

Dissertation

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Spatio-temporal control of Wnt/β-catenin signaling during fate commitment of human neural progenitor cells

Vorgelegt von:

Benjamin M. Bader geb. in Hagenow

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Begutachtet von:

Prof. Dr. Dieter G. Weiss, Universität Rostock (Rostock, Deutschland) Prof. Dr. Ernest Arenas, Karolinska Institute (Stockholm, Schweden)

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"When you employ the microscope, shake off all prejudice, nor harbour any favourite opinions; for, if you do, 'tis not unlikely fancy will betray you into error, and make you see what you wish to see."

Henry Baker (1743), Cautions in viewing objects

picture on the previous page: human neural progenitor cells ReNcell VM after three days of differentiation. β -catenin (green), Tuj3 (green, neuronal marker), nuclei (blue). 3D-rendering projection using Imaris-software, boxes represent isolated objects after surface-rendering of the nuclear staining which allows the quantification of fluorescence signals in 3D; Single cell: isolated neuron from larger image, 3D-surface rendering of different sections of a neurite. numbers correspond to the β -catenin fluorescence intensities in each section.

Contents

AI	bbrev	iations	IV					
Sι	ımma	ary	VI					
1	Intr	oduction	1					
	1.1	Overall context: cell replacement therapies for Parkinson's disease	1					
	1.2	Wnt-signaling	4					
		1.2.1 History	4					
		1.2.2 Wnt proteins	4					
		1.2.3 Wnt receptors: Fz and LRP	5					
		1.2.4 Wnt/ β -catenin signaling	5					
		1.2.5 Non- β -catenin signaling $\ldots \ldots \ldots$	7					
		1.2.6 Wnt-signaling modulators	8					
		1.2.7 Cross talk of Wnt-signaling pathways	10					
	1.3	The midbrain - origin of dopaminergic neurons in vivo	10					
		1.3.1 Midbrain development	10					
		1.3.2 Dopaminergic neurons	11					
		1.3.3 Wnt-signaling molecules in the developing midbrain	12					
		1.3.4 Wnt function in midbrain development and DA neurogenesis	12					
	1.4	Image cytometry	16					
		1.4.1 goes 3D \ldots	17					
		1.4.2in neuroscience	17					
		1.4.3in stem cells research	18					
		1.4.4and signal transduction and protein translocation processes	18					
		1.4.5and systems biology	19					
	1.5	Conclusion	20					
	1.6	Motivation and aims of the work	21					
2	Ехр	erimental procedures	22					
	2.1	Neural progenitor cells from human fetal ventral midbrain - ReNcell VM	22					
	2.2	2 Maintenance and differentiation of ReNcell VM cells						
	2.3	Microscopic characterization of ReNcell VM differentiation	23					
		2.3.1 Fixation and immunostaining for fluorescence microscopy	23					
		2.3.2 Microscopic quantification of neuronal differentiation	23					
	0.4	2.3.3 Cell type dependent analysis during ReNcell VM differentiation	23					
	2.4	Flow cytometric characterization of ReNcell VM differentiation	24					
		2.4.1 Fixation and immunostaining for flow cytometry	24					
	05	2.4.2 Flow cytometric quantification of neuronal differentiation	25					
	2.5	Development of a quantitative image-analysis approach	26					
		2.5.1 Image acquisition by confocal faser scanning microscopy	20					
	ົງເ	2.5.2 5D-mage processing and signal quantification	21					
	$\angle.0$	Statistical allalyses	21					

	2.7	Test for antibody-specificity	27
		2.7.1 β -catenin RNA interference	27
		2.7.2 Nucleofection of siRNAs	28
	2.8	Life cell imaging studies	28
		2.8.1 Plasmid preparation and nucleofection	28
		2.8.2 Visualization of YFP- β -catenin constructs	29
		2.8.3 Fluorescence Recovery After Photobleaching (FRAP)	29
	2.9	Protein biochemistry	30
		2.9.1 Cell lysates and SDS-PAGE	30
		2.9.2 Immunoblotting	30
	2.10	Activation and Inhibition of ReNcell VM differentiation	31
	2.11	Investigation of ReNcell VM proliferation: EdU-Assay and MTT-test	32
	2.12	In vivo investigations	33
		2.12.1 Mouse brain cryosections and immunohistochemistry	33
		2.12.2 Preparation of DIG-RNA probes for RNA in situ hybridization (ISH)	33
		2.12.3 Hybridization of DIG-RNA probes and development for ISH	34
		2.12.4 Image acquisition and image processing from stained cryosections	35
3	Resi	ults	36
Pa	nrt 17	4 - Sub-cellular quantification of β -catenin and translocation analysis	36
	3.1	Controlled differentiation of ReNcell VM in vitro	36
		3.1.1 ReNcell VM differentiate into neurons, astrocytes, oligodendrocytes	36
		3.1.2 A small population of ReNcell VM neurons is dopaminergic	38
	3.2	3D-image cytometry as a tool to quantify sub-cellular protein distribution	39
		3.2.1 3D image reconstruction and surface rendering for cell type and organelle-	
		dependent protein quantification	40
		3.2.2 Nuclear β -catenin concentration increases during ReNcell VM differentiation	41
		3.2.3 Nucleo-cytoplasmic shuttling of β -catenin is differentially regulated during	
		differentiation of ReNcells VM	44
		3.2.4 Immature neurons show increased nuclear β -catenin levels compared to	
		non-neuronal cells	45
	3.3	Summary $1/4$	46
Pa	nrt 2/-	4 - The influence of modification of Wnt/ eta -catenin signaling on differentiation 4	47
	3.4	Activation of β -catenin dependent Wnt-signaling $\ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots$	47
		3.4.1increases nuclear β -catenin levels in ReNcell VM	47
		3.4.2stimulates neuronal differentiation of ReNcell VM	48
		3.4.3has a stronger effect on neurons than on non-neuronal cells \ldots	50
	3.5	Inhibition of β -catenin-dependent Wnt-signaling $\ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots$	51
		3.5.1 decreases nuclear $\beta\text{-catenin}$ levels in ReNcell VM	51
		3.5.2 decreases neuronal differentiation of ReNcell VM \ldots	52
		$3.5.3$ $\dots {\rm does} \ {\rm not} \ {\rm show} \ {\rm a} \ {\rm stronger} \ {\rm effect} \ {\rm on} \ {\rm neurons} \ {\rm than} \ {\rm on} \ {\rm non-neuronal} \ {\rm cells} \ .$	53
	3.6	Influence of Wnt/ β -catenin signaling on dopaminergic differentiation in vitro $~$.	54

Π

	3.7	Summary 2/4	56
Pa	rt 3/4 tion 3.8 3.9	 4 - Wnt/β-catenin signaling and its influence on the interplay between prolifera- and differentiation The role of Wnt/β-catenin on committed neuronal progenitor cells	57 57 59 61 64
Pa	rt 4/4 3.10 3.11 3.12 3.13	 4 - β-catenin kinetics during dopaminergic differentiation in vivo Wnt/β-catenin-signaling in the developing brain	 65 66 66 66 68 72
4	 Disc 4.1 4.2 4.3 4.4 4.5 4.6 4.7 	The human neural progenitor cell line ReNcell VM is a suitable tool to study Wnt/β -catenin signaling during neurogenesis	 73 73 74 74 75 75 78 80
5	Cond	clusions	85
Bil	oliogr	raphy	86
Ac	know	ledgements 1	L 07
Cu	rricul	lum Vitae 1	11
Su	pplen	nent	14

Abbreviations

A2B5	marker for restricted glial progenitor cells
ABC	<u>active</u> beta catenin, dephosphorylated at GSK3 β sites
AHD-2	aldehyde dehydrogenase
AOBS	acousto-optical beam splitter
AP	alkaline phohsphatase
APC	adenomatous polyposis coli
bFGF	basic Fibroblast Growth Factor
BMP	Bone morphogenic factor
BrdU	$5-\underline{\text{Bromo-2'-deoxy-U}}$ ridine
BSA	bovine serum albumine
CamK	$\underline{Calcium/calmodulin-dependent \ protein \ \underline{K}inase, \ effector \ of \ WNT/Ca^{2+}-pathway}$
CDC	<u>cell</u> <u>division</u> <u>cycle</u> , effector of Wnt/Rho and WNT/ Ca^{2+} -pathway
CE	convergent extension
СК	casein kinase
CRD	<u>cystein-rich-domain</u>
DA	dopaminergic
DAPI	4',6- <u>Dia</u> midino-2- <u>phenyli</u> ndole
$dH_2O \ \ldots \ldots$	distilled water
DIG-RNA	$\underline{\text{Dig}}$ oxigenin-UTP/RNA
Dkk1	Dickkopf-1, a Wnt antagonist
DMEM	$\underline{\mathbf{D}}$ ulbecco/Vogt $\underline{\mathbf{M}}$ odified $\underline{\mathbf{E}}$ agle's minimal essential $\underline{\mathbf{M}}$ edium
DMSO	$\underline{\text{Dim}}$ ethyl $\underline{\text{Sulfoxide}}$
Dsh/Dvl	Disheveled
DTT	Dithiothreitol
Е	$\underline{\mathbf{E}}$ mbryonic day
EDTA	$\underline{\mathbf{E}}$ thylendiamin-tetraacetat
EdU	5- <u>e</u> thynyl-2'- <u>d</u> eoxy <u>u</u> ridine
EGF	$\underline{\mathbf{E}}$ pidermal $\underline{\mathbf{G}}$ rowth $\underline{\mathbf{F}}$ actor
EGFP	Enhanced green fluorescent protein
Fgf	<u>F</u> ibroblast growth factor
FITC	5-fuorescein isothiocyanate
FP	<u>floor</u> <u>plate</u>
FRAP	<u>Fluorescence recovery after photobleaching</u>
Fz	Frizzled
GAPDH	<u>g</u> lycer <u>a</u> ldehyde-3- <u>p</u> hosphate <u>deh</u> ydrogenase
GFP	green fluorescent protein
GSK	<u>g</u> lycogen synthase kinase
HBS	<u>H</u> epes <u>B</u> uffered Saline
$\mathrm{Hh}~\ldots\ldots\ldots$	Hedgehog
Hu C/D \ldots	neuronal RNA-binding protein, marker for young neurons, ANA-1

IZ	intermediate zone
Jnk	c-Jun <u>N</u> -terminal kinase
LDL	low density lipoprotein
Lef	Lymphoid enhancer factor
LRP	low density lipoprotein receptor-related protein
MMTV	<u>mouse mammary tumor virus</u>
mRNA	$\underline{\text{messenger }} \underline{\text{RNA}}$
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZ	marginal zone
NFAT	<u>n</u> uclear factor of <u>activated</u> <u>T</u> -cells, involved in Wnt/Rho-pathway
Nurr1	<u>Nuclear receptor related 1 protein</u> , marker for DA precursor cells
O.C.T	\underline{O} ptimal \underline{C} utting \underline{T} emperature compound
OD	optical density
PBS	<u>phosphate</u> <u>buffered</u> <u>saline</u>
PBST	<u>PBS</u> containing <u>T</u> ween20
PCP	planar cell polarity
PD	Parkinson´s disease
PDL	$\underline{p}oly-\underline{D}-\underline{l}ysine-hydrobromid$
PDZ	$\underline{PSD95}/dic$ large tumor suppressor $\underline{DlgA}/ZO-1$ homologous
РКС	<u>protein kinase C</u>
PLC	<u>Phospholipase</u> \underline{C}
$\operatorname{PSA-NCAM} \ \ldots$	marker for restricted neuronal progenitor cells
Rac, Rho $\ldots\ldots$	small GTP-binding proteins, involved in Wnt/Rho-pathway
RNAi	<u>RNA</u> interference
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
Shh	Sonic hedgehog
siRNA	small interfering <u>RNA</u>
SN	substantia nigra
$t_{1/2}$	half-recovery time of FRAP
TCF	$\underline{\mathrm{T-Cell}}$ - $\underline{\mathrm{Factor}}$
TH	tyrosine hydroxylase, marker for DA neurons
v-myc	oncogene, transcription factor
VM	ventral midbrain
VZ	ventricular zone
WIF-I	\underline{W} nt-inhibitory-factor
Wnt	wingless-related MMTV integration site
YFP	yellow fluorescent protein

Summary

Signaling pathways that are involved in embryonic development exhibit complex regulatory mechanisms for the temporal and spatial distribution of signaling molecules. The Wnt/ β -catenin pathway is known to be involved in neural stem cell self-renewal and neuronal differentiation - especially of dopaminergic (DA) neurons in the midbrain. Further understanding of this neurogenesis is a prerequisite for cell replacement therapies in neurodegenerative diseases such as Parkinson's disease in which DA neurons are lost. The complex spatio-temporal control of Wnt/ β -catenin signaling proteins, especially of the key protein β -catenin, is crucial for neuronal differentiation. However, a quantitative and kinetic description and correlation between signaling protein-redistribution and neuronal differentiation remain to be elucidated until now.

In this thesis, the role of Wnt/ β -catenin signaling during neuronal differentiation was studied. Both, the *in vitro* model of the human neural progenitor cell line ReNcell VM as well as the *in vivo* model of the embryonic mouse midbrain were under investigation. To elucidate the spatio-temporal distribution of β -catenin, a 3D-image analysis approach was developed which was applied *in vitro* and *in vivo*. Together with quantitative live cell imaging and cell cycle analyses, the results presented herein indicate that Wnt/ β -catenin signaling...

- 1. ...is directly connected with the degree of neuronal differentiation in ReNcell VM
- 2. ...activity includes a mechanism controlling the kinetics of nuclear β -catenin import
- 3. ...activation affects neurons stronger than non-neurons during the time of fate commitment
- 4. ...mediates cell cycle exit or re-entry during the first hours after initiation of differentiation
- 5. ...influences the number of proliferating cells that are committed to the neuronal lineage already
- 6. ...steadily increases during dopaminergic differentiation which was shown for the first time in a quantitative spatio-temporal manner both *in vitro* and *in vivo*

In total, the data from both *in vitro* and *in vivo* experiments presented in this thesis, demonstrate how precisely Wnt/ β -catenin signaling is controlled during the transition from stem cells over committed progenitor cells to mature neurons and how it guides stem cells during the process of fate commitment. This study substantiates the presently limited knowledge about neurogenesis in human cell models and closes the gap between the mechanistic knowledge based on rodent models and the use of human neural stem cells for cell replacement strategies in neuro-degenerative diseases. It, therefore, provides information which increase the knowledge necessary for improved therapies for Parkinson's Disease in the future.

1 Introduction

1.1 Overall context: cell replacement therapies for Parkinson's disease

The term neurodegenerative disease is used for a wide range of acute and chronic conditions in which neurons and glial cells are lost in the central nervous system. In acute cases such as in response to ischemic stroke or spinal cord injury, numerous types of neurons and glial cells die within a restricted brain area over a short time period. In chronic cases, there is either a selective loss of a specific cell population, such as dopamine (DA) neurons in Parkinson disease (PD) and motor neurons in amyotrophic lateral sclerosis (ALS), or a widespread degeneration of many types of neuron, such as occurs in Alzheimer disease (AD), over a period of several years (Lindvall and Kokaia, 2010; Lees et al., 2009).

Motivation for research on DA neurogenesis. Almost 200 years ago, in 'An Assay about the Shaking Palsy' James Parkinson described a progressive neurodegenerative disease with characteristic symptoms such as tremor, rigidity, slow movements (bradykinesia) and postural instability (published 1817, reprinted in (Parkinson, 2002)). Today, Parkinson's disease (PD) is the most-frequent neurodegenerative disease after Alzheimer's disease. Approximately 0.3 % of the general population is affected by PD and the probability increases with age (Weintraub et al., 2008a). About 1.8 % of the european population older than 65 years is affected by PD (de Rijk et al., 2000). Above 85 years, the prevalence increases to 4-5 % (Weintraub et al., 2008a). In the United States, there are currently up to 1 million with diagnosed PD, which is greater than the combined number of cases of multiple sclerosis, ALS, and muscular dystrophy. Approximately 60,000 Americans are diagnosed with Parkinson's disease each year, and this number does not reflect the thousands of cases that go undetected. In respect to the demographic development, the economic burden of the disease is substantial, related to direct/indirect costs and medical resource utilization (Foundation, 2010; Weintraub et al., 2008a).

DA pathology. Konstantin Tratiakoff was the first to link the pathology of PD with a loss of noradrenergic neurons and decreased pigmentation in the region of the substantia nigra pars compacta in Parkinsonian patients (Tretiakoff, 1919). This degeneration is the main characteristic of the PD-pathology which results in decreased levels of dopamine in the striatum which is the region of dopaminergic (DA) neuronal projection. This loss of striatal innervation not only results in the above mentioned motor dysfunctions but also leads to depression and insomnia (for review see Weintraub et al. (2008c,b)). The loss of dopamine was first measured in vivo by Carlsson and Waldeck (1958) who described that dopamine was present in the brain and its role as neurotransmitter (Carlsson et al., 1958). One of the most remarkable findings of Carlsson was to show that depletion of dopamine by reserpine caused similar symptoms which could be reversed by application of the precursor of dopamine: levodopa (Seiden and Carlsson, 1963). For this work, he was awarded with the Nobel Price in 2000. His contribution provoked clinicians to treat Parkinsonian patients with levodopa (L-DOPA) (Cotzias, 1968; Cotzias et al., 1968) which until today is the most effective treatment to reduce PD symptoms. Besides L-DOPA, other treatments to alleviate the symptoms are available including other dopamine agonists and deep brain stimulation of the subthalamic nucleus.



Figure 1: Stem cell-based therapies for PD. PD leads to the progressive death of DA neurons in the substantia nigra and decreased DA innervation of the striatum, primarily the putamen. Stem cell-based approaches could be used to provide therapeutic benefits in two ways: first, by implanting stem cells modified to release growth factors, which would protect existing neurons and neurons derived from other stem cell treatments; and second, by transplanting stem cell-derived DA neuron precursors neuroblasts into the putamen, where they would generate new neurons to relieve disease-induced motor impairments. Source: Lindvall and Kokaia (2010).

However, there is no cure available, partly due to the fact that little is known about the reason for the degeneration. One possible mechanism is that a combination of increased dopamine, hyper-active calcium channels and α -synuclein trigger the cellular death: calcium channels lead to an increase of dopamine inside the cell, dopamine then reacts with α -synuclein to form inactive complexes and then the complexes gum up the cell's ability to dispose of toxic waste that is produced in the cell over time. The waste eventually kills the cell (Mosharov et al., 2009). Yet, this knowledge has not led to a possible treatment. Thus, a definite cure is still highly demanded.

Cell replacement therapy. The strategy of cell replacement therapy (CRT) is one promising hope for this. In CRT, the lost DA neurons are replaced by transplanted DA neurons which were differentiated *in vitro*. The pioneer work has been achieved with fetal ventral midbrain (VM) tissue which was transplanted into rat models of PD (Björklund et al., 1980b,a). Transplantation into human patients was first tested almost 20 years ago (Freed et al., 1992a,b). Nowadays, stem cell-derived cells are predifferentiated *in vitro* to various stages of maturation, e.g., into neuroblasts (i.e. immature neurons). These are grafted into the brain and could induce functional improvement by releasing therapeutic molecules that are neuroprotective or modulate inflammation (Lindvall and Björklund, 2004; Lindvall and Kokaia, 2010). Clinical trials with intrastriatal transplantation of human embryonic mesencephalic tissue, which is rich in postmitotic DA neuroblasts, have provided proof of principle that neuronal replacement can work in PD patients (Fig. 1). The newly differentiated DA neurons reinnervate the denervated striatum and become functionally integrated, restoring striatal DA release and give rise to clear symptomatic relief in some patients (see e.g. Lindvall and Björklund (2004)) and are still functional after a decade (Piccini et al., 1999).

Stem cells. There is a strong need for other sources of DA neurons, because availability of human embryonic mesencephalic tissue is limited (partly due to ethical questions) and variability of functional outcome after transplantation is high. DA neuroblasts for preclinical transplantation have been generated *in vitro* from stem cells from several different sources and species, including humans (for a more comprehensive review on this topic see Lindvall and Kokaia (2010); Lees et al. (2009)). One of these human stem cell models is the ReNcell VM cell line which is derived from 10 weeks old human fetal tissue from the ventral midbrain (VM) and immortalized by stable v-myc oncogene transduction (Donato et al., 2007; Hoffrogge et al., 2006). Differentiate of these neural progenitor cells into neurons was described to be considerably faster than reported for other neuronal progenitor or stem cell lines such as N-tera2 (Schwartz et al., 2005) or PC12 (Greene and Tischler, 1976) which need several weeks to differentiate. Additionally, ReNcell VM differentiate into neurons with DA, at least catecholaminergic, phenotype. In total, the human origin, the fast neuronal differentiation and the capacity of DA neurogenesis (despite the genetic modification for immortalization) demonstrate that this cell line is a suitable tool to study the mechanisms of human DA differentiation *in vitro* and was, therefore, used in this thesis as the major cell model. A similar human cell line from ventral midbrain with capacity for DA neurogenesis was recently described by Tønnesen et al. (2010) which, however, needs a differentiation protocol disproportionately longer than in ReNcell VM.

Besides embryonic human cell lines such as ReNcell VM, one of the most promising approaches today is the use of induced pluripotent stem (iPS) cells which are derived from somatic cells of patients (Takahashi and Yamanaka, 2006). However, the use of viruses encoding the reprogramming factors represents a major limitation of the current technology due to its potential to induce malignant transformation. Interestingly, most recent findings revealed that the long process of complete re-programming is not necessary for de-differentiation and neuronal re-differentiation (Vierbuchen et al., 2010). This nicely indicates that research on iPS cells is still at the beginning. Yet, the proof of principle to re-program human fibroblasts into functional DA neurons has been achieved (Soldner et al., 2009). Nonetheless, the knowledge to differentiate pluripotent stem cells into DA neurons, however, is still based on research using embryonic stem cells. The information about the extrinsic and intrinsic signaling mechanism regulating their development is essential to generate functional DA neurons from an undifferentiated state. Both animal models and embryonic cell lines represent suitable means to study this process *in vitro* and *in vivo* and, therefore, provide a fundamental basis for a future cure for PD.

This thesis concentrates on the role of one of the above-mentioned extrinsic factors and the associated signaling cascade which controls neuronal and dopaminergic differentiation *in vitro* and *in vivo*: the Wnt/ β -catenin signaling pathway.

1.2 Wnt-signaling

1.2.1 History

The pioneer work of Wnt signal transduction research was performed in the late 1980s and early 1990s. In these years the gene products of the *Drosophila* wingless (wg) and mouse *int1* genes were found to belong to a large and evolutionarily highly conserved family of extracellular signaling molecules (Rijsewijk et al., 1987). However, the *Drosophila wg* was first described by Sharma (1973) through its function in wing and haltere development during embryogenesis (Sharma and Chopra, 1976). The name 'Wnt' is derived from a combination of the names wingless and Int1 (Nusse et al., 1991). The mouse *int-1* was first identified as an oncogene playing a role in mouse mammary carcinomas: upon insertion of the mouse mammary tumor virus (MMTV) into the *int-1* locus it contributes to the formation of mammary carcinomas (Nusse and Varmus, 1982; Nusse et al., 1984). Based on protein sequence homology Rijsewijk et al. (1987) showed that *int-1* and *wg* were homologous. This time coined the term 'Wnt' as <u>wingless-related MMTV integration site</u> (Baker, 1987; Cabrera et al., 1987; Rijsewijk et al., 1987; Nusse et al., 1991).

For many years, the biochemical mechanisms that control the activity of β -catenin-mediated gene activation were in the focus of research (reviewed by Barker and van den Born (2008); Huang and He (2008)). The fact that β -catenin was frequently found to be mutated in human cancers was the driving force for this research. This mutation causes hyperactive Wnt/ β -catenin signaling in nearly all intestinal cancers and in a variety of other diseases. Nowadays, the attention has shifted back to the cell membrane level, where signal transduction is initiated by the binding of Wnt proteins to membrane receptors.

1.2.2 Wnt proteins

The mammalian Wnts comprise a large family of 19 secreted, glycosylated and lipidated protein ligands that affect diverse processes in spatio-temporal patterns such as embryonic induction, generation of cell polarity, and the specification of cell fate (Logan and Nusse, 2004). For up-to-date information, see The Wnt Homepage updated by R. Nusse (2010). Wnts are defined by their amino acid sequence rather than by functional properties (Nusse and Varmus, 1992; Miller, 2002). Shared features of all Wnts include a signal sequence for secretion, several highly charged amino acid residues and many glycosylation sites. The primary amino acid sequence of Wnts suggests that they should be soluble. However, secreted Wnt proteins are hydrophobic (through attachment of palmitate) and in most cases found directly associated with the cell membrane (Willert et al., 2003; Hausmann and Basler, 2006; Takada et al., 2006).

The attempt to group individual Wnt proteins into classes in respect to specific activities resulted in the subdivision of Wnts into 'canonical' and 'non-canonical'. This nomenclature was based on the ability of 'canonical' Wnts only to induce an ectopic axis in *Xenopus* embryos (McMahon and Moon, 1989) and to cause the morphological transformation of mouse C57MG mammary cells (Wong et al., 1994). Both events correlate with an increase in the level of β -catenin which results in an increased β -catenin-dependent activation of T-cell specific transcription factor-related (TCF) genes (Shimizu et al., 1997). The distinction between the two groups of ligands appeared clean-cut: canonical Wnts bound to Frizzled and activated β -catenin/ TCF, whereas non-canonical Wnts bound to Frizzled and activated small Rho GTPases, c-Jun N-terminal kinase (Jnk) and other β -catenin-independent signaling events like formation of cell polarity (Strutt et al., 1997; Theisen et al., 1994) or convergent extension movements during vertebrate development (Heisenberg et al., 2000; Wallingford et al., 2001). However, our today's knowledge about the intrinsic properties of individual Wnt proteins tells us, that the devision into canonical and non-canonical Wnts seems no longer to be correct. A better division would be Wnt/ β -catenin and non- β -catenin signaling as described below.

1.2.3 Wnt receptors: Fz and LRP

In mammals, 10 Frizzled (Fz) receptors were described, each containing a seven-pass transmembrane domain and an extracellular N-terminal Wnt-binding domain (cystein-rich-domain (CRD)) and a C-terminal Dsh-binding domain (KTXXXW-motive, binding to PDZ-domain of Dsh) (yu Wang et al., 2006; Umbhauer et al., 2000). The Dsh-binding domain is present in all Fzs. As a member of the G-protein coupled receptor family, Fz also activates G-proteins, a mechanism which is linked to non-canonical Wnt-signaling (Slusarski et al., 1997a; Bikkavilli et al., 2008) as is presented in more detail in section 1.2.5. Fz binds also other proteins which have the CDR-domain like the group of secreted Frizzled-related proteins (sFRPs) as described in section 1.2.6. Due to their structural similarities some Fzs are dispensible for development which indicates overlapping functions among the members of the Fz family (van Amerongen and Berns, 2006).

In mammals, the two low density lipoprotein (LDL) receptor-related proteins (LRP), namely LRP5 and LRP6, were described to contribute to the activation of Wnt/ β -catenin signaling. LRP5/6 are the only members of the family of LDL receptors capable of activating Wnt-signaling. However, other LRPs (LRP1 and LRP4) were shown to antagonize Wnt/ β -catenin signaling through Fz-receptor inactivation by sequestration or endocytosis (Zilberberg et al., 2004; Ohazama et al., 2008). The epidermal growth factor (EGF) repeats that are required for Wnt-binding, also bind to the canonical Wnt-antagonist Dickkopf (Dkk) (Mao et al., 2001) as described in section 1.2.6. The intracellular domain contains 5 PPP(S/T)P motifs which are phosphorylated when Wnt binds to the EGF domain. This phosphorylation is necessary for the downstream action within Wnt/ β -catenin signaling (Tamai et al., 2004).

