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Wnt/β-catenin Signaling in Midbrain Dopaminergic Neurons

av

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AKADEMISK AVHANDLING

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To Narinder, for lighting the flame
ABSTRACT

Parkinson’s disease (PD) is caused by a progressive degeneration of the dopaminergic (DA) neurons of the substantia nigra. However, the mechanisms underlying the degenerative process remain elusive, with no cure for PD presently available. Cell replacement therapy (CRT), based on the transplantation of new DA neurons into PD patients’ brains, represents a promising treatment strategy that requires in depth knowledge of the signals regulating the generation of DA neurons during embryogenesis.

The Wnt signaling pathway regulates neural patterning, cell fate determination, proliferation, differentiation, and neuronal maturation during vertebrate development. In this thesis, evidence is presented that Wnt/β-catenin signaling is an important component of dopaminergic (DA) neuron development. Treatment of ventral midbrain (VM) precursor cultures with purified Wnt5a increases DA differentiation. Similarly, inhibition of GSK-3β or overexpression of β-catenin increases the conversion of Nurr1+ precursors into DA neurons. Expression profiling of Wnt components during midbrain development revealed that Frizzled (Fz) 9 is absent in newborn DA neurons while highly expressed in DA precursors. Upon closer examination we found that Fz9 inhibits Wnt5a signaling in DA cells, suggesting that the effects of Wnt5a during DA development require low levels of Fz9.

A widespread clinical application of CRT has been hindered by an inadequate availability of fetal tissue and poor graft survival within the host brain. Here, we demonstrate that treatment of VM precursor cultures with the JNK inhibitor SP600125 causes a great increase in the number of DA neurons, through reduced apoptotic signaling. The observed survival effect exhibits a degree of specificity for the DA lineage since the total neuronal pool is not significantly altered. When used in transplantation experiments in a rodent model of PD, SP600125 doubles the amount of surviving DA neurons. Combined, these results indicate that inhibition of JNK signaling can significantly reduce the number of DA neurons needed for grafting and improve the survival of grafted cells within the host brain, thereby fortifying CRT as a viable option in the treatment of PD.

Finally, the E3 ubiquitin ligase Parkin which is linked to familial forms to PD is identified as a novel regulator of Wnt/β-catenin signaling. Accordingly, β-catenin levels are increased in parkin null animals. Stabilization of β-catenin in differentiated primary VM neurons initially results in increased levels of cyclin E and proliferation, followed by increased levels of cleaved PARP and a loss of DA neurons. These findings suggest that Parkin could serve to protect DA neurons against Wnt/β-catenin-induced toxicity.

In summary, this thesis unveils several novel aspects of Wnt/β-catenin signaling in DA neurons, thereby opening up new avenues for the understanding and treatment of PD.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numeral:


II. Nina Rawal, Gonçalo Castelo-Branco, Kyle M. Sousa, Julianna Kele, Kazuto Kobayashi, Hideyuki Okano, and Ernest Arenas.


III. Gonçalo Castelo-Branco*, Nina Rawal*, and Ernest Arenas.


IV. Nina Rawal, Clare Parish*, Gonçalo Castelo-Branco*, and Ernest Arenas.

Inhibition of JNK Increases Survival of Transplanted Dopamine Neurons in Parkinsonian Rats. (2006) Under revision.


*Equal contribution
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>m</td>
<td>Mouse</td>
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<tr>
<td>E</td>
<td>Embryonic</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>AP</td>
<td>Anteroposterior</td>
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<tr>
<td>DV</td>
<td>Dorsoventral</td>
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<tr>
<td>DA</td>
<td>Dopaminergic</td>
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<tr>
<td>FP</td>
<td>Floor Plate</td>
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<tr>
<td>MHB</td>
<td>Midbrain Hindbrain Boundary</td>
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<tr>
<td>Otx2</td>
<td>Orthodenticle homologue 2</td>
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<tr>
<td>Gbx2</td>
<td>Gastrulation brain homeobox 2</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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<tr>
<td>Pax2</td>
<td>Paired box 2</td>
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<tr>
<td>En</td>
<td>Engrailed</td>
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<tr>
<td>Fgf8</td>
<td>Fibroblast growth factor 8</td>
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<tr>
<td>VZ</td>
<td>Ventricular Zone</td>
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<tr>
<td>Aldh2</td>
<td>Alddehyde dehydrogenase 2</td>
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<tr>
<td>SN</td>
<td>Substantia nigra</td>
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<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
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<tr>
<td>SNpc</td>
<td>Substantia Nigra pars compacta</td>
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<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
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<tr>
<td>Shh-N</td>
<td>Sonic Hedgehog N-terminal</td>
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<tr>
<td>Nurr1</td>
<td>Nuclear Receptor Related 1</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine Transporter</td>
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<tr>
<td>Fz</td>
<td>Frizzled</td>
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<tr>
<td>sFRP</td>
<td>Soluble Frizzled Related Protein</td>
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<tr>
<td>CRT</td>
<td>Cell Replacement Therapy</td>
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<tr>
<td>GSK-3β</td>
<td>Glycogen Synthase Kinase 3β</td>
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<td>Dkk</td>
<td>Dickkopf</td>
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<td>Hepatocyte Nuclear Factor  g3β</td>
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<td>Planar Cell Polarity</td>
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<td>CamKH</td>
<td>Ca(^{2+})-calmodulin dependent KinaseII</td>
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<tr>
<td>NF-AT</td>
<td>Nuclear Factor of Activated T-cells</td>
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<tr>
<td>Dvl</td>
<td>Dishevelled</td>
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<tr>
<td>Min</td>
<td>Minimal neoplasia</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
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<tr>
<td>EMT</td>
<td>Epithelial Mesenchymal Transition</td>
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<td>Hh</td>
<td>Hedgehog</td>
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<td>Ihh</td>
<td>Indian Hedgehog</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
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<tr>
<td>DM</td>
<td>Dorsal Midbrain</td>
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<td>MZ</td>
<td>Marginal Zone</td>
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<td>VMAT</td>
<td>Vesicular Monamine Transporter</td>
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<td>PD</td>
<td>Parkinson’s Disease</td>
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<tr>
<td>GDNF</td>
<td>Glial Derived Neurotrophic Factor</td>
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<td>ES</td>
<td>Embryonic Stem</td>
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<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
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<td>Lewy Body</td>
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<tr>
<td>UCHL-1</td>
<td>Ubiquitin C-terminal Hydroxylase 1</td>
</tr>
<tr>
<td>I3M</td>
<td>Indirubin-3-Monoxyxone</td>
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<td>Kenpaullone</td>
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<tr>
<td>Tuj1</td>
<td>βIII-tubulin</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>DIV</td>
<td>Days In Vitro</td>
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<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
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<tr>
<td>L-DOPA</td>
<td>Levodopa</td>
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<tr>
<td>LRP</td>
<td>Low density lipoprotein Receptor related Protein</td>
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1 INTRODUCTION

1.1 Development of dopaminergic neurons

1.1.1 Early patterning of the central nervous system

During vertebrate embryogenesis, the process of neural induction causes a sheet of epithelial cells to acquire neural characteristics. At mouse (m) embryonic days (E) 6-7, during late gastrulation, the epithelial sheet, termed the neural plate, closes to form the neural tube. This event marks the beginning of the development of the central nervous system (CNS). Throughout embryogenesis, proliferating progenitor cells in the neural tube migrate away and differentiate into neurons, astrocytes, and oligodendrocytes - the three main cell types of the CNS. CNS development has been compared to a Cartesian grid where each point in the grid (each cell) can be identified and defined through its coordinates along two axes: The anteroposterior (AP) axis runs from head to tail of the embryo while the dorsoventral (DV) axis runs from the stomach to the back (Figure 1a). One of the first events during neural specification is the spatially restricted expression of transcription factors and soluble factors that pattern the neural tube and create what will later become the forebrain, midbrain, hindbrain, and spinal cord. Following the Cartesian analogy, each point of the grid will have a unique profile and determine when and where specific types of neurons, including dopaminergic (DA) neurons, are born (Lumsden and Krumlauf, 1996; Prakash and Wurst, 2006; Wolpert, 2002).

1.1.2 Early specification of dopaminergic neurons

Midbrain DA neurons are born in the proximity of two signaling hubs; the isthmus and the floor plate (FP). The isthmus, a structure found in all vertebrate species analyzed so far, is created around mE7.5 at the midbrain hindbrain boundary (MHB). The isthmus is one of two signaling centers along the AP axis, and is formed at the boundary between regions expressing the transcription factors orthodenticle homologue 2 (Otx2) (anterior) and gastrulation brain homeobox 2 (Gbx2) (posterior) (Acapora et al., 1997; Broccoli et al., 1999; Wassarman et al.,
The other signaling center along the AP axis, the anterior neural ridge, regulates forebrain development (Houart et al., 1998; Shimamura and Rubenstein, 1997). Along the DV axis, the FP contains specialized cells that secrete Sonic Hedgehog (Shh) along almost the entire neural tube. In the midbrain, a subset of the Shh-secreting cells of the FP also express HNF-3β (Hynes et al., 1995a). Shh regulates patterning along the DV axis by regulation of transcription factors such as Nkx2.2 (Briscoe and Ericson, 2001; Jessell, 2000; Litingtung and Chiang, 2000).

By mE8, Paired box 2 (Pax2), Lmx1b, and Wnt1 are expressed across the MHB and later on the expression of Engrailed-2 (En-2), Pax5, and Pax8 also overlaps in this region (Adams et al., 2000; Hynes and Rosenthal, 1999; Wurst and Bally-Cuif, 2001). The soluble factor Fibroblast growth factor (Fgf) 8 is secreted by the MHB but its expression is restricted to the Gbx2-expressing domain (Crossley et al., 1996). In a seminal experiment using Fgf8 soaked beads it was established that Fgf8 and Shh together are necessary and sufficient to induce DA neurons at ectopic locations (Ye et al., 1998). Accordingly, ectopic expression of HNF-3β has been reported to induce the formation of a dorsal floor plate, which in turn induces the expression of Shh, resulting in the formation of ectopic DA neurons (Hynes et al., 1995b). Thus, factors from the isthmus and FP cooperatively regulate the early events leading up to DA neurogenesis (Figure 1b).

