From MEDICAL BIOCHEMISTRY AND BIOPHYSICS

Karolinska Institutet, Stockholm, Sweden

IDENTIFICATION OF NOVEL WNT/PCP SIGNALING REGULATORS AND THEIR ROLE IN MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT AND PARKINSON'S DISEASE

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Stockholm 2018

Cover: Communication between a dopaminergic neuron and a glial cell by Alena Salašová

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Published by Karolinska Institutet.
Printed by Eprint AB 2018
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ISBN 978-91-7676-999-7

IDENTIFICATION OF NOVEL WNT/PCP SIGNALING REGULATORS AND THEIR ROLE IN MIDBRAIN DOPAMINERGIC NEURON DEVELOPMENT AND PARKINSON'S DISEASE

Thesis for doctoral degree (Ph.D.)

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Public defense: Wednesday 28th March 2018, 9:30 AM
Samuelsson Lecture Hall, Tomtebodavägen 6, KI Solna

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"The harder you fall, the heavier your heart; the heavier your heart, the stronger you climb; the stronger you climb, the higher your horizon."

— modified from Criss Jami

I would like to dedicate this work to my loving parents, Alena Salašová and Petr Salaš, who have never doubted my dreams, and always supported me with a large dose of optimism and courage when I was going through hard challenges.

Tuhle práci bych ráda věnovala svým milujícím rodičům, Aleně a Petru Salašovi, kteří mě vždy podporovali v cestě za svými sny, a kteří nikdy nešetřili porcí optimismu a kuráže ve chvílích nejtěžších.

ABSTRACT

Wnt signaling controls a wide spectrum of complex cell responses during prenatal development, in the adulthood and during disease. In this doctoral study, we have identified and explored novel regulatory components of Wnt/Planar Cell Polarity (PCP) pathway and their function in various cellular processes during embryogenesis and central nervous system (CNS) development. We paid special attention to molecular mechanisms underlying the morphogenesis of the ventral midbrain (VM) and development of midbrain dopaminergic (mDA) neurons, a brain area that is strictly regulated by Wnt signaling. We also touched upon possible clinical applications of our findings in neurodegenerative disorders, such as Parkinson's disease (PD).

We used a large number of traditional biochemical tools as well as more advanced methodologies such as proteomics and phospho-proteomics, RNA-scope *in situ* hybridization, confocal microscopy, electron microscopy and CRISPR/Cas9 technology (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9). We have also used different models such as cell lines and primary cultures, as well as genetically modified organisms, including *Xenopus laevis* (Frog), *Danio rerio* (zebrafish) and mouse embryos. To better understand the functional complexity of the Wnt/PCP signaling, we examined a number of transgenic mice models, which allowed us to uncover the function of Wnt/PCP protein complexes in the mammalian CNS. Finally, some of our observations were confirmed by using human prenatal brain tissue (study II). Please find below the main highlights of each study included in this thesis:

In study I, we explored the molecular mechanism by which the crucial Wnt signaling integrator Dvl and the cell cycle protein kinase NEK2 regulate the progression of cells from the G2 to the M phase. We identified Dvl as a NEK2 substrate and described that they mediate disassembling of centrosomal linker proteins from the centrosome, a process essential for duplicating the centrioles and polarization of the mitotic spindle during mitosis. Such findings are of tremendous importance in cancer research and in the context of ciliopathies which show defects in the centrosomal structures.

In study II, we investigated the expression of mammalian Wnts in developing choroid plexi. We discovered that biologically active Wnt5a is secreted to the cerebral spinal fluid (CSF) by the epithelial cells of the hindbrain, but not the telencephalic choroid plexus, in both mouse and in human embryos. We further describe that secreted Wnt5a forms a complex with high-density lipoprotein particles containing ApoE and ApoJ, but is not found in exosomes. Analysis of the Wnt5a deficient mice revealed a possible function of Wnt5a in the choroid plexus to inhibit progenitor proliferation in the neighbor ventricular zone. Our results suggest that Wnt5a gradients in the developing mammalian brain might be formed by diffusion of Wnt5a-lipoprotein complexes through the CSF.

In study III, we tackled a molecular mechanism behind the Wnt5a signal transduction in the ventral midbrain. Analysis of *Wnt5a*$^{-/-}$, *Wnt5a overexpressing*, *Wnt5a*$^{-/-}$*;Ror2*$^{-/-}$ and *Ror2*$^{-/-}$*;Vangl2*$^{-/-}$ mice identified a function of the Wnt5a-Ror2/Vangl2 signaling axis in the VM morphogenesis and in mDA neuron development. Our study shows that correct Wnt5a expression levels are crucial for VM morphogenesis, mDA neurogenesis and mDA neuron maturation. Moreover, we found a novel phenotype of bilateral asymmetry in *Ror2*$^{-/-}$*;Vangl2*$^{-/-}$ animals which suggests that Vangl2 alone or in a complex with Ror2 controls the correct position, proliferation and differentiation of mDA progenitors into mDA neuroblasts and neurons. Our results additionally identify a novel role of Wnt/PCP signaling in controlling mDA neurogenesis, which may be of interest for the development of novel regenerative approaches to treat neurodegenerative diseases which affect mDA neurons, such as Parkinson's disease.

In study IV, we performed a proteomic analysis of the core Wnt/PCP receptor Ror2, and discovered several novel binding partners which were verified in mDA cells and in the developing ventral midbrain. We selected SorCS2, a proneurotrophin receptor from the VPS10-domain containing sortilin receptor family, as a top candidate because of its specific expression in the mouse midbrain floorplate and its functional involvement in mDA neuron wiring. By using *X. laevis* and *D. rerio*, we found that the Ror2-SorCS2 receptor complex is required during embryogenesis to regulate convergent extension, somitogenesis and brain development. We also suggest that SorCS2 has the capacity to internalize Ror2 and its other co-receptors in a Wnt/PCP-dependent manner *in vitro* and *in vivo*, via an unknown pathway. These data reveal that the two pathways previously considered to be independent, Wnt/PCP and proneurotrophin receptor signaling, functionally interact. Moreover, our results identify SorCS2 as a novel regulator of Wnt/PCP signaling in vertebral embryogenesis.

In study V, we investigated whether Leucine-rich repeat kinase 2 (Lrrk2), the protein product of the *park8* gene, which is mutated in more than 40% of patients with inherited PD, can interact with the Wnt/PCP pathway by using a proteomic screening. We describe that Lrrk2 interacts with a number of Wnt/PCP components in dopaminergic cells, in the VM of E18.5 mice embryos, and in a human cell line. Particularly, we show the capacity of Lrrk2 to inhibit Wnt/β-catenin signaling *in vitro* and *in vivo* in *X. laevis* embryos. We observed that these regulatory changes depend on the presence of Prickle1 and Dvl. Our results thus provide novel insights into the molecular mechanisms by which Lrrk2 and Wnt signaling interact, and describe Lrrk2 and Prickle1 as novel dual regulators of Wnt/PCP and Wnt/β-catenin signaling. Moreover, we suggest that the pathogenesis of PD may involve an alteration in the balance between these two Wnt signaling pathways.

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I. Cervenka I, Valnohova J, Bernatik O, Harnos J, Radsetoulal M, Sedova K, Hanakova K, Potesil D, Sedlackova M, **Salasova A**, Steinhart Z, Angers S, Schulte G, Hampl A, Zdrahal Z, Bryja V: Dishevelled is a NEK2 kinase substrate controlling dynamics of centrosomal linker proteins. Proc Natl Acad Sci U S A. 2016 Aug 16;113(33):9304-9

II. Kaiser K, Gyllborg D, **Salašová A**, Molina FL, Laguna-Goya R, Potěšil D, Barker RA, Gato Casado Á, Bryja V, Arenas E, Villaescusa JC: WNT5A is transported via lipoprotein particles in the cerebrospinal fluid and regulates progenitor proliferation (MANUSCRIPT)

III. Gyllborg D, **Salašová A**, Toledo EM, Gao B, Yang Y, Villaescusa JC, van Amerongen R, Arenas E: Ror2 and Vangl2 control dopaminergic neurogenesis and multiple aspects of cell polarity in the midbrain floor plate (MANUSCRIPT)

IV. **Salašová A**, Yokota C, Kasper Kjaer-Sorensen,Vestergaard B, Navis A, Thomasen P, Bernatik O, Varas M, Ernfors P, Bryja V, Nykjaer A, Arenas E: Proneurotrophin receptor SorCS2 is a novel regulator of WNT/PCP pathway during embryogenesis (MANUSCRIPT)

V. **Salašová A**, Yokota C, Potěšil D, Zdráhal Z, Bryja V, Arenas E: A proteomic analysis of LRRK2 binding partners reveals interactions with multiple signaling components of the WNT/PCP pathway. Mol Neurodegener. 2017 Jul 11;12(1):54.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

VI. Boström J*, Sramkova Z*, **Salašová A***, Johard H, Mahdessian D, Fedr R, Marks C, Medalová J, Souček K, Lundberg E, Linnarsson S, Bryja V, Sekyrova P, Altun M, Andäng M: Comparative cell cycle transcriptomics reveals synchronization of developmental transcription factor networks in cancer cells. PLoS One. 2017 Dec 11;12(12):e0188772.

VII. Månsson-Broberg A, Rodin S, Bulatovic I, Ibarra C, Löfling M, Genead R, Wärdell E, Felldin U, Granath C, Alici E, Le Blanc K, Smith CIE, **Salašová A**, Westgren M, Sundström E, Uhlén P, Arenas E, Sylvén C, Tryggvason K, Corbascio M, Simonson OE, Österholm C, Grinnemo KH. Wnt/β-Catenin Stimulation and Laminins Support Cardiovascular Cell Progenitor Expansion from Human Fetal Cardiac Mesenchymal Stromal Cells. Stem Cell Reports. 2016 Apr 12;6(4):607-617

*** Contributed equally**

CONTENT

1 Introduction ... 1
1.1 Wnts, the inner GPS of the animal kingdom ... 1
1.1.1 Cell cycle regulation ... 2
1.1.2 Planar cell polarity and convergent extension ... 3
1.1.3 Migration and cell fate decisions in developing CNS ... 5
1.2 Wnt signaling pathways ... 8
1.2.1 Wnt signaling complexity in a living organism ... 8
1.2.2 Wnt/β-catenin signaling pathway ... 8
1.2.3 Non-canonical Wnt signaling pathways: Wnt/PCP ... 9
1.3 Wnt/PCP mediators ... 10
1.3.1 Wnts – the general features ... 10
1.3.2 Wnt5a - The key to the Wnt/PCP door ... 11
1.3.3 Ror2 - Receptor tyrosine kinase-like orphan receptor 2 ... 12
1.3.4 Celsr1 - Cadherin EGF LAG seven-pass G-type receptor 1 ... 13
1.3.5 Ptk7 – Inactive tyrosine-protein kinase 7 ... 13
1.3.6 Dishevelled – the multitasking organizers ... 14
1.3.7 Prickle1 ... 15
1.4 Wnt signaling and dopaminergic circuits ... 16
1.4.1 Signaling centers during the brain development ... 16
1.4.2 Role of Wnts in the development of midbrain dopaminergic neurons ... 20
1.4.3 Wnt signaling in the CNS ... 23
1.4.4 Wnts and Parkinson's disease ... 25
2 Aims of the study ... 27
3 Results & discussion section ... 28
3.1 Study I: Dishevelled is a NEK2 kinase substrate controlling dynamics of centrosomal linker proteins ... 28
3.1.1 Introduction ... 28
3.1.2 Results and discussion ... 28
3.2 Study II: WNT5A is transported via lipoprotein particles in the cerebrospinal fluid and regulates progenitor proliferation ... 32
3.2.1 Introduction ... 32
3.2.2 Results and discussion ... 32
3.3 Study III: Ror2 and Vangl2 control dopaminergic neurogenesis and multiple aspects of cell polarity in the midbrain floor plate ... 35
3.3.1 Introduction ... 35
3.3.2 Results and discussion ... 36

3.4 Study IV: THE Proneurotrophin receptor SorCS2 is a novel regulator of THE WNT/PCP pathway during embryogenesis 38
3.4.1 Introduction 38
3.4.2 Results and discussion 38
3.5 Study V: A proteomic analysis of LRRK2 binding partners reveals interactions with multiple signaling components of the WNT/PCP pathway 43
3.5.1 Introduction 43
3.5.2 Results and discussion 44
4 Concluding remarks and perspectives 47
5 Acknowledgements 49
6 References 57

LIST OF ABBREVIATIONS

AD	Alzheimer disease
A-P axis	Anterior-to-posterior axis
AP-1/2	AP-1 complex subunit sigma-1/2
aPKC	Atypical protein kinase C
AQP1	Aquaporin 1
BLBP	Brain lipid-binding protein
BMP	Bone morphogenic protein
CDKRab2	Cyclin dependent kinase 5 regulatory subunit associated protein 2
CE	Convergent extension
Celsr1	Cadherin EGF LAG seven-pass G-type receptor 1
CEP	Centrosomal protein of (number of the protein)
ChP, HbChP	Choroid plexus, hindbrain choroid plexus
CK1	Casein kinase 1
C-NAP1	Centrosomal Nek2 associated protein 1
CNS	Central nervous system
CRD	Cysteine-rich domain
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9
CSF	Cerebral spinal fluid
Dvl	Dishevelled/Dishevelled in invertebrates
E10	Embryonic day 10
ECM	Extracellular matrix
En-1/2	Engrailed-1/2
FGF	Fibroblast growth factor
Fucci	Fluorescent ubiquitination-based cell cycle indicator
Fzd/Fz	Frizzled
GFP	Green fluorescent protein
Glut1	Glucose transporter 1