1.2.4 Wnt/ β -catenin signaling

The Wnt/ β -catenin pathway strictly controls the levels of the cytoplasmic protein β -catenin, which has two distinct and crucial roles in cell adhesion and activation of Wnt/TCF target genes in the nucleus (Huelsken and Behrens, 2002). β -catenin is constitutively expressed by the cells. Its role in cell adhesion is to establish the link between E-cadherin and the actin cytoskeleton. Within this complex it is immobile and does not influence Wnt-signaling. However, in the absence of a Wnt signal free cytoplasmic β -catenin is efficiently captured by a scaffold protein called Axin as illustrated in Fig. 2. Axin is a part of a protein complex usually referred to as the destruction complex, also including adenomatous polyposis coli (APC) and the kinases case in kinase (CK)-1 α and glycogen synthase kinase (GSK)-3 β . APC has a crucial role in the efficient anchoring of β -catenin. CK1 α and GSK3 β sequentially phosphorylate highly conserved serine and threonine residues in the N-terminus of the bound β -catenin (Ishizaki et al., 1996). This phosphorylation enables the E3 ubiquitin ligase/ β -Trcp to target β -catenin for rapid proteasomedependent degradation (Coleman et al., 2001). In the absence of β -catenin, a protein called Groucho binds to TCF/lymphoid enhancer factor (Lef) proteins that bind the promoters of Wnt target genes in the nucleus (Sebbagh et al., 2001; Doran et al., 2004).



Figure 2: The Wnt/ β -catenin pathway: In the absence of active Wnt (A), free cytoplasmic β -catenin (β -cat) is degraded, and Wnt target genes are repressed. Degradation is mediated by a destruction complex including adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), casein kinase (CK) and Axin. (B) If Wnt-signaling is active, β -catenin degradation is reduced. Hence, β -catenin accumulates, enters the nucleus, binds to T-cell factor (TCF)- and lymphoid enhancer binding protein (Lef)-family transcription factors and activates transcription. The components shown are described in more detail in the text; other Abbreviations: CBP, CREB-binding protein; DKK, Dickkopf; Dsh, Dishevelled (=Dvl); GBP, GSK3-binding protein; LRP, LDL-receptor-related protein; P, phosphorylation; sFRP, secreted Frizzled-related protein.

The activation of Wnt/ β -catenin signaling occurs after binding of Wnt proteins (including Wnt1, Wnt3a, Wnt7, Wnt8) to specific surface receptor complexes: the seven-pass transmembrane Wnt receptor Frizzled (Fz) and the single-pass transmembrane co-receptor low-density lipid receptor (LRP)-5 or LRP6 (Fig. 2B). This binding activates the phosphorylation of Dishevelled (Dsh) proteins which then also interact with the Fz-receptor (Hammond et al., 1997). This binding stimulates the aggregation of LRP5/6 at the membrane which facilitates its phosphorylation by CK1 α . As a consequence, Axin is recruited to the membrane by Dsh and β -catenin is no longer phosphorylated and, hence, degraded (Ishizaki et al., 1996; Nolen et al., 2004) (this unphosphorylated β -catenin is often referred to as active β -catenin). Thus, active β -catenin accumulates and enters the nucleus, where it interacts with TCF/Lef proteins and activates them to act as potent transcription factors (Behrens et al., 1996). Together with other co-activator proteins they activate the transcription of Wnt target genes involved in cell cycle

1 INTRODUCTION

like Cyclin-D1 or c-myc (Tetsu and McCormick, 1999; He et al., 1998), Wnt-antagonism like Axin2 or Dickkopf (Yan et al., 2001; Niida et al., 2004), stem cell differentiation like Oct4 or Nanog (Cole et al., 2008; Pereira et al., 2006) or neurogenesis like Neurogenin 1 or NeuroD1 (Hirabayashi et al., 2004; Kuwabara et al., 2009). For an up-to-date list of Wnt target genes please see 'The Wnt Homepage' by R. Nusse (2010). For more comprehensive discussion about Wnt/ β -catenin signaling please also see (Huang and He, 2008; Macdonald et al., 2007; Logan and Nusse, 2004; Moon et al., 2004; Polakis, 2000).

1.2.5 Non- β -catenin signaling

Non- β -catenin signaling (non-canonical) refers to Wnt-pathways which are not involved in β catenin stabilization. In comparison to the canonical pathway which mediates cell fate and axis duplication non-canonical Wnt-signaling mediates gastrulation and contributes to planar cell polarity (PCP, which controls the cells orientation in epithelial planes), convergent extension (CE, which controls the cellular expansion in a 3-dimensional space), migration (through cytoskeleton rearrangements) and transcription as was shown by Schambony and Wedlich (2007) and Murphy and Hughes (2002). During non-canonical Wnt-signaling Wnt-ligands still bind to Fz and activate Dsh. However, further downstream effectors do not involve GSK3 β and β -catenin.

Two main groups of non-canonical Wnt-pathway are found in the literature: Wnt/PCP and Wnt/Ca²⁺. Nevertheless, Wnt/PCP-signaling depends on small Rho GTPases such as Rho, Rac and cdc42, which are known to be effectors of different pathways which led to another separation of PCP-signaling into RhoA and Wnt/JNK-signaling (Tahinci and Symes, 2003; Habas et al., 2003; Dejmek et al., 2006; Schambony and Wedlich, 2007).

Wnt/Ca²⁺-pathway. The first indication of the existence of β -catenin-independent Wnt pathways was the observation that overexpression of Wnt5a or Wnt11 in *Xenopus* oocytes leads to increased intracellular calcium levels while having no effect on the β -catenin concentration (Slusarski et al., 1997b; Kühl et al., 2000). The same effect was shown in zebrafish embryos, where injection of Wnt5a and Wnt11 mRNA increased intracellular [Ca²⁺] which then activated calcium/calmodulin-dependent kinase (CamKII) and protein kinase C (PKC) (Westfall et al., 2003; Kühl et al., 2000) (for review see Kohn and Moon (2005)). Wnt-activated Ca²⁺-release depends on the activation of phospholipase C (PLC) which is triggered by G_i/G₀-protein signaling as was shown by Slusarski et al. (1997a) with pertussis toxin that blocked this pathway. If active, CamKII controls the transcription factor NFAT (nuclear factor of activated T-cells) to translocate into the nucleus where it activates target gene transcription (Murphy and Hughes, 2002; Dejmek et al., 2006) (Fig. 3A).

Wnt/JNK-pathway. The Wnt/JNK-pathway and the Wnt/RhoA-pathway are involved in the regulation and generation of planar cell polarity (PCP) (reviewed by Fanto and McNeill (2004); Mlodzik (2002)) referring to the generation of uniform orientation of a population of cells within a single epithelial plane. This has been studied intensively in *Drosophila* where the body plan has numerous examples of PCP, including uniform orientation of hairs on the wing and body wall and the orientation of ommatidia in the eye. The key proteins in PCP are Fz and Dsh,

8

but in this case, activation of Dsh does not lead to stabilization of GSK-3 β . Instead, the PCP pathway activity is controlled by the activation of C-Jun N-terminal kinase (JNK) and of small GTPases (heterotrimeric G proteins) as illustrated in Fig. 3B. Besides Fz, the Wnt-receptor Ror2 also can bind Wnts (i.e. Wnt5a) which also leads to the activation of JNK-signaling (Liu et al., 2008). In the literature Ror2-signaling is sometimes described as a distinct non-canonical pathway. However, many evidences indicate that it also directly effects CE and PCP as shown in Ror1/2^{-/-}-knockout mice with altered A/P body axis and inner ear hair orientation (Nomi et al., 2001; Yamamoto et al., 2008). The Wnt-ligands that were shown to signal through the non-canonical pathways include Wnt5a, Wnt4 and Wnt11. Overexpression of any of these Wnt ligands is followed by an increase in intracellular calcium or an increase in the activation of CamKII or PKC (Du et al., 1995; Moon et al., 1993). Moreover, none of these genes induces axis duplication which is an exclusive effect of the canonical pathway.

Wnt/Rho-pathway. Also being involved in the regulation of cell polarity (PCP), the Wnt/Rhopathway depends on the activation of RhoA (Fig. 3C). This is achieved by Fz/Dsh/DAAM association after binding of Wnt ligands (Habas et al., 2001). RhoA activates the Rho-Kinase which then leads to the rearrangement of the actin cytoskeleton and focal adhesions (Ridley and Hall, 1992; Leung et al., 1995). This indicates its role in CE movements which was shown in *Xenopus* and zebrafish, where RhoA rescued CE defects of Wnt5a and Wnt11 mutants (Tahinci and Symes, 2003; Unterseher et al., 2004; Zhu et al., 2006).

A final aspect of the different non-canonical pathways is the possibility that the PCP and Wnt-Ca2+ pathways cannot be entirely separated from each other. The fact that similar Wnt-Fzinteractions activate numerous downstream pathways each in a different context is particularly intriguing. The non-canonical pathways are involved in a number of different aspects of the development. One major example is the nervous system where the development of the neural tube during neurulation, neuronal migration or neuronal polarity (reviewed by Montcouquiol et al. (2006)), all of which relate in one form or another to the generation of polarized cell types.

1.2.6 Wnt-signaling modulators

The complexity of regulation of Wnt signaling pathways is mirrored by the number of different receptors as mentioned above. In addition to this there are numerous secreted extracellular modulators such as Wnt-inhibitory factor I (WIF-I), Dickkopf (Dkk) and secreted Frizzled related proteins (sFRP). These factors do not only inhibit but also act agonizing and interact with Wnt-signaling pathways.

The binding of **WIF-I** to canonical Wnts like XWnt8 prevents axis duplications in *Xenopus* embryos which shows that WIF-I antagonizes Wnt/ β -catenin signaling (Hsieh et al., 1999). Similar observations were shown by Wissmann et al. (2003) describing elevated intestine tumor frequency after WIF-I down-regulation and reversely the absence of WIF-I as the reason for epithelial intestine tumors (Cebrat et al., 2004), which both indicate uncontrolled Wnt/ β -catenin-mediated proliferation.

The **Dickkopf** family consists of four members (Dkk1-4). Dkk1 is able to block XWnt8induces head induction in *Xenopus* embryos (Glinka et al., 1998), demonstrating its role as an antagonist for Wnt/ β -catenin signaling (Fedi et al., 1999). In contrast to this, Dkk2 is able to



Figure 3: Non-canonical (β -catenin-independent) pathway. (A) vertebrate WNT/Ca²⁺pathway: Wnt activates PLC via Dsh or directly via Fz which upregulates Ca²⁺. As a second messenger, Ca²⁺ is involved in numerous cellular processes. (B) Wnt/JNK-pathway: Wnt binds Ror2 and thereby activates JNK via PI3K and Cdc42, or it binds to Fz to activate JNK via Rac1. Until now, it is not clear whether the two pathways are different, parallel or one. Rac1 regulates the rearrangement of the actin cytoskeleton, while JNK is able to act as a transcription factor to activate target genes via c-jun. (C) Wnt/Rho-pathway: Wnts activate via RhoA, via DAAM and Dsh. RhoA activates Rho-Kinase which regulates actin cytoskeleton rearrangements. Abbreviations: PLC, Phospholipase C; NFAT, nuclear factor of activated T-cells; CamK, calcium/calmodulin-dependent protein kinase; CDC, cell division cycle; DAAM, Dishevelled-associated activator of morphogenesis; Dsh, Dishevelled; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; Rac and Rho, small GTP-binding proteins.

activate Wnt/ β -catenin signaling as was shown in Fz8 and LRP5/6-overexpression approaches (Wu et al., 2000; Brott and Sokol, 2002). The action of Dkk1 and Dkk2 also depends on binding to Kremen1/2, which potentiate the antagonistic effect on Wnt/ β -catenin signaling (Mao et al., 2002). However, Dkk1 was demonstrated *in vitro* to influence non-canonical Wnt-signaling, too (Lee et al., 2004). It also activates JNK-signaling during gastrulation *in vivo*, indicating a role for both β -catenin-dependent and -independent signaling (Caneparo et al., 2007).

In mammals, the family of secreted Frizzled related proteins (sFRP) consists of five members. They contain a CRD-domain (N-terminal like in Fz) which is able to bind to Wnt and, therefore, are regarded as Wnt-antagonists (Lin et al., 1997; Leyns et al., 1997; Dennis et al., 1999; Uren et al., 2000). However, sFRP1 is able to bind to the CRD-domain of Fz, thereby activating Wnt/ β -catenin signaling (Melkonyan et al., 1997; Uren et al., 2000; Yokota et al., 2008).

1.2.7 Cross talk of Wnt-signaling pathways

As evident from the schematic illustration of Fig. 3, there is a high degree of cross talk between the non-canonical Wnt-pathways. However, this is only a snapshot of all interaction possibilities. Just to mention some examples: the Wnt/Ca²⁺-pathway interferes with the canonical (β -catenin) pathway via the activation of the nemo-like kinase, which phosphorylates TCF and thus inhibits β -catenin signaling (Ishitani et al., 2003). The JNK-pathway can be inhibited by overexpression of RhoA as shown by Nagao et al. (1999). Wnt/Ca²⁺ signaling is linked to Wnt/Rho-signaling via the potentiation of PCK α activity as suggested by Slater et al. (2001) to be mediated by RhoA and cdc42. Interestingly, nuclear localization was shown to be modulated by the Wnt/JNK-pathway mediators Rac1 and JNK2 (Wu et al., 2008).

All findings about Wnt-signaling cross-talk nicely show that the discrimination of distinct pathways should be extended to the term 'Wnt signaling network'. Most Wnts can activate different downstream effectors, depending on the receptors, co-receptors and intra and extracellular modulators and mediators. Our knowledge about this Wnt-signaling network is growing rapidly and more and more Wnts are shown to activate pathways they were not thought to be involved in. The cooperation of ligands, receptors, inhibitors and pathway mediators to integrate signals in a correct way becomes obvious when discussing the *in vivo*-mechanisms of embryonic development. Temporal and spatial expression patterns of all these components control the program responsible for initial embryonic cell devision, tissue and organ generation, and finally, the development of an organism with countless cells that are specifically organized. This organization leads to structures too complex for us to understand them completely (yet). One of them is the brain.

1.3 The midbrain - origin of dopaminergic neurons in vivo

The development of the central nervous system (CNS) is influenced by a variety of extracellular signaling molecules including Wnt, Hedgehog (Hh), Notch, fibroblast growth factor (Fgf), bone morphogenic factors (BMPs) and transforming growth factors (TGFs). These factors regulate in a spatio-temporal and cell type-dependent manner processes like proliferation, differentiation, migration and communication of a steadily increasing number of cells and a multitude of cell-types. During gastrulation, the mesodermic notochord signals to the ectoderm to form a neural plate. The neural tube is formed by primary and secondary neurulation through infolding and hollowing, respectively. Four neuromeres are formed along the neural tube, beginning from anterior with the prosencephalon, mesencephalon, rhombencephalon and most posterior the spinal cord. The mesencephalon forms the midbrain which is the place of birth and differentiation of DA neurons.

1.3.1 Midbrain development

During midbrain development proper proliferation and differentiation depends on a temporal and spatial control of morphogenic gradients. Through these gradients the cells are able to orientate within the brain in a temporal and spatial manner which commits the cells fate. One of these morphogens is the Wnt family of proteins. Wnts are not only for crucial developmental processes during early epiblast formation and gastrulation (for review see Gadue et al. (2005); Rohde and Heisenberg (2007); Tada and Kai (2009)), but also involved in a the development of numerous organs (Dale et al., 2009) and hence involved in many diseases (Johnson and Rajamannan, 2006) which are, however, outside the scope of this thesis.

Besides Wnts, other morphogens like Otx2/Gbx2, Fgf and Sonic hedgehog (Shh) precisely regulate midbrain development (for review see Puelles (2007)). The spatio-temporal expression of Otx2 and Gbx2 establishes the boundary between midbrain and hindbrain (Vernay et al., 2005). Fgf8 is necessary for anterior/posterior (A/P) patterning and the midbrain/hindbrain boundary (Suzuki-Hirano and Shimogori, 2009) and also positively influences dopaminergic differentiation in the midbrain (Baizabal and Covarrubias, 2009). Shh controls the dorsoventral (D/V) axis of the midbrain being exclusively expressed in the floor plate (FP) of the ventral midbrain (VM). Therefore it defines the ventral identity in the neural tube and the midbrain/hinbrain boundary (Briscoe et al., 2000; Bayly et al., 2007). It also controls differentiation of DA neurons in an early stage of midbrain development (Hynes et al., 1995). However, during this process of DA neurogenesis, Shh antagonizes Wnt/ β -catenin signaling in the FP of the midbrain. This interplay of Shh and Wnt-signaling is crucial for induction and proliferation of DA progenitors: high dosage of Shh reduces Wnt-mediated proliferation and DA neuron production in midbrain cultures (Joksimovic et al., 2009). These findings demonstrate how the dynamic interplay of canonical Wnt/ β -catenin signaling and Shh may orchestrate floor plate neurogenesis.

Wnt-signaling in the midbrain is mainly influenced by Wnt1 and Wnt5a. Wnt1 is expressed in the E11.5 midbrain but is restricted to the dorsal midline in the roof plate (RP), at the midbrain/hinbrain boundary and at two stripes flanking the ventral midline in the FP (Wilkinson et al., 1987; McMahon and Bradley, 1990; Prakash et al., 2006). Wnt5a is expressed throughout the VM at early stages (E9.5) and is restricted to the FP by E11.5-E13.5. Loss of Wnt5a results in an increase of proliferation of DA progenitors and the number of DA precursors (Andersson et al., 2008), which also reflects the antagonism between Wnt1 and Wnt5a being involved in proliferation and differentiation, respectively.

1.3.2 Dopaminergic neurons

DA neurons of the ventral midbrain in particular are dying in Parkinsons's disease (PD). Predominantly, DA neurons from the substantia nigra (SN, A9 population) are lost during this disease. DA neurons are also found in the A8 (retrorubral field) and A10 (ventral tegmental area) populations. During embryogenesis they are present throughout the ventral midbrain and the first two prosomeres of the diencephalon at E11.5 (Marín et al., 2005). The birthplace is believed to be located in the region where Shh of the FP and Fgf8-expression of the midbrain/hindbrain boundary are close together. This is plausible since several studies have shown that Shh and Fgf8 both are sufficient to induce DA neurogenesis (Hynes et al., 1995; Stull and Iacovitti, 2001; Kim et al., 2003).

In the developing ventral midbrain, proliferating DA-progenitor cells reside in the ventricular zone (VZ) and as they differentiate into DA-neurons migrate through the intermediate zone (IZ) down to the marginal zone (MZ) (Fig. 4) (Kawano et al., 1995; Puelles et al., 2003). A variety of marker proteins are present in each phase and help to distinguish each stage: proliferating VZ progenitor cells are positive for Sox2, aldehyde dehydrogenase (AHD-2), Otx-2 and Msr1/2, here proliferation is controlled by Shh, IZ precursor cells are positive for L-aromatic amino acid

decarboxylase (AADC), Lmx1b (a homeodomain transcription factor important for DA neuron fate specification (Andersson et al., 2006)), Nurr1 and paired-like homeodomain transcription factor 3 (Pitx3) and MZ DA neurons are positive for Lmx1b, Nurr1, Pitx3 and TH (Teitelman et al., 1983; Wallén et al., 1999; Zetterström et al., 1996; Chen et al., 1998a,b; Puelles et al., 2003; Andersson et al., 2006).

1.3.3 Wnt-signaling molecules in the developing midbrain

As mentioned before, the temporal and spatial interplay of different signaling cascades is the driving force for proper (mid)brain development. To understand how Wnt-signaling influences the mechanisms of proliferation and differentiation within the midbrain, it is important to know which signaling molecules are expressed at the different stages of embryogenesis: around E11.5 Wnt1, Wnt4, Wnt5a, Wnt7a, Wnt7b and Wnt9a are expressed in the FP of the mouse VM (Vendrell et al., 2009). Wnt5a and Wnt7a are already present at E9.5 (Parr et al., 1993).

The expression of Wnt-receptors in the E11.5 midbrain is limited to Fz1/2/3/6/7/9/10. Fz1-3 are ubiquitously expressed in the midbrain, Fz6/7/9 in the VM, Fz9/10 in the dorsal midbrain (DM); Fz4/5/8 were not found in the midbrain by *in situ* hybridization (Fischer et al., 2007; Rawal et al., 2006). Interestingly, Fz9 was found to be only expressed in the DA progenitor cells and not in the mature TH+ neurons of the VZ/VM, indicating a differentiation stage-dependent expression (Rawal et al., 2006). The co-receptors of $Fz/Wnt/\beta$ -catenin signaling LRP5/6 are expressed throughout the developing embryo showing a high redundancy between both of them (Kelly et al., 2004). Ror1/2 are also differentially regulated and present at early (E9.5-E10.5) stages as described by several authors (Al-Shawi et al., 2001; Matsuda et al., 2001).

The expression of Wnt modulators like sFRPs in the midbrain is limited to later stages of embryogenesis (E14.5-E15.5) where a general weak presence of sFRP1 was detected in the whole brain. Generally sFRPs like sFRP1 and sFRP2 are present in the forebrain and hindbrain (Leimeister et al., 1998; Hoang et al., 1998).

These findings represent only a small part of the complex spatio-temporal expression pattern of Wnt-signaling components. It is obvious that Wnt-signaling proteins are highly regulated during VM development. Hence, increased knowledge about the temporal and spatial distribution of these proteins will lead to a better understanding of neurogenesis, showing the need for further mapping of the Wnt components during brain development. Especially the knowledge about the spatio-temporal control of Wnt-signaling proteins and the the available tools for quantitative sub-cellular, temporal and differentiation stage-dependent analyses are limited — a topic which is, therefore, in the scope of this thesis.

1.3.4 Wnt function in midbrain development and DA neurogenesis.

Wnt1 in midbrain development. The involvement of Wnts in midbrain development has been demonstrated in $Wnt1^{-/-}$ -knockout mice, resulting in a partial deletion of the midbrain and hindbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Danielian and McMahon, 1996) and more interestingly in a significant loss of DA neurons (Prakash et al., 2006). However, not all DA neurons were lost in these experiments, which led to the suggestion that another Wntsignaling mechanism was involved in DA differentiation. McMahon et al. (1992) revealed that this decrease of DA neurons was mediated by the loss of Engrailed 1/2 (En1/2) which led to the



adapted from Castelo-Branco and Arenas (2006)

Figure 4: Development of DA neurons in the ventral midbrain (VM). (1) A/P and D/V patterning if ventral midbrain at E10.5: floor plate cells in ventricular zone (VZ) express Sonic hedgehog (Shh, green) while neuroepithelial cells in the isthmus region release FGF-8 (blue). The intersection between the two gradients (arrows) corresponds to the birth place of DA neurons (red). (2) temporal development and DA fate decision in the VM: (2A) neural precursors in the medial VZ express aldehyde dehydrogenase (AHD-2) and Otx-2. Shh induce the proliferation of these progenitors. (2B) Through radial glia guidance, these precursors migrate into the intermediate zone (IZ) and begin to express L-aromatic amino acid decarboxylase (AADC), at E10.5 they start to express Nurr1 which is followed by cell cycle exit. (2C) the postmitotic precursors arrive in the marginal zone (MZ) at the pial surface and start expressing DA neuron markers like tyrosine hydroxylase (TH), Pitx-3 and Lmx-1b. At E13 they reach their final location (i.e. the future substantia nigra *pars compacta* or other DA-nuclei). V, ventricle. For a more comprehensive summary of this topic please see (Castelo-Branco and Arenas, 2006).

deletion of the midbrain and hindbrain (McMahon et al., 1992; Danielian and McMahon, 1996). The underlying mechanism of Wnt1 initiating DA neurogenesis was suggested by Prakash et al. (2006) showing that Wnt1 induces Otx2 which represses Nkx2-2 in the FP. In the absence of Wnt1 Nkx2-2 represses DA neurogenesis and rather promotes serotonergic neurogenesis. Besides this mechanism the authors showed that Wnt1 also activates Fgf8-mediated DA differentiation. Additionally, the time of Wnt1-expression overlaps with the first appearance of DA neurons in the VM (Castelo-Branco et al., 2003). The authors treated primary VM cultures with Wnt1 which resulted in an increased number of DA neurons up to three-fold. The reason for this was an increase in proliferation of Nurr1+ DA precursors and an increased total number of neurons in the culture (Castelo-Branco et al., 2003, 2004). Reversely, in Wnt1^{-/-}-knockout mice both DA and β III-tubulin neurons are lost exclusively in the FP but remain present in the basal plate (BP, located laterally of the FP) of the midbrain, indicating that Wnt1 is necessary for DA neurogenesis in the FP (Andersson et al., 2008). These findings nicely show the role of canonical Wnts like Wnt1 during the early time of both fate commitment and proliferation of restricted (DA) progenitor cells in the FP/VM *in vivo*.

Crosstalk between Wnt1 and Wnt5a in midbrain development. However, both Wnt1 and Wnt5 were shown to expand the Nurr1+ progenitor population and increase DA differentiation. This suggests that What affect specific sub-populations of either neuronal or glial restricted progenitor cells in the developing midbrain (Wagner et al., 1999). One possible explanation for this is a different receptor-constitution which might result in a different responsiveness to a given What ligand (Rawal et al., 2006). During DA differentiation between E11.5 and E13.5 the only Wnt-receptor that is highly expressed in TH+ neurons is Fz8. However, other Fz-receptors are differentially expressed between E11.5 and E13.5 suggesting a Fz-mediated and stage-dependent control of Wnt-signaling activity (Rawal et al., 2006). Since nearly all Fz-receptors were shown to be present in the TH+ region of the VM, the authors concluded that Wnt/Fz-signaling is more important in newborn DA neurons than in their neighboring environment. Interestingly, Fz9 is only present in DA-precursors (but not in DA-neurons) and is only activating Dsh after its activation with Wnt3a but not with Wnt5a. This suggests that Fz9 only mediated the proliferating effects of Wnt3a but not the differentiating effects of Wnt5a during DA differentiation (Castelo-Branco et al., 2003). LRP5/6 are crucial co-receptors for Wnt/ β -catenin signaling and were thought be involved only in this pathway. However, Bryja et al. (2009) have shown that LRP6 physically interacts with Wnt5a. Recent findings by Castelo-Branco et al. (2010) revealed that the loss of LRP6 leads to a transient reduction of DA neurons in the VM, despite normal patterning and proliferation. Although this binding does not activate β -catenin signaling, it prevents activation of Wnt5a-signaling in vitro. The authors showed that overexpression of LRP6 blocked activation of downstream Wnt5a-signaling, which indicated that the presence of this canonical co-receptor prevented Wnt5a-mediated non-canonical Wnt-signaling. For midbrain development in vivo this implies, that the receptor constitution of both Fz and LRPs also regulates the cells responsiveness on either Wnt1 or Wnt5a in a developmental stage-dependent manner.



Figure 5: What in the development of DA neurons. During VM development and before the birth of the DA neurons, Wht-1 and Wht-5a (to a lesser extent) induce the proliferation of neural precursor cells. On their way through the intermediate zone the progenitors begin to express Nurr-1 and exit the cell cycle due to inhibition of Wht-1 signaling partly by Wht-5a, which, at this stage, is able to induce DA differentiation. Wht-1 is also involved in this process although to a lesser extend. Wht-3a is not expressed in the developing VM, as it would inhibit the DA differentiation, either by promoting precursor proliferation or inducing differentiation to an alternative cell fate. For a more comprehensive summary of this topic please see (Castelo-Branco and Arenas, 2006).

Wnt5a in midbrain development. The positive effect of Wnt5a on DA differentiation is not due to the increased number of proliferating cells (which is the task of Wnt1). However, treatment of VM cultures with Wnt5a increased the pool of proliferating BrdU+/Nurr1+ DA precursors which indicates a differentiation stage-dependent effect (Castelo-Branco et al., 2003). This is in agreement with the exclusive presence of Fz9 on Nurr1+ DA precursors as stated above. Additionally, Wnt5a-treatment also increases the ratio of TH+ DA neurons to β III-tubulin+ neurons as was shown by Schulte et al. (2005). Reversely, Wnt5a-inhibition with a specific antibody blocked this effect (Castelo-Branco and Arenas, 2006). Investigation of Wnt5a^{-/-}knockout mice revealed that a loss of Wnt5a increased proliferation of IZ progenitors and the pool of Nurr1+ DA precursors (Andersson et al., 2008). These findings reflect the antagonistic relationship between Wnt1 and Wnt5a-signaling, being responsible for proliferation and DA maturation, respectively. Nevertheless, despite an increased pool of precursors, the number of DA neurons (Nurr1+/TH+) was constant compared to the WT. The authors suggested that the transition from Nurr1 DA precursors to Nurr1+/TH+ DA neurons was disturbed, which resulted in a relative loss of DA neurons at E12.5 (Andersson et al., 2008).

