From Karolinska Institutet, Stockholm, Sweden

Regulation of Midbrain Dopaminergic Neuron Development by Wnts, sFRPs and bHLH Proteins

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Stockholm 2007

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ISBN *978-91-7357-242-2*

To Mum, Niclas and Felicia for keeping the flame burning

"I should like to work like the archeologist who pieces together the fragments of a lovely thing which are alone left to him. As he proceeds, fragment by fragment, he is guided by the conviction that these fragments are parts of a whole which, however, he does not yet know. He must be enough of an artist to recreate, as it were the work of the master, but dare not build according to his own ideas. Above all, he must keep holy the broken edges of the fragments; in that way only may he hope to fit new fragments into their proper place and thus ultimately achieve a true restoration of the master´s creation."

– Hans Spermann, 1938

ABSTRACT

Parkinson´s disease (PD) is a chronic neurodegenerative disorder. The main pathology is characterized by progressive degeneration of the dopaminergic (DA) neurons of the substantia nigra leading to loss of striatal dopamine innervation. The knowledge about the fundamental mechanisms behind the degenerative process has been limited. During recent years a promising approach is evolving based on DA cell replacement therapy. However, in order to find a cure for PD an increased understanding is required about the intrinsic and extrinsic signals involved in the development of DA progenitor cells during embryogenesis. This thesis identifies novel players in midbrain development and investigates the mechanisms by which three major signaling modulators, Wnts, soluble Frizzled Related Proteins (sFRPs) and basic-Helix-Loop-Helix (bHLH) family of proteins, regulate DA neuron development.

Proneural genes belong to the bHLH family of transcription factors and are crucial regulators of neurogenesis and subtype specification in many areas of the nervous system. Their function in DA neuron development has been unknown. In this thesis it is reported that the proneural genes Neurogenin2 (Ngn2) and Mash1 have an intricate pattern of expression in the ventricular zone (VZ) of the ventral midbrain (vMB), where DA neurons are generated. To examine the function of these genes, mice were analyzed in which one or two of these genes were deleted (Ngn1,Ngn2 and Mash1) or substituted (Mash1 in the Ngn2 locus). Our results demonstrate that Ngn2 is required for the differentiation of Sox2+ VZ progenitors into Nurr1+ postmitotic DA neuron precursors in the intermediate zone (ImZ), and that it is also required for their subsequent differentiation into DA neurons in the marginal zone (MZ). Although Mash1 normally has no detectable function in DA neuron development, it could partially rescue the

generation of DA neuron precursors in the absence of Ngn2. These results demonstrate that Ngn2 is uniquely required for the development of vMB DA neurons.

The Wnt signaling pathway regulates several developmental processes in the mammalian CNS; neural patterning, cell fate determination, proliferation, differentiation, neuronal maturation, cell migration and axon guidance. Our results present evidence that Wnt-1, -3a, and -5a expression is differentially regulated during vMB development. Wnt-3a promoted the proliferation of precursor cells expressing the orphan nuclear receptor-related factor 1 (Nurr1) but did not increase the number of DA neurons. Conversely, Wnt-1 and -5a increased the number of rat vMB DA neurons in rat embryonic day 14.5 precursor cultures by two distinct mechanisms. Wnt-1 predominantly increased the proliferation of Nurr1-precursors. In contrast, Wnt-5a primarily increased the proportion of Nurr1 precursors that acquired a neuronal DA phenotype. These findings indicate that Wnts are key regulators of proliferation and differentiation of DA precursors during vMB neurogenesis and that different Wnts have specific and unique activity profiles.

Furthermore temporal expression profiles of Wnt components during critical phases of MB development revealed Frizzled (Fz) 9, a Wnt receptor, to be highly expressed in DA progenitors but not in newborn DA neurons. A possible function of Fz9 during early MB development might be to regulate proliferation of DA progenitors and inhibit differentiation, since Fz9 reduced Wnt5a signaling in DA cells *in vitro*.

Finally, we set to examine the function of sFRPs in the developing ventral midbrain. sFRPs are a secreted family of factors that sterically hinder the Wnt ligand-Fz receptor complex to form and thereby block the Wnt signling pathway.

sFRP1-3, but not 4 were expressed in the developing vMB, during DA neurogenesis. We therefore examined whether sFRP1-3 could work as Wnt antagonists in a dopaminergic cell line. We found that high doses of sFRP1 and sFRP2, but not sFRP3,

acted as competitive antagonists of Wnt signaling. Treatment of vMB precursor cultures with sFRP1 resulted in DA neuron cell death, an effect that is compatible with Wnt1 blocking. However, treatment with sFRP2 lead to increased proliferation of progenitors and increased number of DA neurons, an effect incompatible with a Wnt blocking activity. Analysis of the sFRP2$^{-/-}$ mice also suggested that sFRP2 does not block Wnt function. On the contrary, we found that sFRP2 is partially required for several sequential steps in DA neuron development, including DA neurogenesis, differentiation of DA precursors into neurons and neuritogenesis. Thus, our results unravel several novel functions of sFRPs and identify sFRP2 as a novel player in DA neuron development.

In summary, these results presented identify several novel players in midbrain DA neuron development and reveal new functions of proneural bHLHs, Wnts and sFRPs, thereby extending our knowledge and identifying factors that may be used to develop novel DA cell replacement therapies for the treatment of PD.

LIST OF PUBLICATIONS

I. **Kele, J.*, Simplicio, N.*, Ferri, A.L., Mira, H., Guillemot, F., Arenas, E. and Ang, S.L.** Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. Development 133(3): 495-505. 2006

II. **Castelo-Branco, G.*, Wagner, J.*, Rodriguez, F.J.*, Kele, J., Sousa, K., Rawal, N., Pasolli, H.A., Fuchs, E., Kitajewski, J. and Arenas, E.**. Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc Natl Acad Sci U S A 100(22): 12747-52. 2003

III. **Rawal, N., Castelo-Branco, G., Sousa, K.M., Kele, J., Kobayashi, K., Okano, H. and Arenas, E.** Dynamic temporal and cell type-specific expression of Wnt signaling components in the developing midbrain. Exp Cell Res 312(9): 1626-36. 2006

IV. **Kele, J., Anderson, E, Bryja, V., Bonilla S., Rubin, J. S., Shimono A., and Arenas, E.** SFRP2 is required for midbrain dopaminergic neuron development. *Manuscript*

* Denotes equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
A-P	Anterior-posterior axis
APC	Adenomatous polyposis coli
BMP	Bone morphogenic protein
BrdU	Bromodioxyuridine
CAMKII	Calcium/calmodulin-dependent kinase
CBP/p300	cAMP response element binding protein
CDK	Cyclin-dependent kinase
CK1	Casein kinase1
CM	Conditioned media
CNS	Central nervous system
CRD	Cysteine rich domain
DA	Dopaminergic
DAT	Dopamine transporter
DCC	Deleted in Colorectal Cancer
dMB	Dorsal Midbrain
D-V	Dorsal-ventral axis
Dvl	Dishevelled
En-1	Engrailed-1
ES	Embryonic stem cells
Fgf8	Fibroblast growth factor 8
Fz	Frizzled
Gbx2	Gastrulation brain homeobox 2
GDNF	Glial cell-derived neurotrophic factor
Gli1	Glioma associated oncogene homolog 1
GPCR	GTP-binding protein coupled receptors
GSK3β	Glycogen synthase kinase 3β
HEB	HeLa E-box Binding protein
Hes1/3	Hairy enhancer of split 1/3
Hnf-3β	Hepatocyte nuclear factor 3β
ICD	Intra Cellular Domain
JNK	c-jun NH_3-terminal kinase
Lmx1a/b	LIM homeobox transcription factor 1
LRP5/6	Low density related lipoprotein receptor
LXR	Liver X receptor
Mash1	Mammalian achaete-scute and atonal homolog
MZ	Marginal Zone
mE	Mouse embryonic stage
MHB	Mid Hindbrain-boundary
Msx1	Msh-like 1 homeobox
Mz	Marginal Zone
NCC	Neural Crest Cells
nE	Neuroepithelial cell
NFAT	Nuclear factor of activated T cells
Ngn1/2	Neurogenin 1/2
NMR	Nuclear Magnetic Resonance

NR	Nuclear receptor
NRE	Nuclear receptor response element
NSC	Neural Stem Cell
NTN1	Netrin 1
Nurr1	Orphan nuclear receptor-related factor 1
Otx-2	Orthodenticle homologue 2
Pax-2	Paired-box 2
pCAF	P300/CBP-associated factor
PCP	Planar cell polarity
PD	Parkinson's Disease
PH3	Phosphohistone 3
Pitx2/3	Paired-like homeodomain transcription factor-2/3
PKC	Protein kinase C
RNAi	RNA interference
Ror2	R or-family receptor tyrosine kinase
sFRP	soluble Frizzled-Related Protein
Shh	Sonic hedgehog
SN	Substantia nigra pars compacta
Sox2	SRY-related HMG box gene 2
TCF	T-cell factor
TGF-β	Transforming growth factor β
TH	Tyrosine hydroxylase
Tuj1	β tubulin III
vMB	Ventral midbrain
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WIF-1	Wnt inhibitory factor-1
β-TrCP	β-transducin repeat containing protein

1 INTRODUCTION

The mammalian brain is an intricate organ, with over a hundred billion cells that are interconnected via electrical and hormonal signaling systems to control a multitude of functions, from very basic mechanisms to behavior and awareness. Many studies have focused on midbrain dopamine (mDA) neurons since they are involved in key neural processes, from initiation of motor responses, to pleasure and reward. A major disease associated with the loss of dopaminergic neurons is Parkinson's disease. Over the years, various symptom-reliving treatments have developed but a definite cure has yet to emerge. Extensive efforts over the last 20 years have focused around developing a cell replacement therapy for damaged cells in the midbrain dopamine system. Successful attempts on transplanting immature midbrain fetal tissue have been performed in both animal models and Parkinson's disease patients. Such cell replacement therapies have been hindered by limited fetal tissue availability. It has therefore been suggested that other cells such as stem cells, that can propagate and can differentiate into DA neurons and can be standardized, would be a better cell source for transplantation. However this approach requires sufficient knowledge on DA neuron development in order to control the fate of the stem cells and guide them into the desired phenotype. To achieve this we need an increased understanding of the cascades of events and the interplay between different signaling pathways which allow cells to make multiple decisions. An emerging view that has become apparent in recent years is that signaling molecules have to be placed in context of the intricacies of the embryological processes and the diversity of cell types in the nervous system. Successful cell-replacement strategies will most probably require both the understanding of the cell biology and the secreted signaling factors and transcription factors involved in generating a mDA neuron.

1.1 Gastrulation/Induction: Deciding between epidermis and neural plate

The vast complexity of the central nervous system (CNS) is intriguing given that it is formed from a simple cellular layer, the neural plate, in the course of a process known as *gastrulation*. This process is initiated by morphological arrangements of the early embryo forming the primitive streak and an elongating region of proliferating and migrating cells. This is followed by ingression of cells from the surface ectoderm through the primitive streak into the interior of the embryo to give rise to the mesodermal and endodermal germ layers. As the streak extends, the developing embryo undergoes consecutive convergent extension movements and invaginations to establish an internal endoderm, an intermediate mesoderm and an external ectoderm. The early ectoderm can be divided into three sets of cells: the internally positioned neural tube, which will form the brain and the spinal cord, the externally positioned epidermis of the skin, and the neural crest cells that eventually will migrate away and give rise to skin and the future peripheral nervous system (PNS).

The neural plate and the head fold are formed when the ectoderm receives signals from underlying mesoderm and endoderm instructing the primitive streak to regress. The neural plate rolls up, detaches from the non-neural ectoderm and finally zips along the dorsal midline of the embryo to form a tube-like structure and the notochord. Cells surrounding the neural plate become elongated and move upward, fold, fuse together and pinch off to form the neural tube. In the so called default model, neural induction was believed to result from inhibition of bone morphogenic protein (BMP) activity (Smith et al. 1992; Lamb et al. 1993; Hemmati-Brivanlou et al. 1994; Sasai et al. 1994;

Sasai et al. 1995; Furthauer et al. 1999) which otherwise would direct the ectoderm to become epidermis. However, more recent findings in *Xenopus* and chick give a more complex view of neural induction than indicated in the "default model" (Hemmati-Brivanlou et al. 1997). These studies suggest that neural induction consists of several steps and that the modulation of BMP and other signaling pathways, such as retinoic acid (Sive et al. 1990; Ruiz i Altaba et al. 1991; Conlon 1995), Fibroblast growth factors (Fgfs), Wnts and Churchill (Lamb et al. 1995; Wilson et al. 2001b; Sheng et al. 2003; Delaune et al. 2005), seem to depend on spatial and temporal regulation before and during gastrulation (Stern 2005).

A critical event in the early patterning of the vertebrate CNS is the generation of distinct domains of progenitor cells at different positions along the rostrocaudal and dorsoventral axes of the neural plate. In mammals, even before the posterior portion of the tube has closed (mouse embryonic day (mE) 6-7), neural plate closure is initiated at several places along the rostral-caudal axis. Early specification of progenitor cells along the rostral-caudal axis occur, relying on the expression of a combinatorial code of soluble signals and transcription factors from both the neural tube and non neural tissues (Lumsden et al. 1996; Nordstrom et al. 2002). By mE9.0, gastrulation is completed and the main regions of mouse CNS can be distinguished morphologically along the rostral-caudal axis: forebrain, midbrain, hindbrain and the spinal cord.

1.2 How the midbrain is made

Several populations of DA neurons exist in the brain. However, the vast majority of these DA neurons are situated in the midbrain. Along the neural tube local organizers give identity and initiate patterning of the CNS. Midbrain patterning is initiated in an

orchestrated fashion by two signaling centers; (i) the isthmic organizer (at the midbrain-hindbrain boundary; MHB) along the anterior-posterior (A-P) axis and (ii) the floor plate (the ventral-most longitudinal subdivision of the neural tube), along the dorsal-ventral (D-V) axis.

The induction of midbrain DA neurons depends on the morphogenic gradient established between floor plate derived glycoprotein Sonic hedgehog (Shh), which emanates from ventral midline cells, and the activity of the fibroblast growth factor (Fgf) family member 8 (Fgf8). Fgf8 is secreted by the isthmic organizer before mE9.5, creating a permissive environment for correct positioning/expression of early midbrain patterning genes (Ye et al. 1998). Additionally, Wnt1 (Prakash et al. 2006) and the transforming growth factors-β (TGF β; (Farkas et al. 2003)) are also involved in the patterning process. These extrinsic factors specify A-P and D-V identity of the midbrain, resulting in a spatio-temporal activation of a combination of transcription factors and additional soluble signals.

1.3 Midbrain identity and DA neuron induction

1.3.1 Isthmic activity

The isthmus develops at the rim of the future MHB (around E7.5). This organizer is required for the development of all cell types and nuclei in the midbrain as well as for the development of the cerebellum in the anterior hindbrain. Studies have shown that transplanting the isthmic organizer to the diencephalon leads to the formation of an ectopic midbrain (Martinez et al. 1991), whereas transplantation to the hindbrain results in development of an ectopic cerebellum (Marin et al. 1994). The isthmus is

restricted by the expression interface of the rostrally situated ortodenticle homologue2 (Otx2) homeodomain transcription factor and the caudally positioned gastrulation brain homeobox2 (Gbx2). Otx2 expression is restricted to for- and midbrain between E8.5 and E12.5 (Ang 2006). *Otx2* and *Gbx2* act antagonistically to set up the position of the isthmic organizer. Both ectopic expression of Otx2, as well as genetic ablation of Gbx2, shift the isthmic markers and results in expansion of Otx2 expression caudally (Broccoli et al. 1999; Millet et al. 1999). In addition, ablation of Otx2 results in the loss of fore- and midbrain structures (Acampora et al. 1999; Rhinn et al. 1999).

Fgf8 and Wnt-1 are both secreted from the isthmic organizer. Fgf8 is suggested to be the key molecule in mediating isthmic activity. This is demonstrated by ectopic induction of an isthmus/tectum (dorsal part of midbrain) upon Fgf8 expression (Crossley et al. 1995; Crossley et al. 1996; Martinez et al. 1999), whereas Fgf8 inactivation in the MHB results in the loss of tectum and cerebellum (Meyers et al. 1998; Reifers et al. 1998; Chi et al. 2003). Unlike *Fgf8*, *Wnt1* does not have inductive activity but is essential for mid/hindbrain development and has recently been shown to maintain isthmic activity in chick (McMahon et al. 1990; McMahon et al. 1992; Olander et al. 2006).

Gene expression in the isthmic region occurs in a coordinated manner. At mE7.5 the expression of *Lmx1b* (Adams et al. 2000; Guo et al. 2007) and by mE8 the transcriptional repressor engrailed-1 (*En-1*), paired-box 2 (*Pax2*) and Wnt1 can be detected. Fgf8 appears soon after in the anterior hindbrain adjacent to Wnt1, which is expressed in the posterior midbrain. This is followed by the expression of *En-2*, *Pax5* and *Pax8* (Crossley et al. 1995; Hynes et al. 1999; Liu, A. et al. 2001a; Wurst et al. 2001).

Pax2 is necessary for the induction of *Fgf8* in the MHB by partly regulating Pax5 and 8. Pax2 is among the earliest known genes to be expressed throughout the MHB region in late gastrula embryos (Joyner 1996; Li Song et al. 2000). In Pax2 $^{-/-}$ mice, Fgf8 is never induced in the MHB (Ye et al. 2001). Interestingly, Pax $2^{-/-}$ embryos do not exhibit any defects in the Gbx2/Otx2 gene expression in the MHB, and display a normal Wnt-1 expression, indicating that these genes are not involved in the induction of Fgf8 (Ye et al. 2001). However, the compound mutant (Pax2/5) exhibit a severe midbrain phenotype (Schwarz et al. 1997; Schwarz et al. 1999). Recently, evidence of additional transcription factors regulating inductive activity of the isthmus organizer, regionalization and refinement of the midbrain domain have emerged. The LIM homeodomain transcription factor Lmx1b was shown to be essential for the initiation of Fgf8, as deletion of Lmx1b leads to complete loss of Fgf8 expression in MHB (Guo et al. 2007). Conversely, misexpression of Lmx1b in the MHB of chick embryos induces ectopic expression of Fgf8 and Wnt1 and causes an expansion of tectum and cerebellum (Adams et al. 2000; Matsunaga et al. 2002). These results indicate that Lmx1b is required for the Fgf8 expression in MHB. Another important feature of Lmx1b deletion is the initial reduction and subsequent loss of Wnt1, En1 and Pax2 in the MHB of these embryos, which indicates that although Lmx1b is not required for the initial expression of these genes, it is important for the maintenance of the MHB. Furthermore, Gbx2 expression was also downregulated in Lmx1b null mice but the expression of Otx2 in MHB was not affected. Altogether, these results imply that Lmx1b is an important component for the initiation and maintenance of inductive activity in the isthmic organizer during midbrain development (Guo et al. 2007). In spite of the early role of the regulatory homeodomain proteins En1/2, they are not involved in initiating Fgf8 expression, since in the absence of both En1/2, Fgf8 is still

induced in the MHB region (Liu, A. et al. 2001a). However, En1/2 are required for the maintenance of Fgf8 and Wnt1 expression (Thomas et al. 1990; Wurst et al. 1994; Liu, A. et al. 2001a). In addition, several studies have shown an important role of this gene family in the survival of post-mitotic DA neurons (Alberi et al. 2004). Altogether, the above studies imply that transcription factors Lmx1b, Pax2 and En1/2 are important components for the initiation and/or maintenance of the isthmic organizer during midbrain development. Otx2 and Gbx2 are not required for the induction of Fgf8 or Wnt1 expression but appear to have a general role in establishing the organizer (Rhinn et al. 1998; Broccoli et al. 1999; Millet et al. 1999; Rhinn et al. 1999; Li et al. 2001). Fgf8 and Wnt1expression in the isthmic organizer terminates around embryonic day E13.5 at the end of DA neurogenesis (Di Porzio et al. 1990; Xu, J. et al. 2000). Hirata et al., showed that the bHLH genes Hes1 and 3 were also involved in the maintenance of MHB activity. Hes1 and Hes3 double mutants prematurely terminate the expression of specific genes such as Fgf8, Wnt1 and Pax2/5 in cells with isthmic activity (Hirata et al. 2001).