GSK3	Glycogen Synthase Kinase 3
HDL, LDL	High/low-density lipoprotein fraction
HEK	Human embryonic kidney 293 cell line
Hpf	Hours post fertilization
IF	Immunofluorescence
IP-MS/MS	Immunoprecipitation coupled tandem mass spectrometry
IZ	Intermediate zone
JNK	Jun-N-terminal kinase
LOF, GOF	Loss-of-function, gain-of-function
Lrp4/5/6	Low-density lipoprotein-related receptor 4/ 5/6
Lrrk2	Leucine-rich repeat kinase 2
mDA	Midbrain dopaminergic
MEF	Mouse embryonic fibroblasts
mFP	Midbrain floorplate
MHB	Midbrain-hindbrain boundary
MO	Morpholino
MZ	Marginal zone
NEK2	Serine/threonine NIMA protein kinase 2
NMDAR	N-methyl-p-asparate receptor
PCP	Planar cell polarity
PD	Parkinson’s disease
Pk	Prickle
PSD-95	Postsynaptic density protein 95
Ptk7	Inactive tyrosine protein kinase 7
q/RT-PCR	Quantitative/real time polymerase chain reaction
Rab5/11	Ras-related protein Rab 5/11
Ror2	Receptor tyrosine kinase-like orphan receptor 2
Shh	Sonic Hedgehog
SN4741	Substantia nigra 4741 cell line

SNpc	Substantia nigra pars compacta
SorCS2	VSP10 containing-domain receptor SorCS2
TD	Telencephalon-diencephalon boundary
TelChP	Telencephalon Choroid plexus
TH	Tyrosine hydroxylase
Vangl2	Van Gogh like 2
VM	Ventral midbrain
VTA	Ventral tegmental area
VZ	Ventricular zone
WB	Western blotting
Wg	Wingless
WISH	Whole-mount *in situ* hybridization
Wnt	Wingless/Integration
WT, KO, KD	Wild type, knock-out, knock-down
X. laevis	Xenopus laevis

1 INTRODUCTION

Probably no one would have thought in 1982, when the first mammalian Wnt ligand, proto-oncogene *Integration-1* (*int-1*), was identified by Roel Nusse and Harold Varmus [12] that the Wnt field will become so interdisciplinary, and revolutionize many research areas, including cancer research, neuroscience, developmental biology, biotechnology and regenerative medicine. Shortly after their discovery, *Int-1* was aligned to its *Drosophila* homolog *Wingless* which caused the fusion of the two names into *Wnt-1*. Many more Wnt-related proteins have been described in the past 35 years, and due to their high clinical relevance, they have become the research focus for scientists all around the world. I hope that this thesis will take you on a fun exploration of the Wnt signaling world and will simply "Wnt you".

1.1 WNTS, THE INNER GPS OF THE ANIMAL KINGDOM

In mammals, Wnts (*Wingless/Integration*) are a large family of 19 secreted lipid-modified glycoproteins that serve multiple functions in development, tissue homeostasis, and disease. Wnts typically function as morphogens, and are highly conserved throughout the animal kingdom. As such they comprise, together with Sonic-Hedgehog (Shh), Fibroblast growth factor (FGF), Notch or Bone morphogenetic protein (BMP) signaling, one of the most essential pathways that control embryonic development and regeneration. Deregulation of Wnt signaling often leads to lethal phenotypes such as craniofacial defects, spina bifida or exencephaly. Abnormal Wnt signaling has been genetically linked to several developmental disorders such as Robinow syndrome and autism. Postnatally, their dysfunction has been associated to different types of cancer, skeletal malformations, neurological disorders or cardiovascular diseases [5, 13, 14].

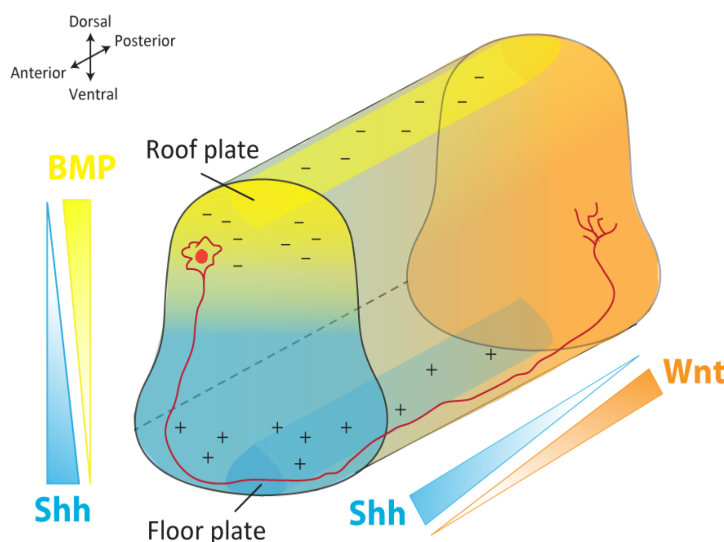


Figure 1: Wnt gradients together with other morphogens place attractive and repulsive cues which guide axons of commissural neurons during spinal cord development [1, 2].

Wnts control a wide spectrum of complex cellular processes during development. In order to create a living organism from a one-cell stage zygote, the cells must undergo many genetic and epigenetic changes, and provide a precise micro-environment composition for correct cell-to-cell communication. That includes placing attractive and repulsive clues in the form of trophic and apoptotic factors, creating morphogen gradients,

and eliminating mispositioned, dysfunctional or unnecessary cells in order to define the right tissue size and its function (**Figure 1**). Generally, the following cellular processes are essential during the CNS development: cell proliferation, cell survival, stem cell renewal, apoptosis, cell migration, neuronal diversification, and synaptic connectivity; and Wnts regulate all of them [11, 13, 15].

1.1.1 Cell cycle regulation

Cell cycle progression and its strict regulation are essential for the life of each cell. Postmitotic cells do not divide and they stay in a quiescent, G0 phase. Proliferating cells are usually in the interphase which is composed of G1, S, G2 phases, and is characterized by heavy transcriptional and translational levels as well as multiple mitogen and DNA quality check points. These phases are followed by mitotic division that requires massive cytoskeletal reorganization, which is strictly regulated by the so-called centrosomal cycle. A centrosome is a cytoplasm organelle of animal eukaryotic cells which organize microtubule nucleation, mitotic spindle organization and polarization, as well as formation of the basal body of the primary cilia. It also participates in cell signaling and cell polarity. In the interphase, centrosome is composed of two, mother and daughter centrioles. The centrioles are cylindrical structures composed of nine specialized microtubules symmetrically arranged around the central core. The microtubules are surrounded by a protein mass called a centrosomal linker which is constituted of γ-tubulin, centrosomal proteins family (CEPs), pericentrin, centrosomal Nek2 associated protein 1 (C-NAP1), CDK5 regulatory subunit associated protein 2 (CDK5RAP2), and Rootletin. Together, they clasp the centrioles until the M phase. In the M phase, the centrosome is duplicated. The linker proteins are phosphorylated by Polo-like kinase1, acetyltransferase Mst2, and serine/threonine NIMA protein kinase 2 (NEK2), leading to a cleavage of Rootletin and removal of C-NAP1 from centrosomes. Centrioles can thus separate and migrate to the opposing poles of the cells where they begin polymerization of α and β-tubulin, and subsequent production and bipolar orientation of the mitotic spindle. Microtubule polymers of the mitotic spindle bind the kinetochore of each chromatid. Microtubules subsequently depolarize which translocate sister chromatids to the opposing side of the cells. This is followed by the cytokinesis. The centrosome must be very often relocated during other cellular processes, for instance during the cell migration. A mature centrosome also builds an anchoring basal body of immobile primary cilia in postmitotic cells which must be disassembled if they re-enter the cell cycle [16-18].

Wnt signaling regulates cell proliferation, stem cell renewal and neurogenesis, processes fully dependent on the cell cycle. That is why defective Wnt signaling is strongly involved in regeneration and carcinogenesis [19, 20]. It has been shown that the so-called Wnt/β-catenin signaling controls expression of the proto-oncogene c-myc and the cell cycle kinase cyclin D1 which both regulate the G1 phase. The activation of Wnt/β-catenin signaling by

the CDK14/Cyclin Y complex is further required for G2/M transition and mitotic events [21]. It has been shown that Axin1 localizes in centrosomal structures in a complex with γ-tubulin and regulates the microtubule nucleation in Wnt signaling dependent manner [22, 23]. Dishevelled (Dvl or Dsh in *Drosophila*), an important mediator of Wnt signaling pathways, is localized in centrosomal structures where it controls the polarization of the basal body of the primary cilia [24], the orientation of the mitotic spindle [25] and the primary cilia disassembly [26]. It has been also shown that the non-canonical Wnt receptor Ror2, regulates cell cycle progression in reactive astrocytes [27]. These data provide evidence that different Wnt signaling components and pathways control various events during the cell cycle in different cell types [16].

1.1.2 Planar cell polarity and convergent extension

Tissue polarity is one of the most spectacular phenomena in living organisms, which determines the patterning and organization of the body plan. Planar cell polarity (PCP) refers to the process by which cells coordinate their alignment within a plane in a polarized manner across a tissue. This process leads to an asymmetry between an apical and basal side of the cells, and sets the anterior-to-posterior body axis. In other words, it is a cellular compass. Many proteins have been described to control PCP, for example proteins of the Cadherin family, G-protein coupled receptors (GPCRs), different components of the Wnt signaling pathways, atypical protein kinase C (aPKC), endocytotic proteins from Rab family and others. The establishment of PCP is crucial during embryogenesis and early postnatal development. However, PCP maintenance is also essential for tissue homeostasis and repair in order provide correct stimulatory and inhibitory signals to the surrounding cells. In humans, deregulated PCP signaling has been associated with many pathologies, typically birth defects, ciliopathies, and even neurological disorders such as autism [11].

PCP is governed by signals that control the enriched localization of polarity-mediating protein complexes. The asymmetric enrichment appears either intracellularly (polarization within a cell), or extracellularly at cell-cell junctions, which mediates the polarization of the entire tissue (**Figure 2A**). So-called PCP proteins also direct the orientation of the subcellular structures. Wnt morphogens and some of their transmembrane receptors have an irreplaceable function in regulating PCP, and we thus call this pathway the Wnt/PCP pathway. The mechanism of the asymmetric distribution of Wnt/PCP proteins was described in a great detail in *Drosophila* where it regulates hair orientation in the wing. In the wing blades, the Van Gogh protein (Vang; Vangl2 in vertebrates) accumulate on the proximal side of the cell together with Prickle1. They are complementary to the distal side which has accumulated molecules of Frizzled, Dishevelled and Diego proteins. The cells are interconnected with the cadherin-containing protein Flamingo (Celsr in vertebrates). Such asymmetry governs the polarization of each epithelial cell in the fly's wing, and the correct orientation of the single trichome (hair). The polarity of the tissue is orchestrated by

additional protein gradients including Wingless (Wg), and Fat-Dachsous (Ds)-Four-jounted (fj) signaling axis [11], as schematized in the **Figure 2B**, although, this description is very simplified. In mammals, mechanisms of the Wnt/PCP signaling are mostly studied in the polarized epithelial cells [11]. A good example is mechanosensory cells in the cochlea of the inner ear which grow stereocilia on their apical side. The loss of PCP signaling components in these cells causes degeneration of the cilia and deafness [28, 29].

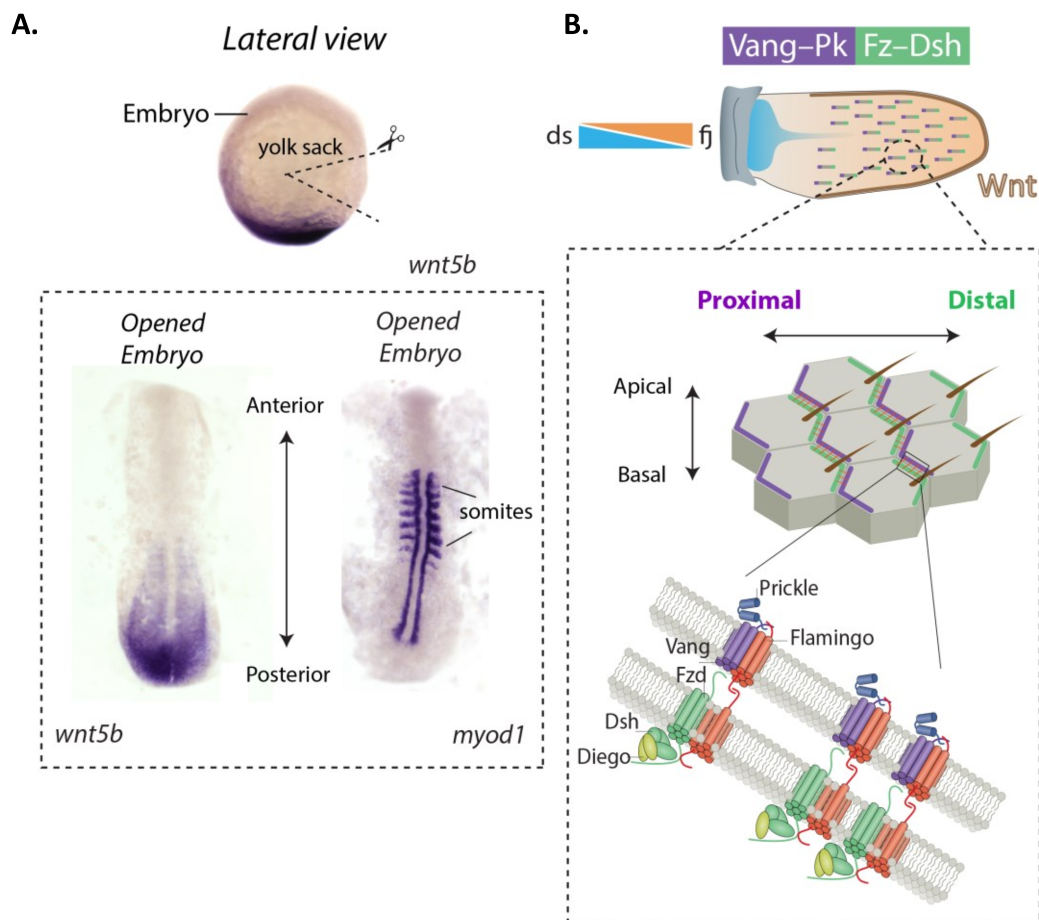


Figure 2: Wnt/PCP proteins control planar polarity within a cell and a tissue which governs the body plan. **A.** Polarized expression of *Wnt5b* during convergent extension in zebrafish embryos determines the A-P body axis. **B.** Asymmetric distribution of protein complexes and Wnt concentration gradients determine the apical-basal polarity and proximal-distal polarity of the epithelial cells in Drosophila's wing. Modified from [11].