All these findings indicate the synergistic effects between Wnt1 (canonical) and Wn5a-(noncanonical)-signaling. Loss of Wnt5a alone does not decrease the number of DA neurons but increases the pool of Nurr1+ DA precursors. Loss of both Wnt5a and Wnt1 decreased the number of DA neurons additively. This shows that Wnt5a contributes to DA neurogenesis and that Wnt1 is responsible for proliferation of the restricted DA (Nurr1+) precursors *in vivo* (Castelo-Branco and Arenas, 2006; Andersson et al., 2008). After Wnt1-controlled proliferation of the DA progenitors and precursors, Wnt5a-signaling follows with the differentiation program for transition from Nurr1 precursors to Nurr1/DA neurons as illustrated in Fig. 5. However, the initial onset of the differentiation process (and therefore the fate decision) seems to be controlled by Wnt1. In agreement with this, our group recently revealed that the endogenous amount of Wnt5a mRNA and proteins increased during differentiation of the human embryonic midbrain culture ReNcell VM (personal communication, O. Mazemondet et al. (2010) and A. Rohn (2009)). Addition of recombinant Wnt5a at the onset of differentiation showed increasing effects on the Hu C/D positive neuronal population in ReNcell VM.

From these observations it becomes evident that *in vitro* protocols that target high yields of DA differentiation should include both activation of Wnt/ β -catenin signaling (Wnt3a) and non- β -catenin signaling (Wnt5a). Recent data from Cajánek et al. (2009) presented evidence that the inhibition of Wnt/ β -catenin signaling at the right moment of differentiation is also required to enhance DA neurogenesis in embryonic mouse stem cell cultures.

It remains to be elucidated whether this knowledge from the embryonic mouse midbrain *in vivo* and especially the interplay between Wnt1 and Wnt5a can be transfered into human *in vitro* models. It is obvious that a precise temporal interplay of different signaling pathways at different stages during neuronal differentiation is crucial for proper brain development and any disturbance inevitably leads to severe dysfunctions. Therefore, the need for precise, spatio-temporal and quantitative analyses is apparent. Modern image cytometry provides tools to address this lack of resolution and offers a means to investigate rather qualitative observations in a quantitative manner.

1.4 Image cytometry

The art of combining molecular, morphological and phenotypic information at the single-cell level with cellular mechanisms and dysfunctions — an art known as microscopy — nowadays represents the key to modern cytomics: a collection of the complex and dynamic cellular reactions that underly physiological processes. Thus, it describes the structural and functional heterogeneity of the cellular diversity of an organism. Projects like the 'Human Cytome Project' combine state-of-the-art techniques and technologies from different fields (Tsien, 2003; Tárnok, 2004). Methods from molecular biology (genome) and protein biochemistry (proteome) are necessary but not sufficient for the understanding of the cytome, one example of which is the complex mechanism of controlling stem cell fate decision. Spatial relations and the molecular morphology of cells and tissues are accessible only to microscopic analysis (Ecker and Tarnok, 2005). In this context, multi-parametric methods like fluorescence microscopy are of particular interest because they allow the analysis of protein expression, interaction, distribution and morphological characteristics (Gao et al., 2004; Lenz et al., 2004).

As long as researchers investigated cells through microscopes, they tried to yield quantitative data from these observations. With the advent of computer-assisted digital image acquisition about 30 years ago, quantitative image analyses stepped into the next generation. In parallel, the optical principal of confocal microscopy, which was patented in 1957 already, was improved until it became a standard and fully mature technology at the end of the 1980s (Minsky, 1957; Pawley, 2006). In laser scanning confocal microscopy a pinhole aperture enables an optical section of narrow depth to be imaged by only allowing fluorescent light from the focal plane to reach the detectors (whereas a traditional widefield microscope also acquires information from outside the plane of focus) (Goldman and Spector, 2005). The pioneers Cremer and Cremer (1978) were the first to use a focused lasers beam for the scanning process, which scanned the

three dimensional surface of a fluorescent object point-by-point. At the beginning of the 1990s, computer chips like the i860 offered the prospect of fast 3D-reconstructions of confocal image series and the software to make this a routine technique (Whimster et al., 1995). Ever since, both computers and confocal microscope systems improved tremendously, illustrating the need for quantitative image cytometry in more than two dimensions.

1.4.1 Cytometry goes 3D

In many biological applications two-dimensional representations of microscopic images are still state of the art. However, if we want to understand the interaction of proteins in a spatial relation, the third dimension is highly important and relevant. The function of a single protein or protein complex is directly related to its sub-cellular location. Thus, the detection and localization of proteins in relation to other sub-cellular structures is an important task in biological studies. The use of highly specific staining methods and fluorescent markers that emit light at different wavelengths in combination with fluorescence microscopy permit detailed studies of the spatial distribution and localization of proteins. One challenge is the circumstance that biological samples are three-dimensional, and therefore, signals often spread across 3-dimensional volumes.

Researchers can choose from a number of techniques for biological fluorescence microscopy, and the performance of these methods has been studied in the literature (Murray et al., 2007; Bolte and Cordelières, 2006). The most common approach in many biological applications today is the use of techniques like spot scanning/laser scanning or spinning disk confocal microscopy. The use of these confocal techniques for volumetric (3D) imaging is the most common approach in many biological applications today. Localization and quantification of fluorescence signals from *in situ* detected proteins within their cellular compartments has been an active research field over the years (Boland et al., 1998; Markey et al., 1999; Huang and Murphy, 2004). Glory and Murphy (2007) published a comprehensive comparison of image analysis-based methods for determination of intracellular localization of fluorescent-labeled molecules within cells. With these methods it is possible to locate signals in relation to major organelles in the cells and to search for temporal location patterns using various classification techniques by MATLAB programming. With the power of bioinformatics, the sub-cellular localization of a protein can be predicted based on its amino acid sequence (Chou and Shen, 2007). Nevertheless, the predicted localization still has to be visualized *in situ*, so that the results can be verified. This is only one example for the modern synergy of information sciences and life sciences, which especially becomes important in the fields of high-throughput screening (HTS) and high-content analysis (HCA) for biomedical and drug research. HTS and HCA are mainly used by pharmaceutical companies or genome-wide screening projects and therefore outside the scope of this thesis. For a comprehensive summary on the use of image cytometry in HTS please see review by Lang et al. (2006).

1.4.2 Image cytometry in neuroscience

In the field of neuroscience, quantitative image cytometry is mainly used for *in vitro* quantification of complex cellular biochemistry and anatomy like dendritic tree morphology, protein aggregation, neurotransmitter internalization, neuron and synapse number, cell migration, proliferation and apoptosis (Dragunow, 2008). However, image cytometry can also be applied to images of sections of brain tissue, making it applicable to both *in vivo* and *in vitro* neuroscience. For example, automatic image quantification was used to identify and count neural progenitors in sections of the adult human brain, identifying for the first time the human rostral migratory stream (Curtis et al., 2007). Automated or semi-automated systems can measure many different aspects of neurites, including the amount of outgrowth occurring in the same cells over time, the number of processes emerging from each cell, cross points from adjacent neurites, and their branching (Ramm et al., 2003; Price et al., 2006; Keenan et al., 2006; Vallotton et al., 2007). For these approaches software tools are available ranging from free open source software like ImageJ (NIH) over easy-to-use and intuitive but powerful tools like Imaris (Bitplane) to sophisticated programs like MetaMorph (Molecular Devices) combined with MATLAB (MathWorks).

However, most laboratories in the academic sector still perform these quantifications for *in vitro* studies either by manual counting (which is slow, subjective and laborious) or biochemically (without cellular resolution). Many academic laboratories cannot afford high-throughput automated microscopy platforms, or automation might not be possible in particular applications. Therefore, it is important that sample preparation, staining procedures and image acquisition are standardized as far as possible to yield the best possible results when applying quantitative image cytometry. For an overview on this topic please see the summary by Theodosiou et al. (2007), who provided an excellent review on methods for (semi-) automated image analysis.

1.4.3 Image cytometry in stem cells research

Presently, the stem cell field is already benefiting from cellular imaging, such as for monitoring stem cell fate in vitro and in vivo. Today, there is hope that the use of cell therapy for transplantation (Hyun, 2010; Lindvall and Kokaia, 2010), treating myocardial infarction (George, 2010; Boilson and Gulati, 2010) or neuro-degenerative diseases like Alzheimer's disease (Taupin, 2009) or Parkinson's disease (Parish and Arenas, 2007; Manfredsson et al., 2009; Sharp and Keirstead, 2009) will be successful. However, these stem cell therapies require a clear understanding of how stem cells or progenitor cells differentiate in order to avoid tumorigenesis. It is well known that the use of multiple but specific neuronal differentiation protocols for human stem cells in parallel could lead to the generation of oligodendrocytes, astrocytes and neurons in the same petri dish in vitro (reviewed in Trounson (2002); Cazillis et al. (2006)). Subpopulation identification, therefore, is essential and only possible through microscopic means. Cellular imaging and screening of cell subpopulations using cellular imaging can quickly and reproducibly yield a vast amount of data from only a few thousand cells per well. In summary, the explicit understanding of how stem cells differentiate (signaling pathways, protein translocation) and the subsequent evaluation of the cell's fate (phenotyp) are excellent examples for the need of quantitative image cytometry.

1.4.4 Image cytometry and signal transduction and protein translocation processes

Signal transduction involves temporal and spatial changes in signal strength and localization. New microscopy platforms are emerging which provide flexibility in terms of resolution. When used at low resolution, they are able to quantify phenotypic changes such as changes in cell

19

number and cell shape (as described above). In high-resolution mode, sub-cellular changes can be quantified such as protein translocation across sub-cellular compartments. For image cytometry, commonly found examples of cytoplasmic/nuclear translocation of a transcription factor are the detection and quantification of nuclear factor- κB (NF- κB) and the related p38 (Kwon et al., 2007) or FOXO3 (Zanella et al., 2008, 2010). These proteins translocate into the nucleus in response to inflammatory stimuli or stress. Quantifying this process by imaging was successfully used to profile drug compound selectivity between these signaling pathways (Bertelsen and Sanfridson, 2005; Bertelsen, 2006). Today, life cell imaging is revolutionizing the field of translocation studies. Here, the mechanism of NF- κ B nuclear/cytoplasmic shuttling is also the model system for the advancement in this field. Using single cell life cell imaging, Mullassery et al. (2008) have shown that NF- κ B is oscillating between nucleus and cytoplasm. This group around Michael White has used iterative real-time single cell imaging and mathematical modeling to show that the NF- κ B system is oscillatory and uses delayed negative feedback to direct nuclear to cytoplasmic cycling of transcription factor(s) that regulate gene expression (Nelson et al., 2004; Mullassery et al., 2008). However, this level of temporal and spatial resolution requires suitable fluorescent probes, highly advanced microscopy platforms and a model system which can be analyzed in this context.

One of these model systems is the (canonical) Wnt/β -catenin pathway (section 1.2) where the nuclear accumulation of β -catenin is a suitable example for the use of quantitative image cytometry. Especially the context of colon carcinogenesis is in the focus of quantitative image cytometry because (due to aberrations in the APC gene) β -catenin dramatically accumulates in the nuclei of the stem cells in the crypts which is followed by uncontrolled proliferation and hence tumor growth (Camac et al., 2007). The Wnt/ β -catenin-signaling pathway is also relevant during neuronal differentiation although, to our knowledge, it was not investigated quantitatively in a temporal and spatial approach, neither *in vitro* nor *in vivo*. A topic which is directly in the scope of this thesis and will be discussed in greater detail throughout this manuscript.

1.4.5 Image cytometry and systems biology

As mentioned before, dynamic experiments have the ability to reveal new and useful information about inherently complex biological control systems. However, understanding how this information fits together and how perturbations affect system dynamics can be a complex task, especially when considering the regulatory mechanisms of observed oscillatory phenomena which can behave in non-intuitive ways (Nelson et al., 2004).

Understanding how mammalian cells function requires a dynamic perspective. However, owing to the complexity of signaling networks, these non-linear systems can easily elude human intuition. The central aim of systems biology is to improve our understanding of the temporal complexity of cell signaling pathways, using a combination of experimental and computational approaches. Live-cell imaging and computational modeling are compatible techniques which allow quantitative analysis of cell signaling pathway dynamics (Mullassery et al., 2008).

In addition to measuring individual parameters, cellular imaging technologies can be promising tools for studying cell systems biology to understand physiology and disease at a whole-cell level. In this approach, a complex network of cell types, treatments and readouts are analyzed by machine-learning algorithms. The aim is to discriminate as many disease-modulating agents and drugs as possible while taking a minimal set of measurements (Butcher, 2005, 2007). This methodology requires that relevant information is selected by processing large amounts of data into computer models of regulatory networks.

Cellular imaging combined with powerful computing techniques could, therefore, revolutionize our understanding of the complexity of the mode of action of drugs, as well as our understanding of the dysfunctions of diseased cells (Lang et al., 2006). It has the potential for individualized disease course prediction and, hence, for the development of novel, individualized strategies for curative therapy and new therapeutic concepts (Tárnok, 2004).

1.5 Conclusion

In summary the use of modern image cytometry to investigate the complex spatio-temporal processes of protein translocation in differentiating stem cells represents a cutting-edge approach. The scope of application in the field of regenerative medicine with respect to cell-replacement-therapies in Parkinson's disease is, therefore, very promising to increase the knowledge in this field and, hence, the background of this thesis.

The exact temporal sub-cellular redistribution of key proteins during Wnt/β -catenin signaling is a central mechanism crucial for many developmental processes and diseases and its investigation will give insights into the mechanism involved in early neuronal differentiation and the decision process during progenitor cell/neuron-transition.

1.6 Motivation and aims of the work

In a wider sense, the goal of the work was to increase the presently limited knowledge about human neural stem cell models to improve cell replacement therapies for neuro-degenerative diseases such as Parkinson's disease. Here the focus lay on the sub-cellular protein translocation processes during neuronal differentiation in respect to Wnt-signaling.

Hence, we asked the following questions:

- 1. Is the human neural progenitor cell line ReNcell VM suitable to investigate Wnt-signalingdependent neuronal differentiation?
- 2. Is the extent of Wnt/β -catenin signaling directly connected to the extent of neuronal differentiation? and if so,
- 3. What is the underlying mechanism for this correlation?
- 4. Is the sub-cellular Wnt-dependent protein translocation process comparable between the human ReNcell VM model and the developing embryonic mouse midbrain?

Based on these questions the specific aims of this study were:

- To characterize the human neural progenitor cell line ReNcell VM in terms of differentiation capacity.
- To establish an easy-to-use method to quantify proteins in a sub-cellular, cell type-dependent and temporal manner.
- To analyze the correlation between Wnt/β -catenin signaling activity and efficacy of neuronal differentiation.
- To investigate the underlying mechanism of this correlation.
- To compare this human cell model with the well known model of the embryonic mouse midbrain with respect to sub-cellular β -catenin localization.

2 Experimental procedures

2.1 Neural progenitor cells from human fetal ventral midbrain - ReNcell VM

2.2 Maintenance and differentiation of ReNcell VM cells

All experiments were carried out using the human neural progenitor cell line ReNcell VM (Millipore, Schwalbach, Germany, SCC008; ReNeuron Inc., Guildford, UK) derived from 10 weeks old human fetal tissue from the ventral midbrain (VM) and immortalized by stable *v-myc* oncogene transduction. These cells exhibit a cell cycle length of 20-25 hours, readily differentiate into neurons and glia and have been described as a model to study cellular processes occurring during the development of degenerative diseases such as Huntington's, Alzheimer's and Parkinson's diseases (Hoffrogge et al., 2006; Donato et al., 2007).

The cells were only used up to passage 25 to avoid the influence of possible changes in differentiation characteristics. Newer passages ($\geq P8$) were obtained from 10 % DMSO (Dimethyl sulfoxide, Sigma-Aldrich, Hamburg, Germany, D4540) stocks that were frozen in DMEM:F12 (Invitrogen, Karlsruhe, Germany, 21331-046) and kept in liquid nitrogen. The cells were expanded similar to the protocol described in Donato et al. (2007) at 37 °C/5 % CO₂ in DMEM:F12 supplemented with B27 neural cell supplement (Invitrogen, Karlsruhe, Germany, 17504-044), L-Glutamine (2 mM; Invitrogen, Karlsruhe, Germany, 25030-024), Heparin (10 units/ml; Sigma-Aldrich, Hamburg, Germany, H3149), Gentamicin (50 μ g/ml; Invitrogen, Karlsruhe, Germany, 15750-037) in the presence of human recombinant Epidermal Growth Factor (EGF; 20 ng/ml; Sigma-Aldrich, Hamburg, Germany, E9644) and basic Fibroblast Growth Factor (bFGF; 10 ng/mL; Invitrogen, Karlsruhe, Germany, 13256-029) - referred to as growth medium. The cell culture flasks (TPP) were covered with laminin to facilitate cell adhesion (10 μ g/ml in DMEM:F12; 1 μ g/cm²; Trevigen/Biozol, Eching, Germany, 3400-010-01) and washed with Hanks' Balanced Salt Solution (HBSS w/o Ca&Mg; Invitrogen, Karlsruhe, Germany, 14170-088). Glas coverslips (Kindler, Freiburg, Germany) were flame-treated for sterilization and instantly coated with poly-D-lysine-hydrobromid (PDL, stock 25 µg/ml, 0.1 µg/cm², at 37 °C/5% CO₂; Sigma-Aldrich, Hamburg, Germany, P7280). After overnight incubation PDL was washed away with sterile dH_2O and the coverslips aseptically irradiated with UV-light for 20 minutes, air-dried and coated with laminin for 4 hours at 37 $^{\circ}C/5$ % CO₂. Sub-confluent cell layers were passaged using Trypsin-EDTA (in HBSS w/o Ca & Mg, Invitrogen, Karlsruhe, Germany 25300-054; benzonase, 25 U/ml, Merck, Darmstadt, Germany 1.01654.0001; in DMEM:F12) followed by quenching of trypsin in trypsin inhibitor solution (0.55 mg/ml trypsin inhibitor,Sigma-Aldrich, Hamburg, Germany T6522; 1 % human serum albumine (HSA), Grifols, Langen, Germany, 720611; in DMEM:F12). Maintaining sub-confluency up to a level of 70-80 %was crucial to avoid spontaneous differentiation. In most cases, the cells were grown for 4 days before the start of differentiation.

ReNcells VM differentiate after withdrawing the growth factors (i.e. EGF and bFGF) starting at a cell confluence around 70-80 %. The cells were washed with warm HBSS once and differentiation medium was added (growth medium without growth factors). Most experiments were conducted within the first three days after initiation of differentiation.

2.3 Microscopic characterization of ReNcell VM differentiation

2.3.1 Fixation and immunostaining for fluorescence microscopy

Cells were fixed in phosphate buffered saline (PBS, 10x: 0.137 mM NaCl, 2.7 mM KCl, 8.0 mM Na₂HPO₄, in dH₂O, pH 7.4, all Sigma-Aldrich, Hamburg, Germany) containing 4 % paraformaldehyde (PFA) and 4 % sucrose (pH 7.4; Sigma-Aldrich, Hamburg, Germany) for 20 min followed by PFA-quenching in PBS containing 50 mM NH₄Cl (Merck, Darmstadt, Germany) for 10 min at room temperature (RT). Permeabilisation, when required, was achieved by incubation with 0.2 % Triton X-100 (Roth, Karlsruhe, Germany) in PBS for 5 min at RT. Blocking of non-specific binding sites was performed with PBS containing 1 % white gelatin gold (Sigma-Aldrich, Hamburg, Germany 18808) for 60 min. Primary antibodies (Tab. 1) were diluted in blocking solution for 60 min at RT, followed by two 5 min washes with PBS containing 0.2% gelatin. Secondary antibodies (Tab. 1) were incubated for 45 min at RT, followed by two washes. For quantitative image cytometry analyses (see section 2.5.1) attention to exactly the same incubation times was crucial to compare different samples with each other. If required, samples were stained with markers for actin (1 μ g/ml phalloidin-TRITC; Sigma-Aldrich, Hamburg, Germany 77418) or cell nuclei (bisBenzimide 1 μ g/ml; Hoechst33258; Sigma-Aldrich, Hamburg, Germany 861405) at RT for 20 min and 5 min, respectively. Finally, coverslips were washed in PBS containing 0.2 % gelatin, then in PBS alone and shortly in dH₂O and were mounted on glass slides (Menzel, Braunschweig, Germany) using ProLong gold antifade mounting reagent (Invitrogen, Karlsruhe, Germany P63930) overnight.

All images from fixed cells were acquired using a confocal laser scanning microscope (TSC SP2 AOBS, Leica, Wetzlar, Germany), if not stated otherwise in the text. Images were processed with Adobe Photoshop version CS3, ImageJ (NIH/USA) or Imaris software (V6.0-6.3, Bitplane, Zurich, Switzerland). Long term live cell microscopy and acquisition of time lapse videos were performed using Biostation IM (Nikon, Dusseldorf, Germany at 37 °C and 5 % CO₂ for up to 72 hours in Hi-Q4 dishes (Ibidi, Martinsried, Germany). Sequential images from 10-20 different areas were acquired with an interval of 5 min per round (i.e. Fig. 6).

2.3.2 Microscopic quantification of neuronal differentiation

For quantification of the neuronal differentiation capacity the number of immunoreactive cells from the total number of cells (based on the nuclear staining) was counted from 30 randomly chosen and non-overlapping visual areas (63x objective) per coverslip of two wells per experimental condition for each experiment. All experiments were repeated with at least three independent preparations.

2.3.3 Cell type dependent analysis during ReNcell VM differentiation

Neural progenitor cells like ReNcell VM have the capacity to differentiate in many neural cell types. Therefore, the cells were stained with markers for various cell types and visualised by fluorescence microscopy: stem cells, restricted progenitor cells, astrocytes, neurons, neurons with dopaminergic background, oligodendrocytes (Tab. 1 and 2).

antigen	host	dilution	supplier			
primary antibodies						
Active- β -Catenin (ABC), clone 8E7	mouse	1:100	Millipore, Schwalbach, Germany, 05-665			
$\beta\text{-catenin, clone H102}$	rabbit	1:300	Santa Cruz, Heidelberg, Germany, sc-7199			
$\beta\text{-catenin, clone E5}$	mouse	1:300	Santa Cruz, Heidelberg, Germany, sc-7963			
β III-tubulin-FITC (neurons)	mouse	1:80	Abcam, Berlin, Germany, ab25770			
etaIII-tubulin	rabbit	1:80	Sigma-Aldrich, Hamburg, Germany, T2200			
Glial fibrillary acidic protein (GFAP), (clone G-A-5, Cy3)	mouse	1:100	Sigma-Aldrich, Hamburg, Germany, C9205			
Hu C/D (ANA-1)	mouse	1:300	Invitrogen, Karlsruhe, Germany, A-21271			
Nestin (stem cells)	mouse	1:100	BD, Heidelberg, Germany, 611658			
O4 (oligodendrocytes)	mouse	1:50	Chemicon, Nuernberg, Germany, MAB345			
Tyrosine hydroxylase (dopaminergic neurons)	mouse	1:100	Santa Cruz, Heidelberg, Germany, sc-25269			
Tyrosine hydroxylase	rabbit	1:100	Chemicon, Nuernberg, Germany, AB152			
Dopamine transporter (DAT)	rabbit	1:100	Sigma-Aldrich, Hamburg, Germany, D9567			
Pitx3	rabbit	1:500	Cymed, Stockholm, Sweden			
secondary antibodies and isotyp	secondary antibodies and isotype controls					
anti-mouse Alexa 488nm,	goat	1:500	Invitrogen, Karlsruhe,			
594nm, 647nm	-		Germany			
anti-rabbit Alexa 488nm, 594nm, 647nm	goat	1:500	Invitrogen, Karlsruhe, Germany			

 Table 1: Antibodies used for fluorescence microscopy

2.4 Flow cytometric characterization of ReNcell VM differentiation

2.4.1 Fixation and immunostaining for flow cytometry

The cells were trypsinized as described above (section 2.3.1) and resuspended in 1 ml PBS. For intra-cellular immunostainings this suspension was slowly dripped into 10 ml -20 °C methanol and fixed for at least one hour at -20 °C. For antibody-labeling of intra-cellular proteins the methanol was replaced by 200 μ l working buffer (PBS, 0.03 % [w/v] EDTA, 2 % [w/v] bovine serum albumine (BSA, Sigma-Aldrich, Hamburg, Germany A7906), pH 7.4). After one hour

antigon	labol	host	dotails	dilution	supplion			
antigen	label	nost	details	dilution	supplier			
primary antibodies								
Hu C/D	unconjugated	mouse	young neurons	1:300	Invitrogen, Karl- sruhe, Germany			
PSA-NCAM (2-2B)	phycoerythrin	mouse IgM	restricted neu- ronal progenitor cells	1:15	Miltenyi, Ber- gisch Gladbach, Germany			
A2B5	phycoerythrin	mouse	restricted glia	1:15	Miltenyi, Ber-			
(105-HB29)		IgM	progenitor cells		gisch Gladbach, Germany			
secondary an	secondary antibodies, dyes and isotype controls							
anti-mouse	Alexa 488nm	goat	secondary anti- body	1:500	Invitrogen, Karl- sruhe, Germany			
IgM	phycoerythrin	mouse	isotype-control	1:15	Miltenyi, Ber- gisch Gladbach, Germany			
propidium iodide	unconjugated	-	DNA-staining	1:20	BD, Heidelberg, Germany			
7-AAD	unconjugated	-	DNA-staining	1:20	BD, Heidelberg, Germany			

Table 2: Antibodies and dyes used for flow cytometric analyses

blocking in working buffer the primary antibody (Tab. 2) was directly added and incubated for 30 min on a shaker at RT followed by overnight incubation at 4 °C. After washing in 500 μ l hepes buffered saline (HBS: 0.9 % [w/v] NaCl; 0.33 % [w/v] HEPES; pH 7.5; all Sigma-Aldrich, Hamburg, Germany) the cells were incubated with the secondary antibody in 200 μ l working buffer for one hour followed by washing in 500 μ l HBS and final resuspension in 200 μ l HBS. All washing steps were done at 4 °C. The flowcytometric measurement was performed using an EpicS-XL flow cytometer (Beckman Coulter, Krefeld, Germany).

For membrane-associated proteins such as PSA-NCAM or A2B5, the cells were trypsinized, washed in cold PBS and resuspended in cold blocking buffer (0.5 % [w/v] BSA, 2 mM EDTA in PBS). The antibodies were directly diluted in 50 μ l of this suspension to the required concentration (Tab. 2). After ten minutes incubation at 4 °C in the dark, 500 μ l cold blocking buffer was added to dilute the antibodies and fixed by adding 500 μ l PFA solution and overnight incubation at 4 °C (final concentration 2 %, PBS containing 4 % PFA and 4 % sucrose).

2.4.2 Flow cytometric quantification of neuronal differentiation

The cells were stained as described in section 2.4.1 using antibodies against neuronal proteins (Tab. 2). To exclude fluorescence signals originating from unspecific antibody binding, isotype control antibodies were used for the phycoerythrin-conjugated antibodies or the secondary antibody alone was used as a control for the unconjugated Hu C/D antibody. These control measurements were performed for each experimental condition. The signal obtained from the controls was used to select the gate region -1 % false positive cells that was subtracted from the final value. For each experimental condition 20.000 events were analysed. Using the frontside-scatter dotplot, damaged cells were discriminated by gating out cellular debris. Initial experiments to analyse the population of cells with damaged membranes which could have led to false-positive signals were performed by DNA staining with 7-amino-actinomycin D (7-AAD, BD, Heidelberg, Germany, 51-68981E) which only enters damaged cells. These observations led to a neglectable amount of 7-AAD+ cells of 0.23 % \pm 0.12 % showing that the procedure *per se* is not damaging the cells excessively. For flow cytometric analyses of living cells with PE-conjugated antibodies against membrane epitopes (A2B5 and PSA-NCAM) both 7-AAD and propidium iodid (PI, BD, Heidelberg, Germany, 51-66211E) produced unspecific crosstalk signals into the PE-channel and negatively incfluenced the analysis results so that they were not used in parallel.