1.3.2 The midbrain floorplate has discrete molecular and functional properties

Thus, when the isthmus is established correctly, soluble signals from the roof plate and floor plate divide the midbrain in a dorsal and ventral half separated by the lateral sulcus. These two regions will have their own temporal and developmental competence.

The floor plate organizer constitutes a discrete heterogeneous group of cells located at the neural tube ventral midline, important for specification of neural D-V identity.

The inductive activity of this region is exerted through secretion of Shh. In midbrain, Shh secretion occurs from a subset of FoxA2 (or hepatocyte nuclear factor 3β, HNF3β) positive cells (located in the ventral neuroepithelium) at E7.75-E12 (Hynes et al. 1995a; Wallen, A. et al. 1999; Puelles, L. et al. 2003; Puelles, E. et al. 2004; Zervas et al. 2004). Interestingly, over-expression of FoxA2 transcription factor and Gli, one of the downstream effectors of Shh, result in generation of DA cell markers in the dorsal midbrain, emphasizing the importance of Shh signals in DA neuron induction (Sasaki et al. 1994; Hynes et al. 1997). Thus, DA neurons are generated in the ventral midbrain (vMB) where Shh and Fgf8 intersect at E10.5 (Hynes et al. 1995a; Hynes et al. 1995b; Ye et al. 1998; Hynes et al. 1999). The intersection of Fgf8 and Shh gradients also control the development of DA neurons in the rostral forebrain (Fig 1A).

Although knowledge about early molecular players in DA neuron development is limited, Andersson et al. recently showed that LIM homeodomain protein, Lmx1a (Andersson, E. et al. 2006b) is an important intrinsic determinant expressed at E9 within the DA progenitor domain. This group also identified the transcriptional repressor Msx1/2, that is co expressed with Lmx1a at E9.5 but restricted only to the progenitor zone lining the differentiated DA neurons. In addition at E9, Nkx6.1, a homeodomain (HD) transcription factor, is broadly expressed in ventral progenitor cells (including motor neuron progenitors; (Vallstedt et al. 2001)), and co expressed with Lmx1a. However, from E9.5 and onward Nkx6.1 expression is down regulated in presumptive DA progenitors and this coincides with the initiation of Msx1/2 expression in these cells. Addition of exogenous Shh to chick neural plate explants was able to induce Lmx1a and Msx1 expression, implying the inductive role of Shh in the expression of these two markers in the vMB. Overexpression of Lmx1a in the midbrain of chick embryos was also found to induce a DA phenotype. Conversely,

downregulation of Lmx1a resulted in a repression of the DA phenotype, indicating the role of Lmx1a in DA cell specification. The inductive activity of Lmx1a on DA progenitors is probably through activation of the bHLH panneuronal transcription factor Ngn2 (Andersson, E. et al. 2006b). Ngn2 is expressed at E10.5-10.75 in Sox2-positive proliferative progenitors in the midbrain that later give rise to Nurr1-expressing postmitotic cells, which eventually differentiate into tyrosine hydroxylase (TH)+ DA neurons (Paper I). Overexpression of Msx1 resulted in loss of Nkx6.1 expression and upregulation of Ngn2 followed by induction of DA markers in vMB cells. In addition, Msx1 overexpression lead to loss of floor plate cells and downregulation of Shh. However, the expression of Lmx1a was not affected by Msx1 overexpression. These data suggest that Msx1, which in turn is regulated by Lmx1a, regulates the timing of DA progenitor induction, probably through the induction of the Ngn2 expression and repression of alternative ventral fates in vMB. However, it must be noted that the inductive property of Lmx1a obtained in vMB was absent in the dorsal midbrain. This indicates that, although Lmx1a is an important determinant of DA neuron development, its activity is context-dependent and the presence of additional factors, such as Shh, is required for correct DA progenitor identity. Interestingly, Lmx1b was not ascribed determinant activity for induction of DA progenitors (Andersson, E. et al. 2006b). This can be due to the fact that Lmx1a and Lmx1b have different roles during midbrain development. Instead of regulating DA progenitor induction, Lmx1b maintains, as discussed above, the expression of Pax2, Wnt1 and En1 and can therefore be involved in generating a permissive region for initiation of inductive activities (Adams et al. 2000; Andersson, E. et al. 2006b; Guo et al. 2007), and maintenance of midbrain DA phenotype.

Otx2 is not only involved in region specification at the MHB but also has additional roles in DA progenitor specification and differentiation. Deletion of Otx2 in midbrain by En1Cre; $Otx2^{flox/flox}$ from E9.5 onwards results in expansion of Fgf8 and Shh rostrally and dorsally, respectively, in the midbrain (Puelles, E. et al. 2004; Ang 2006). In these Otx2 null mutant embryos, an ectopic expression of serotonergic neurons can be found at the expense of TH+ cells, as well as an expansion of the type II homeodomain transcriptional regulator, Nkx2-2, (another Shh responsive gene required for the specification of ventral cell populations in hindbrain and spinal cord, (Briscoe et al. 1999; Pattyn et al. 2003a; Pattyn et al. 2003b). The midbrain DA progenitors at E12.5 are rescued in En1Cre; $Otx2^{flox/flox}$; $Nkx2\text{-}2^{-/-}$ embryos. The ectopic expression of Nkx2.2 seen in En1Cre; $Otx2^{flox/flox}$ embryos results also in a loss of Wnt1 expression. Interestingly, Otx2 itself is regulated by Wnt1, as ectopic expression of Wnt1 results in ectopic induction of Otx2 in the floorplate of the rostral hindbrain (Prakash et al. 2006).

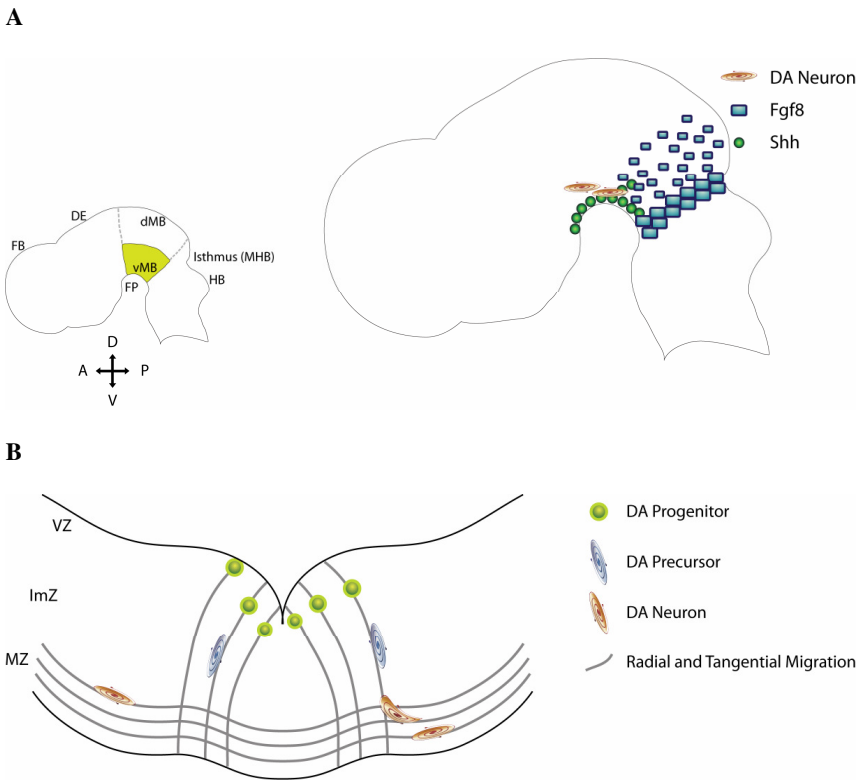

Fig 1. vMB identity and the course of differentiation in the DA lineage.
A. Sagittal view of E10.5 mouse brain. The DA neurons are born in the vMB in the intersection of two signaling molecules, the floorplate derived Shh, determining the location of DA neurons and the isthmus derived Fgf8 that positions them A-P to the MHB.
B. Coronal view of E10.5-11.5 mouse vMB (shown in green in 1 A). The developing DA progenitors will progress through three zones on their way to become fully differentiated neurons. Dividing progenitors are located in the ventricular zone (VZ). As they become postmitotic precursors they start migrating ventrally along radial glia (RG) through the intermediate zone ImZ. When reaching the marginal zone (MZ) they migrate laterally to become terminally differentiated DA neurons. The transition from vMB neuroepithelial cells toward vMB neurons, is partially thought to happen via an intermediate RG state. The (RG) VZ progenitor is instructed to become a neurogenic progenitor dividing next to the VZ. In the ImZ it will generate and instruct the newly generated daughter cells to become a postmitotic ImZ precursor cell that in turn migrates to the MZ to become a dopaminergic neuron. Thus, RG is thought to act as a neurogenic unit in the vMB.
Forebrain(FB); Hindbrain (HB); DE (Diencephalon); Floorplate (FP) Mid-hindbrain-boundary (MHB).

1.3.3 The Dopaminergic lineage

At early stages of embryonic development, neuroepithelial progenitor cells in the ventricular zone (VZ) undergo symmetric division – two identical neuroepithelial cells

are generated at each division, thereby expanding the population founder cells that will ultimately through asymmetric division, produce cells with different fate potential, (Kriegstein et al. 2006). Accordingly, the midbrainDA lineage can be divided into specific subsets based on successive molecular parameters; progenitors, the fast dividing apical progenitors, committed DA precursors, immature DA neurons and terminally differentiated and mature DA neurons.

1.3.3.1 Sox2 proliferating progenitors

The permissive region generated by early signaling from organizing centers induces progenitor cells to divide in the midbrain ventricular zone (VZ), give rise to postmitotic DA precursors in the intermediate zone that differentiate into newborn DA neurons. In the apical layer, lining the midbrain VZ, phosphohistone (PH)3+ dividing progenitors are found followed by the symmetrically dividing Sox2+ and Ki67+ proliferating cells (Paper I). As progenitor cells divide they give rise to postmitotic precursor cells that emerge in the intermediate zone. These cells downregulate Sox2 expression and initiate the expression of the nuclear orphan receptor, Nurr1 (Zetterstrom et al. 1997), and the early generic neuronal marker Tuj1 (Paper I). These markers define the DA precursors at E10.5.

1.3.3.2 From Nurr1 precursor to DA neuron

In *Nurr1* null embryos, DA neurons have either been reported to not be generated (Zetterstrom et al. 1997) or to be generated but fail to be maintained (Saucedo-Cardenas et al. 1998). However, in both cases defects in DA synthesis and transmitter release were identified, indicating that Nurr1 partly determine the neurotransmitter

phenotype by regulating several proteins required for DA synthesis and regulation. Later during differentiation (E10.5-11 and onward), Nurr1+ cells express tyrosine hydroxylase (TH; the rate-limiting enzyme of dopamine synthesis; (Zetterstrom et al. 1997; Castillo et al. 1998; Saucedo-Cardenas et al. 1998; Smits et al. 2003) and other specific markers of DA phenotype, such as vesicular monoamine transporter (VMAT2), DAT (dopamine transporter) (Sacchetti et al. 1999; Smidt et al. 2003), Aromatic acid decarboxylase (AADC), and c-Ret (Wallen, A. A. et al. 2001; Nunes et al. 2003; van den Munckhof et al. 2003; Smidt et al. 2004). However, Nurr1 has also been attributed other functions. Transient Nurr1 overexpression in MN9D cell lines induced cell cycle arrest in G1 phase, and resulted in a dramatic decrease in cell proliferation followed by morphological acquisition of a mature phenotype (Castro et al. 2001). Moreover, Nurr1 has been shown to physically interact with p57, a CDK inhibitor, and to promote maturation (Joseph et al. 2003). Conversely, retroviral overexpression of Nurr1 in primary ventral mesencephalic cultures led to significantly increased apoptosis and attainment of an immature TH+/βIII-tubulin+ phenotype (Kim, H. J. et al. 2007). The present view, however, is that Nurr1 is a post mitotic marker needed to acquire a DA phenotype (Sakurada et al. 1999; Wagner et al. 1999; Kim, J. H. et al. 2002; Kim, J. Y. et al. 2003).

The midbrain specific Pitx3, a homeodomain transcription factor, also becomes expressed in late differentiated DA neurons. The expression of Pitx3 appears to be regulated by Lmx1b. Lmx1b $^{-/-}$ embryos display both Nurr1 and TH positive DA cells but show deficiency in expression of Pitx3 and die at mE16 (Smidt et al. 2000). This show, in addition to its earlier mentioned role, that Lmx1b is important for complete differentiation and survival of TH+ DA cells.

1.3.3.3 The migrating young DA neuron finds its place

As cells exit the cell cycle and become postmitotic, they are thought to migrate radially along radial processes perpendicular to the ventricular zone, and later spread tangentially in lateral direction (Hanaway et al. 1971; Kawano, H. et al. 1995; Hall et al. 2003). Eventually these cells arrive at their final destinations in the marginal zone (Fig 1B) forming the specific midbrain DA neuronal groups: the ventral tegmental area (VTA ; A10 neurons), the substantia nigra pars compacta (SNc; A9 neurons) and the retrorubral field (RRF: A8). Reports from the developing rat brain have exhibited a necessity for midbrain DA neurons to interact with radial glia (RG) between embryonic day E12 and 20 for migration to take place (Shults et al. 1990). In the young neuroepithelium, RG stretch their processes from the apical surface in the VZ to the pial surface in the marginal zone (MZ) to direct neuronal migration. In addition, RG have been reported as neurogenic units that themselves can give rise to differentiating DA neurons (Hall et al. 2003). Lineage tracing studies are needed in order to link the molecular determinants and functions to DA neurons.

2 BASIC HELIX LOOP HELIX PROTEINS

During the initial phases of nervous system development, progenitor cells in the neural tube proliferate and divide symmetrically to give rise to identical multipotent neuroepithelial cells. These progenitors subsequently divide asymmetrically to generate cells that are fated to differentiate into a neuron, sometimes following additional cycles of cell division and later give rise to glia. These processes are in part regulated by a subset of transcription factors belonging to the basic helix-loop-helix (bHLH) family. The bHLH family of transcriptional regulators are key players in a wide range of developmental processes in mammals which, in addition to neurogenesis, also include hematopoiesis, myogenesis, sex determination and gut development (Jan et al. 1993; Weintraub 1993; Hassan et al. 2000; Massari et al. 2000; Vervoort et al. 2001).

A subset of bHLH transcription factors, which includes members of the NeuroD, Neurogenin, Mash, Olig, Id and Hes families, have long been recognized for their ability to positively regulate the neuronal fate decision in both invertebrates and vertebrates (Lee, J. E. 1997). These "proneural" genes were first identified in *Drosophila* as key regulators of neural lineage development (Garcia-Bellido 1979; Villares et al. 1987; Murre et al. 1989; Jarman et al. 1993; Brunet et al. 1999; Guillemot 1999), because of their involvement in promoting the generation of progenitors and initiating the development of neural lineage, such as neurons and glia. The vertebrate bHLH (Guillemot et al. 1993; Lee, J. E. 1997; Casarosa et al. 1999; Horton et al. 1999) were either isolated by RT-PCR based on sequence conservation with their Drosophila counterparts or by yeast two-hybrid screens based on their ability to dimerize with other bHLH proteins (Lee, J. E. 1997; Guillemot 1999). Proneural

bHLH factors and neuronal differentiation bHLH factors are transcriptional trans-activators. As such, these proteins bind DNA as heterodimeric complexes together with E proteins (E12, E47, E2-2, HEB). The basic domain of these bHLH factors mediate interactions with DNA sequences known as an E boxes (see below; (Bertrand et al. 2002)).

2.1 bHLH structure and function

The bHLH domain is about 60 amino acids long and encompass a DNA-binding basic region followed by two helices separated by a variable loop region (HLH) (Massari et al. 2000). The formation of homodimeric or heterodimeric complexes between different family members is acchived by the HLH domain that promotes dimerization. The two basic domains, brought together through dimerization, bind DNA sequences that contain a specific hexanucleotide motif, CANNTG, referred to as E box. Up to date over 400 bHLH proteins have been identified in various organisms, from yeast to humans (Ledent et al. 2002) of which a large family with 125 and 102 members are encoded in the human and mouse genome, respectively.

Based on shared structural and biochemical properties, bHLH families have been ordered in different classes named class A, B, C, D, E and F (Atchley et al. 1997). Class A proteins are ubiquitously expressed, whereas class B bHLHs are tissue or celltype specific. Class A bHLH proteins can form homodimers or heterodimers with class B bHLH proteins. Class D corresponds to HLH proteins that lack a basic domain, are therefore unable to bind DNA, and act as antagonists. Class E includes proteins related to the Drosophila Hairy and Enhancer of split bHLH (HER proteins)

(Dawson et al. 1995) and bind preferentially to sequences referred to as N-boxes (CACNAG).

2.2 Repressor and Activator bHLH Genes

bHLH proteins can be further divided into repressors and activators, based on their function. The repressor-type bHLH genes include Hes genes, homologs of Drosophila hairy and Enhancer of split, and Id (Inhibitors of differentiation) genes, and the Drosophila emc (extra macrochaetae) homologs. Activator-type bHLH genes include Mash1, Math and Neurogenin (Ngn), homologs of Drosophila achaete–scute complex and atonal, respectively.

2.2.1 Repressor bHLH Genes

The Hes family comprise seven members, amongst them Hes1, Hes3 and Hes5 are highly expressed by neural stem cells (Akazawa et al. 1992; Sasai et al. 1992; Allen et al. 1999). Hes factors form a dimer and bind to DNA through a conserved bHLH domain located in the amino-terminal region (Sasai et al. 1992). Hes genes comprise a carboxterminal sequence that interacts with the corepressor TLE/ Grg, a Drosophila Groucho homolog, and recruits the histone deacetylase, thereby inactivating the chromatin and transcription (Paroush et al. 1994; Grbavec et al. 1996). Thus, Hes genes are classified into the repressor-type bHLH genes. The activator-type bHLH genes such as Mash1 represent target genes for Hes factors. Hence, Hes1 represses Mash1 expression by binding directly to the promoter (Ishibashi et al. 1994; Chen, H. et al. 1997). The bHLH activator E47 and activator-type bHLH form a heterodimer and promote neuronal differentiation from neural stem cells. E47 and Hes1 forms a non-

functional heterodimer and inhibits formation of Mash1-E47 heterodimer (Sasai et al. 1992). Accordingly, two different mechanisms are used by Hes1 to antagonize Mash1 activity: inhibiting the activity at the protein–protein interaction level and also repressing the expression at the transcriptional level (Fig 2). In addition Hes genes also promote gliogenesis (Furukawa et al. 2000; Hojo et al. 2000; Ohtsuka et al. 2001).

