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WNTS IN DEVELOPMENT: FOCUS ON MIDBRAIN

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On the Cover:
On the cover is a picture of a ventral portion of the developing mouse midbrain. The ventral midbrain contains dopaminergic neurons that express tyrosine hydoxylase protein. Detection of tyrosine hydroxylase with fluorescent antibodies (in green) reveals the presence of dopaminergic neurons. Developing dopaminergic precursors express Nurr1 (in red, in the nucleus) as they begin to mature, and when they are mature (green) TH^+ neurons. This thesis has, among other subjects, studied the role of *Wnt5a* (in blue) in the development of dopaminergic neurons. As one can see, cells express *Wnt5a* before becoming $Nurr1^+$ or TH^+, but some $Nurr1^+$ and TH^+ cells also express *Wnt5a*.

"…perhaps the most important principle of all was stated by Viktor Hamburger, who affirmed that "Our real teacher has been and still is the embryo—who is, incidentally, the only teacher who is always right" (…) Anyone who gets past gastrulation and middle school must be respected as a survivor."

-All I Really Needed to Know I Learned during Gastrulation, Scott F. Gilbert (Gilbert, 2008)

ABSTRACT

Studies in the field of embryonic development reveal the temporal and spatial requirement of specific genes/proteins in the formation of a given tissue. The specific knowledge of which genes/proteins regulate development of the ventral midbrain (VM) provides a guideline for the *in vitro* differentiation of specific cell types therein, such as dopaminergic (DA) neurons. Large-scale *in vitro* production of DA neurons is a prerequisite for cell replacement therapies in Parkinson's disease (PD, a neurodegenerative disease in which DA neurons are lost). Ideally, this differentiation could be accomplished by direct application of extracellular proteins. One family of signaling proteins, which may fill such a function, is the Wnt family of proteins.

Previous results from our laboratory demonstrated a role for Wnt1 and Wnt5a in the development of DA neurons *in vitro*, while multiple other laboratories had shown that Wnt1 was required for midbrain development *in vivo*.

In this thesis work, we investigated signaling through Wnt5a and LRP6 (a Wnt receptor thought to bind Wnt1/Wnt3a, but not Wnt5a), as well as the roles of Wnt1, Wnt5a, LRP6, sFRP1 and sFRP2 (secreted proteins which reportedly bind Wnts to antagonize their function) in the development of the midbrain and DA neurons.

The extracellular domain of LRP6 bound Wnt5a, and inhibited Wnt5a or Wnt11 signaling. *LRP6* loss-of-function (LOF) studies, in mouse and *Xenopus,* revealed typical convergent extension (CE) defects which were rescued by *Wnt5a* or *Wnt11* LOF. Conversely, *Wnt5a* or *Wnt11* gain-of-function (GOF) were rescued by *LRP6* GOF. Thus, LRP6 acts as a negative regulator of Wnt5a/Wnt11 signaling *in vivo*. In a DA neuron cell line, overexpression of *LRP6* decreased active Rac1 levels, while Wnt5a activated Rac1 and was required for Wnt5a-induced DA neuron differentiation.

Despite the capacity of Wnt5a to induce DA neurons *in vitro*, *Wnt5a*$^{-/-}$ mice did not display an overall loss of DA neurons. Instead, a transient increase in proliferating progenitors and Nurr1+ (NR4A2+) precursors led to a bottleneck in differentiation, such that the ratio of differentiated cells was lower in *Wnt5a*$^{-/-}$ mice. The *Wnt5a*$^{-/-}$ VM displayed typical convergent extension (CE) defects, with a flattened medial ventricular zone, and a laterally expanded Shh domain. This CE defect resulted in an anteroposterior shortening and lateral expansion of the DA neuron nucleus. Examination of *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice revealed a phenocopy of this CE defect, but no similarity in effects on proliferation or NR4A2+ cell numbers, indicating that CE and differentiation may be separately regulated. In support of this, neither sFRP1 nor sFRP2 induced DA neurons in primary VM cultures.

The *Wnt1*$^{-/-}$ midbrain displayed a medial loss of floorplate DA markers, including Lmx1a. The DA population was instead bisected and displaced laterally, forming bilateral populations in the basal plate region, with ~10% remaining DA neurons. Haploinsufficiency of *LRP6*, or ablation of *Wnt5a*, in the *Wnt1*$^{-/-}$ background was sufficient to reduce the number of DA neurons by an additional 70% compared to *Wnt1*$^{-/-}$ alone. Thus, *Wnt5a* may partially compensate loss of *Wnt1*, while the mild *Wnt5a*$^{-/-}$ DA phenotype may be a result of robust compensation by *Wnt1*.

Finally, we developed an improved VM neurosphere DA differentiation protocol combining Wnt3a and Wnt5a treatments. Sequential treatment with Wnt3a and Wnt5a robustly increased the number of DA neurons, compared to either Wnt alone.

In sum, the data presented in this thesis describes multiple Wnt ligand and receptor interactions, in regulation of diverse processes such as CE or DA differentiation. Hopefully, these insights into Wnt signaling, especially in the realm of DA neurogenesis, will contribute to improved therapies for PD.

LIST OF PUBLICATIONS

I. Vitezslav Bryja*, Emma R. Andersson*, Alexandra Schambony*, Milan Esner, Lenka Bryjová, Kristin K. Biris, Anita C. Hall, Bianca Kraft, Lukas Cajanek, Terry P. Yamaguchi, Margaret Buckingham and Ernest Arenas, **The extracellular domain of LRP5/6 inhibits non-canonical Wnt signaling *in vivo***, 2009, Molecular Biology of the Cell, (20): 924-936

II. Emma R. Andersson, Nilima Prakash, Lukas Cajanek, Eleonora Minina, Vitezslav Bryja, Lenka Bryjova, Terry P. Yamaguchi, Anita C. Hall, Wolfgang Wurst, and Ernest Arenas, **Wnt5a Regulates Ventral Midbrain Morphogenesis and the Development of A9–A10 DA Cells *In vivo***, 2008, PLoS ONE,(3): e3517

III. Emma R. Andersson*,1, Lenka Brjyjova*, Vitezslav Bryja, Carmen Ramirez, Carmen Salto, and Ernest Arenas, **Canonical and non-canonical Wnt signaling synergize in the generation of DA neurons**, *Manuscript*

IV. Julianna Kele*, Emma R. Andersson*, Vitezslav Bryja, Carlos Villaescusa, Sonia Bonilla, Jeffrey S. Rubin, Akihiko Shimono, and Ernest Arenas, **sFRP1 and sFRP2 in DA neuron development**, *Manuscript*

*denotes equal contribution

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
A/P	Anteroposterior
AP	Alar plate
APC	Adenomatous polyposis coli
BMP	Bone morphogenetic protein
BP	Basal plate
BrdU	Bromodioxyuridine
β-Trcp	beta-transducin repeat containing protein
ca	constitutively active
Ca^{2+}	Calcium
CAMKII	Calcium/calmodulin-dependant protein kinase
cdc42	cell division cycle 42 homolog
CE	Convergent extension
CK	Casein kinase
CNS	Central nervous system
CRD	Cysteine-rich domain
Cre	Cyclization recombinase
Cripto	Cryptic family 1, a.k.a. Cfc1
CRT	Cell replacement therapy
D/V	Dorso-ventral
DA	Dopaminergic
Daam1	Dishevelled associated activator of morphogenesis 1
DAT	Dopamine active transporter
DDR1	Discoidin domain receptor family, member 1
DEP	Dishevelled/EGL-10/Pleckstrin
DIX	Dishevelled-Axin
Dkk	Dickkopf
dn	dominant negative
DNA	Deoxyribonucleic acid
Dvl or Dsh	Dishevelled
E	Embryonic day
EGF	Epidermal growth factor
En	Engrailed
EphB2	Eph receptor B2
ES cell	Embryonic stem cell
Fc	Fragment crystallizable (tail region of antibody)
Fgf8	Fibroblast growth factor 8
flox/floxing	Flanked by LoxP (locus of X-over P1)
Foxa2	Forkhead box A2 , previously known as Hnf3β
FP	Floor plate
FRL1	Fibroblast growth factor receptor ligand 1, Cripto homolog
Fzd or fz	Frizzled (in mammals or *Drosophila*)
Gbx2	Gastrulation brain homeobox 2
GFP	Green fluorescent protein

G_i/G_o	Two types of G proteins
Gli	Glioma associated oncogene homolog
GOF	Gain-of-function
GPC4/6	Glypican 4/6
GPCR	G protein coupled receptors
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
HA	Hemagglutinin
Hh	Hedgehog
ICD	Intracellular domain
ICM	Inner cell mass
IP	Immunoprecipitation
iPSC	induced pluripotent stem cell
IZ	Intermediate zone
JNK	c-jun NH-terminal kinase
Kny	Knypek
Kremen	Kringle containing transmembrane protein 1
L-DOPA	Levodopa
LEF	Lymphocyte enhancing-binding factor
Lmx1a/b	LIM homeobox transcription factor 1 a/b
LOF	Loss-of-function
LRP	Low density lipoprotein receptor-related protein, arrow in *Drosophila*
MAPK	Mitogen-activated protein kinase
Mash1	Mammalian achaete-scute and atonal homolog, aka Ascl1, achaete-scute complex homolog 1
mdDA neuron	Mesodiencephalic DA neuron
MHB	Midbrain-hindbrain boundary
MHP	Medial hinge point
MMTV	Mouse mammary tumor virus
MO	Morpholino
mRNA	messenger RNA
Msx1	Msh homeobox 1
MZ	Marginal zone
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
Ngn1/2	Neurogenin 1/2, a.k.a. Neurog1/2
Nkx2-2	NK2 transcription factor related, locus 2; a.k.a Nkx2.2
Nr4a2	Nuclear receptor subfamily 4, groupA, member 2; a.k.a. Nurr1
Otx2	Orthodenticle homolog 2
P1, P2	Prosomere 1, prosomere 2
PCP	Planar cell polarity
PD	Parkinson's disease
PDZ	PSD-95/dic large tumor suppressor (DlgA)/ZO-1 homologous
Pitx3	Paired-like homeodomain transcription factor 3
PKC	Protein kinase C
PS-Dvl	Phosphorylated and mobility-shifted Dishevelled
PTK	Protein tyrosine kinase
QPCR	Quantitative real time polymerase chain reaction

R1	Rhombomere 1
Rac	RAS-related C3 botulinum substrate
Rho	Ras homolog gene family
RNA	Ribonucleic acid
ROK	Rho-kinase
Ror2	Receptor tyrosine kinase-like orphan receptor 2 (Ror2)
RP	Roof plate
Ryk	Receptor-like tyrosine kinase
SARP	Secreted apoptosis-related protein, now known as sFRP
sFRP	secreted frizzled-related protein
shh	Sonic hedgehog
siRNA	small interfering RNA
SN	Substantia nigra
Sox2	SRY-box containing gene 2
TCF	T-cell specific transcription factor, a.k.a. Tcf7l2
TGF	Transforming growth factor
TH	Tyrosine Hydroxylase
TrkA	Tyrosine kinase receptor A; a.k.a. neurotrophic tyrosine kinase, receptor, type 1 NTRK1
Tuj11	Tubulin, beta 3; a.k.a. TUBB3
VANG	Van Gogh
Vangl	VANG-like
VM	Ventral midbrain
VTA	Ventral tegmental area
VZ	Ventricular zone
Wg	Wingless
WIF1	Wnt inhibitory factor
Wise	Context-dependent activator and inhibitor of Wnt signaling protein Wise, a.k.a. SOST or SOSTDC1
Wnt	Wingless-related MMTV integration site

1 Introduction

1.1 Context: Parkinson's Disease and Cell Replacement Therapy

Parkinsonism is a progressive neurodegenerative disease, characterized by slowness of movement (bradykinesia), tremor, rigidity, and postural instability, described by James Parkinson in 1817 (published as a monograph in 1817, reprinted in (Parkinson, 2002)).

In his 1919 thesis, Konstantin Tretiakoff described a loss of substantia nigra pigmentation in the autopsies of Parksinsonian patients (Tretiakoff, 1919). Almost 40 years later, Arvid Carlsson first demonstrated a new method of measuring *in vivo* dopamine levels (Carlsson and Waldeck, 1958). Carlsson's work went on to describe that dopamine was present in the brain (Carlsson et al., 1958) and that dopamine was an actual neurotransmitter rather than merely a precursor of noradrenaline. Importantly, Carlsson discovered that administration of reserpine (which depletes monoamine neurotransmitters, including dopamine) caused Parkinson-like symptoms which could be alleviated by administration of levodopa, a precursor of dopamine (Seiden and Carlsson, 1963). For this work, Carlsson won the 2000 Nobel Prize. Thus, the loss of substantia nigra dopaminergic (DA) neurons was implicated in the pathology of Parkinson's disease (PD). Carlsson's results prompted clinicians (including George C. Cotzias and Oliver Sacks) to administer levodopa to Parkinsonian patients (Cotzias, 1968; Cotzias et al., 1967) which even today, remains the most prescribed therapeutic avenue for the treatment of PD. The symptomatic improvements were so dramatic, that a subsequent book by Oliver Sacks (Awakenings, 1973) was produced as an Academy award-nominated film (Awakenings, 1990).

PD is estimated to affect 0.3% of the general population (in the United States of America). The probability of being affected by PD greatly increases with age, with most patients being diagnosed in their early 60's. In the age-group 65-75 years the incidence of PD reaches 1-2%, and in individuals older than 85 PD incidence is estimated at 4-5% (for a review see (Weintraub et al., 2008a)).

The reduced dopamine production and loss of striatal innervation in PD results in cognitive (depression, insomnia) and motor (bradykinesia, tremor, rigidity, and postural instability) dysfunctions (for reviews see (Weintraub et al., 2008b, c)). The cost to the individual, to the family, and to society, is immense since the loss of both motor control and cognition imposes a great challenge on both the patient and the caretakers.

Currently, several treatments are available to alleviate the symptoms of PD, but there is no cure. Levodopa, dopamine agonists, and deep-brain stimulation (DBS) of the subthalamic nucleus are a few of the available treatments (Weintraub et al., 2008b). Unfortunately, these treatments only provide temporary symptomatic relief, often need to be combined, and may result in unwanted side effects such as dyskinesias that can be as debilitating as PD itself. Despite the pathological cause of PD being discovered in

1919, and the positive effects of levodopa being discovered 50 years ago, little progress has been made towards identifying a definitive cure.

One hope for a cure would be cell replacement therapy (CRT): to replace the lost DA neurons with transplanted DA neurons engineered in the lab. Successful improvement of PD symptoms has been achieved using fetal ventral midbrain (VM) tissue in both rat models of PD (Bjorklund et al., 1980a; Bjorklund et al., 1980b) and, to a certain extent, in human patients (Freed et al., 1992a; Freed et al., 1992b; Lindvall et al., 1989). However, the limited availability of fetal tissue and the ethical questions surrounding its use have made this therapeutic option less feasible. Embryonic stem (ES) cells or induced pluripotent stem cells (iPSCs, (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007)) offer promising alternatives (Figure 1).

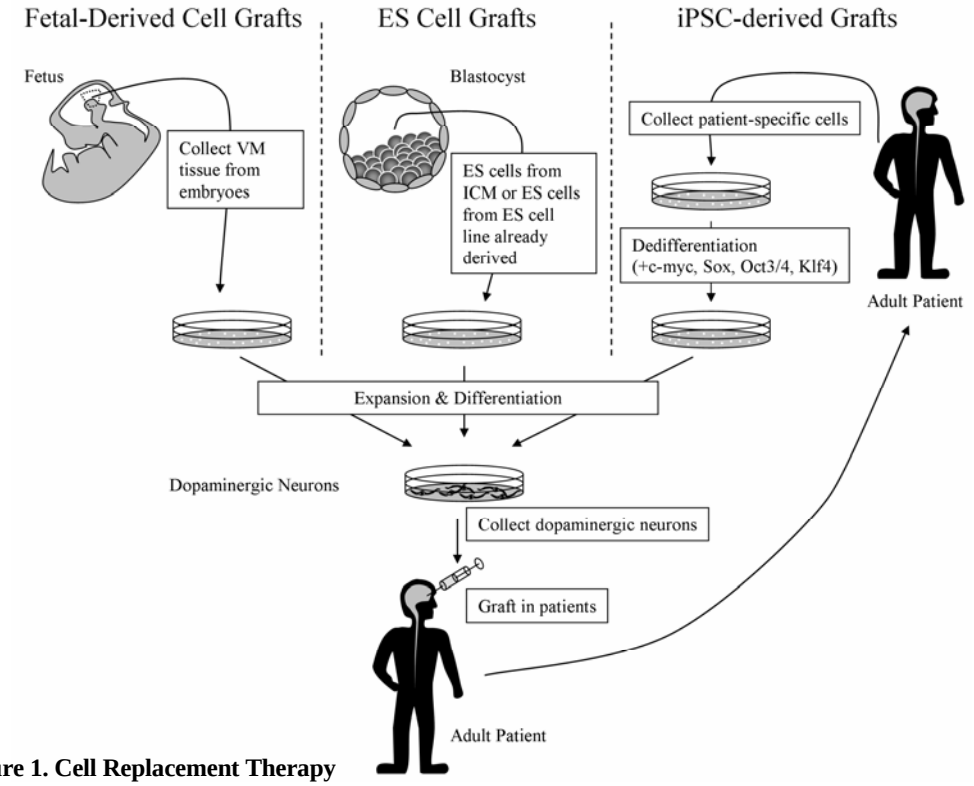

Figure 1. Cell Replacement Therapy
There are several alternate sources of cells for CRT in Parkinson's disease (PD). Neural cells from the ventral midbrain (VM) of fetuses have improved PD in human patients, and both fetal-derived cells and embryonic stem (ES) cell grafts have shown promise in rat models of PD. Patient-specific induced pluripotent stem cell (iPSC) grafts offer yet another alternative, which remains to be tested in PD models. Currently, iPSCs are generated through transfection of c-kit, Oct3/4, Sox and Klf4, and perhaps future studies will reveal extrinsic factors that regulate their expression.