2.5 Development of a quantitative image-analysis approach

2.5.1 Image acquisition by confocal laser scanning microscopy

If not stated otherwise, most of the microscopic studies were performed by confocal fluorescence microscopy using a TCS SP2 laser-scanning microscope (Leica, Wetzlar, Germany). This system included a 405 nm diode laser, an argon-krypton laser with 458, 476, 488, 514 nm laser lines and two helium-neon lasers with 543, 594 and 633 nm laser lines. The system also contained acousto-optical tunable filters (AOTF), an acousto-optical beam splitter (AOBS) and four prism spectrophotometer detectors that permitted simultaneous excitation and detection of multiple fluorochromes. However, to avoid cross-talk of fluorescence emitted fluorescence into another channel, each section was scanned sequentially for every channel. For the quantitative analysis three dimensional XYZ-scans were performed by using identical microscopical hardware parameters (gain, offset, pinhole size, frame and line average, zoom settings, analogue and digital laser power, picture size and resolution) throughout a time series. The distance between confocal sections was constantly chosen with a value of 285 nm at a resolution of 512x512 pixels using a 63x 1.4NA DIC Oil HCX Plan-Apo lens and a pinhole size of < 1 airy unit (AU), which in general gives the best signal/noise ratio (SNR). Photomultiplier detection ranges were set as follows for: Hoechst (410-590 nm), Alexa488/FITC (495-560); Alexa594/TRITC (600-700 nm). Line and frame average were set to 2 and 4, respectively. Identical section distances in all stacks resulted in different stack sizes of 20 to 30 sections, depending on the cell culture thickness. This also standardized the 3D-voxel size, which was important for the subsequent image quantification using Imaris software. For statistical reasons at least three image stacks from randomly chosen, non-overlapping areas of each coverslip were acquired. For the quantitative comparison of neuron/non-neuron fluorescence signals at least three areas with neurons were selected. For the quantitative analysis of nuclear β -catenin the used antibody combination was as follows: rabbit-anti-human β -catenin + Alexa594 goat-anti-rabbit and co-staining for neurons with mouse-anti-human Hu C/D + Alexa488 goat-anti-mouse or β III-tubulin-FITC (compare Tab. 1).
2.5.2 3D-Image processing and signal quantification

Confocal image series were 3D-reconstructed (maximal projection in 3D) and analyzed using Imaris software by creating surface objects on the basis of intensity values per channel and the contrast around the object. A suitable example is the rendering of cell nuclei with a high signal/noise ratio and a high contrast at the nuclear edges resulting in a 3D-layer surface around the nucleus. To precisely compare 3D-images originating from different biological samples the intensity threshold and surface smoothing option (in most cases 300 nm if not stated otherwise) were set constant throughout the samples of one differentiation experiment. The cell nuclei were rendered on the basis of the Hoechst staining. If multiple touching nuclei were to be rendered the software automatically splited touching objects on a diameter and size basis which was usually set to a value of 6 μ m but also adjusted if necessary. For better reproducibility mitotic and apoptotic cells were excluded depending on the size and signal intensity (more intense DNA-staining in mitotic and apoptotic nuclei). Neurons were separated from non-neuronal cells depending on the fluorescence intensity of the neuron staining. Analyses of each experimental condition were performed in triplicates. The quantification results of the mean and sum fluorescence intensities and the volume in μ m³ per object were exported into Excel format.

2.6 Statistical analyses

Statistical analyses (unpaired, two-tailed Student's t test) were achieved using Prism5 (Graph-Pad Software, La Jolla, USA). p < 0.05 was considered a statistically significant difference (*), p < 0.01 (**), p < 0.001 (***). The results were presented as mean \pm SEM or ratio \pm scaled SEM, respectively. The numbers of independent biological repetitions as well as of repetitions within one experiment were defined as "N" and "n", respectively. For quantitative image analyses, mean intensity values for the nuclei of a given image-set for a given time point were averaged and SEM's were calculated. If required, normalization of test (x) and control (y) means \pm SEM's (σ) was performed. Normalization/scaling of SEM's was achieved by Gaussian error propagation using the following equation:

$$\Delta f = \sqrt{\left(\frac{\sigma f}{\sigma x}\right)^2 \times \sigma_x^2 + \left(\frac{\sigma f}{\sigma y}\right)^2 \times \sigma_y^2}$$

Averaging of multiple experiments was performed by averaging of mean values and propagation of SEM's (σ_n) via the following equation:

$$\Delta f = \sqrt{\sigma_1^2 + \sigma_2^2 + \dots \sigma_{n-1}^2 + \sigma_n^2} / n$$

Using these equations, the fluorescence signal distribution of multiple nuclei in every single image was taken into account in the final data representation.

2.7 Test for antibody-specificity

2.7.1 β -catenin RNA interference

RNA interference was achieved to show that the β -catenin staining with primary and secondary antibodies (Tab. 1, section 2.3.1) was specific enough for fluorescence signal quantification.

Small interfering RNAs (siRNA's) were used to down regulate β -catenin mRNA (CTNNB1; GeneID: 1499; coding sequence: CCA-CAG-CUC-CUU-CUC-UGA-GUA-A; GC 52%; target accessions: NM001098209.1, NM001098210.1, NM001904.3; validated stealth RNAi siRNA, Invitrogen, Karlsruhe, Germany CTNNB1-VHS50819). The "Stealth RNAi siRNA Negative Control Hi GC" from Invitrogen, Karlsruhe, Germany (12935-400) was used as a negative control. A plasmid coding for enhanced green fluorescent protein (EGFP, "pMAX-GFP" Lonza, Basel, Switzerland) served as positive transfection control.

2.7.2 Nucleofection of siRNAs

Based on the nucleofection technique introduced by Amaxa/Lonza a dual-shock electroporation procedure was established and optimized using the standard electroporation device GenePulser II (BioRad, Munich, Germany) for successful electroporation of ReNcell VM. Transfection was not possible with lipofection-mediated techniques or by $Ca_2(PO_4)_2/DNA$ -co-precipitation. The cells were washed in HBSS, trypsinized, washed in warm PBS and $9x10^5$ cells were resuspended in 100 µl intracellular nucleofection buffer (140 mM Na₂PO₄, 5 mM KCl, 10 mM MgCl₂, pH 7.4, sterile filtered). Between 0.1 - 1 nmol of siRNA's or 2 μg EGFP-plasmid were added and incubated for 5 min at RT. Nucleofection was performed in a 0.2 cm electroporation cell (Lonza, Basel, Switzerland) with two subsequent electric shocks in a GenePulser II: (1) 1000 V for 0.1 - 0.2 msec; GenePulser parameters: U = 0.95 kV, capacity = 10 μ F; (2) 100 V for 15-30 msec; GenePulser parameters: U = 0.1 kV, capacity = 0.5 μ F x 1000 at "high cap" setting. After electroporation 500 μ l of growth medium were added quickly and the cells were directly seeded into 24-well plates on PDL/laminin-coated glas coverslips. The seeding density was chosen empirically and depended on the time between transfection and start of differentiation or experiment. A density of $1.2-2 \times 10^5$ cells/cm² of the initially counted cell suspension was usually used per well. The cells were PFA-fixed 24 and 48 hours after transfection and immunostained with primary and secondary antibodies (Tab. 1, section 2.3.1). RNA interference was screened with identical confocal microscope settings used for the signal quantification (sections 2.5.1 and 2.5.2).

2.8 Life cell imaging studies

2.8.1 Plasmid preparation and nucleofection

Plasmids were isolated from *E. coli* -80 °C glycerol stocks. Newly obtained plasmids were transfected into chemo-competent *E. coli* (One shot Top10, Invitrogen, Karlsruhe, Germany C4040) according to the manufacturer's instructions. Mini or maxi preparation of plasmid-DNA was performed using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, Karlsruhe, Germany K2100-06) according to the manufacturer's instructions. DNA concentration was determined according to its optical density (OD) at 260 nm (OD260). DNA purity was evaluated by normalizing OD260 to OD280, where a ratio of 1.8 corresponded to pure DNA. Plasmid size and quality were confirmed by restriction analysis using 2 restriction enzymes to cut the insert out of the plasmid backbone.

Transfection into ReNcell VM was performed by nucleofection using 2×10^6 cells by the procedure described in section 2.7.2. Alternatively the Amaxa transfection kit V (Lonza, Basel,

Switzerland, VCA-1003) and the Amaxa nucleofector device (protocol X001) were used according to the manufacturer's instructions. Transgene screening was performed microscopically using a TCS SP2 AOBS (Leica, Wetzlar, Germany) laser-scanning microscope or a Zeiss Imager Z1 with AxioCam Mrm (Zeiss, Jena, Germany).

2.8.2 Visualization of YFP- β -catenin constructs

For live cell imaging studies ReNcell VM cells were transfected with plasmids coding for fulllength human β -catenin coding sequence for aa 2-781, fused to EYFP-cDNA behind a strong CMV (cytomegalovirus) promoter (kindly provided by J. Behrens, Erlangen, Germany, described in Krieghoff et al. (2006)). Plasmids were amplified, purified and transfected as described in section 2.8.1. For live cell imaging the transfected ReNcell VM cells were seeded into laminincoated 4-well LabTek chamber slides (Nunc, Langenselbold, Germany, 177399). The cells were visualized using an inverse confocal laser scanning microscope (TSC SP2 AOBS). Cells transfected with the positive control plasmid pMAX-GFP or pEYFP-plasmids were visualized using a 20 mW argon laser at 488 nm or 514 nm, respectively. The AOBS photomultiplier-range for YFP-detection was set from 520 nm to 600 nm and from 495 nm to 575 nm. For longer cell survival during microscopy the cells were kept at 37 °C, 5 % CO₂ in a humidified chamber.

2.8.3 Fluorescence Recovery After Photobleaching (FRAP)

For FRAP experiments up to 4 μ g plasmids were transfected into ReNcell VM cells. Differentiation was initiated 24 hours after transfection (section 2.2). Cells were selected for analysis by virtue of their β -catenin fluorescence intensity. Photobleaching was performed with a confocal laser scanning microscope (TCS SP2 AOBS).

Cells expressing YFP fusion proteins with sufficiently high fluorescence intensity above background noise were visualized and monitored using the 514 nm laser at 4 % power and bleached at 100 % power. For nuclear bleaching ten subsequent bleach frames at 0.625-second intervals were applied (total bleach time 6.25 sec) followed by 25 frames at 0.85-second intervals, 30 frames at 10-second intervals and 15 frames at 30-second intervals. Before bleaching five frames were recorded to measure the average pre-bleach intensity which was set to 100 %. The average fluorescence intensity inside the regions of interest (ROI) was measured using Leica confocal Software (LSC, Leica, Wetzlar, Germany). ROIs without cells were selected to subtract background noise, unbleached cells inside the scanning window were selected to exclude the influence of bleaching events due to image acquisition. This was achieved by normalizing the intensity value for every time-stamp to the respective value from the control-ROI.

To rule out a significant contribution of new protein synthesis to the fluorescence recovery in these experiments, total cell fluorescence during the recovery time was analyzed, which was found to increase by less than 3 % within 10 minutes. Due to technical constraints, the first image in the bleach experiments could only be taken about 0.8 seconds after the end of the bleach pulse, which resulted in some recovery before the first image was acquired.

2.9 Protein biochemistry

2.9.1 Cell lysates and SDS-PAGE

The cells were grown in T75 cell culture flasks. Culture medium was removed, cells were washed with PBS and harvested from the dish with a cell scraper in ice-cold PBS. Cells were collected in 15 ml tubes and centrifuged with 140 g for 5 minutes at 4 °C (Labofuge400R, Heraeus). The supernatant was removed and the cells were resuspended in cold PBS including one part of 4x SDS sample buffer (sodium dodecyl sulfate; pH 6.8: 250 mM Trizma base, 30 mM EDTA, 40 % glycerol, 4 % 2-mercaptoethanol, 4 % SDS, 0.01 % bromphenolblue (all from Sigma-Aldrich, Hamburg, Germany)), subsequently boiled at 95 °C for 5 min (Thermomixer R, Eppendorf, Hamburg, Germany) and stored at -20 °C.

The proteins were separated by vertical gel electrophores using 7.5 % or 10 % polyacrylamid gels (for 10 % separation gels: 13.33 ml 30 % acrylamide/0.8 % bisacrylamide (Serva 10675 and 29195), 10 ml 4x LGS (1.5 M Trizma base pH 8.8, 0.4 % [w/v] SDS), 16.47 ml dH₂O, 140 μ l TEMED (Sigma-Aldrich, Hamburg, Germany T8133) and 64 μ l 12.5 % ammonium peroxide sulfate (APS, Acros, Nidderau, Germany 20153-0010); 7.5 % separation gels were down scaled to the required acrylamide concentration; stacking gels: 0.575 ml 30 % acrylamide/bisacrylamide, 1.25 ml 4x UGS (0.5 M Trizma base, pH 6.8, 0.4 % SDS), 3.109 ml dH₂O + 46 μ l TEMED + 20 μ l 12.5 % APS). The samples migrated in running buffer (25 mM Trizma base, 192 mM glycine (Sigma-Aldrich, Hamburg, Germany, G7126), 0.1 % SDS) at constant 20 mA per gel for about 70-90 minutes using a Mighty Small 200 Hoefer electrophoresis device (GE Healthcare, Solingen, Germany, 80-6147) and a Power Pac 300 (BioRad, Munich, Germany). The gels were either stained with Coomassie or were transferred onto nitrocellulose membranes (Hybond-ECL nitrocellulose membrane, Amersham, Munich, Germany, RPN78D) for immunoblotting (section (0.125%) 2.9.2). For Coomassie staining the gels were incubated in Coomassie staining solution (0.125\%) [v/v] Coomassie R250 (Serva, Heidelberg, Germany, 17525), 50 % [v/v] methanol (AppliChem, Darmstadt, Germany, A0688), 10 % [v/v] acetic acid (Roth, Karlsruhe, Germany, 3738.2)) for one hour followed by fixation and destaining in destain solution I (50 % [v/v] methanol, 10 %[v/v] acetic acid) for one hour and destain solution II (5 % [v/v] methanol, 7 % [v/v] acetic acid) overnight.

2.9.2 Immunoblotting

After the separation in SDS gels, the proteins were transferred onto NC-membranes to label them with antibodies for the detection and quantification of specific proteins. NC membranes, filter papers (Whatman, Dassel, Germany, 426890) and the protein containing acrylamide gels were equilibrated in blot buffer (200 mM glycine, 25 mM Trisma base, 20 mM methanol) for 10 minutes and assembled with the blotting device (Transblot SD Semi Dry Transfer Cell, BioRad, Munich, Germany) according to the manufacturer's instructions. The proteins were transferred to the NC-membrane at constant 12 V for 30 minutes using a Power Pac 200 (BioRad, Munich, Germany). The transfer quality was controlled by overall protein staining with Ponseau S solution (3 % [v/v] trichloroacetic acid, 0.2 % [v/v] Ponceau S; Sigma-Aldrich, Hamburg, Germany P7170) for 3 minutes followed by dH₂O wash to remove excessive Ponceau S. Images were taken using a conventional flat-bed scanner at a resolution of at least 300 dpi.

antigen	host	dilution	supplier
primary antibodies			
anti-human β -catenin,	mouse	1:300	Santa Cruz, Heidelberg,
clone E5			Germany, sc-7963
dephosphorylated β -catenin,	mouse	1:500	Calbiochem, Darmstadt,
clone 8E4			Germany, 219350
GAPDH, clone FL-335	rabbit	1:500	Santa Cruz, Heidelberg,
			Germany, cs-25778
secondary antibodies			
anti-rabbit-IgG POD	goat	1:80.000	Sigma-Aldrich, Hamburg,
			Germany, A-9169
anti-mouse-IgG ECL	goat	1:10.000	GE Healthcare, Solingen,
horseradish POD			Germany, na931v

 Table 3: Antibodies used for immunoblotting

Before antibody labeling, membranes were incubated in TTBS (10 mM Trisma base, 149 mM NaCl (AppliChem, Darmstadt, Germany, A3597), 0.1 % [v/v] Tween 20 (Sigma-Aldrich, Hamburg, Germany P2287)) containing 5 % [w/v] 'instant nonfat dry milk' for one hour to block unspecific binding sites. After washing 3x 10 minutes in TTBS, primary antibodies (Tab. 3, diluted in TTBS) were incubated for 1 hour at RT or overnight at 4 °C followed by 3x 10 minutes washing with TTBS. Secondary antibodies conjugated with horseradish peroxidase (Tab. 3, diluted in TTBS) were incubated for one hour at RT followed by 3x 10 minutes washing with TTBS and a final in TBS wash (TTBS w/o Tween20). Afterwards, the NC membranes were exposed to ECL solution (Amersham ECL Reagents, GE Healthcare, Solingen, Germany, RPN2209) for 5 minutes and the resulting chemiluminescence signals were visualised using light sensitive films (Hyperfilm ECL, GE Healthcare, Solingen, Germany, 28906836) which were developed (Kodak Professional D-19 Developer, Sigma-Aldrich, Hamburg, Germany, P5670 and fixative, Filmfabrik Wolfen, Germany) and scanned for quantification. Quantification of protein signals was performed using ImageJ/Gel-plugin (NIH/USA) to determine differences between samples. After scanning, images were saved as TIF format and analysed without image enhancement. Whole lanes or single bands were selected manually followed by automatic measurement of grey levels. Relative signal intensities were compared using MS Excel after normalization to the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.10 Activation and Inhibition of ReNcell VM differentiation

Activation of Wnt/ β -catenin-signaling. Activation of the β -catenin-dependent Wnt-pathway (canonical) was achieved with recombinant human Wnt3a (R&D Systems, Wiesbaden, Germany, 5036-WN) which was dissolved in PBS containing 0.1 mM EDTA, 0.5% CHAPS (Sigma-Aldrich, Hamburg, Germany), pH 6.8. This buffer alone also served as negative/solvent control. Downstream activation in a ligand-independent manner was performed with the GSK3 β -inhititor SB216763 (stock solution diluted in DMSO which also served as negative/solvent control, Sigma-

Aldrich, S3442).

Inhibition of Wnt/ β -catenin-signaling. Inhibition of the β -catenin-dependent Wnt-pathway was achieved with recombinant human Wnt-inhibitory-factor-1 (WIF-I; R&D Systems, Wiesbaden, Germany, 1341-WF) or with recombinant human dickkopf related protein 1 (Dkk-1; R&D Systems, Wiesbaden, Germany, 1096-DK). PBS containing 0.1 mM EDTA, 0.5% CHAPS, pH 6.8 served as negative/solvent control.

2.11 Investigation of ReNcell VM proliferation: EdU-Assay and MTT-test

Proliferation assay (EdU)

EdU (5-ethynyl-2'-deoxyuridine, Invitrogen, Karlsruhe, Germany, E10187) is a novel easy-touse alternative for the BrdU-assay (5-Bromo-2'-deoxy-Uridine) to directly measure active DNA synthesis or S-phase synthesis of the cell cycle. EdU is a nucleoside analogue of thymidine and is incorporated into DNA during DNA synthesis. The detection is achieved by a coppercatalyzed reaction between an azide group conjugated to a fluorescent dye (Alexa 594 or 488 nm, Invitrogen, Karlsruhe, Germany, A10270 and A10266) and an alkyne group of EdU, leading to a covalent link. For this reaction a buffer was used (100 mM Tris (Sigma-Aldrich, Hamburg, Germany), 2 mM CuSO₄ (Merck, Darmstadt, Germany), 100 mM ascorbic acid (Sigma-Aldrich, Hamburg, Germany) in dH₂O) in which 1 μ M Alexa-azide was added and directly incubated on Triton-X100-permeabilized cells (section 2.3.1) for 30-40 min at RT in the dark followed by standard immunostaining procedures (section 2.3.1). EdU was used at a concentration of 5 μ M and pulsed/incubated for three hours before fixation if not stated otherwise. For microscopic analyses the number of EdU+ cells out of 1000 cells (based on the nuclear staining) was counted from randomly chosen, non-overlapping visual areas per coverslip of two wells per experimental condition for each experiment. All experiments were repeated on at least three independent occasions. For flow cytometric EdU-analyses the cells were trypsinized, washed in cold PBS, stained for membrane-associated proteins (if required) and PFA-fixed as described in section 2.4, permeabilized with 0.2 % Triton-X100/PBS for 5 minutes, washed in cold PBS and incubated with EdU-reaction buffer, including Alexa488-azide for 30 minutes in a shaker (75 rpm) at 4 °C in the dark. After washing twice in PBS the cells were directly measured.

Proliferation and cytotoxicity assay - MTT

The MTT-test is a colorimetric test to measure metabolic functionality of cells based on the reduction of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan by an enzymatic reaction in living cells. Hence, it is a means to quantify cytotoxicity by measuring the OD at 550 nm. However, it is also possible to correlate the signal intensity with cell proliferation (Mosmann, 1983).

The cells were seeded in 96-well plates in a sub-confluent cell density. The reagents (SB216763 and DMSO, Wnt3a and PBS/CHAPS) were diluted in 200 μ l differentiation medium per well to start ReNcell VM differentiation. Two hours before cell lysis 50 μ g/ml MTT (Sigma-Aldrich, Hamburg, Germany, M5655) was added. After two hours incubation the medium was aspirated and the cells were lysed in 200 μ l lysis buffer (DMSO, Sigma-Aldrich, Hamburg, Germany;

2.4 % [v/v] or 0.4 M acetic acid, Roth, Karlsruhe, Germany 3738.2; 1 % [w/v] or 35 mM SDS, Invitrogen, Karlsruhe, Germany, 5525UA). The OD at 550 nm was measured versus a blank (lysis buffer only) with a photometer (Anthos Reader 2010 Typ 1750, Anthos, Krefeld, Germany). Eight wells per experimental condition were measured for each experiment. All experiments were repeated on at least three independent occasions.

2.12 In vivo investigations

2.12.1 Mouse brain cryosections and immunohistochemistry

Pregnant female mice (C57BL/6 strain, Charles River, Sulzfeld, Germany) were killed via cervical dislocation at embryonic day (E) 11.5, the embryos were collected and transfered into ice-cold PBS, fixed in 4% PFA for 4 hours, cryoprotected in PBS containing 20% sucrose for at least one day (until embryos were sunk), embedded in O.C.T. compound (Optimal Cutting Temperature compound) using standard embedding casettes type 'base mold' (both Sakura Tissue-Tek, Zoeterwoude, The Netherlands) frozen on dry ice and stored at -20 °C. Serial coronal sections (14 μ m thick) of the embryonic brains were obtained on a cryotome (HM 560, Leica, Wetzlar, Germany) and collected on microscope slides (StarFrost-Plus, Menzel, Braunschweig, Germany, J1800AMNZ).

The dried sections were surrounded with a PAP-pen (Sigma-Aldrich, Hamburg, Germany, Z672548-1EA) to produce a hydrophobic border around the sections, washed 3 x 5 min with PBST (0.5 % Tween20 (Sigma-Aldrich, Stockholm, Sweden) in PBS), incubated for one hour in blocking buffer (PBS, pH 7.4, 5 % [v/v] normal goat serum (Jackson Immuno Research, Gothenburg, Sweden, 005-000-121), 0.1 % [w/v] BSA, 0.3 % [v/v] TritonX-100) to block unspecific binding sites for subsequent immunostaining. Alternatively, the sections were directly used for *in situ* hybridization (ISH, see section 2.12.2) and immunostained afterwards. For ISH the use of freshly fixed, frozen and directly cut material yielded better signals due to limited RNA-stability. Primary antibodies (Tab. 4) were diluted in blocking buffer and incubated overnight at 4 °C in a humidified chamber. The sections were washed 3 x 5 min with PBST and incubated in the dark with the secondary antibodies (Tab. 4) in blocking buffer for two hours at RT in a humidified chamber. After washing with PBST for $3 \ge 5$ min the cell nuclei were labeled with TO-PRO-3 iodide (DNA-staining, 0.5-1 µM, Invitrogen, Huddinge, Sweden, T3605, excitation at 647 nm) or Hoechst33258 (1 μ g/ml, Sigma-Aldrich, Stockholm, Sweden, 861405, excitation at 405 nm) for 5 min, mounted in PBS containing 80 % glycerol and covered with glas coverslips (Menzel, Braunschweig, Germany). Alternatively, the sections were mounted in DABCO mounting medium containing 0.5-1 μ g/ml DAPI (4',6-diamidino-2-phenylindole; both Sigma-Aldrich, Stockholm, Sweden) for nuclear staining.

2.12.2 Preparation of DIG-RNA probes for RNA in situ hybridization (ISH)

RNA *in situ* hybridization was performed with small changes according to Conlon and Herrmann (1993) and Castelo-Branco et al. (2003). In brief: The plasmid Wnt1-5' ISH probe (from McMahon lab, 3350 bp, PGEM4-vector with complete Wnt1-cDNA insert (1450 bp), ampicillin resistence, T1-RNA-polymerase forward promoter, Sp6-RNA-polymerase reverse promoter) was linearized by HindIII-digest (5 μ l plasmid, 2 μ l buffer, 1 μ l enzyme (Fermentas, Helsingborg,

antigen	host	dilution	supplier		
primary antibodies					
Active- β -Catenin (ABC), clone 8E7	mouse	1:250	Millipore, Solna,		
			Sweden, $05-665$		
$\beta\text{-catenin, clone }14$	mouse	1:200	BD, Stockholm,		
			Sweden, 610153		
$\beta\text{-catenin-FITC},$ clone 14	mouse	1:200	BD, Stockholm,		
			Sweden, 610155		
Nurr1, clone E20	rabbit	1:250	Santa Cruz, Heidelberg,		
			Germany, SC-990		
Tyrosine hydroxylase	mouse	1:500	Immunostar, Hudson,		
			USA, 22941		
Tyrosine hydroxylase	rabbit	1:500	Pel Freeze, Milwaukee,		
			USA, P40101-0		
secondary antibodies and isotype controls					
anti-mouse Alexa 488nm,	goat	1:500	Invitrogen, Huddinge,		
555nm, 647nm			Sweden		
anti-rabbit Alexa 488nm,	goat	1:500	Invitrogen, Huddinge,		
555nm, 647nm			Sweden		
digitonin-AP	mouse	1:2000	Abcam, Stockholm,		
			Sweden		

 Table 4: Antibodies used for immunhistochemistry on cryosections

Sweden), 12 μ l dH₂O) for 2 hours at 37 °C and controlled on a 1 % agarose gel. After restriction 180 μ l dH₂O and 200 μ l phenol were added, vortexed and centrifuged shortly to separate the two phases. To the aquaeous phase 100 μ l phenol and 100 μ l chloroform were added, vortexed and centrifuged shortly. To the aquaeous phase 20 μ l 5 M NaCl (=1:10) and 500 μ l ethanol (=2.5 volume) were added and incubated overnight at -20 °C for RNA precipitation. After 30 minutes centrifugation at 20.000 g the supernatant was removed, the RNA-pellet washed with 70 % ethanol, air-dried and resuspended in 20 μ l dH₂O. To obtain detectable antisense RNA for cellular Wnt1-mRNA, 5 μ l of the linearized plasmid was transcribed by T1-RNA-polymerase reaction using digoxigenin-UTP (DIG-RNA, Sigma-Aldrich, Stockholm, Sweden) for 3 hours at 37 °C (5 μ l DNA, 4 μ l buffer, 2 μ l 0.1 M DTT, 4.6 μ l dH₂O, 2 μ l DIG-RNA (Roche, Stockholm, Sweden), 2.4 μ l RNA polymerase (Invitrogen, Huddinge, Sweden) and controlled afterwards on a 1 % agarose gel. After adding 100 μ l TE-buffer, 10 μ l 4 M LiCl and 300 μ l ethanol, the RNA was precipitated overnight at -20 °C, centrifuged at maximum speed (i.e. 20.000 g), washed in 70 % ethanol, air dried and resolved in 100 μ l TE buffer. If not stated otherwise, all chemicals were from Merck, Stockholm, Sweden.