Wnt morphogens and their receptors control PCP by forming protein gradients. These concentration gradients provide molecular fingerprints that can be decoded by the neighboring cells. It is believed that the combination of concentration gradients of different ligands and receptors thus create a topographical map, which helps cells to navigate, migrate, determine and maintain their specific function in the organism [30, 31]. These results suggest that Wnt signaling works in a combinatorial manner.

Besides the polarized clustering of the PCP proteins, the cells must actively maintain the enriched intracellular and transcellular protein complexes which they either recycle or remove if they are misplaced or dysfunctional. Endocytosis seems to play an important role in the protein sorting, trafficking and lysosomal degradation of Wnt/PCP proteins. It has been shown that some of the Wnt/PCP proteins such as Prickle1, Dvl2, Celsr1, Ptk7 or Vangl2 are internalized upon their interaction with Rab5, Rab11, AP-1/2, dynamins and other endocytic proteins, which subsequently affect the planar cell polarity and the synaptic plasticity [32-37].

Wnt/PCP signaling also governs one of the most important processes during embryogenesis called **convergent extension** (CE). Convergent extension is a series of strictly regulated spatiotemporal events during gastrulation, neurulation, axis elongation and organogenesis which occur in invertebrates and vertebrates. It triggers and drives a massive, collective rearrangement and migration of progenitor cells of the germ layer towards the dorsal side of gastrula. The cells narrow and "converge" to form an embryonic body axis, providing anterior-to-posterior orientation, thus the basis of the body plan. Simultaneously, the cells proliferate and migrate along the axis, which leads to embryonic elongation (=extension) (**Figure 3**). A typical phenotype of defective CE movements is reduced embryo length. Unfortunately, the regulation of CE movements is not well understood because of its spatiotemporal complexity and variations among species [38, 39]. Mechanisms of CE have been extensively studied in *Xenopus laevis* (frog) and *Danio rerio* (zebrafish) embryos. These models provide great advantages to study such processes due to the rapid production of a large number of eggs, fertilization outside of the mother, fast development and embryos' transparency.

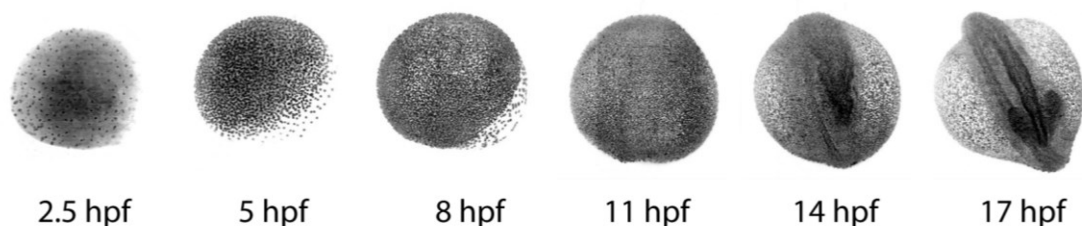


Figure 3: A 3D-imaging which tracked individual dividing cells during convergence and extension movements in a zebrafish embryo. Hpf stands for "hours post fertilization". The formation of the body axis is already visible at 11hpf, and the head at 17hpf. The gastrulation lasts until 10hpf. The photos were modified from [6].

1.1.3 Migration and cell fate decisions in developing CNS

Cell motility is a fundamental cell behavior that is highly dependent on the dynamic remodeling of the cytoskeleton, extracellular matrix and transcriptional changes. Defects in migration during CNS development may lead to abnormal brain wiring, causing brain malformation, cognitive dysfunctions or seizures. Wnt signaling pathways regulate some of

the known mechanisms necessary for cell migration, including cell adhesion, chemotaxis, primary cilia movements, development of fillopodia and lamellipodia at the leading edge, or the collapse of the growth cone [40-43]. Abnormal cell motility caused by hyperactive Wnt signaling has been seen in metastatic stages of many invasive tumors [44, 45].

In the developing CNS, long distance migration (sometimes several millimeters) and correct positioning of neuronal and non-neuronal cells is crucial for cell identity and brain connectivity. In the adult nervous system, cell migration is mostly seen after an injury when astrocytes and microglia migrate to inflamed areas, to repair the wound. Cell motility is regulated on multiple levels. Extracellularly, cell migration is stimulated by many factors such as Wnts (Wnt1, Wnt5a, Wnt2, Wnt4), neurotrophins (GDNF, BDNF), semaphorins (Semph3A, 4D/E), and cytokines (CXCL12), and by the interaction of cell surface adhesion molecules, such as cadherins, with extracellular matrix (ECM) adhesion molecules, for instance laminins. Repulsive cues have even higher importance in cell migration than attractive clues as they prevent cells from migrating to the wrong areas. The extracellular cues are transduced either via specialized receptors, changes in the ion channels or by internalization of protein complexes (e.g. via endocytosis). Intracellularly, the signals can be transduced in multiple ways, often through activation of small GTPases (Rac1, Cdc42 and RhoA), cytoskeletal proteins (myosinII, tubulin, actin), cyclin dependent kinases (Cdk5/p35), microRNAs, transcription factors and many other signaling molecules and pathways [10]. *In vitro* studies have shown that stem cells and progenitor cells can sense and prefer different structural patterns and softness of the material altering their niche, features which are currently being investigated in order to develop engineered biomaterials and to improve cell and tissue repair and transplantation [46].

Neuronal progenitors migrate at different times dependent on the neuronal type, brain area and animal species. Generally, we distinguish two models of CNS migration, radial and tangential migration (**Figure 4**), which have been mostly described in cortical areas. Tangential cell migration is an event where cells can migrate in different directions based on their active communication with the environment. This migration is the most common and is typical for integrating interneurons into the brain circuits. The migrating cells extend branched processes to sense the extracellular clues, which guide the leading edge and the axonal outgrowth. The projection of the processes is followed by branch stabilization, centrosome relocalization into the axon, and nucleokinesis. Cells thus undergo a translocation of the soma, which is glial-independent [10].

On the other hand, radial migration has been characterized by the physical interaction of postmitotic neuroblasts with radial glia cells. In the developing brain, radial glia cells typically express Glast, Nestin, and brain lipid-binding protein (BLBP). Their soma is usually located at the ventricular zone whereas their processes are stretched across the developing tissue, and is in contact with the pial surface. Due to such positioning, they serve as scaffold

that is used by neuroblasts to climb along the radial glia processes and reach their final location. It has been shown that radial glia have the capacity of undergoing neurogenesis and thus producing neuronal precursors, such as dopaminergic neuroblasts in ventral midbrain. It is believed that there are no radial glia cells in the adult brain [47, 48]. Radially migrating neuroblasts usually display bipolar morphology. Nevertheless, they undergo a transient phase when they obtain a multipolar morphology, possess many thin retracting processes, and seek the positional information independently from the radial glia. It has been hypothesized that this behavior is critical for the determination of correct neuronal identity and decision making whether to stay or to continue in radial migration [10].

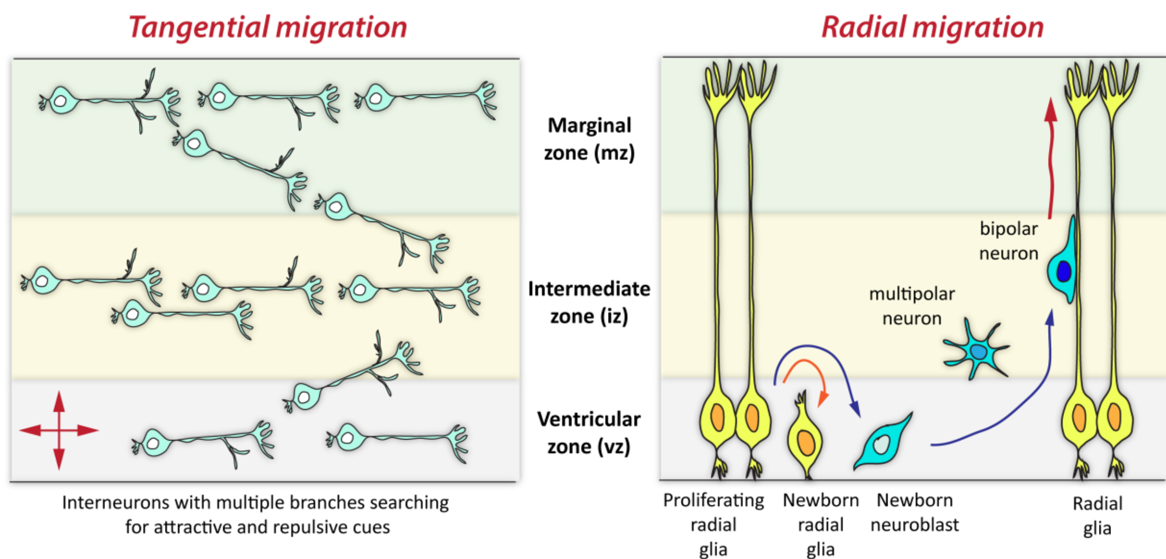


Figure 4: A scheme of tangential and radial migration. Drew according to [10].

Cell migration itself is not just a mechanical process involving translocation of a cell from the place A to the place B. It has been shown that migrating cells are undergoing cell fate changes and maturation steps, which are controlled by environmental factors that they get in contact with during the migration, such as Wnts and other morphogens, as well as growth factors and ECM. These factors lead to transcriptional and epigenetic changes resulting in cell differentiation and specification in different brain areas. These processes have become a large focus of attention for translational researchers who try to understand such mechanisms *in vivo* and recapitulate them *in vitro* [49]. The correct understanding of stemness and the sequential events which are necessary for cell differentiation can be used in regenerative medicine in order to prepare high quality cell grafts for cell transplantation therapies or for triggering tissue regeneration *in vivo,* by e.g. small molecules or gene transfer. There is a large need for such knowledge in order to develop applications for neurological disorders such as Parkinson's disease, stroke or spinal cord injury, where we need to replace the missing pool of physiologically functioning neurons [9].

1.2 WNT SIGNALING PATHWAYS

1.2.1 Wnt signaling complexity in a living organism

There is a large number of Wnt ligands (up to 19) which are able to bind to more than 15 different receptors with distinct preference, which makes Wnt signaling very complex and it has been challenging to uncover the precise molecular mechanisms by which cells transduce the Wnt signals [4, 50]. Moreover, many Wnt regulators interact with other signaling pathways which include MAPK/ERK [51-53], Notch [54-56] or BMP [57-59]. R-spondins, Syndecans and Heparan Sulphate Proteoglycans have also been shown to directly modulate Wnt signaling pathways [4]. It is therefore believed that the right ratio and high complexity of Wnt signaling enables cells to recognize and translate various extracellular clues, and subsequently control dynamic and highly refined cellular- and tissue-specific events such as cell polarity or cell migration [4, 11, 14]. For this reason it is very important to evaluate the results from Wnt signaling studies in the context of the tissue, cellular events and activation levels.

Historically, we distinguish two main branches of Wnt signaling pathways, a canonical also called Wnt/β-catenin signaling pathway, and non-canonical, β-catenin independent Wnt signaling pathways which include Wnt/Planar Cell Polarity (PCP) and Wnt/Calcium (Ca^{2+}) pathways. Interestingly, the activation of non-canonical Wnt signaling pathways inhibit the Wnt/β-catenin pathway and vice versa, indicating that these two signaling branches are in balance with one another (**Figure 5**) [60-62].