### 1.1.3 Late specification of dopaminergic neurons

By E10 in the mouse, proliferating DA progenitors reside in the neuroepithelium of the ventricular zone (VZ) and they are identified by the expression of aldehyde dehydrogenase 2 (Aldh2) and Otx2 (Puelles et al., 2003; Wallen et al., 1999). The proliferation of the VZ DA progenitors is regulated by Shh (Puelles et al., 2003). The inverted fountain model states that the VZ DA progenitors migrate ventrally, towards the marginal zone of the midbrain, where they subsequently migrate laterally again to populate the separate DA pools of the ventral tegmental area (VTA) (medial) and the substantia nigra pars compacta (SNpc) (lateral) (Figure 1c) (Bayer et al., 1995). During the migration towards the marginal zone, at mE10.5, the DA progenitors start expressing orphan nuclear receptor related 1 (Nurr1), thereby exiting cell cycle and becoming post-mitotic (Castro et al., 2001; Wallen et
al., 1999). By mE11.5, cells that have reached the marginal zone begin expressing the enzyme tyrosine hydroxylase (TH) (Alberi et al., 2004; Kawano et al., 1995). TH catalyzes the conversion of L-tyrosine into L-DOPA and is the most commonly used marker of DA neurons. Along with TH, newborn DA neurons also express the transcription factors Pitx3, En-1, and Lmx1b (Alberi et al., 2004; Smidt et al., 2000; Smidt et al., 1997), (see below). Recent findings on the role of Pitx3 during DA specification have however challenged the inverted fountain model, in favor of the so-called corona model. The latter states that VZ DA progenitors migrate radially towards the marginal zone, and that the DA neurons of the VTA originate from the medial part of the VZ while the DA neurons of the SNpc originate from more lateral portions of the VZ (Hanaway et al., 1971; Smidt et al., 2004) (Figure 1d). From mE12.5 until birth, DA neurons develop axonal connections rostrally in order to reach their target neurons in the striatum and cortex (Voorn et al., 1988).

**Figure 1** Specification of VM DA neurons. (a) Sagittal view of an E11.5 mouse brain. FB-forebrain, DE-diencephalon, MB-midbrain, HB-hindbrain, VM-ventral midbrain, DM-dorsal midbrain. The embryo is patterned along the anteroposterior (AP) and dorsoventral (DV) axes. (b) Magnified sagittal view of the midbrain. Sonic hedgehog (Shh) is secreted throughout the neural tube by the cells of the floor plate (FP). Fibroblast growth factor (Fgf) 8 is secreted by the isthmus. Together, they control VM DA neurogenesis. (c) Magnified transverse view of the VM. DA progenitors reside in the ventricular zone (VZ). In the inverted fountain model, they migrate medially (pink arrows) before they migrate down toward the marginal zone (MZ). They subsequently migrate laterally (green arrows) to their final positions in the substantia nigra (SN) and ventral tegmental area (VTA). (d) In the corona model, the DA progenitors migrate radially toward the MZ (pink arrows) thus suggesting that SN DA neurons originate from more lateral positions within the VZ.
1.1.4 Factors governing dopaminergic neuron development

As for most cell types of the CNS, DA development is regulated by both intrinsic and extrinsic signals. One might envision that one of the roles of the extrinsic factors presented below is to regulate the activity of the transcription factors presented subsequently.

**Shh**

Sonic Hedgehog is expressed in the FP and acts as a ventralizing morphogen throughout the neural tube. The Shh gradient regulates the expression of genes that in turn control the generation of several cell types (Ericson et al., 1997; Orentas et al., 1999; Yamada et al., 1993; Yamada et al., 1991). Shh is thus a central player during CNS development. Early on, addition of Shh to midbrain explants was reported to induce DA neurons (Hynes et al., 1995a; Wang et al., 1995). Additionally, analysis of transgenic mice expressing the N-terminal form (biologically active form) of Shh (Shh-N) or the Shh effector gene *Gli-1* revealed ectopic DA neurogenesis in the dorsal midbrain (DM) (Hynes et al., 1997). Interestingly however, the same effects were not observed in the dorsal hindbrain, indicating that additional factors present in the midbrain were required for the generation of midbrain DA neurons.

**Fgf8**

Fibroblast growth factor (Fgf) 8 is secreted by the isthmus during AP patterning. Due to the severe gastrulation defects in Fgf8 null mice, the discovery of its importance during DA development required conditional knockouts and gain-of-function studies with Fgf8 soaked beads. Ye and colleagues described that Fgf8 and Shh could induce DA neurons together (Ye et al., 1998). Combined, these molecules are thought to provide the necessary positional information required along the DV and AP axes respectively, enabling DA progenitors to take on a DA phenotype.
Nurr1

Nuclear receptor related 1 (Nurr1) is part of the NGF1B and Nor1 subfamily of the steroid-hormone super family of receptors. In the CNS, Nurr1 expression can be detected in post-mitotic DA precursors in the VM at E10.5, and in hippocampus, cortex and spinal cord. Nurr1 expression is maintained throughout adult stages (Wallen et al., 1999; Zetterstrom et al., 1997; Zetterstrom et al., 1996a; Zetterstrom et al., 1996b). Knockout studies of Nurr1 revealed a selective loss of TH+ DA neurons at birth, while other catecholaminergic cell groups appear unaffected. Nurr1 null DA progenitors migrate to the marginal zone and acquire a ventral neuronal phenotype, as assessed by Pitx3 and Lmx1b expression (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Smidt et al., 2000; Wallen et al., 1999). However, they do not express DA markers such as c-ret, dopamine transporter (DAT) or vesicular dopamine transporter type 2 (VMAT2), indicating that they fail to acquire a mature DA phenotype (Smits et al., 2003; Wallen et al., 2001). Further examination has pointed to a role in DA neuron survival, thus implying that Nurr1 is important both during the development and the maintenance of a DA phenotype (Saucedo-Cardenas et al., 1998; Wallen et al., 1999).

Lmx1a

Lmx1a is expressed in the ventral midline from E9 and at E10.5 it co-localizes with Nurr1 expression in DA precursors located above the marginal zone. In contrast to the family member Lmx1b, Lmx1a expression is restricted to DA progenitors, and it is maintained in post-mitotic DA neurons. Lmx1a has been reported to induce a DA phenotype and to suppress alternative fates through induction of Msx1 (see below). Both these factors are reported to act downstream of Shh in vivo. Surprisingly however, derivation of DA neurons from embryonic stem (ES) cells, through overexpression of Lmx1a and Msx1, requires the concomitant addition of Shh (Andersson et al., 2006b), indicating that Shh regulates the activity of additional factors required for DA development.
Expression of the homeodomain transcription factor \textit{Lmx1b} is first detected at mE7.5, indicating that it mediates events prior to DA specification. Later on, Lmx1b is expressed in the midbrain and analysis of \textit{Lmx1b} null animals has revealed reduced levels of Pitx3 while other markers such as Nurr1 and TH remain normal. TH expression is however lost after E16, suggesting that Lmx1b might be important for DA survival. Conversely, Lmx1b levels are unchanged in \textit{Nurr1} null animals. Combined, these findings suggest that Nurr1 and Lmx1b constitute two independent pathways, both of which are necessary for the generation of DA neurons (Smidt et al., 2000).

Interestingly, Lmx1b is necessary for the induction and/or maintenance of Wnt1 expression in the MHB, suggesting that Wnt1 acts downstream of Lmx1b and might thus mediate the observed effects of Lmx1b on Pitx3, in the independent pathway generating DA neurons (Adams et al., 2000; Matsunaga et al., 2002).

Expression of proneural genes such as Neurogenin (Ngn) 1 and 2 are sufficient for the initiation of generic neurogenic programs \textit{in vivo} (Blader et al., 1997; Ma et al., 1996; Mizuguchi et al., 2001). However, the role of Ngn5 in midbrain development has not been studied. Data from our laboratory and others shows that Ngn2 is required for the development of DA neurons, since ablation of \textit{Ngn2} results in an extensive loss of DA neurons (Andersson et al., 2006a; Kele et al., 2006). Ngn2 is
thought to control the differentiation of proliferating Sox2+ progenitors into Nurr1+ precursors (Kele et al., 2006).

**Pitx3**

The transcription factor Pitx3 is expressed in the midbrain at E11.5 and confined to the SN and VTA DA neurons during adult stages (Smidt et al., 1997). Several Pitx3 mutants have been analyzed (Nunes et al., 2003; Smidt et al., 2004; van den Munckhof et al., 2003) and the partially non-overlapping results indicate that several issues need to be further examined. At present, it appears that Pitx3 is necessary for the differentiation or maturation of SN DA neurons, through a direct or indirect regulation of the TH gene (Prakash and Wurst, 2006).

**Engrailed 1 and 2**

*Engrailed* (En) 1 and 2 are expressed in the caudal midbrain and rostral hindbrain during embryogenesis and are restricted to SN and VTA DA neurons in the adult (Davidson et al., 1988; Simon et al., 2001). *En-1* and -2 single knockouts do not display any striking phenotype, indicating that the two genes exhibit a degree of redundancy. However, in the *En1/2* double knockout, DA neurons are born but die around E14, suggesting that En, like Lmx1b, is involved in the maintenance of a DA phenotype. *In vitro* analyses have indicated that En inhibits apoptosis (Alberi et al., 2004) but the mechanism of action remains to be confirmed *in vivo*.

### 1.2 Wnt signaling

#### 1.2.1 The Wnt signaling pathway

The Wnt signaling pathway is highly conserved across many species. The name Wnt derives from the Drosophila gene *wingless* (wg), which is involved in segment polarity during development, and the murine gene *int-1*, which is required for the development of the forebrain, midbrain, cerebellum, and neural crest (Amoyel et al., 2005; Brault et al., 2001; Ciani and Salinas, 2005; Heisenberg et al., 2001; Houart et al., 2002; Lagutin et al., 2003; McMahon and Bradley, 1990; Megason and McMahon, 2002; Thomas and Capecchi, 1990). To date, 19 Wnt genes have
been identified in mammals (7 in Drosophila) and they normally contain between 350-400 amino acids (Nusse, 2006). As is typical of extracellular proteins, Wnts contain cysteine residues that form disulfide bonds and prior to secretion they are glycosylated and palmitoylated (Wainwright et al., 1988; Willert et al., 2003). These modifications render the Wnts hydrophobic, poorly soluble, and prone to localization close to cell membranes. The discovery that Wnts are palmitoylated helped devise a strategy for the first successful purification of Wnt proteins (Willert et al., 2003), which in turn has revolutionized the field of Wnt biology. However, not all Wnts have proven equally easy to purify. These difficulties most likely reflect the differential palmitoylation and/or glycosylation status of individual Wnt ligands.

