escherichia coli* and separation of DNA in agarose gels were done according to Sambrook *et al.* (Angers et al., 2006; Parish et al., 2008; Sambrook and Russell, 2001).

For retroviral production, 8 - 10 x 10^4 Phoenix gag-pol packaging cells/cm^2 were platted in 10 cm Petri dishes. Phoenix gag-pol cells were then transiently transfected with 24 μg of retroviral vector and 2,5 μg of pVSV-G (Vesicular stomatitis virus glycoprotein) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the medium was changed and cells were placed at 32°C. Forty-eight hours after transfection, culture supernatant containing VSV-G pseudotyped viral particles was harvested, passed through 0,45 μm supor membrane (PALL Life Sciences) to remove cell debris, and used for infection or stored at -80ºC until further use.

For viral transduction, cells were incubated with the viral supernatant containing polybrene (hexadimethrine bromide: 2 μg/ml, Sigma) for 12-24 hours, immediately after dissection and at DIV 1. After 2 DIV, the small spheres were briefly centrifuged at 500 rpm for 2 minutes to remove the viral supernatant, and fresh N2 medium supplemented FGF2, B27 and BDNF was added. Cells were grown as neurospheres for 5 days, with addition of fresh media every 2-3 days, and differentiated as described in section 2.1. GFP-transduced control cultures were expanded and differentiated in the same way. These experiments were performed in biosafety level 2 laboratories.

3.4. *shRNA lentiviral production and transduction*

Third-generation replication-incompetent lentiviral vectors (pLKO.1-CMV-tGFP) encoding for either non-targeting shRNA or Tiam1 shRNA (TRCN0000042594 and TRCN0000042595, Sigma), were produced by transient transfection of HEK293T cells with Lipofectamine 2000, using a four-plasmid system, as previously described (Bryja et al., 2007a; Cajanek et al., 2009; Hottinger et al., 2000; Schulte et al., 2005). The viral particles were pseudotyped with the vesicular stomatitis virus G-protein encoded by the pMD.2G plasmids described previously (Dull et al., 1998; Lee et al., 1999; Torres et al., 1996; Wong et al., 1994). The packaging construct used in this study was the pMDLg/RRE (Chen et al., 2003; Dull et al., 1998; Malbon and Karoor, 1998). The Rev gene was inserted in the pRSV-Rev plasmid, to decrease the risk of recombination and production of replication-competent retroviruses. Forty-eight hours later, the supernatant was collected and filtered through a 0.45 μm filter. High-titer stocks were

obtained by centrifugation for 2 hours at 50000 G. The pellet was resuspended in PBS and 1% BSA and stored frozen at -80°C until further use. The titers of the lentiviral vector encoding GFP were determined on HEK 293T cells. The cells were plated at a density of 1×10^5 cells per cm^2. Serial dilutions of the viral stocks were added, and the number of GFP-infected cells was analyzed 48-72 hr later. Titers were calculated by counting the frequency of green cells per well and dividing it by the dilution factor. The titers of the vectors used in this study were in the range of 1×10^7 to 1.5×10^7 transducing units per mL. A multiplicity of infection of 1-2 was used for VM primary cells.

VM tissue was dissected and plated as previously described. The cells were transduced immediately after dissection in N2 media containing Shh, FGF2, FGF8 and polybrene (2 μg/ml). After 24 hours in media containing lentivirus, the cells were switched into fresh media, and grown as neurospheres for 5 days. Fresh media was added every 2–3 days for the duration of the culture period. At day 5, spheres were collected and seeded at a density of 50 spheres/cm^2 on PDL/laminin pre-coated plates in N2 media supplemented with BDNF and GDNF (20 ng/ml each) and differentiated for 3 days. The efficiency of the silencing was determined by qPCR. These experiments were performed in biosafety level 2 laboratories.

3.5. *Immunoprecipitation, western blotting, and densitometry analysis*

Cells extract were obtained 24-36 hours after transfection, HEK293A cells were harvested for total cell extract in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.4, 1mM EDTA, 0.5% NP-40, protease inhibitor cocktail (Roche) or RIPA + 0.05 % SDS buffer (for Rac1 immunoprecipitations). Cell extracts were cleared from cell debris by centrifugation (16000g/15min/+4°C) and supernatants were incubated with protein G sepharose (GE Healthcare) (1h/+4°C/orbital shaker) to pulldown non-specific interactors. Subsequently, pre-cleared extracts obtained after centrifugation (200g/5min/+4°C) were incubated with 1 μg (10 μg in case of IPs for MS analyses) of following antibodies: mouse monoclonal anti-Rac1 (clone 23A8, Millipore), rabbit polyclonal anti-Tiam1 (sc-872), mouse monoclonal anti-MYC (sc-40), mouse monoclonal anti-Dvl3 (sc-8027) (Santa Cruz Biotechnology), rabbit polyclonal anti-FLAG (F7425, Sigma), for 30min/+4°C/ orbital shaker and subsequently with protein G sepharose.

Following overnight incubation, samples were rigorously washed with RIPA buffer and subsequently analyzed by western blotting. Sample preparation and western blotting was done as described before (Cajanek et al., 2009; Dull et al., 1998). The secondary antibodies used were: HRP-conjugated anti-mouse secondary antibody (GE Healthcare), and HRP-conjugated anti-rabbit secondary antibody (Sigma) (1:5000). Luminescence was detected by either film exposure (Agfa), or ChemiDoc XRS system (Biorad) using the ECL system (GE Healthcare). Non-saturated images, within dynamic range of the CCD camera were chosen as representative and used for densitometric analyses.

3.6. *GTPase pull-down assay*

Cells were harvested in GTPase lysis buffer (150 mM NaCl, 10 mM Tris-Cl pH 7.4, 10 mM $MgCl_2$, 1% Triton-X100, 0.1% SDS, 1 mM DTT, protease inhibitor cocktail (Roche)). The pulldowns were performed with recombinant GST-PAK-CRIB as described before (Bryja et al., 2008; Dull et al., 1998). For experiments with Tiam1 siRNA, the SN4741 cell were seeded out (750,000 cells/6-well plate), transfected first with either control or Tiam1 siRNA 24 hours after seeding, then transfected with indicated expression constructs after additional 24 hours, processed 24 hours after the last transfection, and subsequently analyzed by western blotting. In experiments involving Dvl overexpression MYC-Rac1 was co-transfected, changes in Rac1 activity were subsequently measured for MYC-Rac1. A densitometry analysis was performed using Image J software, where indicated. Level of activated Rac1 (GTP-Rac1) was normalized to level of Rac1 in total cell lysate (TCL).

3.7. *RT-PCR and quantitative real-time PCR (qPCR)*

Total RNA from either SN4741 cells or VM cultures was extracted using RNeasy extraction kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For reverse transcription, 100 ng - 1 μg of total RNA was initially treated with 1 unit of RQ1 RNase-free DNase (Promega) for 40 min. The DNase was inactivated by the addition of 1 μl of 0.02 M EDTA and incubation at 65ºC for 10 min. Random primers (0.5 μl; Life

Technologies, Grand Island, NY) were then added, and the mixture was incubated at 65ºC for 10 min. Each sample was then equally divided in two tubes: a cDNA-reaction tube (RT+) and a negative-control tube (RT-). A master mixture (1X first-strand buffer (Life Technologies)/0.01 M DTT (Life Technologies)/0.5 mM deoxynucleoside triphosphates (Promega)) was then added to both RT+ and RT- tubes and incubated at 25ºC for 10 min and then at 42ºC for 2 min. Two-hundred units of SuperScript II RT (Invitrogen) was then added only to the RT+ tubes, and all samples were incubated at 42ºC for 50 min. SuperScript II was inactivated by incubation for 10 min at 70ºC. Both reactions RT+ with cDNA and RT– were then diluted 5-10 times for further analysis.

Real-time RT-PCR was performed 96-well plates, in triplicate for each sample with 2 µl of cDNA (1:5 - 1:10 dilution) in a total volume of 10 µl per well. Each PCR consisted of 0.3 µM each of the forward and reverse primers and 1× SYBR green PCR Master Mix (Applied Biosystems). The PCR was performed with the Applied Biosystems 7900HT Fast Real-Time PCR System and the reaction started with 50°C for 2 min and 95°C for 10 min for enzyme activation, followed by 40 cycles, which each consisting of 95°C for 15 s for melting and 60ºC for 1 min for annealing/extension. A melting curve was obtained for each PCR product after each run to confirm that the SYBR green signal corresponded to a unique and specific amplicon.

GenBank mouse and human cDNA sequences were used in PRIMER EXPRESS 1.0 (Applied Biosystems) and PRIMER 3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) for primer design. The following oligonucleotides were used: Wnt1 primers (forward: CTT CGG CAA GAT CGT CAA CC, reverse: GCG AAG ATG AAC GCT GTT TCT), TH primers (forward: AGT ACT TTG TGC GCT TCG AGG TG, reverse: CTT GGG AAC CAG GGA ACC TTG), Nurr1 primers (forward: CAG CTC CGA TTT CTT AAC TCC AG, reverse: GGT GAG GTC CAT GCT AAA CTT GA), Pitx3 primers (forward: TTC CCG TTC GCC TTC AAC TCG, reverse: GAG CTG GGC GGT GAG AAT ACA GG), Otx2 primers (forward: TGT AGA AGC TAT TTT TGT GGG TGA, reverse: GAG CAT CGT TCC ATC TAA CTT TTT), Ngn2 primers (forward: CGG GTC AGA CGT GGA CTA CT, reverse: GAG GAC GAG AGA GGG AGA CC), FoxA2 primers (forward: CAT CCG ACT GGA GCA GCT A, reverse: CAT AGG ATG ACA TGT TCA TGG AG), Lmx1a primers (forward: GAC AAG AAG CTC TAC TGC A, reverse: CTC ATA GTC CCC TTT GCA GA), Lmx1b primers (forward: GCT GTG CAA GGG TGA CTA T, reverse: GCT GCC AAT GTC TCT CGG A), En1 primers (forward: GAC TCA CAG

CAA CCC CTA GT, reverse: GCC GGT GGC TTT CTT GAT CTT), Shh primers (forward: GAG CAG ACC GGC TGA TGA CTC A, reverse: CTC GAC CCT CAT AGT GTA GAG A), Tiam1 primers (forward: GAG CCA GAG GGA GGC GTG GA, reverse: TGG CAT CCT GAG GGG ACG GG) and Rac1 primers (forward: CGT CCC CTC TCC TAC CCG CA, reverse: CTT TCG CCA TGG CCA GCC CC). Quantum RNA classical 18S internal standard kit (Ambion, Austin, TX) was used as an internal control. Apart from 18S, all the remaining primers were purchased from Eurogentec (Seraing, Belgium), DNA Technology (Aarhus, Denmark) and Eurofins MWG Operon (Ebersberg, Germany).