1.2.2 Wnt/β-catenin signaling pathway

The mechanisms of the Wnt/β-catenin signaling pathway are relatively well understood. It has been shown that Wnt/β-catenin signaling is typically activated by Wnt1, Wnt3a or Wnt8 ligands. Our current knowledge about the signal transduction involves Wnt ligands binding to a family member of the seven-pass transmembrane receptors Frizzled (Fzd) and its co-receptor, low-density lipoprotein-related receptor 5 or 6 (Lrp5/Lrp6). The Wnt-Fzd-Lrp5/6 protein complex is called the signalosome. In the absence of Wnt stimulation, the signalosome is not formed, and β-catenin is phosphorylated on multiple sites by the β-catenin destruction complex, which is composed of Axin1, Glycogen Synthase Kinase-3β (GSK3β), Adenomatous Polyposis Coli (APC) and Casein Kinase 1α (CK1α). The phosphorylated β-catenin is subsequently recognized and ubiquitinated by the β-Trcp E3 ubiquitin ligase, which labels β-catenin for its degradation in the proteasome. Upon formation of signalosomes, Fzd and Lrp5/6 are phosphorylated on their intracellular domains by polymerizing Disheveled 1, 2 and 3 molecules (Dvl1, 2, 3) and CK1 isoforms. These changes are recognized by the destruction complex which is recruited to the membrane and cannot longer phosphorylate β-catenin. Consequently, β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it binds to a family of

transcription factors TCF/LEF. Together they control the expression of several target genes such as c-*myc* or *cyclin d1* [50, 63].

1.2.3 Non-canonical Wnt signaling pathways: Wnt/PCP

Non-canonical Wnt signaling consists of several pathways whose signaling mechanisms vary, and are less understood. The most typical ligands for controlling these pathways are Wnt5a, Wnt7a/b and Wnt11. These ligands can activate two main pathways: the Wnt/PCP pathway that controls planar cell polarity and signals downstream through small GTPases, and the Wnt/Ca^{2+} pathway that uses changes in calcium levels for its signal transmission [4].

The Wnt/PCP pathway has been implicated in many fundamental processes such as convergent extension (CE) movements, determination of the anterior-posterior axis and tissue morphogenesis. Many proteins on different regulatory levels have been identified to govern the Wnt/PCP pathway but the molecular mechanisms are not clear. Generally, it is accepted that the activation of Wnt/PCP signaling involves the binding of specific Wnt ligands to Fzd and the recruitment of several co-receptors, such as the Receptor tyrosine kinase-like orphan receptor 2 (Ror2) and its interacting partner Van Gogh like 2 (Vangl2) [31]. These interactions are followed downstream by phosphorylation of Dvl and activation

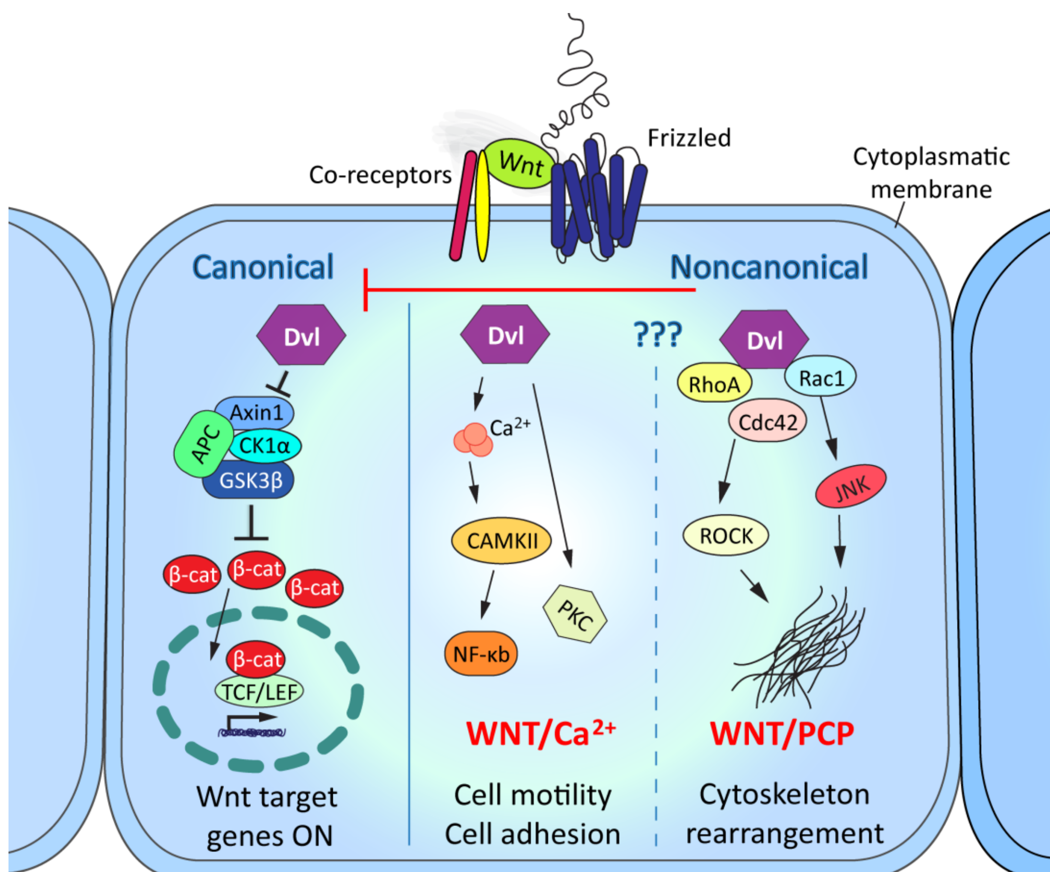


Figure 5: A scheme of the three main Wnt signaling pathways. The activation of Wnt signaling pathways is determined by binding of various Wnt ligands to specific receptor complexes which mediates different downstream activation of the Wnt signaling pathways. Drew according to [3, 4].

of the small GTPases, Rac1 and RhoA, or Jun-N-terminal kinase (Jnk), leading to cytoskeleton remodeling and changes in gene expression [64-66].

It has been recently suggested that the Wnt/PCP pathway might be also independent from Fzd receptors, in contrast to the canonical signaling where the binding of Fzd to its co-receptor Lrp5/6 is required for the signal transduction [4, 67]. In recent years, the Wnt/PCP pathway has been sub-divided into specific signaling axes according to the specific signaling component involved, for example the Wnt5a-Ror2-Dvl axis [67-69].

Due to the regulatory diversity and the high, cell-context dependence of the Wnt/PCP pathway, there is no standardized and sensitive biochemical assay to measure the activity of the Wnt/PCP signaling. This has been one of the biggest complications of this research field and has led to the predominant use of biological assays to examine the activity of this pathway. In the next paragraphs, I will introduce you to some of the core Wnt/PCP signaling components that I have worked with during my PhD projects.

1.3 WNT/PCP MEDIATORS

1.3.1 Wnts – the general features

Wnts are cysteine-rich ligands that undergo several posttranslational modifications before being secreted and fully active, with glycosylation and acetylation (= lipidization) being the most prominent. Precise modifications differ for each Wnt. For example, glycosylation appeared to be crucial for Wnt3a and Wnt5a secretion and activity [70] but has only a minor effect on Wnt1 [71]. Acetylation probably helps Wnts to locally diffuse in a tissue creating concentration gradients which elicit cell and tissue patterning by providing a diverse spectrum of precise signaling “barcodes” during embryogenesis [11, 72]. Therefore, it has been suggested that the glycosylation and acetylation probably affect binding properties of Wnts to different proteins present in the extracellular matrix, which might represent another signaling mechanism of Wnt regulation [73]. Unfortunately, not much is known about such interactions.

Wnts are acetylated by the enzyme o-acetyltransferase called Porcupine that covalently adds palmitic acid to the conserved serine residues in the lumen of the endoplasmatic reticulum [73, 74]. Acetylation is important for the intracellular transport of Wnts from the endoplasmatic reticulum, their secretion, and biological activity. Notably, acetylation turns Wnts into hydrophobic, insoluble molecules which must be likely transported in the water-based extracellular space in a paracrine manner, and over long distances via binding to soluble proteins. It has been suggested, mostly by studies in *Drosophila*, that Wnt transport is mediated either via direct binding to Wnt-protein carries such as albumin [75] or Swim [76], to lipoprotein particles [77], or by their incorporation inside of exosomes or exosomal-like vesicles, which was observe in the *Drosophila* brain and in epididymal fluid in mice [45,

78, 79]. Another proposed mechanism includes transport of Wnts via specialized filopodia called cytonemes during neural plate formation in zebrafish [80]. Nevertheless, the precise mechanisms of Wnt transport remain to be discovered.

Solubility of Wnts has been very challenging since it is not possible to isolate them without a detergent. That is why it has not been possible to purify biologically active, recombinant Wnts *in vitro,* except of Wnt1, Wnt3a and Wnt5a, which are more soluble forms [73].

1.3.2 Wnt5a - The key to the Wnt/PCP door

Wnt5a is one of the most studied Wnt ligands as it is one of the most essential activators of planar cell polarity in multiple organs during development [30, 31, 81-83]. Wnt5a is expressed across the postnatal brain in different brain areas where it controls axonal guidance, dendritogenesis, synaptogenesis and synaptic plasticity [13, 84-86]. It has been shown that Wnt5a mediates maturation of the synaptic bouton via enriching the postsynaptic density protein PSD-95 clusters on the postsynaptic side [86, 87]. Wnt5a also signals via concentration-dependent gradients (**Figure 6**), that if disturbed, may cause signaling alterations [30, 31, 88].

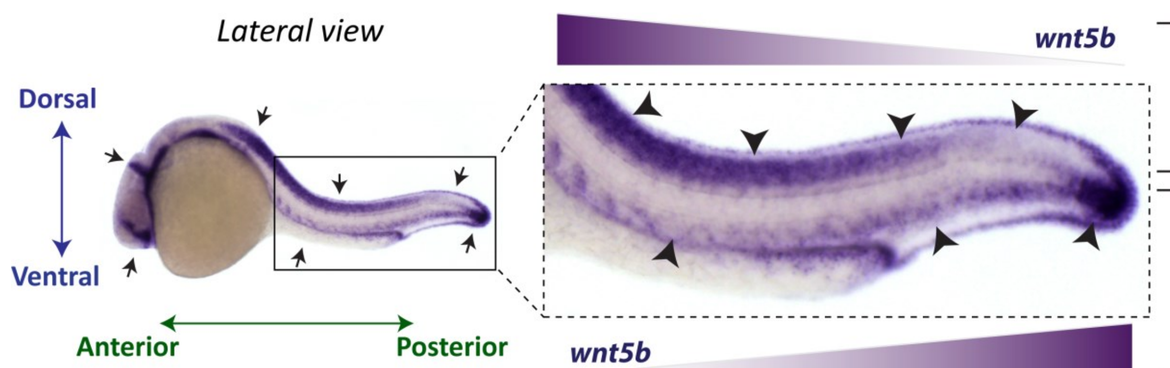


Figure 6: Distinct patterns of *Wnt5b* expression in a zebrafish embryo at 24hpf reveal the formation of concentration gradient in the trunk (arrow heads), and distinct expression in the brain areas (arrows). Wnt5b is a fish orthologue of Wnt5a.

Wnt5a null mice suffer perinatal lethality caused by asphyxia (severe hypoxia caused by abnormal breathing). They also display many abnormal defects in the developing skeleton and CNS such as extremely short spine, tail and limbs, or craniofacial and neuronal defects [82, 89, 90]. Interestingly, the Wnt5a overexpression caused defects in the skin which were similar to the ones observed in Wnt/β-catenin signaling loss of function [91] suggesting a mutual role of Wnt5a to modulate distinct Wnt signaling responses. Wnt5a has been intensively studied not only for its interchangeable role during development but also for its clinical relevance in different types of cancer [43, 92, 93], inflammation [69], Alzheimer disease [94], amyotrophic lateral sclerosis (ALS) and multiple sclerosis [95, 96], chronic pain

[97, 98] and congenital developmental disorders including brachydactyly type B and Robinow syndrom [81, 99, 100].

1.3.3 Ror2 - Receptor tyrosine kinase-like orphan receptor 2

Ror2 is a single-pass transmembrane receptor that together with its homolog Ror1 belongs to a tyrosine kinase family. Ror1 and Ror2 share most of their structure, and are suggested to be biochemically and functionally redundant. Interestingly, Ror receptors have been shown to bind multiple Wnt ligands, namely Wnt1, Wnt2, Wnt3, Wnt3a, Wnt4, Wnt5a and Wnt5b, Wnt6, Wnt7a, Wnt8, Wnt11, though it has been suggested that Wnt5a is the main ligand for Ror2 [101, 102].

Ror2 is an important mediator of Wnt/PCP signal transduction that regulates CE and neural tube closure during early development [31, 67, 103]. Postnatally, Ror2 mediates axonal guidance and synaptogenesis [104-107]. It has been reported that Ror2 controls cell cycle progression of reactive astrocytes after a brain injury [27]. *Ror2 null* mice show defects in the skeleton, heart, lung and external genitalia [108, 109]. Deregulation of Ror2 and Wnt5a expression has been correlated to different types of invasive tumors, and thus they have become the novel targets for cancer treatment [43, 93, 110]. Similarly to Wnt5a, Ror2 has been genetically linked to brachydactyly B and Robinow syndrome [111, 112].

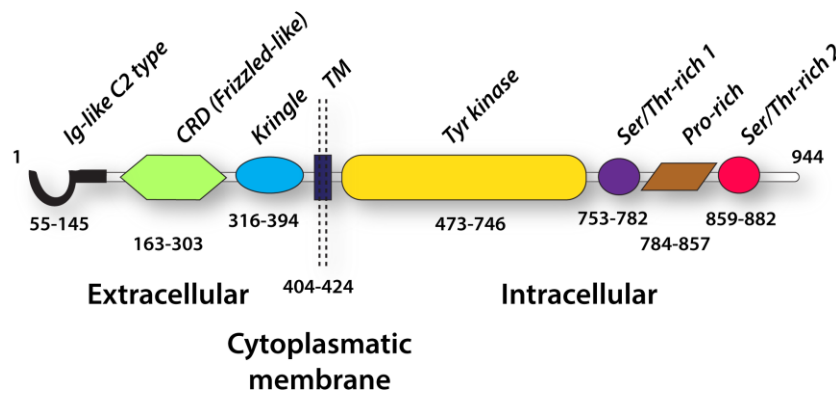


Figure 7: Ror2 structure.