In contrast to Hes factors, Ids inhibit gene transcription by sequestration. bHLH factors (including members of the Mash, Neurogenin, NeuroD, and Olig families; (Norton 2000)) that entail E proteins for their activity are hindered by Ids that form dimers with the E proteins. In addition, Ids promote cell cycle progression by interacting with components of the cell-cycle machinery (Lyden et al. 1999; Kondo et al. 2000; Wang, S. et al. 2001).

2.2.2 Activator bHLH genes and Neurogenesis

Proneural bHLH factors are able to initiate the development of neural lineages and guide transition from proliferation to neurogenesis (Fig 2). These processes demand coordinated activity of proneural versus antagonizing bHLH proteins, culminating in terminal neuronal differentiation. Thus, neurogenesis is brought about by two categories of bHLH activity: proneural bHLH proteins that initiate neurogenesis (e.g. Ngns and Mash), and neural differentiation bHLH factors (e.g. NeuroD) that are involved in mediating terminal differentiation. In addition, the expression of certain proneural bHLH factors is important for determination of neuronal subtype identity.

Mash1 and Ngns (along with their homologues) are the most extensively studied bHLH proteins. In vertebrates, the idea that Mash1 and Ngns proteins initiate

neurogenic programs in a variety of progenitor cells was initially derived from *in vitro* studies, where it was shown that bHLH factors are sufficient to convert *Xenopus* ectodermal cells into neurons. Gain-of-function studies performed in these animals showed that over expression of *Xenopus* Ngn1 resulted in ectopic neurogenesis, ectopic neuronal differentiation, and Notch signalling (Ma et al. 1996). Similar results were obtained in mammalian cortical progenitors, where Ngn1 over expression promoted differentiation of cortical progenitors into neurons (Sun et al. 2001). In addition, Mash1 overexpression in P19 carcinoma cells promoted neuronal differentiation and induced expression of pan-neuronal markers, such as Tuj1 and neurofilament M (Lo et al. 1998; Farah et al. 2000; Sun et al. 2001). Also, a recent report revealed Mash1 to synergize with additional factors to regulate various steps of neurogenesis (Castro et al. 2006).

Additional *in vivo* studies (Ma et al. 1996; Blader et al. 1997; Mizuguchi et al. 2001) using knockout strategies have revealed that proneural bHLH genes also contribute to the specification of diverse neuronal subtypes (Bertrand et al. 2002).

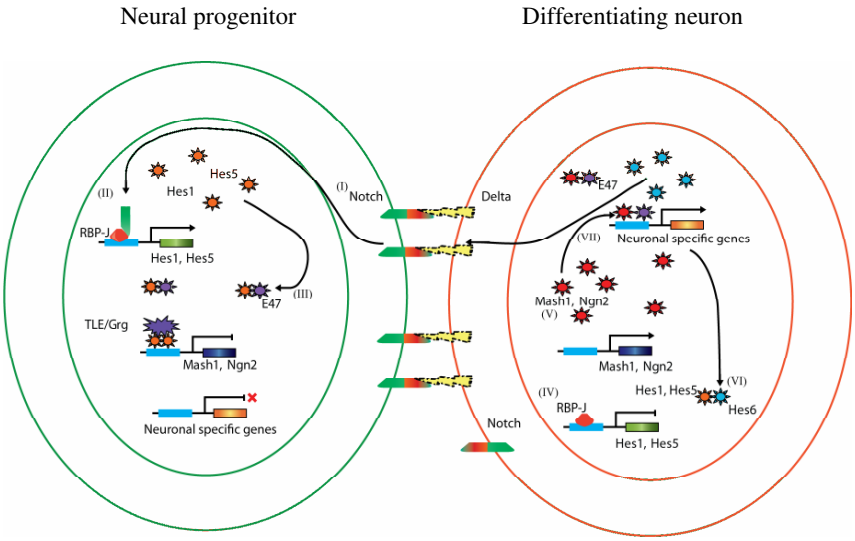

Fig 2 Repressor/Activator bHLH genes and neurogenesis
One important role of Notch and Hes signaling in CNS development is to maintain a balance between the number of developing neurons and the number of remaining progenitors by a mechanism known as ***lateral inhibition*** (Beatus et al. 1998; Giagtzoglou et al. 2003). Initially, proneural genes are expressed in progenitor cells. Through lateral inhibition and Notch signaling, this initial pattern is refined and proneural gene expression becomes restricted to cells that later will be differentiated. (**I**) Proneural bHLH genes upregulate expression of the Notch ligands on the neighbouring cell, which bind to the Notch receptor on the progenitor cell. (**II**) Notch is processed upon acivation and releases the ICD that is translocated to the nucleus and forms a transcriptional activator complex with the DNA-binding transcriptional repressor protein RBP-J. (**III**) This complex induces Hes1 and -5 expression, that in turn inhibit the activator type bHLH genes by 1) sequestering E47 and 2) by repressing their transcription when binding to their promoters and recruiting the co-repressor TLE/Grg. (**IV**) In the differentiating neuron, RBP-J represses Hes1 and-5 expression and no Notch activation takes place. (**V**) Expression of the activator type bHLH occurs, that in turn (**VI**) promotes the expression of Hes6 that inhibits Hes1 activity and reinforces neurogenesis. (**VII**) Neuronal specific genes, such as the Notch ligand Delta, are induced by the activator type bHLH genes. Schematic illustration adapted from (Kageyama et al. 2005).

2.3 Sox, bHLH and Cell Cycle

The HMG-box SoxB1 family genes, including Sox1, 2 and 3, have been recently identified as factors responsible for instructing and maintaining stem and neural progenitor cells. In ES cells, Sox2 has been found to maintain self-renewal (Rodda et al. 2005). Upon differentiation, ES cells predominantly generate neural cells (Zhao, S. et al. 2004b) and an enhancer of Sox2 has been found to respond to neural inducing signals (Uchikawa et al. 2003). SoxB1 genes are also expressed in cells committed to a

neural identity and are thus found in the neural plate throughout ontogeny (Pevny et al. 2005), and in adult neurogenic regions (Ellis et al. 2004; Komitova et al. 2004). In the nervous system, SoxB1 genes are thought to maintain neural progenitor cells as they regulate the expression of nestin in neural primordial cells (Tanaka, S. et al. 2004) binding to a Sox consensus sequence in the Hes1 promoter (Kan et al. 2004). This function of SoxB genes could be accomplished by either maintaining the neural progenitors in the cell cycle or by inhibiting neuronal differentiation (Bylund et al. 2003; Graham et al. 2003). Mitotic progenitors initially express proneural genes at low levels (Bertrand et al. 2002; Bylund et al. 2003). In turn, positive feed back loops result in high levels of proneural genes in a subpopulation of progenitor cells, in analogy with the cells being irreversibly committed to differentiation (Bertrand et al. 2002) (Fig 2). This highlights an important feature of bHLH function, apart from neural fate determination of progenitors, they promote the terminal and irreversible exit from the cell cycle. Arrest of cell-division is crucial for separation of progenitor cells from extrinsic fate-determining factors and for their direction towards differentiation. Ngns and Mash1 appear to be involved in this transition. Ectopic Ngn1 expression decreases the proportion of proliferating progenitors, indicating that Ngn1 can promote cell cycle arrest (Lo et al. 2002). In addition, missexpression of Ngn2 in chick neural tube promotes premature cell cycle exit and neuronal differentiation of motoneurons (Mizuguchi et al. 2001; Novitch et al. 2001). When progenitors from wild-type mice are compared to those from mice lacking both Ngn2 and Mash1, it is clear that the presence of Ngn2 and Mash1 limits the number of cell divisions (Nieto et al. 2001). Progenitors derived from Ngn2/Mash1 double mutant mice give colonies that are three times larger than those derived from wild-type mice.

Although a role for proneural bHLH factors in cell cycle withdrawal is suggested by the mentioned studies, it is not clear whether the effects on cell cycle withdrawal are direct or simply a consequence of differentiation. For example, regulation of the cell cycle by proneural genes is likely to involve the activation of cyclin-dependent kinase (Cdk) inhibitors, such as p16, p21 and p27 (Mutoh et al. 1998; Farah et al. 2000) (Vernon et al. 2003). However, it is not yet clear whether the regulation of cell cycle through Cdk-inhibitors is done directly or through activation of neuronal differentiation genes. Direct activation has been observed to take place in some lineages at the level of neuronal differentiation genes. A study of NeuroD gene function showed that NeuroD in combination with a co-factor (p300) activated p21 Cdk-inhibitor expression in HeLa cells which inhibited cell proliferation, while in NeuroD deficient mice, p21 failed to be expressed, leading to elevated cell numbers and increased mitosis (Mutoh et al. 1998).

2.4 Terminal differentition and bHLH

Transient expression of proneural bHLH factors induces a second sustained wave of bHLH differentiation genes which mediate the terminal differentiation of neurons (Kageyama et al. 1997; Lee, J. E. 1997). They belong to the NeuroD/ Nex family and include NeuroD, NeuroD2 (NeurD related factor, NDRF) and Nex (Math2). bHLH differentiation proteins are, similary as proneural bHLH factors, E box binding transcriptional activators that, are sufficient to induce cell cycle arrest and neuronal differentiation in culture when overexpressed (Lee, J. E. et al. 1995; Farah et al. 2000). Members of the NeuroD/Nex family are specific to cells that are (or will become) neurons; their expression begins in the immature neuron rather than in neuroepithelial cells and is maintained during neuronal differentiation (Lee, J. K. et al. 2000b). NeuroD is required for the proliferation, differentiation and survival of the granule cells in the

cerebellum and hippocampus (Miyata et al. 1999; Liu, M. et al. 2000), and mutation of Nex or NeuroD2 in the mouse also results in defects in the differentiation and survival of cerebellum and hippocampal neurons (Schwab et al. 2000; Olson, J. M. et al. 2001) which are clearly distinct from the loss of progenitors observed in mice that lack Mash1 and Ngn. Interestingly, no phenotype has been reported in the neocortex for the single knockouts (NeuroD, NeuroD2, or Nex2) or the NeuroD/Nex1 double knockout (Schwab et al. 1998; Miyata et al. 1999; Schwab et al. 2000; Olson, J. M. et al. 2001), which may indicate that members of the NeuroD family function in redundant manner. Alternatively, there may be other yet unidentified transcription factors that mediate the terminal differentiation.

Proneural and differentiation bHLH factors act in sequential steps. In *Xenopus*, the *Xenopus* homologs of Ngn1, Math3 and NeuroD are expressed in cascades. Ectopic expression of Ngn1 is able to induce the expression of both Math3 and NeuroD. Moreover, although Math3 and NeuroD can cross-activate each other, they are unable to induce Ngn1 expression (Ma et al. 1996; Perron et al. 1999). A similar result were also obtained in mouse, where Ngn1 or Ngn2 is required for expression of Math3 and NeuroD in cranial sensory neurons (Fode et al. 1998; Ma et al. 1998), whereas Mash1 acts upstream of Ngn1 and NeuroD in olfactory sensory epithelium (Cau et al. 2002), thus indicating that the NeuroD family genes act downstream of vertebrate proneural genes.

2.5 Neuron OR Glia?

During development, neural stem cells change their competency over time (Qian et al. 2000; Temple 2001; Hatakeyama et al. 2004; Placzek et al. 2005), giving rise to three

major neural lineages: first neurons, followed by oligodendrocytes and astrocytes (Bayer et al. 1991; Qian et al. 2000). These cell types are differentiated in specific temporal orders at defined positions. However, their spatio-temporal regulation is poorly understood. During the past years several studies have revealed that multiple bHLH transcription factors are involved in controlling lineage determination (Bertrand et al. 2002). In addition, several more recent models have evolved from studies in the spinal cord regarding the lineage relationship between neurons and glia (Zhou et al. 2002; Gabay et al. 2003; Pringle et al. 2003; Sugimori et al. 2007).

Proneural bHLH factors not only promote neural fates but they also inhibit gliogenesis (Tomita et al. 2000; Nieto et al. 2001; Sun et al. 2001). Mice deficient for Mash1 and/or Ngn2 show premature and excessive number of glial precursors, and their cortical progenitors rather adopt an astrocytic fate (Nieto et al. 2001). A similar phenotype is also observed in mice deficient for both Mash1 and Math3, where cells that normally differentiate into neurons instead adopt a glial fate (GFAP) (Tomita et al. 2000). Moreover, Ngn2 is able to repress oligodendrocyte specification in the spinal cord and its down regulation is a crucial step in the fate switch from neurons to oligodendrocytes in the ventral spinal cord (Zhou et al. 2001). These results suggest that proneural genes are necessary to avoid premature glial lineage determination. Interestingly, a severe reduction in the number of oligodendrocyte progenitors in the spinal cord of Mash1 null mutant mice is seen, implying an important role of Mash1 proneural factor not only in neurogenesis but also in the onset of oligodendrogeneis (Sugimori et al. 2007). The transcription factors Pax6, Nkx2.2 and Olig2, in combination with bHLH proneural factors patterning, seem also to be involved in temporal control of both neurogenesis as well as gliogenesis in the developing spinal cord. Mash1's activity in promoting oligodendrocyte differentiation is modulated by the activity of Nkx2.2 and

Olig2, while Pax6 seems to be involved in differentiation of spinal neurons. Astrocyte differentiation is regulated by the Id1 and Hes1 expression without any coordination with these patterning factors (Sugimori et al. 2007).

2.6 How do the proneural genes manage to inhibit gliogenesis?

Proneural genes might inhibit glia differentiation through sequestration of transcriptional coactivators away from glial promoters, preventing the expression of glial genes (Sun et al. 2001). The factors BMP and ciliary neurotrophic factor (CNTF) synergistically stimulate transcription of the astrocyte marker GFAP (Bartlett et al. 1998). GFAP expression is induced through binding of a protein complex, consisting of STAT 1/3 (activated by CNTF signaling), Smad1 (activated by BMP signaling), and CBP/p300 (ubiquitously expressed transcriptional coactivators), to its promoter (Nakashima et al. 1999). Ngn1 inhibits the assembly of this protein complex by binding to both CBP/p300 and Smad1, interfering with the ability of CBP/p300-Smad1 binding to STATs. This effect is independent of DNA binding and is distinct from the proneural functions of Ngns. Thus proneural factors may act by promoting the acquisition of one fate while inhibiting alternative fates.

The Notch pathway is usually viewed to inhibit differentiation and maintain progenitors in an undifferentiated state. However, it serves other functions at different stages of development and in different cell types. One such function of the Notch pathway and Hes activity is in gliogenesis (Furukawa et al. 2000; Hojo et al. 2000; Morrison et al. 2000; Tanigaki et al. 2001). It has been shown that cell-cell contact *in vitro* of cortical stem cells promotes an astrocyte fate, whereas isolated cells are more likely to become neurons, implying the existence of an instructive mechanism, which could be Notch

(Tsai et al. 2000). Indeed, Notch activation instructs glial differentiation, as seen in neural crest stem cells (NCSC) (Morrison et al. 2000). NCSC exposed to Notch ligand, even in the presence of BMP, undergo glial differentiation. This implies that a switch, triggered by Notch ligand, occurs in these cells to end neuronal differentiation and instead initiate gliogenesis (Morrison et al. 2000) Notch is also able to instruct astrocyte differentiation in stem cells from adult hippocampus. Notch signaling can promote expression from GFAP promoter, even in the absence of CNTF, and this promotion does not require STAT3 activation or STAT binding sites in the GFAP promoter. This indicates that the promotion of glia by Notch does not depend on signaling through the STAT pathway and is not entirely based on an inhibition of Ngn's sequestration of the CBP/p300-Smad1 complex from the STAT binding site. Thus, Notch may promote gliogenesis in additional ways beyond the inhibition of the proneural gene expression (Tanigaki et al. 2001). Downstream effectors of Notch signaling, Hes proteins are also involved in gliogenesis. In the retina, forced expression of Hes1 or Hes5 promotes the conversion of retinal progenitors into Muller glia (Furukawa et al. 2000; Hojo et al. 2000), whereas disruption of the Hes5 gene causes a decrease in the number of Müller glia that form. Similarly, in progenitors derived from the spinal cord, overexpression of Hes1 causes the differentiation into astrocytes (Wu, Y. et al. 2003). Thus, Notch may act both directly and indirectly, via induction of Hes factors, to promote astrocyte formation in the cortex. Altogether, these results may suggest that early during development before onset of gliogenesis, Notch activation promote maintenance of undifferentiated state of progenitors, whereas at later times, when gliogenesis is about to start or has started, it promotes gliogenesis.

2.7 Midbrain and bHLH

In recent years a huge body of evidence, evolving from work in the developing cortex and spinal cord, has given insight into the many mechanisms underlying bHLH factor activity. These findings have established the foundation for understanding bHLH functions in other regions of the developing nervous system, including the midbrain (Paper I); (Andersson, E. et al. 2006a). The proneural bHLH factors Ngns and Mash1 have been shown to play important roles in the neurogenesis and differentiation of midbrain DA neurons. During midbrain development at mE11.5, Ngn1and Ngn2 are expressed in Sox2+ proliferating progenitors at the ventral MB ventricular zone. Mash1 is expressed at higher level more laterally in the ventricular zone. In addition, Ngn2 is also expressed in the intermediate zone, lining the ventricular zone at the ventral midline, where Ngn2 positive precursor cells also express Nurr1. In the intermediate zone, no Mash1 expression is detected. In the marginal zone, where Nurr1/TH+ differentiated DA cells lie, no Ngn2 or Mash1 expression is seen (Paper I);(Andersson, E. et al. 2006a). When Ngn2 was deleted in the embryos, a near complete loss of DA neurons at mE11.5, assessed by the expression of TH and Pitx3, was observed (Paper I). In contrast to data obtained from sensory neurons, where a compensatory role of Ngn1 in absence of Ngn2 was shown (Ma et al. 1999), Ngn1 does not appear to play a critical role in the midbrain neurogenesis, as its ablation, either single or double knockouts, does not affect the outcome of TH expression in the marginal zone of ventral midbrain of mE14.5 embryos (Paper I). Similar results were obtained in another study where the role of Ngn2 during the development of midbrain DA neurons was examined (Andersson, E. et al. 2006a). In this study, a loss of medially located midbrain DA precursors, as well as reduction in DA neurons, during later stages of development was seen in Ngn2 mutants. The reduction of DA precursor cells was

probably mediated by the arrest of migration out of the ventricular zone and into marginal zone (Andersson, E. et al. 2006a). Moreover, in Ngn2 null embryos a small population of TH+ neurons (17%) was observed in the marginal zone at the end of the DA neurogenic period (mE14.5; Paper I). This small population seems to be dependent on Mash1 expression, as ablation of both Ngn2 and Mash1 results in complete loss of TH+ cells. When Ngn2 expression was swapped by Mash1 in knock-in Ngn2 KIMash1/ KIMash1 embryos, there was an additional partial rescue of TH expressing neurons. Mash1$^{-/-}$ embryos did not show any difference in DA phenotype compared to wild types (Paper I). Altogether, these results indicate that Ngn2 is important for neurogenesis of DA progenitors and their differentiation into TH-positive DA neurons. Mash 1 appears to be dispensable for neurogenesis and differentiation of ventral midbrain DA neurons, although it partially can rescue the generation of DA precursors in the absence of Ngn2. This partial rescue seen in Ngn2 ablated embryos can possibly be due to the fact that Mash1 is involved in allowing the progenitor cells to divide while retaining neurogenic potential (see below; (Lo et al. 2002; Kim, H. J. et al. 2007). Ngn2 may control neurogenesis and TH expression by directing the progenitor cells out of the cell cycle (Thompson et al. 2006; Kim, H. J. et al. 2007). This was shown to be true, at least *in vitro*, for neural progenitor cells isolated from ventral midbrain. Overexpression of Ngn2 in these cells led to cycle arrest even in the presence of mitogens, and generation of βIII-tubulin-positive neurons. However, these cells were positive for DA neuronal markers and expressed TH only in presence of Nurr1 activity (Andersson, E. K. et al. 2007; Kim, H. J. et al. 2007). Overexpression of Mash1 in these progenitor cells did not result in cell-cycle exit. Instead, cells continued dividing long after the mitogenic factors were withdrawn and did not give rise to neurons (Kim, H. J. et al. 2007). These data suggest that Ngn2 direct the fate of DA progenitors towards neurons, but the synergistic activity of other factors important for

differentiation of DA precursors, such as Nurr1, are also needed to induce dopaminergic phenotype. Importantly additional factors such as the LXR α & β family members has been shown to be involved in directing neuronal versus glial cell-fate choices in vMB progenitors (Sousa and Hall, manuscript).