Relative mRNA expression levels was obtained using 2^{-ddCt} method as fold change versus control (Livak and Schmittgen, 2001) by subtracting the Ct value of each sample by the value of the housekeeping gene encoding 18s rRNA (dCt values), and then subtracting the control. 2^{-ddCt} values were linearized through log2 transformation prior to statistical analysis. In the Wnt1 gene regulation study, the different populations were grouped according to the dCt values: low Wnt1 experiments had dCt>20.6, intermediate Wnt1 had dCt between 16-20.6 , and high Wnt1 had dCt<16.

3.8. *Immunocytochemical analysis*

For immunocytochemistry experiments, cells were fixed in 4% paraformaldehyde (15-30 minutes at room temperature), blocked in 5% normal donkey or goat serum and 0,25% Triton X-100 for 1 hour at room temperature and incubated in the appropriate primary antibody with 2% serum and 0,25% Triton X-100 overnight at 4ºC. Cells were then washed three times with PBS and then incubated with the respective secondary antibody with 2% serum and 0,25% Triton X-100 for 1 hour at room temperature. After two more washes with PBS, cell nuclei were stained by Hoescht or DAPI for 10 minutes at room temperature. The following antibodies were used: rabbit polyclonal anti-TH (1:500, Pel-Freez), mouse monoclonal anti-TH (1:400, Sigma), mouse anti-GFP (1:75, Millipore), rabbit anti-GFP (1:800, Invitrogen), mouse monoclonal anti-ßIII tubulin (Tuj, 1:1000, Promega), mouse monoclonal anti-Rac1 (1:100, Millipore), rabbit polyclonal anti-FLAG (1:1000, Sigma), rabbit polyclonal anti-MYC, mouse monoclonal anti-MYC (both 1:1000, Santa Cruz Biotechnology), mouse monoclonal anti-FLAG (1:2000, Sigma),

rabbit polyclonal anti-Dvl2 (1:500, sc-13974 Santa Cruz Biotechnology), Alexa Fluor 488 goat anti-mouse or anti-rabbit and Alexa Fluor 555 donkey anti-mouse or anti-rabbit (all 1:1000, Molecular Probes, Invitrogen).

The DAergic neurons numbers were counted as either number of TH-positive cells per area of Tuj1-positive cell clusters (10 randomly selected observation fields per well, Tiam1 siRNA experiments) or number of TH-positive cells per area of DAPI-positive clusters (all spheres within the well analyzed, Wnt1 gene regulation and Tiam1 shRNA experiments). Area of DAPI-positive clusters was measured in pixels^2, and subsequently converted into μm^2 (1 square pixel = 0.6209 square μm, for 10x objective and 2x zoom). Two to three wells per condition were analyzed in each experiment. Fluorescent labeling was examined using a Zeiss LSM5 Exciter inverted confocal scanning laser microscope and Olympus FV1000 Confocal Microscope.

3.9. *Statistical analysis*

Statistical analysis (Student's t-test, Mann-Whitney test, one-way ANOVA followed by Neuman-Keuls multiple comparison test, correlation and linear regression) was performed using Prism 4 and 5 (GraphPad Software). $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were considered as a statistically significant difference. Results are presented as mean $\pm$ standard error of the mean (SEM) of three to ten independent experiments.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1. *Wnt1 dose-dependently regulates genes involved in midbrain dopaminergic development*

4.1.1. Rationale

Midbrain DAergic neurons are generated in three developmental stages from approximately E7.5–14.5 in mouse: patterning, specification, and neuronal differentiation (Ang, 2006). Elucidating the function of transcription factors, signaling molecules, and their interactions during VM development has been essential for the ability to manipulate stem cells and iPSCs for cell-based therapies (Kim et al., 2002; Lee et al., 2000; Soldner et al., 2009). However optimal differentiation to authentic mesDAergic neurons requires further understanding of the molecular mechanisms underlying mesDAergic neuronal development. Toward this goal, numerous laboratories investigated mesDAergic neuron development, resulting in identification of important signaling molecules (e.g., SHH and Wnt1) and key transcription factors (e.g., FoxA2, Lmx1a, Lmx1b, Msx1, Ngn2, Nurr1, and Pitx3) [reviewed in (Ang, 2006; Smidt and Burbach, 2007)]. However, molecular interactions/networks between these extrinsic factors and intrinsic transcription factors are not well understood.

Wnts are acknowledged to be master regulators of fetal brain development and continue to direct neuronal growth and survival in the adult brain [reviewed in (Inestrosa and Arenas, 2010; Lie et al., 2005)]. Wnt1, an activator of Wnt/ß-catenin pathway, is known to be essential for proper development of the midbrain DAergic system (Castelo-Branco and Arenas, 2006; Inestrosa and Arenas, 2010; Prakash and Wurst, 2006). Previous studies have shown that Wnt1 null embryos have a loss of the entire midbrain (McMahon and Bradley, 1990; Panhuysen et al., 2004; Prakash and Wurst, 2007) and that it is necessary for the ectopic induction of mesDAergic neurons by FGF8 and SHH (Prakash et al., 2006). Moreover, Castelo-Branco *et al.* have shown that *Wnt1* was expressed in the VM at the time of DAergic neurogenesis, and that the treatment of mouse primary VM cultures with partially purified Wnt1 conditioned media increased the

number of DAergic neurons *in vitro* (Castelo-Branco et al., 2003). Finally, Wnt1/ß-catenin signaling was shown to regulate the expression of the mesDAergic neuron determinant Lmx1a (Chung et al., 2009). Although substantial advances have been made on the past years in understanding how Wnt1 establishes the initial DAergic domain in the VM, we are still far from knowing all gene regulatory cascades behind *Wnt1* control of DAergic development.

In this study, we focused on analyzing Wnt1's gene regulation on VM DAergic progenitor cells in proliferation and differentiation. We identified a positive correlation between *Wnt1* and *Shh, Lmx1a, Otx2, En1, Nurr1* in proliferating progenitors and a negative correlation with *Ngn2* in differenting cells. Furthermore, we observed a dose and time-dependant *Wnt1-Lmx1a* and *-Otx2* regulation, crucial for proper mesDAergic development. On the other hand, we identified a role for *Wnt1* in the prevention of continued neurogenesis in the later stages of DAergic differentiation, by negatively regulating *Ngn2*. Finally, we provided evidence that *Wnt1* expressed in DAergic progenitors plays a role in the long-run differentiation process, by directly correlating with the number of differentiated DAergic neurons.

4.1.2. Wnt1 regulates the expression of several crucial genes involved in mesDAergic specification and differentiation in a time-dependent manner

In in order to elucidate *Wnt1*'s role in gene regulation in the developing VM *in vitro*, *Wnt1* was retrovirally overexpressed in E10.5 mice ventral midbrain neurospheres precursor cultures and gene expression was examined. To clearly see the effect of transgene expression without masking their effect by culture conditions, we only used Wnt1 as a DAergic-inducing factor, in the presence of FGF2, B27 and BDNF. After 5 days in proliferation, some cells were collected for analysis and the reamaining were further differentiated and analyzed at day 8, after three days of neuronal differentiation. The expression of *Wnt1* and potential mesDAergic regulators/target genes were analyzed at these time points by quantitative real-time PCR (qPCR). The levels of *Wnt1* mRNA were plotted against those of our genes of interest (GOI) and linear regression analysis were performed. A regression analysis attempts to describe the dependence of a variable on one explanatory variable, finding the best line that predicts Y from X; it implicitly

assumes that there is a one-way causal effect from the explanatory variable to the response variable, regardless of whether the path of effect is direct or indirect. Correlation quantifies the degree to which two variables are related. Correlation does not fit a line through the data points. It simply computes a correlation coefficient (Pearson r) that tells you how much one variable tends to change when the other one does. When Pearson r is 0, there is no relationship. When Pearson r is positive, one variable goes up as the other goes up. When Pearson r is negative, one variable goes up as the other goes down. The R^2 values (or coefficient of determination) are estimates of how good fit the line (correlation) is. They represent the percentage of variation of the data explained by the fitted line. The p (probability) values measure the probability that the values are not derived by chance. These p values are not a measure of "goodness of fit" *per se*, rather they state the confidence that one can have in the estimated values being correct, given the constraints of the regression analysis (*ie.*, linear with all data points having equal influence on the fitted line).

Of all the genes analysed by qPCR, Wnt1 forced expression correlates with the mRNA expression of *Shh, En1, Lmx1a, Otx2, Nurr1, Ngn2* in a time dependent-manner, but not *TH, Pitx3, Lmx1b* or *FoxA2*. The lack of an effect on the expression of these genes at both time points suggests that they are not regulated by Wnt1 alone, but does not rule out the possibility that Wnt1 in combination with another factors(s) or concentrations of factor(s) may be required to regulate that target gene. Conversely, the effects that we report imply that a regulation exists in the particular cell preparation tested and may not be representative of all cell types or time-points during development.

Interestingly, *Shh* mRNA levels were found to significantly correlate with *Wnt1* levels both in proliferation (Pearson r = 0.6602, R^2 = 0.4359, p = 0.0378) and differentiation (Pearson r = 0.6199, R^2 = 0.3843, p = 0.0315). The linear regression lines are shown in Figure 7.

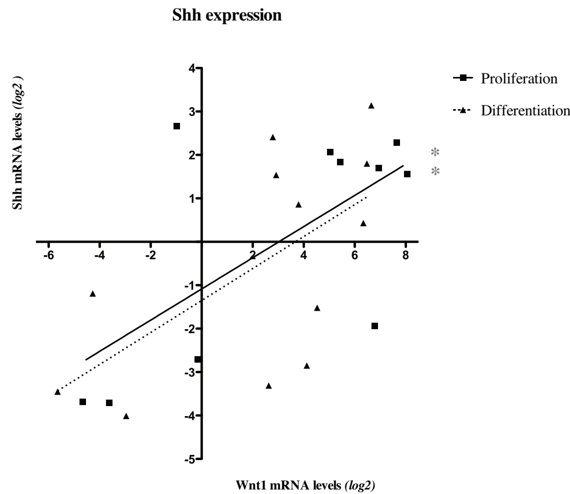

Figure 7 - *Shh* mRNA levels significantly correlate with *Wnt1* levels during proliferation and differentiation. The best-fit linear regression lines are represented in the figure; *p<0.05, linear regression.