2.12.3 Hybridization of DIG-RNA probes and development for ISH

The freshly cut and dried cryosections from fixed embryonic brains (section 2.12.1) were incubated in hybridization buffer (0.19 M NaCl, 10 mM Tris pH 7.2, 5 mM $NaH_2PO_4*2 H_2O_4$

pH 6.8, 50 mM EDTA, 50 % [v/v] formamide, 20 % [v/v] 50x dextran, 0.1 % [v/v] Denhardts solution, 10 % [v/v] tRNA (all Sigma-Aldrich, Stockholm, Sweden), in RNase-free autoclaved dH_2O , prewarmed to 70 °C, 200 μ l per slide) containing 30 μ l/ml DIG-RNA probe. To avoid evaporation, the viscous solution was covered with a coverslip and incubated overnight at 70 $^{\circ}$ C in a humidified chamber containing fresh washing buffer (50 % [v/v] formamide, 2x SSC pH 7.5, 0.1 [v/v] % Tween20 in dH₂O). After carefully removing the coverslip the sections were washed 4x 20 minutes in washing buffer at exact 65 $^{\circ}$ C followed by 3x 20 minutes washing with 1xMABT at RT (5xMAB: 50 mM maleic acid, 0.75 M NaCl, pH 7.5; freshly added 0.1 % [v/v] Tween20). Blocking of unspecific binding sites was performed for one hour at RT using blocking buffer (2 % [w/v] blocking agent (Boehringer, Stockholm, Sweden), 20 % [v/v] sheep serum, 20 % [v/v] 5xMAB, 0.1 % [v/v] Tween20 in dH₂O). Afterwards, the blocking solution was removed and 200 μ l of the digitonin-AP-antibody solution (1:2000 in blocking buffer; Abcam, Stockholm, Sweden; AP, alkaline phohsphatase) was added, covered with parafilm to avoid evaporation and incubated at 4 °C overnight in a humidified chamber. After 6x 20 minutes washing in MABT and 3x 10 min in B3 solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.1 % [v/v] Tween20 in dH₂O) at RT the ISH was developed for at least four hours at RT in the dark using 800 μ l of developing solution per slide (20 μ l/ml nitro-blue tetrazolium chloride (NBT)/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (BCIP)). After obtaining detectable signals the developing reaction was stopped in PBS. The sections were directly mounted in 80 % glycerol/PBS or immunostained additionally (section 2.12.1). If not stated otherwise, all chemicals were from Merck, Stockholm, Sweden.

2.12.4 Image acquisition and image processing from stained cryosections

Immunohistochemistry (IHC) images of stained cryosections were acquired with confocal laser scanning microscopes: for three-color imaging: Zeiss-510 (488, 543 and 633 nm lasers, emission filters: #13: BP 505-530, #20: BP 575-640, #26: BP 660-710); for four-color imaging: Olympus FV1000 (405, 470, 561 and 630 nm lasers, emission filters: LP 455, BP 515-550, BP 660-740). Similar to the image acquisition from fixed and stained cell cultures as described in section 2.5.1, the distance between confocal sections was constantly set to 750 nm at a resolution of 1024x1024 pixels using a 20x 0.75NA lens (Olympus) or a 25x 0.8NA water immersion lens (Zeiss). For four-color-staining procedures, sequential scanning was used to avoid fluorescence cross-talk. For fluorescence signal quantification studies at least three 14 μ m sections per brain were analyzed. If not stated otherwise, data for cell type comparison between Nurr1+ and TH+ cells were obtained from the same brain. All experiments were repeated with embryonic brains from at least three pregnant females and three littermates each. Image processing was performed as described in section 2.5.2 using Imaris software V6.3 with following differences: surface smoothing factor = 500 nm, particle diameter for automatic surface/nuclei splitting = 5 μ m, to improve cell-type separation the masking-feature was used to isolate Nurr1+/TH+ nuclei for isolated quantification of positive and negative nuclei.

In situ hybridization (ISH) for Wnt1 was visualized with a Zeiss Axioskop microscope with a HBO100 mercury arc lamp; images were collected with a C4742-95 Hamamatsu camera and processed with Adobe Photoshop version CS3 and Imaris software.

3 Results

Part 1/4 - Sub-cellular quantification of β -catenin and translocation analysis

3.1 Controlled differentiation of ReNcell VM in vitro

It has been described that ReNcell VM, originating from from ten-week gestation fetal human midbrain tissue, show characteristic proliferation, morphology and karyotype of a cell line when cultivated under the influence of growth factors (i.e. bFGF, EGF). Upon growth factor removal the cells differentiate into astrocytes, oligodendrocytes and neurons which exhibit electrophysiological activity and partly a dopaminergic (DA) background (Donato et al., 2007).

However, neuronal differentiation in ReNcell VM has not been described quantitatively in a kinetic approach, being a prerequisite for the time-dependent investigations described in this thesis. For that reason, fluorescence microscopic and flow cytometric analyses were performed to investigate neurogenesis in general and more precisely differentiation into DA neurons.



Figure 6: Long term microscopy of differentiating ReNcell VM: First morphological changes occur within the first 24 hours after initiation of differentiation, cell branching and formation of long protrusions begin between 36 and 48 hours, between 48 and 72 hours a stable meshwork of cell branches is formed; bar = 50 μ m; for 72 hours movie please also see suppl. S1.

3.1.1 ReNcell VM differentiate into neurons, astrocytes, oligodendrocytes

To gain an overview about the cell culture growth characteristics, 72 hours non-stop long time live cell imaging was performed to investigate the change in cell morphology during differentiation. First cell protrusions and increased cell branching were found within the first 12 hours. During the first two days of differentiation a network of cell branches developed which stabilized during the following day (Fig. 6 and suppl. S1). To investigate more precisely which cell types appear at which stage of differentiation, immunostaining was performed of fixed cells with antibodies

36



Figure 7: Neuro and gliogenesis in ReNcell VM. (A-D) Mature and immature neurons stained for β III-tubulin and Hu C/D at 6 and 72 hours after initiation of differentiation. As a neuronal RNAbinding protein Hu C/D is earlier expresses than β III-tubulin. (E) After 72 hours of differentiation single O4+ oligodendrocytes appear. (F) During differentiation a complex network of astrocytic branches is formed. Astrocytes represent the majority of cell population after 72 hours of differentiation; Gliary acetic fibrillary protein (GFAP, astrocytes, red), β III-tubulin (neurons, green); nuclei (Hoechst, blue); major frame grid = 10 μ m.



Figure 8: Rapid neurogenesis during ReNcell VM differentiation. (A) Flow cytometric analysis of neurons at 72 hours of differentiation: intensity plot of Hu C/D + Alexa488GAM fluorescence (grey area) vs. unspecific Alexa488GAM fluorescence (dashed line); smaller peak indicates neuronal sub-population; smaller image: forward/side scatter plot shows differentiating cells at 72 hours; cell debris (grey) outside the gate region was excluded from analysis; FS, forward scatter (cell size); SS, side scatter (cell granularity). (B) Flow cytometric analysis of Hu C/D+ cells: neuronal differentiation reaches peak at 72 hours; N \geq 3, 20.000 cells each.

for specific cell types and differentiation stages at different time points during the first three days of differentiation.

Immunostaining of early or late neuronal proteins like the neuronal RNA-binding protein Hu C/D or β III-tubulin indicated the presence of neurons already 3-6 hours after initiation of differentiation as shown in Fig. 7A,C. β III-tubulin+ neurons were present from 12 hours but sometimes after 6 hours, already. Neurites stretching 100s of μ m from the soma appeared after 48-72 hours of differentiation (Fig. 7B). The presence of astrocytes and oligodendrocytes was confirmed by labeling of GFAP and O4, respectively (Fig. 7E,F). The neuronal population was quantified by flow cytometry and microscopic cell counting of immunostained cells. Flow cytometric analyses of Hu C/D+ neurons revealed continuous neuronal differentiation for the first 72 hours reaching a percentage of approximately 4.5 % of the total cell number (Fig. 8). The spontaneous differentiation described by Donato et al. (2007) was also observed but occurred very rarely and, hence, was neglected for the analyses.

Together these results confirmed and showed quantitatively that ReNcell VM differentiate at a high degree of reproducibility. Additionally, ReNcell VM have the potential to differentiate into neurons with with dopaminergic, at least catecholaminergic phenotype, indicated by positive staining of tyrosine hydroxylase (TH) and Pitx3. To investigate this sub-population in more detail, microscopic quantification of TH+ neurons was performed.

3.1.2 A small population of ReNcell VM neurons is dopaminergic

To investigate DA differentiation under standard culture conditions, the cells were positively stained for TH which indicates a dopaminergic phenotype. It should be stated that co-staining for other DA markers such as dopamine transporter (DAT) with the antibody from Sigma-Aldrich was negative during the first 72 hours and staining for the DA precursor cell marker Nurr1 with the antibody from Santa Cruz produced unspecific signals and stained all nuclei weakly. However, preliminary results from Pitx3-staining revealed positive signals indicating that TH+ cells indeed were dopaminergic (data not shown). The number of TH+ neurons was quantified at different time points during the first days of differentiation using fluorescence microscopy. The results showed that first TH+ neurons appear between 6-12 hours of differentiation with an increasing frequency of approximately 0.25 % after three days, reflecting 5-10 % of the overall neuronal population (Fig. 9). DA differentiation has been shown to be influenced by Wnt/ β -catenin signaling in the ventral midbrains (VM) of mice *in vivo* (for review please see Castelo-Branco and Arenas (2006)). To closer investigate this mechanism in a human *in vitro* cell model, we quantitatively described the influence of Wnt/ β -catenin signaling during neuronal and DA differentiation in ReNcell VM. For this reason, a quantitative 3D-imaging approach was developed using confocal images to follow Wnt-signaling proteins through sub-cellular compartments.



Figure 9: Dopaminergic differentiation in ReNcell VM: (A) The first tyrosin hydroxylase (TH) positive cells appear between 6 and 12 hours of differentiation; the earliest TH+ cells show a progenitor cell-like morphology changing continuously into neuronal morphology (B). (C) Microscopic quantification of TH+ cells during the first 72 hours of differentiation shows that the percentage of DA neurons reflects approximately 5-10 % of the overall neuronal population (Hu C/D cells, Fig. 8); nuclei (Hoechst, blue) TH (green); N=3, n=2 each; major frame grid = 10 μ m.

3.2 3D-image cytometry as a tool to quantify sub-cellular protein distribution

To understand the mechanisms underlying neuronal differentiation in respect to protein redistribution during active Wnt/β -catenin signaling, a temporally resolved, cell type-dependent and sub-cellular quantification of the involved signaling proteins is from utmost importance. This becomes even more evident by using a cell line with different directions of differentiation, e.g. glia and neuronal cells.

Previous investigations on the activity of Wnt/ β -catenin signaling in ReNcell VM showed that upon inhibition of GSK3 β with LiCl or small molecules like SB216763, the cells activated downstream Wnt-related gene transcription (personal communication C. Lange et al. (2008) and O. Mazemondet et al. (2010)). Therefore, we quantified β -catenin during the first 72 hours of ReNcell VM differentiation using SDS-PAGE and immunoblotting. This approach gave clues about the action of endogenous Wnt-signaling indicated by a two-fold increase of total and active/dephosphorylated β -catenin as illustrated in Fig. 10.

These results suggest that differentiating ReNcell VM exhibit endogenous β -catenin related signaling. Nevertheless, a spatio-temporal and especially a cell type-dependent investigation about the accumulation of β -catenin remained to be elucidated. For this reason a microscopic image cytometry approach was developed with the goal to quantify fluorescence signals in different cell compartments and cell types.



Figure 10: β -catenin during ReNcell VM differentiation. Quantification of protein bands after immunoblotting indicates a two-fold increase during the first three days of differentiation. The comparison between total and dephosphorylated β -catenin does not show significant differences. Dephosphorylated amino acids at the GSK3 β phosphorylation sites corresponds to active β -catenin; as illustrated, random gel-loading was performed to reduce the systemic error which occurs when loaded in the same order; N=5, n=2 each.

3.2.1 3D image reconstruction and surface rendering for cell type and organelle-dependent protein quantification

3D-image cytometry approach. The major advantage of microscopic 3D-image analyses is the possibility to describe metabolic events quantitatively and multi-parametrically. For the quantitative, cell type-dependent and temporal description of protein translocation processes during Wnt/ β -catenin signaling, we chose the nuclear accumulation of β -catenin as an example to develop an easy-to-use semi-automatic and reproducible image cytometry approach as illustrated in Fig. 11. For this task, confocal images of randomly chosen areas were acquired. 3D-reconstruction was achieved using the Imaris software platform from Bitplane. This system allows to create 3D-surfaces around objects based on the signal threshold and signal-to-noise ratio (SNR) in a selected channel of a multi-color image stack. Since DNA-stained nuclei showed an excellent SNR, 3D-surfaces were generated semi-automatically with high reproducibility. Within the 3D-volumes the mean-fluorescence intensities were quantified for every channel and nucleus, resulting in a collection of mean-values that represent the average intra-nuclear protein quantity at the moment of cell fixation.

Increasing reproducibility. To increase experimental and statistical certainty, at least three randomly chosen fields were selected per coverslip, containing between 30 and 50 cells each. This was also necessary because the staining intensity varied slightly between different

areas of a coverslip. Additionally, increased reproducibility was achieved (i) by identical staining procedures in terms of exactly similar incubation times during immuno-staining, (ii) by identical microscope hardware settings and (iii) by identical 3D-rendering software settings as described in section 2.5. Attention to these points resulted in the possibility to compare fluorescence signals originating from different time points of a series or from differently treated samples.

Comparison of different cell types. Co-staining for specific cell marker proteins facilitated the comparison between the cell types of interest. By labeling early neuronal markers (Hu C/D) or late markers (β III-tubulin), neurons were discriminated from non-neuronal cells (i.e. glia cells or undifferentiated) resulting in a cell type-dependent and temporal quantification of nuclear β catenin levels as shown in Fig. 11C-D. Considering a complex network of glial and neuronal cell branches and neurites growing on top of each other, the use of confocal imaging, 3D-rendering and 3D-quantification is highly advantageous and recommended. This becomes obvious during the morphological changes between 48 and 72 hours of differentiation as shown in Fig. 6 and 7F.

Investigating antibody specificity. For control reasons to test the antibody specificity RNA interference of β -catenin was performed by transfection of mRNA-specific siRNAs into proliferating cells. The results confirmed a high antibody specificity after immunostaining of the cells 48 hours after transfection. The signal intensity of various cells was decreased significantly in the transfected cell culture (Fig. 12). These down-regulatory effects were not detectable in mock-transfected cells.

Based on these data can be concluded that the used polyclonal β -catenin antibody was specific enough to monitor intra-cellular β -catenin redistribution by using the 3D-image cytometry approach described above.

3.2.2 Nuclear β -catenin concentration increases during ReNcell VM differentiation

Using this approach nuclear β -catenin signals were quantified at different time points during the first 72 hours of ReNcell VM differentiation. The results revealed that the amount of nuclear β -catenin increased continuously more than two-fold during the investigated time as plotted in 13. This is supported by a parallel biochemical study, which showed in terms of incline and magnitude consistent quantitative and temporal results obtained from nuclear extracts collected by sub-cellular fractionation (data not shown, personal communication by O. Mazemondet). In this biochemical study the increase of nuclear β -catenin was found to be increased 2.5-fold after 48 hours and 2.7-fold after 72 hours (Mazemondet et al., 2010). In comparison with the increase of total β -catenin shown by Western blotting (Fig. 10), the nuclear increase was more pronounced (2.12-fold ± 0.49 vs. 1.62-fold ± 0.3 , $n \geq 5$), suggesting a controlled nuclear β -catenin import. To test if the kinetics of the β -catenin shuttling rate is differentially controlled during differentiation, we turned to quantitative life-cell imaging to investigate the cytoplasmic/nuclear shuttling kinetics of β -catenin.



Figure 11: 3D fluorescence signal quantification: (A) 3D-reconstruction (total projection) of confocal image stack acquired from differentiating ReNcell VM stained for β -catenin (red) and nuclei (blue, Hoechst); (B) 3D-surface rendering based on the intensity of the nuclear staining; surface objects are generated automatically. The mean fluorescence intensity per volume was used to analyze β -catenin in nuclear volumes during differentiation. (C-D) Cell type-specific quantification: neurons (green, Hu C/D) are excluded from the analysis automatically on the nuclear signal intensity of their neuronal staining; only nuclear β -catenin signals of non-neuronal cells are quantified. (E-F) Nuclear signal quantification in neurons automatically separated by intensity filters as stated above. (D and F) show isolated intranuclear β -catenin staining; major frame grid = 10 μ m.



Figure 12: RNA interference shows high specificity of β -catenin antibody: 48 hours after nucleofection of siRNA's very low amounts of β -catenin (green) were detectable in several cells as indicated by the three example pictures (dotted lines). This down regulation of endogenous β -catenin was not detectable in cells transfected with control-siRNAs; nuclei: blue (Hoechst), major frame grid = 5 μ m.



Figure 13: Signal quantification by 3D-surface rendering. (A) Low abundance of nuclear β catenin signals at six hours of differentiation. (B) After 12 hours the amount of nuclear β -catenin has increased, also indicated by the nuclear accumulation (dotted line in (C)); bar = 10 μ m; (D): 3Dsignal quantification of nuclear fluorescence signals reveals a more than two-fold increase of nuclear β catenin levels during the first 72 hours of differentiation, indicating activation of Wnt/ β -catenin-signaling; normalized to t=0; N=6, n=3.

3.2.3 Nucleo-cytoplasmic shuttling of β -catenin is differentially regulated during differentiation of ReNcells VM

To quantify the kinetics of protein translocation processes in living cells, the method of fluorescence recovery after photobleaching (FRAP) was established and improved in the lab. After transfection of YFP- β -catenin fusion constructs into ReNcell VM by nucleofection, compartment bleaching of the whole nucleus was performed comparable to the approach of Krieghoff et al. (2006). The curves plotted in Fig. 14 show that the speed of nuclear β -catenin import was elevated 3-5 hours after initiation of differentiation. Approx. 20 hours later this up-regulation has decreased again below the initial levels. Calculation of the half-recovery times $t_{1/2}$ confirmed these observations. This time corresponds to the time when half of the initial fluorescence is recovered indicating the speed of β -catenin mobility. For the differentiation time of 3-5 hours $t_{1/2}$ was significantly smaller (2.65 min) compared to 4.14 min and 4.64 min for t=0 and t=24 hours, respectively. Approximately 15 % of nuclear β -catenin was not recoverable and was defined as immobile fraction which corresponded to bound β -catenin.



Figure 14: Nuclear β -catenin influx rate is increased after initiation of differentiation: Fluorescence recovery after photobleaching analyses of YFP- β -catenin-transfected ReNcell VM. (A) Prebleach image; (B) Nuclear staining (Hoechst); (C) Post-bleach image; (D) Cell with recovered fluorescence in bleached area, t=8 min; Nuclear bleaching area was selected from nuclear staining; bar = 5 μ m; (E) Mean recovery curves of bleached nuclei show faster recovery after 3-5 hours of differentiation compared to proliferating cells and cells at 24 hours of differentiation indicated by a steeper incline of the curve and a shorter half-recovery time. Plateau phase was reached after approx. 8 minutes. Halfrecovery time t_{1/2} corresponds to the time when half of the *initial* intensity is recovered, indicating the speed of β -catenin mobility (black circles). Immobile fraction corresponds to bound β -catenin which was not replaced by new YFP-tagged molecules; N \leq 3; n \leq 4; (***) Two-way ANOVA for time course with p \leq 0.001; 0 hours vs. 24 hours: p=0.71 (n.s.).

This suggests that the extent of Wnt/ β -catenin signaling is differentially regulated during the first 24 hours of ReNcell VM differentiation partly through the mechanism of nuclear β -catenin import. The first hours after initiation of differentiation, therefore, seem to be relevant for the

direction of commitment to the neuronal or glial lineage. As a result we returned to the image quantification approach to compare the nuclear β -catenin concentration between neurons and non-neuronal cells.

3.2.4 Immature neurons show increased nuclear β -catenin levels compared to non-neuronal cells

Microscopic analyses of protein distribution not only allowed to discriminate different proteins in different compartments but also different cell types. By immunostaining of neuronal proteins such as Hu C/D, β III-tubulin or TH with subsequent semi-automatic signal quantification, the comparison of neurons with non-neuronal cells was performed by separation of the respective nuclei. In neuronal nuclei the staining intensity of Hu C/D and other markers was significantly elevated which facilitated the isolation and separate quantification.

Temporal investigations revealed that nuclear β -catenin was significantly elevated in the erliest neurons (6-12 hours, Hu C/D+/ β III-tubulin–, termed 'immature neurons') compared to non-neurons as depicted in Fig. 15A,B and plotted over time in 15C (also see suppl. S3 for normalization to t=0). After 24 hours this effect was reversed and the maturated neurons exhibited less nuclear β -catenin than the non-neuronal cells. This difference was even more prominent after immunostaining and quantification of active nuclear β -catenin that is dephosphorylated at the GSK3 β -phosphorylation sites (Fig. 15D). Active β -catenin is the form activating the TCF/LEF transcription factor machinery and its increase corresponds to active Wnt-signaling. It should be noted that the monoclonal anti-mouse antibody for active/dephosphorylated β -catenin showed very heterogeneous staining results in terms of reproducibility when used in human cells which decreased the statistical certainty. The data plotted in Fig. 15D correspond to two experiments (N=2, n=3) with homogeneous staining results of most cover slips. However, the trend of an initial increase of nuclear ABC is obvious.

Consistent with the results from the FRAP analyses, these data argue for a temporally limited upregulation of Wnt/ β -catenin signaling during the first 12-24 hours of ReNcell VM differentiation. This especially was evident in the Hu C/D+ neurons between 6 and 12 hours also appearing during this critical time which suggests, that Wnt/ β -catenin signaling is involved during the cell fate decision process in human neural progenitor cell line ReNcell VM.



Figure 15: Comparative analysis of neuronal and non-neuronal nuclear β -catenin: (A,B) Immature neurons (green, arrows, 9 hours of differentiation) show stronger staining intensities of nuclear β -catenin compared to non-neuronal cells (undifferentiated and glia cells); purple staining corresponds to co-localized nuclear Hoechst staining (blue) and increased nuclear β -catenin (red); (C) This stronger accumulation is temporarily limited to the first hours of differentiation and then decreases below the nuclear β -catenin level of non-neuronal cells at later stages (24-72 hours), please also see suppl. S3 for data representation normalized to t=0. (D) This effect is even more prominent with active, dephosphorylated β -catenin signals, β -catenin: red, neurons: green (β III-tubulin-FITC), nuclei: blue (Hoechst), bar = 10 μ m; unpaired t-test to control: *, p ≤ 0.05 ; **, p ≤ 0.01 ; N=3, n=3 each.

3.3 Summary 1/4

The results shown in sections 3.1 - 3.2.4 described the human neural progenitor cell line ReNcell VM as a suitable cell model to investigate Wnt/β -catenin signaling during neuronal differentiation as indicated by the following evidences:

- reproducible cell line characteristics
- fast differentiation into neurons (partly dopaminergic) and glia cells within 72 hours
- during differentiation Wnt/β -catenin signaling is active, shown by total and, more importantly, nuclear β -catenin accumulation
- this nuclear β -catenin accumulation is differentially controlled during the first 24 hours shown by up-regulated nuclear import kinetics after 3-5 hours of differentiation
- the first detectable neurons exhibit significantly elevated β -catenin levels compared to non-neurons during the first 12 hours of differentiation

These findings led us to the prediction that Wnt/β -catenin signaling is linked to neuronal differentiation in ReNcell VM through a temporal increase of nuclear β -catenin. To closer elucidate this link, extrinsic up and down-regulation of Wnt/β -catenin signaling was performed by either upstream or downstream activators and inhibitors, respectively.

Part 2/4 - The influence of Wnt/ β -catenin signaling modification on differentiation

3.4 Activation of β -catenin dependent Wnt-signaling

Nuclear β -catenin accumulation during early ReNcell VM differentiation implicated a possible relationship between signaling activity and the mechanism initiating neuronal differentiation. To confirm this, ReNcell VM cells were treated with activators of Wnt/ β -catenin signaling as illustrated in Fig. 16. Addition of recombinant human Wnt3a activates the cascade at the membrane level. Indirect activation further downstream by inhibition of GSK3 β with small molecules (here SB216763) led to increased β -catenin accumulation and hence a stronger downstream activation of target genes.



Figure 16: Activation of Wnt/ β -catenin signaling by (A) indirect activation through inhibition of GSK3 β with SB216763, a small molecule (grey), and (B) by addition of recombinant human Wnt3a acting upstream at the receptor level.

3.4.1 Activation of Wnt/ β -catenin signaling increases nuclear β -catenin levels in ReNcell VM

As stated above, it was shown that both LRP and Dvl are activated after initiation of ReNcell VM differentiation which indicates endogenous Wnt-ligand production and secretion followed by pathway activation. Adding recombinant Wnt molecules would therefore lead to an increased activation of the Wnt/ β -catenin pathway and subsequently influence both nuclear β -catenin levels and neuronal differentiation, eventually. To test this hypothesis, human recombinant Wnt3a was added into the differentiation medium at time point 0. Using the 3D-image analysis approach described above (section 3.2), nuclear β -catenin signals were quantified during differentiation under the influence of either Wnt3a or SB216763. The results showed significantly increased



Figure 17: Activation of Wnt/ β -catenin increases nuclear β -catenin concentration: (A) upstream activation with additional recombinant Wnt3a (50 ng/ml in CHAPS buffer) elevates nuclear β -catenin levels significantly already within the first hours of differentiation; normalized to control; N=5, n=3 each. (B) downstream activation by GSK3 β -inhibition with SB216763 (3 μ M in DMSO) showed a response similar to Wnt3a by promoting nuclear β -catenin-accumulation; please also see suppl. S4 for data representation normalized to t=0, page 113. N=3, n=3 with \approx 50 cells each; unpaired t-test to CHAPS and DMSO control, respectively: *, p \leq 0.05; **, p \leq 0.01; ***, p \leq 0.001.

nuclear β -catenin concentrations compared to control cells (CHAPS/DMSO-treated) in both Wnt3a and SB216763-treated cells. In both approaches, this increase reached a peak after six hours already, followed by decreasing levels that nearly reached control values after 72 hours (Fig. 17, also see suppl. S4 for normalization to t=0).

3.4.2 Activation of Wnt/β-catenin signaling stimulates neuronal differentiation in ReNcell VM

It was shown *in vivo* that activation of Wnt/β -catenin signaling with Wnt1 increased the number of neurons in differentiating mouse embryonic midbrain cultures (Castelo-Branco et al., 2003). To test whether this correlation between signaling activation and neuronal differentiation is present in differentiating ReNcell VM, human recombinant Wnt3a was added into the differentiation medium at time point 0. Temporal quantification of the neuronal number by flow cytometric analyses of Hu C/D+ neurons revealed that an initial addition of Wnt3a elevated the neuronal percentage 2.8-fold as plotted in Fig. 18A.

To confirm this correlation between pathway activation status and degree of neuronal differentiation the β -catenin antagonist GSK3 β was inhibited which, therefore, activated the pathway independently of Wnt-ligands. For this task 3 μ M SB216763 was added which also resulted in significantly increased neuronal differentiation (Fig. 18B).Cytotoxic effects from either CHAPS buffer (Wnt3a solvent) or DMSO (SB216763 solvent) could be excluded as indicated in Fig. 19. For Wnt3a-treated cells, microscopic quantification of Hu C/D+ and β III-tubulin+ neurons confirmed the flow cytometry results (data not shown). Due to strong autofluorescence of SB216763 which disturbed the accuracy of flow cytometric measurements, these cells were analyzed by microscopy only.

This approach convincingly showed that extrinsic over-activation of the endogenous Wnt/β catenin signaling led to significantly increased neuronal differentiation in ReNcell VM. Taken together, this activation approach indicated a temporal interplay between initially increased nuclear β -catenin concentration and a subsequent increase of the percentage of neurons. To closer investigate this correlation in respect to the findings that immature neurons exhibit stronger nuclear β -catenin accumulation (Fig. 15), the response to Wnt3a and SB216763 on neurons and non-neurons was compared cell type-specifically.



Figure 18: Both upstream and downstream activation of Wnt/ β -catenin signaling elevates neuronal percentage: (A) Flow cytometric analysis of Wnt3a-treated cells (50 ng/ml) indicates significantly enhanced neuronal differentiation compared to control cells. (B) Neuronal differentiation is also significantly promoted after inhibition of the β -catenin antagonist GSK3 β by 3 μ M SB216763. Control cells for Wnt3a and SB216763 were treated with CHAPS and DMSO-containing medium, respectively (see section 2.10), SB216763-treated cultures were quantified by microscopy as described in section 2.3.2; N=3, paired t-test to control: *, p \leq 0.05.