3 THE WNT PATHWAY

Wnts comprise a large family of secreted, cysteine-rich, lipid modified glycoproteins that affect diverse processes. During embryonic development these processes include generation of cell polarity, cell fate specification, axis formation, patterning, proliferation, morphogenesis, cell motility and synaptogenesis (Cadigan et al. 1997; Logan et al. 2004; Bennett et al. 2005; Ciani et al. 2005; Lowry et al. 2005). Wnts also play a role in adult homeostasis (Reya et al. 2005).

3.1 Wnt signaling

Wnt mediated signals can be transduced through three different pathways: (1) the classical canonical *Wnt/ β-catenin* mediated pathway (2) the *Wnt/Ca2+* pathway and (3) the *(PCP)* Planar Cell Polarity pathway (see Fig 3).

3.1.1 Canonical Wnt signaling

The ***Wnt/ β-catenin*** mediated pathway is initiated by Wnt binding with its seven transmembrane receptor, Frizzled (Fz), and the low-density-lipoprotein (LRP) 5/6 co-receptors. When bound, the terniary complex activates casein kinase1ε, which phosphorylates the cytoplasmic mediator Dishevelled (Dvl) proteins (Zeng, X. et al. 2005; Bryja et al. 2007b) This results in the recruitment of β-arrestin that forms a ternary complex with Dvl and Axin (Bryja et al. 2007a) and inhibits the activity of the glycogen synthase kinase-3β (GSK-3β) destruction complex. This blocking in turn avoid GSKβ phosphorylation of β-catenin, that otherwise would be ubiquitylinated and targeted for destruction in the proteasom. Thus, dephosphorylation (stabilization) takes

place in the cytosol followed by translocation of β-catenin to the nucleus, where it binds transcription factors of the TCF/LEF family. Subsequently, transcription of TCF/LEF target genes can takes place (Shimizu et al. 1997; Kishida et al. 1999; Peters et al. 1999). Wnt-1,-2,-3, 7a/b and Wnt-8a/b has been identified as players in the canonical pathway (Moon et al. 1997).

3.1.2 Non canonical Wnt signaling

Non-canonical Wnts, eg Wnt4, -5a, -6 and Wnt11 (Moon et al. 1997), activate diverse intracellular pathways and describes Wnt signals that still interact with Fz receptors to activate Dvl. However, upon ligand binding GSKβ or β-catenin is not the downstream mediator.

3.1.2.1 The Wnt/Ca2+ pathway

The initial observation of β-catenin independent pathways was made in *Xenopus* oocytes where overexpression of Wnt5a or Wnt11 resulted in an elevated level of intracellular calcium without affecting β-catenin stability (Sheldahl et al. 1999; Kuhl et al. 2000b). This and further studies showed, that upon Wnt binding to Fz, activates protein kinase C (PKC) and via Ca++/calmodulin dependent kinase II (CamKII), activates calcineurin (CN) and subsequent transcriptional activation of the nuclear factor of activated T cells (NFAT) (Slusarski et al. 1997; Kuhl et al. 2000a; Saneyoshi et al. 2002). D-V patterning of the early embryo is regulated in response to this pathway mediated by Wnt5a (Slusarski et al. 1997; Saneyoshi et al. 2002; Weeraratna et al. 2002).

3.1.2.2 The PCP pathway

The PCP pathway is also referred to as convergent-extension pathway, is associated with many polarization processes resulting in the formation of uniform orientation of cells in a single plane (Darken et al. 2002; Goto et al. 2002; Shariatmadari et al. 2005) and involves (i) the activation of GTPases (Rho, Rac and Cdc42) that result in cytoskeletal reorganization and cell adhesion (Habas et al. 2001; Jessen et al. 2002; Habas et al. 2003; Lu et al. 2004) or (ii) the activation of Jun N-terminal kinase (JNK). The signaling diversity in this pathway is achieved upon the various amount of signaling molecules detected, including Vangl, Celsr, and PTK7, all of them interacting with either Fz or Dvl (Dabdoub et al. 2003).

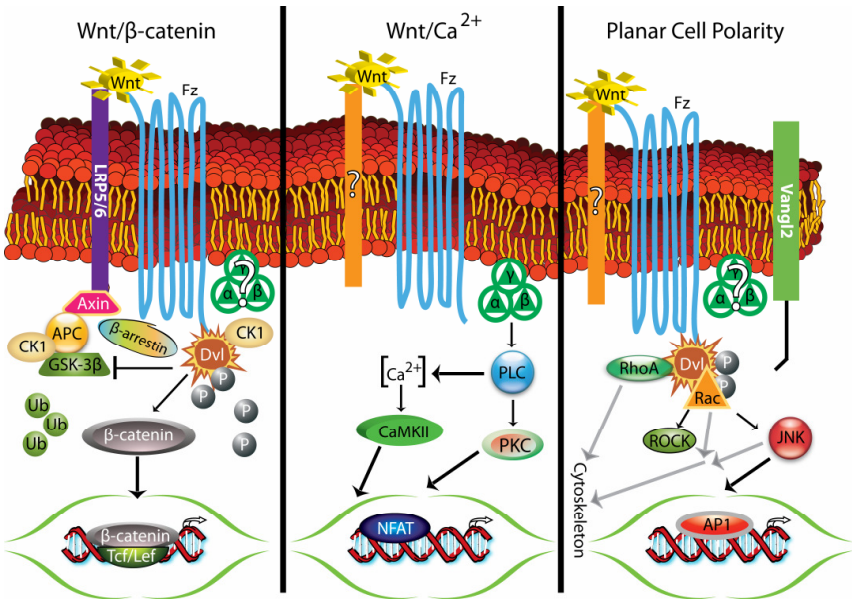

Fig 3.The Wnt signaling pathways: The **Wnt/β-catenin** pathway signals via stabilized β-catenin either in the cytoplasm, or by translocation to the nucleus. The accumulation of stabilized β-catenin in the cytoplasm is a result of a dephosphorylation event leading to sequestration from the GSK3β destruction complex. In turn, the **Wnt/Ca^{2+} pathway** signals are mediated in the absence of LRP, by activating phospholipase Cβ (PLC) that leads to raised intracellular Ca^{2+} levels and by sequential events influences cellfate and cell adhesion processes. In the **PCP pathway** different Wnt-Fz combination signal via alternative Dvl-mediated process, in the absence of LRP, using GTPase Rho and Rho-associated kinase (ROCK).

More recently, the Wnt signaling has began to move away from the stringent classification of the Wnts as either canonical or non-canonical as several lines of evidence illustrate crosstalk between the various intracellular components (Mudher et al. 2001; Maye et al. 2004; Le Floch et al. 2005; Schulte et al. 2005; Mikels et al. 2006) (Paper II).

Understanding how the Wnt signaling pathways are regulated has been of critical importance not only to explain processes of development and adult homeostasis, but also because of its involvement in a number of disease states; namely cancer and numerous neurodegenerative diseases (Polakis 2000; Clevers et al. 2006; De Ferrari et al. 2006; de Iongh et al. 2006; Fox et al. 2006; Krishnan et al. 2006).

3.2 Wnt ligands

3.2.1 Glycosylation and Lipid Modifications

Secreted Wnt ligands, of which 19 have been identified in mammals, are expressed in overlapping, temporal-spatial patterns (Miller et al, 2001;www.stanford.edu/~ rnusse/wntwindow.html).The crystal structure for Wnt proteins has yet to be solved, but insights in the shared features of all Wnts, including the highly conserved spacing of the cysteine residues (Mason et al. 1992). Post transcriptional modifications, including glycosylation (Nusse et al. 1992) and palmitoylation, account for their hydrophobic nature and preferential location associated/anchored to cell membranes (via lipid rafts, implicated as platforms for signal transduction and cell activation; (Simons et al. 2000) and extracellular matrix (ECM; (Kadowaki et al. 1996; Reichsman et al. 1996). Interestingly, in addition to membrane targeting and glycosylation, the palmitoylation appears to be involved in Wnt Frizzled (Fz) interactions (Cong et al. 2004). Thus, lipid

modification has shown to be essential for the biological activity serving many diverse purposes, whereas blocking or removal of the palmitate moiety results in a biologically inactive/inert and no longer hydrophobic protein (Willert et al. 2003; Zhai et al. 2004; Schulte et al. 2005). This feature might partly explain the difficulties encountered upon purification attempts of active protein. Thus, with this newly gained knowledge, recent attempts have resulted in the successful purification of Wnt3a, Wnt5a and Wnt2 (Willert et al. 2003; Schulte et al. 2005) (Sousa et al., submitted). Moreover, Porcupine acyl-transferase, a conserved multipass transmembrane resident endoplasmic reticulum (ER) protein, has been suggested to lipid modify Wnt proteins within the ER, a finding that may be important in order to purify other members of the Wnt family. However, this remains to be biochemically confirmed (Hofmann 2000; Tanaka, K. et al. 2002; Nusse 2003)

3.2.2 Wnt secretion and extracellular transport

Wntless (Wls/Evi/Mom-3, (Thorpe et al. 1997), another multipass transmembrane protein, was recently identified in the secretory pathway as necessary to promote release of Wnt from producing cells. Two independent genetic screens suggest that: 1) Wls/Wntless acts in a cell non-autonomous fashion, supported by a RNAi-mediated knock down study that inhibited the activation of Wnt-responsive luciferase reporter in co-cultured responding cells (Banziger et al. 2006)., 2) In the absence of Wls, the transport to the cell surface is inhibited and thereby Wnt fails to be secreted (Bartscherer et al. 2006). Although, the phenotypes observed in Wls are very alike, those observed in the porcupine mutant differ in spatial distribution and function (Banziger et al. 2006; Mikels et al. 2006). Unlike porcupine, Wls is not present in the ER but rather in the Golgi and/or on the plasma membrane, the secretory activity of

Wls does not depend on the palmitoylation grade of the Wnts and the glycosylation status of the retained Wnt protein is not altered upon knockdown of Wls.

The wide variety of actions that Wnts have been ascribed to is partly thought to be through their ability to act as secreted morphogens. Interestingly, despite their hydrophobic nature, they not only act locally, creating a concentration gradient across a certain tissue, but also influence cells in a long-range manner as far as 30 cell diameters away from producing cells to attain positional information (Neumann et al. 1997). The mechanisms underlying long range movement of molecules with such strong membrane affinity are unclear. However, recent findings indicate that this might partly be achieved by the retromer complex, a cluster of proteins functioning in basal-to-apical transcytosis (Verges et al. 2004), that act within the Wnt-producing-cells generating Wnt forms that can be transported outside the cells (Panakova et al. 2005; Coudreuse et al. 2006; Prasad et al. 2006).

3.3 Frizzled receptors (Fz)

To date, 10 Frizzled receptors have been identified (Mikels et al. 2006). *In vivo* they exhibit a dynamic spatial-temporal distribution pattern (Kim, A. S. et al. 2001b) (Paper III) and have been suggested by several studies to be involved in cell fate determination (Fz9 in the hippocampus, Fz5 in retina and neocortex and Fz3 in the neural crest (NCC) (Deardorff et al. 2001; Zhao, C. et al. 2004a; Van Raay et al. 2005). The seven-pass transmembrane receptor, Fz2, was the first Wnt receptor demonstrated to mediate Wnt signals. Treatment of Drosophila Fz2 overexpressing S2 cells with Wingless (Wg) led to stabilization of the Drosophila β-catenin ortholog, Armadillo (Bhanot et al. 1996).

Fz proteins possess a conserved extracellular cysteine-rich N-terminal domain (CRD) that account for high affinity binding of multiple Wnts (Hsieh et al. 1999b; Dann et al. 2001; Wu, C. H. et al. 2002). However, more recent reports indicate that alternative lower affinity Wnt binding sites exist and that the need for CRD might be context dependent (Chen, C. M. et al. 2004; Povelones et al. 2005). Few binding affinity assays have been performed between Wnt ligand and Fz receptors due to the lack of purified Wnt protein. However, the importance of ligand affinity as a downstream determinant of Wnt signaling has been partially revealed. Putative Fz signaling mechanisms have been suggested at the cell membrane (Carron et al. 2003) whilst at the cytoplasmic side an NMR study showed direct interaction between Dvl PDZ domain and the conserved C-terminal region of Fz (Chen, W. et al. 2003; Wong, H. C. et al. 2003). A clear cut mechanism underlying Fz signal transduction is tentative.

3.3.1 Is there convincing evidence, besides the topology, to classify Fzs as GPCRs?

Genetic (Katanaev et al. 2005) and biochemical evidence (Wang, H. Y. et al. 2004) has suggested G-proteins to be activated in response to Fz, initiating a biochemical cascade that hydrolyses GTP to GDP. This in turn can activate or inhibit adenylyl cyclase to regulate cAMP levels. The Moon and Malbon laboratories have also presented several lines of evidence in favor of Fz being GPCRs. (Liu, T. et al. 2001b).

A recent study has introduced a new player in the Wnt/ β-catenin pathway. The multifunctional scaffold protein β-arrestin, known to mediate GPCRs signaling, has been shown to be part of a trimeric complex with Axin and Dvl, that is necessary for Wnt/ β-catenin signaling during embryonic development *in vivo* (Bryja et al. 2007a).

3.4 LRP coreceptors

Upon Fz binding, canonical Wnts concomitantly bind to two single-span transmembrane receptors, the low-density lipoprotein receptor-related protein (LRP) 5 and -6, forming a ternary complex. These comprise multiple EGF repeats important for binding to Wnt, triggering an intracellular phosphorylation event necessary for recruitement of the scaffolding protein axin to the membrane, hindering its participation in the β-catenin destruction complex (Pinson et al. 2000; Tamai et al. 2000; Wehrli et al. 2000; Mao et al. 2001; Tamai et al. 2004). LRP5 homozygous mutants are viable (Gong et al. 2001) whereas LRP6 null mice display developmental defects including partial deletion of the dorsal MHB region similar to that observed in Wnt1 null mice. Redundancy of the two receptors has been suggested based on: (1) An overlapping expression pattern *in vivo,* (2) An increased LRP5 expression in the developing midbrain of LRP6 null mice (Castelo-Branco et al., manuscript in preparation). (3) Mice depleted of both LRP5 and-6 exhibit embryonically lethal gastrulation defects (Kelly et al. 2004).

3.5 Wnt modulation/antagonism

Wnt antagonists comprise two functional groups based on mechanisms of action, (i) the soluble frizzled related protein (sFRP) class and (ii) the Dickkopf (Dkks) class.
The Dkks class encompass four members (Dkk1-4) that contain two CRDs and share structural similarity with the colipase family (Aravind et al. 1998; Krupnik et al. 1999), implying a common function. Dkk exert their inhibitory activity upon formation of a ternary complex with LRP and Kremen, sequestering thus the LRP receptor away from the Wnt/Fz complex (Bafico et al. 2001; Mao et al. 2001; Semenov et al. 2001; Mao et

al. 2002). However, in the absence of Kremen, Dkk has been shown to function as an activator (Mao et al. 2003). In contrast, the sFRP class of antagonists (comprising of sFRP, Wnt inhibitory factor and Cerberus), bind directly to Wnts altering the ability to form Wnt/Fz complexes, and thereby antagonizing both canonical and non canonical signaling.

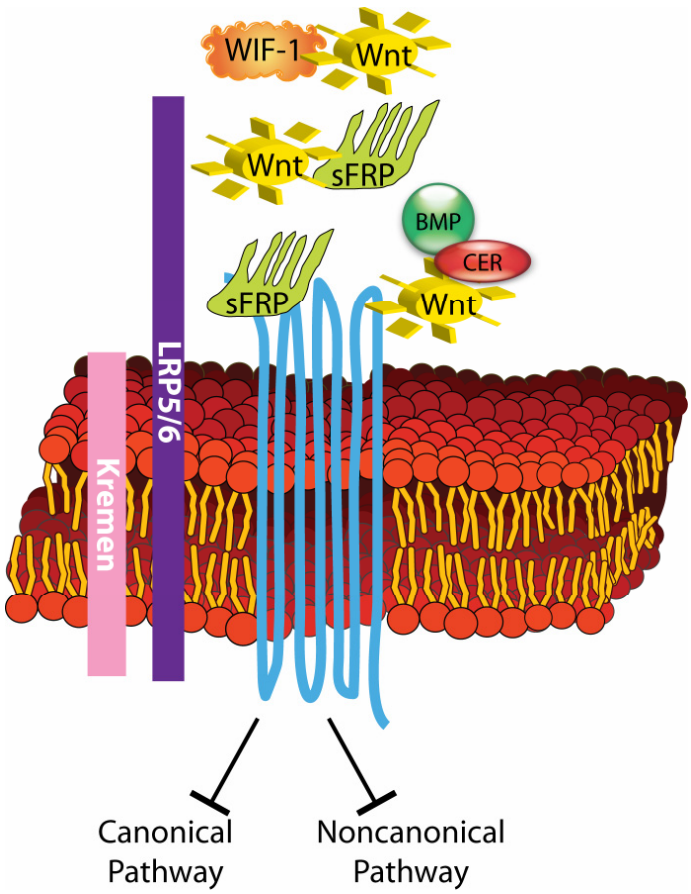

Fig 4. Wnt pathway antagonists/modulators. sFRPs, Cerberus (CER) and WIF-1 prevent the Wnt-Fz complex to form and thereby blocking both canonical and non-canonical signaling pathways.

3.5.1 sFRP family

The sFRP family consists of eight identified members where a unifying nomenclature exists for five of them (sFRP1-5). Based on sequence homology sFRP1 (FRP, SARP2, FrzA), sFRP2 (SARP1) and sFRP5 (SARP3) form a subclass shown to suppress the canonical Wnt/β-catenin pathway by down regulation of both β-catenin levels and Myc target gene expression in cultured cells (Suzuki et al. 2004). The two most distantly related sFRPs, sFRP3 (FrzB, FrzB-1, fritz) and sFRP4 (FrzB-2) form a second subclass. A third subclass exists but these have not been identified in mammals (Sizzled, Sizzled2 and Crescent). .