We also found that *Lmx1a* and *Otx2* strongly correlate with *Wnt1* mRNA levels, both during proliferation as in differentiation (linear regression lines are shown in Figure 8 and 9). Linear regression of *Lmx1a* on *Wnt1* is significant during proliferation (Pearson r = 0.8889, R^2 = 0.7901, p = 0.0006) and differentiation (Pearson r = 0.7432, R^2 = 0.5523, p = 0.0056). Linear regression of *Otx2* on *Wnt1* is significant during proliferation (Pearson r = 0.9038, R^2 = 0.8169, p = 0.0003) and differentiation (Pearson r = 0.6467, R^2 = 0.4183, p = 0.0230).

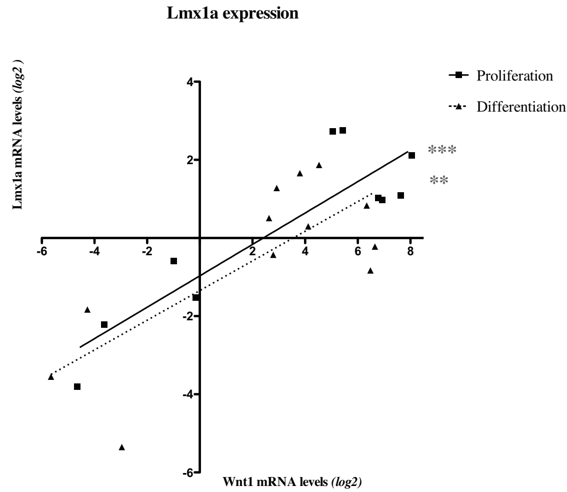

Figure 8 - *Lmx1a* mRNA levels significantly correlate with *Wnt1* levels during proliferation and differentiation. The best-fit linear regression lines are represented in the figure; **p<0.001, ***p<0.0001, linear regression.

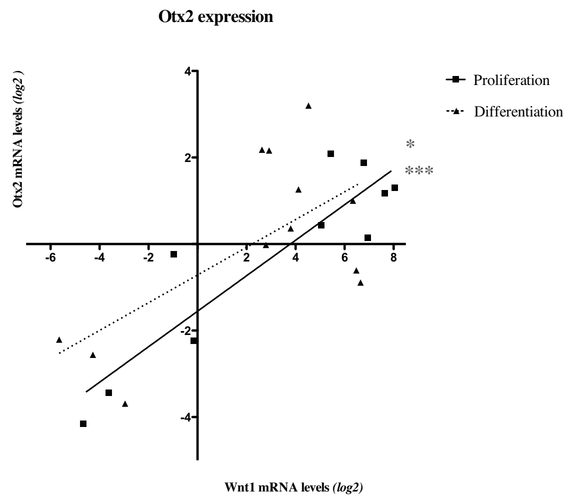

Figure 9 – *Otx2* mRNA levels significantly correlate with *Wnt1* levels during proliferation and differentiation. The best-fit linear regression lines are represented in the figure; *p<0.05, ***p<0.0001, linear regression.

On the other hand, *En1 and Nurr1* mRNA levels were found to be significantly correlated with *Wnt1* during proliferation (*En1*: Pearson r = 0.7164, R^2 = 0.5132, p = 0.0198; *Nurr1*: Pearson r = 0.7990, R^2 = 0.6384, p = 0.0056), but not during differentiation (*En1*: Pearson r = 0.4216, R^2= 0.1777, p= 0.1966; *Nurr1*: Pearson r = 0.5289, R^2 = 0.2797, p = 0.0771) (linear regression lines are shown in Figure 10 and 11, respectively).

Finally, *Ngn2* showed a significant negative correlation with *Wnt1* mRNA levels during differentiation (Pearson r = -0.6804, R^2 = 0.4630, p = 0.0149), but not during proliferation (Pearson r = -0.6172, R^2 = 0.3810, p = 0.0766) (linear regression line is shown in Figure 12).

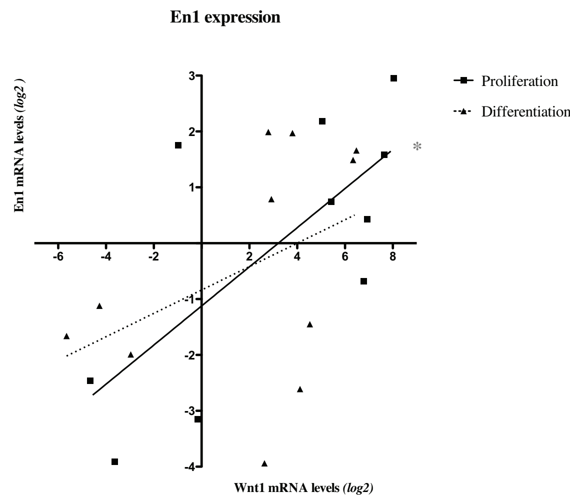

Figure 10 - *En1* mRNA levels significantly correlate with *Wnt1* levels during proliferation. The best-fit linear regression lines are represented in the figure; *p<0.05, linear regression.

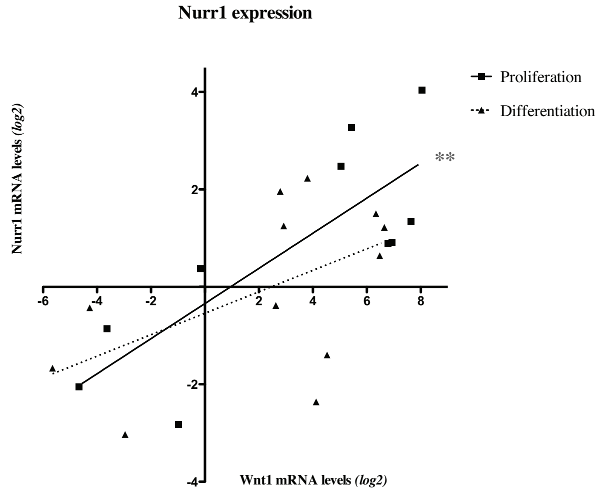

Figure 11 – *Nurr1* mRNA levels significantly correlate with *Wnt1* levels during proliferation. The best-fit linear regression lines are represented in the figure; **p<0.001, linear regression.

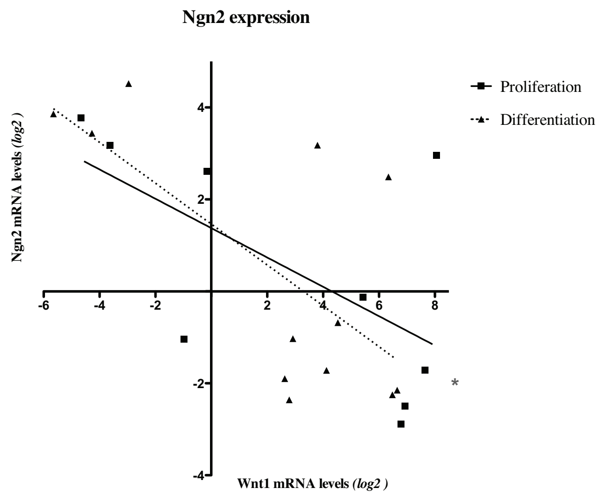

Figure 12 – *Ngn2* mRNA levels show a significant negative correlation with *Wnt1* levels during differentiation. The best-fit linear regression lines are represented in the figure; *p<0.05, linear regression.

4.1.3. *Wnt1* regulates the expression of *Lmx1a, Otx2,* and *Ngn2* in a dose-dependent manner

Since *Wnt1* overexpression in VM neurospheres gave different *Wnt1* mRNA levels, experiments were grouped as low, intermediate or high *Wnt1* levels (according the dCt values assessed by qPCR, normalized to house-keeping gene and linearized by $\log_2$ data transformation). Three statistically different Wnt1-dose populations were examined at two developmental stages (day 5 of proliferation and day 8, after differentiation for 3 days) and further analyzed for the capacity of different Wnt1 doses to regulate gene expression (Figure 13).

These three different *Wnt1* level populations were assessed for different DAergic markers, revealing a statistically significant regulation of *Lmx1a* mRNA levels by *Wnt1*, both in proliferation and differentiation (one-way ANOVA, p = 0.0021 in proliferation and p = 0.0005 in differentiation). While *Wnt1* levels had a dose-dependant effect on *Lmx1a* expression during proliferation, intermediate levels of Wnt1 were sufficient to upregulate *Lmx1a* at the highest levels during differentiaiotn (Figure 14).

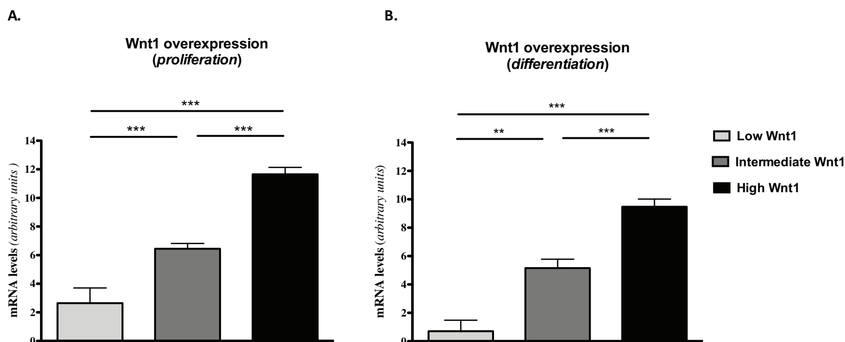

Figure 13 – *Wnt1* mRNA levels in the three groups during proliferation (A) and differentiation (B). A. Experiments with low (n = 4), intermediate (n=10) and high Wnt1 mRNA levels (n = 6) after proliferation for 5 days; **B.** Experiments with low (n = 3), intermediate (n=8) and high Wnt1 mRNA levels (n = 9) after differentiation from day 5 to 8. ** p<0.001, *** p<0.0001, One-way ANOVA followed by Newman-Keuls Multiple Comparison test.