Figure 19: Cytotoxicity/MTT-test of differentiating ReNcell VM treated with solvent agents. (A) Treatment with CHAPS buffer as a solvent control for Wnt3a has no influences on cell survival. A concentration of 0.002 % corresponds to 50 ng/ml Wnt3a which was used in the activation experiments. (B) Similarly, treatment with DMSO as the solvent control for SB216763 has no effect on viability, either. N=3, n=8 each.



Figure 20: Immature neurons show an increased nuclear β -catenin accumulation when Wnt/ β -catenin signaling is active. (A) 3D-image quantification of nuclear β -catenin in neurons vs. non-neuronal cells during Wnt3a-treatment. During the first 12 hours of differentiation the difference of nuclear β -catenin levels between neurons and non-neurons is significantly increased after initial Wnt3a-treatment. (B) downstream activation with SB216763 showed a similar but slightly lower effect than Wnt3a, suggesting a different receptor composition on neurons compared to non-neurons; compare Fig. 15; N=3, n=3 each; unpaired t-test to CHAPS and DMSO control, respectively: *, p ≤ 0.05 ; **, p ≤ 0.01 ; ***, p ≤ 0.001 .

3.4.3 Immature ReNcell VM neurons react stronger on stimulation of Wnt/ β -catenin signaling than non-neuronal cells

The comparison of neurons with non-neuronal cells in respect to nuclear β -catenin concentration showed that neurons exhibit elevated nuclear accumulation during the first 12 hours of differentiation (section 3.2.4). Due to this observation and the finding that activators of Wnt-signaling increased nuclear β -catenin levels as well as neurogenesis (section 3.4.1), the neuronal response to these activators was investigated selectively. In comparison to non-neuronal cells, neurons showed significantly increased levels of nuclear β -catenin after Wnt3a between 6-12 hours of differentiation as depicted in Fig. 20A. Treatment with SB216763 also increased nuclear β -catenin specifically in neurons between 6-9 hours but not as strong as Wnt3a (Fig. 20B). At later differentiation stages (24-72 hours) the treatment did not show significant differences compared to control.

Due to the evidence that signaling activation rather had an effect on immature neurons during the first 12 hours, this would also include the pool of committed neuronal progenitor cells. Further investigations regarding this topic are described in sections 3.8.1 - 3.8.3.

To verify the hypothesis about the connection between Wnt/β -catenin signaling and the early decision processes during neurogenesis, the reverse experiment of this activation approach was performed by inhibition of Wnt-signaling with upstream inhibitors.

3.5 Inhibition of β -catenin-dependent Wnt-signaling

As described above, activation of Wnt/β -catenin signaling increased very early (between 6-12 hours of differentiation) the concentration of nuclear β -catenin. More importantly, this activation resulted in a significantly increased percentage of neurons. To test whether blocking of Wnt/β -catenin signaling had the opposite effect, inhibition experiments with either Wntinhibitory-factor-I (WIF-I) or Dickkopf-1 (Dkk-1) were performed as illustrated in Fig. 21.



Figure 21: Inhibition of Wnt/ β -catenin signaling by treatment with either Wnt-inhibitory-factor-I (WIF-I) or Dickkopf-1 (Dkk). Both proteins prevent the activation of the LRP/Frizzled-receptor complex through different mechanism: WIF-I directly blocks free Wnt-ligands in a rather unspecific way, Dkk blocks the LRP-co-receptor and, therefore, specifically Wnt/ β -catenin signaling.

3.5.1 Upstream inhibition of Wnt/β-catenin signaling reduces nuclear beta-catenin levels in ReNcell VM

To test whether the levels of nuclear β -catenin were also affected by inhibitory factors, nuclear β catenin signal intensities were quantified during WIF-I or Dkk-1-treatment. The results showed significantly decreased levels of nuclear β -catenin during the first 12 hours after initial addition of WIF-I. This decrease was also detectable in Dkk-1-treated cells but limited to the first 3-6 hours only. After the initial down-regulation the amount reached control levels again (Fig. 22).

To investigate whether this inhibition also effects neuronal differentiation Hu C/D+ neurons were quantified by flow cytometry after treatment with WIF-I or Dkk-1.



Figure 22: Inhibition of Wnt/ β -catenin decreases nuclear β -catenin concentration: (A) Upstream inhibition with WIF-I (50 µg/ml in CHAPS buffer) decreases nuclear β -catenin levels significantly during the first 3-6 hours after treatment and initiation of differentiation; N=4, n=3 each. (B) Treatment with Dickkopf-1 (50 ng/ml Dkk-1) showed a WIF-I-similar response, but the decrease was limited to the first 3 hours; N=3, n=3 each; unpaired t-test to CHAPS control: *, p ≤ 0.05 ; **, p ≤ 0.01 .

3.5.2 Upstream inhibition of Wnt/β-catenin signaling decreases neuronal differentiation of ReNcell VM

WIF-I and Dkk-1-treatment revealed a significant decrease of the neuronal percentage of approximately 50 % with both inhibitors. WIF-I showed a stronger effect than Dkk-1 to delay neurogenesis (Fig. 23). Latest flow cytometric analyses from our group confirmed these findings (Masters thesis, personal communication A. Rohn (2009)).

In terms of efficacy to negatively influence neuronal differentiation, the difference between WIF-I and Dkk-1 is in agreement with the down-regulation of nuclear β -catenin import as described in the previous section. These results support the indication that Wnt/ β -catenin is an important mediator of neuronal differentiation in the human neural progenitor cell line ReNcell VM. WIF-I was more potent in terms of inhibiting neurogenesis compared to Dkk-1, although both proteins showed a reduction in the same order of magnitude. The different efficacies of the two inhibitors can be explained by different effective concentrations of the proteins and by the amount used in the assays or by specificity aspects of WIF-I being an inhibitor for multiple Wnt-ligands.

In accordance to the findings of the activation experiments, the negative manipulation of Wnt/β -catenin signaling directly affected nuclear β -catenin concentrations and neuronal differentiation in ReNcell VM. These data again indicate the temporal interplay between signaling activation and neurogenesis: first, early control of nuclear β -catenin concentration followed by the respective effect on neuronal differentiation. To investigate the inhibitory effect on nuclear β -catenin in neurons specifically, we compared neuronal with non-neuronal fluorescence signal intensities in cells under the influence of WIF-I and Dkk-1.



Figure 23: Upstream inhibition of Wnt/ β -catenin signaling reduces neuronal percentage: Microscopic analyses indicate that (A) treatment with the general Wnt-inhibitory-factor-I (WIF-I, 50 μ g/ml) reduces significantly neuronal differentiation in differentiating ReNcell VM; (B) The specific antagonist of Wnt/ β -catenin signaling Dickkopf-1 (Dkk, 50 ng/ml) also negatively influences neuronal differentiation; N=3, paired t-test to CHAPS-treated control: *, p ≤ 0.05 ; **, p ≤ 0.01 .

3.5.3 Immature ReNcell VM neurons do not react stronger on inhibition of Wnt/ β -catenin signaling than non-neuronal cells

Both, WIF-I and Dkk-1-treatment decreased the amount of nuclear β -catenin and the number of neurons, significantly. Combining this with the observation that ReNcell VM-neurons have a higher responsiveness to Wnt-signaling activators within this time frame (6-12 hours) (section 3.4.3), we compared nuclear β -catenin between neurons and non-neurons.

Treatment with WIF-I diminished the difference of nuclear β -catenin between neurons and non-neurons that was present around 6 hours of differentiation in control cells as shown in Fig. 24A and suppl. S5. At later time points (12-72 hours) WIF-I did not show any significant effect. Treatment with Dkk-1 slightly reduced the difference only at 6 hours but did not shown any significant change for the complete time of investigation (Fig. 24B and suppl. S5). In comparison to the considerable difference of nuclear β -catenin accumulation between neurons and non-neurons after activation of Wnt/ β -catenin, neurons show a much weaker response to Wnt-inhibitory factors.

This is consistent with the hypothesis that the transition from restricted (= committed) neuronal progenitor cell to neuron is controlled by the degree of Wnt/ β -catenin activity. One reason for this is linked to the experimental approach to perform the quantification in post-mitotic neurons (Hu C/D+) which were committed already.



Figure 24: Nuclear β -catenin in neurons is not affected differently when Wnt/ β -catenin signaling is inhibited. (A) 3D-image quantification of nuclear β -catenin in neurons vs. non-neuronal cells during WIF-I-treatment. The early difference between neurons and non-neurons was diminished by WIF-I treatment only at 6 hours. during later stages WIF-I did not change this difference. (B) After addition of Dickkopf-1 (Dkk) the difference between neurons and non-neurons was not changed significantly throughout the time of investigation; please also see suppl. S5 for data representation normalized to t=0, page 113; N=3, n=3 each; unpaired t-test to CHAPS and DMSO control, respectively: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

As already mentioned, the putative underlying mechanism is likely to control the pool of immature neuronal cells before expressing marker proteins for mature neurons. Therefore, we hypothesized that Wnt/β -catenin signaling controls the pool of restricted progenitor cells and more precisely restricted neuronal progenitors using a mechanism that we, therefore, closer elucidated and that is described below in sections 3.8.1 - 3.8.3.

With the knowledge about the temporal control of Wnt/ β -catenin signaling which seems to control neuronal differentiation in ReNcell VM, we closer investigated the small population of TH+ DA neurons as introduced in section 3.1.2. This approach was to compare the spatio-temporal kinetics of Wnt/ β -catenin signaling during general neurogenesis with dopaminergic neurogenesis.

3.6 Influence of Wnt/ β -catenin signaling on dopaminergic differentiation in vitro

The correlation between early β -catenin accumulation and neuronal differentiation was investigated using Hu C/D+ and β III-tubulin+ neurons. To closer elucidate DA differentiation in ReNcell VM, we tested whether this correlation was also present in differentiating TH+ DA neurons and how this type of neurons would react on activation of Wnt/ β -catenin signaling.

We analyzed whether upstream activation of Wnt/ β -catenin signaling by Wnt3a also increased the number of TH+ neurons. The results from temporal analyses indicate that 50 ng/ml Wnt3a increase the percentage of DA neurons right from the first moment of their appearance (Fig. 25A). However, due to the small number of TH+ cells and the quantification approach using fluorescence microscopy, the systemic error was too high to yield statistical certainty. Nonetheless, the results showed that DA differentiation by trend is elevated after Wnt3a-treatment.

In correlation to the analyses of the general neurogenesis, we used our 3D-image quantification approach to measure the nuclear β -catenin concentration in the nuclei of TH+ cells during the



Figure 25: Upstream activation of Wnt/ β -catenin signaling increases percentage of dopaminergic (DA) neurons and nuclear β -catenin in DA ReNcell VM-neurons: Microscopic analyses indicate that (A) treatment with Wnt3a (50 ng/ml) increases the number of DA neurons in differentiating ReNcell VM; due to the small ratio of positive cells and the method used for quantification the difference remains to be statistically verified; N=3. (B) 3D-image analyses of nuclear β -catenin in TH+ DA neurons vs. TH– cells shows increased β -catenin-levels from 48 hours onwards. After initial Wnt3a-treatment (50 ng/ml) this difference is more prominent and occurs after 24 hours already; N=3, paired t-test to CHAPS-treated control: *, p ≤ 0.05 .

first 72 hours of differentiation. The results indicated that under control conditions accumulation of nuclear β -catenin started between 24 and 48 hours of differentiation. Under the influence of 50 ng/ml Wnt3a this process started between 12 and 24 hours already (Fig. 25B) showing that DA neurogenesis in ReNcell VM is affected by Wnt/ β -catenin signaling.

Comparing this data with the previous observations from the overall neuronal population, DA differentiation is (despite the high standard deviation) also increased after activation of Wnt/ β -catenin signaling with Wnt3a. This could be related to the accelerated nuclear β -catenin accumulation process observed 12-24 hours earlier than under control conditions when treated with Wnt3a. However, one obvious difference between TH+ DA neurons and the overall (Hu C/D+) neuronal pool is the time window of nuclear β -catenin accumulation. In TH+ DA neurons this occurred 12-24 hours later than in Hu C/D+ neurons. These observations suggest that Wnt/ β -catenin signaling is more active at later stages during DA differentiation. Due to the fact that the yield of DA neurons in ReNcell VM was too low to analyze enough cells for statistical certainty and to further elucidate the process of spatio-temporal β -catenin redistribution we turned to the *in vivo* model of the embryonic mouse brain. Additionally, this approach was also to investigate the nuclear β -catenin accumulation in a cell stage-dependent manner in the embryonic mouse midbrain. The data are presented in sections 3.10 - 3.12.

3.7 Summary 2/4

The results shown in sections 3.4 - 3.6 describe that the extent of Wnt/β -catenin signaling activity influenced ReNcell VM progenitor cells during the early phase of neuronal differentiation as indicated by the following evidences:

- activation of Wnt/ β -catenin signaling with SB216763 or Wnt3a
 - significantly increased nuclear β -catenin levels
 - led to significantly more neurons after 48-72 hours
 - specifically increased nuclear β -catenin in the first neurons during 6-12 hours of differentiation
 - showed a similar but delayed effect on nuclear β -catenin accumulation in DA neurons
 - slightly increased the number of DA neurons
- inhibition of Wnt/β -catenin signaling with WIF-I or Dkk1
 - significantly decreased nuclear β -catenin levels during the first 3-6 hours only
 - led to significantly less neurons after 48-72 hours
 - did not show a strong influence on already differentiated neurons during 6-12 hours of differentiation

These findings suggest that neurogenesis in ReNcell VM is modulated by a precise temporal controlling mechanism of Wnt/ β -catenin signaling activity. Especially in the very early time window after initiation of differentiation, an elevated signaling activity was evident in the neuronal population. Inhibition of the signaling-pathway decreased nuclear β -catenin levels only during the first hours. Additionally, the inhibition did not influence immature neuronal cells in terms of nuclear β -catenin concentration. Together, this suggests that Wnt/ β -catenin signaling influences the progenitor population during their commitment phase. To elucidate this hypothesis, we closer investigated the events controlling cell cycle exit and initiation of differentiation which occurred during the first 12-24 hours after growth factor-withdrawal.

Part 3/4 - Wnt/β-catenin signaling and its influence on the interplay between proliferation and differentiation

3.8 The role of Wnt/ β -catenin on committed neuronal progenitor cells

On the bases of the data shown above, we hypothesized that Wnt/β -catenin signaling might influence the pool of committed neuronal progenitor cells (sections 3.4 (activation) and 3.5 (inhibition)). The influence of both activation and inhibition occurred only during the very early time window of neuronal differentiation between 6-12 hours which then was followed by a change of the neuronal percentage. This suggests that Wnt/β -catenin signaling effects the cell population which undergoes the decision process to become neurons, i.e. restricted (= committed) neuronal progenitor cells.

3.8.1 Proliferation of ReNcell VM stops after 12 hours of differentiation

Empirically we found that some ReNcell VM still undergo proliferation during the first hours of differentiation. Recent flow cytometric data from our group showed that the number of cells in G2/M and S phase decreased continuously and stopped between 12-24 hours nearly completely (conference proceedings Jaeger et al. (2009)). To answer the question how Wnt/ β catenin signaling influenced this proliferating population, further studies at the single cell level were performed during this time window.

Using long term life cell imaging (non-stop 0-72 hours), we observed an abrupt stop of cell division events illustrated in Fig. 26A and suppl. S2 (video file). Quantification of these events revealed that the majority of ReNcell VM stop proliferation between 12 and 15 hours (Fig. 26B) which is in accordance with the flow cytometric analyses. Closer investigations on the pool of DNA-synthesizing cells (S-phase) was performed using the EdU-incorporation assay as described in section 2.11 and illustrated in Fig. 26C. Temporal microscopic quantification of EdU+ S-phase cells revealed a constant decline of the number of DNA-synthesizing cells from 50 % at t=0 to 25 % after 12 hours followed by a sharp drop to 3 % at 15 hours and 1 % after 24 hours. To correlate the proliferation status with the differentiation status, immunostaining for nestin+ progenitor cells was performed. As indicated in Fig. 27 the staining intensity of nestin and the number of nestin+ cells decreased constantly during the first 72 hours.



Figure 26: ReNcell VM stop proliferation 12 hours after initiation of differentiation: (A) Mitotic event in phase contrast; time between images = 5 minutes; images taken from a 72 hours time series; please also see video file suppl. S2; (B) Quantification of these mitotic events shows a sharp decline in the number of dividing cells between 12 and 15 hours and a nearly complete stop of proliferation within the first 24 hours of differentiation; (C) Quantification of EdU+ cells (3h pulse) during differentiation indicates a constant decrease of the number of proliferating cells and also shows a sharp decline after 12 hours of differentiation; (D) Proliferating cells incorporate EdU (red) into their DNA (blue) during S-phase; β -catenin (green); major frame grid = 10 μ m; N=3 with n≈1000 cells counted each.



Figure 27: ReNcell VM decrease abundance of the stem cell marker nestin during differentiation: the number of nestin+ cells (red) as well as the staining intensity decreases constantly during the first 72 hours of differentiation; image acquisition with Zeiss Imager Z1 and AxioCam Mrm using equal image acquisition times; nuclei: blue; bar=50 μ m.

3.8.2 Activation of Wnt/ β -catenin signaling extends time window of proliferation

To investigate the effect of downstream Wnt/ β -catenin signaling on cell proliferation, the cells were treated with SB216763 followed by MTT-tests. As described in section 2.11 this assay provides relative information about metabolic activity or indirectly the cell number. Treatment with the GSK3 β -inhibitor SB216763 in a concentration range between 1 and 10 μ M increased the overall metabolic activity and/or cell number significantly after 12 hours already (with 10 μ M). The use of higher concentrations between 50 and 100 μ M decreased the metabolic activity and hence the cell number below the level of DMSO-treated control cells. A significant cytotoxic effect of this GSK3 β -inhibition was present from 24 hours and 48 hours onwards with 100 μ M and 50 μ M respectively, as shown in Fig. 28. Preliminary results from Wnt3a-treated cells (50 ng/ml) confirmed the observations of an increased cell number after 24 hours (data not shown).

To closer investigate this effect on the single cell level and to answer the question whether the cell cycle is altered, SB216763 and Wnt3a-treated cells were pulsed with EdU for three hours before cell fixation. The results revealed that 3 μ M SB216763 increased the number of EdU+ (S-phase) cells between 6 and 24 hours which was statistically significant. This difference was especially obvious at 15 and 24 hours where the number of EdU+ cells was increased more than two-fold (2.33- and 2.25-fold). Wnt3a treatment showed a similar effect and elevated the number of EdU+ cells between 9 and 15 hours which was verified statistically, too (Fig. 29). Again, at the 15 hours-time point the difference was most prominent (1.76-fold). This indicated that the

above-mentioned higher metabolic activity (MTT-test) reflected an increase of the cell number which was mediated by an extended time window of proliferation.



Figure 28: Activation of Wnt/ β -catenin extends time window of proliferation: MTT-test of SB216763-treated cells normalized to DMSO control. Downstream activation of Wnt/ β -catenin signaling with 1-10 μ M SB216763 results in elevated MTT-reaction reflecting an increased number of cells and, hence, longer ReNcell VM proliferation. Higher concentrations of SB216763 (50-100 μ M) show stronger cytotoxic effects than DMSO (100 μ M SB216763 \cong 1 % DMSO); unpaired t-test to DMSO control: *, p ≤ 0.05 ; **, p ≤ 0.01 ; ***, p ≤ 0.001 ; N=3, n=8 each.



Figure 29: Upstream and downstream activation of Wnt/ β -catenin signaling extend time window of proliferation during early differentiation: (A) GSK3 β -inhibitor SB216763 (3 μ M) increases number of DNA synthesizing/proliferating cells (EdU incorporation into DNA, 3 hours EdU pulse). (B) The same effect, but delayed, is detectable after upstream activation with 50 ng/ml Wnt3a; unpaired t-test to control: *, p ≤ 0.05 ; **, p ≤ 0.01 ; ***, p ≤ 0.001 ; N=4.

3.8.3 Restricted neuronal progenitor cells show ongoing proliferation

To test the hypothesis whether in ReNcell VM restricted neuronal progenitor cells undergo proliferation, the composition of the restricted (= committed) neuronal and glial progenitor cell pool was analyzed by flow cytometry. Restricted neuronal progenitor cells were immunostained for PSA-NCAM, an early neuronal marker present in both immature progenitor cells and mature neurons (for review see Bonfanti (2006)). Restricted glial progenitor cells were stained for A2B5 (A2B5+ bipotential glial progenitor cells can give rise to both oligodendrocytes and type-2 astrocytes in culture (for review see Nishiyama et al. (2009))). The analysis revealed that at 12 hours approximately 1.5 % of the cells were PSA-NCAM+ restricted neuronal progenitor cells (compare to 0.5 % of Hu C/D+ neurons at 12 hours). This number increased more than 4-fold to 6.5 % at 24 hours. The number of A2B5+ restricted glial progenitor cells at 12 hours was nearly four times higher (5.25 %) than the immature neuronal population (Fig. 30A-F). This amount reflected the overall ratio of neurons to glia at the end of the differentiation process where the majority of the cells were astrocytes.

At 12 hours, co-staining for β III-tubulin revealed PSA-NCAM+/ β III-tubulin– and PSA-NCAM+/ β III-tubulin+ cells. PSA-NCAM+/ β III-tubulin– cells represented the population inside the early commitment-phase (Fig. 30G), double-positive cells represented a more mature neuronal population as indicated in Fig. 30H. Especially after 72 hours the cells exhibited high abundance of both PSA-NCAM and β III-tubulin (Fig. 30I-J).

To detect the cells that were double-positive for EdU and PSA-NCAM, differentiating cells were analyzed by flow cytometry after a 6-hours EdU-pulse. Quantification of EdU+ cells after 12 hours confirmed the microscopic analyses (Fig. 29 and revealed that approximately 24 % of the cells were in S-phase (Fig. 31A-B). EdU/PSA-NCAM double-staining and two-color flow cytometry revealed that a small sub-population of EdU+ cells were positive for PSA-NCAM (Q2 in Fig. 31C): 0.6 % of the overall population and 4.2 % of the EdU+ population. These results were verified by immunostaining and fluorescence microscopy as illustrated in Fig. 31D. The cells with clearly polarized neuronal morphology (Fig. 31D) did not show double-positive staining, indicating a later post-mitotic stage of neuronal maturation. At 12 hours of differentiation the first double-positive cells appear after a 6-hours-EdU-pulse and *a fortiori* present after 8 or 10-hours-EdU-pulses.

Based on these findings we concluded that our hypothesis of a proliferating restricted progenitor cell pool that could be affected/increased by Wnt/ β -catenin signaling is plausible. Especially the time between cell cycle exit (G1/G0-transition) and first appearance of neuronal proteins seems to be the time window for the cell fate commitment influenced by Wnt/ β -catenin signaling. However, further analyses are necessary to investigate the temporal relation between time of differentiation, proliferation and expression of neuronal proteins in more detail.





Figure 30: Restricted progenitor cell population during differentiation: (A-C) flow cytometric analyses of restricted neuronal progenitor cells labeled with PSA-NCAM-PE indicate a sub-population which is growing steadily during the first day of dif- $(\mathbf{D}-\mathbf{F})$ The number of ferentiation. A2B5+ glial restricted progenitor cells is larger than the PSA-NCAM+ population. Dotted lines in intensity plots corresponded to fluorescence of isotype control; $N_{C,F}=3$. (G) At 12 hours, some PSA-NCAM+ cells (red) do not express neuronal markers such as β IIItubulin (green). (H) In contrast, cells with neuron-like morphology also show β III-tubulin expression. (**I-J**) At 72 hours of differentiation, PSA-NCAM expression is highly abundant in β IIItubulin+ neurons; nuclei: blue; major frame grid = 10 μ m.


Figure 31: Restricted neuronal progenitor cells undergo proliferation. (A-C) Flow cytometric analyses of EdU-incorporation at 12 hours of differentiation after a six hours pulse; (A) signals from non-specific isotype control and Alexa488azide staining with 1 % false positive events. (B) EdU+ population at 12 hours. (C) Restricted neuronal progenitor cells labeled with PSA-NCAM-PE are indicated in blue in the quadrants Q1 and Q2 (corresponding to positive cells like these in Fig. 30A), red cells in Q4 correspond to EdU+ cells also shown in (B). Events in Q2 correspond to EdU+/PSA-NCAM+ cells, indicating a proliferating PSA-NCAM+ subpopulation of appox. 0.6 % of the total cell population; SS, side scatter. (D) At 12 hours, first PSA-NCAM+ cells (green) exhibit EdU-incorporation (red) after six hours EdU pulse. This double-positive population corresponds to the cells in Q2 in (C). (E) Cells with highly polarized neuron-like cell shapes are not double positive indicating a more differentiated cell stage.

3.9 Summary 3/4

The activity of Wnt/ β -catenin signaling which directly correlates with the neuronal differentiation of ReNcell VM (see sections of Wnt-manipulation 3.4-3.6) seemed to be mediated through a mechanism controlling the cells during the early differentiation phase of the first 12-15 hours. We showed, that during this time

- ReNcell VM still proliferated (EdU+ S-phase cells) but stopped proliferation nearly completely after 15 hours of differentiation.
- the abundance of the marker for proliferating stem cells nestin was decreased continuously.
- the number of proliferating cells was significantly increased after Wnt3a or SB216763treatment which also increased the total cell number at later stages quantified by MTT-test and EdU assay.
- a sub-population of restricted neuronal progenitor cells (PSA-NCAM) was EdU+ and, thus, proliferating.
- around 4 % of the proliferating population are restricted neuronal progenitor cells which were quantified by flow cytometry and confirmed by fluorescence microscopy.

The importance of the spatio-temporal distribution of β -catenin and the extent of Wnt/ β catenin signaling activity is evident when combining the results from both differentiation and proliferation studies. Especially the timing and the degree of signaling during the transition from stem cell over neuronal progenitor cell to mature neuron seems to be one of the driving forces for the decision process in cell lines like ReNcell VM.

To validate these results and to compare these observations of the human cell line with the *in vivo* situation, we turned to an *in vivo* animal model - the embryonic mouse midbrain.

Part 4/4 - β-catenin kinetics during dopaminergic differentiation in vivo

3.10 Wnt/ β -catenin-signaling in the developing brain

To verify the results from the *in vitro* assays, we investigated how β -catenin is controlled in a spatio-temporal manner in the developing embryonic mouse midbrain. The midbrain is the location of DA neurogenesis and, therefore, a suitable model for comparison with ReNcell VM. One major advantage of the embryonic mouse midbrain is the fact that the stem cells of the floor plate differentiate into DA neurons with a high percentage and in a very reproducible manner. Since ReNcell VM show a very small frequency of DA neurons, the embryonic midbrain is a suitable model to translate the *in vitro* findings into the *in vivo* situation and more importantly into DA neurogenesis.



3.11 Nuclear β -catenin levels increase during migration and differentiation of dopaminergic cells in vivo

The advantage of 3D-image cytometry in comparison to Western blotting approaches becomes even more evident when investigating tissues with multiple cell types. Using the image quantification approach described in section 3.2 nuclear β -catenin levels were quantified in the floor plate (FP) of the embryonic mouse midbrain at embryonic day 11.5 (E11.5). Between E10.5 and E11.5 first Nurr1+ DA precursors cells and Nurr1+/TH+ DA neurons appear in the FP. Therefore this time point is comparable with the time window observed in ReNcell VM.

Whole embryo cryosections of 14 μ m thickness (corresponding to one cell layer, Fig. 32B) were collected, stained and visualized as described in section 2.12. At E11.5 approx. 15-20 sections were collected at the level indicated by the dashed line in Fig. 32A. Immunostained cells of the area between ventricular zone (VZ) and marginal zone (MZ) of the midbrain floor plate were visualized by confocal microscopy. Fluorescence signals of nuclear β -catenin were quantified in a cell type-dependent manner as illustrated in Fig. 32C (here Nurr1+ vs. Nurr1– nuclei).

To investigate nuclear β -catenin levels in a differentiation stage-dependent manner, we first analyzed Nurr1+ DA precursor cells as shown in the section below, and then analyzed TH+ cells as described in section 3.11.2.