Figure 2 The Wnt signaling pathway. (a) The canonical pathway signals through β-catenin that can signal in the cytoplasm, or enter the nucleus and initiate transcription together with TCF/LEFs. F-Frizzled, LRP-Low density lipoprotein receptor related protein, Dkk-Dickkopf, K-Kremen, αβγ-αβγ-G-protein subunits, Dvl-Dishevelled, APC-Adenomatous Polyposis Coli, GSK-3β-Glycogen synthase kinase 3β, Ub-ubiquitin, P-phospho (b) The planar cell polarity signals through JNK, Rac and Rho and regulates cell movement through modulation of the cytoskeleton or AP1-mediated transcription. JNK-cJun-N-terminal kinase, AP1-activating protein 1, P-phospho. (c) Activation of the Ca²⁺ pathway causes influxes of Ca²⁺ which in turn activates Protein kinase C (PKC), Ca²⁺-calmodulin dependent kinase II (CaMKII), and/or nuclear factor activated in T-cells (NF-AT). NF-AT is a transcription factor that can initiate transcription of target genes.
**On the cell surface**

Wnt signaling is initiated by the binding of a Wnt ligand to a Frizzled (Fz) receptor located in the cell membrane. Fzs are seven-pass transmembrane receptors that have been suggested to signal through G-proteins (Cadigan and Liu, 2006; Huang and Klein, 2004). Overexpression of constitutively active G\(\alpha\)-subunits activates Wnt/\(\beta\)-catenin reporter genes (Liu et al., 2001b) and reduction of G\(\alpha\)o gene activity compromises Wg signaling in Drosophila (Katanaev et al., 2005). However, biochemical confirmations of these findings remain to be presented and thus, the precise relationship between Fzs and G-proteins is still a matter of debate. To date, 10 human and murine Fzs have been identified (Nusse, 2006) and several reports have demonstrated that different members of the Fz family can regulate cell fate determination, including Fz5 in the retina, Fz9 in the hippocampus, and Fz3 in neural crest (Deardorff et al., 2001; Van Raay et al., 2005; Zhao et al., 2005). However, due to lack of purified Wnt proteins, the binding affinities of native Wnt-Fz complexes remain undetermined. Wnt-Fz binding can be modulated by different types of soluble ligands such as soluble Frizzled related proteins (sFRPs), Cerberus, and Wnt Inhibitory Factor (Kawano and Kypta, 2003).

In addition to binding Fzs, Wnts can simultaneously bind low density lipoprotein receptor related protein (LRP) 5 and 6 that are also present in the cell membrane (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). LRPs are single-span transmembrane receptors that contain multiple EGF repeats that are important for Wnt binding. LRP6 is expressed in the developing midbrain and loss of the protein results in a partial deletion of the midbrain (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000) and a delayed differentiation of DA neurons (G. Castelo-Branco, unpublished).

Dickkopfs (Dkks) belong to a family of soluble factors that have been shown to antagonize Wnt signaling by binding to LRPs and Kremen receptors. However, Dkks and Wnts do not bind the same part of the LRP receptor, indicating that the mechanism of inhibition is not competition for receptor binding. Instead, the formation of LRP-Dkk-Kremen ternary complexes disengages the LRP receptor from the Wnt-Fz complex, precluding signal transduction by Wnt (Bafico et al., 2001; Mao et al., 2002; Mao et al., 2001; Semenov et al., 2001). Intriguingly, in
the absence of Kremen 2, Dkk2 has been demonstrated to function as an activator of canonical Wnt signaling (Mao and Niehrs, 2003) (Figure 2a). Wnts have also been shown to bind the tyrosine kinase receptors Ryk and ROR2. Similarly to the LRPs, Ryk can form a complex with a Wnt ligand and a Fz receptor, resulting in activation of the canonical Wnt signaling. Ryk signaling has been reported to control axon guidance in the spinal cord (Cheyette, 2004; Liu et al., 2005b; Yoshikawa et al., 2003). The ROR2 receptor has been associated with the non-canonical PCP pathway (see below). Interestingly, both Ryk and ROR2 have been described to signal downstream of Wnt5a and ablation of ROR2 results in a phenotype similar to that of the Wnt5a null (Oishi et al., 2003; Yoshikawa et al., 2003).

Inside the cell - canonical β-catenin signaling

The complexity of molecular players on the cell surface is further illustrated inside the cell by the existence of at least three different downstream pathways. The canonical pathway involves stabilization of the cytoplasmic protein β-catenin. In the absence of Wnt ligand, β-catenin is phosphorylated by a destruction complex consisting of Axin, Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase-3β (GSK-3β) (Behrens et al., 1998; Itoh et al., 1998). Previous studies have shown that phosphorylation of β-catenin by GSK-3β is preceded by a priming phosphorylation by Casein Kinase 1 (CK) 1 (Amit et al., 2002; Liu et al., 2002; Matsubayashi et al., 2004; Yanagawa et al., 2002). These phosphorylation events are followed by ubiquitylation by the E3 ubiquitin ligase β-TrCP (Kitagawa et al., 1999; Winston et al., 1999), targeting β-catenin for degradation in the proteasome. Binding of Wnt to Fz receptors triggers GSK-3β to phosphorylate the LRP receptor (Zeng et al., 2005), which in turn can recruit the scaffold protein Axin to the cell membrane. Destabilization of Axin results in the release of β-catenin from its destruction complex, making it active for signaling. Active β-catenin signals by entering the nucleus and inducing transcription of target genes, through interactions with transcriptional factors of the TCF/LEF family. In the absence of Wnt, TCF/LEFs act as transcriptional repressors together with the Groucho
family of corepressors, and histone deacetylases (Cavallo et al., 1998; Chen and Struhl, 1999). When active β-catenin enters the nucleus, it converts TCF/LEFs to transcriptional activators in a coregulatory exchange that replaces Groucho with the histone acetylase CBP/p300 (cyclic AMP responsive element binding protein) (Hecht et al., 2000; Takemaru and Moon, 2000). Interactions between the TCF/LEF/β-catenin complex and chromatin might subsequently be mediated by Pygopus and Bcl-9 (Legless) (Kramps et al., 2002; Parker et al., 2002). Accordingly, mutations of the Pygopus or Legless genes give rise to Wg-like phenotypes in Drosophila (Thompson et al., 2002). The outcome of the canonical β-catenin signaling cascade is the transcription of target genes such as cell cycle genes cyclin D1 and c-myc, and midbrain genes such as En1, c-ret and Ngn1 (Nusse, 2006) (Figure 2a).

Non-canonical planar cell polarity signaling

The second pathway downstream of Wnt is often referred to as the planar cell polarity (PCP) or convergent-extension pathway (Vinson et al., 1989). During the development of the neural tube, groups of cells move in the same direction in a coordinated manner and these movements are regulated by PCP signaling. Fzs activate cJun-N-terminal Kinase (JNK), which in turn activates its downstream effector cJun. cJun subsequently activates the transcription factor AP-1 and initiates transcription of target genes. Wnt5a has been reported to regulate cell polarity in Drosophila and convergent-extension in Xenopus (Moon et al., 1993), and to activate JNK in vitro (Yamanaka et al., 2002). Thus, activation of JNK signaling is used as a measure of PCP signaling. However, the contribution of JNK signaling to PCP is still under debate and other possible mediators are the small GTPases Rac and Rho (Fanto and McNeill, 2004) (Figure 2b).

Non-canonical Ca²⁺ signaling

The Wnt/Ca²⁺ pathway has been reported to regulate events such as DV patterning of the early embryo (Saneyoshi et al., 2002), and cardiogenesis (Pandur et al., 2002), through the activation of protein kinase C (Sheldahl et al., 1999) and Ca²⁺-calmodulin dependent kinase II (CaMKII) (Kuhl et al., 2000). CaMKII can activate
calcineurin which in turn activates the transcription factor nuclear factor of activated T-cells (NF-AT) (Saneyoshi et al., 2002). Interestingly, Wnt5a has been found to signal through the Ca\textsuperscript{2+} pathway (Slusarski et al., 1997; Weeraratna et al., 2002) and mediate the aforementioned effects on DV patterning. Along these lines, Dishevelled, and Prickle, a PCP gene, can activate Ca\textsuperscript{2+} signaling (Sheldahl et al., 2003; Veeman et al., 2003) indicating that there might be points of overlap between the non-canonical PCP and Ca\textsuperscript{2+} pathways (Figure 2c).

New Wnt signaling components are continuously being identified and they increase the complexity to the pathway. Of particular importance are the key players in the center of the web:

**Dishevelled**

Dishevelled (Dvl) functions as a signaling activator and is the final common denominator before the three intracellular pathways diverge. Dvl is thus a central component of Wnt signaling and this fact is illustrated by the finding that different Dvl domains are involved in the different downstream pathways. While the DIX and PDZ domains regulate the canonical \(\beta\)-catenin pathway (Axelrod et al., 1998; Boutros et al., 1998; Moriguchi et al., 1999; Penton et al., 2002; Rothbacher et al., 2000), the PDZ and DEP domains are involved in the non-canonical PCP and Ca\textsuperscript{2+} pathways (Axelrod et al., 1998; Boutros et al., 1998; Heisenberg et al., 2000; Moriguchi et al., 1999; Sheldahl et al., 2003; Tada and Smith, 2000; Wallingford et al., 2000). Dvl acts downstream of Fz and direct interactions have been reported (Wong et al., 2003). Furthermore, Fzs have been shown to promote hyperphosphorylation of Dvl (Rothbacher et al., 2000; Takada et al., 2005; Umbhauer et al., 2000; Willert et al., 1997). The hyperphosphorylation, resulting in activation of Dvl can be mediated by several kinases, such as CK1 and -2, and Par-1 (Cong et al., 2004a; Ossipova et al., 2005; Sun et al., 2001; Willert et al., 1997). In the context of canonical Wnt signaling, phosphorylated Dvl can bind Axin and inhibit its activity (Fagotto et al., 1999; Kishida et al., 1999; Li et al., 1999; Smalley et al., 1999), which in turn activates canonical Wnt signaling.

The precise role of Dvl in the PCP pathway remains unclear. However, Dvl relays the signals induced by Fz onto the small GTPases Rac and Rho, resulting in
modulations of the actin cytoskeleton (Habas et al., 2003; Tahinci and Symes, 2003). In the Wnt/ Ca\(^{2+}\) pathway, Dvl has been reported to cause an influx of Ca\(^{2+}\) and to activate protein kinase C and CamKII (Sheldahl et al., 2003; Wallingford and Habas, 2005).

**Axin**

Axin was named for its inhibitory effect on secondary axis formation, i.e canonical Wnt/β-catenin signaling (Zeng et al., 1997). Moreover, certain mutations in Axin result in increased levels of β-catenin and activated Wnt signaling (Peifer and Polakis, 2000). Axin is a scaffold protein that binds several of the key modulators of Wnt signaling, LRPs, GSK-3β, APC, CK1, and β-catenin (Hart et al., 1998; Kishida et al., 1999; Kishida et al., 1998; Mao et al., 2001; Nakamura et al., 1998; Tolwinski et al., 2003; Wehrli et al., 2000). Dvl inactivates Axin by inducing its re-localization to the cell membrane (Cliffe et al., 2003), releasing β-catenin from the destruction complex. Axin is known to bind LRP (Mao et al., 2001) and truncated forms of LRP can stabilize β-catenin independently of Dvl (Cadigan and Liu, 2006; Cong et al., 2004b; Li et al., 2002; Liu et al., 2005a; Schweizer and Varmus, 2003), suggesting that LRP can recruit Axin to the cell membrane independently of Dvl. In brief, it appears that both LRP and Dvl contribute to the inactivation of Axin. While inhibiting the canonical pathway, Axin has also been shown to activate non-canonical JNK signaling (Zhang et al., 1999). Interestingly, the number of Axin molecules within the cell is up to 5000 times less than those of the other components of the destruction complex (Lee et al., 2003), indicating that Axin might be the limiting component that regulates the assembly and disassembly of the destruction complex.