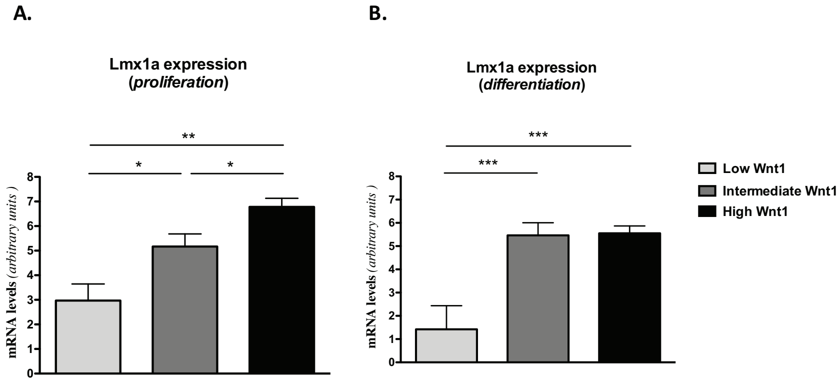

Figure 14 – *Lmx1a* is regulated by *Wnt1* in a dose-dependent manner during proliferation (A) and by intermediate levels of *Wnt1* during differentiation (B). * p<0.05, ** p<0.001, *** p<0.0001, One-way ANOVA followed by Newman Keuls's Multiple Comparison test.

Instead, intermediate concentrations of *Wnt1* were sufficient to regulate *Otx2* expression both during proliferation and differentiation (one-way ANOVA, p = 0.0006 in proliferation and p = 0.0037 in differentiation). Higher concentrations of *Wnt1* did not influence *Otx2* expression (Figure 15).

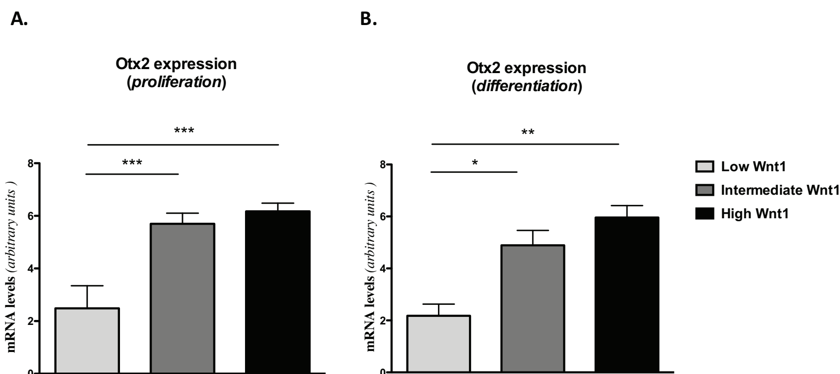

Figure 15 – *Otx2* is regulated by *Wnt1* in a dose-dependent manner in during proliferation (A) and by intermediate levels of *Wnt1* during differentiation (B). * p<0.05, ** p<0.001, *** p<0.0001, One-way ANOVA followed by Newman Keuls's Multiple Comparison test.

In contrast, *Ngn2* mRNA levels were not regulated during proliferation but were regulated by *Wnt1* during differentiation. While low *Wnt1* levels resulted in high *Ngn2* expression, high *Wnt1* levels gave low levels of *Ngn2* (one-way ANOVA, p = 0.0041). These reults suggest that in the absence of mitogens Wnt1 may play a role in terminating neurogenesis by reducing Ngn2 expression (Figure 16).

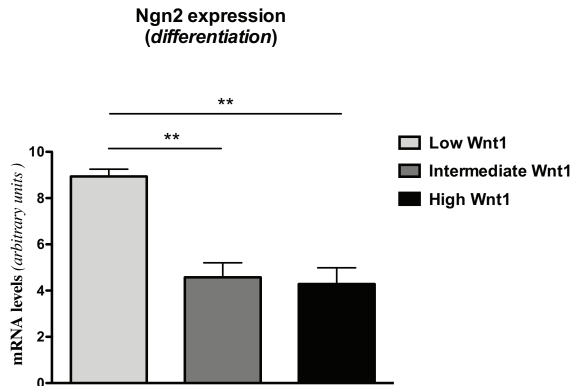

Figure 16 – *Ngn2* is regulated by *Wnt1* in a dose-dependent manner during differentiation. **p<0.001, One-way ANOVA followed by Newman Keuls's Multiple Comparison test.

4.1.4. *Wnt1* regulates the differentiation of TH-positive dopaminergic neurons.

To verify whether *Wnt1* could play a role in the differentiation of midbrain DAergic neurons, the number of TH-positive neurons was examined by immunocytochemistry in *Wnt1*-transduced ventral midbrain neurospheres that had been expanded for 5 days and differentiated for 3 more days. Interestingly, the number of TH-positive cells at day 8 significantly correlated with *Wnt1* mRNA levels in proliferating neurospheres at day 5 (Pearson r = 0.9746, R^2 = 0.9499, p = 0.0254), but not in differentiated neurospheres at day 8 (Pearson r = 0.8749, R^2 = 0.7654, p = 0.1251) (Figure 17).

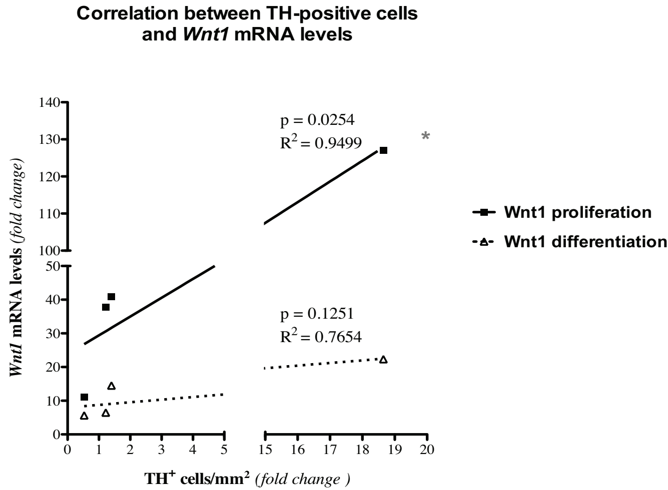

Figure 17 - *Wnt1* mRNA expression during proliferation correlates with the number of TH-positive dopaminergic neurons obtained from differentiated VM neurospheres. *p<0.05, linear regression.

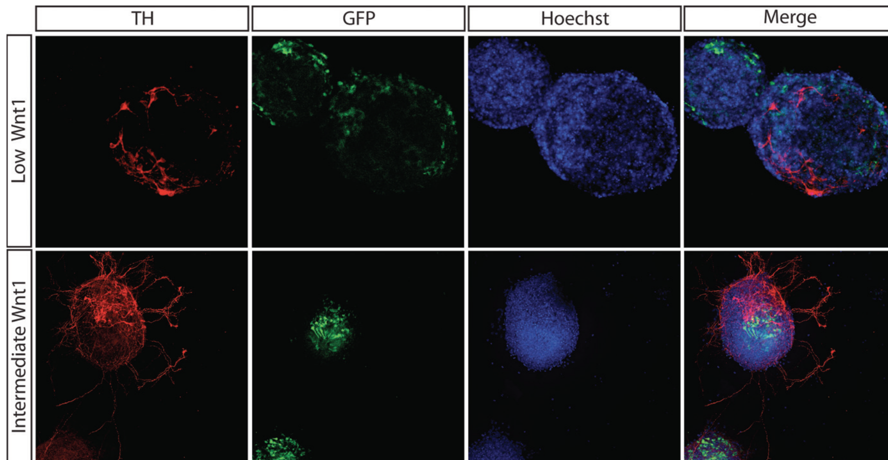

Figure 18 – Intermediate Wnt1 mRNA levels increase the generation of TH-positive neurons in VM neurosphere cultures expanded for 5 days and differentiated for 3 days, compared to low-dose Wnt1 cultures. TH-positive cells (in red), GFP (in green) and Hoechst (in blue).

Combined, our results suggest that, in the present culture conditions, Wnt1 plays an important role at early developmental stages, by promoting the specification (expression of *Lmx1a* and *Otx2*) and allowing neurogenesis (absence of negative regulation of *Ngn2* at day 5), resulting in the generation of a greater number of TH-positive cells. Immunocytochemistry analysis showed that intermediate levels of *Wnt1* were sufficient to induce significant differences in the number of TH-positive cells (Figure 18).

4.1.5. Discussion

The specification of distinct neuronal cell-types along the CNS is controlled by inducing signals whose expression in distinct areas provides neuronal progenitors with a precise expression code of transcription factors. The discovery that certain transcription factors, specifically Otx2, Lmx1a and Lmx1b, Engrailed 1 and Engrailed 2 and Neurogenin 2, are all expressed in mesDAergic progenitors, has allowed progenitors to be identified for the first time by a combinatorial transcription factor code (Andersson et al., 2006a; Andersson et al., 2006b; Kele et al., 2006; Puelles et al., 2004; Simon et al., 2001). Midbrain DAergic development was shown to be initiated by SHH, FGF8, and Wnt1 signaling, defining the initial arena where DAergic progenitors develop (McMahon and Bradley, 1990; Prakash et al., 2006; Ye et al., 1998). As described before, SHH secreted from the notochord directly induces FoxA2 in the VM, which in turn regulates SHH itself (Jeong and Epstein, 2003), Ngn2 and Nurr1, inhibits alternate fates (Nkx2.2+ cells) (Ferri et al., 2007), and promotes the survival of mesDAergic neurons. More recently, a study by Chung *et al* has reported that Wnt/β-catenin regulates an Lmx1a-Lmx1b autoregulatory loop controlling the specification and differentiation of DAergic neurons from ES cells (Chung et al., 2009). In order to gain further insight into this process we have investigated whether Wnt1 regulates these and other pathways or genes in endogenous midbrain progenitors isolated from the VM.