In order to generate DA neurons from undifferentiated cells, and in order to direct their efficient engraftment, it is imperative to know which extrinsic and intrinsic signals regulate their development *in vivo*. Ideally, our knowledge would be sufficient such that patient-specific iPSCs could be derived from the patient, differentiated into DA neurons and re-engrafted into the patient using only extrinsic signals. This approach would diminish the need for genetic manipulation, thus reducing the risk of DNA damage and cancer, while providing a cure for PD.

This thesis focuses on the *in vitro* and *in vivo* functions, in DA neuron and midbrain development, of some members of one such family of extrinsic factors: **Wnts**.

1.2 Introduction to Wnt Signaling

1.2.1 History

The name "Wnt" (pronounced /wĭnt/) derives from a combination of two gene names: *wingless* and *int* (Nusse et al., 1991). The *Drosophila* gene *wingless* (*wg*) was first identified for its function in wing and haltere formation during embryogenesis (Sharma, 1973; Sharma and Chopra, 1976). Murine *int-1*, on the other hand, was first identified due to its role in mammary carcinomas: activation of the *int-1* gene by proviral insertion of the mouse mammary tumor virus (MMTV) into chromosome 15, either 3' or 5' to the *int-1* locus, led to mammary carcinomas (Nusse et al., 1984). Based on protein sequence homology (54%), Rijsewijk and colleagues deduced that *wg* and *int-1* were homologues (Rijsewijk et al., 1987) and later several Wnt pioneers coined the term Wnt (wingless-related MMTV integration site (Nusse et al., 1991)).

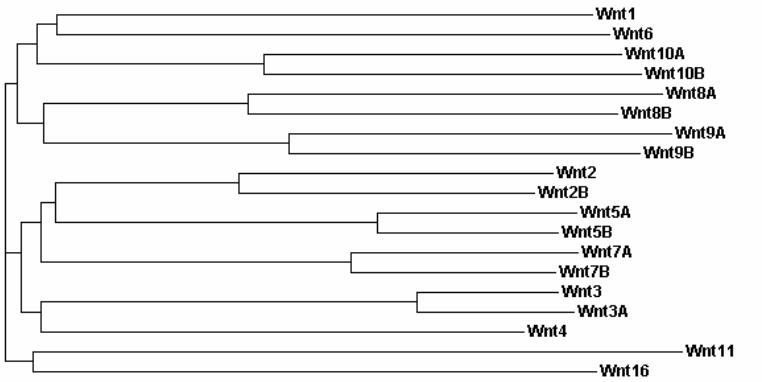

Figure 2. Wnt protein phylogram
Wnt protein sequences were aligned using KAlign, and the resulting alignment was run in ClustalW to obtain a phylogram. Based on protein sequences, β-catenin activating Wnts such as Wnt1 and Wnt3a do not seem more closely related to one another than to Wnt5a and Wnt11, non β-catenin activating Wnts. The following sequences were used: Wnt1: NP_067254.1; Wnt2: NP_076142.2; Wnt2b: NP_033546.2; Wnt3: NP_033547.1; Wnt3a: NP_033548.1; Wnt4: NP_033549.1; Wnt5a: NP_033550.2; Wnt5b: NP_033551.2; Wnt6: NP_033552.2; Wnt7a: NP_033553.2; Wnt7b: NP_033554.2; Wnt8a: NP_033316.1; Wnt8b: NP_035850.1; Wnt9a: NP_647459.1, Wnt9b: NP_035849.2; Wnt10a: NP_033544.1; Wnt10b: NP_035848.1; Wnt11: NP_033545.1; Wnt16: NP_444346.2

Wnts comprise a family of 19 secreted, glycosylated and lipidated proteins (for an up-to-date summary, see The Wnt Homepage: (Nusse, 2008)). Wnts activate multiple different pathways, seemingly in a cell-specific and context-dependent manner. These pathways regulate multiple processes including proliferation, differentiation, migration, planar cell polarity (PCP), and convergent extension (CE). Despite a lack of distinctive sequence similarities (Figure 2) or structural motifs, original nomenclature divided Wnts into two groups based on functional assays: "canonical" or "non-canonical" Wnts, depending on their ability to transform C57MG mammary cells (Wong et al., 1994) or induce body axis duplication in *Xenopus* (Sokol et al., 1991). This classification is losing relevance, since some Wnts formerly considered non-canonical (unable to transform cells or induce axis duplication) have been discovered to induce axis duplication (Wnt5a with Fzd5 (He et al., 1997)) or activate β–catenin (Wnt5a with Fzd4 (Mikels and Nusse, 2006)) if a specific receptor/co-receptor is supplied.

Furthermore, C57MG mammary cell transformation by Wnt1 has been suggested to be mediated, in part, by down-regulation of Wnt5a (Olson and Papkoff, 1994) and is mimicked by Wnt5a antisense (Olson and Gibo, 1998), making it difficult to distinguish "canonical" effects from suppression of "non-canonical" effects.

Canonical Wnt signaling involves the stabilization of β-catenin and is therefore more precisely referred to as Wnt/β-catenin signaling (for review, see (Huang and He, 2008)). Non-canonical non-β–catenin Wnt signaling encompasses several different pathways, each of which can be specified by a characteristic activated downstream component e.g. calcium (Ca^{2+}) release, or Rac activation (for a summary, see (Semenov et al., 2007)). Typically β–catenin-activating Wnts include Wnt1 and Wnt3a (Sokol et al., 1991), while non-β–catenin-activating Wnts include Wnt5a and Wnt11 (Moon et al., 1993). Based on protein homology, Wnt5a/Wnt11 and Wnt1/Wnt3a do not seem more closely related within their class (e.g. Wnt1-Wnt3a) than outside their class (e.g. Wnt5a-Wnt11) (Figure 2).

Certain Wnt pathway components have been identified that are presently considered unique to a specific pathway. Other pathway components such as Frizzled (Fzd) or Dishevelled (Dvl) are shared by several pathways, and the list of shared components is growing. Many efforts within the field have been focused on identification of mechanisms/components contributing to signaling specificity. In some cases, such as with Fzds, it appears that some components promote distinct branches of signaling e.g. Wnt/β–catenin signaling or Wnt/Rac signaling.

1.2.2 Wnt receptors and co-receptors

1.2.2.1 Frizzled Receptors

Frizzleds (Fzd in mammals, fz in *Drosophila*) were first discovered in *Drosophila* due to their involvement in PCP (Vinson and Adler, 1987). Disruption of *fz* resulted in disorganization of the hairs on the *Drosophila* body, hence the name "frizzled". Surprisingly at the time, another member of the family, fz2, was found to bind wingless (*Drosophila* Wnt) and be sufficient to transduce Wnt signals to activate β–catenin (Bhanot et al., 1996). Disruption of both *fz* and *fz2* was necessary to obtain a phenotype mimicking *wg* ablation (Chen and Struhl, 1999). Interestingly, the affinity of fz2 for wingless is an order of magnitude higher than the affinity of fz1 for wingless (Rulifson et al., 2000).

There are 10 Fzds reported in mammals, each containing a 7 transmembrane domain and an extracellular N-terminal, Wnt-binding, cysteine-rich domain (CRD) (Wang et al., 2006a). The CRD seems to be dispensable for signaling per se (Chen et al., 2004), but probably contributes to the formation of Fzd homodimers, heterodimers, higher order protein complexes, or presentation of Wnt to other portions of Fzd (Povelones and Nusse, 2005). Within the C-terminal intracellular domain, a KTXXXW motif, immediately after the last transmembrane domain, is necessary for Fzd binding to the PSD-95/dic large tumor suppressor (DlgA)/ZO-1 homologous (PDZ) domain of Dvl and activation of Wnt/β-catenin signaling (Umbhauer et al., 2000). The KTXXXW

motif is present in all Fzds, even those that do not reportedly contribute to Wnt/β-catenin signaling. Another PDZ-binding domain, a T/SXV motif (Kim et al., 1995), is found further towards the C-terminus in Fzd1, Fzd2, and Fzd7 .

Due to their structure, Fzds have been identified as members of the G-protein coupled receptor (GPCR) family, but were initially thought to signal independently of G-proteins. Later studies have shown G-protein involvement in Fzd signaling (Bikkavilli et al., 2008; Slusarski et al., 1997a).

Attempts to identify Fzds dedicated to a specific Wnt pathway have produced interesting results, but no unifying theory so far. A bioinformatics approach, based on identification of G-protein-coupling selectivity ($G_{i/o}$, G_q, G_s), has grouped Fzds into four groups: Fzd2, Fzd4, Fzd6, Fzd9, and Fzd10 are predicted to be coupled to the $G_{i/o}$ subclass; Fzd5 and Fz8 to the G_q subclass; Fzd1 and Fzd7 to both subclasses; and Fzd3 to all three $G_{i/o}$, G_q and G_s subclasses (Wang et al., 2006a). Protein Kinase C (PKC) has been implicated in Fzd phosphorylation, and is involved in PCP. Injection of Fzd mRNAs into *Xenopus* embryos demonstrated distinct activities of Fzds on PKC translocation. Fzd2, Fzd3, Fzd4 and Fzd6 induced PKC translocation while Fzd1, Fzd7 and Fzd8 did not (Sheldahl et al., 1999). Binding specificity has also been tested in several ways, for example Fzd4, Fzd5, Fzd7 and Fzd8 bind XWnt8 (*Xenopus* Wnt8, most similar to murine Wnt8a, then Wnt8b), while Fzd1, Fzd2, Fzd3, Fzd6, Fzd9 and Fzd10 do not (Smallwood et al., 2007). Overexpression of Fzd1 and Fzd7, but not Fzd2 or Fzd5, induces Rac1 activation (Habas et al., 2003). However, most of these groupings do not seem to be directly related to protein sequence similarities (see sequence similarities in Figure 3).

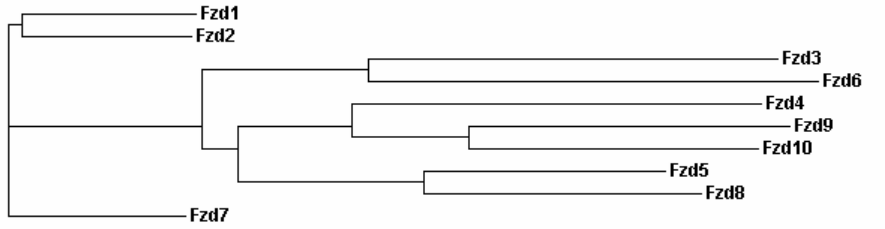

Figure 3. Frizzled protein phylogram
Fzd protein sequences were aligned using KAlign, and the resulting alignment was run in ClustalW to obtain a phylogram. Functional assays, bioinformatical analyses and binding studies have grouped Fzds into different sets, which are not directly reflected in protein sequence similarity (see text for details). The following sequences were used: Fzd1: NP_067432.2; Fzd2: NP_065256.1; Fzd3: NP_067433.1; Fzd4:NP_032081.3; Fzd5: NP_001036124.1; Fzd6: NP_032082.2; Fzd7: NP_032083.3; Fzd8: NP_032084.1; Fzd9: NP_034376.1; Fzd10: NP_780493.1

Frizzleds have also been reported to bind other CRD-containing proteins such as secreted Frizzled-related proteins (sFRPs, please see the sFRP section for more information).

Finally, ablation of some *Fzds* does not appear to affect survival: *Fzd1*, *Fzd2*, *Fzd7*, and Fzd8 seem to be dispensable for development, implying a degree of overlap in function among multiple Fzds (van Amerongen and Berns, 2006). Future generation of double mutant mice, e.g. *Fzd1* and *Fzd2* which are sequentially closely related, would provide more information as to whether Fzd1 and Fzd2 truly are so different functionally, or whether, in development, they contribute to the same processes (consider also, that

their expression patterns in the developing VM are identical (Fischer et al., 2007)). *Fzd3;Fzd6* double mutant mice have already revealed their redundant functions in regulating PCP, with neural tube defects not seen in either single mutant (Wang et al., 2006b).

1.2.2.2 LRP

Depletion of maternal and zygotic *arrow* phenocopies depletion of *wingless*, indicating that expression of *arrow* is required for Wnt/β–catenin signaling in *Drosophila* (Wehrli et al., 2000). In mammals, two LRP receptors (LRP5 and LRP6) are involved in activation of Wnt/β–catenin signaling and show a high level of homology with *Drosophila* arrow (for review, see (He et al., 2004)). This subclass of LDL receptors is dissimilar to other LDL receptors (Figure 4A) and so far, no other LDL receptors have been implicated in Wnt signal activation. However, repression of Wnt signaling by LRP1 (antagonism of Wnt-3a signaling through Fzd1 sequestration, (Zilberberg et al., 2004)), or LRP4 (repression of Wnt signaling through endocytosis of LRP/Fzd proteins upon BMP/Wise binding, (Ohazama et al., 2008)) has been suggested.

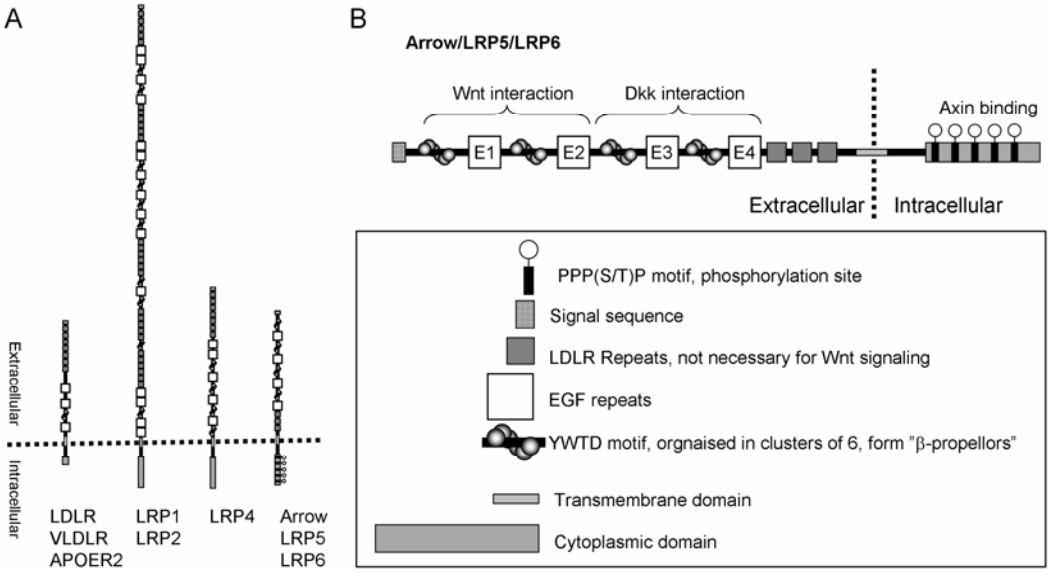

Figure 4. LRP5/6/arrow structure compared to other LDL Receptors

A. LDL receptors generally contain multiple LDLR repeats, hence the name. This is especially true for LRP1 and LRP2. B. Arrow, LRP5 and LRP6, on the other hand, have three LDLR repeats, which are adjacent to the transmembrane domain and dispensable for Wnt signal transduction. Two EGF repeats (E1/E2) are required for Wnt binding and two EGF repeats are required for Dkk binding. In the intracellular domain, five PPP(S/T)P motifs are phosphorylated upon Wnt binding and are required for Wnt/β-catenin signaling.