**GSK-3β**

Glycogen Synthase Kinase 3 (GSK-3) was originally identified as the kinase phosphorylating and inactivating glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984), thereby regulating insulin signaling. There are two mammalian GSK-3 isoforms: α and β, encoded by distinct genes. Both isoforms are ubiquitously expressed and exhibit 97% homology within the kinase domain while
other domains differ substantially (Doble and Woodgett, 2003; Woodgett, 1990). It is now known that GSK-3β is involved in several signaling pathways and that it is a key player in Wnt signaling (Frame and Cohen, 2001; Grimes and Jope, 2001; Woodgett and Cohen, 1984). In the absence of Wnt, GSK-3β phosphorylates β-catenin and targets it for degradation. It was recently shown that GSK-3β and CK1 are the kinases responsible for the phosphorylation of LRP6, which in turn activates the Wnt pathway (Davidson et al., 2005; Zeng et al., 2005). Thus, GSK-3β appears to play a dual role in the regulation of canonical Wnt signaling.

Ablation of GSK-3β is embryonically lethal, due to degeneration of liver tissue (Hoeflich et al., 2000). Thus, despite structural similarities, the two isoforms α and β are not completely redundant. Interestingly, GSK-3β has also been described to regulate microtubule formation via Dvl, independently of β-catenin (Ciani et al., 2004).

**APC**

The *Adenomatous Polyposis Coli* (APC) gene is mutated in more than 80% of all colon cancers (Kinzler and Vogelstein, 1996), and is dysfunctional in familial adenomatous polyposis, an inherited disease that predisposes to colon cancer (Lal and Gallinger, 2000). Ablation of APC is embryonically lethal although the cause of death is unclear. Heterozygote animals display the Min (minimal neoplasia) phenotype, i.e., they develop tumors shortly after birth. APC acts as an inhibitor of Wnt signaling by binding to Axin and β-catenin, within the destruction complex. It can shuttle between the cell membrane, the cytoplasm, and the nucleus where one of its main functions appears to be the regulation of β-catenin/TCF-mediated transcription (Henderson, 2000; Neufeld et al., 2000).

**Pathway modulators**

Several molecules have been described that activate or inhibit one or more of the Wnt pathways, thereby regulating the biological read-out of Wnt signaling. Par-1 and Frequently rearranged in advanced T-cell lymphomas (FRAT) 1 have been implicated as activators of Wnt/β-catenin signaling while Axin, Idax, and Naked inhibit canonical signaling through different mechanisms (Hino et al., 2001; Sun et
al., 2001; Yost et al., 1998; Zeng et al., 1997; Zeng et al., 2000). Ltap (Strabismus) has been shown to activate JNK signaling (Park and Moon, 2002).

**Cytoplasmic β-catenin signaling**

β-catenin can be found in different pools within the cell. Apart from the pool that regulates transcription, β-catenin also interacts with cadherins and α-catenin in the formation of adherens junctions (Bienz, 2005). The cadherin-catenin complex mediates epithelial-mesenchymal transitions (EMT), an important event during morphogenesis. EMT is also associated with tumor development, through loosening of both adherens and tight junctions (Thiery and Sleeman, 2006). Loss of adhesion could thus liberate β-catenin that could act in Wnt signaling, highlighting the close connection between adhesion and cancer. Interestingly, it was recently shown that β-catenin can indeed switch from a function in adhesion to transcription through direct competition between α-catenin and Bcl9-2 for the binding to β-catenin (Brembeck et al., 2004). Thus, although β-catenin/cadherin signaling is a separate matter, the balance between the two pools of β-catenin can influence the activity of the Wnt pathway.

**Cross-talk**

Wnt2 was recently reported to signal through GSK-3β and cJun/AP-1 (Le Floch et al., 2005), and Dvl has been reported to regulate the metabolism of amyloid precursor protein through activation of both protein kinase C and JNK (Mudher et al., 2001). Both these reports suggest that it is possible to activate components of two pathways simultaneously. Furthermore, non-canonical Wnts, such as Wnt-11, have been shown to inhibit canonical signaling (Maye et al., 2004). Thus, the notion of strict boundaries between the different intracellular Wnt pathways already appears to be an oversimplified view of Wnt signaling.

**Cross-talk with other pathways**

The Notch, Hedgehog, Ephrin, TGF-β, and Wnt pathways control several crucial steps during embryogenesis throughout adulthood and are all implicated in tumor
formation. Cross-talk between the Wnt pathway and the other signaling pathways is an emerging concept that adds an additional level of complexity to cell signaling.

**Wnt-TGF-β**

Studies in Xenopus have shown that a combined activation of the Wnt and TGF-β pathways is critical for the formation of the Spemann’s organizer (a signaling center important for amphibian embryogenesis) and they have been shown to act cooperatively on the Mxvl promoter (Attisano and Labbe, 2004; Crease et al., 1998; Cui et al., 1996; Labbe et al., 2000; Nishita et al., 2000; Schohl and Fagotto, 2002; Willert et al., 2002; Zorn et al., 1999). This cross-talk has been suggested to be mediated through direct interactions between Smads and TCF/LEFs (Labbe et al., 2000; Nishita et al., 2000; Takaku et al., 1998). Both pathways are heavily implicated in neoplasias and accordingly, Smad4/APC compound mutants develop cancer more often than single mutants (Takaku et al., 1998).

**Wnt-Notch**

The Notch signaling pathway is known to regulate cell fate determination, proliferation, survival, and boundary formation during development (Hansson et al., 2004). Wnt has been reported to inhibit Notch signals through a direct interaction at the level of Dvl (Strutt et al., 2002). However, phosphorylation of the intracellular domain of Notch by GSK-3β is thought to protect Notch from degradation (Foltz et al., 2002). Furthermore, ablation of Notch protein results in increased levels of β-catenin in the skin (Nicolas et al., 2003). At a transcriptional level, increased Wnt signaling has been reported to trigger oncogenic conversion of primary human breast epithelial cells through upregulation of the Notch ligands Dll3 and Dll4 (Ayyanan et al., 2006), and to control Notch signaling during embryonic patterning through regulation of the Notch ligand Dll1 (Hofmann et al., 2004).

**Wnt-Hh**

The Hedgehog (Hh) signaling pathway and its key player Shh is known to interact with the Wnt pathway at several levels of the signaling cascade. Apart from GSK-3β, which modulates both pathways (Kalderon, 2002), Wnt5a has been reported to
be a Shh target gene in developing hair follicles (Reddy et al., 2001), and several Wnt family members are targets of Gli genes that act downstream of Shh (Mullor et al., 2001). Conversely, ablation of β-catenin results in a loss of Shh expression in the skin (Huelsken et al., 2001). Contradicting reports of agonistic effects between the Wnt and Hh pathways, an analysis of patients with APC mutations revealed reduced levels of Indian Hedgehog (Ihh-another member of the Hh family), indicating that Ihh antagonizes Wnt signaling in colon cancer cells (van den Brink et al., 2004). Thus, the final outcome of Wnt-Hh cross-talk appears highly context-dependent.

Wnt-Ephrin
The Ephrin ligands and Eph receptors constitute a large family known to regulate cell compartmentalization and organized migration during development, through bidirectional signaling (Pasquale, 2005; Poliakov et al., 2004). Wnt/β-catenin signaling has been reported to control cell positioning in the adult intestinal epithelium through regulation of EphrinB/EphB expression (Batlle et al., 2002). Moreover, loss of Eph expression has been reported to aggravate the tumor development in APC mutant mice (Batlle et al., 2005). Interestingly, Dvl has been shown to mediate Ephrin-induced cell repulsion, the major outcome of bidirectional Ephrin/Eph signaling, through activation of RhoA (Tanaka et al., 2003).

1.2.2 Wnt activity during embryogenesis
Wnt/β-catenin signaling is considered to be both necessary and sufficient for the establishment of the AP axis during neural patterning in Xenopus (Kiecker and Niehrs, 2001) and in chick (Nordstrom et al., 2002). Induction of a secondary axis is thus used as a read-out of canonical Wnt/β-catenin signaling. Wnt morphogen gradients suppress anterior fates (McGrew et al., 1997) and loss of Wnt expression results in an expansion of forebrain markers such as Otx (Erter et al., 2001; Lekven et al., 2001). Wnts are also implicated in DV patterning, where they act dorsalizing (Gunhaga et al., 2003). However, Wnt5a has been reported to promote ventral cell fates (Saneyoshi et al., 2002) during Xenopus development. Later on during
development, Wnts regulate the development of the forebrain, midbrain, hindbrain, neural crest, and spinal cord (Amoyel et al., 2005; Brault et al., 2001; Ciani and Salinas, 2005; Heisenberg et al., 2001; Houart et al., 2002; Lagutin et al., 2003; McMahon and Bradley, 1990; Megason and McMahon, 2002; Thomas and Capecchi, 1990). Wnt activity has also been reported to control synaptogenesis, dendritogenesis, and axon guidance. A gradient of Wnt4 is thought to induce ventral movement of axons in the spinal cord (Lyuksyutova et al., 2003) while Wnt7b has been shown to increase dendrite arborization in hippocampal neurons. Interestingly, Wnt7b is thought to signal via a Dvl-JNK-Rac pathway that functions independently of the canonical pathway (Rosso et al., 2005). Furthermore, release of Wnt7a from post-synaptic cerebellar granule cells causes axonal remodeling of pre-synaptic mossy fibers, thereby regulating the synaptic connectivity between the cells of the cerebellum (Hall et al., 2000).

On a cellular level, canonical Wnt signaling is mainly associated with proliferation (Kioussi et al., 2002; Megason and McMahon, 2002). Direct overexpression of $\beta$-catenin results in an expansion of neural tissue while conditional ablation of $\beta$-catenin reduces brain size (Chenn and Walsh, 2002; Zechner et al., 2003). More specifically, $\beta$-catenin appears to control the decision between proliferation and differentiation since loss of $\beta$-catenin results in reduced proliferation and a concomitant increase in differentiation (Zechner et al., 2003). Interestingly, the two reports examined different parts of the CNS, indicating that control of proliferation by $\beta$-catenin might be a general phenomenon during CNS development. However, several reports have also demonstrated the importance of Wnt/$\beta$-catenin signaling for cellular differentiation (Hirabayashi et al., 2004; Huelsken et al., 2001; Lee et al., 2004). Combined, these findings illustrate that Wnt signaling contributes to patterning, cell fate determination, proliferation, differentiation, and maturation during embryogenesis.

1.2.3 Wnt signaling in the developing midbrain

Shh and TGF-$\beta$ are both expressed throughout the FP and can be considered as general ventralizing signals. Thus, one might predict the existence of factors that
offer more defined positional information within the midbrain and are also expressed asymmetrically between the ventral and dorsal midbrain. One of the best examples of such a factor is Wnt1, which is expressed throughout the presumptive midbrain and later on is restricted to the dorsal midline of the midbrain and diencephalon. Early studies of Wnt1 null mice revealed a loss of DA neurons and a deletion of the MHB, suggesting that Wnt1 is involved in the early specification of DA neurons (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Interestingly, expression of En1 was shown to rescue the Wnt1 null phenotype, indicating that one of the key roles of Wnt1 is to maintain En expression (Danielian and McMahon, 1996). More recent analyses of Wnt1 null mice have shown that Wnt1 is required for the ectopic induction of DA neurons by Shh and Fgf8, and for the expression of Pitx 3 in DA neurons (Prakash et al., 2006).