3.11.1 β -catenin in nuclei of DA precursor cells (Nurr1+)

Microscopic investigation of nuclear β -catenin staining revealed a higher intensity in the cells of the marginal zone compared to the cells of the upper ventricular zone. Co-staining for Nurr1 showed that the cells with the highest β -catenin intensity were co-localized with Nurr1+ cells (Fig. 33A-D). This difference was even more prominent with active dephosphorylated β -catenin (ABC) in the same sections (E-H).

Quantification of nuclear β -catenin signals confirmed these qualitative observations. The results revealed that Nurr1+ nuclei exhibited 24 % more nuclear β -catenin than Nurr1- nuclei. In parallel sections from the same brains were stained for ABC and the mean intensities were also quantified. The data showed that Nurr1+ nuclei exhibited 32 % more nuclear ABC than Nurr1- as plotted in Fig. 33J. These results were obtained from experiments using different sections from the same brain and from experiments using the same section to stain both total and active β -catenin (Fig. 33). Both approaches showed the same ratio between positive and negative nuclei. However, double-stained sections represented the more suitable approach to compare the difference between total and active β -catenin concentrations.

After this quantification in Nurr1+ DA precursors, we analyzed TH+ DA neurons to investigate this accumulation in a later stage of differentiation.

3.11.2 β -catenin in nuclei of DA neuronal cells (TH+)

Nurr1+ DA precursor cells differentiate into TH+ DA neurons during their migration from the ventricular zone of stem cells to the marginal zone which then migrate laterally out of the floor plate. To further investigate the role of Wnt/ β -catenin signaling during this process nuclear β -catenin signals were quantified in TH+ cells for comparison with Nurr1+ DA precursor cells.



Figure 33: Nurr1+ DA precursor \mathbf{cells} show elevated levels of nuclear β -catenin. (A-H) Half of an E11.5 midbrain floor plate stained for β -catenin (green, FITC), active dephosphorylated β -catenin (ABC, yellow, Alexa647), Nurr1 (red, Alexa555) and nuclei (blue, Hoechst); nuclei of Nurr1+ regions (dotted line) were quantified separately and compared to nuclei of Nurr1– regions for both types of β -catenin. Elevated β -catenin levels in the region of Nurr1+ cells are evident. This difference is even more prominent when stained for ABC (compare D & H); major frame grid = 10 μ m; (**J**): quantitative 3D-image analysis of nuclear β -catenin and ABC in Nurr1+ DA precursor cells compared to Nurr1- cells, unpaired t-test: *, p \leq 0.05; N=5 with at least 3 brain sections each.

The separate analysis of TH+ cells in the marginal zone (dotted line in Fig. 34) also showed the differences of β -catenin staining between TH+ and TH– cells similarly to the observations obtained from the Nurr1+ precursor population. The region of TH+ cells exhibited a higher staining intensity than the region of TH– cells. The separate quantification of nuclear fluorescence signals in TH+ vs. TH– nuclei revealed a difference of 38 % for β -catenin-staining and 42 % for ABC-staining (Fig. 34G), which is 14 % and 10 % more than in the Nurr1+ population, respectively.

These results indicated that the amount of nuclear β -catenin increased during the differentiation process from stem cells (Nurr1–) over Nurr1+ DA precursor cells to TH+ DA neurons. Since the Nurr1+ population also contain TH+ cells, we investigated whether Nurr1+/TH– precursors exhibit an intermediate amount of nuclear β -catenin reflecting the transition stage between proliferating progenitors of the VZ and TH+ DA neurons.

Therefore, both Nurr1 and TH were stained together with β -catenin in the same sections as illustrated in Fig. 35. Using the Imaris masking-feature Nurr1+/TH– were separated from TH+/Nurr1+ nuclei and nuclear β -catenin signals were compared between both populations and between each of them and the Nurr1– stem cells of the ventricular zone. The comparison of all Nurr1+ nuclei with Nurr1– nuclei (Fig. 35-1) confirmed the findings presented above (Fig. 33). The comparison of nuclei from TH+ DA neurons with nuclei from Nurr1– progenitor cells (Fig. 35-2) revealed a difference of 65 %, which is 23 % more than in the previous observations (42 %, Fig. 34G). This is due to the circumstance that in previous measurements the population of TH– nuclei included Nurr1+/TH– nuclei which exhibited elevated levels of nuclear β -catenin already. Therefore, the comparison of Nurr1+/TH– nuclei with those of Nurr1– progenitor cells (Fig. 35-4) revealed a difference of 20 % only which was smaller than the comparison of all Nurr1+ with Nurr1– nuclei (Fig. 35-1). Reversely, this was due to the circumstance that the Nurr1+ with Nurr1– nuclei (Fig. 35-1). Reversely, this was due to the circumstance that the Nurr1+ with Nurr1- nuclei (Fig. 35-1). Reversely, this was due to the circumstance that the Nurr1+ with Nurr1- nuclei (Fig. 35-3).

These results from cell type-specific analyses suggest that the nuclear β -catenin concentration and hence Wnt/ β -catenin signaling is controlled in a differentiation stage-dependent manner during DA differentiation *in vivo*. Nurr1– stem cells exhibit low levels of nuclear β -catenin which increase once Nurr1 is expressed and even more after the transition to the TH+ status. To search for the underlying mechanism of this process, we investigated the ligands of Wnt/ β catenin signaling on the mRNA-level *in vivo* as described below.

3.12 Nurr1+ dopaminergic precursor cells show Wnt1 expression

Activation of Wnt/ β -catenin signaling requires ligands. One of those ligands that is also present in the embryonic midbrain is Wnt1. Using RNA *in situ* hybridization (ISH) we tested whether Wnt1 is expressed in close proximity to either Nurr1+ or TH+ cells. Co-staining of Wnt1-ISH and Nurr1 revealed that Wnt1 was expressed in the region of Nurr1+ cells. It also overlaped with the region called 'Nurr1-mountain' (arrow in Fig. 36) where Nurr1+ cells accumulate due to proliferation during their ventral migration into the marginal zone.

These findings show that Nurr1+ DA precursors themselves express Wnt1 and exhibit au-



Figure 34: TH+ DA neurons show increased levels of nuclear β -catenin compared to nuclei of TH– cells. (A-F) 14 μ m cryosection of E11.5 midbrain floor plate (FP) stained for β -catenin (green), active β -catenin (ABC, yellow), tyrosine hydroxylase (TH, red) and nuclei (blue). TH+ nuclei in the marginal zone (dotted line) are separately quantified and compared to TH– nuclei for both types of β -catenin. TH+ cells exhibit elevated β -catenin levels. This difference is even more prominent with ABC; major frame grid = 10 μ m. (G) Quantitative 3D-image analysis of nuclear β -catenin shows higher protein concentration in TH+ compared to TH– cells. This difference is even more prominent when stained for ABC, unpaired t-test to negative population: *, p ≤ 0.05 ; **, p ≤ 0.01 ; N=4 with at least 3 sections each.



Figure 35: Wnt/ β -catenin signaling is differentially controlled during DA differentiation. Comparative quantification of nuclear β -catenin signals (green, FITC) between DA neurons (yellow, dotted line, tyrosine hydroxylase (TH), Alexa647) and their precursors (Nurr1, red dotted line, Alexa555) shows higher intensities in the nuclei (blue, Hoechst) of the TH+ population as depicted in the graph. This indicates that nuclear β -catenin accumulation and therefore Wnt/ β -catenin signaling activity increases during DA differentiation in a differentiation stage-dependent manner; half of midbrain floor plate; 14 μ m cryosection; unpaired t-test: *, p ≤ 0.05 ; **, p ≤ 0.01 ; ***, p ≤ 0.001 ; n=3; major frame grid in images = 10 μ m.



Figure 36: Wnt1 expression in area of Nurr1+ DA precursors. Half of an E11.5 midbrain floor plate (rostral part) stained for β -catenin (green, FITC), Nurr1 (red, Alexa555) and nuclei (blue, Hoechst). RNA *in situ* hybridization for Wnt1 shows that Nurr1+ DA precursor cells are co-localized with the area of Wnt1-expression, indicating the role of Wnt/ β -catenin signaling Nurr1/TH-transition and DA differentiation. As early as the cells reach the area of "Nurr1-mountains" (arrow, first Nurr1 expressing cells migrated here from ventricular zone) they co-localize with Wnt1-expression (black staining); major frame grid = 10 μ m.

to crine Wnt/ β -catenin signaling also shown by nuclear β -catenin accumulation as described in the previous sections. This suggests a combined mechanism of proliferation and differentiation control during maturation of DA precursor cells into mature TH+ DA neurons.

3.13 Summary 4/4

To evaluate the characteristics of cellular differentiation observed ReNcell VM *in vitro*, we investigated the neuronal and dopaminergic differentiation processes *in vivo* using embryonic mouse brains.

- First, we successfully transfered our 3D-image quantification approach into this model beginning with cryo-sectioning of E11.5 embryos followed by immunostaining, image acquisition and 3D-image quantification of nuclear fluorescence signals.
- Comparative quantification of nuclear β-catenin in DA progenitors, Nurr1+ DA precursors and TH+ DA neurons revealed that the amount of nuclear β-catenin increased during DA neurogenesis *in vivo*.
- In situ hybridization of Wnt1-mRNA revealed that Nurr1+ cells also express Wnt1, indicating the reason for the increased β -catenin levels through autocrine Wnt/ β -catenin signaling.

These observations are highly consistent with the *in vitro* findings in terms of the spatiotemporal kinetics of β -catenin distribution and indicate the influence of Wnt/ β -catenin signaling for a trans-activation processes during DA maturation.

4 Discussion

4.1 The human neural progenitor cell line ReNcell VM is a suitable tool to study Wnt/ β -catenin signaling during neurogenesis

The results presented in this thesis show that the human neural progenitor cell line ReNcell VM derived from embryonic midbrain is a suitable cell line model to investigate the interplay between Wnt/ β -catenin signaling and neuronal differentiation *in vitro*. Previous observations on the properties of these cells showed characteristic proliferation, morphology and karyotype of a cell line when cultivated under the influence of growth factors (i.e. bFGF, EGF) (Hoffrogge et al., 2006; Donato et al., 2007). However, neuronal differentiation in ReNcell VM has not been described quantitatively in a temporal approach which is the prerequisite for the understanding of neurogenesis. A lot of evidence is found in the literature that neuronal differentiation in the ventral midbrain is controlled by Wnt-signaling (reviewed by Castelo-Branco and Arenas (2006)). Therefore, a quantitative description of neuronal differentiation with respect to the activity of Wnt/ β -catenin signaling was achieved in this study.

To avoid the influence of other signaling cascades which might influence neuronal differentiation, a minimal differentiation medium was used compared to the differentiation protocol described by Donato et al. (2007) who either added dibutyryl-cyclic-AMP and glial cell line-derived neurotrophic factor (GDNF) or used a pre-aggregation differentiation protocol to enhance neuronal differentiation. The results confirmed that ReNcell VM differentiated into the described cell types readily within three days only. However, the temporal quantification revealed that neuronal neuronal differentiation is considerably faster (less than 3 days) than reported for other neuronal progenitor or stem cell lines such as N-tera2 (Schwartz et al., 2005) or PC12 (Greene and Tischler, 1976) which need several weeks to differentiate. For the temporal analysis Hu C/D+ cells were quantified. As a neuronal RNA-binding protein (Graus et al., 1987; Graus and Ferrer, 1990), Hu C/D is a marker expressed in neurons prior to β III-tubulin which is present from 12 hours onward. In ReNcell VM, the neuronal percentage of 4-5 % corresponds to the physiological condition of the midbrain being a part of the brain area of basal ganglia, diencephalon and brainstem in which a neuron/glia-ratio of 1/11.35 was reported (Azevedo et al., 2009). Further quantification in ReNcell VM showed that 5-10 % of the overall neuronal population were TH+ dopaminergic (DA) neurons which in this case was 0.25 % of the total cell number after 72 hours. The temporal quantification revealed that TH expression started approximately one day after the first appearance of neurons. This corresponds to the *in vivo* situation of DA neurogenesis in the developing midbrain where progenitor cells of the ventricular zone floor plate are restricted to the DA lineage first (Nurr1+ precursors), followed by transition into TH+/Nurr1+ DA neurons: a process that is directly influenced by canonical and non-canonical Wnt-signaling pathways in vivo (for review see (Castelo-Branco and Arenas, 2006)).

The quantitative and temporal description indicated that the human cell line ReNcell VM differentiates very fast compared to other cell lines and with a high degree of reproducibility. Therefore, this cell line is a feasible model to investigate the temporal progression of events involved in Wnt/ β -catenin signaling and its role during neuronal differentiation. At the onset of this work, this was to our knowledge not investigated in a human model system and the

temporal and spatial aspects of cytoplasmic-nuclear β -catenin translocation during neuronal differentiation have not been described until now.

4.2 Wnt/β-catenin signaling is controlled in a spatio-temporal manner in differentiating ReNcell VM

Upon initiation of differentiation ReNcell VM exhibited increased levels of both total and dephosphorylated, active β -catenin which was quantified by immunoblotting. Additionally, the cells reacted at the membrane level to external stimuli such as recombinant Wnt3a. This was presented by our group showing an activation of LRP-co-receptor and Dvl and by increased endogenous TCF-dependent gene transcription revealed by TOP/FLASH assay (personal communication Mazemondet et al. (2010)). This demonstrated that differentiating ReNcell VM exhibited endogenous β -catenin-related signaling and were able to react to external stimuli. The advantage of the image cytometry approach to investigate β -catenin-regulation in a spatiotemporal and cell type-dependent manner which is described in this thesis is obvious when using a progenitor cell line which differentiates in different directions, namely glia and neuronal cells. More sophisticated and complex approaches of sub-cellular quantification were described by Glory and Murphy (2007) who used an image analysis-based method for the determination of intracellular location of fluorescent-labeled molecules within cells using high-end MATLAB programming. In comparison to this the use of the Imaris software is accessible to a wider group of users due to its highly intuitive and automatic 3D-rendering system. The results of this easyto-use and quantitative approach revealed that nuclear β -catenin levels increased more than two-fold within 72 hours. This indicated a controlled nuclear β -catenin import. These findings were validated by SDS-PAGE and quantitative Western blotting of nuclear extracts which also exhibited a two-fold increase after three days and a similar temporal incline (Mazemondet et al., 2010), which shows that the 3D-image quantification approach is a suitable means to quantify sub-cellular protein localization. However, using this approach it was also possible to compare protein localization between several cell types throughout the differentiation process which is not feasible using standard biochemical techniques.

4.3 Wnt/β-catenin signaling is differently regulated between neurons and non-neurons

The comparison of nuclear β -catenin between neurons and non-neurons unexpectedly revealed two major time windows: (i) between 6 and 12 hours of differentiation fluorescence signals were significantly elevated in the earliest, immature neurons (Hu C/D+/ β III-tubulin– cells between 6-12 hours termed as 'immature neurons'), (ii) which was reversed after 24 hours and even decreased below the level of non-neuronal cells during the following days. In the time window between 6-12 hours the initial increase was even more prominent with active (dephosphorylated) nuclear β -catenin which indicated that this form is preferentially imported into neuronal nuclei. Consequently, this led to the conclusion that Wnt/ β -catenin-signaling (indicated by nuclear β -catenin import) is more active in this immature neuronal population which appears during this critical time window of 6-12 hours, too. Hu C/D is one of the first post-mitotic marker proteins in neurons (Graus et al., 1987; Graus and Ferrer, 1990). Therefore, Hu C/D+/ β IIItubulin– neurons between 6 and 12 hours represent the population of cells which just finished

75

their neuronal commitment. Taken together, this new type of quantitative time- and cell typeresolved data argue for a regulated mechanism controlling nuclear β -catenin import during neuronal differentiation and suggest a time-dependent activation of Wnt/ β -catenin signaling during ReNcell VM differentiation. Thus, nucleo-cytoplasmic kinetics of β -catenin shuttling might be differently controlled during the early stage of differentiation. This suggests that Wnt/ β -catenin signaling plays a role during the cell fate decision process in the human neural progenitor cell line ReNcell VM. To address this question, quantitative life-cell imaging was performed to investigate the cytoplasmic/nuclear shuttling kinetics of β -catenin.

4.4 The kinetics of nuclear β -catenin import are regulated in a time-dependent manner during ReNcell VM differentiation.

The rate of fluorescence recovery after photobleaching (FRAP) was used as a parameter to interpret the velocity of nuclear β -catenin import. These measurements showed that the import kinetics increased significantly after 3-5 hours of differentiation compared to proliferating cells and decreased again after 24 hours of differentiation. This is in agreement with the results from the comparative analysis of nuclear β -catenin levels discussed above. Since the first neurons appeared during the first 12 hours already this time window seems to play a significant role during differentiation-related Wnt/ β -catenin signaling. This indicates that the extent of Wnt/ β -catenin signaling is differently regulated between neurons and non-neurons during the first 24 hours, partly through the mechanism of nuclear β -catenin import which was presented in this thesis for the first time in a quantitative manner during differentiation of neural progenitor cells. To closer elucidate this mechanism in a cell type-dependent FRAP-approach, labeling with extracellular marker protein such as the neuronal marker PSA-NCAM is highly recommendable for further investigations in the future.

4.5 Neurogenesis in ReNcell VM is connected to the activity of Wnt/β-catenin signaling

Upstream activation of Wnt/ β -catenin signaling with Wnt3a revealed a significant increase in nuclear β -catenin and a subsequent increase in the percentage of neurons which indicated a direct relationship between signaling activity and neuronal differentiation. This is in agreement with findings of Castelo-Branco et al. (2003) who treated primary midbrain cultures with Wnt1 leading to a three-fold increase in the neuronal number. Interestingly, immature ReNcell VM neurons between 6 and 12 hours reacted significantly stronger on the activation compared to non-neurons (glia, undifferentiated cells). This correlates with the temporal control observed by FRAP discussed above and supports the hypothesis that the degree of Wnt/β -catenin signaling is an important mediator for neuronal differentiation in ReNcell VM especially in this early time window. Treatment with SB216763, a small molecule reacting as antagonist at the ATP-binding site of GSK3 β (Coghlan et al., 2000), promoted both nuclear β -catenin accumulation as well as neurogenesis, and also induced a stronger response in neurons. However, the overall effect was smaller than in Wnt3a-treated cells. This suggests that ReNcell VM neurons exhibit a different Wnt-receptor constitution compared to non-neurons because the specific downstream reaction $(\beta$ -catenin accumulation) is stronger after Wnt3a-induced activation. In contrast, SB216763 activated the pathway receptor-independently which led to a similar response between neurons and non-neurons. The constant decline of nuclear β -catenin after the initial peak might be due to a temporally limited Wnt-excitability of the cells which can also be related to the receptor composition.

Together, this suggests that neurogenesis in ReNcell VM is controlled by a different receptor constitution between neurons and non-neurons. This is plausible because, *in vivo*, the neuronal differentiation of midbrain neurons is controlled by both β -catenin-dependent and independent Wnt pathways in a time-dependent manner. It has been described that Wnt/ β -catenin signaling is replaced by β -catenin independent Wnt signaling (Wnt5a) which rather contribute to morphological maturation after the decision process (Castelo-Branco et al., 2003; Andersson et al., 2008), a process which requires a change in the receptor constitution. In the embryonic mouse midbrain Wnt-Fz-receptor expression is differentially controlled during embryogenesis. For example Fz9 is only found in DA progenitor cells but not in mature DA neurons (Rawal et al., 2006). This is in accordance with data from our group showing that in ReNcell VM the expression of endogenous Wnt5a and Wnt-receptors increased significantly during the first 12-24 hours of differentiation (Mazemondet et al., 2010; Rohn, 2009). Taken together, these data provide evidence for a mechanism which determines the gradual response of ReNcell VM to the degree of Wnt/ β -catenin signaling activity in a temporal and cell type-dependent manner.

In addition the reverse experiment was performed by inhibiting Wnt/ β -catenin signaling using its specific antagonist Dickkopf (Dkk-1) as well as the Wnt-inhibitory factor WIF-I. Both inhibitors reduced the amount of nuclear β -catenin and the percentage of neurons significantly. Interestingly, the effect of both inhibitors on nuclear β -catenin was limited to the first 6-9 hours which again highlights the importance of this time window for neuronal differentiation in ReNcell VM. WIF-I was more potent in terms of inhibiting neurogenesis compared to Dkk-1, although both proteins showed a reduction in the same order of magnitude. The different efficacies can be explained by the specificity of WIF-I being an inhibitor for multiple Wnt-ligands, which rises the question whether β -catenin-independent Wnt-pathways also influence neuronal differentiation of ReNcell VM. However, WIF-I decreased nuclear β -catenin levels which, therefore, reflects the specific inhibition of the Wnt/ β -catenin pathway.

The early difference of nuclear β -catenin observed between neurons and non-neurons was altered by WIF-I only at the 6 hours-time point. Nonetheless, compared to the strong neuronal response after Wnt-pathway activation, the inhibitory effects of both inhibitors were considerably weaker and not active over the whole time of investigation. The reason for this weaker response to the inhibitory factors might be due to the fact that inhibition by WIF-I and Dkk-1 decreased the amount of nuclear β -catenin significantly already in all cells during the first 9 hours. This decrease occurred within the time window in which Hu C/D+ neurons showed higher levels of nuclear β -catenin compared to non-neuronal cells. It is, therefore, obvious that the concentration in nuclei with already low β -catenin content cannot be decreased much further by inhibitory factors. For illustration please see suppl. S5 showing that nuclear β -catenin levels were decreased already 30 % below the basal level of proliferating cells when treated with WIF-I or Dkk-1. This is consistent with our suggestion that the transition from a restricted (= committed) neuronal progenitor cell to a neuron is controlled by the degree of Wnt/ β -catenin activity.

Interestingly, a recent publication from Cajánek et al. (2009) showed that continuous Dkk-1 treatment increased neuronal and DA differentiation in mouse embryonic stem cells (mESCs).

These cells differentiated under the influence of Sonic Hedgehog (Shh) and Fibroblast growth factor 8 (Fgf8) which are both sufficient to induce DA neurogenesis without the influence of Wnt-signaling (Hynes et al., 1995; Stull and Iacovitti, 2001; Kim et al., 2003). Nonetheless, other recent findings by Joksimovic et al. (2009) indicated that the temporal interplay of Shh and Wnt-signaling is highly important for induction and proliferation of DA progenitors in embryonic midbrain cultures. These examples agree with other evidences in the literature suggesting both positive and negative effects of the Wnt/ β -catenin signaling in neural specification of mESCs (Aubert et al., 2002; Otero et al., 2004; Verani et al., 2007). Nevertheless, this clearly shows that the temporal interplay between Wnt/ β -catenin activation and its inhibition at the right moment is crucial for proper neurogenesis, at least in rodent midbrain cultures.

In total, the data from FRAP and signaling activation or inhibition presented in this thesis show for the first time that Wnt/β -catenin signaling influences neuronal differentiation of ReNcell VM progenitor cells and especially the subpopulation of immature neurons during the time window of the first 6-12 hours.

With the knowledge about the relationship of Wnt/β -catenin signaling and neuronal differentiation in ReNcell VM, the small population of TH+ DA neurons was investigated. The selective analysis of TH+ DA neurons in ReNcell VM supported the relationship between activity of Wnt/β -catenin signaling and neurogenesis. Interestingly, the effect on Wnt3a-mediated nuclear β -catenin accumulation in DA neurons was time-shifted into the later time window of 24-72 hours which suggests that Wnt/β -catenin signaling is more active at later stages during DA differentiation. This time shifted effect is plausible because DA differentiation starts later, too. Nonetheless, the first TH+ neurons did not show elevated nuclear β -catenin levels which supports the suggestion that Wnt/β -catenin signaling is also active at later stages of DA differentiation. Unfortunately, the lack of suitable markers for DA precursors such as Nurr1, Pitx3 etc. (which were described for DA neurogenesis in vivo (Teitelman et al., 1983; Wallén et al., 1999; Zetterström et al., 1996; Chen et al., 1998a; Puelles et al., 2003; Andersson et al., 2006)) complicated a differentiation stage-dependent analysis of nuclear β -catenin levels and allowed only the investigation of mature TH+ cells. Also, due to the fact that the yield of DA neurons in ReNcell VM was too low to analyze enough cells for statistical certainty. Here, the need for improved differentiation protocols is evident. Many assays have been described that promote DA neurogenesis in vitro which have the potential to increase DA neurogenesis in primary cells or cell lines, which are partly Wnt-signaling-related, too (for review see Castelo-Branco and Arenas (2006) or (Schwartz et al., 2005; Donato et al., 2007; Sousa et al., 2009; Tønnesen et al., 2010)) and several other stem cell models for DA neurogenesis have been described in the literature (see reviews by Lindvall et al. (2004); Lindvall and Kokaia (2010)). Due to these circumstances and to further elucidate the process of spatio-temporal β -catenin redistribution during DA differentiation we turned to the *in vivo* model of the embryonic mouse brain as discussed in section 4.7. Before turning to the *in vivo* data, it is necessary to discuss the influence of Wnt/ β -catenin signaling on the restricted neuronal progenitor cells which will be also discussed in respect to the *in vivo* data below.

4.6 Wnt/ β -catenin signaling controls proliferation in ReNcell VM

As already mentioned, the data presented in this thesis give rise to hypothesize that in ReNcell VM active Wnt/ β -catenin signaling increase the pool of cells during their commitment phase (restricted neuronal progenitor cells) which is followed by an increased neuronal percentage after 72 hours. Both activation and inhibition of Wnt/ β -catenin signaling indicated that differentiating ReNcell VM were mainly influenced during the early commitment phase (3-12 hours) by the degree of signaling activity which was indicated by the amount of nuclear β -catenin. In the developing mouse midbrain (which is the closest model to ReNcell VM) Wnt/ β -catenin signaling leads to increased proliferation of DA precursors and, therefore, more DA neurons but also more neurons in general (Castelo-Branco et al., 2003, 2004).

In ReNcell VM we empirically found that some cells still undergo proliferation during the first hours of differentiation. Recent flow cytometric data from our group showed that the number of cells inside G2/M and S-phase decreased continuously and stopped nearly completely between 12-24 hours of differentiation (personal communication O. Mazemondet et al. (2010) and A. Jaeger et al. (2009)). These indications were supported and expanded by the single cell data presented above. It indicates that cell proliferation overlapped with the time of neuronal and glial commitment. This nicely supports the importance of the first 12 hours for the differentiation process in ReNcell VM as discussed above. Therefore, we hypothesized that besides its influence on neuronal differentiation Wnt/ β -catenin signaling also triggers proliferation of a certain set of cells that are inside the commitment phase. The cell cycle checkpoint G1/G0 seems to be affected by this process. Re-entering G1/S and, hence, a new cell cycle would increase the cell number – also if is committed already. In contrast, the cell cycle exit at G1/G0 would trigger the differentiation process and stop of proliferation.

Signaling activation with SB216763 led to a significantly increased metabolic activity which was measured by MTT-test. Usually used as a cytotoxicity test, the signal intensity can be correlated with the number of cells. Hence, activation of Wnt/ β -catenin signaling with SB216763 directly stimulated proliferation of ReNcell VM. Nonetheless, a complete inhibition of GSK3 β with more than 50 μ M of SB216763 led to a significant decrease of the number of cells indicating the essential role of GSK3 β in numerous other pathways and mechanisms including cellular homeostasis described in the literature (Rayasam et al., 2009; Sun et al., 2009).

The mechanism involved in this increase in the cell number was investigated by the EdUincorporation assay. The data provide evidence that activation of Wnt/ β -catenin signaling elevated the number of cells re-entering the cell cycle at G1/S from the beginning of differentiation. This was indicated by a higher number and prolongated presence of EdU+ S-phase cells. Combined, these data argue for a strong influence of Wnt/ β -catenin signaling on the proliferating cell population during the first 12 hours. The increased cell number at later stages (24-72 hours) observed in the MTT-assay was, therefore, due to the circumstance that activation of Wnt/ β -catenin signaling increased the number of cells re-entering the cell cycle directly after initiation of differentiation.