The LRP5/6/arrow receptor has been extensively studied, and several domains necessary for Wnt signal reception and transduction have been mapped (Figure 4B). LRP5/6/arrow contains 4 epidermal growth factor (EGF) repeats, which are required for binding of Wnt ligand (E1-E2) or Dickkopf (Dkk, binds E3-E4) Wnt signaling modulators (Mao et al., 2001a). Other extracellular domains in LRP5/6/arrow include LDL repeats (Brown et al., 1998), and YWTD motifs (for reviews, see (He et al., 2004;

Jeon and Blacklow, 2005)). Within the intracellular domain are 5 PPP(S/T)P motifs. These 5 motifs are phosphorylated upon LRP binding of Wnt and are necessary and sufficient for Wnt/β–catenin signaling (Tamai et al., 2004).

LRP is required for the transduction of the Wnt signal itself, and is not merely involved in presentation of Wnt ligand to Fzd receptors, which are also necessary for signal transduction. Importantly, the intracellular domain (ICD) of LRP is sufficient to induce Wnt/β-catenin signaling and LRP lacking extracellular domains acts as a constitutively active LRP. Conversely, the extracellular domain acts as a dominant negative molecule, sequestering Wnts without transducing an activating signal, when lacking the ICD (Brennan et al., 2004; Liu et al., 2003; Mao et al., 2001b).

Initially, it was hypothesized that LRP conferred Wnt signaling specificity and directed signaling towards the Wnt/β-catenin pathway (Wehrli et al., 2000). This hypothesis is borne out in several observations. *LRP6* overexpression is sufficient to confer Wnt responsiveness in cells, including Wnts which do not normally activate Wnt/β-catenin (Dejmek et al., 2006). Moreover, *LRP6*$^{-/-}$ mutant mice display a pleiotropic phenotype, partially resembling multiple "canonical" Wnt knockout mice (Pinson et al., 2000): a less morphologically defined midbrain-hindbrain barrier (MHB), similar to the MHB and midbrain deletion seen in *Wnt1*$^{-/-}$ mice (McMahon and Bradley, 1990); axis truncation as in the *Wnt3a*$^{-/-}$ mice (Nakaya et al., 2005), and limb patterning defects similar to those seen in *Wnt7a*$^{-/-}$ mice (Parr and McMahon, 1995).

However, mice with mutations in *LRP6* also display other phenotypes (Carter et al., 2005; Pinson et al., 2000), such as neural tube closure defects, that are more closely reminiscent of PCP/CE defects seen in mice mutated for components of non-β-catenin Wnt signaling (Murdoch et al., 2001; Murdoch et al., 2003; Wallingford and Harland, 2002). It is important to keep in mind though, that in PCP/CE, gain of function (GOF) and loss of function (LOF) often produce similar effects (Schambony and Wedlich, 2007; Unterseher et al., 2004). Therefore the *LRP6*$^{-/-}$ CE/PCP phenotype could be the result of either GOF or LOF in CE/PCP. (For more information on this, please see Paper I in which we dissect out whether, and how, LRP6 modulates CE/PCP).

1.2.2.3 Ryk

Ryk (related to tyrosine kinase) was identified in screens for protein tyrosine kinases (PTKs), as a PTK with an aberrant intracellular catalytic domain (Hovens et al., 1992). Although it is unclear exactly how Ryk signals, replacement of the extracellular domain of Ryk with the extracellular domain of TrkA (another PTK) binding a known ligand (nerve growth factor) demonstrated that Ryk could activate mitogen-activating protein kinase (MAPK) signaling (Katso et al., 1999). The extracellular domain of Ryk contains a domain with similar motifs to Wnt Inhibitory Factor (WIF, see more in "Modulators of Wnt signaling"), and therefore Ryk was proposed as a Wnt-binding receptor (Patthy, 2000). Indeed, Ryk can bind Wnt1, Wnt3a, Wnt5a and Wnt11, and Dvl (Keeble et al., 2006; Kim et al., 2008; Lu et al., 2004). Ryk displays functional interactions with both Wnt3a and Wnt5a: Ryk can mediate Wnt5a effects on axon

guidance (Keeble et al., 2006) as well as activate a Wnt-reporter in response to Wnt3a (Lu et al., 2004).

Ryk$^{-/-}$ mice display a cleft palate, shortened limbs and postnatal mortality due to respiratory failure and feeding problems (Halford et al., 2000). This cleft palate phenotype is similar to that of *EphrinB2*$^{-/-}$ mice and was originally described in the context of EphB2 signaling crosstalk (Halford et al., 2000), but it should be noted that this phenotype also resembles *Wnt5a*$^{-/-}$ mice (He et al., 2008), *Wnt9b*$^{-/-}$ mutant mice and loss of *WNT3* in humans, indicating that there are multiple possible pathways which could interact with Ryk in palate development.

1.2.2.4 Ror

There are two known receptor tyrosine kinase-like orphan receptors (Ror1 and Ror2). While *Ror1*$^{-/-}$ mice appear normal until they die at birth due to respiratory failure (Nomi et al., 2001), *Ror2*$^{-/-}$ mice grossly phenocopy *Wnt5a*$^{-/-}$ mice, with dwarfism and shortened limbs (Nomi et al., 2001; Oishi et al., 2003). *Ror1;Ror2* double mutant mice display an exacerbated phenotype, with more severe anteroposterior (A/P) shortening (Nomi et al., 2001). Both Ror1 and Ror2 are capable of binding Wnt5a (Fukuda et al., 2008; Oishi et al., 2003). Binding of Ror1 to Wnt5a results in activation in NFκB (Fukuda et al., 2008), while Ror2 binding of Wnt5a activates JUN NH_2-terminal kinase (JNK) (Oishi et al., 2003), and results in GSK3 phosphorylation of Ror2 (Yamamoto et al., 2007). Ror2 can bind Wnt5a, Fzd2 or Fzd5 (but not Fzd8) through its CRD (Oishi et al., 2003).

1.2.2.5 Knypek

Zebrafish *Knypek* (60% identical to murine *glypican 6* - *GPC6*) contains a CRD-like domain, is required for zebrafish CE, and genetically interacts with *Wnt11* (Topczewski et al., 2001). Knypek/GPC4/6 binds Wnt5a, Wnt11 and Fzd7 (Ohkawara et al., 2003; Topczewski et al., 2001) and is thought to stabilize the interaction between Wnt and Fzd.

1.2.2.6 Other Receptors

Many other receptors have been implicated in Wnt signaling, including (but not limited to):

- Discoidin domain receptor 1 (DDR1), which is phosphorylated in response to Wnt5a (Jonsson and Andersson, 2001);
- Fibroblast growth factor receptor ligand 1 (FRL1, a *Xenopus* Cripto homologue) which transduces a Wnt11 signal in *Xenopus* to activate β–catenin signaling, and can bind Wnt5a but not Wnt8 (Tao et al., 2005);
- Kremens bind to Dkk which in turn bind LRP. Kremen/Dkk thus scavenges LRP receptors away from Fzds, blocking Wnt/β–catenin signaling (Mao et al., 2002);

1.2.3 Dishevelled

Dishevelled (Dvl in mammals, dsh in *Drosophila*) was first discovered in *Drosophila*, where loss of *dsh* leads to segment polarity defects (Noordermeer et al., 1994). Later it was discovered that *dsh* was also implicated in PCP in the wing (Theisen et al., 1994). Three *dsh* homologues (*Dvl1-3*) have since been identified in mice and humans (for review, see (Wallingford and Habas, 2005).

Dvls contain three conserved domains, a DIX (Dishevelled/Axin) domain, a PDZ (PSD-95/dics large/ZO-1 homologous) domain and a DEP (Dishevelled/EGL-10/Pleckstrin) domain. These three domains have been differentially implicated in different Wnt signaling pathways (Figure 4, 6), and probably permit Dvl to serve as a branching point for Wnt signal transduction. The DIX and PDZ domains are necessary for β–catenin activation, while the PDZ and DEP domains are necessary for non β–catenin signaling pathways (Boutros et al., 1998; Sheldahl et al., 2003).

Previously, hyperphosphorylation of Dvl (producing phosphorylated and mobility-shifted Dvl: PS-Dvl) was assumed to be a hallmark of active Wnt signaling (Gonzalez-Sancho et al., 2004). However, recent work by Bryja *et al.* has revealed that the hyperphosphorylation of Dvl by CK1 and CK2 is not required for Rac1 activation by Wnt11 (Bryja et al., 2008). Phosphorylation of Dvl may thus act as a switch between individual branches of Wnt signaling.

Signaling specificity may also arise from subcellular localization of Dvl. Membrane-associated Dvl is correlated with PCP signaling, while cytoplasmic or nuclear Dvl is correlated with Wnt/β-catenin signaling (Axelrod et al., 1998). However, translocation of Dvl to the membrane is also sometimes seen in β-catenin signaling (Boutros et al., 2000). Other reports have shown that apicobasal subcellular distribution determines Dvl signaling specificity (Wu et al., 2004).

1.3 Wnt/β-catenin Signaling

In the absence of a "canonical" Wnt ligand, cytosolic β–catenin is phosphorylated and targeted for degradation by a destruction complex comprising adenomatous polyposis coli (APC), casein kinase 1α (CK1α), glycogen synthase kinase-3β (GSK3β), and the scaffolding protein Axin (Figure 5A). Sequential phosphorylation of β–catenin by CK1α and GSK3β primes β–catenin for ubiquitination by β-Trcp and thereby proteosomal degradation (for reviews, see (Huang and He, 2008; Macdonald et al., 2007).

Wnts (including Wnt1, Wnt3a and Wnt8) bind to Fzd and LRP receptors to initiate signaling. The binding of Wnt to LRP and Fzd ultimately leads to the stabilization of β–catenin (Figure 5B). Some data suggests that the physical proximity of LRP and Fzd, induced by Wnt binding of each, activates the signaling cascade, as shown by different fusion constructs of LRP or Fzd inducing complex formation and Wnt signal activation, even in the absence of Wnt (Cong et al., 2004; Holmen et al., 2005). However, this

mechanism does not explain how LRP deletion constructs, lacking an extracellular domain, would alone activate Wnt/β-catenin signaling (Brennan et al., 2004; Liu et al., 2003; Mao et al., 2001b), unless the ICD itself localizes close to the Fzd ICD. Instead, constitutive oligomerization of overexpressed LRP ICD has been reported, leading to phosphorylation of the ICD. This oligomerization activates Wnt/β-catenin signaling independently of Dvl, which is otherwise required for oligomerization of wild type (WT) LRP (Bilic et al., 2007).

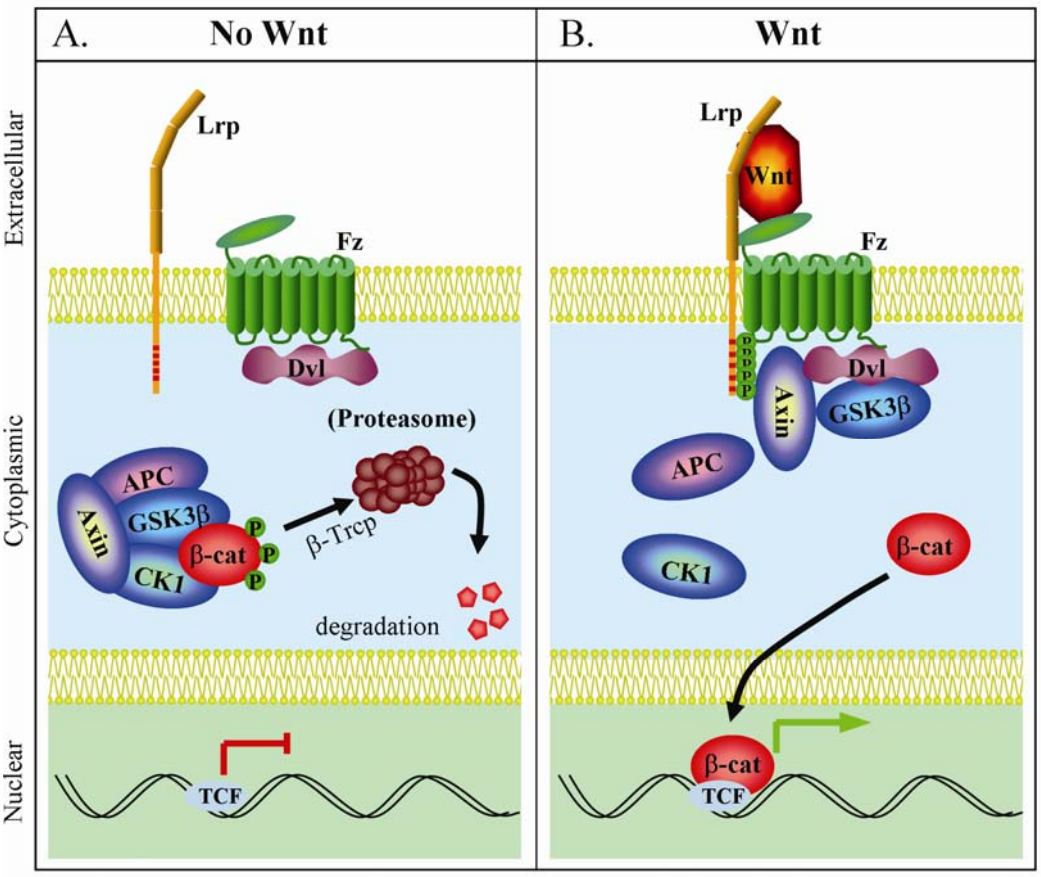

Figure 5. Wnt/β-catenin Signaling

(A) In the absence of Wnt, β-catenin is phosphorylated by a destruction complex composed of Axin, APC, GSK3β, and CK1α and thus targeted for proteosomal degradation. (B) Binding of Wnt to LRP and Fzd receptors initiates signaling and leads to inhibition of the destruction complex. Dephosphorylated and activated β-catenin accumulates in the nucleus where it binds TCF/LEF transcription factors to activate transcription of Wnt target genes. Oligomerization of Wnt signaling components has been reported, but is omitted in this scheme for the sake of simplicity.

There are multiple mechanisms suggested for the inactivation of the β–catenin destruction complex. Upon binding of Wnt, the LRP receptor is phosphorylated by GSK3β and CK1γ, which leads to the recruitment of Axin away from the destruction complex (Davidson et al., 2005; Zeng et al., 2005). Axin is also recruited to the membrane by Dvl, which may contribute to its degradation (Cliffe et al., 2003). GSK3β is inhibited by CK1ε-phosphorylated/activated Dvl. By any of these mechanisms, or a combination thereof, the Axin/APC/GSK3β/CK1α degradation complex is inactivated. β–catenin is no longer phosphorylated/degraded, and therefore accumulates in the nucleus where it binds to transcription factors T-cell factor/lymphoid enhancer-binding

factor (TCF/LEF, (Brunner et al., 1997; Molenaar et al., 1996; Roose et al., 1998)) to activate transcription of target genes such as siamois (Brannon et al., 1997), cyclin-D1 (Tetsu and McCormick, 1999) and c-myc (He et al., 1998), which are otherwise repressed.

1.4 Non-β–catenin Wnt Signaling

Non-canonical, non-β–catenin activating, Wnt signaling collectively refers to Wnt signaling which does not involve β–catenin stabilization (Figure 6). This should not be mistaken for a lack of impact on Wnt/β–catenin signaling, indeed many non-canonical pathways show antagonism with the Wnt/β–catenin signaling pathway.

Non-β–catenin pathways mainly contribute to the processes of PCP (the orientation of cells within an epithelial plane), and CE (the narrowing and lengthening of a 3-dimensional structure), migration, and in some cases, transcription (Schambony and Wedlich, 2007). Much of the literature groups non-β-catenin Wnt signaling in terms of Wnt/PCP or Wnt/Calcium. However, Wnt/PCP signaling involves activation of several small Rho GTPases such as Rho, cdc42 and/or Rac, which have different intracellular targets and appear to constitute separate pathways (Beane et al., 2006; Dejmek et al., 2006; Habas et al., 2003; Kim and Han, 2005; Rosso et al., 2005; Schambony and Wedlich, 2007; Schlessinger et al., 2007; Tahinci and Symes, 2003; Zhu et al., 2006).