The sFRP, and their governing inhibitory activity, were discovered by several parallel lines of investigation during the 1990s. FrzB/sFRP3 was the first member to be identified in a differential screen for dorsalizing factors together with Cerberus (Bouwmeester et al. 1996) and in skeletal patterning (a Frizzled motif associated with bone development, FrzB, (Hoang et al. 1996)). In this latter study, a GenBank data search revealed a significant homology to the characteristic N-terminal region of Drosophila Frizzled (involved in binding Wnt ligands) but lacking the receptor seven transmembrane domain. Further studies in *Xenopus* embryos, provided support for a function of sFRP3 and a direct interaction with Wnt. (Leyns et al. 1997; Lin, K. et al. 1997; Wang, S. et al. 1997a; Wang, S. et al. 1997b). Thus, sFRP3 was shown to: 1) be expressed in the gastrula organizer (Spermann´s) complementary to Xwnt8 localisation, 2) co-immunoprecipitate with Xwnt8 and bind to cells overexpressing a membrane bound form of Wnt-1 but do not inhibit Wnt3a, -5a or 11 and, 3) block ectopic Xwnt8 function and Wnt1 induced β-catenin stabilization in 293 cultured cells (Leyns et al. 1997; Lin, K. et al. 1997; Wang, S. et al. 1997a; Wang, S. et al. 1997b). The remaining

members of the family were identified by several different approaches (Finch et al. 1997; Melkonyan et al. 1997; Rattner et al. 1997; Hu et al. 1998; Chang et al. 1999). In addition sFRP1 interaction with Wnt1, XWnt8 and Wnt2, but not Wnt5a, was observed in *Xenopus* (Xu, Q. et al. 1998; Dennis et al. 1999).

The mechanism underlying how sFRPs exert their regulatory function is far more complex and remains under debate within the Wnt society. At first and foremost, how is the binding achieved? Second, is Fz, Wnt or Fz+Wnt the binding partner/s? Third, is sFRP always acting as a Wnt antagonist?

3.5.2 sFRP structure – "so similar still so different". What are the underlying mechanisms of action?

Interestingly, despite the secreted/soluble nature of sFRPs, several studies documented sFRPs at the plasma membrane and/or in the ECM following synthesis by cultured cells. The C-terminal portion of sFRP comprises a domain that has weak sequence similarity with the axon guidance molecule Netrin. A few cysteine residues and several conserved parts of hydrophobic residues characterize this region (Banyai et al. 1999), suggesting that it may have matrix stabilizing properties upon interaction with Wnts (Roth et al. 2000). These positive C-terminally situated residues have been reported to be responsible for sFRP1 and sFRP3 binding to heparan sulfate proteoglycans upon secretion (in common with some Wnts, (Reichsman et al. 1996)), and thereby stabilizing sFRP-Wnt complexes (Hoang et al. 1996; Finch et al. 1997; Üren et al. 2000). However, when sFRP2 and 3 were engineered with a glycolipid tag to remain anchored to the cell membrane, they conferred Wg binding to the cell surface (Rattner et al. 1997). This raises the possibility that, having seemingly more common features

with Wnts than with Fz, sFRPs might be regulated by mechanisms similar to Wnts. Still, being far easier to purify than Wnts, it is arguable if they go through any, glycosylation or lipid modification prior to secretion. Nevertheless, some of the previously mentioned experiments were performed with unpurified reagents thus the evidence of sFRP/Wnt binding was indirect. Accordingly, the Rubin laboratory not only showed convincing evidence by ELISA, co-precipitation and cross-linking experiments that purified sFRP1 directly bound Wg, but also that sFRP1 exhibited biphasic properties on Wg signaling. Increasing Wg dependent stabilization of Armadillo at low doses and, inhibiting at high concentrations (Üren et al. 2000). While there has been doubt whether the CRD ((Lin, K. et al. 1997), using Wnt1) or the C-terminal region ((Üren et al. 2000), using Wg) is required for functional interaction with Wnt, the use of different ligands and/or different binding affinities among sFRPs and Wnt may also account for the contradiction. Prominently, a crystallographic study revealed the potential for CRD to dimerize (Dann et al. 2001) and the CRD of sFRP1 to bind a truncated form of Fz (Hfz6-CRD) and itself *in vitro* (Bafico et al. 1999). Thus, sFRPs might operate in a dominant negative mode binding to Fz, creating a non functional complex or in competing fashion with Fz for Wnt ligands.

3.6 Intracellular Wnt transducers

3.6.1 Dishevelled (Dvl)

The Dvl family comprises three members, Dvl1-3, with different primary sequences, but all holding several hallmark motifs and docking sites (Wharton 2003). The precise mechanism of Dvl function is not known but the three conserved primary domains of Dvl scaffolds have been well described and are involved in the different downstream

Wnt signaling pathways. The major landmarks of the protein are: (i) at the N-terminus a Dishevelled and Axin (DIX) binding domain (Zeng, L. et al. 1997; Luo et al. 2004) (ii) in the mid region a post-synaptic density-95, discs-large and Zonula occludens-1 (PDZ) domain; and (iii) located about midway between the PDZ domain and the C-terminus of Dvl a Dishevelled, Egl-10, Pleckstrin (DEP) domain. The PDZ domain serves as docking site for various number of proteins such as protein kinases (e.g., casein kinase 1 and casein kinase 2), phosphatases (e.g. PP2C), adaptor proteins (e.g β-arrestins), and Fzs (Kay et al. 2004), whereas the DEP domain is responsible for protein-protein, interaction connecting Dvl to the small molecular weight RhoA family of GTPases through its interaction with Daam1 (Boutros et al. 1998). However, the protein binding domains outside these well conserved sites are less well defined. Dvl associated proteins suspected of direct functional interactions with Dvl can be divided in four groups:(1) GPCR (e.g β-arrestins); (2) β-catenin signaling (e.g Axin, Nkd, Frat, Idax, GBP/Frat, Frodo, GSK3 β, and β-TrCP); (3) signaling via the planar cell polarity/PCP pathway (Strabismus, Van Gogh, Trilobite, Daam1, RhoA, Rho-associated protein kinase [ROCK], Rac, c-Jun N-terminal kinase [JNK], and Prickle),; (4)Protein kinases/phosphatases (e.g., Ck1, Ck2, GRK2, Par-1, PAK, PP2C,PKC, PKA) as more general players (Malbon et al. 2006). The compound mutants (Dvl1/2) have seemingly most severe phenotypes, followed by the Dvl2 null (cardiac morphogenesis, somite segmentation and neural tube closure) . The least severe mutant was Dvl1, which is viable. These results suggested redundant mechanisms between the Dvl genes (Lijam et al. 1997; Hamblet et al. 2002).

3.6.2 β-catenin and the destruction complex

The main role of the destruction complex is to keep levels of β-catenin low in the absence of Wnts. The scaffolding protein, Axin, is the center of the complex, creating an arena for binding, and bringing into proximity, the core components: CK1(-α, -δ) and GSK3 (-α,- β the two kinase families) and APC (the adenomatous polyposis coli, a tumor suppressor) (Lee, E. et al. 2003a). In the absence of Wnt ligand, β-catenin enters the complex by initially binding to Axin and APC, positioning the N-terminal part of β-catenin nearby CK1 and GSK3 for subsequent and sequential phosphorylation.This is followed by binding of the E3 ligase, β-TrCP1, to the phosphorylated N-terminus of β-catenin and subsequent ubiquitinylation by an E2 ligase to be targeted to the proteasome for degradation. How the ubiquitinated β-catenin leaves the complex, remains unknown (Kimelman et al. 2006).

Upon binding of Wnt ligand, β-catenin becomes stabilized thus predominantly present in an unphosphorylated state and is translocated to the nucleus by Axin, APC and Pygopus (Kramps et al. 2002; Wiechens et al. 2004) to initiate TCF/LEF mediated transcription. CyclinD1, En-1/2, c-myc, Pitx2, neurogenin-1, c-ret, Fgf-18 and fibronectin are among some of the target genes that TCF/LEF family of transcription factors regulate (Logan et al. 2004). The wide spectrum of genes activated by β-catenin are in concordance with observation describing its function, such as proliferation in the CNS, thus over-expression of β-catenin leads to neural tissue expansion whereas conditional ablation results in reduced brain size (Chenn et al. 2002; Zechner et al. 2003). Other functions of β-catenin in the brain include promoting the differentiation of midbrain DA precursors into DA neurons (Paper II) and dendritic morphogenesis (Yu, X. et al. 2003). Interestingly, conditional gene activation in the Axin2 expressing domain in early neural development exhibited a severe impairment in midbrain where

the ventricles failed to fully extend at E10.5-11.5 and several midbrain structures were missing at E13.5 (Yu, H. M. et al. 2007). This is in line with the function and importance of Wnt/ β-catenin pathway during embryonic midbrain development.

3.7 Wnt ligands in neural development

3.7.1 Wnts - in neural induction

As implicated previously, based on experiments in *Xenopus* and *chick,* BMP inhibition does not by default induce neural fates (Wilson et al. 2001b; Delaune et al. 2005). In chick, two separate pathways have been suggested to be initiated by Fgf: one by which Fgf induces neural fates independent of BMP inhibition and another through which Fgf represses BMP transcription, a pathway that additionally requires the inhibition of the Wnt pathway (Fig 4).

Evidence for this model came from experiments where epiblast cells, were exposed to *canonical Wnt3a* and *Wnt8c* which led to blocking of the neural inducing activity of Fgfs, an effect that could be reverted by adding a soluble form of Fz8 (Fz8-CRD). Furthermore, explants exposed to the *non-canonical Wnt5a*, still generated Sox2+/Sox3+/Otx2+ neural cells but not the epidermal Msxl/2+/GATA2+ cells, i.e. neural fates were no longer blocked (Wilson et al. 2001a; Wilson et al. 2001b). Conversely, Baker et al. (Baker et al. 1999) has shown the opposite effect in *Xenopus* where Wnt signaling seems to promote neural induction. The conflict is thought to be resolved by differential timing of these events: Wnt signaling is required in early (pre-gastrula) stages of development, while inhibition of Wnt may be important for acquisition of neural fate at later stages (Bainter et al. 2001; Wilson et al. 2001b; Stern 2005). However, a cocktail of Fgfs, BMP antagonists, Noggin and Wnt antagonists, were unable to induce expression of the neural marker Sox2 in chick epiblasts in intact

embryos, implying that additional *in vivo* signals might be required for neural induction in chick (Linker et al. 2004).

Nevertheless, *in vitro* neural determination and differentiation achieved upon Wnt antagonism (sFRP2), could be reverted by treatment with *Wnt-1* or lithium chloride through *β –catenin* stabilization and *GSK3β* inhibition, respectively (Aubert et al. 2002). Furthermore, the early role of canonical Wnts were also confirmed in *Wnt3a* null mice and in mice double mutant for TCF-1 and LEF-1 showing an excess of neuroectodermal tissue, at the expense of paraxial mesoderm (Takada et al. 1994; Yoshikawa, Y. et al. 1997; Galceran et al. 1999).

3.7.2 Patterning to connectivity - from a Wnt perspective

Results obtained in *Xenopus* and chick, using β-catenin-mediated activation, show that an early endogenous gradient of Wnt signaling underlies the specification of cell fates during neuraxis formation (Kiecker et al. 2001; Yamaguchi, T. P. 2001; Nordstrom et al. 2002). This might explain the initial classification of Wnt proteins in two functional groups, based on ability (i.e Wnt1 and Wnt3a) or disability (i.e.Wnt4 and Wnt5a) to induce a secondary D-V axis in *Xenopus* embryos and having a morphologically transforming capacity following ectopic Wnt expression (Olson, D. J. et al. 1994; Wong, G. T. et al. 1994; Du et al. 1995; Torres et al. 1996; Shimizu et al. 1997; Slusarski et al. 1997). Thus axis duplication assays in *Xenopus* have served as the hallmark for activation of the canonical Wnt/ β-catenin pathway.

For proper A–P identity of the neural tube, the regulation of Wnt signaling is accomplished at two levels: (i) through extracellular antagonists (eg. secreted Wnt antagonists, such as sFRP3/FRZB1 (Leyns et al. 1997) and Cerberus) that bind to Wnts

or to their receptors, and (ii) through intracellular factors that are expressed or activated in anterior regions of the neural tube. Thus, to generate anterior neural structures the posteriorizing function of Wnts must be inhibited rostrally as demonstrated by overexpression of Cerberus (Bouwmeester et al. 1996; Glinka et al. 1997). Conversely, loss of Wnts results in the expansion of forebrain markers (Erter et al. 2001; Lekven et al. 2001).

Following an early Wnt gradient, responsible for establishing the rostro-caudal identity of the neural plate and subsequent local inhibition of Wnt signaling, result in sequential regionalization of the forebrain and other areas such as midbrain, hindbrain, neural creas and spinal cord in a temporal-spatial manner (Ciani et al. 2005). However, D-V patterning, with Wnts acting as dorsalizing factors, takes place simultaneously with the A-P refinement of the neural tube that seems to require repression of the Wnt/ β-catenin signaling (Yamaguchi, Y. et al. 1989; Gunhaga et al. 2003). This has been further confirmed in the zebrafish mutant headless as these embryos lack eyes, forebrain and have only parts of the midbrain with a simultaneous rostral expansion of midbrain and hindbrain markers (Kim, C. H. et al. 2000; Dorsky et al. 2003).

Neural tube closure is regulated by the Wnt–PCP pathway. In humans, abnormal PCP signaling can result in neural tube defects. Recent evidence indicates that the PCP pathway regulates neural tube closure by controlling convergent extension movements in the dorsal neural tube (Wallingford et al. 2002). Furthermore Dvl1:Dvl2 double mutant mice show neural tube defects from the midbrain to the tail and is similarly observed in the loop-tail-mutant mouse carrying spontaneous mutations in the *Drosophila* homologue *Strabismus* and in the cadherin EGF LAG seven-pass G-type receptor 1 mutant, homolog of the *Drosophila* PCP gene *flamingo*. Both these PCP

genes are known to interact with Dvl indicating a role of Dvl in the PCP pathway (Lijam et al. 1997; Kibar et al. 2001; Hamblet et al. 2002; Park et al. 2002; Curtin et al. 2003).

Axon guidance, Dendrite morphogenesis and Synaptogenesis: are also processes dependent on Wnt activity. An elegant report by the Tessier-Lavigne and Zhou laboratories has shown that a decreasing anterior–to-posterior gradient of Wnt4 is established in order to guide commissural axons in the spinal cord with the requirement of the Fz3 receptor (Lyuksyutova et al. 2003). Interestingly, dendritic branching and growth has been shown to be regulated by Wnt7b, expressed in hippocampus, through the alternative Dvl-JNK-Rac pathway (Rosso et al. 2005) whereas synaptic connectivity between cells in the cerebellum is accomplished by axonal remodeling of pre-synaptic mossy fibers achieved upon Wnt7a secretion from post-synaptic cerebellar granule cells (Hall et al. 2000).

3.8 Wnts revisited in MB development

Wnt1 and -5a have been shown to have pivotal roles during midbrain DA development (Wagner et al. 1999; Hall et al. 2003; Castelo-Branco et al. 2006) (Paper II).
As indicated earlier, Wnt1 is expressed throughout the presumptive midbrain and at later stages is being restricted to the dorsal midline of the MB and diencephalon. It has a prominent early role not only in formation of MHB but also in specification of DA neurons. Analysis of the *Wnt1* null mice at E9.5 exhibit loss of caudal midbrain and rostral hindbrain, leading to severe defects in MB and cerebellum at later stage (McMahon et al. 1990; Thomas et al. 1990). En1 expression in the MHB region is initially unaffected, but is lost at a later stage before cell death takes place. Importantly,

En1 expression under the Wnt1 promoter rescues most of the dopaminergic phenotype of the *Wnt1* null indicating *En1* to be downstream of Wnt1 and, pointing to Wnt1 as a key regulator in maintaining En1 expression (Danielian et al. 1996).
Moreover, although the domain of Wnt1 expression is located anterior to that of Fgf8 in the MHB, Wnt1 does not affect the induction of Fgf8 but is rather thought to be required for the refinement and position of Fgf8 expression possibly through En-1 (Ye et al. 2001). In turn Fgf8 regulates Wnt1 expression in the prospective midbrain (Chi et al. 2003), creating a loop to maintain the MHB. (Prakash et al. 2006). In addition, specific inactivation of β-catenin in the MHB mimics the Wnt1 null phenotype, indicating a possible role of canonical signaling in regulating MHB patterning (Brault et al. 2001).

The discovery that the canonical Wnt signaling pathway is of importance for MB DA development, was first demonstrated in E10.5 vMB DA precursor cells, *in vivo*, monitored for expression and transcriptional activity of β-catenin (Paper II). At E10.5 these cells exhibited co-localization of β-catenin and the DA precursor marker, Nurr1 and displayed TCF/LEF activity in TOPGAL reporter mice, indicating canonical Wnt signaling before birth of TH+ neurons. Further compiled evidence from spatio-temporal expression patterns revealed several members of the Wnt family to be expressed during MB DA neuron development indicating involvement of other Wnt signaling pathways (Paper II; Paper III). Interestingly, treatment of vMB primary precursor cultures with Wnt5a conditioned media, displayed a predominant role of Wnt5a, a non canonical Wnt, in DA neuron development.
Several additional studies have been performed confirming Wnt5a as an important factor during midbrain development (Schulte et al. 2005) (Paper II; Parish et al., submitted). The underlying mechanism by which Wnt5a exert its function remains

elusive. Thus, further *in vivo* studies in the CNS needs to be surveyed, as the only well documented results from the Wnt5a null mice at present is the reduction in caudal structures and increased β-catenin signaling in the distal limb, indicating inhibition of β-catenin stabilization by Wnt5a (Yamaguchi, T. P. et al. 1999; Topol et al. 2003).

3.9 Wnt5a - canonical and/or non canononical, through Ror2 or not, these are the questions?

The finding that knockout mice for the orphan tyrosine kinase receptors Ror1 and Ror2 exhibited similar phenotypes to that of Wnt5 null mice suggested a possible direct interaction (Yamaguchi, T. P. et al. 1999; Yoda et al. 2003). Moreover, Ror2 and Wnt5a show overlapping expression patterns and exert synergistic effects upon JNK signal activation by interacting both physically and functionally (Oishi et al. 2003). Previous reports from zebrafish and *Xenopus* had placed Wnt5a as mediator of signal transduction through the Ca^{2+} -dependent pathway, and blocker of secondary axis formation upon co-expression with a canonical Wnt (Torres et al. 1996; Malbon et al. 1998; Chen, W. et al. 2003). These findings indicated the ability of one Wnt signal to act at least in two distinct pathways. How this was accomplished was indeed further demonstrated in a recent report by Mikels and Nusse (Mikels et al. 2006), where purified Wnt5a elicited a fast dose-responsive decrease in Wnt3a-induced (canonical) SuperTopflash (STF) reporter activity in HEK293 cells. The authors suggested this taking place in a potentially post-translational mode as surprisingly no changes could be detected in β-catenin stabilization nor in Ca^{2+} -dependent signaling. Nevertheless, in accordance with the Wnt5a and Ror2 null mice phenocopying one another, the Ror2-CRD exhibited direct binding to Wnt5a and was shown to be necessary for Wnt5a mediated suppression. Thus, these results and further regulatory complexity in recently

identified co-receptors (RYK/Derailed, (Kroiher et al. 2001; Yoshikawa, S. et al. 2003; Lu et al. 2004; Bovolenta et al. 2006); FRL1/Cripto, (Tao et al. 2005) and Wnt binding domains proteins, such as CRD (Povelones et al. 2005) and WIF (Hsieh et al. 1999a; Liepinsh et al. 2006), may partly explain how Wnt signaling diversity is achieved and can account for the wide range of implications in developmental processes and disease.

3.9.1 sFRP expression in development – taking several routes?

Dynamic expression patterns of the sFRPs in chick, mouse and *Xenopus* show an additional level of complexity (Leimeister et al. 1998; Baranski et al. 2000; Esteve et al. 2000; Jaspard et al. 2000; Ladher et al. 2000; Terry et al. 2000; Kim, A. S. et al. 2001b). sFRP mRNA are usually found in distinct, partly or completely overlapping domains in the developing embryo. Usually these patterns are complementary to Wnt expressing regions, indicating interactions and potential inhibitory effects (Kawano, Y. et al. 2003) (paper IV).