Ror2 contains multiple domains. The extracellular part of Ror2 is composed of an immunoglobulin C2 domain, followed by cysteine-rich domain (CRD), also called Frizzled-like domain), and a membrane-proximal Kringle domain [113]. These domains are anticipated to be involved protein-protein interactions. Ror2 is anchored in the cytoplasmic membrane by a transmembrane domain. Intracellularly, Ror2 contains a large tyrosine kinase domain, and three predicted domains, Serine/Threonine domain 1 and 2, and a Proline-rich domain, thus the domains responsible for the kinetic activity of Ror2. It has been shown that CK1ε binds to the Proline-rich domain, and subsequently phosphorylates its Serine/Threonine rich domain 2. The phosphorylation at the Ser/Thr domains leads to auto-phosphorylation of the

Tyrosine kinase domain, which is a predicted prerequisite for full activation of Ror2 (**Figure 7**) [61, 114, 115].

It has been shown that Wnt5a induces homodimerization of Ror2 and formation of a ternary complex with Fzd. This is subsequently followed by recruitment and phosphorylation of Ror2 by Dvl [58, 67, 116], Gsk3β [117] and/or Ck1ε [115, 118]. Ror2 binds to Fzd2 [119] through its CRD domain but it can also bind Wnt5a, and transduce the signal without the presence of Fzd [103, 120]. Ror2 also forms heterodimers with other Wnt/PCP receptors such as Vangl2 [31] and Ptk7 [121, 122]. The Ror2-Vangl2 receptor complex has been shown to create receptor gradients in addition to the Wnt5a gradient in the developing mouse limb bud, by which they control limb development *in vivo* [31, 88]. In addition, $Ror1^{-/-}$;$Ror2^{-/-}$ mice phenocopy Wnt5a mutant animals [67, 120] suggesting that Ror1 and Ror2 function as the main receptors of Wnt5a-dependent signaling *in vivo,* independently of Fzd receptors [123]. Nevertheless, the precise molecular mechanism by which Ror2 transduces the Wnt5a signal has not been solved yet.

1.3.4 Celsr1 - Cadherin EGF LAG seven-pass G-type receptor 1

Celsr1, also known as Flamingo in *Drosophila*, is a large seven-pass transmembrane receptor composed of 3014 amino acids. Celsr1 together with its two homologs Celsr2 and Celsr3, are typical regulators of Wnt/PCP signaling [124-126] in multiple tissues such as inner ear, skin, brain or tooth [127-130]. As such, it has the capacity to inhibit Wnt/β-catenin signaling [125]. Celsrs are a family of atypical cadherins with an enormous ectodomain that is composed of 9 cadherin repeats, 6 epidermal growth factor EGF-like domains, 2 laminin G repeats, 1 hormone receptor motif (HRM), and a G-protein-coupled receptor proteolytic site (GPS). This is followed by seven-pass transmembrane domains and a cytoplasmic tail. Celsr1 is also classified as part of the cell adhesion receptor family of G-protein-coupled receptors [126]. Celsr1 is involved in CE movements [131, 132], anterior-posterior patterning and cell polarity [133, 134], cortical neurogenesis in mice [129], neuronal migration of branchiomotor neurons in zebrafish hindbrain [135], as well as axonal outgrowth in *Drosophila* and *c. elegans* [134, 136]. Almost nothing is known about mechanisms of Celsr1 signaling. It has been suggested that Celsr1 is a Wnt5a receptor, functioning in cooperation with Fzd, Vang and Dvl, and together they regulate processes such as dendrite outgrowth and axonal branching [136, 137].

1.3.5 Ptk7 – Inactive tyrosine-protein kinase 7

Ptk7 is another single-pass transmembrane receptor that is involved in planar cell polarity, neural tube closure and neural crest migration [138-141]. The function of Ptk7 is often deregulated in different types of tumors where it likely controls cell proliferation, cell motility and angiogenesis [142-144]. Ptk7 has an atypical protein structure. It contains an incomplete intracellular tyrosine kinase domain, which is considered to be kinase-dead but

is actively involved in downstream signaling [145]. Ptk7 is a strong regulator of Wnt/PCP pathway, and as such it inhibits Wnt/β-catenin activity [146-148]. In the presence of Wnt5a, Ptk7 can bind to Fzd7 and recruit Dvl to the plasma membrane in *Xenopus* embryos [138]. Two publications have shown that overexpressed Ptk7 physically interacts with Ror2 and that upon Wnt5a stimulation this receptor complex controls cell movements and tissue morphogenesis in *X. laevis* development [121, 122]. In planarians, Ptk7 and wntP-2 control the trunk-tail positional identity during regeneration [149].

Interestingly, a few studies have observed that Ptk7 also positively regulates Wnt/β-catenin signaling pathway [62, 150] through an unknown mechanism. In *X. laevis* embryos, Ptk7 morphants phenocopy embryos depleted for Wnt3a and Lrp6, and show reduced Wnt/β-catenin activity. Furthermore, Ptk7 can physically interact with Lrp6 and subsequently inhibit the Wnt/PCP pathway [62], suggesting a reciprocal role of Ptk7 in both Wnt/PCP and Wnt/β-catenin signaling. Moreover, Berger *et al* suggested that Ptk7 localization is affected differently by different Wnt ligands. They showed that canonical Wnts such as Wnt8, Wnt2b and Wnt3a together with Fzd7 mediate caveolin-dependent lysosomal degradation of Ptk7, whereas non-canonical proteins Wnt5a, Wnt11 and Ror2 do not. They hypothesized that Ptk7 rather inhibits canonical Wnt signaling by outcompeting the ligand-binding which disables Wnts to bind to their Wnt receptors [34]. Nevertheless, the regulation of Ptk7 signaling and its dual role between Wnt signaling pathways remains largely unclear.

1.3.6 Dishevelled – the multitasking organizers

Dishevelled proteins (Dvl/Dsh) are core mediators of Wnt/β-catenin and β-catenin independent signaling pathways. We recognized three different Dvl genes in mammals, Dvl1, Dvl2 and Dvl3. The structure and the domain features of Dvls are much conserved in the animal kingdom, and even though different paralogs have been found in distinct species (one Dsh in Drosophila, and more than 4 Dshs in zebrafish) they overall share the basic functionality in Wnt signaling. This suggests a synergistic function conserved across the species and a biochemical redundancy within a tissue. Nevertheless, the expression of Dvl paralogs largely depends on the species, development stage, tissue, and the isoforms themselves. Dvl1 is considered more specific for CNS development, whereas Dvl2 and Dvl3 are more important for the mesodermal tissue. Nevertheless, genetic mutations in Dvl2 and Dvl3 are linked to neural tube defects, pointing at the contributions of all isoforms in CNS development [151-154]. Overall, we simply do not understand how are Dvls regulated, and how exactly they activate, and sometimes inhibit, the Wnt signaling [118, 155, 156].

Dvls are characterized by their ability to polymerize, both at endogenous levels and after overexpression, via their Dishevelled-and-Axin (DIX) domain, a process regulated in a very dynamic manner [157, 158]. It has been shown that Dvl can crosstalk with multiple proteins in the cellular membrane, cytosol and even in the nucleus. Such interactions usually occur

through the DEP and PDZ domains, whereas the phosphorylation sites are placed at the regions of proline-rich and basic domains (**Figure 8**) [155]. It is believed that the efficiency of Dvl polymerization and their interaction/ release to/from their specific binding partners at any given moment governs the Wnt activation and the downstream signaling specificity [60, 155, 159-161]. This was also supported by the observations that Dvl loss-of-function often recapitulates some (not all!) features found in mutants of the Wnt/PCP regulators [31, 124], such as neural tube closure failure, skeletal malformations, cardiac outflow and craniofacial defects [152-154]. These features are also found in patients with congenital diseases that often carry mutations in Wnt signaling genes such as Robinow syndrome [99, 100, 111, 162].

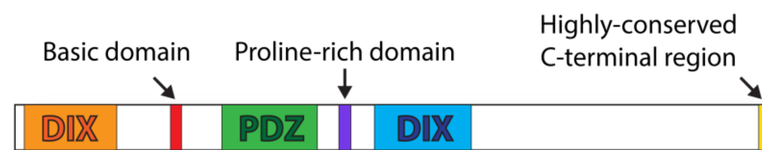


Figure 8: A scheme of the Dishevelled structure.

1.3.7 Prickle1

Prickle1 is a cytosolic protein downstream of the Wnt/PCP signaling pathway that is important for apical-basal cell polarity [162-164]. Nevertheless, its precise function and molecular signaling in the Wnt/PCP pathway is rather unknown. It has been shown that Prickle1 controls cell movements during gastrulation, cell morphogenesis and neuronal migration [165-169]. It was also suggested that Prickle1 controls oligodendrocyte differentiation [170]. At the molecular level, Prickle1 can bind to Dvl and cause its ubiquitination and degradation which leads to the downregulation of Wnt/β-catenin signaling [171]. This interaction has been proposed to be a mechanism by which Prickle1 regulates the asymmetric localization of Fzd and Dvl across cell-cell contacts from the proximal to the distal side of the cell [172-174].

Mutations in Prickle1 have been associated to seizures [175], progressive myoclonus epilepsy [176, 177] and autism [178], suggesting that its deregulation may result in altered CNS development and/or synaptic plasticity. This hypothesis is supported by the fact that mouse Prickle1 can promote neurite outgrowth in postmitotic neurons in the developing neocortex and in neuroblastoma [179-181], as well as axon outgrowth in sensory peripheral neurons in *Drosophila* [182]. Moreover, Prickle1 has been found to interact with Synapsin1, a protein important for synaptogenesis and vesicle trafficking [178]. Even though there are hints suggesting that Prickle1 is important for formation and modulation of CNS, its precise function and molecular signaling mechanism/s are rather unclear.

1.4 WNT SIGNALING AND DOPAMINERGIC CIRCUITS

1.4.1 Signaling centers during the brain development

The brain is an ectodermal structure that starts being shaped during gastrulation. The first neural tissue is the neural plate, which is formed by a flat sheet of neuroepithelial cells. During convergent extension, the neural plate starts to fold (neurulation), until the two edges fuse dorsally, to form the neural tube, the future brain and spinal cord. The neural tube then undergoes neuronal patterning by the action of the so-called signaling centers. These centers are located in specific positions, such as the floor plate or the midbrain-hindbrain boundary and secrete specific combinations of signaling molecules, which provide spatiotemporal information along the tube that determines the anterior-posterior and the dorsal-ventral identity [183]. In this chapter, I will talk about the floor plate, the midbrain-hindbrain boundary and the choroid plexus, three signaling centers that are conserved in vertebrates [183-186].

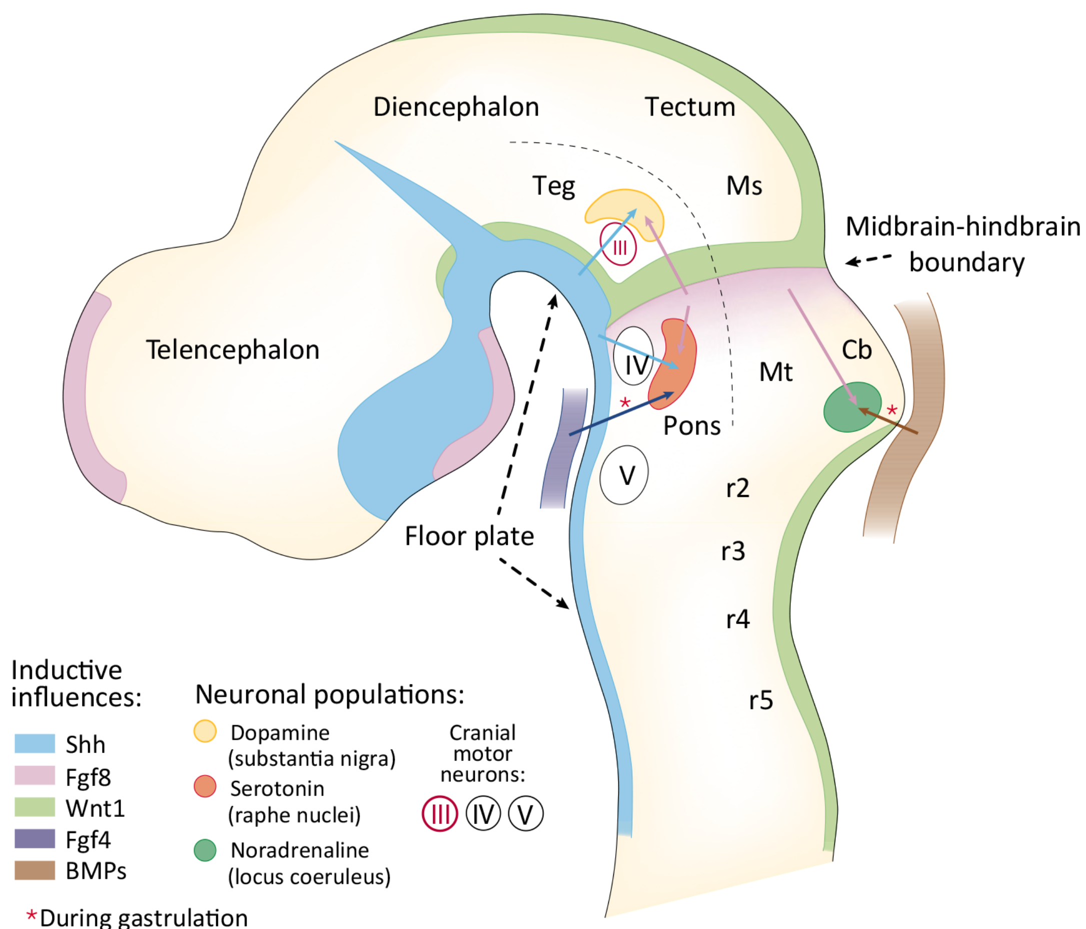


Figure 9: Mouse brain during embryogenesis. The spatiotemporal signals that control neurogenesis, specification and neuronal maturation during the brain development are secreted in concentration-dependent manner from the signaling centers such as floor plate and midbrain-hindbrain boundary. Modified from [5].