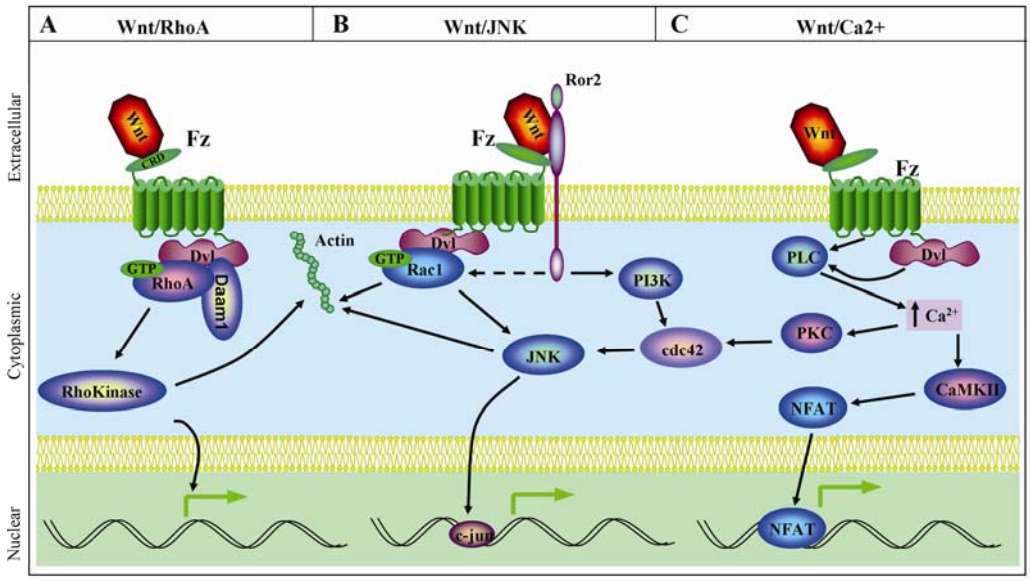

Figure 6 Non-canonical Wnt Pathways
A. Wnt/RhoA: Wnts can activate RhoA, which requires the recruitment of Daam1 by Dvl. RhoA activates RhoKinase which regulates actin cytoskeleton rearrangements. B. Wnt/JNK: Wnts can bind Ror2 to activate JNK via PI3K and cdc42, or Fzd to activate JNK via Rac1. At present, it is unclear whether these two cascades are distinct, parallel or one. Dashed lines demonstrate that Ror2 has not been shown to activate JNK via Rac1. Rac1 regulates actin cytoskeleton rearrangements, while JNK activates transcription of JNK target genes via c-jun. C. Wnt/Ca^{2+}: Wnt can activate PLC directly or via Dvl, which upregulates intracellular Ca^{2+}. Elevated calcium activates PKC, and NFAT via CamKII. NFAT activates transcription of target genes. Cross talk between Wnt/Ca2+ and Wnt/Rac is likely in that PKC activates JNK, possibly via cdc42 activation.

One indication that RhoA and Rac pathways are separate stems from the observation that RhoA LOF CE defects cannot be rescued by Rac1, and conversely, Rac1 LOF defects cannot be rescued by RhoA (Tahinci and Symes, 2003). Therefore, this thesis will describe Wnt/Rho and Wnt/JNK signaling pathways separately, rather than together under the umbrella term "Wnt/PCP".

While focus in the Wnt field has been on the role of RhoA in PCP, roles for RhoA, cdc42 and Rac1 have also been described in transcriptional regulation (Hill et al., 1995) and cell cycle progression (Olson et al., 1995).

1.4.1 Wnt/RhoA

Wnt1, Wnt5a and Wnt11 are capable of activating RhoA (Endo et al., 2005; Zhu et al., 2006). The activation of RhoA via Fzd requires the association of Daam1 to Dvl and RhoA (Habas et al., 2001). RhoA then activates Rho Kinase (Leung et al., 1996; Leung et al., 1995), leading to the assembly of actin-myosin filaments and focal adhesion complexes (Ridley and Hall, 1992) (Figure 6A),. Thus, RhoA is required for CE movements in *Xenopus* and zebrafish (Tahinci and Symes, 2003; Unterseher et al., 2004; Zhu et al., 2006), and can rescue the CE defects seen in Wnt5a and Wnt11 zebrafish mutants (Zhu et al., 2006).

The Wnt/Rho pathway is summarized in Figure 6A.

1.4.2 Wnt/JNK

Wnt activation of JNK has been observed in several systems, although the intermediate signaling components are reportedly distinct (Boutros et al., 1998; Habas et al., 2003; Rosso et al., 2005; Schambony and Wedlich, 2007; Yamanaka et al., 2002; Zhang et al., 2002).

While the most recent classification of non-β-catenin pathways grouped Wnt/RhoA and Wnt/Rac1 (which activates JNK) as a composite "PCP" pathway, and separately described Wnt/Ror2 as a pathway activating JNK (Semenov et al., 2007), several lines of evidence suggest that, apart from the components of the Wnt/PCP pathway defined in *Drosophila* (Fz, Dsh, Strabismus/Vangl2, Flamingo/Celsr1 and Prickle, (Seifert and Mlodzik, 2007)), Ror2 may be involved in PCP and/or CE in vertebrates:

1. Dorsal overexpression of *Ror2* in *Xenopus* causes a shortened body axis and defects in head structures, including cyclopia; which are typical PCP/CE defects (Hikasa et al., 2002).
2. *Ror2* synergizes with *Fzd7* and *Wnt11* to inhibit CE (Hikasa et al., 2002).
3. Ror2 regulation of CE in Keller explants mimics Wnt5a regulation; *Ror2* MO or *Wnt5a* MO strongly disrupt explant constriction, but have less of an effect on elongation (Schambony and Wedlich, 2007).
4. *Ror2*$^{-/-}$ mice display defects in inner ear hair cell orientation (Yamamoto et al., 2008), an established PCP defect.
5. *Ror1*$^{-/-}$*;Ror2*$^{-/-}$ mice phenocopy *Wnt5a*$^{-/-}$ mice, with severe A/P body axis and limb shortening (Nomi et al., 2001).

6. In *Caenorhabditis elegans*, *Ror* (*CAM-1*) has been described to synergize with *Vangl* (*VANG*) and *Wnt* (*EGL-20*) in the regulation of cell polarity (Green et al., 2008), although this did not result in JNK activation.
7. Finally, the Wnt/Ror2 classification did not include Fzd binding, while Ror2 has been found to bind the CRD of Fzd2 and Fzd5, but not Fzd8 (Oishi et al., 2003).

Therefore, rather than separately discussing a Wnt/PCP pathway, and a Wnt/Ror2 pathway, when both activate JNK, these are grouped in a Wnt/JNK pathway (Figure 6B).

Ror2 binds Wnt5a, leading to the activation of JNK signaling (He et al., 2008; Liu et al., 2008; Nishita et al., 2006; Oishi et al., 2003). Ror2 does not bind Wnt3a directly (Oishi et al., 2003), but nonetheless potentiates Wnt3a activity (Li et al., 2008).

While activation of JNK by Wnt5a in *Xenopus* does not require Rac1, and instead requires cdc42 (Hikasa et al., 2002; Schambony and Wedlich, 2007); Wnt5a activation of JNK in mammalian cells (HEK293T) has been shown to require Rac1 activity, but not RhoA or cdc42 (Habas et al., 2003). In one of these reports, the rescue of Ror2 GOF by dominant negative cdc42 was only partial, thus it is possible that other small RhoGTPases contribute to the Ror2 GOF phenotype. It remains to be seen whether these differences in intermediate signaling components reflect species-specific routes of the same pathways, or entirely different pathways in multiple species.

The Wnt/JNK pathway is summarized in Figure 6b.

1.4.3 Wnt/Ca $^{2+}$

Calcium (Ca^{2+}) is an intracellular messenger that mediates numerous processes including exocytosis, muscle contraction, transcription and proliferation (for review see (Clapham, 2007)). Injection of *Wnt5a* (Slusarski et al., 1997b) or *Wnt11* (Westfall et al., 2003) mRNA in zebrafish embryos triggers release of intracellular Ca^{2+}, leading to the activation of Ca^{2+}/calmodulin-dependant kinase II (CamKII) and protein kinase C (PKC) (for review, see (Kohn and Moon, 2005)). CaMKII regulates NFAT, which translocates to the nucleus upon Wnt stimulation and thus activates transcription of NFAT target genes (Murphy and Hughes, 2002). The activation of Ca^{2+} release in response to Wnt is dependent on G_i/G_o-protein signaling, as demonstrated by sensitivity to pertussis toxin (G_i/G_o/GPCR inhibitor) blocking of this pathway (Slusarski et al., 1997a).

The Wnt/Ca^{2+} pathway is summarized in Figure 6C.

1.5 Signaling Cross-Talk

A high degree of cross talk permeates the different Wnt signaling pathways, indicating that Wnt pathways form a signaling network, rather than single signaling pathways. The Wnt/Ca^{2+} pathway has been shown to activate nemo-like kinase, which inhibits β–catenin signaling by phosphorylation of TCF (Ishitani et al., 2003). Dominant-negative (dn) RhoA inhibits JNK activity (Nagao et al., 1999). Furthermore, cross-talk between

the Wnt/Rho and Wnt/Ca^{2+} pathway is suggested by RhoA and cdc42, but not Rac1, potentiation of PKCα activity (Slater et al., 2001). Rac1 activation of JNK was reported to specifically regulate nuclear localization of β–catenin (Wu et al., 2008). In this report, dominant negative (dn) Rac1, or siRNA against *Rac1*, blocked Wnt3a-induced Lef1-Luciferase reporter activity, but did not affect Wnt3a-induced stabilization of β–catenin in the cytoplasm. This is an important distinction, since dephosphorylation of β–catenin is usually taken as a measure of β–catenin signaling. In a further twist, the nuclear localization of β-catenin was regulated by JNK2 phosphorylation of specific serine residues in β–catenin.

Multiple other non-β-catenin pathways seem to be activated by Wnt signaling, but at present have not been described as fully, or in the context of VM or DA neuron development.

1.6 Modulators of Wnt signaling

Apart from the plethora of receptors (10Fzds, LRP5/6, Kremen1/2, Ror1/2, Ryk etc) that transduce Wnt signaling, there are multiple secreted modulators of Wnt signaling. Extracellular Wnt-interacting proteins include Wnt inhibitory factor 1 (WIF1), Dickkopf (Dkk), and secreted Frizzled related protein (sFRP). Most of the extracellular modulators of Wnt signaling have been ascribed roles as antagonists of Wnt signaling. However, accumulating data indicates that many of these factors can also act as agonists in certain contexts, or interact with non-Wnt signaling pathways.

1.6.1 Wnt Inhibitory Factor (WIF) and Dickkopf (Dkk)

WIF1 binds XWnt8 and blocks XWnt8-induced axis duplication (Hsieh et al., 1999). However, injection of *WIF1* mRNA itself into *Xenopus* embryos induces a partial secondary axis. WIF1 is suggested to be a Wnt antagonist due to its blocking of XWnt8, but the fact that it can induce a secondary axis implies that it may also be able to agonize Wnt/β–catenin signaling under certain conditions. The extracellular domain was sufficient to block XWnt8-induced axis duplication, indicating that this extracellular domain binds Wnt and is sufficient to inhibit Wnt. Surprisingly, the *Drosophila* homologue of WIF1, Shifted, modulates hedgehog diffusion rather than wingless signaling (Glise et al., 2005; Gorfinkiel et al., 2005).

There are 4 members of the Dickkopf family of proteins (Dkk1-4), which can be grouped by sequence similarity into two groups: Dkk1/2/4 and Dkk3 (Fedi et al., 1999; Glinka et al., 1998; Krupnik et al., 1999). *Dkk1* can block *Xwnt8*-induced head induction in *Xenopus*, but induces a head itself when co-injected with a BMP antagonist (Glinka et al., 1998). In line with this, *Dkk1*$^{-/-}$ mice lack anterior head structures (Mukhopadhyay et al., 2001). As such, the role of Dkk1 *in vivo* is consistent with reports of Wnt/β-catenin antagonism (Fedi et al., 1999), while Dkk2 has been reported to activate Wnt/β–catenin signaling in the presence of overexpressed *Fzd8* (Wu et al., 2000) or LRP5/6 (Brott and Sokol, 2002).

Dkk1 and Dkk2 bind LRP6 with high affinity (Bafico et al., 2001; Mao et al., 2001a), thus modulating Wnt-β-catenin signaling. Dkk1 and Dkk2 also bind Kremen1 and Kremen2, which potentiates Dkk1/2 blocking of β-catenin signaling (Mao et al., 2002). Originally, Dkks were considered to be dedicated to specific modulation of β-catenin signaling (Niehrs, 2006; Semenov et al., 2001). However, *in vitro* Dkk regulation of non-β-catenin pathways has been reported (Lee et al., 2004). Furthermore, Caneparo et al. described a role for *Dkk1* in gastrulation movements, demonstrating that Dkk1 blocks activin-induced explant elongation and binds Knypek to potentiate Wnt activation of JNK (Caneparo et al., 2007). Therefore, it would appear that Dkk1 is capable of simultaneously repressing Wnt/β–catenin and activating Wnt/JNK signaling.

1.6.2 Secreted Frizzled-Related Proteins (sFRPs)

The family of secreted Frizzled related proteins (sFRP1/2/3/4/5 in mammals) can be separated into two subgroups based on sequence homology: sFRP1/2/5, and sFRP3/4 (Bovolenta et al., 2008). sFRPs contain an N-terminal CRD and a C-terminal domain with weak homology to Netrin. Both domains have been reported to bind to Wnts, although it is generally assumed that the CRD is the more relevant Wnt-binding domain (Leyns et al., 1997; Lin et al., 1997; Lopez-Rios et al., 2008). As with the other extracellular Wnt modulators, and based on many reports showing inhibition of Wnt signaling (Dennis et al., 1999; Leyns et al., 1997; Lin et al., 1997; Lopez-Rios et al., 2008; Uren et al., 2000; Wang et al., 1997a; Wang et al., 1997b; Xu et al., 1998), sFRPs are generally regarded as Wnt antagonists. However, the CRD of sFRP1 can interact with Fzd (Bafico et al., 1999), and sFRP1 can activate Wnt/β-catenin signaling (Uren et al., 2000; Yokota et al., 2008), or increase β-catenin levels (Melkonyan et al., 1997).

Injection of *sFRP1* into *Xenopus* animal caps mimics injection of *Wnt5a*: blocking activin-induced elongation (Xu et al., 1998). While *sFRP1* is capable of blocking *XWnt8*/activin induced elongation, it does not show a clear interaction with *XWnt5a* (Xu et al., 1998). sFRPs were separately identified around the same time for their opposing roles in apoptosis. sFRP1 (a.k.a SARP2) sensitized cells to apoptotic stimuli, whereas sFRP2 (SARP1), conferred resistance to apoptotic stimuli (Melkonyan et al., 1997). This indicated different roles for sFRP1 and sFRP2. However, double *sFRP1;sFRP2* mutant mice revealed deficiencies in A/P axis elongation and thoracic somitogenesis, which were not seen in the individual mutants, and which revealed functional redundancy between these two *sFRPs* (Satoh et al., 2006). *sFRP1; sFRP2;sFRP5* triple mutants revealed that *sFRPs* genetically interact with both "canonical" Wnt signaling (*Dkk*) and "PCP" signaling (*Vangl*) in compound mutant mice (Satoh et al., 2008).

1.7 Wnt Signaling Conclusion

It is clear from the wealth of data that Wnts are capable of activating or inhibiting a vast array of signaling pathways, depending on which receptors, co-receptors and intracellular signaling mediators are present. The original canonical/non-canonical nomenclature referring to Wnts per se is no longer valid, since most Wnts can be manipulated to activate pathways they were previously not thought to be able to induce. Furthermore, the extensive cross-talk between the different pathways redefines the extent of each pathway. More recent reviews of the field argue that Wnt signal output is

governed by Wnt receptors and that said receptors, rather than Wnts, govern signaling output specificity.

Rather than going from one extreme (Wnts dictate specificity) to the other (receptors dictate specificity), surely a middle-ground, in which ligand, receptors and pathway components cooperate to integrate these signals into a complex signaling network, is more akin to the *in vivo* situation. Also, it is possible that graded responses contribute to the output of a specific signal. From an evolutionary perspective, it would seem a poor strategy to have 19 Wnt ligands, if receptors alone governed the signaling output… This vast signaling network would then integrate Wnt signals into the network of other signals being processed.

1.8 Development of the Central Nervous System – a Wnt perspective

The fusion of two haploid gametes, two halves, to make one fertilized egg-cell, and the subsequent development of this cell into a complex organism composed of 10-100 trillion cells, of numerous different types and organized in complex structures such as the brain and vasculature, is one of the miracles of development.