As a secreted factor, it is not surprising that sFRPs act in conjuction with other secreted factors in the control of morphogenetic zones and in facilitating boundary formations in the developing embryo delimiting the range of Wnt activity. Thus, these effects are paralleled by inhibitory actions on other developmental signaling pathways and may form part of an antagonistic machinery providing coordinated control of several pathways. Interstingly, both sFRP1 and 2 have been implied in crosstalk between the Hedgehog and Wnt pathway since both are upregulated in response to Shh, inhibiting Wnt activity (Lee, C. S. et al. 2000a; He, J. et al. 2006). In the developing hindbrain, sFRP2 was shown to be expressed in even numbered rhombomeres where neural crest cells (NCCs) migrated from when programmed cell death (PCD) occurred in odd

numbered rhombomeres. Importantly, ectopic expression of sFRP2 lead to inhibition of PCD, whereas downregulation of sFRP2 led to increased PCD. The conclusion was that sFRP2 protected NCCs from PCD by antagonizing the Wnt induced activation of BMP-mediated apoptosis in the hindbrain (Ellies et al. 2000). Further evidence from chick and zebrafish show that Wnt antagonism blocks the acquisition of early dorsal character and in mouse that sFRP2 might account for the patterning in the forebrain boundaries through Pax6 and Wnt7b (Kim, A. S. et al. 2001a; Houart et al. 2002; Nordstrom et al. 2002). In addition sFRP1 interaction with Wnt7b has been demonstrated to attenuate the non-canonical Wnt signaling pathway resulting in inhibition of axon guidance (Rosso et al. 2005).

However, the Shimono lab has recently reported the first compelling *in vivo* evidence suggesting that Wnt regulation by sFRP1 and sFRP2 is required for embryonic patterning. Double null mutations in SFRP1 and 2 lead to severe shortening of the thoracic region as a consequence of reduction in A-P axis, due to abnormal cell migration at the end of gastrulation and incomplete somite segmentation, due to altered Notch oscillations. However, single knocked out mice do not exhibit any early embryonic defects (Satoh et al. 2006), suggesting the ability of one SFRP1 and 2 to compensate for each other.

3.9.2 Agonism or antagonism? Opposing gradients; Different cellular responses and Alternative pathways

Even futher levels of complexity are achieved upon sFRP mediated modulation. Their suggested biphasic mode of action might open up the possibility for them to alternate between being agonists (at low concentrations) or antagonists (at high doses;

(Üren et al. 2000) (Paper IV). In addition, several *in vitro* experiments have demonstrated sFRP1, 2 and 3 to exert opposing cellular responses used at the same concentrations (Melkonyan et al. 1997; Yoshino et al. 2001; Galli et al. 2006) (Paper IV). Furthermore opposing gradients of sFRP5;sFRP2 and sFRP1;sFRP3 have been reported in the retina and in the developing mouse telecephalon, respectively (Chang et al. 1999; Kim, A. S. et al. 2001b). Also, alternative routes seem to be involved since sFRP1 has been suggested to modulate retina cell differentiation and axonal growth by alternative β -catenin independent pathways (Esteve et al. 2003; Rodriguez et al. 2005).

4 AIMS OF THE THESIS

Albeit the growing number of factors involved in dopaminergic development, the number of extrinsic and intrinsic signals regulating the developing ventral midbrain, and the knowledge about their function are still limited.

This thesis set to identify and investigate the function of novel intrinsic and extrinsic components involved in the development of ventral midbrain dopaminergic neurons.

General aim:

Study the function of Proneural bHLH proteins (Paper I) and Wnt ligands (Paper II+III), their receptors (Paper III), and their modulators sFRPs (Paper IV) in the developing midbrain.

Specific aims:

- Characterize the expression patterns of Mash1, Ngn1 and Ngn2 (Paper I)
- Analyze single, compound or substitution mutant mice for these three proneural genes (Paper I).
- Identification of Wnt ligands expressed in the developing ventral midbrain (Paper II+III).
- Study the function of Wnt1, Wnt3a and Wnt5a in primary precursor cultures of the ventral midbrain (Paper II).

Investigate the expresion of receptors and intracellular signaling components of the Wnt pathway (Paper III).

- Identification of the expression of sFRP1-4 in the developing vMB (Paper IV).
- Characterization of the function of SFRP1, 2 and 3 *in vitro* (Paper IV).
- Analysis of the sFRP2 null mutant mice (Paper IV).

5 RESULTS AND DISCUSSION

5.1 Paper I

5.1.1 Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons.

Despite the growing body of evidence and understanding of the mechanisms that underlie dopaminergic neuron development there are still critical periods of development and biological processes that remain poorly understood. One of such process is dopaminergic neurogenesis. To date, the only gene that has been found to regulate DA neurogenesis is *Nurr1* (Zetterstrom et al. 1997; Castillo et al. 1998; Saucedo-Cardenas et al. 1998), expressed in postmitotic dopaminergic precursors just prior to their differentiation into dopaminergic neurons. Taken in account our previous *in vitro* data, this developmental step is also suggested to be positively regulated by Wnt-1, Wnt-5a and the Wnt effector β-catenin (Castelo-Branco et al. 2004), Paper II). In agreement with a possible function of Wnt signaling in neurogenesis, β-catenin has been shown to activate the expression of proneural bHLH genes (Israsena et al. 2004). Interestingly, both β-catenin and its partner Lef1 bind directly to the Ngn1 promoter in embryonic neural progenitors. Moreover, a consensus GSK3 phosphorylation site is present in proneural bHLH proteins (Moore et al. 2002; Israsena et al. 2004). This highlights one more time the requirement of both intrinsic and extrinsic factors as we have proposed in previous studies on Nurr1 (Wagner et al. 1999; Sousa et al. 2007). However, up to the beginning of paper I, the function of the molecular players directly involved in the vMB DA neurogenic process had not been studied. We therefore focused on proneural bHLH genes since they had been found to function both as

proneural (Bertrand et al. 2002) and as neuronal subtype specification genes in several neural systems (Hirsch et al. 1998; Lo et al. 1998; Fode et al. 2000; Parras et al. 2002; Pattyn et al. 2004).

In the mammalian PNS, for example, *Ngns*, but not *Mash1*, promote a sensory neuron identity (Perez et al. 1999; Lo et al. 2002). Accordingly, in the Ngn2 null embryos, there is a neurogenic defect in a subset of dorsal root ganglia (DRG) sensory neurons. Ablation of both Ngn1 and 2 leads on the other hand to complete loss of sensory neurons (Ma et al. 1999). Alternatively, Mash1 promotes neurogenesis in the autonomic nervous system from neural crest cells (Lo et al. 2002).

In the forebrain, neurogenins (Ngn1 and Ngn2), as well as Mash1, are expressed at high levels in dorsal and ventral domains, respectively (Fode et al. 2000). Mash1 is expressed in the ventral region of the telencephalon and specifies the GABAergic neurons, while Ngn2 is expressed in the dorsal region and specifies the glutamatergic neurons (Fode et al. 2000; Parras et al. 2002; Schuurmans et al. 2004). In the absence of Ngn2, Mash1 is ectopically expressed in the dorsal telenephalon, leading to ectopic GABAergic neurogenesis in the dorsal forebrain. Accordingly, absence of Mash1 leads to loss of progenitor cells in the ventral telencephalon, which also results in loss of GABAergic interneurons (Casarosa et al. 1999). When both Ngn2 and Mash1 are absent, this phenotype is exacerbated. In this case, a failure of progenitor specification in both the ventral and dorsal telencephalon drastically reduces the number of cortical neurons that develop (Fode et al. 2000). Similarly, when the coding sequence of Mash1 is swapped into the Ngn2 locus using a knockin strategy, cortical progenitors from the dorsal telencephalon are misdirected in their fate and become GABAergic neurons. In addition, many Ngn2-deficient neurons take the unusual route from the dorsal to ventral

region of the telencephalon, instead of migrating radially within the cortex (Chapouton et al. 2001). These findings indicate that proneural bHLH activity is required for the specification of precursors in the telencephalon and emphasize the importance of Mash1 in the specification of GABAergic neurons. However, when Ngn2 is expressed from the Mash1 locus ($Mash1^{KINgn2/\ KINgn2}$), neurons of the ventral telencephalon do not become glutamatergic and differentiate normally (Parras et al. 2002). These findings indicate that while proneural bHLH factors play a role in neuronal subtype specification in the telencephalon, their activities are dependent upon cellular context. Furthermore, although the activator-type bHLH can regulate the neuronal subtype identity in addition to the pan-neuronal phenotypes, they alone are not sufficient to generate diverse types of neurons. Both in the developing retina and spinal cord, specification of the different layers of cells and specification of motor neurons, respectively, require co-expression of homeodomain proteins together with the activator-type bHLH (Hatakeyama et al. 2001; Inoue et al. 2002; Lee, S. K. et al. 2003b).

5.1.2 Ngn2 is required for DA neurogenesis but not for cell cycle exit of Sox2+ VZ progenitors

Examination of proneural gene expression led to the division of the MB VZ in three separate zones from medial to lateral, depending on the expression levels of Ngn2 and Mash1. High levels of Ngn2 and moderate of Mash1 in the midline, followed by high levels of Ngn2 and low of Mash1 in the basal plate and high levels of Mash1 lateraly. We found that the HMG-box transcription factor Sox2, that is expressed in early neuroectoderm as well as in VZ from neurulation (Wood et al. 1999), also labels VZ neuroepithelial cells in the MB. Interestingly, two different Sox2+ cell types could be

distinguished in the vMB VZ: Large apical double Ki67 positive PH3 positive cells and smaller Ki67 positive PH3 negative neuroepithellial cells.

bHLH proteins have also been reported to regulate cell cycle exit. Ectopic expression of Ngn2 decreases the number of proliferating precursors (Mizuguchi et al. 2001; Lo et al. 2002). Conversely, cortical progenitors isolated from double Ngn2 and Mash1 mutant mice give larger colonies than those isolated from wild type mice (Nieto et al. 2001). Moreover, Ngn2 has been reported to downregulate Sox1-3 gene expression in the spinal cord (Bylund et al. 2003). Our results, showing an extensive overlap in the expression domains of Sox2 and proneural bHLH in the VZ, would not support the notion that Ngn2 downregulates Sox2 in the developing midbrain. However, it is possible that the neurogenic activity of Ngn2 could be inhibited by Sox2 in the VZ were both genes are co-expressed. In this model, the expression of Ngn2 would allow the initiation of neurogenesis and Sox2 would contribute to cell cycle exit. Indeed, we found that in the absence of Ngn2, very few Sox2+ VZ cells give rise to Nurr1+ ImZ cells, but surprisingly, most of them exit the cell cycle at the right time, and proliferate to the same extent as wild type cells. Thus, the impaired capacity of Sox2+ progenitors to generate Nurr1+ postmitotic dopaminergic precursors, and to migrate into the ImZ, resulted in an accumulation of Sox2+ progenitors in the neuroepithelium of Ngn2 nulls. This phenotype was predominantly restricted to the most rostral levels of the vMB at E11.5, but extended throughout the vMB neuroepithelium at 14.5, when the accumulation of Sox2+ cells was very evident. Thus, our results indicate that Ngn2 is required for neurogenesis, but not for cell cycle exit. In other words, postmitotic cells that are not TH+ cells are generated in the absence of Ngn2, suggesting that additional mechanisms must control cell cycle exit. One candidate that could control this function in the absence of Ngn2 is Mash1. However an accumulation of

Sox2+/Ki67+/GLAST+/GFP+ precursors in VZ was also detected in the double mutants (not shown), indicating that other factors are involved.

5.1.3 Alternative mechanisms for cell cycle exit and DA neurogenesis: Msx1, and Pax2

Recently, Andersson et al. suggested that Msx1, in addition to its suppressive role of alternative cell fates in spinal cord motor neurons (Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2002), was able to induce cell-cyle exit and differentiation through its aptitude to promote Ngn2 activity. Moreover, consistent with the idea that the ventral midline initially is occupied by glial-like floor-plate cells (Placzek et al. 2005), analysis of the ShhE-Msx1 transgenic mice further suggest a putative involvement of Msx1 in promoting a glial-to-neuronal switch at the ventral midline (Andersson, E. et al. 2006b). Interestingly, the medial vMB domain exhibits Mash1 and Ngn2 expression, unlike floor-plate cells in other regions. This raises the intriguing possibility that ventral midline progenitors in the vMB may undergo neurogenesis. The mechanism by which Msx1 would exert its action is unclear. However, it may be through suppression of Ngn2 repressors such as the Id proteins, that have been found to promote cell cycle progression in the cortex (Ferguson et al. 2002), and shown to be directly induced by the Wnt/β-catenin signaling pathway in colon carcinomas (Rockman et al. 2001). In addition, SoxB2 family genes, that work as repressors of progenitor fate, controlling cell cycle exit, could also be potential factors. Other additional or alternative factors could be members of the Pax family. In particular Pax6, has been found to promote neurogenesis in the cortex (Heins et al. 2002) and has been implicated in controlling timing and differentiation in the developing spinal cord (Sugimori et al. 2007). In the MB, the phenotype of the Pax2/5 double ko (Schwarz et al. 1999) imply a possible role

of these two members of the Pax family in controlling timing and differentiation of MB DA neurons. Preliminary data from our laboratory indicate an upregulation of Ngn2 and Hes1 expression in E10.5 vMB cultures by overexpressing Pax2 or 5 (Dr. Anita Hall, personal communication). This suggests that Pax6 and Pax2 may not only have cross-repressive functions to maintain the diencephalic-midbrain boundary, but also have opposing roles later in DA development, as an upregulation of Pax6 can be identified in Ngn2 null in the midline (unpublished W.Lin and S-L.Ang). Thus, our results suggest that Ngn2 is dispensable for the cell cycle exit of Sox2+ vMB precursors and that this process is regulated by several genes.

Importantly, the requirement of Ngn2 could not be compensated by Ngn1 (expressed in zone 2 in Ngn2 mutant mice at E11.5), but could partially be rescued by endogenous Mash1 or by Mash1 expressed under the control of the Ngn2 promoter. Interestingly, Mash1 also partially rescued the generation of Nurr1+ postmitotic cells in the ImZ of Ngn2 mutant embryos at E14.5, confirming previous findings that Ngn2 function is required by some, but not all, VZ progenitors to generate postmitotic precursors in the ImZ.

5.1.4 Ngn2 ablation delays the DA differentiation of Nurr1+ precursors: a second role for Ngn2

The spatial distribution of Ngn2 (in the VZ overlapping with Mash1 and in Nurr1+ postmitotic precursors of the ImZ), raises the possibility of a second role for Ngn2 in the development of DA precursors. Interestingly, inactivation of Ngn2, but not of Mash1, dramatically reduced the number of TH+ cells to approximately 15% of wildtype levels at E11.5. By the end of the neurogenic period (E14.5), more Nurr1+

precursors had been produced (40% of control), but the numbers of TH+ cells did not increase to the same extent as at E11.5. Normal onset of expression of TH in postmitotic Nurr1+ precursors occurs within half a day. Hence, the delay in expression of TH in Ngn2 nulls suggests an additional role of Ngn2 in regulating this differentiation step. Consistent with the idea of a delayed TH phenotype acquisition, a further proportionate increase in TH+ cells was observed in Ngn2 mutants at E17.5. Interestingly, the remaining TH+ cells seen in the Ngn2 mutants were consistently positioned laterally in the presumptive TH domain, still labeling with mature DA markers and acquiring a DA phenotype. Accordingly, studies by Björklund's laboratory confirmed that the reduction affected specifically the mesDA neurons and not the Isl+ cholinergic neurons (Andersson, E. et al. 2006a). Interstingly, the new DA neurons were equally distributed between SN and VTA and, exhibited appropriate innervations of forebrain areas (Andersson, E. et al. 2006a). Mash1 and Ngn2 double mutants showed a similar reduction in the number of TH+ neurons, when compared with Ngn2 single mutants, indicating that Mash1 was not required for the differentiation of Nurr1+ precursors into TH+ cells. This finding is consistent with the lack of expression of Mash1 in postmitotic precursors. However, in $Ngn2^{KIMash1/KIMash1}$ embryos, expression of Mash1 in postmitotic DA precursors partially accelerated their differentiation into TH+ neurons at E14.5 and E17.5, suggesting that Mash1 is also able to substitute for this later role of Ngn2 when ectopically expressed in postmitotic precursors.

Alternatively, the delay might be due to downregulation of additional factors that are required by Nurr1 precursors for differentiation into DA neurons by E14.5. Components of the Wnt pathway may represent such candidates, as their expression is developmentally regulated during vMB neurogenesis (paper II+III). Wnt1 could contribute to the early role of Ngn2 by enriching the Nurr1+ progenitor pool whereas

Wnt5a could be involved in the later event promoting the differentiation of Nurr1+ precursors into TH+ neurons. Preliminary results from analysis of Ngn2 expression in the Wnt1 null mice at E11.5 show a loss of Ngn2 expression in the most ventral domains. Conversely, no obvious difference in Wnt1 expression is observed in the Ngn2 null mice, indicating that Wnt1 is upstream of Ngn2. However, a possible Wnt5a expansion can be detected dorsally in these mice confirming the maturation role of Wnt5a in DA development and indicating a possible role in the remaining lateral population (unpublished data). Further involvement of the Wnt/ β-catenin pathway in concert with proneural bHLH function have been suggested by several studies; co-immunoprecipitation assays show that β-catenin binds directly to the Ngn1 promoter and activating the expression of proneural bHLH genes in neural progenitors (Hirabayashi et al. 2004; Israsena et al. 2004); gain and loss of function studies in mouse telencephalon and neural crest cells exhibiting a loss of Ngn2 expression upon β-catenin ablation (Hari et al. 2002; Backman et al. 2005) whereas constitutively active β-catenin in the neural crest cells results in upregulation of Ngn2 (Lee, H. Y. et al. 2004). In addition Wnt3a together with Fgf8 have been shown to induce the dorsal telencephalic marker Ngn2 in chicken telencephalic explants while simultaneously repressing ventral telencephalic markers such as Nkx 2.1 (Gunhaga et al. 2003). Taken together, a clear link can be established however further work remains to be done in order to elucidate the molecular interplay between the components of the Wnt pathway and proneural bHLH function in developing DA neurons.

5.1.5 Mash1 functions permissively in the MB conversely to other neural systems

Ngn2 loss of function mutant resulted in both a loss of pan-neuronal markers such as microtubule associated protein-2 (MAP2) and Tuj1 and, also lead to the disappearance of Nurr1 in the ImZ. None of these effects were observed in Mash1 mutant mice, indicating that Ngn2 plays a proneural function in the developing ventral midbrain. It has previously been described that the proneural effects of Mash1 and Ngn2 in the cortex are equivalent (Nieto et al. 2001). However, the proneural effects of Mash1 in the ventral hindbrain could only partially be rescued by Ngn2 ($Mash1^{Ngn2/Ngn2}$) (Pattyn et al. 2004).