Wnt1 is expressed in the mouse VM from E8.0-E14.5 (Ellisor et al., 2009; Wilkinson et al., 1987), being confined to a ring encircling the neural tube at the rostral border of the MHB in the caudal midbrain, the roof plate of the mes- and diencephalon, and two stripes adjacent to the floor plate of the midbrain, this latter expression domain

overlaping with the region where mesDAergic progenitors first appear (Prakash et al., 2006). *In vivo* studies have shown that Wnt1 has an early patterning activity in the midbrain, controlling *Otx2*, which in turn represses *Nkx2.2* and maintains the Nkx6.1-expressing domain through dorsal antagonism on Shh. This strongly suggests that Otx2 is required to provide midbrain neuronal precursors with a specific differentiation code suppressing that of the anterior hindbrain. Failure of this control affects the identity code and fate of midbrain progenitors (Prakash et al., 2006; Puelles et al., 2004). Indeed, Fgf8 and Shh signaling require *Otx2* in the ventral midbrain in order for these signals to be properly interpreted. As expected, not only could we detect a correlation between the expression of *Wnt1* and the levels of *Otx2*, but we also found that *Otx2* expression levels increase in response to intermediate Wnt1 levels both in proliferating as well as in differentiating progenitors. Noteworthy, is the ability of Wnt1 to induce *Otx2* even during differentiation in our cultures. A potential role for Otx2 in later stages of DAergic development is still controversial: in *Nestin-Cre;Otx2$^{flox/flox}$* conditional mutant embryos, loss of Otx2 protein from E10.5 onwards resulted in loss of expression of the proneural gene *Ngn2* in mesDAergic progenitors, suggesting that *Otx2* is also required for the generation of mesDAergic neurons, presumably via regulating the expression of *Ngn2* (Vernay et al., 2005). However, mesDAergic development recovered in *En1cre;Otx2$^{flox/flox}$;Nkx2.2$^{-/-}$* mutant embryos prior to neuronal differentiation, at E9.5 (Prakash et al., 2006). On the other hand, Ngn2 is mostly expressed in the VZ of the ventral midbrain and in very few postmitotic Nurr1-positive cells, where it is required both for neurogenesis and for the acquisition of generic neuronal properties, but not for their terminal differentiation (Andersson et al., 2006a; Kele et al., 2006). In agreement with previous results, we have found that *Ngn2* expression, is negatively regulated by Wnt1 levels during differentiation indicating that Wnt1 allows neurogenesis in proliferating progenitors, and high levels reduce their capacity to undergo neurogenesis during differentiation. However, since *Wnt1* levels decrease during differentiation and low levels correlate with higher *Ngn2* expression, we interprete this finding to indicate that the decrease in *Wnt1* levels during differentiation may contribute to maintain Ngn2 expression.

In agreement with these findings, previous studies have shown that conditioned-medium from Wnt1-expressing cells have shown to increase proliferation and neurogenesis of ventral mesencephalic precursor cells in general, and promoted the generation of TH-expressing cells from Nurr1-positive precursors in culture (Castelo-

Branco et al., 2003). In our work, we have seen that *Nurr1* expression levels correlate with *Wnt1* but we could not confirm the finding in our dose-response analysis. We interpret this finding to indicate that *Nurr1* might be a further downstream indirect target of *Wnt1* that may require other genes or different expression levels than the ones in our cultures (Figure 19). Indeed, it has been previously reported that Nurr1 is downstream of *Lmx1a* (Andersson et al., 2006b), but it is unclear whether it is a direct target. Similarly, *En1* correlated with *Wnt1* levels but did not show a dose-response. Provided that *En1* expression is maintained by *Otx2* (Rhinn et al., 1998), and it is lost in *Wnt1*$^{-/-}$ embryos (Davidson et al., 1988; Davis and Joyner, 1988; McMahon et al., 1992), our findings may reflect the fact that *En1* is also a downstream target (Figure 19).

How Shh is regulated and which genes are controlled by Shh during VM development is also a controversial topic. Whereas Andersson *et al.* have showed that *Shh* and *Lmx1a* are coexpressed in the ventral midline at early stages and that ventral induction of *Lmx1a* depends on *Shh* signaling (Andersson et al., 2006b), Chung *et al.* demonstrated that acute Shh treatment or inhibition led to no significant changes in *Lmx1a*, *TH* or *Nurr1* mRNA levels, suggesting that these genes are not direct targets of the Shh signaling (Chung et al., 2009). However, it is possible that Shh indirectly regulates these genes through further downstream targets. In our study we have seen that *Shh* mRNA levels correlate with *Wnt1* levels in precursors proliferation and persists throughout differentiation. Interestingly, *FoxA2*, a known direct downstream target of the Shh signaling pathway (Sasaki et al., 1997), did not show a significant correlation with *Wnt1* levels in our study, suggesting that additional factors or higher levels of Shh are required. With regard to *Lmx1a*, overexpression in chick embryos and deletion in mice (Andersson et al., 2006b) identified *Lmx1a* as a crucial determinant of mesDAergic neuron fate development. This study showed that the overexpression of *Lmx1a* in the VM of chick embryos promoted the generation of DAergic neurons over that of other neuronal subtypes. Chung *et al.* suggested that the Wnt1-Lmx1a pathway is formed independently of the SHH-FoxA2 pathway, although they functionally interact with each other during mesDAergic development (Chung et al., 2009). Interstingly, our findings show that Wnt1 dose-dependently regulates Lmx1a expression both in proliferating and differentiating VM progenitors, indicating that the regulation of Lmx1a is a key and carefully regulated function of Wnt1 in VM development (Figure 19). This finding, together with the regulation of *Otx2* and *Ngn2* by Wnt1, places Wnt1 as a central piece in the regulation of DAergic specification and neurogenesis. This view is supported by the

finding that high levels of Wnt1 during proliferation increases the number of DAergic neurons in our cultures.

Our present data suggest that Wnt1, by controlling several target genes involved in DAergic neuron development and maintenance, might be a promising target in the treatment of PD. We propose that Wnt1 activation in specific cell types at a certain time-point and in a specific dose, may become a valuable strategy to improve the DAergic differentiation of pluripotent stem cells or to modulte the function of diseased DAergic neurons. Nevertheless, many questions still remain open, and elucidation of their answers is currently one of the most important tasks in the field.

Figure 19 - Genetic network of the *Wnt1* signaling pathway in the midbrain

The emerging genetic network of the *Wnt1* signaling pathway reveals an important role for *Wnt1* in ventralization (regulation of *Shh*; in yellow) and maintaining floorplate identity while inhibiting alternate fates (regulation of *En1* and *Otx2*; in yellow), inhibition of late of neurogenesis (negative regulation of *Ngn2*; in red), and DAergic phenotype specification (regulation of *Lmx1a* and *Nurr1*; in blue). Bold boxes indicate Wnt1-dosage-dependent gene regulation and the dotted boxes indicate Wnt1-low-dose-dependent gene regulation. The thin line boxes depict genes that are Wnt1-dosage independently regulated. Arrowheads indicate positive regulation and blunt-ended arrows indicate negative regulation, regardless of whether they are direct or indirect. Please refer to the Results and Discussion section for details and related references.

4.2. *Tiam1 regulates the Wnt5a/Dvl/Rac1 signalling pathway and the differentiation of midbrain dopaminergic neurons*

4.2.1. Rationale

This study focused on Wnt signaling pathways independent of ß-catenin, in particular on Wnt5a-mediated activation of the small GTPase, Rac1 [reviewed in (Lai et al., 2009; Schlessinger et al., 2009)]. Ever since Castelo-Branco *et al.* demonstrated a Wnt5a effect on the generation of TH-positive neurons from Nurr1-positive precursors in mouse primary neuron culture (Castelo-Branco et al., 2003), one of the goals of the Arenas Lab has been to understand the mechanisms and consequences of Wnt5a action on mesDAergic differentiation. Since then, Wnt5a, has shown to promote differentiation of precursors into DAergic neurons in various culture systems including primary VM precursors and ES cells (Bryja et al., 2007b; Castelo-Branco et al., 2003; Hayashi et al., 2008; Parish et al., 2008; Sanchez-Pernaute et al., 2008; Schulte et al., 2005). Further insights into the function of Wnt5a were brought by its purification (Schulte et al., 2005), which allowed a further analysis of the mechanisms of Wnt5a signaling.

To date, the precise molecular mechanisms underlying the pro-differentiation effects of Wnt5a and the signaling pathways activated by Wnt5a in DAergic cells, are still not clear. Our lab showed that purified Wnt5a does not activate the Wnt/ß-catenin pathway in DAergic cells, but promoted DAergic differentiation via a Wnt/ß-catenin-independent pathway (Bryja et al., 2007b; Schulte et al., 2005). Moreover, we have recently demonstrated that the pro-differentiation effects of Wnt5a require the activity of the Rac1 GTPase (Andersson et al., 2008). Rac1 belongs to the Rho family of small GTPases and its activity is controlled by guanine exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) [reviewed in(Jaffe and Hall, 2005; Rossman et al., 2005)]. While Wnt ligands are known to trigger activation of various small GTPases from the Rho and Ras families in various cell types (Schlessinger et al., 2009), evidence for the employment of specific GEF(s) in Wnt5a/Rac1 signaling is missing, and the mechanism of small GTPase activation in context of Wnt signaling is poorly understood.

Rac1 has been proposed to interact and be regulated by the cytosolic protein Dishevelled (Dvl) (Habas et al., 2003; Rosso et al., 2005), a critical component of Wnt signaling. Recent data from our lab has characterized the Dvl-Rac1 interaction and shown that the N-terminal part of Dvl is required for complex formation between Rac1 and Dvl, as well as for the Dvl-mediated Rac1 activation (Cajanek et al., submitted). As initial attempts to immunoprecipitate Rac1 and identify its binding partners by mass spectrometry did not lead to the identification of any candidate GEF of Rac1, we then set for identifying endogenous interactors of Dvl that could be involved in the activation of Rac1. Dvl3 was immunoprecipitated in mouse embryonic fibroblasts (MEFs) and subsequent LS-MS/MS was performed. In addition to previously described Dvl partners (Gao and Chen, 2010) such as Nucleoredoxin (Funato et al., 2006) or Ror2 (Witte et al., 2010), Tiam1, a GEF known to activate Rac1 (Habets et al., 1994), was found.

In this study we identify Tiam1 (T-cell lymphoma invasion and metastasis 1), a Rac1 GEF (Habets et al., 1994), as a novel binding partner of Dvl. Moreover, using loss of function approaches we demonstrated a functional requirement of Tiam1 for Rac1 activation induced by Wnt5a or Dvl, as well as for generation of DAergic neurons from DAergic progenitors in neurospheres cultures. In sum, we propose Tiam1 as a novel regulator of Wnt/Dvl/Rac1 pathway in DAergic cells.