Development of the central nervous system (CNS) is directed by a set of extracellular signaling molecules including Wnt, Notch, fibroblast growth factors (Fgf), Hedgehog (Hh), transforming growth factors (TGFs) and bone morphogenic proteins (BMPs). Together, these master regulators coordinate the proliferation, migration, differentiation and connectivity of a multitude of cell-types within the CNS.

1.8.1 From a single cell to a midbrain in 250 words or less

After fertilization of the ovum by a sperm cell, the resulting zygote proliferates to produce a ball of cells called a morula. In the first step of differentiation, the morula cavitates to produce a blastocyst. The outer shell of cells compose the trophoblast, which will contribute to formation of the placenta, and the cells within the sphere are the inner cell mass (ICM), which will contribute to development of the embryo and certain extra-embryonic tissues. Embryonic stem (ES) cells can be derived from this ICM which is pluripotent. The ICM differentiates into the epiblast and the hypoblast. The epiblast gives rise to embryonic tissues, whereas the hypoblast gives rise to extra-embryonic tissues. Formation of a primitive streak in the epiblast leads to ingression of cells into the primitive streak, forming new layers of cells beneath, endoderm and mesoderm, in a process known as gastrulation. The notochord, which is formed by the mesoderm, signals to the ectoderm above to form a neural plate. Primary or secondary neurulation results in formation of a neural tube from the neural plate by infolding or hollowing, respectively. The neural tube forms 4 neuromeres, from anterior to posterior: prosencephalon, mesencephalon, rhombencephalon and the spinal cord. The mesencephalon gives rise to the midbrain, in which DA neurons are born and differentiate. Figure 7 summarizes some of the contributions of Wnts to these processes.

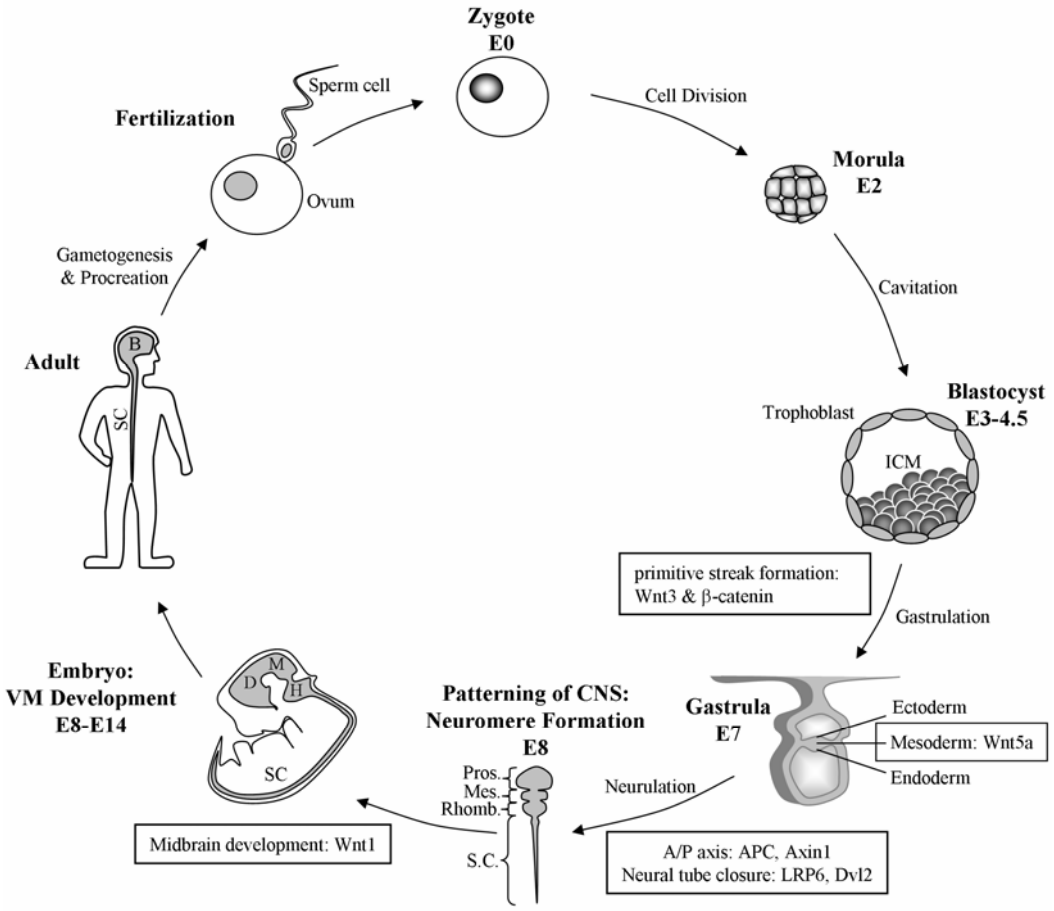

Figure 7. The Circle of Life

Wnt signaling is required for several developmental processes leading to the formation of the midbrain and DA neurons. The corresponding mouse stage is given for each stage as embryonic day (E) e.g. E0, E2 etc. The requirement of Wnt components gleaned from knockout studies are labeled in boxes. Many Wnt knockout phenotypes are severe, and preclude analysis of later developmental processes. A/P = anteroposterior, B = brain, CNS = central nervous system, D = diencephalon, E = embryonic day (mouse), H = hindbrain, ICM = inner cell mass, Mes = mesencephalon, M = midbrain, Pros = prosencephalon, Rhomb= Rhombencephalon, SC = spinal cord, VM = ventral midbrain.

In order for cells to proliferate and differentiate appropriately, it is essential for them to interpret their temporal and spatial position: anterior to posterior, dorsal to ventral, medial to lateral. These cues are supplied by morphogenic gradients of proteins such as Wnts. Wnts also play other important roles during development, for example during primitive streak formation (*Wnt3* expression delineates presumptive primitive streak (Rivera-Perez and Magnuson, 2005), and can induce primitive streak-like cells *in vitro* (Nakanishi et al., 2008; ten Berge et al., 2008)), or gastrulation itself (*Wnt5a* and *Wnt11* zebrafish mutants display CE defects that arise during gastrulation (Heisenberg et al., 2000; Kilian et al., 2003)). Numerous other processes during development are governed by Wnts, but are outside the scope of this thesis.

1.8.2 Midbrain Patterning and Development

Development of the midbrain, and the cell types within, requires temporal and spatial coordinates, specifying where in the anteroposterior (A/P, or rostrocaudal), dorsoventral (D/V) or mediolateral axes each cell resides (Figure 8A).

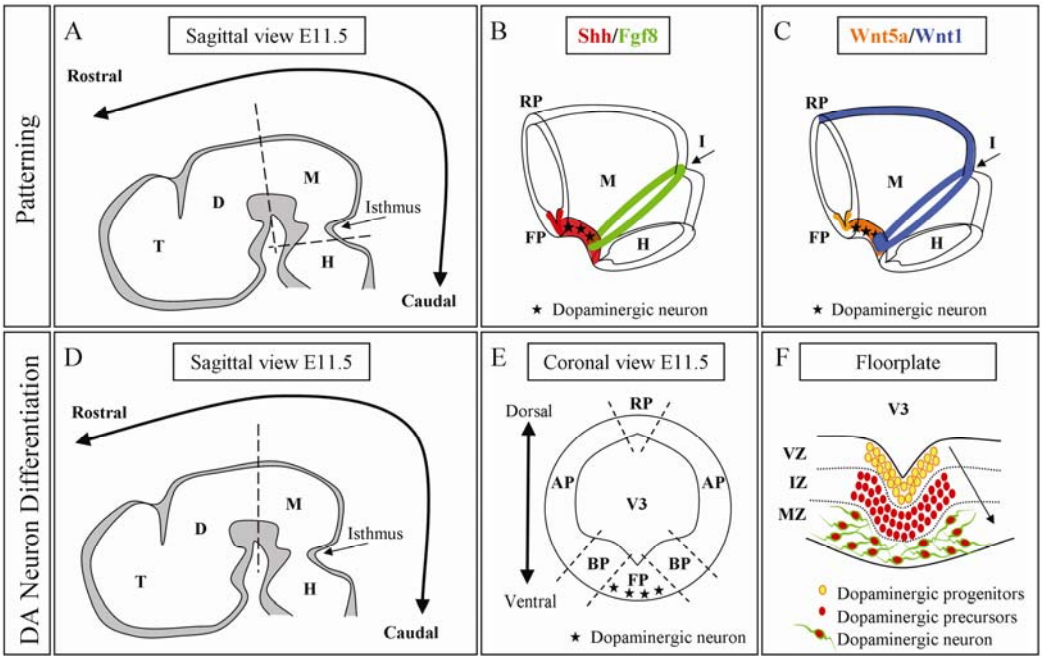

Figure 8 Patterning and DA Neuron Differentiation

A. A scheme of an E11.5 sagittal brain section, depicting the rostrocaudal, or A/P, axis. The midbrain (M) is situated anterior to the isthmus, between the diencephalon (D) and hindbrain (H). Dashed lines demonstrate which portion of the brain: midbrain, isthmus and R1, is depicted in B and C. **B.** 3-dimensional representation of the Shh-expressing floorplate (FP), and the Fgf8-expressing band around the isthmus (I). DA neurons (stars) arise in the intersection between Shh and Fgf8. **C.** A similar pattern of expression and subsequent intersection is formed by Wnt5a in the FP and Wnt1 in the isthmus. Wnt1 is also expressed in two stripes flanking the ventral midline, and in the roofplate (RP). **D.** A dashed line shows the midbrain level represented for the coronal sections in E and F. **E.** The midbrain is dorso-ventrally divided into several compartments, from dorsal to ventral: RP, alar plate (AP), basal plate (BP) and FP. DA neurons are born and develop in the FP. **F.** DA progenitors in the ventricular zone (VZ) migrate through the intermediate zone (IZ) to the marginal zone (MZ), differentiating as they progress through these zones. Abbreviations not included in text: T = telencephalon, V3 = 3rd ventricle

1.8.2.1 Otx2/Gbx2

Midbrain development is precisely regulated by the interaction of several intrinsic and extrinsic factors (for review, see (Liu and Joyner, 2001a; Puelles, 2007)). *Otx2* and *Gbx2* are transcription factors expressed prior to gastrulation (~E7.5) in anterior (Simeone et al., 1992; Simeone et al., 1993) and posterior epiblast (Wassarman et al., 1997), respectively. The expression of *Otx2* subsequently becomes restricted to the prosencephalon and mesencephalon by E9.5, while *Gbx2* is expressed in the rhombencephalon and developing spinal cord. Thus, the midbrain-hindbrain boundary is demarcated by the interface between *Otx2* and *Gbx2*. Loss of either *Otx2* (chimaeras (Rhinn et al., 1998) or hypomorphs (Tian et al., 2002)) or *Gbx2* (Millet et al., 1999) results in rostral expansion of *Gbx2* or caudal expansion of *Otx2*, respectively. A specific requirement of *Otx2* in the patterning of the VM has been shown in *Nestin*^{Cre} conditional *Otx2* knockout mice, in which the isthmus is correctly established. In these mice DA neurons are reduced in number, with a concurrent increase in ectopic serotonergic neurons (Vernay et al., 2005).

1.8.2.2 Fgf8

The A/P axis of the midbrain is specified by the expression of *Fgf8*. *Fgf8* is a secreted molecule, expressed in the anterior hindbrain at E8.5, which becomes restricted to a narrow ring of expression encircling the isthmus by E9 (Figure 8B, (Crossley and Martin, 1995; Heikinheimo et al., 1994)). Interestingly, there are (at least) two splice variants of *Fgf8*: *Fgf8a* and *Fgf8b*. Fgf8b is 11 amino acids longer than Fgf8a, and this additional peptide sequence greatly increases the affinity of Fgf8b for the Fgf receptor (Fletcher et al., 2006; Guo and Li, 2007; Olsen et al., 2006). The requirement of *Fgf8 in vivo* was shown in an elegant allelic series of *Fgf8* mice, in which null alleles revealed a requirement for *Fgf8* in gastrulation and an *Fgf8* hypomorph revealed the requirement of *Fgf8* in regulation of midbrain and cerebellum development (Meyers et al., 1998). Conditional *Fgf8* mice (*Foxg1*^{cre} mediated excision of Fgf8 in anterior neural structures) have confirmed the requirement of *Fgf8* in development of the midbrain (Kawauchi et al., 2005). Conversely, Fgf8-soaked beads are capable of inducing an ectopic isthmus (Martinez et al., 1999).

1.8.2.3 Shh

The D/V axis of the midbrain, and neural tube, is specified by the expression of *Shh* (Briscoe et al., 2000; Briscoe et al., 1999; Epstein et al., 1999). *Shh* is expressed in the floorplate (FP), and as such specifies a ventral identity in the neural tube (Figure 8B). Loss of *Shh* signaling compromises development of the FP. This is clear in that *Shh* induces expression of *Gli* transcription factors, also necessary for VM development: *Gli2*$^{-/-}$ mice lack a FP and DA neurons (Matise et al., 1998). Shh is necessary and sufficient to induce DA neurons (Hynes et al., 1995). Interestingly, a recent paper by Joksimovic et al. showed that *Shh*-expressing cells give rise to DA neurons and described an antagonism between Wnt/β-catenin and Shh as a mechanism explaining the unique neurogenic potential of the FP (Joksimovic et al., 2009)

1.8.2.4 Wnt1 and Wnt5a

Wnt1 expression begins in the 1-somite embryo (ca E8), and is found in a region consistent with presumptive midbrain, but does not seem to be expressed in the FP region (McMahon et al., 1992). Initially, *Wnt1* is broadly expressed in the mesencephalon, but becomes progressively restricted in the midbrain to the dorsal midline, a narrow ring immediately anterior to the *Fgf8* ring at the isthmus, and two stripes of expression flanking the ventral midline (Figure 8C, (Prakash et al., 2006; Wilkinson et al., 1987)).

Wnt5a is broadly expressed in the midbrain at E8.75, and is expressed in the VM at the time of DA neurogenesis (Castelo-Branco et al., 2003; Yamaguchi et al., 1999), but the role of *Wnt5a* in midbrain development had not been described prior to publication of Paper II.

Interestingly, the textbook intersection of *Fgf8*/*Shh* is mimicked by a similar intersection of *Wnt1*/*Wnt5a*, also in the isthmus/floorplate region (Figure 8C, and more on this in the "Wnts and DA neurogenesis section").

1.8.3 DA neurons: Origin

DA neurons, which are lost in Parkinson's disease (PD), are found in the ventral midbrain, prosomere (P) 1, or P2 (Marin et al., 2005), and as such are sometimes referred to as meso-diencephalic DA (mdDA) neurons, in order to distinguish them from other DA neurons in the CNS. The DA neurons include the A8 (retrorubral field), A9 (substantia nigra, SN) and A10 (ventral tegmental area, VTA) populations. It is the SN cells which are especially vulnerable in PD.

Although DA neurons can be found throughout the ventral midbrain (VM) and through P1 and P2 of the diencephalon at E11.5 (Marin et al., 2005), as assessed by the expression of tyrosine hydroxylase (TH), the birthplace of DA neurons is believed to be in the intersection between the *Shh*-expressing floorplate and *Fgf8*-expressing isthmus (Ye et al., 1998) (Figure 8B). This study and several others have shown that Fgf8 and Shh are necessary and sufficient to induce DA neurons (Hynes et al., 1995; Kim et al., 2003).

The regional and temporal identity of DA progenitors has been addressed in several elegant studies, including a comprehensive study by Zervas *et al.* which utilized genetic inducible fate mapping studies to demonstrate that E12.5 mdDA neurons arise from E7.5 (but not E9.5) *Gli1*-expressing cells, as well as from E7.5 or E10.5 *Wnt1*-expressing cells (Zervas et al., 2004). This is confirmed by the observation that *Shh*-expressing cells give rise to DA neurons (Joksimovic et al., 2009). A subsequent study by Bonilla *et al.* showed that *Glast*-expressing FP radial glia, labeled at E10.5, are a source of DA neurons at E12.5 (Bonilla et al., 2008). However, *Gli1* and *Wnt1* can both be found in the entire ventral mesencephalon at around E7.5-E8, and *Wnt1* and Glast are expressed throughout the ventral mesencephalon at E10.5, thus it is difficult to determine exactly where in the A/P axis the cells are born.

DA progenitors in the ventricular zone (VZ) of the VM FP proliferate and migrate towards the marginal zone (MZ) through the intermediate zone (IZ) (Kawano et al., 1995). Several markers aid in the identification of each stage of differentiation (Figure 8E, F). The entire lineage of differentiating DA cells is Lmx1a+.