This indicates that the endogenous activity of Wnt/β -catenin signaling effects the cell proliferation of neuronal progenitor cells using a mechanism of cell cycle re-entry. Taken this into account, and including the fact that a signaling activation increased the percentage of neurons after 72 hours, it can be hypothesized that a certain sub-population of restricted neuronal progenitor cells (i) still proliferated and if so (ii) was selectively being kept in S-phase when treated with Wnt3a or SB216763. This suggestion is also in agreement with the finding that immature Hu C/D+ neurons between 6 and 12 hours showed the strongest response on the activation of Wnt/ β -catenin signaling. The suggested mechanism is also plausible because in the embryonic mouse ventral midbrain, activation of canonical Wnt-signaling was shown to increase the number of proliferating DA precursor (Nurr1+) cells (Castelo-Branco et al., 2003).

Restricted neuronal progenitor cells as a target for Wnt/ β -catenin signaling in vitro. The data presented in this work provide evidence that in ReNcell VM a population of proliferating committed neuronal progenitor cells exits. This would, therefore, be comparable to the above mentioned effect in the embryonic VM shown by Castelo-Branco et al. (2003). Due to the fact that the antibody for committed DA precursor cells (Nurr1) was highly unspecific in ReNcell VM, we used the general marker protein PSA-NCAM, a well known marker for neuronal progenitor cells being expressed as early the cells switch to the neuronal lineage (for review see Bonfanti (2006)). Despite the very small number of PSA-NCAM+/EdU+ cells (4 % of all EdU+ cells), their presence suggests that the above-mentioned Wnt/ β -catenin signaling-mediated mechanism is also plausible in ReNcell VM. This is supported by the morphological data presented herein which showed that EdU+/PSA-NCAM+ cells did not show a neuron-like morphology and no β III-tubulin and in contrast, cells with strong polarized neuronal morphology did not show EdU-staining. The latter indicated a morphologically mature post-mitotic stage of neuronal differentiation, which was also supported by the presence of the post-mitotic marker β III-tubulin.

It is known that restricted neuronal progenitor cells *in vivo* are influenced by both canonical (Wnt1) and non-canonical (Wnt5a) signaling. One example is the pool of Nurr1+ DA precursors in the embryonic mouse ventral midbrain (VM). Here the population size is influenced by both Wnt1 and Wnt5a. Castelo-Branco et al. (2003, 2004) convincingly showed in primary embryonic mouse midbrain cultures that the pool of Nurr1+ DA precursors is expanded when treated with Wnt1 in vitro. This observation was supported by the presence of higher β -catenin levels and close proximity to Wnt1-expression in the E10.5 VM in situ. The authors also showed that treatment with Wnt5a also expanded the population of Nurr1+/BrdU+ cells (S-phase) which reflects that this population is effected by both Wnt1 and Wnt5a-signaling. However, Wnt5a was also shown to mediate the transition of DA precursor to DA neurons because it also increased the percentage of DA neurons (Schulte et al., 2005). These findings can be translated into the in vitro situation in human neural progenitor cells such as ReNcell VM as indicated by (i) the presence of a proliferating pool of restricted neuronal progenitors, (ii) a potential stimulating effect of Wnt3a on the PSA-NCAM+/EdU+ cell population, (iii) the increased percentage of neurons after 72 hours. In agreement with the findings from mouse embryonic midbrain cultures, data from our group revealed that the endogenous amount of Wnt5a-mRNA increased during differentiation of ReNcell VM (personal communication O. Mazemondet et al. (2010) and A. Rohn (2009)). Addition of recombinant Wnt5a at the onset of differentiation showed stimulating effects on the size of the Hu C/D+ neuronal population.

Based on these findings it can be concluded that the hypothesis of a proliferating restricted progenitor cell pool that could be increased by Wnt/β -catenin signaling is plausible. Preliminary

data indicated that activation of Wnt/ β -catenin signaling increased the number of EdU+/PSA-NCAM+ cells (data not shown) which is directly in agreement with the *in vivo* situation in the embryonic mouse midbrain (Castelo-Branco et al., 2003). Further studies on the population of EdU+/ β III-tubulin-/PSA-NCAM+ cells will give a more precise insight into the population of proliferating restricted neuronal progenitor cells. In combination with signaling activation, these observations will clarify the mechanism how Wnt/ β -catenin signaling controls the proliferation of the precursor population in the human neural progenitor cell model ReNcell VM. Additionally, quantification of the nuclear β -catenin accumulation in both PSA-NCAM+/EdU- and PSA-NCAM+/EdU+ cells will increase the knowledge about the spatio-temporal control of Wnt/ β -catenin signaling in these populations.

In total, the findings from proliferation-tests, FRAP and signaling activation or inhibition presented in this thesis show for the first time that in ReNcell VM Wnt/β -catenin signaling influences differentiation of the neural progenitor cells and especially the subpopulation committed to become neurons during the time window of the first 6-12 hours. However, the relationship between the early increase of signaling intensity and the downstream activation of target genes remains to be elucidated. Also, the mechanism of how the cells fate is decided is not clear, yet. This requires further cell type- and time-dependent approaches to investigate the molecular mechanism influencing the cell fate decision process. For this, transfection of TCF-GFP constructs and subsequent quantification of nuclear β -catenin in differentiating cells and singlecell quantitative real-time PCR of selected cells represent only two possibilities to elucidate these molecular events. Nonetheless, the extensive time- and cell type-dependent quantification experiments presented herein document the spatio-temporal-dependent events which trigger neurogenesis in human midbrain cultures. In general, these processes seem to be similar to those in the embryonic mouse midbrain. However, the spatio-temporal sequence of the events shown here in vitro remained still to be confirmed in vivo in the mouse model. Also, the low yield of DA neurons in ReNcell VM showed the need to investigate a model in which the *in vitro* findings could be translated into DA neurogenesis. Therefore, we turned to the embryonic mouse midbrain with the goal to investigate the activity of Wnt/β -catenin signaling in a spatial, cell type and differentiation stage-dependent manner: On the one hand to support and confirm the in vitro data, on the other to investigate the process of sub-cellular protein translocation in vivo.

4.7 Wnt/β-catenin signaling is controlled in a differentiation stage-dependent manner in the developing midbrain

The midbrain is the location of DA neurogenesis and, therefore, a suitable system for comparison with the human midbrain progenitor cell model investigated in this thesis. One major advantage of the embryonic mouse ventral midbrain (VM) is the fact that the stem cells of the floor plate differentiate into DA neurons with a high frequency and in a very reproducible manner. In addition, much is known about how Wnt-signaling influences DA neurogenesis in the VM (for review see Castelo-Branco and Arenas (2006)). Since ReNcell VM exhibit a very small percentage of DA neurons (approximately 0.3 % after 72 hours), the embryonic midbrain is a more suitable model to translate the spatio-temporal relationship between Wnt-signaling and neurogenesis obtained from *in vitro* findings into the *in vivo* situation and more importantly

into DA neurogenesis.

We show, that our image cytometry approach could be adapted from *in vitro* cell cultures to stained cryosections representing the *in vivo* situation. In general, tissues exhibit increased unspecific signal intensities compared to cell cultures due to extra-cellular matrix proteins. However, the section thickness of 14 μ m corresponded to approximately one cell layer which reduced the amount of unspecific signals but allowed to quantify β -catenin signals in intact nuclei. The results were obtained at embryonic day (E) 11.5 because here undifferentiated progenitor cells, Nurr1+ DA precursors cells and Nurr1+/TH+ DA neurons are present and represent all major stages of DA differentiation (Castelo-Branco and Arenas, 2006). Therefore, this time point is comparable to the time window of 72 hours observed in ReNcell VM.

The data from nuclear β -catenin quantification indicated that the activity of Wnt/ β -catenin signaling increased during DA neurogenesis in a differentiation stage-dependent manner which we showed for the first time *in vivo*. This was reflected by the levels of nuclear β -catenin which increased from Nurr1– to Nurr1+ to Nurr1+/TH+ cells. This difference was even more prominent with active β -catenin (ABC) in the same sections. One explanation for this is the fact that cytoplasmic β -catenin also plays a role in E-cadherin-mediated cell-cell communication (Moon et al., 2004) which increased the cytoplasmic "background". In contrast, active dephosphory-lated β -catenin is responsible for activating canonical target genes and hence accumulated only in cells that activated Wnt/ β -catenin signaling (Moon et al., 2004) which is in agreement with the observation.

Therefore, these findings correlate with the *in vitro* data which showed that (i) nuclear accumulation of ABC was stronger than total β -catenin, (ii) the nuclear β -catenin import kinetics were elevated in early differentiating progenitor cells, (iii) PSA-NCAM+ restricted neuronal progenitor cells were influenced by manipulation of Wnt-signaling.

These data indicate that Wnt/β -catenin signaling is more active in the population of Nurr1+ DA precursors of the intermediate and marginal zones (IZ, MZ) than in the progenitor cells of the ventricular zone (VZ). This is in agreement with the observation that Wnt1-treatment increases both proliferation and DA differentiation of Nurr1+ DA precursors in mouse embryonic stem cell cultures (Castelo-Branco et al., 2003). Reversely, in Wnt1^{-/-}-knockout mice both DA and β III-tubulin+ neurons are lost in the floor plate (Andersson et al., 2008). Nevertheless, the progenitor cells of the VZ are also expandable by Wnt1-treatment indicating that proliferation of this population is also controlled by Wnt/ β -catenin signaling. Here, it is important to state that Shh-signaling mainly controls the proliferation of this VZ population and also antagonizes Wnt1-signaling (Joksimovic et al., 2009) which indicates that first Shh and then Wnt1 trigger this progenitor cell proliferation, followed by a later trans-activation of Wnt1-signaling which might influence initiation of DA differentiation and maturation. In total the data presented in this thesis substantiate the importance of Wnt/ β -catenin signaling for midbrain (DA) neurogenesis at the level of Nurr1+ precursor cells.

The reason for the stronger β -catenin accumulation and dephosphorylation in FP/VM Nurr1+ precursors was presented by RNA *in situ* hybridization (ISH). To our knowledge, we showed for the first time that Wnt1 directly co-localized with Nurr1+ cells, especially in the rostral part of the VM and mainly in the region called "Nurr1-mountains". Here Nurr1+ cells accumulate due to proliferation during downward-migration into the marginal zone (Castelo-Branco and Arenas, 2006). These findings indicate that Nurr1+ DA precursors themselves express Wnt1 and exhibit autocrine Wnt/ β -catenin signaling which was also shown by nuclear β -catenin accumulation as discussed above. This suggests a combined mechanism of proliferation and differentiation control during maturation of DA precursor cells into mature TH+ DA neurons. This is the first correlation of nuclear β -catenin accumulation and autocrine Wnt1-signaling during embryonic midbrain neurogenesis in a cell type- and differentiation stage-dependent manner. Previous data convincingly showed, that Wnt1 is expressed in the E11.5 midbrain restrictively at the dorsal midline in the roof plate, at the midbrain/hinbrain boundary and at two stripes flanking the ventral midline in the FP (Wilkinson et al., 1987; McMahon and Bradley, 1990; Prakash et al., 2006). To our knowledge, it has not been shown yet, that Wnt1-expression directly overlaps with the population of Nurr1+ cells which is presented herein. The closest finding corresponding to this was demonstrated by Castelo-Branco et al. (2003) who showed in sagittal E10.5 midbrain sections Wnt1-expression in the rostral VM partly overlapping with TH and Nurr1+ cells. However, this was presented with low magnification and not shown in the same sections. Nonetheless, the authors persuasively described that MZ cells exhibited TCF-dependent gene transcription. This observation directly correlates with the quantitative data presented in this thesis which, therefore, complements the picture of Wnt/β -catenin signaling in the developing VM.

Hence, our findings of increased nuclear β -catenin levels in IZ Nurr1+ precursors revealed that this population exhibits stronger Wnt/ β -catenin signaling than VZ progenitor cells, partly due to autocrine Wnt1-signaling. It also indicates that the activity of Wnt/ β -catenin signaling might control the number of DA progenitors which start to express Nurr1+ DA precursors by controlling their proliferation and, thus, establishes the basis for maturation of DA neurons (which is controlled by non-canonical Wnt5a-signaling). However, once the cells start to express Nurr1+ they exit the cell cycle so that the Nurr1- population is the one influenced by the proproliferative effects of Wnt/ β -catenin signaling (also see Castelo-Branco and Arenas (2006)). Translating this into the human ReNcell VM model, the suggestion as introduced above that the pool of PSA-NCAM+ restricted neuronal progenitor cells is the critical population influenced by Wnt/ β -catenin signaling is plausible: increased proliferation of these cells leads to an increased percentage of neurons which was observed after Wnt3a-treatment.

Unexpectedly, TH+ DA neurons exhibited even higher nuclear β -catenin levels than Nurr1+ precursors. It is known that canonical Wnt1-signaling is active in the Nurr1+ precursors but not in mature TH+ DA neurons. During the transition from Nurr+/TH– DA precursors to Nurr+/TH+ DA neurons non-canonical activity of Wnt5a-signaling increases and Wnt1signaling decreases (Castelo-Branco and Arenas, 2006; Andersson et al., 2008). However, our results indicated that the amount of nuclear β -catenin also increased steadily during DA maturation reaching a maximum in Nurr1+/TH+ DA neurons. To increase the resolution in terms of the differentiation stage, all possible combinations of Nurr1 and TH-positive or negative cells were compared with each other and confirmed that the amount of nuclear β -catenin increased in a differentiation stage-dependent manner (compare Fig. 35).

This spatio-temporal and differentiation stage-dependent redistribution of β -catenin has, to our best knowledge, not been described yet and suggests two important roles for Wnt/ β -catenin



Figure 37: Multiple roles for Wnt/ β -catenin signaling during neuronal and DA differentiation: (A) In the human neural progenitor cell line ReNcell VM, active Wnt/ β -catenin signaling promotes neuronal differentiation indicated by increased nuclear β -catenin levels in early stages of differentiation, especially present in immature neurons between 0 and 12 hours and by elevated neuronal percentage after 72 hours. One putative mechanism is suggested by the stimulating effect on cell proliferation during the early stage between 0 and 12 hours. Here, committed neuronal progenitor cells present a possible group of cells which selectively re-enter the cell cycle due to Wnt-signals. In DA neurons nuclear β -catenin levels are increased at later stages (48-72 hours) suggesting a Wnt-signaling-mediated trans-activation which is important for maturation, migration and survival of DA neurons. However, extrinsic Wnt-activation leads to increased DA neurogenesis, too, which is in agreement with the situation in the embryonic midbrain *in vivo*. (B) During DA differentiation *in vivo* nuclear β -catenin signaling: enhanced proliferation stage-dependent manner which indicates a dual role for Wnt/ β -catenin signaling: enhanced proliferation of Nurr1+ DA precursors and a later trans-activation required for maturation, migration and survival. Hence, both *in vivo* and *in vitro* systems support each other in terms of spatio-temporal control of Wnt/ β -catenin signaling and expand our present understanding of its role during DA neurogenesis.

signaling during DA differentiation and maturation. On the one hand, it influences the proliferation of (Nurr1+) restricted precursor cells which supports the observations in ReNcell VM described above (PSA-NCAM+/EdU+ cells). On the other hand, a divergent Wnt/β -catenin pathway could guide TH+ DA neurons during their migration and further maturation. In the literature, these processes have not been associated with DA neurogenesis, yet. Nonetheless, it is known that Wnt/β -catenin triggers axon remodeling, synaptic differentiation and morphologic maturation in general (Hall et al., 2000) and that many components of this signaling pathway are closely associated with the microtuble cytoskeleton (for review see Salinas (2007)). Therefore, the loss of VM neurons in knockout mice with diminished Wnt/β -catenin signaling, might also be mediated by a disturbed morphological maturation and migration of TH+ DA neurons of the FP/VM. Nonetheless, this loss is mainly due to the lack of progenitor cell proliferation. This hypothesis about the later canonical Wnt-mediated trans-activation remains still to be elucidated e.g. in heterozygous knockout mouse models such as $Wnt1^{+/-}$, expecting a decreased nuclear β -catenin accumulation in both Nurr1+ and TH+ cells accompanied by altered cellular morphology and migration. The overall increase in the region of Nurr1+/TH+ cells (and not only in the nuclei) arises the possibility of a combined nucleo-cytoplasmic mechanism, mediating both cytoskeleton rearrangements and TCF/Lef-dependent gene transcription. In addition, the analysis of GFP-TCF reporter mice in the FP/VM will provide evidence whether the nuclear β -catenin accumulation in TH+ neurons induces TCF/Lef-dependent gene-transcription.

These in vivo observations are highly consistent with the in vitro findings in terms of spatiotemporal kinetics of β -catenin distribution which are presented in this thesis. Similarly to the observation in DA neurons in ReNcell VM, mouse midbrain DA neurons also exhibit increased accumulation of nuclear β -catenin at later differentiation stages. These concordant findings between the mouse model and human neural progenitor cells are summarized in Fig. 37 and suggest that the role of Wnt/ β -catenin signaling during DA neurogenesis has to be extended – away from proliferation-control towards later trans-activation effects on DA maturation.

Therefore, our findings from FRAP, signaling activation or inhibition and cell cycle analyses in ReNcell VM are supported by the well investigated mouse model. In this thesis, it is shown for the first time in a quantitative manner that Wnt/β -catenin signaling influences the neuronal differentiation of the human neural progenitor cells ReNcell VM and especially the subpopulation committed to become neurons during the time window of the first 6-12 hours. The presented spatio-temporal data are presently used to improve and validate/falsify mathematical models to simulate aspects of the Wnt/β -catenin pathway with the focus on nucleo-cytoplasmic shuttling of β -catenin – a process which has opened new questions and directions of research already and exemplifies the modern synergy of quantitative screening approaches (as presented herein) and system biology (Schmitz et al., 2008). Nonetheless, the relationship between the increased signaling intensity in the early time window and the downstream activation of target genes remains to be elucidated. This requires further cell type- and time-dependent approaches to investigate the molecular mechanism influencing the cell fate decision process. Here, the use of TCF-GFP constructs and single cell analyses with cell type-specific markers (neuron, glia, progenitor) will provide a closer insight at what time of differentiation and in which cell-types TCF/Lef-dependent gene transcription is active. Followed by single cell real-time PCR, the downstream target genes can be monitored cell type-specifically.

5 Conclusions

The extensive time- and cell type-dependent quantification experiments presented herein document the spatio-temporal-dependent events which trigger neurogenesis in human midbrain cultures. In general these processes are similar to those in the embryonic mouse midbrain which also were pioneered in this work in a quantitative cell type-dependent manner. This study substantiates the presently limited knowledge about neurogenesis in human cell models and closes the gap between the mechanistic knowledge based on rodent models and the use of human neural stem cells for cell replacement strategies in neuro-degenerative diseases. It, therefore, provides information which potentially will be beneficial for improved therapies for Parkinson's Disease in the future.

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Curriculum vitae and list of publications

Personal Information	
Name:	Benjamin Bader
Date & Place of Birth:	18.11.1981 in Hagenow, Germany
Education	
Jan 2007 - Mar 2010	PhD thesis in the DFG research training school dIEM oSiRiS, in
	the lab of Prof. Dr. Dieter G. Weiss, University of Rostock
Oct 2000 - Aug 2006	Studies of Biology and Diploma at the University of Rostock
Sep 1992 - June 2000	High School "Robert-Stock" in Hagenow, Abitur
Sep 1988 - Aug 1992	Comprehensive School "POS II" in Hagenow, Germany
Practical Experience	
since April 2010	NeuroProof GmbH, Rostock-Warnemünde, Germany
Jan 2007 - March 2010	Member of the group of Prof. Dr. Dieter G. Weiss, University of
	Rostock, Germany
May 2009 - Jul 2009 and	research visits in lab of Prof. Dr. Ernest Arenas, Molecular Neu-
Mar 2010	robiology, MBB, Karolinska Institute, Stockholm, Sweden
Aug 2006 - Dec 2006	scientific assistant in group of Prof. Dr. Mats-Olof Mattsson
	(DGF "mercator" guest professor at Institute of Biological Sci-
	ences, University of Rostock, Rostock, Germany)
Sept 2005 - Aug 2006	Diploma thesis: "The role of Myosin-Va in organelle positioning
	in dendritic spines of primary cortical neurons in vitro"
Feb 2005	training on stem cell culture techniques at ReNeuron Inc., Guild-
	ford, England

Publications

- <u>Bader, B. M.</u>, Lemcke, H. and Weiss, D.G. (2010) Wnt/β-catenin signaling controls expansion of restricted neuronal progenitor cell during differentiation of human neural progenitor cells. (manuscript in preparation).
- Mazemondet, O., Hubner, R., Frahm, J., Koczan, D., <u>Bader, B. M.</u>, Weiss, D. G., Uhrmacher, A. M., Frech, M., Rolfs, A., Luo, J. (2010) Quantitative and kinetic profile of Wnt/β-catenin signaling components during human neural progenitor cell differentiation (submitted).
- <u>Bader</u>, B. M., Kuznetsov, S. A., Rolfs, A. and Weiss, D. G. (2010) Differentiation of human neural progenitor cells is connected to Wnt/beta-catenin signaling in a spatio-temporal manner (submitted).
- Uhrmacher, A. M., John, M., Mazemondet, O., Unger, A., Rharass, T., <u>Bader, B. M.</u> and Rolfs, A. (2009) Computer Science Meets Cell Biology - GRK dIEM oSiRiS - University of Rostock, *Rostocker Informatik-Berichte* (32).

Conference talks

- 1. Annual meeting of German Society for Cytometry (2009) in Leipzig, Germany, oral presentation chosen from abstracts: Quantitative image cytometry: nuclear beta-catenin shuttling during differentiation of human and mouse midbrain progenitor cells.
- 2. Baltic Winter School (2008) in Binz/Rügen, Germany, oral presentation chosen from abstracts: Systems biology of neural differentiation.
- 3. 5th International Stem Cell School (2008) in Berlin, Germany: Neuronal progenitor cell fate: following Wnt-pathway components during glial and neuronal differentiation.
- Annual meeting of German Society for Cytometry (2008) in Bremen, Germany, oral presentation chosen from abstracts: Signal protein translocation in differentiating human neural progenitor cells: 3D image quantification for a systems biology approach.

Teaching, activities, memberships

- Responsible for visitor program for research seminars of the DFG PhD training school dIEM oSiRiS: i.e. Prof. Dr. Robert Murphy, Carnegie Mellon University, Pittsburgh, USA, July 2007; Prof. Dr. Jürgen Behrens, University of Nürnberg, August 2007
- Supervisor for diploma/masters thesis: "The influence of Wnt-signaling on the differentiation of embryonic neural progenitor cells" by Alexandra Rohn, University of Rostock 2009, 9 months, written in german)
- Tutor for major student lab internships (6 x 5 weeks, 4 x 10 weeks) in cell biology and cell physiology, University of Rostock, 2005, 06, 07, 08, 09
- supervisor for annual 2 weeks light microscopy students course, Light Microscopy Center of the University of Rostock, 2004, 05, 06, 07, 08, 09
- Society member of Deutsche Gesellschaft für Zellbiologie (**DGZ**) (German Society for Cell Biology), Gesellschaft für Biochemie und Molekularbiologie e.V. (**GBM**) (German Society for Biochemistry and Molecular Biology) and International Society for Advancement of Cytometry (**ISAC**)

Abstracts, Conference contributions, Posters

 <u>Bader, B. M.</u>, Arenas, E. and Weiss, D. G. (2009) Quantitative image cytometry: nuclear β-catenin shuttling during differentiation of human and mouse midbrain progenitor cells. In: Martin Schlegel, Elmar Endl, S. M. C. S. (Ed.), Proceedings: 19th Annual Conference of the German Society for Cytometry. No. 19th in Annual Conference of the German Society for Cytometry. DGfZ, Leipzig, Germany, p. p18.

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- <u>Bader, B. M.</u> and Weiss, D. G. (2009). Nuclear beta-catenin import during differentiation of neural midbrain progenitor cells. Proceedings of EMBO Workshop: Wnt Signaling in Development and Disease August, Arolla, Switzerland.
- Schmitz, Y.*, <u>Bader, B. M.</u>*, Weiss, D. G., Wolkenhauer, O. and Rateitschak, K. (2008) Systems biology of neural differentiation. Abstract book: Baltic Winter School, Binz/Rügen, Germany.
- 4. <u>Bader, B. M.</u>, Redlich, B., and Weiss, D. G. (2008). Neuronal progenitor cell fate: following Wntpathway components during glial and neuronal differentiation. Proceedings of 5th international stem cell school on regenerative medicine, Berlin, Germany.
- Schmitz, Y., <u>Bader, B. M.</u>, Weiss, D. G., Wolkenhauer, O. and Rateitschak, K. (2008). Elucidating the role of nucleo-cytoplasmic shuttling of β-catenin antagonists by mathematical modelling. Summer School on Systems Biology for Medical Applications, Tenerife, Spain.
- Bader, B. M., Redlich, B., Weiss, D. G. (2008). Quantitative 3D analysis and evaluation of confocal images for the study of translocation of Wnt-signalling molecules in human neural progenitor cells. In: *European Journal of Cell Biology*. Abstracts from Annual Meeting of The German Society for Cell Biology, Marburg, Germany; Volume 87S1. Wiley InterScience, p. 18, ISSN 0171-9335
- 7. <u>Bader, B. M.</u> and Weiss, D. G. (2008) Signal protein translocation in differentiating human neural progenitor cells: 3D image quantification for a systems biology approach. In: DGfZ/ESCCA joint abstracts from The 18th Annual Meeting of The German Society of Cytometry (DGfZ) and The 8th Euroconference of Clinical Cell Analysis by The European Society for Clinical Cell Analysis (ESCCA), Bremen, Germany. Vol. 74B of *Cytometry Part B: Clinical Cytometry*. Wiley InterScience, p. p383, DOI: 10.1002/cyto.b.20462
- Bader, B. M., Schmitz, Y., Redlich, B., Rateitschak, K., Wolkenhauer, O. and Weiss, D. G. (2008). Quantitative 3D image analysis to study the beta-catenin translocation during differentiation of human neural progenitor cells. Abstract book: 2nd International Congress on Stem Cells and Tissue Formation, p218, Dresden, Germany.
- Jaeger, A., <u>Bader, B. M.</u>, Weiss, D. and Kriehuber, R. (2008) Apoptosis in the human neural progenitor VM197 cell line - a life or death decision during the early differentiation process. Abstract book: 2nd International Congress on Stem Cells and Tissue Formation 2008, p251, Dresden, Germany.
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- <u>Bader, B. M.</u>, Weiss, D. G. (2007) The role of Wnt-pathway mediated changes in the cytoskeleton/motorenzyme-system upon differentiation of neural progenitor cells. In: *Zehn plus eins*. Dagstuhl, Aachen: Verlag Mainz, pp. 161-162, ISBN: 3-86130-882-7
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Appendix / Supplement

S1: Video - 72 hours long-term live-cell-microscopy: morphologic changes.

Video to Fig. 6 on page 36: Long term microscopy of differentiating ReNcell VM: first morphological changes occur within the first 24 hours after initiation of differentiation, cell branching and formation of long protrusions begin between 36 and 48 hours, between 48 and 72 hours a stable meshwork of cell branches is formed:

www.biologie.uni-rostock.de/tierphysiologie/Downloads/Bader-Dissertation-2010-S1.avi (378 Mb) or CD

S2: Video - 72 hours long-term live-cell-microscopy: mitotic events.

Video to Fig. 26 on page 58: ReNcell VM stop proliferation 12 hours after initiation of differentiation: Mitotic event in phase contrast; time between images = 5 minutes; images taken from 72 hours time series; Quantification of these mitotic events shows a sharp decline in the number of dividing cells between 12-15 hours and a nearly complete stop of proliferation within the first 24 hours of differentiation:

www.biologie.uni-rostock.de/tierphysiologie/Downloads/Bader-Dissertation-2010-S2.avi (69 Mb) or CD

S3: Nuclear β -catenin in ReNcell VM neurons and non-neurons.



Figure 38: Nuclear β -catenin in neurons and non-neurons under control conditions. Data representation of Fig. 15C page 46 when normalized to t=0.



S4: Nuclear β -catenin in neurons and non-neurons treated with Wnt3a & SB216763.

Figure 39: Nuclear β -catenin in untreated cells, treated neurons and treated non-neurons. Data representation of Fig. 17 page 48 when normalized to t=0.

S5: Nuclear β -catenin in neurons and non-neurons treated with WIF-I & Dkk-1.



Figure 40: Nuclear β -catenin in untreated cells, treated neurons and treated non-neurons. Data representation of Fig. 24 page 54 when normalized to t=0.