4.2.2. Tiam1, a Rac GEF, interacts with Dvl

To confirm the Dvl-Tiam1 interaction, Tiam1-FLAG and Dvl2-EGFP were overexpressed in SN4741 cells, and subsequently analyzed for their subcellular localization by confocal microscopy. In cells transfected with Tiam1-FLAG only, this was localized both in cytosol and the membrane (Figure 20A). On the other hand, when Dvl2-EGFP was co-expressed, the membrane-localized Tiam1-FLAG was not significantly mobilized, but the cytosolic pool was efficiently relocated and co-localized with Dvl2-EGFP polymers in cytosolic puncta (Figure 20B). These findings supported the idea of a Dvl-Tiam1 protein-protein interaction in the cytosolic puncta.

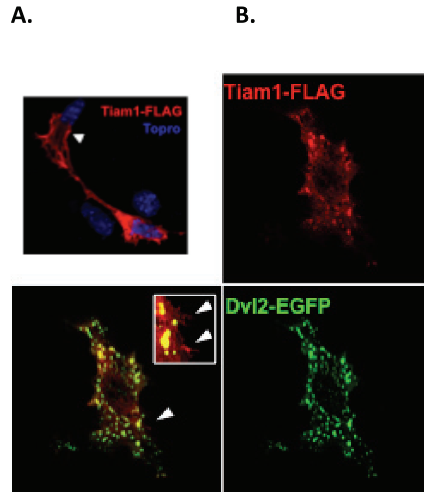

Figure 20 – A. Tiam1-FLAG protein (red) localizes both in the cytosol and at the membrane (arrow) upon overexpression in SN4741 cells. Topro3 was used to counterstain cell nucleus. **B. Co-expression of Dvl2-EGFP (green) and Tiam1-FLAG (red) in SN4741 cells lead to a cytoplasmatic co-localization of Dvl2-EGFP and Tiam1-FLAG in puncta.** Arrowheads show that a small pool of Tiam1-FLAG that did not co-localize with Dvl2-EGFP remained in the membrane.

In order to verify whether Dvl and Tiam1 were part of the same protein complex, co-immunoprecipitation was performed in HEK293A cells. Tiam1 was found to interact with both Dvl2-HA (Figure 21) and Dvl3-FLAG (Figure 22), as detected by their co-immunoprecipitation with Tiam1-FLAG. Rac1 had previously shown to co-immunoprecipitate with both Dvl2-MYC and Dvl3-FLAG with similar efficiency (unpublished data). Hence, these results confirmed that both Dvl2 and Dvl3 do form a complex with Tiam1.

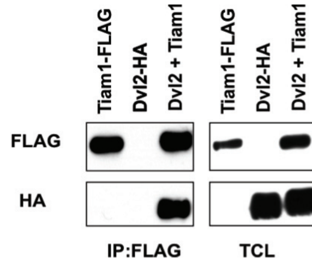

Figure 21 – Dvl2-HA was found to form a complex with Tiam1-FLAG, as assessed by immunoprecipitation with Tiam1-FLAG in HEK293A cells (first panal last lane). The right panel shows tota cell lysates (TCL).

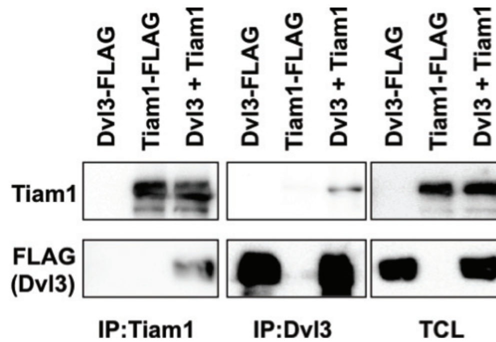

Figure 22 - Dvl3-FLAG was also immunoprecipitated by Tiam1-FLAG in HEK293A cells. The complex formed by Tiam1-FLAG and Dvl3-FLAG was detected by immunoprecipitation with either anti-Dvl3 or anti-Tiam1 antibodies.

4.2.3. Tiam1 facilitates Dvl-Rac1 interaction and is required for Rac1 activation

We next examined whether Tiam1 played a role in the formation of the Dvl-Rac complex and the activity of Rac1. Co-immunoprecipitation of Dvl2-MYC with Rac1 was enhanced when Tiam1-FLAG was overexpressed (Figure 23). Quantification of these results (n=5) indicated that the expression of Tiam1 increased the Dvl2-Rac1 co-immunoprecipitation by 46%, as compared to the condition without Tiam1 expression (Figure 24). Thus, these results suggest that presence of Tiam1 facilitates the Dvl-Rac1 interaction.

As previous data suggested that Tiam1 forms a complex with Dvl, we decided to test if Tiam1 was involved in the transduction of signal between Dvl and Rac1. By overexpressing Tiam1 in both HEK293A and SN4741 cells, Tiam1 was identified as sufficient to induce Rac1 activation (Figure 25). Moreover, overexpression of Dvl2-MYC or Dvl3-FLAG increased the levels of activated MYC-tagged Rac1-GTP (Figure 26 and 27).

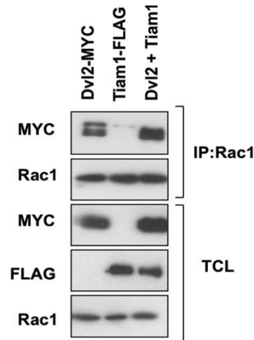

Figure 23 – Dvl-MYC was pulled down by Rac1 (first lane) and this co-immunoprecipitation was enhanced by co-expression of Tiam1-FLAG (third lane).

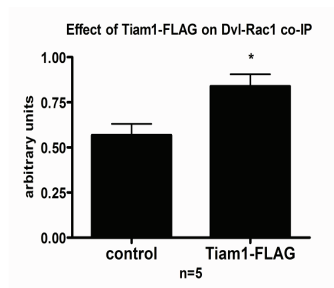

Figure 24 - Densitometric quantification of the relative amount of Dvl2-MYC co-immunoprecipitating with Rac1 in control or Tiam1 co-transfected cells. Values were normalized to the total level of Dvl-MYC (total cell lysate). * $p<0.05$, Mann-Whitney test.

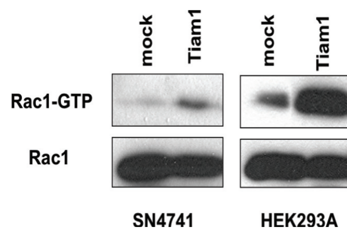

Figure 25 - Tiam1-FLAG was sufficient to increase GTP-Rac1 level, compared to control in both SN4741 and HEK293A cell lines. Cells were transfected with a Tiam1-FLAG or mock plasmid and analyzed for the level of GTP-Rac1 24 hours after transfection.

In order to check whether Tiam1 was required for Rac1 activation in the context of Wnt/Dvl/Rac1 pathway, Tiam1 was knocked down in SN4741 DAergic cells using Tiam1 siRNA. Interestingly, when Tiam1 protein level was reduced neither Dvl2-MYC nor Dvl3-FLAG overexpression was sufficient to induce Rac1-activation (Figure 26 and 27).

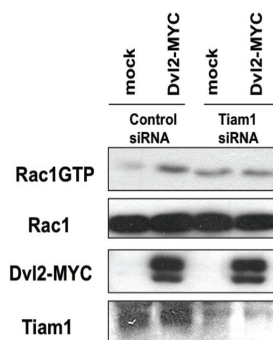

Figure 26 - Dvl2-MYC increased the level of Rac1-GTP compared to mock transfected cells, but not in cells where Tiam1 expression was knocked down by siRNA. SN4741 cells were transfected with control or Tiam1 siRNAs and subsequently with Rac1- MYC, and Dvl2-MYC or mock vector.

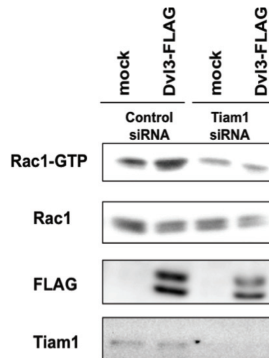

Figure 27 – Dvl3-FLAG increased the levels of MYC-tagged Rac1-GTP compared to mock transfected SN4741 cells, but not in cells where Tiam1 expression was knocked down by siRNA. SN4741 cells were transfected with control or Tiam1 siRNAs and subsequently with Rac1-MYC, and Dvl3-FLAG or mock vector.

In addition, the relative increase in Rac1-GTP levels after Dvl2-MYC and Dvl3-FLAG overexpression were significantly decreased to almost basal levels after Tiam1 knockdown (Figure 28).

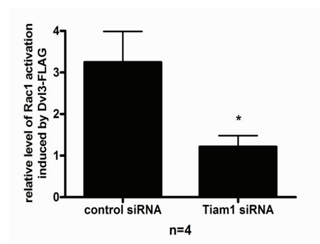

Figure 28 – Densitometric quantification of the relative level of Rac1-GTP (normalized to total MYC tagged Rac1) induced by Dvl3-FLAG in control or Tiam1 siRNA conditions. * $p<0.05$, Mann-Whitney test.

To test whether Tiam1 is required for Wnt ligand-depedent activation of Rac1, SN4741 DAergic cells were treated with Wnt5a and subsequently analyzed for Rac1 activation. The ability of Wnt5a to trigger increase in Rac1-GTP levels was impaired when Tiam1 expression was knocked down by siRNA (Figure 29 and 30). These results together demonstrate that Tiam1 is required for Rac1 activation in DAergic cells either by Dvl overexpression or acute stimulation by Wnt5a. Thus, Tiam1 was identified as a novel regulator of the non-canonical Wnt/Dvl/Rac1 signaling pathway in DAergic cells.

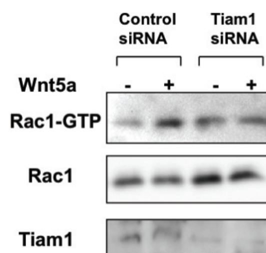

Figure 29 – Wnt5a ability to trigger Rac1 activation is impaired when Tiam1 expression is knocked down. SN4741 cells transfected with control or Tiam1 siRNA and 24 hours after they were stimulated with Wnt5a (100ng/ml) or vehicle (PBS-CHAPS) for 2.5 hours and subsequently analyzed for Rac1 activation (level of Rac1-GTP).

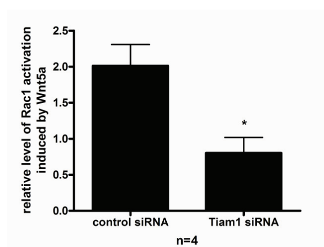

Figure 30 – Densitometric quantification of the relative level of Rac1-GTP (normalized to total Rac1) induced by Wnt5a stimulation in control siRNA versus Tiam1 siRNA (second versus fourth lane). *p<0.05, Mann Whitney test.