Floor plate (FP). The floor plate is found at the most ventral part of the entire neural tube, from the anterior brain to the spinal cord. The FP contributes to ventral-dorsal patterning, cell specification and cell migration, by sequential secretion of morphogen and creation of signaling gradients (**Figure 9**). The FP is the main source of Shh and Netrin1, and together with BMPs which are derived from the roof plate, control cell polarization and identity along the ventral-dorsal axis of the neural tube. The FP also expresses Slit and Robo proteins, which regulate ipsilateral organization of the commissural neurons by stopping their axons from crossing the midline, and thus creating bilateral symmetry of the neural tube [183, 187, 188].

The FP first contains neuroepithelial stem cells that differentiate into radial glia cells, which act as the main signaling center during development. It is for this reason that the FP is considered a glial structure. To our knowledge, FP radial glia cells can undergo neurogenesis only in the ventral midbrain but not in the other regions. As a consequence, the midbrain FP also contains neurons [184, 187, 189].

Gene expression patterns in the FP change during embryogenesis, depending on their position in the AP axis in the neural tube. In the ventral midbrain, the floor plate is the main source of not only Shh, but also Wnt1 and Wnt5a, which provides the additional signals and instruction for neurogenesis, and maturation of midbrain dopaminergic (mDA) neurons, which will subsequently acquire A9/*substantia nigra* and A10/ventral tegmental area identity and will integrate, into cell subtype specific neural circuits [49, 89, 185]. Nevertheless, we do not yet understand the precise cellular and molecular mechanism orchestrated by the FP during the VM development.

Midbrain-hindbrain boundary (MHB). During vertebrate embryogenesis, the midbrain ends caudally as a constriction, which is connected to the hindbrain via midbrain-hindbrain boundary (also called isthmic organizer). The MHB constriction is initiated soon after the neural tube closure. That includes shortening of the cells, laminin-dependent basal constriction, inflation and adhesion of the ventricle at the midline, and peripheral midbrain layer (PML) formation. Consequently, any defects in the MHB lead to a loss or abnormal development of the midbrain, hindbrain and cerebellum [5, 190-192].

The MHB is characterized by the specific expression of Wnt1 in the anterior, midbrain side of the MHB, and Fgf8 in the posterior, hindbrain side. It also expresses transcription factors Pax-2, Pax-5 and Engrailed-1 (En-1), which further contribute to the development of mDA neurons [5, 193-195]. The function of this embryonic signaling center is not only to secret morphogens, such as Wnt1 and Fgf8, and thus to provide spatiotemporal information, but importantly, it also builds a physical barrier between two distinct brain regions. The position of the MHB is determined and maintained by expression of two mutually repressive signals, the homeobox proteins Otx2 in the midbrain side and Gbx1/2 in the hindbrain side (**Figure 10**). In zebrafish, the activation and the expression of Otx2 and Gbx1/2 is regulated by

gradients formed by Wnt8a, which is secreted by lateral mesodermal precursors. The loss of Wnt8a moves the position of the MHB posteriorly. However, Gbx1/2 and Otx2 maintain the barrier function, avoiding thus alterations in migration and axonal pathfinding [5, 190, 196]. Loss-of-function experiments in zebrafish have also revealed that Wnt1/Fgf8 expression is crucial for the MHB morphogenesis, and sub-sequential development of the midbrain, hindbrain and cerebellum [197-200].

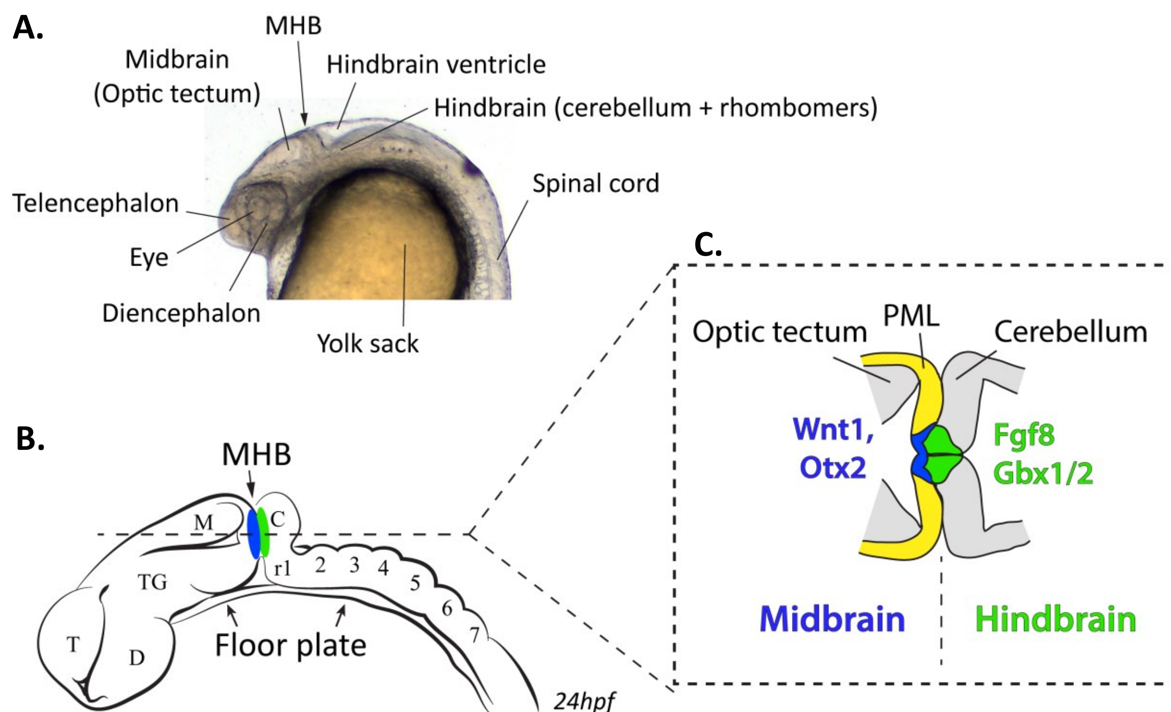


Figure 10: The anatomy of the zebrafish brain with the structurally distinguished MHB at 24hpf. A. The bright field photo of live, transparent embryo. **B.** A scheme of the fish brain at this stage. **C.** A scheme of MHB transverse section with the distinct expression patterns separating the midbrain region from hindbrain. TG = tegmentum, r1-7 = rhombomeres, PML = a peripheral midbrain layer [7].

Some studies suggested that by creating concentration gradients, Wnt1 and Fgf8 control the anterior-posterior orientation of the neural tube during patterning. This is supported by the fact that Wnt1 and Fgf8 are expressed already during gastrulation at the blastoderm margin and nascent paraxial mesoderm, which probably define the correct MHB position before contracting the neural tube and creating the actual boundary [5, 183, 190]. The MHB will later give rise dorsally to the cerebellum and part of tectum; and ventrally to diverse cell types including mDA neurons [5, 191, 201, 202].

Choroid plexus (ChP). The cerebrospinal fluid (CSF) is the so-called third circulation system in mammals. The nervous system uses this system to deliver nutrients, oxygen and ions to the brain parenchyma, and exchange them with metabolites and toxins which need to be removed in order to maintain the homeostasis in the tissue [203]. The CSF is produced by filtering plasma from the blood, mostly via choroid plexus (ChP). The ChP is a highly

vascularized, folded structure growing inside each of the 4 cerebral ventricles. The ChPs are composed of a monolayer of polarized epithelial cells that contain microvilli on their apical side (facing the ventricle). The basal membrane of the epithelial cells separates them from a neighboring inner stroma, which is composed of connective tissue and contains leukocytes. Leukocytes migrate into the ChP stroma through the fenestrated endothelium of the choroidal capillaries (**Figure 11**) [204].

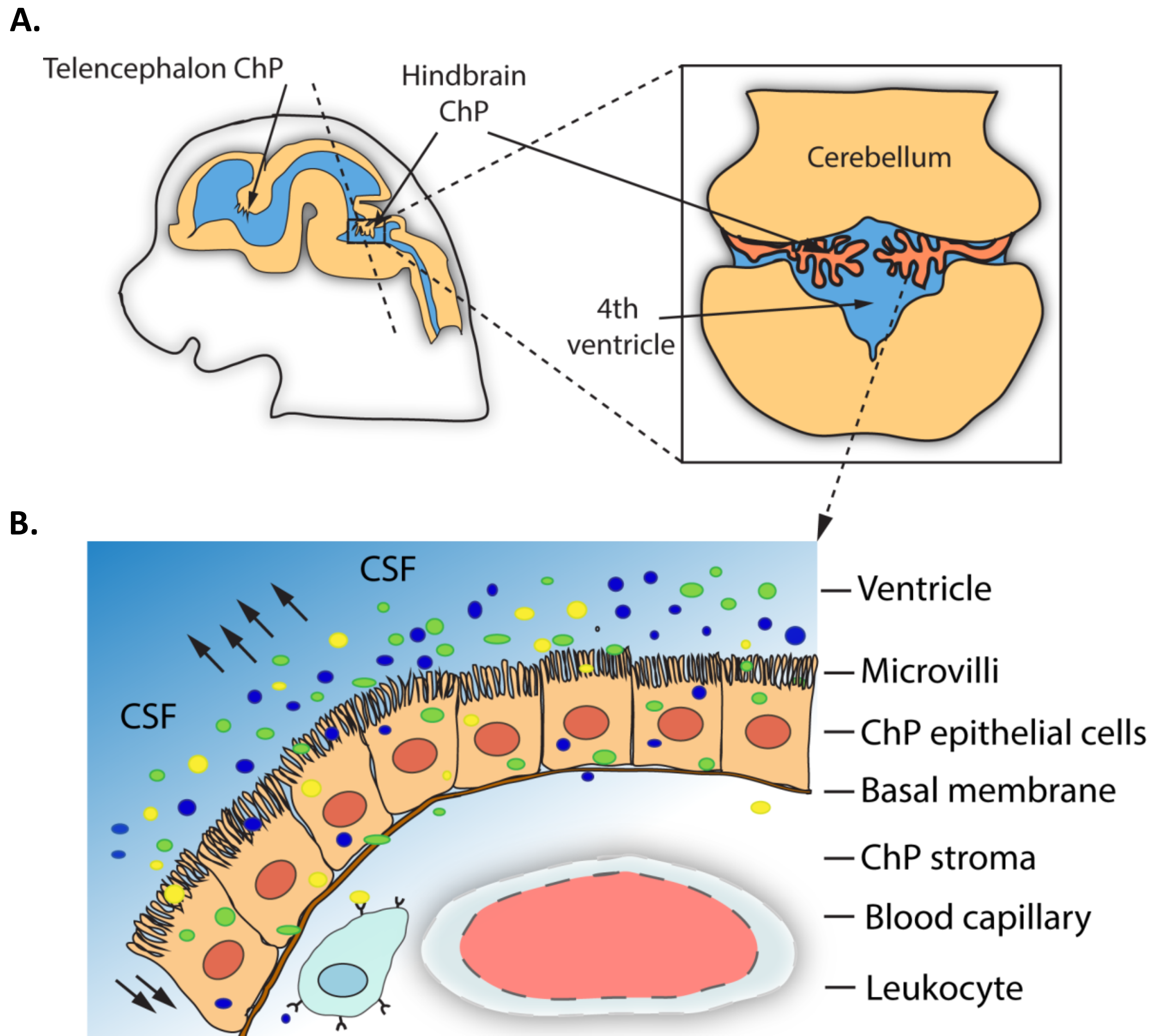


Figure 11: A scheme of hindbrain ChP in mouse E17.5 embryos. A. A sagittal view of the mouse brain with a coronal view at the HbChP. **B.** Ciliated epithelial cells of choroid plexus are interconnected with tight junctions. There is a constant exchange of the trophic factors between the epithelial cells and leukocytes in the ChP stroma.

ChP plexi differentiate from distinct lineages in roof plate at different times of the development, with the earliest being the hindbrain ChP in the 4th ventricle, which can be structurally recognized already around E12 in mice [186, 205]. It has been shown that the maturation of the ChPs from distal to proximal side within ChP involves gradients of Aquaporin 1 (AQP1) and glucose transporter 1 (Glut1), which were shown to regulate proliferation in the ChP root zone. Thus it is proposed that AQP1 and Glut1, together with

Calbindin 1 and Proliferating cell nuclear antigen (PCNA) serve as functional markers of correct ChP development and maturation in mouse and human [186, 206].