Ngn2 has also been reported to contribute to the specification of sensory neurons in the peripheral nervous system (Perez et al. 1999) and motor neurons in the ventral spinal cord (Mizuguchi et al. 2001; Novitch et al. 2001). Further, Ngn2 has been shown to be able to rescue the early differentiation of Mash1 null sympathetic neurons (Hirsch et al. 1998; Lo et al. 1998) but not the specification of spinal cord motor neurons (Mizuguchi et al. 2001). Our results indicate that deletion of Ngn2 alone results in the loss of subtype specific genes in the dopaminergic lineage such as Nurr1, Pitx3 and TH. These alterations could only be partially rescued by endogenous or ectopic expression of Mash1 in Ngn2 null cells, suggesting that at least part of the function of Ngn2 in the vMB is specific. Unlike Ngn2, an instructive role has also been attributed to Mash1 in other systems, as it can re-specify neuronal lineages when expressed in *Ngn2*$^{-/-}$ precursors in the cortex and the hindbrain (Bertrand et al. 2002; Parras et al. 2002). Mash1 has also been implicated in the specification of GABAergic neurons in the dorsal midbrain (Miyoshi et al. 2004). However, our findings in the ventral midbrain do not allow to extend that concept to ventral territories since we have not detected a re-

specification of *Ngn2*$^{Mash1/Mash1}$ cells in the ventral midbrain into GABA or serotonin neurons, two phenotypes specified by Mash1 (Fode et al. 2000; Pattyn et al. 2004). Thus, our findings suggests that the function of *Mash1* in the ventral midbrain, unlike in other neural tissues, is less instructive and more permissive, and is therefore more similar to that described for *Ngn2* in other systems (Bertrand et al. 2002; Parras et al. 2002).

These results indicate that the activity of Mash1 or Ngn2 depend to a large extent on the cellular context and on additional transcription factors. Thus, a critical step for the future will be to identify the factors that interact with Ngn2 during vMB neurogenesis and dopaminergic neuron specification. In this context, it should be noted that DA neuron identity was not completely blocked by deletion of Ngn2 since 10-15% of TH+ cells were observed in the Ngn2 null or after simultaneous deletion of Ngn2 and Ngn1 or Mash1 at E14.5. Thus additional factors are likely to be involved in the specification of DA neurons. Alternative and complementary neurogenic pathways have been recently described for cortical neuron specification (Schuurmans et al. 2004). First, a Ngn1 and -2 dependent process, and second, a Ngn-independent but *Pax6* and *Tlx* dependent process resulting in the generation of early born cortical plate lower layer or late born upper layer glutamatergic neurons, respectively. It remains to be determined whether a similar mechanism involving other Pax genes, such as *Pax2*,*Pax5 and/or Pax6* or even *Nurr1,* could contribute to this process.

Taking into account the timing of the activation of bHLH proteins in the ventral midbrain, occurring at the time of the expression of postmitotic neural markers, two independent reports have demonstrated that Ngn2 and other bHLH proteins do not only activate the neurogenic machinery but also regulate genes critically involved in cell

migration (Hand et al. 2005; Ge et al. 2006) This suggests an exciting hypothesis that proneural bHLH proteins, and specifically Ngn2 in the midbrain, may control (and perhaps be controlled by) pathways that are activated at the same time in the same cell, such as those regulating cell migration. In agreement with this possibility, in the $Ngn2^{-/-}$ mice progenitor cells that exited the cycle did not differentiate into TH+ neurons and did not migrate away from the VZ, where they accumulated. This result suggests a possible coordination of neurogenesis and migration by Ngn2 in the ventral midbrain.

In summary, we hereby describe a complex pattern of proneural bHLH gene expression in the developing ventral midbrain. We found that only *Ngn2* is required for the development of dopaminergic neurons. The deletion of Ngn2 could only be partially rescued by endogenous Mash1 (differentiation into Nurr1+ cells) or ectopic expression of Mash1 in Ngn2 cells (differentiation into TH+ cells). Thus our findings show that Ngn2 is a key regulator of dopaminergic specification and neurogenesis (see Fig 7 in Paper I) and suggest that overexpression of Ngn2 in dopaminergic progenitors or stem cells may contribute to enhance the dopaminergic differentiation of stem/precursor cells. Such strategies could contribute to the future development of transplantation- or endogenous neurogenesis-based cell replacement strategies for the treatment of neurodegenerative diseases such as Parkinson's disease.

5.2 Papers II+III

5.2.1 Differential regulation of midbrain dopaminergic neuron development by Wnt-1, -3a and -5a.

Wagner (Wagner et al. 1999) reported that mouse NSCs overexpressing Nurr1 can be differentiated into DA neurons when co-cultured with vMB T1 astrocytes. This result suggested that astrocytes not only were a source of mitogens and survival factors, but a source of instructive factors that promote the acquisition of a neuronal DA phenotype. Interestingly, the effects of glia were region-specific and distinct morphological phenotypes were induced in stem cells by co-cultivation. Subsequently, several lines of evidence suggested that hippocampal glia also stimulates neurogenesis (Song et al. 2002). Headlines such as the "Amazing astrocyte" appeared (Svendsen 2002). Evidence, for the identity of the molecular player responsible for part of this "instructive activity" of glia to neurons, came from the observations that embryonic vMB derived glia is highly enriched and secretes Wnt5a protein. Moreover, Wnt-5a blocking antibodies were found to prevent the differentiation of vMB neurospheres into dopaminergic neurons, when the vMB neurospheres were co-cultured with a monolayer of P1 vMB astrocytes and treated with Fgf2, Fgf8 and Shh (Castelo-Branco et al. 2006). These results were consistent with our model (Wagner et al. 1999; Hall et al. 2003) in which poorly soluble factor/s emanating first from radial glia (during early development), and later from astrocytes (postnatally), promote the differentiation of stem cells or primary Nurr1+/TH- precursors into newborn Nurr1+/TH+ dopamine neurons.

Wnt1 is perhaps the best well studied Wnt with regard to its function in the midbrain. As previously mentioned, Wnt1 is required for the formation of the MHB (McMahon et

al. 1990; Thomas et al. 1991). However, it was not until 1996 when midbrain dopaminergic neurons were specifically examined (Danielian et al. 1996; Serbedzija et al. 1996). In the study by Danielian et al., En-1 was knocked into the Wnt1 locus resulting in the rescue of TH+ neurons in the Wnt1 null, but not that of midbrain motor neurons. Recently, a more detailed analysis of the Wnt1 null has revealed that in addition to the initial segmental deletion, the patterning of the floorplate region is altered and that the very few DA neurons born, fail to turn on Pitx3 and subsequently die (Prakash et al. 2006). Furthermore, overexpression of Wnt1 under the engrailed promoter was found to be sufficient to instruct a dopaminergic phenoptype in the ventral hindbrain. This finding was very important because it unraveled a novel function of Wnt 1 as an instructive factor. Moreover, when the capacity of Fgf8 to induce midbrain DA neurons in explants was tested in the Wnt1 mutants, no TH+ cells appeared, suggesting that Wnt1 is required for the effects of Fgf8 on DA progenitors. The function of Wnts in DA neuron development has also been examined in E10.5 TOPGAL reporter mice by β-galactosidase expression in the Nurr1+ domain. The obtained results confirmed that canonical Wnt signaling takes place before/at the birth of DA neurons in the vMB, as assessed by β-galactosidase expression in the Nurr1+ precursor cells. Furthermore, spatial-temporal expression patterns for Wnt1, -3a and -5a were observed to coincide with Nurr1+ and TH+ cells. Treatment of vMB precursor cultures with Wnt1, -3a or -5a partially purified conditioned media, highlighted their importance as players in DA development but also pointed out to the specificity of the effects. Wnt1 primarily increased the number of TH+ neurons through an expansion of the Nurr1+precursor pool. In contrast, Wnt3a (principally expressed in the dMB), enriched the Nurr1+ precursor pool but reduced their differentiation into DA neurons. Wnt5a predominantly promoted the differentiation of the Nurr1+ precursors into a DA neuronal phenotypes. Wnt5a treated cultures showed increased mRNA levels of the

midbrain specific gene Pitx3 and the GDNF co-receptor, c-ret (both required for DA neuron survival). Additionally, Fz8-CRD administration was able to partially inhibit the effects of Wnt5a on DA differentiation (Paper II). The findings above, based on treatment with conditioned media were further confirmed with purified Wnt5a (Schulte et al. 2005). In addition, recent data from Wnt5a overexpressing vMB neurospheres showed that these cells had the transcriptional profile, biochemistry and intrinsic electrophysiological properties of midbrain DA cells. Furthermore, upon transplantation into Parkinsonian mice, these cells could significantly enhance cellular and functional recovery (Parish et al., under revision). Taken together, these results illustrate the importance of Wnts as regulators of DA precursor proliferation and differentiation.

5.2.2 Wnt5a a ”non canonical” Wnt?

A Wnt ligand can be classified as canonical based on their capcity to: 1) Morphologically transform cultured mammary cells; 2) Induce secondary axis duplication in *Xenopus;* and 3) Cause nuclear translocation of β-catenin. However, the finding that Wnt5a, a Wnt originally classified as non-canonical, was able to induce a secondary axis in *Xenopus* (He, X. et al. 1997) challenged both the criterias for classifying a factor as canonical or not, and more specifically the separation between non-/canonical Wnts. Moreover, a recent study by the group of Roel Nusse (Mikels et al. 2006) has provided evidence thatWnt-5a a could regulate canonical Wnt signaling. However, these results were obtained after overexpression of receptors, which may have affected the balance between canonical and non-canonical signaling. To add on to this complex situation, recent studies have shown that stabilized β-catenin can signal in

the cytoplasm , together with cadherins (Yu, X. et al. 2003), implying that nuclear translocation is not an absolute necessity for the Wnt/β-catenin signaling.

In the developing midbrain there is no *in vivo* evidence to indicate that Wnt5a neither stabilizes nor not stabilizes β-catenin. However, *in vitro* data obtained with a midbrain dopaminergic cell line (SN4741), has suggested that Wnt-5a works in a non-canonical fashion. Nevertheless, the recent identification of a cadherin as a transcriptional target of the non-canonical pathway (Schambony et al. 2007), has provided both a link between nuclear and cytosolic Wnt signaling, and a mechanism by which canonical and non canonical pathways can interact. These findings therefore underline the importance of cytosolic signaling by β-catenin, nuclear signaling by non-canonical Wnts and at the same time open up the possibility of devising novel assays to examine non-canonical signaling activity *in vivo*.

5.2.3 New venues

The recent identification of Wntless might give an opportunity to manipulate so that specific Wnts of interest can be abolished. Already Wntless is an interesting tool to identify how other signaling components of the Wnt pathway is modified upon a blocking of Wnt secretion. Importantly, the ability of Wnts to exert, although to less extent, a long range function, can also be applicable for alternative pathways. The recent finding that Wnt5 signals through Ror2 (Yamaguchi, T. P. et al. 1999; Yoda et al. 2003) and the ability of Ror1 and Ror2 (Yamaguchi, T. P. et al. 1999; Yoda et al. 2003) compound mutant to phenocopy Wnt5a, open an intresting venue for addtional knowledge on how Wnt5 operates *in vivo* and *in vitro* in the midbrain. Thus, in addition to the present *in vivo* studies performed in our laboratory on Wnt1 and -5a double knock out mice, the analysis of Ror1 and Ror2 compound mutant, would be interesting.

5.2.4 Dynamic Temporal and Cell-type specific Expression of Wnt signaling components in the Developing Midbrain.

The signaling diversity exerted/achieved by the 19 Wnt ligands, further extended by the plethora of potential Fz receptors , might not be surprising taking in account the magnitude of functions in which Wnt signaling is involved in during the development of a multicellular organism. The emerging knowledge of the Wnt ligand´s structural properties might ease up the process of purifying additional members of the family and thereby extend our knowledge of the exact mechanism underlying the formation of the Wnt ligand-Fz receptor complex and the following signal transducing mechanism. However, identifying new players may seem to add to the existing complexity but it can also be viewed as linking different downstream components and thereby being able to categorize the diversity, unwinding to the level of a graspable comprehensive functional network. The identification of β-arrestin (Bryja et al. 2007a) represents such an example possibly linking Wnt signaling to GPCR dependent pathways.

In order to further our knowledge about Wnt signaling during DA development the temporal expression profiles of the remaining Wnt ligands, all existing Fz receptors and various downstream components were made by a comparison between dMB and vMB. Surprisingly, most *Wnts* and all *Fzs* were represented in addition to many intracellular components implicating Wnt signaling in a wide variety of actions during vMB development. Neuronal and glial precursor-enriched cultures and newborn DA neurons isolated from TH-GFP mice were used in order to identify the expression profile/s of Fzs during DA development. An interesting finding was the expression of *Fz6* and *Fz7* in DA neurons and also in the enriched glial fraction, supporting the idea of a cross talk

between glia and neurons, mediated by Wnts (Wagner et al. 1999; Hall et al. 2003; Castelo-Branco et al. 2006). *Fz9* had the most outstanding and striking expression pattern, being expressed in neuronal progenitors and absent in newborn DA neurons. Fz9 has previously shown to be expressed in the developing telencephalon and in the adult hippocampus (Kim, A. S. et al. 2001b; Zhao, C. et al. 2004a) and it has been reported to be expressed in neuronal progenitors and to play a role in the maintenance of undifferentiated phenotype (Van Raay et al. 2001; Ranheim et al. 2005). Interestingly, spatial analysis of Fz9 at mE11.5 assessed by *in situ* hybridization (ISH) displayed an exclusively restricted expression to the proliferating VZ in the vMB, partly coinciding with Sox2+, PH3+ and Ki67+ cells (data not shown). Furthermore Fz9 was found to reduce Wnt5a but not Wnt3a mediated hyperphosphorylation in a DA cell line originally lacking the endogenous receptor. Thus, Fz9 expression might inhibit the maturation capacity of Wnt5a at early stages of the DA lineage as its expression decreases and allows Wnt5a to exert its effect later on. Analysis of the Fz9 knockout, would possibly give an insight in how Wnt influence the intrinsic signal mechanism before the DA neuron is born.

5.3 Paper IV

5.3.1 sFRP2 is required for the development of ventral midbrain dopaminergic neurons.

SFRPs have a dynamic expression pattern and display a wide range of Wnt–modulating activities during development and in the adult (Kim, A. S. et al. 2001b; Jones et al. 2002; Kemp et al. 2005; Lindsley et al. 2006). The diversity of actions displayed by sFRPs are probably due to their unique structural properties comprising Fz like and

Netrin like domains. Early studies in *Xenopus*, co-expressing Wnts and SFRPs led to inhibition of Wnt-mediated dorsal axis duplication, (Finch et al. 1997; Leyns et al. 1997; Wang, S. et al. 1997b; Xu, Q. et al. 1998) and further biochemical evidence concluded that sFRPs work as inhibitors of the Wnt pathway by forming a complex with Wnt or/and Fz and thereby sterically hindering ligand-receptor interaction (Bafico et al. 1999; Dann et al. 2001). However, recent evidence indicates that sFRP function is highly context and concentration dependent (Melkonyan et al. 1997; Üren et al. 2000; Yoshino et al. 2001; Galli et al. 2006; Wawrzak et al. 2007).

5.3.2 sFRPs: Novel modulators of DA neuron development?

Spatio-temporal mapping of sFRPs revealed a dynamic, partialy overlapping expression pattern of sFRP1,2 and Wnt5a in the vMB. However, complementary patterns of sFRP1 and Wnt1 (Fig 5.) were also identified (Paper II);(Prakash et al. 2006). Their early expression pattern indicates an additional putative function of sFRPs in DA neurogenesis in establishing, maintaining and refining boundaries in the midbrain. sFRP3, conversely to sFRP1-2, is downregulated at the time of birth of DA neurons and can only be detected in the VZ area around the sulcus, the border between ventral and dorsal midbrain. This finding suggests an earlier function for sFRP3, possibly in the ventro-dorsal boundary. In sum, the expression patterns of sFRPs in the ventral midbrain exhibit similarities with other areas of the CNS. These finding suggested that sFRPs in the ventral midbrain may also serve similar functions as sFRPs in other structures, where they form opposing gradients (Chang et al. 1999; Kim, A. S. et al. 2001b) or regulate Wnts (Ellies et al. 2000; Kim, A. S. et al. 2001b).

Fig 5. Schematic picture representing the spatial distribution of sFRP1-3 compared with Wnt1 and Wnt5 expression domains in the vMB at E11.5.

5.3.3 sFRP1 and sFRP2 exert opposing effects in the developing ventral midbrain

In order to assess the function of sFRP in the developing midbrain we first assessed their mode of action. Interestingly, sFRP1, and to less extent sFRP2, acted as antagonists of Wnt signaling (inhibiting Wnt3a-mediated β-catenin activation as well as both Wnt3a and Wnt5a-mediated Dvl2 phosphorylation) in a DA cell line. However, in vMB primary precursor cultures the biological effects of sFRP1 and 2 were opposing. sFRP1 decreased whilst sFRP2 increased the number of TH+/Tuj+ neurons, BrdU+cells and pyknotic nuclei, even in the presence of the DA neuron enriching property of Wnt5a. In accordance with a recent report (Wawrzak et al. 2007) neither activation nor inhibition of β-catenin or Dvl by sFRP2 were detected, indicating that the interaction only occurs when sufficient ligand is available. The similarity of the biological activity of sFRP2 and Wnt1 or Wnt-5a indicated that the observed effects could not be explained by Wnt antagonism, and suggested that alternative signaling mechanisms must be involved. Hence, sFRP2 may serve two functions; block high

levels of Wnt5a signaling and display a biological activity on its own, independent of Dvl and β-catenin. Interestingly, the effects of sFRP2 on proliferation of DA neuronal progenitors and differentiation of DA precursors into neurons were to some extent similar to the effects of Wnt5a alone. These effects could be interpreted as a function of sFRP2, as a positive modulator of Wnt signaling, or, via the involvement of alternative pathways (Ellies et al. 2000; Lee, C. S. et al. 2000a; Brophy et al. 2003; Lee, H. X. et al. 2006). However, since Wnt signaling was not enhanced by sFRP2 alone, our results suggest that an alternative signaling pathway must be activated. On the other hand, the pronounced negative effect of sFRP1 on DA neurons in our system is in agreement with observations in other systems, where sFRP1 has been reported to decrease, while sFRP2 increases cell resistance to apoptotic stimuli (Melkonyan et al. 1997) . In addition, upon co-administration of sFRP1 and sFRP2 on primary vMB precursor cultures, the effect of sFRP2 is cancelled by sFRP1. This result is in agreement with a report on kidney cultures performed at the same developmental stage where sFRP1 blocked the effect of sFRP2 (Yoshino et al. 2001).

5.3.4 sFRP2 does not operate as an antagonist during DA development but rather a modulator of DA development in three sequential steps

Analysis of sFRP2 null mice did not reveal any increase in cell death in the vMB. The phenotype of sFRP2$^{-/-}$ mice was compatible with a stepwise impairment of neurogenesis, which reduced the number of postmitotic DA precursors (Nurr1+) cells generated by the proliferative progenitor (Sox2+) cells and was followed by a subsequent impairment of the differentiation of DA precursors into DA neurons. This part of the sFRP2$^{-/-}$ phenotype and the partial recovery at the end of the neurogenic

period of DA neurogenesis (E14.5) resembles the Ngn2$^{-/-}$ mice, which is partially rescued at E14.5 by Mash1 (Paper I). Given the similarities in the phenotypes, and that other sFRPs and Wnts are expressed at lower levels at E14.5, we speculate that Mash1 is a candidate factor to partially rescue DA neurogenesis in the sFRP2$^{-/-}$ mice. However, since the phenotype of the sFRP2$^{-/-}$ mice differed in other aspects, it is also possible that an alternative mechanism is responsible for the partial recovery. Three main differences distinguish the phenotype of the sFRP2$^{-/-}$ mice and the Ngn2 $^{-/-}$ mice (Andersson, E. et al. 2006a) (Paper I). 1) No accumulation of cells were detected in the neuroepithelium. 2) A decrease in the number of motor neurons was detected. 3) sFRP2 promoted neuritogenesis in DA neurons. This last result is consistent with the role of sFRP1 in axonogenesis (Rodriguez et al. 2005). However, in our study sFRP2, but not sFRP1, promoted neurite outgrowth, suggesting that distinct sFRPs control axonogenesis/neuritogenesis in different neuronal populations. Thus, our findings indicate that sFRP2 does not act as a Wnt antagonist in DA neuron development but rather promotes three sequential steps: DA neurogenesis, differentiation of DA precursors, and DA neuritogenesis. Thus, we hereby report a unique activity profile of sFRP2 as novel regulator of multiple aspects of DA neuron development.