At E11.5,

- VZ progenitors are Sox2+, Msx1/2+,
- IZ precursors are Lmx1b+, NR4A2+, Pitx3+, and
- MZ DA neurons are Lmx1b+, NR4A2+, Pitx3+, and TH+

(Andersson et al., 2006; Chen et al., 1998; van den Munckhof et al., 2003; Zetterstrom et al., 1996).

1.9 Wnts in the Mammalian Midbrain and DA Neurogenesis

1.9.1 Wnt Ligand and Receptor Expression in VM Development

In order to assess Wnt signaling in the VM, and its possible effects on VM morphogenesis and DA differentiation, it is of considerable interest to define the temporal and spatial expression of Wnt signaling components in the VM. Thankfully, much work has been done to map which Wnt ligands, receptors, and co-receptors are present during VM development.

1.9.1.1 Expression of Wnts in the VM

Wnt1, *Wnt4*, *Wnt5a*, *Wnt7a*, *Wnt7b*, and *Wnt9a* are expressed in the murine FP/VM around E11.5 (Murphy, 2008; Rawal et al., 2006). *Wnt5a* and *Wnt7a* are expressed in the VM as early as E9.5 (Parr et al., 1993).

1.9.1.2 Expression of Wnt Receptors in the VM

Fischer *et al.* have extensively mapped the expression of Fzd receptors in the midbrain during development. At E9.5, *Fzd1/2/3* are expressed ubiquitously in the midbrain, *Fzd6/7* are expressed in the VM, and *Fzd9/10* are expressed in the DM, while *Fzd4/5/8* do not appear to be expressed in the midbrain. At E10.5, expression of *Fzds* is similar to that at E9.5, with the exception that *Fzd9* can now be seen in the VM. Coronal sections reveal that *Fzd10* is expressed in the basal plate (BP) but not the FP at E10.5. By E12.5, expression of *Fzd1/2* is high and *Fzd7/8* is moderate in the VM, *Fzd3* is still ubiquitous in the brain, *Fzd4/5* do not appear to be expressed in the VM, *Fzd6* displays a dispersed, punctuate pattern in the VM and *Fzd9/10* are weakly expressed in the VM (Fischer et al., 2007).

Expression of *Ror1* is seen in the VM at E9.5, whereas expression of *Ror2* is much weaker (Al-Shawi et al., 2001; Matsuda et al., 2001; Oishi et al., 1999). By E10.5 *Ror1* expression has diminished in the VM, but can still be detected by QPCR in the rat VM at E13.5, the equivalent of mouse E11.5. *Ror2* expression cannot be detected by QPCR at any stage (E10.5, E11.5, E13.5, E15.5) in rat VM (Rawal et al., 2006). *Ryk* expression can be seen in the dorsal midline at E9 (Kamitori et al., 1999).

1.9.1.3 Expression of Wnt modulators in the VM

sFRP1 is strongly expressed in the forebrain and hindbrain but not detected in midbrain by whole mount in situ hybridization between E8 and E11. *sFRP2* is strongly expressed in the diencephalon and hindbrain but not detected in midbrain, again by whole mount in situ hybridization between E8 and E10.5 (Hoang et al., 1998; Leimeister et al., 1998). *sFRP4* is not detected in whole mounts of the CNS, but in paraffin sections weak general staining is seen, with slightly higher expression in E14.5 cerebral cortex and E15.5 VZ. At E14.5 low levels of *sFRP1* are detected broadly in the brain.

Interestingly, the three Dvls display slightly different distributions in the developing CNS. At E12.5, Dvl1 is broadly expressed, while Dvl2 is preferentially expressed in the VZ and Dvl3 is preferentially expressed in the MZ (Tissir and Goffinet, 2006).

1.9.1.4 Conclusion

Wnt ligands and Wnt signaling components are expressed in complex overlapping and complementary domains during VM development. While much work has already been done, the exact expression domains of each component should continue to be mapped, in order to obtain a precise temporal and spatial atlas of Wnt components in VM development.

1.9.2 Wnt Function in Mammalian Midbrain/DA Development

1.9.2.1 Wnt1 in Midbrain/DA Development

Wnt function in the development of the VM has been especially addressed in the case of Wnt1 (Danielian and McMahon, 1996; McMahon and Bradley, 1990; McMahon et al., 1992; Prakash et al., 2006). Loss of *Wnt1* leads to a deletion of the midbrain and rhombomere 1 (R1), thus DA neurons are lost in the process. Interestingly, a small residual portion of *Wnt5a*–expressing midbrain remains, which led McMahon and colleagues to suggest that certain VM populations may rely on another Wnt than Wnt1 (McMahon et al., 1992).

The *Wnt1*$^{-/-}$ midbrain defect begins at the 5-somite stage (~E8.25), at which point *Engrailed 1/2* (*En1/2*) expression, which is normal between 1-4 somites, is lost in the mesencephalon, but not the metencephalon (McMahon et al., 1992). By the 23-30 somite stage, *En1/2* is also lost in the metencephalon (McMahon et al., 1992). Ultimately, the loss of *En1/2* expression leads to midbrain and hindbrain deletion, as was clearly demonstrated by experiments in which the *Wnt1*$^{-/-}$ midbrain deletion was rescued by *En1* expression under the regulation of a *Wnt1* enhancer (Danielian and McMahon, 1996).

Further confirmation of the role of *En1/2* in *Wnt1*-regulated midbrain morphogenesis is the fact that *En1;En2* double mutants phenocopy the *Wnt1*$^{-/-}$ midbrain deletion and display a loss of TH+ DA neurons (Liu and Joyner, 2001b; Simon et al., 2001).

Coexpression of *Wnt1* and *Wnt3a* suggested that they serve redundant roles in the myelencephalon and spinal cord (McMahon et al., 1992). Later generation of *Wnt1;Wnt3a* double mutant mice revealed defects in neural crest and CNS progenitor expansion, not seen in either single mutant, confirming redundancy of *Wnt1* and *Wnt3a* in dorsal CNS (Ikeya et al., 1997). Interestingly, Ikeya *et al.* also reported a loss of anterior brain structures in *Wnt1;Wnt3a* double mutant mice, indicating a role for *Wnt3a* in anterior CNS formation as well as in the myelencephalon/spinal cord.

Initially, Wnt1 was not though to have any patterning activity, since *Wnt1* overexpression in the mid-hindbrain region increased the size of the inferior colliculi without affecting patterning (Panhuysen et al., 2004). This was refuted by studies showing that ectopic expression of *Wnt1* under the *En1* promoter was sufficient to induce DA neurons in the aberrantly *Otx2+/Gbx2*- hindbrain, which were not due to

increased proliferation, but instead due to repression of *Nkx2-2* (Prakash et al., 2006). This *Otx2/Nkx2-2* regulation of the DA domain was confirmed in the $Otx2^{-/-}$ FP (*Otx2* floxed out by $En1^{Cre}$), where the resulting ectopic expansion of *Nkx2-2* into the FP repressed the development of DA neurons, evidenced by the fact that ablation of *Nkx2-2* rescued the DA defect in the $Otx2^{-/-}$ FP. In conclusion, in the WT brain, *Wnt1* induces *Otx2* which represses *Nkx2-2* in the FP, *Nkx2-2* would otherwise repress the development of DA neurons and instead induce serotonergic neurons. Prakash *et al.* also showed that *Wnt1* is required for Fgf8-induced DA neuron development.

Castelo-Branco *et al.* confirmed that *Wnt1* was expressed in the VM at the time of DA neurogenesis, and showed that treatment of primary VM cultures with partially purified Wnt1 increased the number of DA neurons up to 3-fold. This increase in DA neurons was primarily due to an increase in proliferation and an increase in the total number of Tuj1+ neurons in the culture (Castelo-Branco et al., 2003). However, Wnt1 probably also has other roles in survival or differentiation, since it increased the proportion of TH+/NR4A2+ cells.

1.9.2.2 Wnt5a in Midbrain/DA Development

Wnt5a has been shown to induce an increase in DA neurons. Similarly to Wnt1, Wnt5a-conditioned medium induces an increase in DA neurons from primary VM cultures (Castelo-Branco et al., 2003). However, the effect of Wnt5a on VM cultures is distinct from the effect of Wnt1. Overall, Wnt5a does not increase the number of proliferating cells, but increases the proportion of BrdU+/NR4A2+ cells, indicating a specific effect on DA progenitor or precursor proliferation. Wnt5a also promotes differentiation and increases the proportion of TH+ DA neurons compared to Tuj1+ neurons or NR4A2+ precursors. Purified HA-tagged Wnt5a also increases the number of DA neurons (Schulte et al., 2005). A Wnt5a-blocking antibody blocked the pro-differentiation effects of indirect co-culture of VM glia with primary VM cultures, indicating that glia can direct differentiation of DA neurons through Wnt5a secretion (Castelo-Branco et al., 2006).

Grafting of *Wnt5a*-transfected VM cultures improved behavior in 6-hydroxy-dopamine (6-OHDA)-lesioned rats (animal models of Parkinson's disease), compared to control-transfected grafts (Parish et al., 2008). Wnt5a-treated parthenogenetic primate embryonic stem cells also improved DA neuron differentiation, graft survival and behavioral recovery in 6-OHDA -lesioned rats (Sanchez-Pernaute et al., 2008).

Meninges can also induce DA differentiation of ES cells, in a manner similar to PA6 feeder cells which are often used in ES cell cultures. The pro-DA effect is strongest with E18 meninges which, like PA6 cells, express high levels of *Wnt5a*. This DA differentiation effect can be blocked using a Wnt5a-blocking antibody (Hayashi et al., 2008).

Given the dramatic effect of Wnt5a on DA neuron differentiation *in vitro*, analysis of the role of *Wnt5a in vivo* would be a compelling subject. For more information on the role of *Wnt5a in vivo*, please see Results and Discussion and/or Paper II and Paper III.

1.9.2.3 LRP6 in Midbrain/DA Development

LRP6 is a Wnt-β-catenin receptor, and as such, could be responsible for the positive effects of Wnt1 on DA differentiation. However, *LRP5* and *LRP6* both are widely expressed in the developing embryo and *LRP6* exhibits extensive redundancy with *LRP5* (Kelly et al., 2004; Pinson et al., 2000). While *LRP6* knockouts manifest a less distinct isthmus (Pinson et al., 2000), the phenotype is not as dramatic as the midbrain/R1 deletion seen in *Wnt1*$^{-/-}$ mice (McMahon and Bradley, 1990), indicating that another receptor, such as *LRP5*, is probably partially compensating for loss of *LRP6* in the midbrain-hindbrain boundary (MHB). Analysis of the *LRP5*$^{-/-}$*;LRP6*$^{-/-}$ midbrain is precluded by the fact that *LRP5*$^{-/-}$*;LRP6*$^{-/-}$ mice fail to establish a primitive streak and thus do not develop past gastrulation (Kelly et al., 2004). Therefore it would be of considerable interest to generate VM FP-specific *LRP5;LRP6* knockouts, perhaps by floxing out *LRP5* and *LRP6* with the existing *En1*^{Cre} mice. This mouse could answer the question of whether LRP5/6-binding/β–catenin activating Wnts other than Wnt1 contribute to DA development in the midbrain.

1.9.2.4 sFRPs in Midbrain/DA Development

Both *sFRP1*$^{-/-}$ and *sFRP2*$^{-/-}$ mice survive into adulthood. Each mutant displays some minor abnormalities such as syndactyly (Morello et al., 2008), while double knockouts grossly phenocopy *Wnt5a*$^{-/-}$ mice (Satoh et al., 2006).

Although no midbrain/DA phenotype has been described for *sFRP2*$^{-/-}$ mice, the role of *sFRP2* in repressing *Gli1*-induced *Nkx2-2* in the spinal cord suggests that sFRP2 may be of interest in DA neurogenesis (Lei et al., 2006). This study showed that *Nkx2-2* expression is negatively regulated by *sFRP2* and positively regulated by *Gli*. Furthermore, the CRD of sFRP2 was necessary and sufficient to inhibit the development of *Gli*-induced Nkx2-2+ cells. (See also the discussion on Wnt1 induction of *Otx2*, Otx2 repression of *Nkx2-2* in the VM on page 23).

Prior to this thesis work, no role had been described for sFRP1 or sFRP2 in DA neurogenesis or midbrain development.

1.9.2.5 Other Wnt Components in DA Development

Apart from Wnt5a and Wnt1, which are now relatively well-characterized as pro-DA morphogens, other Wnts or Wnt regulators have been implicated in DA differentiation.

Although DA neurons are born in a different temporal and spatial pattern in Zebrafish; DA progenitors are in the diencephalon and post-mitotic by 12-14 hours post fertilization; it would appear that these DA progenitors are also susceptible to Wnt signaling. Russek-Blum *et al.* found that mRNA injections of *Dkk1*, at the 1-cell stage, specifically increased DA cell numbers, while MO knockdown of *Wnt8b* or *Fzd8a* decreased DA cell number (Russek-Blum et al., 2008). Interestingly, this study

proposed a model in which Wnt8b/Fzd8 signaling restricts DA progenitor pool size already during gastrulation (Russek-Blum et al., 2008).

1.9.3 Conclusion

In sum, the multitude of Wnt ligands and receptors, many of which are expressed in the developing VM, activate a vast array of signaling pathways to elicit cellular responses as diverse as CE or differentiation. The exact constellation of available receptors and signaling components must contribute to fine-tuning the cellular responses to a specific Wnt, and it is with increased knowledge of Wnt presence and function in midbrain development that we will gain insight into the mechanisms behind Wnt-induced DA differentiation.

2 Aims

Ultimately, the goal of research must be to serve mankind (and not only the researcher's curiosity!).

In the broader sense, the goal of this thesis work was to investigate the role of Wnt signaling in DA neuron differentiation, in order to develop therapies/cures for Parkinson's disease.

To bring us a few steps closer to understanding Wnt signaling and its impact on DA neuron differentiation, the following specific questions were asked:

1. Is LRP6 purely a Wnt/β-catenin receptor, or does it interact with non-β-catenin Wnts such as Wnt5a or Wnt11?

2. Does Wnt5a contribute to DA development *in vivo*?

3. Does Wnt1 interact with Wnt5a or "canonical" Wnts in the developing VM?

4. Do Wnt modulators, such as sFRPs, affect DA differentiation or CE?

3 Results & Discussion

3.1 Paper I

The extracellular domain of LRP5/6 inhibits non-canonical Wnt signaling *in vivo*

Prior to beginning this thesis work, the role of LRP5 and LRP6 in mediating Wnt/β-catenin signaling had been extensively characterized by other labs (please see LRP sections for more information). Wnt signaling specificity was assumed to be partially dictated by LRPs, and it was assumed that these exclusively bound and modulated canonical Wnts such as Wnt1 and Wnt3a.

3.1.1 Do LRP6 and Wnt5a interact *in vitro*?

Our first experiments set out to test whether Wnt5a could titrate out Wnt3a-induced cellular responses in our system. Indeed, increasing doses of Wnt5a dampened Wnt3a-induced phosphorylation of LRP6. Conversely, while Wnt5a was capable of repressing Wnt3a/LRP6 signaling, LRP6 was able to repress Rac signaling (which can be activated by Wnt5a). Together, these results indicated an interaction between LRP6 and Wnt5a, and an intriguing question was whether the mutual inhibition could be early in the signaling cascade, possibly even at the level of the LRP6 receptor itself. To our surprise, IgG pulldown of LRP6N-Fc (a tagged construct containing the extracellular domain of LRP6) revealed that the extracellular domain of LRP6 was capable of binding Wnt5a. Immunoprecipitation (IP) of (HA-tagged) Wnt5a revealed that the E1-E2 (Wnt binding) domains were necessary for this binding, while the E3-E4 (Dkk-binding) domains were dispensable. Also, the extracellular domains were necessary for the reduction in active Rac1. Thus, it seemed that LRP6 could bind Wnt5a, and that this interaction might have physiological consequences.

3.1.2 Do *LRP6* and *Wnt5a* interact *in vivo*?

To assess the interaction between LRP6 and Wnt5a *in vivo* we used two model systems. First, we utilized Keller explants, a well-established model for testing PCP/CE effects. Second, we generated and analyzed the phenotype of compound *Wnt5a;LRP6* mutant mice by breeding extant single heterozygous mice.

Xenopus embryos offer many advantages over mammalian systems thanks to the developing zygote/embryo's accessibility, observability and manipulability. Keller explants provide a system in which it is possible to observe gastrulation movements in a 2-dimensional system, rather than try to observe them in the full 3-dimensional embryo (Keller and Danilchik, 1988). A rectangle of dorsal mesendoderm and ectoderm from a pre-gastrulation *Xenopus* embryo is dissected out and cultured, either as a single sheet (open-face explant) or as two sheets sandwiched together (closed sandwich), beneath a coverslip or under a glass bridge. Rather than involuting beneath the ectoderm, the mesoderm elongates and narrows in a process that reflects CE

movements. *Xenopus* is easily manipulated to study the role of a specific gene, by mRNA overexpression or MO knockdown, and subsequent generation of Keller explants from manipulated zygotes can demonstrate said gene's role in PCP/CE.