It was previously thought that the ChP, together with the blood brain barrier, serve only as a circulation barrier between the blood and the CSF. However, recent studies have suggested that the epithelial cells actively secret signaling molecules themselves, and thus control brain development and CSF proteome composition [186, 207, 208]. The epithelial cells of ChP are also known to filter trophic factors and cytokines from the blood to the CSF through their tight junctions. Microvilli and the folding of ChP greatly enlarge the ChP surface increasing the efficacy of the CSF production. The CSF is then transported through the ventricles to the rest of the CNS where its components are captured by the ciliated ependymal cells and other progenitors such as radial glia cells. Moreover, it has been shown that the immune cells localized in the ChP stroma actively communicate with the ChP epithelial cells, e.g. by providing cytokines such as inteferon1/2 [209]. In the healthy brain, the tight junctions between the epithelial cells usually do not allow any cell type to pass, but recent studies proposed otherwise in case of the Th1 lymphocytes [204, 209].

It has been reported that the deletion of Otx2 in the hindbrain ChP causes upregulation of Wnt4 in the CSF and the Wnt4 expression in the hindbrain ChP. This study proposed a role of the ChP in regulating the CSF composition and Wnt signaling. However, it is unclear whether it is Wnt4 or another factor that controls proliferation at a distant site *in vivo* [210]. Importantly, a recent transcriptome analysis of FACS-sorted epithelial cells from lateral/telencephalic and 4th ventricle/hindbrain ChPs revealed that these two structures are molecularly very heterogeneous. Their gene ontology analysis showed that the biggest gene clusters in both data sets encode secreted proteins. Wnt8b was specific for the telencephalic ChP, whereas Wnt5a was specific for the hindbrain ChP as assessed by qPCR. They thus proposed that ChPs may contribute to the so-called regionalization of the developing brain by expressing different morphogens [186].

Notably, alterations in the function of the ChP have been proposed in neurodegenerative diseases such as Alzheimer disease based on transcriptomic analysis [209]. Nevertheless, not much is known about the development or, the mechanisms by which the ChP bestirs the CSF, what signaling molecules are secreted by its epithelial cells, and how do they affect the developing CNS. Moreover, it remains to be determined how lipophilic molecules such as Wnts are transported via the CSF [210].

1.4.2 Role of Wnts in the development of midbrain dopaminergic neurons

The neurotransmitter dopamine belongs to the catecholamine family and is crucial for controlling motor function, reward-motivated behavior, emotional responses, and the release of several hormones. Multiple populations of DA neurons have been identified in distinct brain regions by the presence of typical markers such as the dopamine transporter

(DAT) or a more general marker, tyrosine hydroxylase (TH), an enzyme necessary for dopamine synthesis [49]. The largest and most important dopamine-synthesizing neuron populations are localized in the ventral midbrain (VM), in the *Substantia Nigra pars compacta* (*SNpc*), or A9 region, and the ventral tegmental area (VTA), or A10. It has been shown that the A9 population controls motor function and is particularly vulnerable to stress, and selectively degenerates in PD Parkinson's disease (PD), one of the most common neurodegenerative disorders at present.

A9 and A10 populations are formed in the floor plate from mDA progenitor cells in the ventricular zone (vz), from where they further migrate and differentiate through the intermediate zone (iz) and to the marginal zone (mz). The most critical Wnts for development of these two populations are Wnt1 and Wnt5a, and thus I will focus on them.

Expression of Wnts in the VM: Midbrain DA neurons are born in the VM floor plate between embryonic day 10.5 (E10) and E14 in mice. The formation of the ventral midbrain region is highly dependent on the correct expression of morphogens secreted from the floorplate and MHB, as discussed above. It has been previously shown that Wnt1 controls the anterior-posterior identity, whereas Shh is crucial for the ventral-dorsal specification during the VM patterning. Shh is expressed in the VM between E8.5-E11.5. Wnt1 is first expressed in the in the MHB and in the midbrain roof plate between E10.5-E12.5, and in two distinct stripes in the lateral feature of the midbrain FP [5, 8, 191]. On the other hand, Wnt5a is expressed heavily in the VM from E9.5-E11, and its expression restricts into the midline of VM floor plate between the E11.5-E13.5 [89]. Our group performed a single cell RNA sequencing of mouse and human midbrain [49], as well as bulk RNA-sequencing of mouse midbrain regions [9], and characterized different cell types according to their expression profiles during midbrain development. Interestingly, these studies determined that there are three types of radial glia (Rgl) in the midbrain, and revealed that *Wnt5a* is expressed by Rgl3, Rgl1, and progenitor cells in mouse and human. *Wnt5a* is not by the Rgl2. *Wnt7a/b* is also expressed by Rgl3 in human and by Rgl1-3 in mouse. On the contrary, *Wnt1* was expressed by the different progenitors and Rgl1, but it was not expressed by the Rgl3. The bulk RNA-sequencing further determined that *Wnt3a* is probably expressed by the ependymal cells [9].

Wnt1 and Wnt5a activate distinct Wnt signaling pathways, and thus Wnt1 promotes mostly mDA progenitor pool proliferation, DA neurogenesis and VM patterning, whereas Wnt5a has an important function in mDA differentiation and A-P elongation as shown by Wnt1$^{-/-}$ and *Wnt5a*$^{-/-}$ mice [89, 191, 211]. However, these two pathways regulate each other and often crosstalk, sometimes in a synergistic manner, typically resulting in more severe Wnt/PCP or canonical Wnt phenotypes as shown by LOF experiments in e.g. *Wnt5a*$^{-/-}$ in the skin tissue [91] or in *Wnt5a*$^{-/-}$*;Lrp6*$^{-/-}$ mice as dramatic worsening of the Wnt/PCP defects during embryogenesis [212]. Similarly, the analysis of *Wnt1*$^{-/-}$*; Wnt5a*$^{-/-}$ mice showed that

Wnt1 and Wnt5a functionally cooperate, and their simultaneous LOF resulted in more severe Wnt/PCP phenotype such as A-P shortening, greater loss of DA neuroblasts and neurons, and VM morphogenesis seen as flattened ventricle compared to the single KO animals. TH+ cells were also positioned more dorso-laterally in the basal plate than in the *Wnt1*$^{-/-}$ mice [211]. This spatial, synergistic effect of Wnt1 and Wnt5a on mDA lineage development is now applied in differentiation protocols to derive mDA neurons from stem cells and induced pluripotent stem cells in a more efficient manner [201]. All together, these studies revealed that various Wnts are expressed in the VM by different cell types which likely correspond to their different but equally essential function in controlling the VM morphogenesis and mDA lineage development.

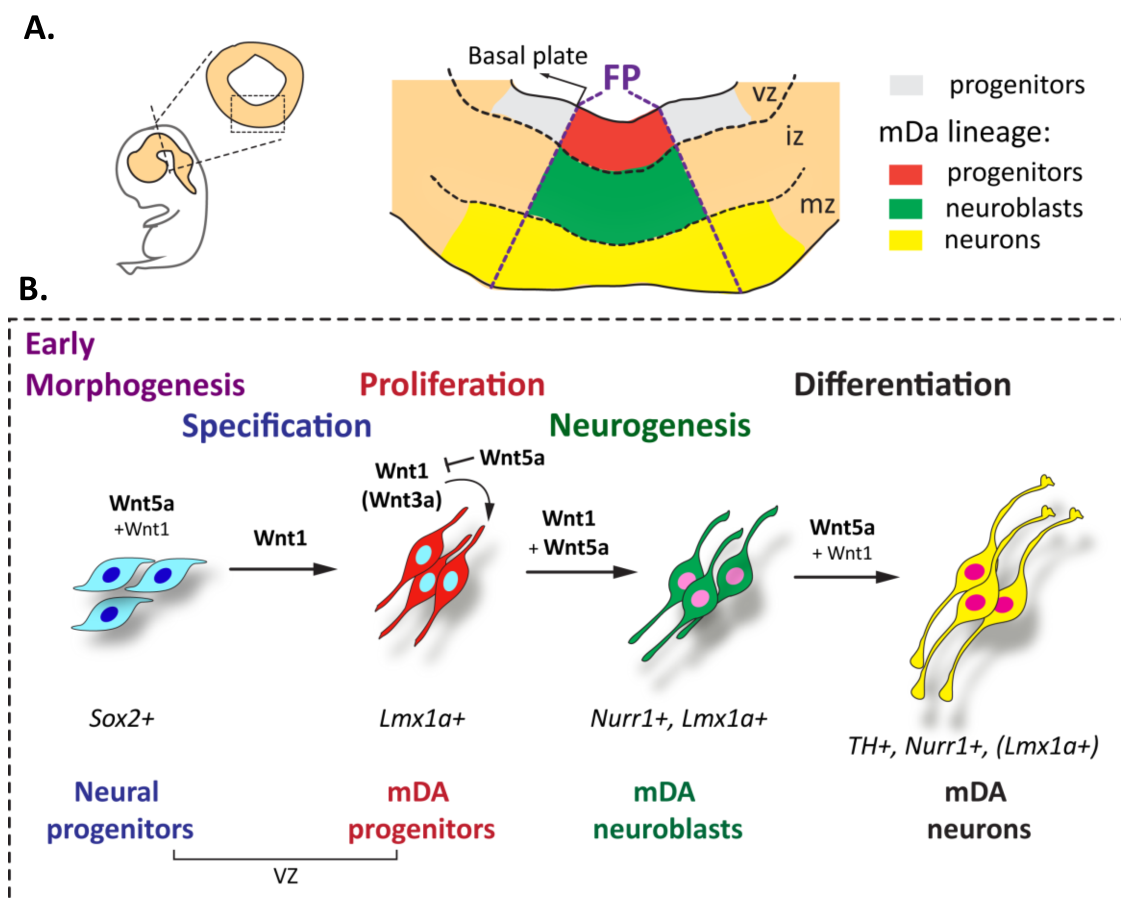


Figure 12: The development of ventral midbrain and mDA lineage is controlled by Wnt1 and Wnt5a. A. A scheme of the coronal section of human VM with highlighted floor plate (dashed purple lines); ventricular (vz), intermediate (iz) and marginal zones (mz; dashed black lines), and regional distribution of mDA lineage. **B.** A scheme of the mDA lineage development and function of Wnts in the particular stages. Wnt1 and Wnt5a show synergistic effect in different developmental events. Wnt1 has a critical role in mDA specification, Wnt5a is a key mediator of mDA differentiation. Drew according to [8, 9].

mDA progenitors are Sox2+, proliferative cells that can be found in the ventricular zone (VZ) of the FP, and which are in contact with the ventricular cavity. These progenitors include first neuroepithelial cells and then radial glia cells. Both cell types have the capacity of undergoing neurogenesis and give rise to postmitotic neuroblasts that will then differentiate

into mDA neurons. During early stages, Wnt1 and Shh control the pattern and expansion of the mDA progenitor pool in the VZ. The mDA progenitors are characterized by the expression of the LIM homeobox transcription factors Lmx1a and Lmx1b that specify the mDA lineage. The expression of *Lmx1a/b* genes is controlled by Wnt1 and Shh, which also regulate the expression of several additional transcription factors essential for mDA neuron development such as FoxA2, Engrailed 1 and 2 and Otx2. While Wnt1 promotes, Wnt5a inhibits the proliferation of mDA progenitors [8, 49, 201].

mDA neuroblasts are the first postmitotic cells in the mDA lineage. These cells are generated by mDA progenitors via neurogenesis, a process that finishes by E14.5. These cells express the nuclear receptor Nurr1/Nr4a2 and are thus Nurr1+, Lmx1a+ double positive. mDA neuroblasts migrate along the radial glia process through the intermediate zone (IZ) towards the marginal zone (MZ). They express the Cxcr4 receptor, and are attracted by the cytokine CXCL12 which is secreted from the meninges [213]. During their migration, neuroblasts start to differentiate into mature TH+ DA cells. While Wnt1 predominantly controls neurogenesis, and the emergence of Nurr1+ neuroblasts, Wnt5a regulates the maturation mDA neuroblasts into mDA neurons *in vivo*. Nevertheless, Wnt1 and Wnt5a both contribute to VM morphogenesis, neurogenesis and differentiation of mDA neuroblasts into mDA neurons. However, the mechanism by which Wnt1 and Wnt5a signaling control and coordinate these functions is not completely understood [8, 211].

Mature mDA neurons: After radial migration, mDA neuroblasts reach the marginal zone (mz) of the mFP, and mature into mDA neurons that can be identified as double Nurr1+ and TH+ cells. They subsequently migrate tangentially towards lateral positions where they postnatally form the *SNpc* and VTA populations. As mDA neurons emerge, their axons start to extend and navigate towards their targets. The A9 population mainly projects to the striatum, forming the nigrostriatal pathway, while the A10 neurons innervate cortical and limbic structures. In mice, these 2 populations account for about 20.000-30.000 mDA neurons, and over 400.000 in humans [214, 215]. The early development of mDA neurons is schematized in **Figure 12**.

1.4.3 Wnt signaling in the CNS

Wnts hold important functions in neuronal maturation and maintenance of the brain circuits, as shown by several *in vitro* and *in vivo* studies discussed in the previous chapter. Different Wnt ligands and their receptors are expressed in various brain areas in the postnatal and adult brain, particularly in those undergoing continuous neurogenesis or active synaptic remodeling such as dentate gyrus of hippocampus (Wnt3a, Wnt7a, Wnt8), olfactory bulb (Wnt1, Wnt3a, Wnt5a, Wnt7a) and cerebral cortex (Wnt2b, Wnt5a, Wnt7a) [216]. The functional activity of Wnts, such as Wnt5a, in these cell types has been linked to neurogenesis, axonal outgrowth, synaptogenesis, dendritogenesis, and synaptic plasticity [13].