4.2.4. Tiam1 is expressed in developing VM and is required for the differentiation of midbrain DAergic precursors into neurons

Since Wnt/Dvl/Rac1 signaling has been previously reported to promote the differentiation of primary ventral midbrain DAergic neurons (Andersson et al., 2008), we now decided to examine the involvement of Tiam1 and examined its expression at mRNA and protein levels. *Tiam1* mRNA was detected by qPCR in the developing mouse VM as early as E10.5, at the onset of DAergic neurogenesis, and increases until E15.5 (Figure 31). Moreover, Tiam1 protein is expressed in the developing VM as early as E10.5 (Figure 32), suggesting a possible function of Tiam1 in midbrain development at these developmental stages.

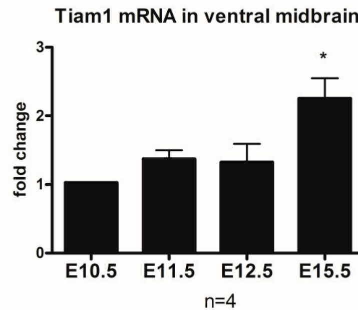

Figure 31 – qPCR analysis showed that *Tiam1* mRNA is expressed between E10.5 and E15.5 in mouse ventral midbrain. *p<0.05, Mann Whitney test (compared to E10.5).

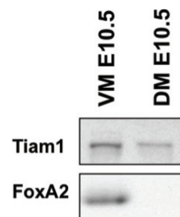

Figure 32 – Western blot analysis confirmed the presence of Tiam1 protein in both ventral midbrain (VM) and dorsal midbrain (DM) samples at E10.5. The floor-plate marker FoxA2 was used to confirm the identity of the VM sample.

As our study indicates that, Tiam1 regulates the function of Rac1 in the context of Wnt/Dvl/Rac1 signaling, we next decided to elucidate the function of Tiam1 in DAergic differentiation. This was assessed by Tiam1 knockdown in differentiating DAergic progenitor cultures. Expanded and subsequently differentiated DAergic progenitor neurosphere cultures were used as a model to study this hypothesis (Parish et al., 2008). In these experiments, midbrain neural stem/progenitor cells were expanded in the presence of Shh, basic FGF and FGF8 for 7 days, being then gently dissociated to smaller cell clusters for higher transfection efficiency with either control siRNA or Tiam1 siRNA, and differentiated for 3 days.

Differentiated progenitor cultures transfected with Tiam1 siRNA showed a *Tiam1* mRNA reduction of about 50% (Figure 33) after 3 days of differentiation, which was comparable with *Rac1* knockout in the SN4741 cell line (Figures 34 and 35). Remarkably, the mRNA levels of the neuronal marker *ß-III-tubulin (Tuj)* and the rate-limiting enzyme in DA sythesis, *TH,* were significantly reduced in Tiam1 knockdown cultures (Figure 33). A similar reduction of *Th* mRNA was observed in knocked down Rac1-differentiated precursor cells (Figure 35). On the other hand, *Nurr1* and *Pitx3* mRNA levels, two transcription factors expressed in postmitotic neuroblasts and mature DAergic neurons, were not significantly decreased by Tiam1 knockdown (Figure 33). These results suggested a role for Tiam1 in the last stages of differentiation, in the acquisition of TH expression by Nurr1-positive and Pitx3-positive postmitotic cells. Indeed, as detected by immunocytochemistry, the number of TH-positive DAergic neurons in the Tuj-positive differentiated neurospheres (normalized by area), were reduced by 50% in the Tiam1 knockdown condition (Figures 36 and 37). These data emphasized the importance of Tiam1 in the generation of midbrain DAergic neurons from neural stem/progenitor cells.

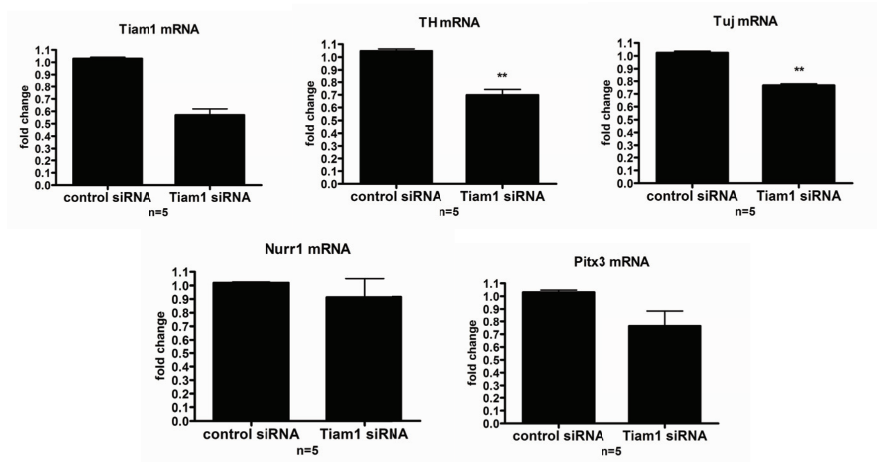

Figure 33 – Tiam1 siRNA decreased the expression of *Tiam1* mRNA, as well as *TH* and *Tuj* mRNAs, but not *Nurr1* or *Pitx3* mRNAs in differentiating ventral midbrain precursors 3 days after transfection. These results suggested an effect of Tiam1 in DA differentiation, similar to that of Wnt5a. **p<0.01, Mann Whitney test.

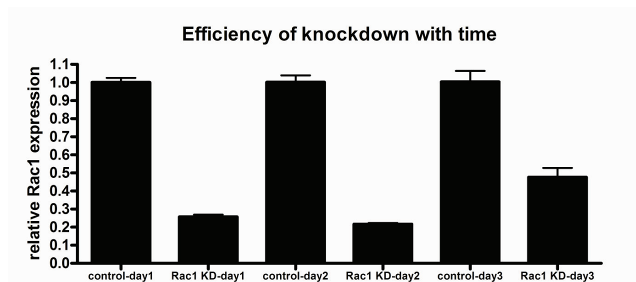

Figure 34 – SN4741 cells were transfected with control or Rac1 siRNAs and analyzed by qPCR for the efficiency of Rac1 knockdown after indicated times post transfection. Rac1 knockdown lasted for 3 days and was very efficient during the first 2 days.

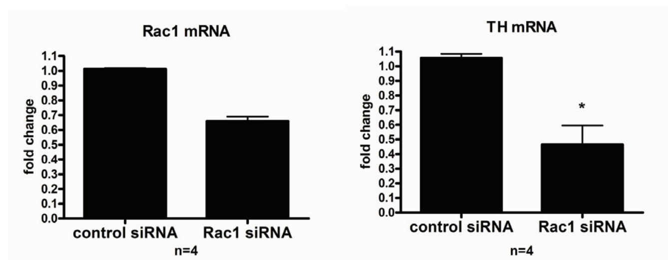

Figure 35 – Rac1 siRNA caused a decrease in expression level of Rac1 and TH mRNA in differentiating ventral midbrain precursors, 3 days after transfection. *p<0.05, Mann Whitney test.

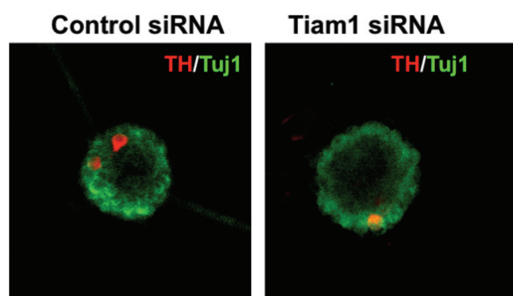

Figure 36 – Immunostaining of control or Tiam1 siRNA VM neurospheres showed similar numbers of Tuj1-positive cells (green) but lower numbers of TH-positive (red) dopaminergic neurons after 3 days of differentiation.

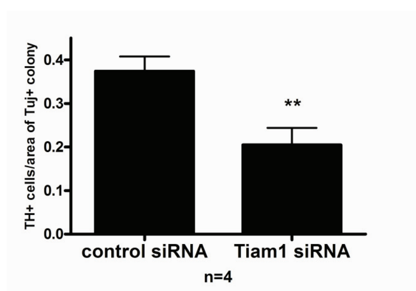

Figure 37 – Quantification of the number of TH-positive cells per area of Tuj-positive neurosphere revealed that Tiam1 siRNA significantly reduced the number of dopaminergic neurons. **p<0.01, Student t-test.

Finally, to confirm whether Tiam1 was indeed downstream of Wnt5a signaling in the midbrain DAergic neuron differentiation context (as observed in the biochemical analysis), Tiam1 was knocked down in Wnt5a-treated differentiating neurospheres by Tiam1 shRNA lentiviral delivery. As expected, Wnt5a treatment (100 ng/ml) increased the number of TH-positive DAergic neurons per area by 58% from 59.2 $\pm$ 3.8 TH-positive cells/mm^2 to 93.6 $\pm$ 8.3 TH-positive cells/mm^2 (Figures 38 and 39). These effects were significantly ablated by Tiam1 shRNA knockdown (61.9 $\pm$ 6.5 TH-positive cells/mm^2).

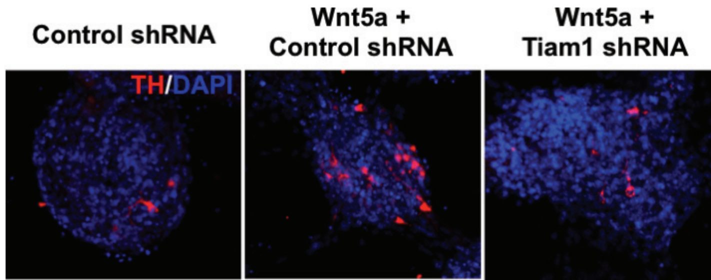

Figure 38 – Treatment with Wnt5a (100 ng/ml for 3 days) **increased the number of TH-positive neurons in neurosphere cultures treated with control shRNA lentivirus,** but not in Tiam1 shRNA treated cultures. DAPI was used for nuclei counterstaining.