Overexpression or knockdown of *LRP5* repressed explant elongation and constriction, while *LRP6* LOF and GOF mostly affected constriction. Neither of these *LRP5/LRP6* effects were dependant on β–catenin since β–catenin overexpression was unable to rescue *LRP5* knockdown. Instead, depletion of *Wnt5a*, *Wnt11* or expression of dominant-negative *Rac1* or *RhoA* rescued *LRP5* knockdown. However, the constriction induced by loss of *LRP6* was rescued by *Wnt11* knockdown, and not *Wnt5a* knockdown. In sum, this indicated that the mechanism behind *LRP5*/*6* LOF CE defects was non-β-catenin Wnt signaling GOF.

Tahinci *et al.* recently demonstrated that both GOF and LOF of *LRP6* result in CE defects in *Xenopus*, and that this phenotype is not seen in β–catenin injected controls. Also, they show that in *LRP6*-injected animal caps, LRP6 is first uniformly distributed, but becomes polarized in response to activin treatment (activin also induces explant elongation, a functional test of CE). In contrast to our findings in Paper I, Tahinci *et al.* show that the intracellular domain (ICD) of LRP6 is sufficient to mediate its CE-antagonistic effect, and that a specific 36 amino acid fragment of the ICD (which does not contain any of the 5 the PPP(S/T)P motifs) is sufficient to mediate this effect. This fragment does not induce Wnt/β-catenin GOF, nor does it oppose *Wnt8*-induced expression of specific β-catenin target genes in animal caps. It reportedly potentiated full-length *LRP6*-mediated blocking of CE, and therefore Tahinci *et al.* proposed that the ICD of LRP6 recapitulated *LRP6* effects on CE. Importantly, these investigations failed to provide any data concerning the interaction between the ICD of LRP6 and Wnt5a or Wnt11. It would be of interest to see whether this 36 amino acid fragment is sufficient to oppose *Wnt11*-induced CE effects, as would be expected if the 36aa fragment is sufficient to mimic full-length *LRP6* effects on CE.

Then, we turned to the mouse to examine whether *Wnt5a* and *LRP6* interacted *in vivo*, in a mammalian system. Mating of $Wnt5a^{+/-}$ and $LRP6^{+/-}$ mice generated the expected frequencies of adult $Wnt5a^{+/-}$; $LRP6^{+/-}$ mice. However, when $Wnt5a^{+/-}$; $LRP6^{+/-}$ mice were intercrossed, the resulting embryos revealed a genetic interaction between *Wnt5a* and *LRP6*. While $Wnt5a^{-/-}$ and $LRP6^{-/-}$ mice die perinatally or soon after birth, $Wnt5a^{-/-}$;$LRP6^{-/-}$ mice could only be found up to E10.5, at which point they were severely under-developed, had failed to turn, and displayed an enlarged heart tube which had not looped. Despite these gross malformations, the exencephaly which is seen in 30% of $LRP6^{-/-}$ mice at E10.5 was rescued in a dose-dependant manner by loss of *Wnt5a*. Therefore, in some cases, Wnt5a and LRP6 act in opposing pathways to control e.g. neural tube closure, but in other cases it would appear that they work together and thus the double knockouts display severe phenotypes not seen in either single mutant.

In both *Xenopus* and mouse models, LOF of *LRP6* was rescued by LOF of *Wnt5a*, *Wnt11* or their signaling components, indicating that LRP6 is a physiologically relevant inhibitor of non-canonical Wnt signaling. Our results support a model in which LRP6 acts to attenuate Wnt5a or Wnt11 signaling *in vivo*, thus restricting non-β-catenin Wnt functions such as CE, summarized in Figure 9.

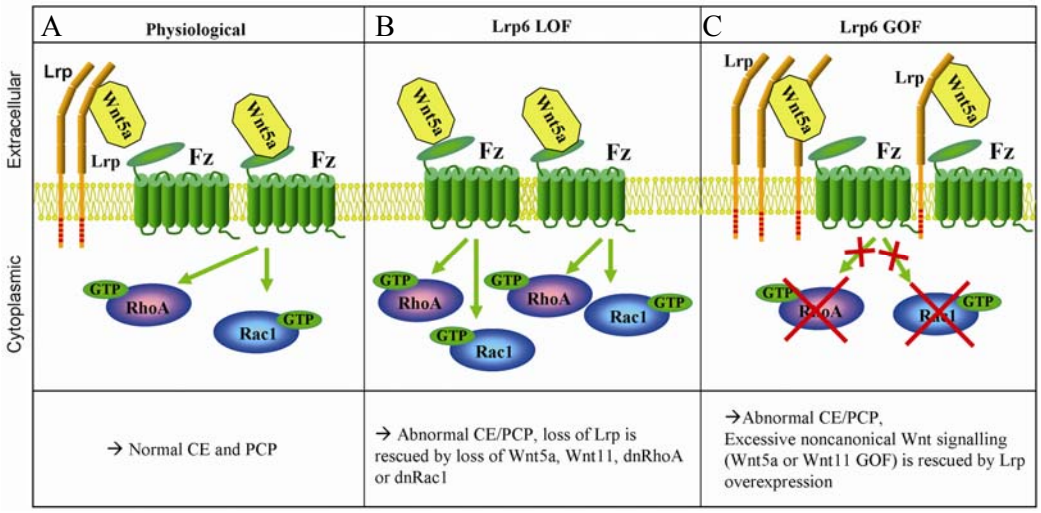

Figure 9. Proposed model of LRP6 inhibition of Wnt5a/Wnt11 signaling
In the wild type mouse, under normal conditions, a balance between Wnt5a and LRP6 allows moderate Wnt5a signaling, as some Wnt5a is scavenged/antagonized by LRP6. In *LRP6*$^{-/-}$ mice, or *LRP5/6* morpholino (MO)-injected *Xenopus* explants, an increase in non-LRP-bound Wnt5a/Wnt11 might lead to an increase in Wnt5a/Wnt11 signaling, as seen by typical PCP/CE defects. This is supported by the fact that the PCP/CE defects are rescued by ablation of Wnt5a, MO knockdown of Wnt5a or Wnt11, and dominant negative forms of RhoA or Rac1. **C.** Conversely, LRP5/6-injected Keller explants, increased scavenging of Wnt5a or Wnt11 would lead to decreased RhoA/Rac signaling. This defect is rescued by overexpression of Wnt5a or Wnt11.

3.2 Papers II&III:

Wnt5a Regulates Ventral Midbrain Morphogenesis and the Development of A9–A10 DA Cells *in vivo*

3.2.1 *Wnt5a* in DA development *in vivo*

Already in 2003, Castelo-Branco *et al.* discovered that Wnt5a promoted the differentiation of Nurr1+ (also known as NR4A2) DA precursors into TH+;NR4A2+ DA neurons (Castelo-Branco et al., 2003), which prompted us to examine the role of *Wnt5a in vivo*. The robust effects that had been seen *in vitro* with conditioned media, as well as with purified recombinant Wnt5a, indicated that a dramatic loss of DA neurons might be found in the *Wnt5a*$^{-/-}$ mice. We were, at first, disappointed; the number of DA neurons appeared normal, or even increased, at various stages.

We therefore decided to rigorously examine the *Wnt5a*$^{-/-}$ VM phenotype, in order to determine what exactly *Wnt5a* was, or was not, doing in the VM during development, and assess whether *Wnt5a* truly was dispensable for DA neurogenesis.

First, we found that *Wnt5a* was expressed in a gradient, with high expression in the rostral VM/P1 boundary, and weakly or not at all in caudal VM at E9.5 and E10.5. Between E11.5 and E13.5, the expression of *Wnt5a* spread caudally into all three VZ/IZ/MZ domains, overlapping with the domain in which DA neurons are born and mature. Wnt5a was known to act in PCP/CE, and usually secreted molecules must establish a gradient to be able to act on PCP/CE. Therefore this graded expression

pattern suggested that we should not only examine DA neurogenesis *per se*, but also PCP/CE in the VM. It is also interesting to note, on the background of the meningeal Wnt5a/DA neurogenesis paper (Hayashi et al., 2008), that *Wnt5a* expression can be seen in the meninges at E18.5, confirming the observation by Hayashi *et al.* that expression of *Wnt5a* is highest in meninges at this stage. QPCRs which were not included in the final version of the manuscript revealed that *Wnt5a* expression in the VM was highest at E9.5, and then decreased at later stages.

After counting every second 14μm, TH-containing, VM/P1 section, we concluded that there was no difference in the number of DA neurons at any stage, except a minor transient increase at E14.5, which was rescued by E17.5/E18.5. Interestingly, the A/P and D/V distribution of the TH+ cells was different in *Wnt5a*$^{-/-}$ mice, which explained the previously reported increase in VM DA neurons (E. Minina, 2006). We interpret this result as a sampling error, as sections from the most intermediate/caudal levels would have clearly given this result. The DA neuron population was shortened in the A/P axis, but laterally expanded; creating the illusion of more TH+ cells if one analyzed limited identical numbers of sections for both WT and *Wnt5a*$^{-/-}$, or examined TH+ cells/section in the *Wnt5a*$^{-/-}$ mice.

Interestingly, quantification of BrdU+, Ki67+ or NR4A2+ cells revealed that there was an increase in VZ progenitor proliferation and NR4A2+ precursors in the entire VM A/P axis, which was not translated into an increase in DA neurons at E12.5. Thus, the proportion of TH+/NR4A2+ cells was actually decreased in the *Wnt5a*$^{-/-}$ mice at this stage, not as a result of decreased TH+ cell number, but as a result of increased NR4A2+ cell numbers.

3.2.2 *Wnt5a* in PCP/CE *in vivo*

During primary neurulation, cells in the medial hinge point (MHP) in the neural plate must assume a pyramidal shape and narrow the midline into a groove, while the adjacent neural folds must elevate and converge to form the neural tube. In PCP/CE mutants, defects in either of these two processes (MHP bending or neural fold convergence) can lead to neural tube defects. For example, *Vangl* mutants display neural tube closure defects, and this is associated with a flattened medial ventricular zone and laterally expanded *Shh* expression (Kibar et al., 2001; Murdoch et al., 2001).

Neural tube closure defects had been noted in *Wnt5a*$^{-/-}$ mice (Qian et al., 2007), but the importance of *Wnt5a* in PCP/CE in the CNS had not been investigated. In fact, CE/PCP defects in specific neuronal populations had not been described in the brain previously (although defects in axonal projections had been linked to PCP/CE).

Our analysis of the *Wnt5a*$^{-/-}$ VM revealed that the midline VZ invagination angle was less acute than in WT, it was “flattened”. Also, the lateral DA population expansion seen in the *Wnt5a*$^{-/-}$ mice was confirmed by all ventral markers examined: *Shh*, *Foxa2*, NR4A2, Lmx1a, *DAT*, *Pitx3*, *Ngn1*, and *Ngn2*. This was unlikely to be a patterning defect, since dorsal markers were unaffected. In sum, VM VZ flattening and *Shh* lateral expansion phenocopied previously reported neural tube phenotypes, and this PCP/CE defect resulted in a later redistribution of the DA neuron population (Figure 10).

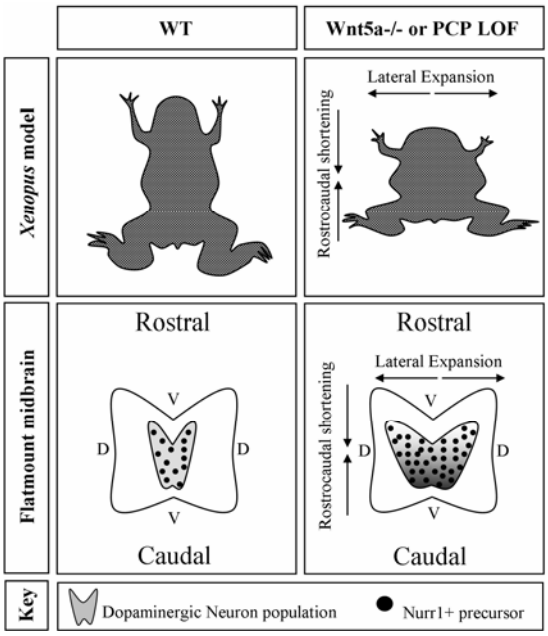

Figure 10. Typical PCP/CE defects
In a wild type (WT) frog, CE movements will lead to a frog with normal morphology. When CE is disrupted, a rostrocaudal shortening and lateral expansion will lead to a shorter, wider frog. Similarly, in the *Wnt5a*$^{-/-}$ midbrain, we found a rostrocaudal shortening and lateral expansion of the DA neuron nucleus, as depicted by a redistribution of the gray coloration of the DA neuron population in this scheme. (The number of Nr4a2 precursors was also increased in *Wnt5a*$^{-/-}$ mice, which is not necessarily linked to PCP/CE)

3.2.3 Wnt5a signaling in DA differentiation

While the Wnt/β-catenin pathway has been well established, the downstream mediators of Wnt5a-induced DA differentiation had not been reported prior to the onset of this thesis work. It had been established that Wnt5a induced hyper-phosphorylation of Dvl (Schulte et al., 2005), but downstream signals were unidentified. We therefore examined whether Wnt5a activated small Rho GTPases, intracellular mediators of CE/PCP, and found that Wnt5a activated Rac1, but not RhoA or cdc42, in a DA neuron cell line (SN4741). Surprisingly, this activation of a known CE effecter was necessary for DA differentiation in primary cultures, as shown by the fact that blocking Rac1 with NSC23766 inhibited Wnt5a-induced DA differentiation. However, similar studies have shown that CK1 inhibition blocks Wnt5a-induced DA differentiation (Bryja et al., 2007b; Parish et al., 2008). Since CK1 activity is dispensable for Rac1 activation in SN cells (Bryja et al., 2008), it is possible that Wnt5a activates multiple pathways that are required for DA differentiation in primary cells (also assuming that the inhibitors in each experiment are specific enough). *In situ hybridization* for *Fzd* receptors in the midbrain at different stages (Fischer et al., 2007), and QPCR comparing TH-GFP+ VM populations with TH-GFP- VM populations (Rawal et al., 2006) has revealed that *Fzds* are differentially expressed in proliferating or differentiating VM, which would allow cells to respond differentially to same Wnt as they differentiate. It also means that using the SN4741 cell line, an immortalized DA neuron, is probably not optimal for assessing Wnt signaling in immature progenitors.

Wnt5a can activate β–catenin or induce axis duplication in the presence of overexpressed Fzd4 or Fzd5, respectively. At present, it is unclear whether *Fzd4* or *Fzd5* is robustly expressed in the VM: both are detectable in the developing rat VM by QPCR, and in mouse TH-GFP+ and TH-GFP- populations at E11.5 and E13.5. However, they are not seen by *in situ hybridization* at E9.5, E10.5, or E12.5 in the mouse (Fischer et al., 2007). Regardless, with the differential VZ/IZ/MZ expression of *Fzds* in the VM, it seems possible that Wnt5a may elicit different cellular responses in

VZ than in MZ. Indeed, Rawal *et al.* found that *Fzd9* was expressed in the VZ at E11.5 (confirmed by Fischer *et al.* at E12.5) and inhibited Wnt5a-induced hyperphosphorylation of Dvl (a caveat is that this was done in SN4741 cells). Therefore, it is possible that in the VZ, in the presence of Fzd9, Wnt5a induces Rac1, which does not require Dvl hyperphosphorylation, but that in the MZ, where *Fzd9* is not expressed, Wnt5a also activates other pathways through hyperphosphorylated Dvl. Because Wnt5a requires both CK1 activity and Rac1 activity to mediate its effect on DA differentiation *in vitro*, it would appear that multiple pathways are activated and required for Wnt5a-induced DA differentiation.

Inhibition of JNK has been shown to increase the survival of TH+ DA neurons (Rawal et al., 2007), and JNK was not activated by Wnt5a during DA differentiation (unpublished). However, since JNK contributes to nuclear translocation of active β–catenin, it remains to be seen what role, if any, JNK plays in DA differentiation (Figure 11). Other unpublished work that was performed for this paper showed that activation of JNK by sorbitol also increased the number of DA neurons in primary cultures. However, sorbitol is not a very selective JNK activator and it is therefore difficult to draw firm conclusions from this.