Studies in our laboratory have shown that individual Wnt ligands differentially regulate the development of DA neurons in vitro. Treatment of VM precursor cultures with Wnt1, -3a, or -5a conditioned medium generated different effects on the number of TH+ DA neurons present in the cultures. While Wnt1 mainly increases the number of DA neurons through an expansion of the Nurr1+ precursor pool, Wnt5a primarily facilitates the differentiation of the Nurr1+ precursors into TH+ DA neurons. Conversely, Wnt3a, which is mainly expressed in the DM, increases the number of Nurr1+ precursors but reduces their differentiation into a DA phenotype. Nurr1/β-catenin co-localization and TCF/LEF activity has been

![Figure 3](image-url)
confirmed in vivo and combined, these studies suggest that Wnts are key regulators of DA precursor proliferation and differentiation (Castelo-Branco et al., 2003) (Figure 3). Interestingly, further studies have shown that VM glia produce and secrete Wnt5a, suggesting that cross-talk between neurons and embryonic glia are an additional component important for DA development (Castelo-Branco et al., 2005; Hall et al., 2003; Wagner et al., 1999). Moreover, the specific activities of individual Wnt ligands highlight the need to further characterize the expression patterns of Wnt signaling components in the developing VM.

1.2.4 Wnt signaling in adult tissues
The seminal identification of hematopoietic stem cells (Becker et al., 1963; Till and McCulloch, 1961) paved the way for the identification of adult stem cells in several other tissues (Fuchs et al., 2004). Interestingly, Wnt activity has been shown to regulate adult stem cell self-renewal events in tissues such as skin, intestine, muscle, and bone marrow (Huelsken et al., 2001; Pinto et al., 2003; Polesskaya et al., 2003; Reya et al., 2003). Thus, although Wnt activity in adult tissues is a young and rapidly growing field, it already appears that Wnts provide necessary proliferative cues for adult stem cells.

1.2.5 Deregulation of the Wnt pathway
The step from regulation of adult stem cell self-renewal to tumor formation is small and Wnt-1 was originally described in virus-induced mouse mammary tumors (Nusse and Varmus, 1982). APC is mutated in 80% of all colon cancers (Kinzler and Vogelstein, 1996) and deregulated Wnt signaling has been linked to cancers of the skin, breast, liver, lung, prostate, and the hematopoietic system (Lustig and Behrens, 2003).

Several other syndromes have also been ascribed to anomalous Wnt signaling: Wnt4 in renal dysfunction (Surendran et al., 2002), β-catenin in pulmonary fibrosis (Chilosi et al., 2003), sFRPs in retinal degeneration (Jones et al., 2000), and Dkk1 in cortical degeneration as seen in Alzheimer’s disease (Caricasole et al., 2004).
Combined, the findings indicate that control of Wnt signaling is crucial for the maintenance of homeostasis in the adult human body.

1.3 The dopaminergic system in the adult brain

The neurotransmitter dopamine (DA) was discovered by Arvid Carlsson (Carlsson et al., 1958) and is synthesized from L-tyrosine. The latter is converted into levodopa (L-DOPA) which in turn is converted into DA. The conversion of tyrosine into L-DOPA is the rate-limiting step in DA synthesis and the reaction is catalyzed by the enzyme tyrosine hydroxylase (TH). Synthesized DA is subsequently packaged in vesicles by the vesicular monoamine transporter (VMAT) located in nerve terminals. Upon stimulation of DA neurons, DA is released into the synaptic cleft where it can bind to DA receptors on pre- and post-synaptic neurons. Synaptic transmission is terminated by the clearance of dopamine from the synaptic cleft, a process mediated by the dopamine transporter (DAT) (Kandel et al., 2000).

The basal ganglia are a modulatory system that assists in the appropriate planning, initiation, and termination of voluntary movement. The basal ganglia are made up of several nuclei including the substantia nigra (SN), the caudate nucleus and the putamen (collectively known as the striatum), the thalamus, and the globus pallidus. The DA neurons of the SN pars compacta (SNpc) receive input from the cerebral cortex and the striatum. In return, DA axons project to the striatum and the globus pallidus, which in turn project to the thalamus. Information from the thalamus is passed onto the neurons of the motor cortex which in turn can instruct muscles to initiate or terminate movement (Figure 4a). Several additional projections and feedback loops to and from the SN make these DA neurons a relay station that can efficiently modulate voluntary movement (Purves, 1997). DA neurotransmission can be subdivided into three separate systems: the mesolimbic system that projects to the limbic structures and modulates emotional experiences; the mesocortical system that projects to the cortex and modulates cognitive functions; and the mesostriatal system that projects to the striatum, and modulates voluntary movement.
The symptoms caused by imbalances of the DA systems collectively illustrate that DA neurotransmission contributes to processes spanning from cognitive function and emotion, to movement of the muscles controlling the eye lids and the tongue.

1.3.1 Dysfunction of the dopaminergic system

**Schizophrenia**
Excessive activity of the mesolimbic DA system is thought to cause the positive symptoms of schizophrenia, while decreased activity of the mesocortical DA system accounts for the negative symptoms (Kandel et al., 2000).

**Parkinson’s disease**
Dysfunction of the mesostriatal DA system is the main underlying cause of Parkinson’s disease (PD). In 1817, James Parkinson wrote “An essay on the shaking palsy” (Parkinson, 2002), the first report on the condition commonly referred to as PD. Interestingly however, descriptions of symptoms and treatments have been found in Indian Ayurvedic scriptures from 3000 years BC. The current incidence is estimated to be approximately 1% of people above 65 years of age (de Rijk MC, 2000). PD is caused by a selective and progressive death of SNpc DA neurons and a subsequent loss of striatal dopamine (Figure 4b). The most common clinical manifestations are tremor, rigidity, and hypokinesia.

**Parkinson’s disease – Treatment options**
PD was the first disease described to be caused by anomalies in neurotransmitter release in the brain, and it raised hopes of finding a rapid and efficient treatment protocol. Before the seminal description of the successful use of L-DOPA (Cotzias et al., 1967), PD patients lived lives trapped in frozen bodies and died from the disease. DA cannot cross the blood brain barrier. Thus, administration of L-DOPA, a precursor that can be converted into DA inside the cell, was an important improvement for PD patients. Today, L-DOPA is the most common treatment option and it is considered to efficiently alleviate symptoms (Olanow, 2004). However, as the remaining neurons degenerate, the initial effectiveness wears off
and patients develop L-DOPA-induced dyskinesias and other severe side effects (Brotchie et al., 2005). With the continuous loss of DA neurons, cognitive functions and autonomous muscle movements are lost. Apart from pharmacological treatment in the form of L-DOPA alone or in combination with other drugs, other treatment options have been limited and include surgical procedures such as deep-brain stimulation. This technique involves the activation of the DA projections to the striatum, thereby stimulating the target tissues. This technique has shown variable success in alleviating symptoms (Brotchie et al., 2005). However, none of these treatment strategies reverse the disease progression and thus, almost two hundred years after its original description, the quest for a cure for PD remains.

Figure 4 The adult dopaminergic system. (a) Magnetic Resonance Image of the human brain (sagittal view) 1-cerebral cortex, 2-striatum, 3-globus pallidus, 4-substantia nigra, 5-motor cortex (Nixon, 2006). (b) Coronal section of an adult rat brain. The DA neurons of the substantia nigra (SN) can be distinguished by TH immunoreactivity (left arrow). In a rodent model Parkinson’s disease, the SN DA neurons are lost (right arrow head).

Neuroprotective strategies aim at maintaining motor function through protection of the remaining degenerating DA neurons in the brain of PD patients. Administration of glial derived neurotrophic factor (GDNF) has been shown to be protective in animal models of PD (Akerud et al., 2001; Beck et al., 1995; Kordower et al., 2000) and a phase I study on humans showed functional improvement upon infusion of GDNF (Gill et al., 2003). Unfortunately, a larger double-blind study revealed no
significant improvement compared to placebo, and thus the beneficial effects of GDNF are still a matter of debate (Lang et al., 2006).

The most promising approach from a long-term perspective is cell replacement therapy. This technique is based on the notion that transplantation of DA neurons into the PD patient’s striatum can induce long-term motor improvements, despite the chronic nature of the disease and the parallel dysfunction of other cell types. The first transplantations of fetal midbrain tissue into the striatum of PD patients were performed in the late 80s (Lindvall et al., 1994). These early reports of long-term functional improvement suggested that the technique could be applied in the clinics; however, subsequent studies could not reproduce the significant benefit (Freed et al., 2001; Olanow et al., 2003) and nearly 20 years later, there is still limited progression of the technique in a clinical setting. Unfortunately, the studies differed in their design, thereby hindering adequate comparisons between them (Winkler et al., 2005). Technical and ethical issues, such as availability of fetal tissue and survival of grafted tissue, have also hampered the widespread use of the technique. The amount of DA neurons that is obtained from fetal preparations is variable and the number needed for functional improvement upon transplantation has been estimated to be approximately 100,000 cells (the equivalent of 6-7 embryos) (Hagell and Brundin, 2001). Combined, these issues have limited the progression of the technique in a clinical setting.

The field of cell replacement therapy has exploded over the past years, largely due to the increased use of embryonic stem (ES) cells. The totipotent ES cell is the most naïve cell type and has the capacity to differentiate into all cell types present in the body. Furthermore, ES cells hold the advantage of not needing to be freshly isolated, thus eliminating the problem of availability. ES cells can be readily expanded. Selection for the neuronal lineage can be achieved through addition of mitogens such as epidermal growth factor and/or Fgf2. In order to obtain DA neurons, ES cells can be grown on top of feeder cells, such as PA6 cells, that induce their differentiation (Kawasaki et al., 2000), or treated with differentiation-inducing factors, via the formation of embryoid bodies (Kim et al., 2002) More recently, a combination of the two protocols, that exploits PA6 cells and mimicks DA differentiation during development via the sequential addition of Shh and Fgf8, brain-derived neurotrophic factor (BDNF), GDNF, TGF-α, cAMP, has been
developed for human ES cells (Perrier et al., 2004). Although protocols based on human ES cells generate substantial amounts of DA neurons in vitro (Perrier et al., 2004), poor survival and loss of the DA phenotype has resulted in low numbers of integrated DA neurons after transplantation (Park et al., 2005; Zeng et al., 2004). Thus, proof of concept for human ES cell-derived DA neurons remains to be demonstrated. A common problem for all types of transplantation is the need for immunosuppressant medication throughout the patient’s life. The ultimate goal would be to use the patient’s own stem cells to circumvent such side-effects but such days appear distant.