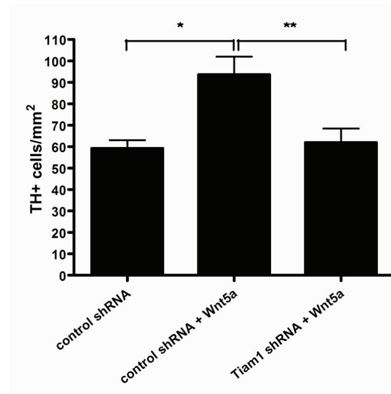

Figure 39 – Quantification of the number of TH-positive cells per area (mm^2) of sphere. * $p<0.05$, ** $p<0,01$, One-way ANOVA followed by Newman Keuls's Multiple Comparison test

In sum, these results suggest that Rac1 and its upstream regulator Tiam1 are involved in the differentiation of midbrain DAergic neurons, and that Tiam1 is a downstream effector of Wnt5a during DAergic differentiation.

4.2.5. Discussion

We have previous reported that Wnt5a activates the small GTPase Rac1 in DAergic cells, linking the DAergic differentiation effect of Wnt5a with a known effector of Wnt/PCP signaling (Andersson et al., 2008). In this study we aimed at clarifying the mechanism by which Wnt5a activated Rac1 in DAergic cells, and particularly tried to identify the GEF(s) responsible for Rac1 activation in the context of the Wnt/Dvl/Rac signaling pathway. Interestingly, while the role of GEFs in the activation of Rho GTPases has been extensively studied, and Rho GTPases were identified as downstream components of Wnt signaling over a decade ago (Fanto et al., 2000), there was very sparse evidence regarding the use of particular GEF in Wnt-driven signaling cascades at the beginning of this study.

Bryja *et al.* identified Dvl as an important regulator of Rac1 activation by Wnt5a (Bryja et al., 2008). This initial characterization of the Dvl-Rac1 interaction set the chase for novel Dvl-binding partners that could potentially activate Rac1. Tiam1, a well known GEF for Rac1 [reviewed in (Collard et al., 1996; Mertens et al., 2003; Michiels et al., 1995)], was identified as a candidate Dvl-interacting protein. Indeed, further overexpression experiments proved that Tiam1 does colocalize with Dvl in cytosolic puncta and that both can be co-immunoprecipitated, suggesting that Tiam1 interacts with Dvl. The ability of Dvl to form polymers has been attributed to its N- terminal DIX domain, and this polymerization ability has been proposed as a prerequisite for efficient Dvl-Rac1 interaction (Nishita et al., 2010). How this complex is affected by the presence/absence of other Dvl-interacting proteins, the phosphorylation status of Dvl and/or acute Wnt ligand stimulation, remains to be elucidated.

In our study we found that Tiam1 knockdown by siRNA decreased the activation of Rac1 induced by Dvl overexpression. In addition, the ability of Wnt5a to trigger Rac1 activation (Andersson et al., 2008) was also impaired by Tiam1 knockdown, confirming the requirement of Tiam1 for Rac1 activation and its role in enhacing Dvl-Rac1 interaction in the context of Wnt/Dvl/Rac signaling and in mediating Wnt5a signaling. However, it should be noted that we were unable to detect the interaction between Tiam1 and Rac1. A possible explanation for this is the unstable character of the interactions between GEFs and small GTPases (Worthylake et al., 2000), which could have prevented from obtaining sufficient levels of Tiam1-Rac1 protein complexes for identification by mass spectrometry. Moreover, the activity of small GTPases seems to

be regulated by protein-protein interaction between GEFs and scaffolding proteins (Buchsbaum et al., 2003; Marinissen and Gutkind, 2005), which fits with our data demonstrating Dvl-Tiam1 interaction.

Tiam1 has previously shown to regulate neurite and axon outgrowth (Kunda et al., 2001; Leeuwen et al., 1997; Tanaka et al., 2004), as well as migration of cortical neurons (Kawauchi et al., 2003), and the formation of dendritic spines (Tolias et al., 2005). In this study we have shown for the first time that Tiam1 is expressed in the developing VM as early as E10.5, at the onset of DAergic neurogenesis, and gradually increases until E15.5. This piece of evidence, together with Tiam1's demonstrated function in Wnt/Dvl/Rac signaling, prompted the assessment of Tiam1 role in DAergic differentiation. When Tiam1 expression was depleted by siRNA in differentiating neurosphere cultures, a decrease in the number of TH-positive DAergic neurons was detected. Moreover, the mRNA levels of *TH* and the neuronal marker *Tuj* was also reduced, as opposed to *Nurr1* and *Pitx3*, both early postmitiotic DAergic markers, which remained unaffected. This provided evidence that Tiam1 could play a role in the differentiation of postmitotic precursors into TH-positive DAergic neurons, which had not been shown before this work. *Tuj* mRNA reduction suggests that Tiam may also regulate other aspects of neuronal development in non-DAergic neurons. Nevertheless, only by combining Wnt5a treatment with Tiam1 knockdown, could Tiam1 function during DAergic differentiation be coupled to the Wnt/Dvl/Rac1 pathway. Indeed, in our experiments where Tiam1 shRNA was delivered to neurospheres precursor cultures treated with Wnt5a, the Wnt5a-induced DAergic differentiation was ablated, suggesting that Tiam1 plays a crucial role in the Wnt5a-induced DAergic neuron differentiation. Interestingly, Wnt5a and Rac1 were previously demonstrated to promote the differentiation of Nurr1-positive postmitotic neuroblasts into TH-positive DAergic neurons (Andersson et al., 2008; Castelo-Branco et al., 2003).

Tiam1 mutant mice, however, do not display developmental defects in the central nervous system resembling Wnt/PCP pathway defects (Malliri et al., 2002). It is likely that other Rac GEFs can compensate for lack of Tiam1 in the developing brain. In the future, it will be of great importance to perform a detailed developmental analysis of the VM in Tiam1 null mice.

In summary, this data is the first evidence for the involvement of a specific Rac1 GEF, Tiam1, in the non-canonical Wnt/Dvl/Rac1 pathway, being required for the Wnt5a-mediated generation of DAergic neurons in VM progenitor cultures. Tiam1 may

therefore be an important drug target for the regulation of the Wnt5a/Dvl/Rac1 pathway. Being able to enhance the GEF activity of Tiam1 might prove to be, in the future, a valuable tool to promote the differentiation of DAergic precursors or stem cell derived-DAergic neurons, as a strategy to fight PD.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

5. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The importance of Wnt signaling during VM and DA neuron development has become a widely accepted fact during the past decade. Early studies showed that the expression of Wnt1 and Wnt5a peak by E10.5–13.5 in the VM, when DAergic neurons arise. Moreover, Wnt1- and Wnt5a-conditioned media were found to increase the number of TH-positive cells in VM precursor cultures *in vitro*, while control-conditioned media and Wnt3a did not (Castelo-Branco et al., 2003). Wnt1 and Wnt5a seem to achieve this increase in DAergic neurons through two different strategies: Wnt1 increased the total number of neurons without affecting the proportion of TH-positive cells out of the total neuronal population, whereas Wnt5a specifically increased the number of TH-positive cells out of the total population of neurons. This suggested that Wnt5a predominantly regulated the maturation and differentiation of the DAergic precursor cells, while Wnt1 increased progenitor proliferation, thereby increasing the total number of neurons, including DAergic neurons (Castelo-Branco et al., 2003). Other reports confirmed the role of Wnt1 in VM proliferation *in vivo* (Panhuysen et al., 2004; Prakash et al., 2006), and revealed an instructive role of Wnt1 in DAergic neuron development *in vivo* (Prakash et al., 2006).

Nevertheless, a detailed knowledge about the developmental pathways regulated by Wnt signaling in DAergic neurons, as well as which key players are involved, is still incipient. Therefore, a more detailed characterization of the molecular cascades regulated by Wnts is critical in order to be able to recreate development *in vitro* as a tool for CRT. The challenge in this thesis was to unravel some of the molecular cascades linking early induction to the differentiation and maintenance of mesDAergic neurons, eventually obtaining a more complete picture of mesDAergic development. Our hope is that when this is accomplished, more effective treatments for mesDAergic-associated neurological disorders, such as PD, can be generated.

In our work we have been able to identify Wnt1 as an important regulator of DAergic development *in vitro*, by controlling specific genes, such as *Lmx1*, *Otx2* and *Ngn2*, which are decisive for DAergic neuron development. In addition, we have shown that early *Wnt1* induction, instructs DAergic differentiation in the later stages. We have also identified Tiam1 as a new regulatory component of Wnt5a-mediated DAergic differentition. As described in this thesis, current evidence strengthens the idea that Wnt signaling might play a key role in the improvement of therapies for PD. It is reasonable

to expect that Wnts and their signalling pathways may become molecular targets for diagnostic and therapeutic purposes. To fullfill this idea in the medical field, there are still many questions that require answers. Recent improvements in techniques, such as transcript expression profiling, ChIP-seq, proteomics, and mesDAergic neuronal cell isolation and culture, will certainly further help unveil the molecular repertoire necessary to generate a mesDAergic neuron.

Finally, the work present in this thesis has expanded our knowledge regarding Wnt signaling in midbrain and DAergic neuron development. We hope this work will hopefully contribute to new protocols, some currently being tested, for differentiation of stem cells and iPS cells into DAergic neurons, with the aim of devising a successful CRT for PD or drug development, using PD-iPS for disease modeling *in vitro* and drug screening. Furthermore, Wnt signaling defects are associated with several cancers and even Alzheimer's disease, meaning that the results presented herein may contribute to improved health, not only in the perspective of cell replacement therapy for PD patients, but also in a wider perspective.

CONCLUSIONS

6. CONCLUSIONS

Based on the work compiled in this thesis, the following conclusions have been drawn:

- *Wnt1* modulates the expression of several crucial genes involved in midbrain DAergic specification (*Shh, Otx2, Lmx1a),* neurogenesis (*Ngn2)* and differentiation (*En1, Nurr1).*

- *Wnt1* plays different roles during proliferation and differentiation of DAergic progenitors by controlling *Lmx1a* in a dose-dependent manner, *Otx2* at a low dose and by negatively regulating *Ngn2* expression during differentiation.

- Exposure of proliferating VM progenitors to *Wnt1,* regulates the differentiation of DAergic neurons.

- Tiam1 is required for Wnt5a/Dvl-induced Rac1 activation.

- Tiam1 is required for the basal and Wnt5a-induced differentiation of DAergic progenitors into DAergic neurons.

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