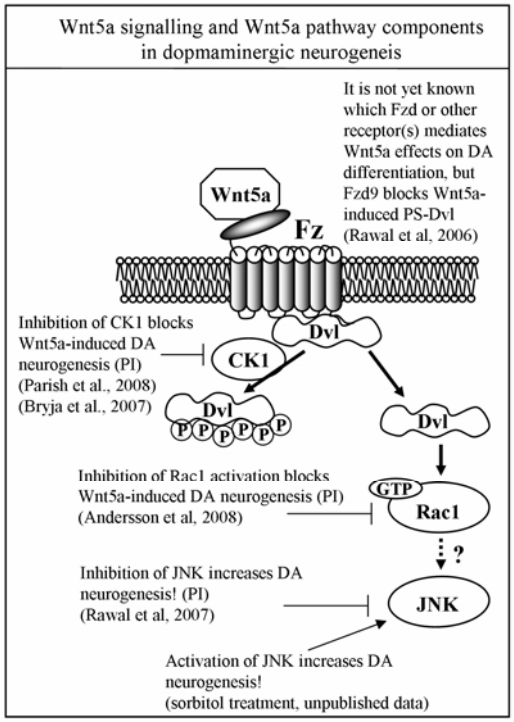

Figure 11. Wnt5a signaling components in DA neurogenesis.
Wnt5a induces Dvl hyperphosphorylation by CK1 to produce phosphorylated and mobility-shifted PS-Dvl. While PS-Dvl is not required for Rac1 activation, blocking of either Rac1 activaty or CK1 activity with pharmacological inhibitors will abrogate Wnt5a-induced DA differentiation. Interestingly, both sorbitol-induced activation and pharmacological inhibition of JNK increases the number of DA neurons. Of these two, the pharmacological inhibition is the more specific. Future studies will hopefully reveal whether active JNK contributes to DA neurogenesis.

Canonical and Non-canonical Wnts synergize in the generation of midbrain DA neurons

3.2.4 The *Wnt1*$^{-/-}$ Floorplate

Extensive studies on the *Wnt1*$^{-/-}$ mice have revealed a deletion of the midbrain and rhombomere 1, and thus a defect in DA neuron number. While previous results from our lab have shown that Wnt1 can increase the number of DA neurons in primary

cultures, there has remained a question of whether other Wnts contribute to the development of DA neurons *in vivo*. Also, the previous experiments (Paper II) showing no difference in DA neuron number in the *Wnt5a*$^{-/-}$ mice, begged the question of whether another *Wnt* was compensating the loss of *Wnt5a*. A small number of TH+ cells remain in the *Wnt1*$^{-/-}$ residual MB, and we set out to investigate whether *Wnt5a* or a "canonical" *Wnt* contributed to these residual cells, by generating and analyzing *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ and *Wnt1*$^{-/-}$*;LRP6*$^{-/-}$ mice.

Most of the reported analyses on the *Wnt1*$^{-/-}$ mice have been done in sagittal sections, precluding the medio-lateral analysis of the VM FP. We first examined whether *Shh* and *Foxa2* were normally expressed in the VM of *Wnt1*$^{-/-}$ mice. While the expression pattern was slightly disrupted, both were expressed in the floor plate (FP) and basal plate (BP) of the VM at E11.5. *Ngn2* and *Mash1* were lost from the FP, and the few remaining TH+ cells were found in the BP area. In fact, all other DA lineage markers examined were found in the BP, including Lmx1a and NR4A2, indicating that *Wnt1* is necessary for the DA neurogenic identity of the FP.

A necessary remaining experiment is to examine the expression of *Nkx2-2*, to confirm the indirect regulation of *Nkx2-2* by *Wnt1*. If this regulation is also seen in the residual midbrain, *ectopic Nkx2-2* might lead to an increase in serotonergic neurons, as reported by Prakash *et al.*, and would also explain the FP loss of DAergic markers. However, the residual FP is extremely thin and mostly Tuj1-negative, indicating that very few, if any, neurons are present in the FP.

3.2.5 Interaction of *Wnt1* with *LRP6* and *Wnt5a*

Wnt1$^{-/-}$*;LRP6*$^{-/-}$ mice displayed a deletion of anterior CNS structures, similar to the previously described *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mice, indicating that LRP6 is probably the receptor for Wnt3a in the anterior CNS. *LRP6*$^{-/-}$ mice are known to have dorsal thalamus defects, and the *Wnt1*$^{-/-}$*;LRP6*$^{-/-}$ mice completely lacked a dorsal thalamus.

Wnt1$^{-/-}$*;Wnt5a*$^{-/-}$ mice did not morphologically look worse than a combination of their separate phenotypes.

Quantification of DA neuron numbers revealed that ablation of either *LRP6* or *Wnt5a* on a Wnt1$^{-/-}$ background reduced the number of DA neurons by an ulterior 70%. The decrease in DA neurons in the *Wnt1*$^{-/-}$*;LRP6*$^{-/-}$ could be due to a developmental delay contributed by the loss of *LRP6* (as found in some single *LRP6*$^{-/-}$ mice), but could also reflect the contribution of another LRP6-binding Wnt to the generation of DA neurons, or, in light of Paper I, a Wnt5a GOF. The loss of DA neurons in the *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice was also intriguing. Loss of *Wnt5a* did not decrease the number of DA neurons in an otherwise WT mouse, but in a *Wnt1*-compromised mouse, loss of *Wnt5a* did decrease the number of TH+ DA neurons. This suggested that perhaps *Wnt5a* does contribute to DA neurogenesis *in vivo*, but that *Wnt1* is sufficient to rescue the loss of *Wnt5a* in the *Wnt5a*$^{-/-}$ single mutant mice. Also, if part of the phenotype of the *Wnt5a*$^{-/-}$ mice is due to *Wnt1* rescue, *Wnt1* may contribute to the increase in proliferation which was observed at E11.5. As reported in Paper II, we did not detect a gain of β–catenin

signaling at E12.5, but it is entirely possible that we missed the correct time period, and that an earlier regulation of β-catenin contributes to increased proliferation in the Wnt5a$^{-/-}$ mice. The fact that the increase in Nr4a2+ cell numbers seen in the *Wnt5a*$^{-/-}$ VM is not seen in the *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ VM (compared to *Wnt1*$^{-/-}$ alone), also suggests that proliferation and NR4A2+ precursor number may be regulated by *Wnt1*. Loss of *Wnt5a* in the *Wnt1*$^{-/-}$ VM rescued the decrease in proliferation in the FP, but had little effect on proliferation in the BP (overlapping with Nr4a2), suggesting that the regulation of proliferation is not specific for DA progenitors/Nr4a2 precursors, and instead is an indirect effect.

Previous *in vitro* data indicated that Wnt1 signaling preferentially promoted proliferation of DA progenitors/precursors, and Wnt5a promoted differentiation of Nr4a2 precursors into DA neurons (Castelo-Branco et al., 2003). Our recent *in vivo* results indicate that both *Wnt1* and *Wnt5a* contribute to DA neurogenesis. We therefore set out to use β-catenin activating Wnt3a and non-β-catenin activating Wnt5a to improve DA differentiation protocols. The idea was to use Wnt3a, during a first passage for proliferation and expansion of the culture, and then Wnt5a in a second phase for differentiation. We tested different combinations of Wnt3a/Wnt5a and found that sequential treatment indeed increased the number of DA neurons more than either treatment alone. Interestingly, intermittent Wnt treatment proved most efficacious, and a Wnt-free step was required to maximize DA neuron yields. Considering our first results, showing that Wnt3a and Wnt5a can compete for LRP6, it is possible that they use some of the same machinery to exert their effects, and that it is necessary to completely remove all Wnt3a to allow Wnt5a to signal.

3.3 Paper IV

sFRPs in DA neuron development

3.3.1 *sFRP* expression in the developing midbrain

The tight regulation of Wnt signaling by Wnt antagonists during neural development prompted us to examine the function of *sFRPs* in the developing VM. Previous studies did not report *sFRPs* in the developing ventral midbrain. We found that expression of both *sFRP1* and *sFRP2* was highest in lateral midbrain, but lower levels of expression of *sFRP2* were detected throughout the VZ of the midbrain, and a low level of *sFRP1* was detected in the entire midbrain.

Furthermore, when we examined the function of *sFRP1* and *sFRP2 in vivo*, we found that *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice phenocopy *Wnt5a*$^{-/-}$ mice, with A/P axis shortening, and limb outgrowth defects. We therefore set out to examine whether sFRPs could be used to modulate DA differentiation *in vitro*, and whether *sFRPs* modulated DA differentiation or CE/PCP in the VM.

3.3.2 sFRP signaling and *in vitro* effects

sFRP1 and 2 have previously been ascribed both identical and contrary roles in Wnt signaling. In SN4741 cells, we found that sFRP1 and sFRP2 both opposed Wnt3a- or Wnt5a-induced phosphorylation of Dvl. The effects of sFRPs on β–catenin phosphorylation status were less clear, so we also performed TopFlash assays with combinations of Wnt3a and sFRPs. In contrast to our biochemical results, we found that sFRP2 increased TopFlash activity by 50% when combined with Wnt3a (N=6, $p<0.05$), but had no effect in the absence of Wnt. (sFRP1 displayed similar tendencies but was not significant at N=4). These conflicting results are hard to explain, but in the first set of experiments, Dvl phosphorylation was assessed at 2 hours; whereas the increase in TopFlash reporter activity by sFRP1 was assessed at 18-24 hours. It is possible that sFRPs inhibit the slow (2 hour) dephosphorylation/activation of β–catenin, but not the fast activation previously reported (Bryja et al., 2007a),. Alternatively, TopFlash activation is not a result of β–catenin dephosphorylation in this experiment. Since *sFRP1* has the same effect in *Xenopus* animal caps as *Wnt5a* (blocking activin-induced CE), and *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice phenocopy *Wnt5a*$^{-/-}$ mice, it is tempting to propose that sFRPs, like Wnt5a, may activate Rac1. If this were true, sFRP activation of Rac1, in association with Wnt3a-induced stabilization of β–catenin, would lead to increased TopFlash Luciferase reporter activity. This possibility remains to be tested.

In primary cultures sFRP1 and sFRP2 had very different effects. Disappointingly, we could not detect any robust effect of sFRP2 on primary cultures, and sFRP1 killed most cells in the cultures. High doses of sFRPs were used in the primary cultures, since we had found that high doses were required to block Wnt3a- or Wnt5a-induced phosphorylation of Dvl. Lower doses of sFRPs had variable effects, and at lower doses sFRP1 was not toxic. Although a range of concentrations were tested (100ng/ml-5μg/ml) TH+ DA neuron numbers were never increased by more than ca 20%.

3.3.3 *sFRPs* in VM Development

Due to our earlier results in the *Wnt5a*$^{-/-}$ VM, we decided to investigate several aspects of VM development in *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice: DA cell numbers, VZ VM angle, the *Shh* domain and the A/P and lateral distribution of the DA population.

One caveat of these experiments is that the *sFRP* knockout mice were generated from separate breedings such that only *sFRP1*$^{-/-}$ and *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice were littermates. For this reason, it is difficult to draw conclusions from non-littermate mice. Stage-matching was limited by defects in A/P elongation and limb formation. For these reasons, we focused our analysis on *sFRP1*$^{-/-}$ and *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ littermate mice.

Analysis of the *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ midbrain revealed an uncoupling between the PCP/CE phenotype and the DA neuron differentiation phenotype. While *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice display the same VZ VM flattening and *Shh*/TH lateral expansion as the *Wnt5a*$^{-/-}$ mice, they did not reveal a clear increase in overall proliferation, NR4A2+ precursors or TH+ DA neurons. However, a modest decrease (compared to the *Wnt5a*$^{-/-}$ mice) was detected in the proportion of TH+/Nurr1+ cells. This could indicate that *Wnt5a* may be regulating PCP/CE and DA differentiation separately in the VM.

Finally, it would be of interest to examine the expression of *Nkx2-2* in the *sFRP2*$^{-/-}$ and *sFRP1*$^{-/-}$*;sFRP2*$^{-/-}$ mice, to assess whether *sFRP2* regulates *Nkx2-2* in the VM, in a similar fashion as in the spinal cord. The described regulation of Nkx2-2 by Wnt1 provides another level at which sFRPs and Wnts may interact. Finally, since *sFRPs* are highly expressed in lateral compartments in the VM, it is more likely that they are affecting cellular processes in these compartments. Investigation of the AP phenotypes of the *sFRP* knockout mice could be more revealing.

4 Conclusions

Based on the work presented in this thesis, I propose the following conclusions:

1. The extracellular domain of LRP6 is capable of binding Wnt5a, and negatively modulates Wnt5a and Wnt11 signaling *in vivo*.

2. Wnt5a is not required for the development of DA neurons in the otherwise wild type VM, but may contribute to differentiation, the NR4A2+Th- → NR4A2+Th+ transition.

3. *Wnt5a* is required for adequate CE in the ventral midbrain.

4. The mild *Wnt5a*$^{-/-}$ DA neuron phenotype may be due to *Wnt1* compensation, since *Wnt1*$^{-/-}$*;Wnt5a*$^{-/-}$ mice have a grosser DA neuron defect than *Wnt1*$^{-/-}$ single mutants.

5. *Wnt1* is required for the neurogenic potential of the FP, and as such, its DA neuron potential.

6. β-catenin-inducing Wnt3a and non–β-catenin-inducing Wnt5a can be combined to improve DA differentiation protocols.

7. *sFRP1* and *sFRP2* are redundantly required for CE in the ventral midbrain.

General Conclusion

The work in this thesis has expanded our knowledge regarding Wnt signaling in midbrain and dopaminergic neuron development. The sum of this work will hopefully contribute to new protocols, some currently being tested, for differentiation of stem cells into DA neurons, with the aim of devising a successful cell replacement therapy. Furthermore, Wnt signaling defects are associated with several cancers and even Alzheimer's disease, meaning that the results presented herein may contribute to improved health, not only in the perspective of cell replacement therapy for PD patients, but also in a wider perspective.

5 Popular Science Summary

During embryonic development, a set of signaling pathways controlled by extracellular molecules supply temporal and spatial information to each cell, such that they may multiply and mature appropriately. Signaling pathways that are used during development to direct the division, migration and maturation of a single cell to a complex organ or organism, may be borrowed in the laboratory to direct naïve cells to adopt a fate of one's choosing. Therefore, the study of development supplies us with a blueprint of which signals direct cells towards specific fates.

This thesis work has aimed to determine how specific molecules, called Wnts, contribute to the development of the midbrain. The midbrain contains dopaminergic neurons, which die in Parkinson's disease. Therefore knowledge of how this part of the brain develops, and how specific molecules direct the development of specific cell types, might help us to develop cures or therapies for Parkinson's disease.

It was previously known that Wnt1 and Wnt5a could increase the number of DA neurons in cultures of midbrain cells. Therefore, we set out to study signaling by Wnt5a and the contribution of these two Wnts to midbrain development. We also studied the role of two secreted molecules, sFRP1 and sFRP2, that are reported to block Wnt signaling.

We found that a receptor, called LRP6, thought to only bind certain Wnt molecules, unexpectedly bound Wnt5a and thereby blocked a signaling pathway which controls morphological changes. This has implications for the signaling pathways possibly activated by Wnt5a, since we now know that Wnt5a can bind more receptors than previously thought.

We also found that Wnt5a controls morphological changes in the midbrain, such that when you knock out this gene (remove it from the animal), the midbrain is shorter and broader. However, since Wnt5a can stimulate the development of DA neurons in cultures, we expected that removing this gene would decrease the number of DA neurons in the mouse. This was not the case.

We then studied how loss of Wnt1 affected midbrain development. We found that, without Wnt1, the ventral part of the midbrain lost its ability to produce DA neurons and is therefore unusually thin. Also, if you remove both Wnt1 and Wnt5a at the same time, you get even fewer neurons, which means that Wnt5a and Wnt1 work together to make DA neurons.

Based on this knowledge, we designed a protocol to generate DA neurons from midbrain cultures using Wnt3a (which is similar to Wnt1 and has the advantage of being commercially available) and Wnt5a. We found that sequential treatment of the cultures with these two Wnts greatly improved the number of DA neurons. This combination may contribute to future protocols for producing DA neurons in the lab to replace the neurons lost in patients with Parkinson's disease.

The two Wnt-inhibiting molecules, sFRP1 and sFRP2, were not found to have an effect on DA neuron development. However, they were found to control the same morphological changes as Wnt5a, since removal of both from the developing embryo resulted in mice that looked like the Wnt5a mice: a shorter and broader midbrain.

In sum, this thesis describes multiple roles of different Wnt-related molecules and how they signal to control different events in development. Hopefully this increased knowledge will contribute to future therapies for Parkinson's disease.

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…….

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