**Parkinson’s disease – Genetics and molecular mechanisms**

Despite the early description of DA cell loss and the presence of Lewy bodies (intracellular protein inclusions), the etiology of PD remains unclear. However, much of the current insight into the molecular mechanisms underlying the neurodegenerative disease has been gained through the identification of genes linked to familial forms of PD (Table 1). Although familial forms account for only 5-10% of all PD cases, their phenotype appears highly similar to that of sporadic PD and has thus warranted in depth studies on the identified genes.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr. Location</th>
<th>Gene</th>
<th>Function</th>
<th>Mutations Identified</th>
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<tbody>
<tr>
<td>PARK 1 and 4</td>
<td>4q21-23</td>
<td>alpha-synuclein</td>
<td>Unknown</td>
<td>Exon duplications and triplications, missense mutations</td>
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<tr>
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<td>6q25.2-27</td>
<td>Parkin</td>
<td>E3 ligase</td>
<td>Exon deletions and duplications, frame-shift, missense, and nonsense mutations</td>
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<tr>
<td>PARK 3</td>
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<td>Unknown</td>
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</tr>
</tbody>
</table>

**Table 1** Nine loci have been linked to heritable forms of Parkinson’s disease (PD). Chr.-chromosomal.
The first major hypothesis in the pathogenesis of PD is based on the finding that inhibitors of mitochondrial complex I (such as MPTP, rotenone, or paraquat) cause a selective loss of DA neurons and protein aggregation, in vitro and in vivo (Manning-Bog et al., 2002; Seniuk et al., 1990). Inhibition of complex I results in a release of free radicals which in turn causes oxidative stress. Oxidative stress has been shown in PD brains (Andersen, 2004) and reduced levels of anti-oxidants is one of the first signs of cell loss in the nigra (Sian et al., 1994). Furthermore, reduced levels of complex I have been found in PD brains (Parker et al., 1989; Schapira et al., 1990). Interestingly, these observations are supported by the finding that overexpression of DJ-1, a gene mutated in autosomal recessive PD, is protective against oxidative stress. The precise function of DJ-1 is unknown but it is localized to the mitochondria and cytoplasm (Canet-Aviles et al., 2004; Taira et al., 2004). The recent identification of PINK1, a protein that localizes to mitochondria and appears to protect against apoptosis (Petit et al., 2005; Valente et al., 2004) further supports the notion that mitochondrial dysfunction is indeed part of PD pathogenesis.

The second major hypothesis takes into consideration the amount of aggregated proteins observed in degenerating DA neurons. Normally, aggregated or dysfunctional proteins are degraded in the proteasome in an ATP-dependent manner. It is speculated that Lewy bodies (LBs), the classical histopathological hallmark of PD, serve to protect the cell against dysfunctional proteins that are not being appropriately degraded. Thus, these observations point to dysfunctions of the ubiquitin-proteasome system (UPS), and are supported by the identification of α-synuclein, parkin, and ubiquitin C-terminal hydrolase 1 (UCHL-1) as genes mutated in familial forms of PD (Leroy et al., 1998; Polymeropoulos et al., 1997; Shimura et al., 2000). α-synuclein is an aggregation-prone protein that constitutes the major component of LBs (Spillantini et al., 1998) while Parkin regulates the proteasomal degradation of proteins through ubiquitylation. UCHL-1 is involved in the recycling of ubiquitin after proteasomal degradation (see below). Interestingly, α-synuclein is not a Parkin substrate in its native state but is thought to become one upon glycosylation, which also alters its conformation and function (Shimura et al.,
These results have yet to be confirmed by other groups but represent a potential link between protein aggregation and proteasomal dysfunction.

The recent identification of *PINK1* and *LRRK2*, two kinases, has expanded the horizon onto cell cycle regulation. *PINK1* has been associated with the tumor-suppressor PTEN (Unoki and Nakamura, 2001), and intriguingly, Parkin has been described to function as a tumor suppressor in the liver and the lung (Picchio et al., 2004; Wang et al., 2004). Thus, oxidative stress caused by mitochondrial dysfunction, protein aggregation caused by dysfunction of the UPS, and cell cycle deregulation appear to be the pieces to that need to be fitted into the PD puzzle.

**Parkinson’s disease – Parkin**

Parkin was originally identified in autosomal recessive juvenile Parkinsonism (Shimura et al., 2000), and missense mutations, deletions, duplications and exonal splicing mutations have all been detected in the *parkin* gene (Lucking et al., 2000; Rawal et al., 2003). These findings indicate that several parts of the *parkin* gene are important for its function, and that its function is indeed important in the prevention of PD. Parkin is an E3 ubiquitin ligase and its N-terminal domain, the ubiquitin like domain (Ubl), is believe to cause tethering of Parkin close to the proteasome (Sakata et al., 2003), while the RING-IBR-RING structure is thought to be important for the interactions with substrates and with the E2 (a ubiquitin conjugating enzyme that carries active ubiquitin). Parkin belongs to the RING-family of E3 ligases (Shimura et al., 2000) and as such, it has been reported to function within a multiprotein Skp1-Cullin1-Fbox/WD repeat (SCF) ligase complex together with F-box/WD repeat protein Fbw7 (Joazeiro and Weissman, 2000; Staropoli et al., 2003). The process of ubiquitylation is initiated by addition of ubiquitin molecules to the target protein, thereby tagging it for degradation in the proteasome (Figure 5). Several Parkin substrates have been identified in vitro, such as the misfolding- and aggregation-prone proteins α-synuclein-interacting protein synphilin, and Pael-R, and the cell cycle protein cyclin E (Chung et al., 2001; Imai et al., 2001; Staropoli et al., 2003). Accordingly, accumulation of the Parkin substrates Pael-R and CDCrel results in SN DA cell death in vivo (Dong et al., 2003; Yang et al., 2003). However, only a few of all putative Parkin substrates have
been confirmed *in vivo* (Ko et al., 2005). Parkin has been linked to the pathogenesis of sporadic PD through the finding that Parkin’s E3 activity is reduced by nitric oxide (Chung et al., 2004; Yao et al., 2004). The biological relevance of these observations is still under debate but nitrosylated Parkin has been detected in human PD (Chung et al., 2004; Yao et al., 2004). Given the above findings, it is somewhat surprising that ablation of functional Parkin protein does not cause a loss of DA neurons, or generate any distinct Parkinsonian phenotype *in vivo* (Goldberg et al., 2003; Itier et al., 2003; Von Coelln et al., 2004). However, it is plausible that alternative E3 ligases may compensate for the loss of Parkin. In any event, the contribution of Parkin to PD pathogenesis remains partially unclear.

**Figure 5** The ubiquitin proteasome system. Ubiquitin (Ub) is activated by a ubiquitin activating enzyme (E1) and transferred to a ubiquitin conjugating enzyme (E2). Parkin, a ubiquitin ligase (E3), and the E2 interacts with normal or dysfunctional proteins and ubiquitin monomers are added to the target protein. The ubiquitin chain targets the protein for degradation in the 26S proteasome. The remains of the degraded protein can be recycled for protein synthesis and the ubiquitin chain is recycled back to free ubiquitin monomers by deubiquitylating enzymes (DUBs) such as UCHL-1.
2 RESULTS

2.1 Wnt signaling during dopaminergic neuron development

2.1.1 Purified Wnt5a increases differentiation of VM precursors into DA neurons.

When joining the laboratory in the fall of 2001, the initial observation that Wnts regulate VM DA development had already been made. I thus embarked on a mission to further explore the role of Wnts in respect of DA development and survival. The development of the field was hampered by the lack of pure Wnt proteins and even though experiments based on conditioned medias had generated several important findings, the issue of specificity remained unaddressed. In 2003, Willert and colleagues successfully purified active Wnt3a, thereby enabling in depth studies on individual Wnt ligands and their biology. The purification protocol is based on the hydrophobicity of Wnt3a and the authors found that palmitoylation was essential for Wnt activity (Willert et al., 2003). With minor changes to the Willert protocol, we successfully purified Wnt5a. This allowed us to address crucial issues related to the effects of Wnt5a during VM development (Paper I). We found that pure Wnt5a increases the number of TH+ DA neurons in VM E14.5 precursor cultures, thereby confirming our observations based on conditioned medias (Castelo-Branco et al., 2003). Moreover, biochemical analysis of Wnt activity in the substantia nigra cell line SN4741 demonstrated that that both Wnt3a and -5a induce hyperphosphorylation of Dishevelled, a central component of Wnt signaling, while only Wnt3a induces stabilization of β-catenin.

2.1.2 Fz9 is expressed in VM neuronal precursors and inhibits Wnt5a signaling in DA cells.

We had previously observed that both Wnt1 and -3a are also present in the developing midbrain and induce proliferation of DA precursors. While Wnt1 increases the number of TH+ DA neurons, Wnt3a appears to inhibit DA...
differentiation (Castelo-Branco et al., 2003). To extend our understanding of the contribution of Wnt signaling during DA development, we examined the expression profile of the remaining Wnt family members, Frizzled (Fz) receptors, and several intracellular signaling components in the developing midbrain and made a comparison between the VM and the DM (Paper II). The observation that most Wnts, all Fzs and many intracellular components were present in the developing midbrain was quite unexpected and indicates that the Wnt family has several roles during VM development and is subjected to tight spatial and temporal regulation. Our results also suggest that several Wnts and/or Fzs might contribute to the final outcome of Wnt signaling during VM development. In order to identify differential Fz expression in the cell types present in the developing VM, we analyzed neuronal or glial precursor-enriched cultures as well as newborn DA neurons isolated from TH-GFP transgenic mice. Interestingly, we found that Fz6 and -7 are expressed by DA neurons and similarly enriched in the glial fraction. Our laboratory has previously hypothesized that glial-neuronal cross-talk is crucial for DA development (Castelo-Branco et al., 2005; Hall et al., 2003; Wagner et al., 1999). Fz6 and-7 could thus contribute to this cross-talk. Across all analyses, Fz9 was the most striking example of a differential pattern of expression. Fz9 is highly expressed in neuronal progenitors and absent in newborn DA neurons. The findings are in accordance with previous reports of Fz9 as a marker of neuronal progenitor populations and the maintenance of an undifferentiated phenotype (Ranheim et al., 2005; Van Raay et al., 2001), which prompted us to further examine the relevance of Fz9 during DA development (Paper II). In situ hybridizations in E11.5 mouse VM revealed that Fz9 is expressed exclusively in the proliferating ventricular zone. At E11.5, TH expression is restricted to the differentiated neurons of the marginal zone, the results suggesting that Fz9 might be incompatible with a DA phenotype. In order to assess the functional significance of Fz9 during DA development, we overexpressed Fz9 in SN4741 DA cells that ordinarily lack endogenous Fz9 expression. Wnt3a, but not -5a, stabilizes β-catenin in these cells and we thus used hyperphosphorylation of Dvl as a read-out of Wnt activity. Both Wnt3a and -5a induce hyperphosphorylation of Dvl in SN4741 cells (Paper I). We found that expression of Fz9 specifically reduced the hyperphosphorylation induced by Wnt5a while it did not alter that induced by Wnt3a (Paper II). We have previously shown
that Wnt3a and -5a have opposing effects on DA differentiation and we thus hypothesized that these effects might, in part, be dependent on the absence or presence of Fz9. In neuronal progenitors the presence of Fz9 is permissive to the proliferative effects induced by Wnt3a (probably mediated by Wnt1 in vivo) while Wnt5a signaling is inhibited by Fz9. Once the expression of Fz9 decreases, Wnt5a can exert its effects on DA differentiation.