5.3.5 Alternative pathways and guidance properties of sFRP2

Several members of the sFRP family were recently found to signal and activate target cells by either Wnt-independent mechanisms (Collavin et al. 2003; Esteve et al. 2003; Lee, H. Y. et al. 2004) or by activating Fz receptors via G_0 proteins (Rodriguez et al. 2005). Thus, the current emerging concept on the function of sFRPs is that, depending on the cell context, sFRPs may work either as Wnt antagonists or as activators of poorly known pathways and functions. Interestingly, in line with evidence from two

laboratories (Kim, A. S. et al. 2001a; Brophy et al. 2003) sFRP2 has been suggested to be a target of Pax2 and-6, respectively. Preliminary data from our laboratory indicate that overexpression of Pax2 or 5 in vMB primary precursor cultures upregulates the expression of both Ngn2 and sFRP2 at E10.5 (Dr. Anita Hall, personal communication). These results highlight another example of interplay between intrinsic and extrinsic signals. The co-regulation of Ngn2 and sFRP2 suggests that these two factors may not only work in concert to regulate DA neurogenesis, but they may perhaps compensate for one each other. However, against this hypothesis is the fact that the same dopaminergic subpopulation (in the midline) is depleted in both knockouts at the initiation of DA neurogenesis. Also, the spontaneous rescue of DA neurogenesis in the sFRP2$^{-/-}$ mice is more pronounced than in the Ngn2 null at E14.5 implying that they may work on different DA population in the lateral domain or work by partiually different mechanisms. This would imply that there are different types of DA neurons intermingled in medial or lateral positions and that these cells are instructed by different mechanisms and are therefore molecularly distinct.

sFRPs have also been involved in axon guidance (Rodriguez et al. 2005) and recent evidence suggests that Ngn2 regulates migration in the cortex by a distinct molecular mechanism rather than mediating its proneural activiy (Hand et al. 2005; Ge et al. 2006). These functions may constitute yet another level of interplay between intrinsic and extrinsic signals. In order for Ngn2 to regulate migration of cortical cells, a phosphorylation event has to take place. Interestingly, phosphorylation of a specific tyrosine residue in the C-terminal region of Ngn2 was found to be required for the activity of the small GTPase, RhoA (Hand et al. 2005). This implies Ngn2 to interact with alternative Wnt signaling pathways that could be modulated via sFRP2, perhaps via Fz receptors and G_0 proteins, as described in Rodriguez et al. (Rodriguez et al. 2005).

Finally, the axon guidance molecule Netrin 1 and its receptor DCC (deleted in colorectal cancer) are highly expressed in DA neurons during developing and in the adult brain (Livesey et al. 1997; Volenec et al. 1998) where they play a role in mDA neuron migration and axon guidance (Fazeli et al. 1997; Flores et al. 2005; Lin, L. et al. 2005) . An interesting hypothesis to be examined is whether sFRP2, known to express a Netrin like domain, interacts with DCC and has a role in organizing the DA circuitry during development and also influences the DA function in the adult.

6 CONCLUSIONS

The interplay between extrinsic versus intrinsic signals is a prerequisite for development to occur. Secreted factors such as Wnts and sFRPs, their transmembrane receptors together with transcription factors of the HMG-box and bHLH family mediate signals controlling cell-fate and differentiation. In this thesis we have characterized the role of some of these components in dopaminergic neuron development. Based on the work hereby presented we conclude:

I) Proneural bHLH and Sox2 proteins are expressed in the developing ventral midbrain.

* Sox2 and Mash1 label ventricular zone progenitors in the developing midbrain.

* Ngn2 is expressed in progenitors of the VZ and precursors in the intermediate zone of the ventral midbrain.

* Ngn2 is a partially required for dopaminergic specification and neurogenesis, but not for cell cycle exit in the ventral midbrain, whereas

* Mash1 has a permissive role during ventral midbrain development.

II) Wnts were identified as important regulators of dopaminergic neurogenesis *in vitro*.

* Wnt1 and Wnt-3a induced the proliferation of dopaminergic precursors whereas

* Wnt5a promoted the differentiation of Nurr1 positive precursors into dopaminergic neurons to a greater extent than Wnt1.

* Wnt-3a did not promote dopaminergic differentiation.

III) At the initiation of DA neurogenesis 13 Wnts and all Frizzleds are expressed in the developing ventral midbrain.

* Fz9 was particularly interesting because it was exclusively expressed in midbrain progenitor cells and inhibited Wnt5a signaling *in vitro*.

IV) sFRP1-3 were identified to be expressed in a dynamic spatial temporal pattern in the developing midbrain and several novel functions were identified.

* SFRP1 block proliferation, differentiation and the effects of Wnt5a in primary ventral midbrain neurons *in vitro*.

* sFRP2 was shown to work as a Wnt3a or Wnt5a antagonist *in vitro* but no evidence was found in the absence of added ligand or *in vivo*.

* sFRP2, by regulating multiple aspect of DA neuron development, such as DA neurogenesis, differentiation of DA precursors into DA neurons, and neuritogenesis in DA neurons, exhibited a unique activity profile.

* sFRP3 has a generic pro-neuornal effect directing primary precursor cells in culture towards a non DA neuronal fate.

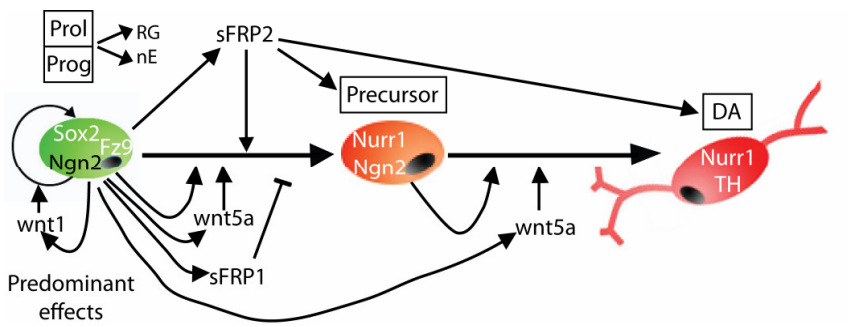

Fig 6. Summary of Paper I, II, III, IV

7 FUTURE PROSPECTIVES

A view well accepted in development is that "form underlies function." Patterning and projection mechanisms organize cells, that are initially undifferentiated, into a highly coordinated neuronal circuits. However, there are several developmental contexts where "function underlies form" (Matsuzaki et al. 2007). This apparent contradiction highlights the coherence between function and form depending on the developmental context.

The results presented in this thesis identify several novel players in midbrain DA neuron development and reveal new functions of proneural bHLH, Wnts and sFRPs, thereby extending our knowledge and identifying factors that may be used to develop novel DA cell replacement therapies for the treatment of PD.

8 ACKNOWLEDGEMENTS

Professor Ernest Arenas Thank you for trust and patience for my "developmental angle of the world", giving me the opportunity to develop my passion for developmental biology, and for giving a platform of freedom, for support, friendship, understanding and for always keeping your door open regardless hours. Your enthusiastic, super optimistic, open minded and dynamic style of leadership has made this journey a unique, challenging and an exiting one and provided a rich atmosphere to grow in.

A big thank you to all the supervisors and collaborators around the world;
Dr Akihiko Shimono lab for the efficient delivery of sFRP KO mice, **Dr Jeff Rubin** for providing me with sFRPs, **Professor Francois Guillemot lab**, especially **Nicolas Simplicio**, **Dr S L Ang lab**, **Professor Anders Björklund lab,** especially Elin Andersson, Josephine Jensen, Lachlan Thompson, Malin Parmar
Professor Paola Bovolenta for acting as opponent **Dr.Jonas Muhr** for being in my committee twice, **Professor Jonas Frisén**, **Dr. Klas Kullander, Dr. Elias Arner, Dr. Ola Hermansson,** for participating in my halftime committee and thesis defense, respectively. **Professor Carlos Ibanez** for all the scientific input during seminaries.
Also a **big thank you to the NNN network** and specially to Dr Gilberto Fisone and Professor Lennart Brodin

---

Professor Stephan Hjort and **Professor Dan Rhöme** being "primers" into science school
Forskarskolan Umeå for that initial year of inspiration that made me stay in science, especially **Professor Maria Fällman** and **Professor Stefan Björklund.**
Professor Gunilla Olivecrona for allowing me taking thoose first steps in protein chemistry
Dr.Staffan Bohm for lightening the initial sparkle for development and axonguidance
Dr. Monica Holmberg for giving good advice and sharing that energy for science

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An excuse on beforehand for all You I have forgotten – do not take this personal! Also for all spelling errors, strange words etc. you might find in your acknowledgement.

People that has keept and keeps the lab "rolling"

Lotta S, Annika A: Thanks for all the early mornings I have been greeted with steaming hot coffee and giggles! **Claudia** Congratulations to the little one first of all and thank you for your warm heart and helpfulness during all these years. **Annika K:** For your professionalism, caring and for just being you! **Johnny** for always fixing whatever the needs to be fixed! **Lena** for all the odd stories! **Eva L. and Kerstin B.** For your kindness and professionalism at MBB

EA group – the "insane in the MB crew" THX!!!

Past members;
Claire : If not for you , none one of you else would be reading this! Thanks for sharing all those ups and downs, even now that you are down under – thanks for being such a wonderful friend! **Davor:** Thank you for your friendship and for being the most sane scientist I know of
Kyle for keeping my spirit up when really needed, for your enormous constructive energy with laughter's and good/bad jokes – TH! Hope there is a front desk at your new lab/home J
Anita, for sharing generously your knowledge, for all your encouragement, and your fantastic personality. Thank you for enriching this journey! C U in London! **Bob** Keeping the grove going, make sure to pass that on to your lovely firstborn.
Helena for being the "priming" both scientifically and individually and for believing in me and my crazy way of thinking **Marcial** for your jokes and happy smile

Goncalo for riding those initial rough scientific waves with me, for sharing crazy times, for inspiration laughter's and good moments of friendship. **Nina** for sharing many intense and fruitful discussions inside and outside the lab, about science, semantics and life along the years **Solange** for the fantastic Brazilian mentality of yours, for always keeping reality in close vicinity and for being caring. C U soon! **Xavier** for the fun times and for sharing many late night lab hours, spanglish dinners **Pontus,** for your easygoing style always with a positive attitude. **Peter Åkerud,** for those initial words of experience and encouragement **Adelheide** for those initial steps in showing me histology and "HOT"ISH **Lisbeth**, for sharing my Gothenburgian roots and dark humor. **Gunnar (Helen &Lukas)**: for the initial link between me and the source of sFRP in the States. Thank you and good luck also to **Joe Wagner, Beth Anne and PEP**

Current members
Diogo: For your friendship, jokes and "easygoing" pace of life, hang in there, there is light at the end of the tunnel and you are carrying a lot of brightness yourself! Ps, there is always the possibility to buy a bottle of expensive wine to brighten up the dark times Ds. **Emma:** Thanks for balancing out my bzzzz-ing, your friendship, warm and relaxed attitude and for sharing the passion for movies, it has been a joy working with you. **Paola**: for your sharp wisdom and being caring, with a huge heart! **Linda**: your laughter "the special Linda way of excitement; stepping up and down", and just being you. **CS** For your laughter and making the lab to our cozy and enchanting living room and for all our discussions about life. It has been enriching You are fantastic! **Clara and Julia:** For being Felicias big sisters in the lab
Sonia: for wonderful spanglish and caring gestures! **Isabel**, for always being supportive and encouraging and for all the late night marathons. **Vita**, for the snowbirding experience, and being "the priming kinase" in the "EA Wnt field" **Lenka** for being such a pure soul, always a little angle in the lab **Lottie** For your fantastic sence of organization, professionalism and being in touch with real life. **Lucas(karate) and Susan:** for all the real(!) beer sessions. **Alejandro**: for always being so polite with a sense of humor; **Spiros:** For the brand new smile in our office!

Members in transit(ion)
Carmen R: Thanks for all the energy from your ultra energetic science style! **Gabriella** for your wonderful pleasant personality and putting things in perspective **Patricia, Maria, Elise, (Terry), Immaculada, Luisa, Rachel,** For providing a additional spice to the lab.

P E Group
Professor Patrik Ernfors: dry sense of humor, inspiring Scientific intensity, all the good time during partys and retreats.
Andreas All the support and encouragement, for creating a pleasant atmosphere **Anna** For sharing the passion for dance and all kinds of moments this past years, spiced with joy and tears. **Jorge** for your helpfulness and thoughtfulness , for all those conversations over the microscope and always having a positive spirit, seeing the best in everyone! **Kalle** For letting me gain an insight in the Kalleistic world, it has been a pleasure **Jens:** For your laid back attitude and knowing how to enyoy life. **Marina** for your good sence of humor and being an "entertainer", keep the attitude and you will make it
Fred for your friendship over the years, sharing moments of laughter, tears, cultural events of all kinds, you're the best! **Mikael:** For being the scientific space cowboy you are, do not change yourself, and also thanks for all the encouraging words during the final leg of this journy. **Christel:** thanks for the friendship and showing me that pigs really can fly! **Gui** For having a fantastic attitude in science and being such a nice person, it has been very plesent sharing office with you during these years. **Esther:** for your intese style of science and morbid sence of humor **Igor** for sharing my passion for developmental biology and helping out **Li** for your friendly smile and always saying hello! **Åsa** for your fantastic laughter and intensity **Karin A** for all the past years with the lots of fun at the NNN meetings, welcome as a member of the "Mothers in Science club" **Miwako** for your scientific perfection and for all those fantastic Japaneese desserts and for knowing how to have fun at a party! **Philippe Nauvellian, Anna Hjelmqvist,** for the early days **Francois** for being such a nice guy, pass that on to the one in the ownen. **Dimitry** For transmitting the best of the Russian culture in the lab. **Richard W**, **Adrian** for all the fun times back in those early days **Susanne** gratis o lycka till med allt, heja GbG

TH group
Paul for given a hand when ever needed with confocality and your fantastic attitude in life and science I will miss U!, **Marton** good luck with your future endevours and keep those good intentions alive **Jan** for being such a nice and helpful guy **Tibor** for your good heart, being helpful and sharing your knowledge unegostistiskt in confocality, statistics or whatever matter you are bothered with **Orsi** for being a friend, all the chats, for your thoughtfulness Ps Although you will be in the land of kilt pipes keep in touch!

PU group
Dr Per Uhlén For making that little odd encouraging Ca^{2+}click in the lab, thanks for letting me run those crazy experiments of mine **Nicolas** for riding the calcium waves with me, for fun times and for your friendship **Seth** for fun times and some intense discussions **Marie** for giving sacrificing your calcium wave for my experiment **Hiromi** for all those giggles **Manuel**

CIB Group
Olle for being Olle ☺ **Susanna, Rizaldy, Henrik J, Andries** for being there as pioneer Phd students **Maurice** for your teasing sense of humour **Alison** – the cool "tassie" with fantastic cookies for all the fun **Muriel** for inspirational Tango **Valerie** for those old days **Maribel** for all your craziness **Chiara** for that big happy smile

OH group
Ola for your broad knowledge inside and outside science, and for being helpful **Karolina, Anna, Shirin, Therese, Ana, Erik, Joshua,** for making that friendly click on the other side of the entrence

Sten Linnarson group
Dr Sten you were on you exit when I started and now when I leave I can bid you welcome back! Nice to C U! **Ats, Peter, Arno and Ellef** and others

Other people making years in science nice
Gustavo, Fernanda and Augustine: For your friendship and encouragement during the years. **Matilda** for fun and nice times during NNN and outside the lab **Ze** for all the groovy times during the years **Jan Stenman** for inspiriting me in those early days of forskarskolan to choose neuroscience and development as speciality – you were right it is ossom!!! **Ulrika Tryggvason** for sharing the midbrain (club) and for the nice times; Boston, parties etc **Elisabeth Andersson** for being helpful **Qiaolin** for your sharing scientific and for pleasant times in Boston **Mattias Ahlénius** for being an early tutor and for sharing my intense passion for science and for the fun times in Thailand. **Dr.Anna Berghard** for your good advices during early days **Jenny** for those practical steps in molecular biology **Helder** for being such a sweet guy and always with a smile lining around.

Friends
"Little Persia" – the Family I can not thank enough;
Hessameh for the unforgettable, unreplaceble "sufficient and enough "bHLH" factor activity "during transition and when transition was not in place "Hes1,2,3,4 and 5" activity or sometimes rarely "Hes 6&7" activity – these are indeed not redundant! For believing in me with your dry sense of humour when moments of doubts were steping in!
Akhtar for your fantastic inspiring hospitality with good food and art – "Leve konsten"
Avid for laughters and "våldgästning är välkommet"
"StorMor*(-mor)"*and ***Emmy***: for getting my blood circulation in place with a little belly dancing and good food
Hessam with you laid back attitude and "love for discomfort;)- thx!"
My favourite cat *Pishi* for your integrity and well chosen moments of affection and dogs
Viktor, *Bamse*: keeping up with the happiness in life!

Anna Carlsson "Pyssling" Norberg for being deeply in touch with reality and being such a true friend although the distance. **Vickan** all the past and future fun times up in Ume **Lena Scott** for popping up most unexpexted on my halftime and being such a support. For the year in Ume.

Marie-Louise "my sister in arms" **Alexandra och Arttu** for being You and being close! **Anna Vallstedt** for your fantastic friendship and support we think alike! **Gabi** my English sister thx for sharing the past and the future **David Hersvik** - miss U – but now me "being on permis" for a little time I hope to see more of U **Peter** "Gyuszi" for the imprint "Jules" for keeping up from childhood until now with your laid back attitude – love´U **Irina** "educational extra mamma" – you were there at the initiation point, **Kim** for all your jokes and happy laughters. **Malin, Cattis o Anna F,**
And the **Essenia crew**

Family
My Father for your visdom in life and for being my initial sparkle in science. Wish we could have shared the "flames".
My Mother Thank you for giving me the start you did and the constant support you give! To have you as my mother has been a most colorful and inspiring experience for life! Love you!
My daughter Felicia: For giving enormous amounts of inspiration and energy to my life and for all the wise and creative statements that you garnish my everyday life with. **Love you to!**
Niclas for your endless courage, intelligence and love, never loosing your sense for a joke and for being an outstanding father and husband – life would not have been the same without You!
Elly for knitting me a piece of art for many important occasions of my life and providing me with a lot of love and support
Rolf for your excellent advice on how to relax and for the warm familiar feeling you radiate
Sofia for being such a wonderful Aunt for Felicia and for your big heart! & **Göran:** just thinking of you put a smile on my face, you really know how to entertain!
Auntie Maria and Uncle Kalman: for contributing to the person I am today and for all the support and love from diapers to doctor (and beyond).
Finally a lot of love to all you in Hungary and other parts of the world: **Gabi** és **Peter**, **Judit** és **Lora**, **Marili** és **Miklos**, **Sándor**, my godfather **Pierre** in Canada

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