2.1.3 Stabilization of β-catenin increases differentiation of VM precursors into DA neurons.

Our laboratory and others have tried to purify other members of the Wnt family, with variable success. It has been speculated that the palmitoylation status and other modifications are highly variable between the different family members, and that these differences might influence the outcome of purification. From a therapeutic view-point, the hydrophobic, sensitive nature of Wnt ligands constitutes a considerable obstacle. However, a way to circumvent the need for pure Wnt protein during the differentiation of stem/progenitor cells into DA neurons would be to activate downstream pathways directly. We thus wished to identify the intracellular pathway mediating the pro-differentiation effects induced by Wnt5a during DA development (Paper III). GSK-3β is a central component in Wnt signaling and at the same time, a point of convergence with signaling pathways such as insulin (Frame and Cohen, 2001). We used two chemical inhibitors of GSK-3β; Indirubin-3-monoxime (I3M), a broad-range inhibitor that also inhibits cyclin dependent kinase 1 (Hoessel et al., 1999; Leclerc et al., 2001), and Kenpaullone (KP) a more selective inhibitor of GSK-3β (Bain et al., 2003). Treatment of VM E14.5 precursor cultures with the inhibitors resulted in an increased neuronal differentiation and maturation, as assessed by β-III tubulin (Tuj1) and microtubule associated protein immunoreactivity. Interestingly, we found that these compounds specifically increased neuronal differentiation into the DA lineage by 3-5 fold, as assessed by TH/Tuj1 immunoreactivity. Further analyses revealed that the specific inhibitor KP did not significantly alter cell survival (as assessed by cleaved caspase 3 immunoreactivity), or proliferation (as assessed by bromodeoxyuridine (BrdU)
incorporation). Instead, the effect on the DA lineage was achieved through increased conversion of Nurr1+ progenitors into TH+ DA neurons. Overexpression of β-catenin generated a similar increase in the number of TH+ neurons, indicating that the effect on DA differentiation was indeed mediated by Wnt/β-catenin signaling.

2.1.4 Inhibition of JNK increases survival of transplanted DA neurons in Parkinsonian rats.

In our analysis of intracellular Wnt signaling, we aimed at blocking cJun-N-terminal kinase (JNK) in order to study the potential role of the JNK/PCP pathway during DA development. JNK has been reported to be downstream of Wnt5a (Oishi et al., 2003; Yamanaka et al., 2002) and we thus hypothesized that inhibition of JNK signaling should reduce the differentiation into DA neurons. We treated rat VM E13.5 and E14.5 precursor cultures with increasing doses of the JNK inhibitor SP600125 for 1 or 3 three days in vitro (1 or 3DIV) and quite unexpectedly, addition of SP600125 resulted in a large increase in the number of TH+ DA neurons. The highest yield of TH+ DA neurons was obtained in the E14.5 cultures while the largest increase was observed in the E13.5 cultures (13-fold increase). In order to address the specificity for the DA lineage, we analyzed the effects on the total neuronal pool, as assessed by Tuj1 counts relative to the total number of cells (visualized by Hoechst 33258 staining). We found that neither the total number of cells nor the total neuronal pool was significantly altered, indicating that SP600125 exhibited specificity for the DA lineage.

JNK signaling is known to mediate neuronal apoptosis (Silva et al., 2005). We therefore examined how SP600125 affected apoptotic signaling in DA neurons. Indeed, we found that SP600125 reduced the level of phospho-cJun in the DA cell line SN4741, suggesting that SP600125 increased the survival of DA neurons. Combined, these findings suggest that the mechanism of action of SP600125 involves the survival of a precursor committed to the DA lineage. We concluded that the observed effects might not be ascribable to Wnt-related actions but that we had identified a way to improve DA cell survival. Successful transplantation of
fetal tissue into PD patients was originally described in the late 80s (Lindvall et al., 1994; Piccini et al., 1999; Wenning et al., 1997) but subsequent studies failed to generate similar function improvement (Freed et al., 2001; Olanow et al., 2003). One of the foremost problems when working with cell replacement therapies in Parkinsonian patients and animal models of disease is the poor survival of grafted cells, and notably the vulnerability of the DA neurons. Based on our in vitro findings, we wished to examine the effect of SP600125 on DA cell survival in primary VM striatal grafts. We utilized the 6-hydroxydopamine (6-OHDA) rodent model of PD and observed a complete ablation of cell bodies as well as fibers and terminals innervating the striatum prior to transplantation. We found that administration of SP600125 through intrastriatal infusion for 5 days resulted in a 2-fold increase in the number of surviving DA neurons within the graft. Moreover, the transplanted DA neurons were found throughout the host striatum. Within 7 days, the grafted DA neurons had developed a mature morphology, indicating that they were well integrated within the host tissue. Importantly, even though SP600125 mediates DA cell survival, this survival does not come at the expense of excessive proliferation in vivo, thereby highlighting the feasibility of the drug in therapeutic use in the treatment of PD. All in all, our study showed that inhibition of JNK signaling increases DA survival in vivo and that it might overcome some of the limitations of cell replacement therapies in the treatment of PD.

2.2 Wnt signaling in dopaminergic neurons

2.2.1 Parkin regulates β-catenin turnover in vivo linking Wnt/β-catenin signaling to dopaminergic neuron degeneration.

Cell degeneration and subsequent loss of functionality within an organ can be compensated by cell division. Our findings regarding Wnt/β-catenin functions during the development of DA neurons lead us to hypothesize that it might also play a role in DA degeneration. Since post-mitotic DA neurons cannot divide, we speculated that the proliferative effects of Wnt/β-catenin would instead result in
apoptosis, thereby aggravating the degenerative process. β-catenin protein levels are regulated through rapid ubiquitylation and subsequent degradation in the proteasome. Two E3 ligases, β-TrCP and Siah-1, are thought to regulate the degradation of β-catenin, through partially independent mechanisms (Kitagawa et al., 1999; Liu et al., 2001a; Matsuzawa and Reed, 2001; Winston et al., 1999) but it is not known whether the PD-linked E3 ligase Parkin could also regulate β-catenin. Parkin has been reported to regulate the turnover of aggregation- and misfolding-prone proteins such as the α-synuclein interacting protein synphilin, and Pael-R (Chung et al., 2001; Imai et al., 2001). Furthermore, Parkin has been shown to protect DA neurons against cyclin E-mediated kainate-induced toxicity (Staropoli et al., 2003). We hypothesized that Parkin might regulate β-catenin turnover in post-mitotic neurons based on the following evidence: (i) the parkin promoter is negatively regulated by the Wnt target gene myc (West et al., 2004) (ii) cyclin E and β-catenin interact directly (Park et al., 2004) (iii) Parkin has been reported to act as a tumor suppressor in tissues where deregulation of Wnt/β-catenin signaling can cause cancer (Lee et al., 2006; Mazieres et al., 2005; Picchio et al., 2004; Wang et al., 2004). We first examined whether Parkin and β-catenin were expressed in the same cellular compartment and found that the patterns of expression predominantly overlapped in the cytoplasm. Using GST pull-down and co-immunoprecipitation experiments we found that the two proteins interacted. Thus, we had established at a physiological level that β-catenin could be a Parkin substrate. We next overexpressed Parkin and found that the steady state levels of β-catenin were reduced. These results were further confirmed by the finding that β-catenin protein levels are significantly increased in parkin null mice. In order to identify the mechanism by which Parkin regulates β-catenin turnover, we performed immunoprecipitation experiments with an anti-β-catenin antibody followed by immunoblotting against ubiquitin. We found that Parkin indeed increased the polyubiquitylation of β-catenin, compared to control. Interestingly, we observed that Fbw7 was necessary for Parkin’s ubiquitin ligase activity. We further showed that Parkin reduced Wnt-induced increases in unphosphorylated, active β-catenin, demonstrating that Parkin can regulate canonical β-catenin signaling. Based on
these findings, we sought to examine the consequences of excessive β-catenin signaling in DA neurons. We found that overexpression of a constitutively active β-catenin (S37A) in VM precursor cultures reduced the number of TH+ DA neurons. Finally, stabilization of β-catenin signaling in differentiated primary VM neurons resulted in increased proliferation followed by apoptosis and a loss of DA neurons. In conclusion, we have identified Parkin as a novel regulator of Wnt/β-catenin signaling, and an imbalance between Parkin and β-catenin in post-mitotic DA neurons may contribute to the pathogenesis of PD.
3 DISCUSSION

3.1 Signaling specificity

In the work presented in this thesis, we observed that virtually all Wnt family members and all Fz receptors are expressed in the developing midbrain. Even though Wnt signaling has been shown to regulate key events such as proliferation, cell fate determination, and differentiation during development, this finding was quite unexpected and underscores the importance and diversity of the Wnt signaling pathway during embryogenesis. These results also raise the question whether an individual Wnt can mediate several processes, or if some Wnts mediate one process, e.g. proliferation, while others mediate differentiation? The presence of several ligands and receptors would perhaps speak in favor of the latter. However, both Wnt1 and -5a can induce proliferation or differentiation depending on the context (Castelo-Branco et al., 2003; Huang et al., 2005; Lee et al., 2004; Murdoch et al., 2003; Prakash et al., 2006), thereby supporting the former argument. Although it appears likely that both ligands and receptors contribute to the specificity and that a given biological outcome is the result of specific ligand-receptor combinations, this issue remains to be addressed in a systematic way. Thus, expression mapping combined with studies on native ligand-receptor interactions will be imperative for a deeper understanding on how Wnt signaling specificity is achieved.

3.2 Canonical vs. non-canonical

Wnt ligands are often categorized into canonical Wnts, such as Wnt3a, that signal through β-catenin, and non-canonical Wnts, such as Wnt5a, that can signal through JNK or Ca$^{2+}$. This attempt to facilitate the understanding of Wnt biology was originally based on the capacity of Wnt ligands to morphologically transform cultured mammary cells or to induce axis duplication in Xenopus (Du et al., 1995; Wong et al., 1994). Other criteria for canonical Wnt signaling have included the nuclear translocation of β-catenin. However, as the complexity of the Wnt signaling cascade is deciphered, these definitions and classifications are complicated by the
observation that Wnt5a also can also induce axis duplication, a classical read-out of canonical signaling (He et al., 1997). Furthermore, it has been shown that β-catenin can signal in the cytoplasm together with cadherins (Yu and Malenka, 2003), indicating that nuclear translocation is not a necessary consequence of Wnt-induced stabilization of β-catenin. Thus, until the complete potential of each Wnt ligand has been fully examined, it might be more accurate to categorize Wnts according to the signaling components they activate and/or the biological outcomes they mediate.

3.3 Wnt5a in the VM – canonical or non-canonical?

In paper I as well as in a previous paper from our laboratory (Castelo-Branco et al., 2003), we show that Wnt5a increases the differentiation of VM precursors into DA neurons. In paper III, we show that stabilization of β-catenin affects the same process, through similar mechanisms involving conversion of Nurr1+ precursors into TH+ DA neurons. Thus, it is logical to hypothesize that the effects of Wnt5a, normally considered a non-canonical Wnt, might be mediated by β-catenin in the VM. So far, our analysis on this matter has been inconclusive. In early experiments before the advent of pure Wnt5a, we observed stabilization of β-catenin in response to the transfection of Wnt5a in primary VM precursor cultures. Unfortunately, the poor transfection efficiency precluded any conclusion as to whether the variable results were due to variability in Wnt5a expression level or whether the biological finding was an artifact. Our more recent studies have shown that Wnt5a does not stabilize β-catenin nor increase the levels of unphosphorylated β-catenin in the DA cell line SN4741. However, comparisons of Fz expression between SN4741 and the developing VM revealed only a partial overlap. Furthermore, SN4741 does not express Kremen II (paper II and G. Schulte, unpublished). Thus, while the SN4741 cell line has been an important tool in our exploration of Wnt biology, it might not be the most suitable system for this particular question. Analyses of a pure VM Nurr1+ cell population treated with recombinant Wnt5a would be needed to gain further insight into this matter in vitro.

If Wnt5a does not stabilize β-catenin in VM precursors, it implies that two pathways contribute to the differentiation of DA neurons. Although this is far from
inconceivable, the concept is relatively unexplored in the literature. Current studies in the laboratory involving the generation of \textit{Wnt1} and \textit{-5a} double knockout animals aim at addressing this issue from a signaling perspective and to give clarity into the role of Wnt5a during VM development \textit{in vivo}.

3.4 Measuring non-canonical Wnt signaling activity
Canonical Wnt activity can be measured at several levels of the signaling cascade; stabilization of total \(\beta\)-catenin, increases in unphosphorylated \(\beta\)-catenin, measurement of transcriptional \(\beta\)-catenin activity (TOPFLASH reporter), altered levels of target gene mRNA/protein, and \textit{in vivo} axis duplication. Unfortunately, this is not the case for non-canonical signaling, where few read-outs and tools are available. JNK and Ca\(^{2+}\) are both involved in fundamental intracellular processes and fluctuations in their steady state levels can be difficult to classify as Wnt-specific effects. Furthermore, the sometimes relatively modest increases sufficient for signaling have proven difficult to measure. Thus, in order to extend our knowledge on the contribution of non-canonical Wnt signaling, one of the most pressing issues is to develop better and more sensitive assays for measurement of non-canonical activity. Identification of key Wnt-specific activation/inactivation steps would allow for measurements of post-translational modifications such as phosphorylation and/or ubiquitylation. Moreover, provided the insight into canonical signaling that has been obtained by studies on model organisms such as Drosophila and Xenopus, further studies on these organisms should hopefully facilitate the identification of new players that are exclusive to non-canonical Wnt signaling. In addition to providing information about new target genes that can be measured at the mRNA/protein level in relatively rapid assays, these models can also provide functional \textit{in vivo} read-outs of non-canonical signaling.

3.5 Fetal tissue – hot or not?
Over the recent years, ES cells have emerged as the new star on the sky of cell replacement therapy. The totipotency of ES cells is a great conceptual and technical
advantage and most protocols developed for fetal tissue have progressively been adapted to ES cells. This could appear somewhat surprising given the ethical debates resulting in restricted use and limited numbers of available ES cell lines in certain countries. Furthermore, until proof of concept has been obtained for human ES cells, this work remains far from a clinical reality. All the work on DA differentiation presented in this thesis is based on fetal tissue and although the hype has died down, fetal tissue and stem cells still hold potential for the following reasons: (i) ES cell lines are known to undergo quite dramatic changes over time, with chromosomal aberrations and altered gene expression as a common consequence. Thus, one of the weaknesses of fetal tissue, namely that the tissue has to be freshly isolated, is at the same time a strength since the tissue is less manipulated. (ii) The risk of complications such as tumor formation is close to nonexistent when using fetal tissue, compared to the relatively high teratoma formation rate observed upon transplantation of undifferentiated ES cells (Parish et al., 2005). Thus, the improvement observed with fetal tissue does not come at the expense of cancer development (iii) While successful in vitro, transplantation of human ES cells has been unsuccessful in vivo so far. Poor survival and loss of the DA phenotype have resulted in low numbers of integrated DA neurons (Park et al., 2005; Zeng et al., 2004). (iv) Proof of concept for functional improvement in human PD has been obtained for transplantation of fetal tissue, thereby making it the only viable cell therapy option available at present.

Given the amount of research currently invested into ES cells, the above mentioned weaknesses will hopefully be improved, making it a feasible and realistic treatment option in the future. Perhaps an optimal protocol might include fresh tissue transiently expanded in vitro prior to transplantation, thereby reducing the amount of tissue needed while maintaining the tissue’s genetic integrity. Until then however, work on fetal tissue deserves more attention than it is currently receiving.

3.6 Wnt/β-catenin signaling in DA degeneration

Our finding that Wnt/β-catenin signaling increases proliferation and apoptosis in primary post-mitotic DA neurons suggests that excessive Wnt signaling might contribute to DA degeneration. The dentification of Parkin as a novel regulator of
\(\beta\)-catenin turnover \textit{in vivo} further strengthens the link to PD. Indeed, we observed a spatially restricted increase in \(\beta\)-catenin in \textit{parkin} null mice, when compared to other brain regions. We interpreted this finding as part of the reason why only DA neurons die in PD despite the fact that all cells lack functional Parkin. However, given the importance of \(\beta\)-catenin during DA development, \(\beta\)-catenin signaling might be more active in degenerating DA neurons than in normal cells and the lack of regulation would thus become more apparent in DA neurons. The consequence would be an excess of proliferative signals resulting in apoptosis. Interestingly, Parkin has been described to act as a tumor suppressor in tissues where deregulated Wnt signaling can cause cancer (Cesari et al., 2003; Lee et al., 2006; Mazieres et al., 2005; Picchio et al., 2004; Wang et al., 2004), suggesting that the role of Parkin might be to control the turnover of the oncogene \(\beta\)-catenin and regulate cell cycle activity. Furthermore, it is noteworthy that the PD-linked genes \textit{UCHL-1}, \textit{PINK1}, \textit{DJ-1} and \textit{LRRK2} have all been associated with cell cycle regulation and it is thus tempting to speculate that the regulation of Wnt signaling by Parkin is part of a general mechanism by which the cell protects itself from excessive proliferation. Aberrant cell cycle activity combined with dysfunction of tumor suppressor genes could thus manifest itself in the form of cancer in tissues with proliferative capacity, whilst resulting in apoptosis in terminally differentiated neurons. Further studies on excessive Wnt signaling combined with Parkin dysfunction, such as crossings of transgenic mice expressing a constitutively active \(\beta\)-catenin with mice lacking \textit{parkin}, will be imperative for our understanding of how cell cycle regulation and apoptosis contribute to the pathogenesis of PD.

\textbf{Concluding remarks}

Over the past years, the Wnt field has exploded, with new players and functions continuously being described. We now know that the Wnt signaling pathway is involved in important processes, including DA development, throughout the life span of a vertebrate organism. However, the mechanisms of action remain ambiguous. Thus, it appears that studies on Wnt signaling will keep scientists busy for a long time to come.
4 CONCLUSIONS

Based on the work presented in this thesis, I wish to draw the following conclusions:

i. Wnt5a increases the differentiation of primary ventral midbrain precursors into dopaminergic neurons.

ii. Most Wnts and all Fzs are expressed in the developing ventral midbrain at the time of birth of DA neurons.

iii. Fz9 can be used as a marker of ventral midbrain neuronal precursors and it inhibits Wnt5a signaling in dopamine cells.

iv. Stabilization of β-catenin in VM precursors increases dopaminergic differentiation and the effects are similar to those observed for Wnt5a.

v. Inhibition of JNK signaling increases the survival of dopamine neurons in vivo and in vitro.

vi. Excessive Wnt/β-catenin signaling in differentiated primary ventral midbrain neurons results in cell cycle re-entry followed by a loss of dopaminergic neurons.

vii. The E3 ubiquitin ligase Parkin regulates β-catenin turnover in vivo and in vitro, thereby linking Wnt/β-catenin signaling to dopamine neuron degeneration.
5 SIGNIFICANCE

The work presented in this thesis is based on the idea that cellular mechanisms important during embryonic development are also important during adult stages. The knowledge obtained during recent years support this notion. Thus, apart from increasing our understanding of the human body, studies on development will hopefully provide tools in the battle against pathology. The Wnt signaling pathway regulates several important processes both during embryogenesis and in the adult human body. However, many aspects of Wnt biology remain elusive. The work presented in this thesis aims at extending our understanding of the role of Wnt signaling in dopaminergic neuron development and degeneration.
6  POPULAR SCIENTIFIC SUMMARY

The nerve cells of the substantia nigra in the brain produce dopamine, a substance that is important for muscle movement. In Parkinson’s disease (PD), the dopamine-producing (DA) cells die, resulting in difficulties to begin and end movements. The typical PD patient therefore has difficulties in walking, moving, and maintaining a straight posture. As the disease progresses and even more cells have died, muscles such as those of the eye lids, tongue, and stomach are affected, causing difficult symptoms. There is no cure for PD at present but one of the most promising treatment strategies is termed cell replacement therapy (CRT). CRT is based on the idea that transplantation of new DA cells into the brain of the PD patient can restore the patient’s movement abilities. However, this treatment strategy demands large numbers of DA cells. DA cells can be derived from stem cells but the signals that are necessary for this transition are still not entirely known. Studies on how DA cells arise during embryonic development aim at answering this question.

The Wnt family of proteins is involved in several important processes during embryonic development and is associated with different cancer types later on in life. In this thesis, evidence is presented that Wnts are also important for the development of DA cells. They can increase the number of cells that have the potential of becoming DA cells (precursor cells) and can also directly push the precursor cells into becoming DA cells.

One of the problems with CRT is poor survival of the DA cells that are transplanted into the brains of PD patients. Data presented in this thesis shows that treatment with the molecule SP600125 increases the survival of DA cells when they are transplanted into an animal model of PD. These results suggest that fewer DA cells would be needed for transplantation and the cells that are transplanted would integrate better into the patient’s brain.

Finally, evidence is presented that Parkin, a protein linked to inheritable forms of PD, can regulate Wnt signaling. These results provide a link between Wnts and PD and suggest that signals that are important during the development of DA cells are also important during their death.

In summary, this thesis uncovers several new aspects of Wnt signaling in DA cells, thereby opening up new avenues for the understanding and treatment of PD.
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REFERENCES