Wnt proteins are localized at both sides of the synaptic bouton. Wnt5a/JNK axis was found to regulate postsynaptic bouton in hippocampal neurons by increasing the clustering of the postsynaptic density protein PSD-95 in the excitatory neurons [87] and $GABA_A$ receptors and their recycling [86]. On the other hand, the exogenous Ror2 is localized in dendrites of hippocampal neurons in close proximity to the synaptic area. There it regulates the dendritic spine morphology which was defected in Ror2-ΔCRD but not in the Ror2 mutants lacking the intracellular domains [105]. Similarly, Wnt7a/b was enriched on the postsynaptic side in pyramidal neurons in the C3 region of the hippocampus, together with the increased synaptogenesis upon the enriched environment [217]. Vangl2 was shown to physically bind N-cadherin and PSD-95 receptor in hippocampal neurons by which it increased synaptogenesis, synaptic markers clustering and dendrite spine formation, as shown also for Prickle2. Moreover, β-catenin competes with Vangl2 for the binding to N-cadherin which can inhibit the signaling. Vangl2 can be internalized via Rab5 [218]. Deregulated Wnt/β-catenin signaling was found impaired in *Drosophila* dopaminergic neurons in a Parkinson's disease model [219].

It has been recently promoted that the activity of various Wnt signaling proteins control the right ratio between the excitatory and inhibitory neurons whose deregulation leads to neurological disorders. It has been shown that Wnt5a increases the dendritic spine formation during development, amplitude of excitatory NMDA currents, intracellular calcium, and excitatory postsynaptic potentials in hippocampal slides [220]. Moreover, Wnt/Ca^{2+} signaling was shown to activate Ror2 which mediated the neuronal excitability via triggering the surface expression of N-methyl-p-asparate receptors (NMDARS), proteins impaired in schizophrenia and AD [107, 220, 221]. Strikingly, conditional *Celsr3*$^{-/-}$ mice show a 50% decrease in excitatory glutamatergic but not in inhibitory neurons in CA1 region of hippocampus resulting in spatial learning, memory and fear deficits. On contrary, *Vang2l*$^{-/-}$ mice showed an increase in synaptic density suggesting the opposing function maybe via asymmetric localization in the synapsis [222].

Importantly, Wnts have been functionally involved in neuroprotection and regeneration of the CNS. Wnt/β-catenin signaling can induce neuronal regeneration of the mammalian retina after injury or during degeneration [223], as well as the glial-dependent regeneration after the spinal cord injury [224]. It has been shown that the pretreatment with Wnt5a has a neuroprotective effect and prevents synaptic damage induced by Amyloid-β25-35 in CA1 region in Alzheimer disease (AD) models, whereas the rats treated with the Wnt5a antagonist SFRP showed learning and memory deficits, similarly to the Wnt modulator Dickkopf-3 [94, 225]. The capacity of Wnt5a to promote multiple aspects of mDA neuron development [226-228] has been applied in differentiating protocols to generate electrophysiologically mature mDA neurons *in vitro* [201]. Such findings have opened novel therapeutic opportunities for Wnts in neurological disorders.

1.4.4 Wnts and Parkinson's disease

Parkinson's disease is one of the most common neurodegenerative disorders. At the diagnosis stage, the PD patients have already lost around 60% of the DA neurons in the *Substantia nigra* causing the typical motor symptoms of this disease, such as resting tremor, rigidity and hypokinesia. At later stages, also other brain regions are affected, and patients may also suffer cognitive impairment, dementia and/or depression. PD is currently considered as a multifactorial disease with large genetic variations, and thus our understanding about the cause and PD progression is still very poor [229]. Since current PD treatments are only symptomatic, more efforts are currently being made to understand mDA neuron biology, its deregulation in PD patients, and the development of targeted therapies to stop the disease progression.

Parkinson's disease includes various forms, but they share the same motor disturbances. At the pathological level, PD is characterized by a progressive loss of mDA neurons located in the *SNpc*, the formation of Lewy bodies containing aggregated α-synuclein filaments and denatured proteins, and the hyper-phosphorylation of microtubule-associated protein Tau protein [229, 230]. Only around 10% of PD cases are considered genetic forms [231]. Abnormally increased oxidative stress and mitochondrial dysfunction, together with protein misfolding, and impairments in the ubiquitin-proteasome and autophagy-lysosomal systems, contribute to PD progression. Deregulated function of several proteins has been found in genetic forms of PD, such as Parkin, Leucine-rich repeat kinase 2 (Lrrk2), Tau, α-synuclein, Serine/Threonine protein kinase Pink1 and Protein/nucleic acid deglycase DJ-1 [232, 233].

Besides the proposed physiological function of Wnt signaling in the CNS, not much is known about the importance of Wnt signaling in PD. Increasing evidence has suggested that Wnt/β-catenin signaling pathways might be deregulated via their defective communication with abnormally functioning PD proteins, such as Lrrk2. Lrrk2 is cytoplasmatic protein involved in autophagy [234-236], vesicle trafficking/sorting via cytoskeletal remodeling [237-239], and in mitochondrial dynamics [240-242], thus several processes impaired in mDA neuron degeneration. Lrrk2 has been found to interact with multiple signaling pathways, including Wnt/β-catenin signaling. It has been shown that overexpressed Lrrk2 forms a protein complex with Dvl1-3 [243], and brings them to the plasma membrane where it further interacts with Lrp6. Together they subsequently trigger the expression of TCF/LEF transcription factors, and thus activate the Wnt/β-catenin pathway. These overexpression experiments were further supported by the co-immunoprecipitation (co-IP) of Lrrk2 with Dvl3, GSK3β, Axin and β-catenin in the adult mouse brain, and downregulation of Wnt/β-catenin signaling in mouse fibroblasts from Lrrk2 KO mice [244, 245]. Moreover, it has been shown that Parkin, an ubiquitin E3 ligase, interacts with β-catenin, and regulates its degradation. It was also found that *Parkin null* mice exhibit high levels of β-catenin, and that acute escalations of β-catenin levels in mDA neurons *in vitro* induce PARP-1 cleavage and

mDA neuronal death [246]. Notably, Pink1 and DJ-1 form an ubiquitin E3 ligase complex with Parkin [247]. Lrrk2 also interacts with Parkin [248] and with Tau in a tubulin-dependent manner [249], suggesting that these proteins could function together and interact or regulate Wnt/β-catenin signaling. Later studies also have shown that Wnt-dependent cell polarity and vesicle recycling might be deregulated in PD patients [60, 245]. More evidence should be thus collected in order to understand the implications of deregulated Wnt signaling in the pathophysiology of Parkinson's disease.

2 AIMS OF THE STUDY

The main focus of this doctoral study was to identify novel regulators of the Wnt/PCP pathway, to describe new mechanisms of the Wnt/PCP signal transduction, and to explore their function during embryogenesis and mDA neurons development. As such, this study provides new insights about Wnt/PCP signaling during embryogenesis, particularly in CNS development, and discusses its possible implications in Parkinson's disease.

These specific aims define each study:

1. **Study I:** How does Wnt signaling regulate mitosis? What is the function of Dvl proteins in this process? What is the mechanism?
2. **Study II:** Is Wnt5a secreted by the choroid plexus? Does Wnt5a regulate development of the choroid plexus? How is Wnt5a transported in the CNS?
3. **Study III:** Are Ror2 and Vangl2 receptors important for ventral midbrain morphogenesis and development of mDA neurons? Do they signal via Wnt5a-Ror2-Vangl2 axis in the ventral midbrain development?
4. **Study IV:** What proteins bind to Ror2 in dopaminergic cells and in the ventral midbrain? What is the mechanism of their signal transduction? What is the function of these protein complexes during embryogenesis and in the ventral midbrain development?
5. **Study V:** Does Lrrk2, a protein with altered function in PD, control Wnt/PCP signaling? What Wnt/PCP components bind to Lrrk2 in dopaminergic cells? How does Lrrk2 crosstalk with Wnt/β-catenin and Wnt/PCP pathways?

To obtain more comprehensive information about the Wnt/PCP signaling, we investigated in detail the biochemistry behind the novel protein interactors identified in this thesis. We combined several advanced approaches such as proteomics, CRISPR/Cas9 system, RNA-scope *in situ* hybridization, confocal microscopy and single cell RNA sequencing together with traditional biochemical methods such as immunoprecipitations, western blotting, immunofluorescence and others. Functionally, we took advantage of *X. laevis and D. rerio* developmental models, which allowed an easy genetic manipulation, relative quantification of the Wnt/PCP activity, and determination of what function the novel protein complexes have during the embryogenesis. In some of our studies, we also used several transgenic mouse models which enabled a more complex analysis about the possible function of these protein complexes in the mammalian CNS. Finally, we also integrated the single cell RNA sequencing data from the developing human VM tissue in order to determine the possible relevance of our findings in human.

3 RESULTS & DISCUSSION SECTION

3.1 STUDY I: DISHEVELLED IS A NEK2 KINASE SUBSTRATE CONTROLLING DYNAMICS OF CENTROSOMAL LINKER PROTEINS

3.1.1 Introduction

Wnt signaling contributes to the cell cycle regulation [21-23, 27]. Dvl, a crucial signaling integrator of Wnt signaling pathways, has been recently found in several centrosomal stuctures [24-26]. Nevertheless, it was not clear if Dvl controls the centrosomal cycle and what is the possible mechanism. In this study, we performed a comprehensive biochemical study using different cell lines, phospho-proteomics, a panel of Dvl mutants, Fucci-based cell sorting [250, 251], and loss and gain of function experiments in order to describe the molecular pathway by which Dvl isoforms, Dvl1, Dvl2 and Dvl3, regulate the centrosome and cell cycle progression.

3.1.2 Results and discussion

By performing immunofluorescence and cellular fractionation, we confirmed that endogenous Dvl1, Dvl2 and Dvl3 co-localizes in the centrosome together with the centrosomal linker proteins Pericentrin (**Figure 13**), C-NAP1, CEP164, CDK5Rap2, γ-tubulin, and Rootletin. By transfecting low levels of Dvl isoforms, we further show that localization of exogenous Dvl is in close proximity with pericentrin, similar to the endogenous protein. We then examined different Dvl truncated mutants in order to distinguish, which Dvl domain is necessary for Dvl localization in the centrosome. We found that the Dvl-DIX domain, a domain required for polymerization of Dvl molecules during Wnt signaling activation, is necessary for Dvl localization in the centrosome. Our

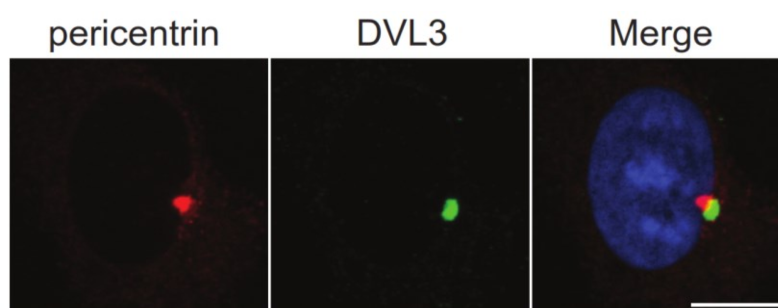


Figure 13: The co-localization of Dvl3 with Pericentrin in HEK293 cells was assesed by IF. Scale bar is 10 µm.

findings are thus in line with the previous observations showing that the Axin-DIX domain is required for its centrosomal localization [22, 23]. Interestingly, the DVL2-M1(F43S) mutant, a multimerization-defective protein which can form dimers with endogenous DVL, remained localized in the centrosome. Therefore we concluded that DVL polymerization is not required for its localization to centrosomes.

Functional screens in *Drosophila* revealed that Dvl is phosphorylated by NEK2 kinase. We confirmed that Dvl co-localizes with NEK2 kinase in the centrosome, and that endogenous and exogenous Dvl binds NEK2 WT via the Dvl-PDZ domain but does not bind to the NEK2 kinase-dead mutant. We next investigated which phosphorylation sites of Dvl are directly regulated the NEK2 kinase activity, and used phospho-proteomics tool and an *in vitro* kinase assay with a panel of specific phospho-Dvl antibodies. We observed that NEK2-dependent phosphorylation of Dvl phospho-sites changed during the cell cycle and affected the subcellular localization of Dvl in the cytosol in "even" distribution of Dvl (S643 phospho-site) or in the centrosome (pT15, pS697 phospho-sites). Moreover, Dvl-pS697 accumulated with the cell cycle progression with a peak in the M phase. We also identified the pS280 phosphorylation site being specific for localization of Dvl in mitotic spindle uniquely during the M phase. These data showed that Dvl is a substrate for NEK2, and that NEK2 phosphorylates Dvl at different sides dependent on the cell cycle phase.

Next, we took advantage of the Fucci system (**f**luorescent **u**biquitination-based **c**ell **c**ycle **i**ndicator), which is a molecular tool based on reciprocal expression of two cyclin proteins, chromatin licensing and DNA replication factor 1 (Cdt1) and its negative regulator Geminin that accumulate in the different phases of the cell cycle [251]. In the Fucci system, these two genes are fluorescently labelled in red and green channels, and as they cycle through the phases in different concentrations, they label cells' nuclei with the distinct colors. We can thus distinguish and visualize the cell cycle phase in the single cell by FACS or confocal microscopy, both in living or fixed cells without applying any synchronization agents that are usually cytotoxic. G1 cells show red nuclei, G1/S-early S phase cells orange, S-phase light green, and G2/M phase cells are bright green. Newly divided cells, and cells in the G0 phase are not fluorescently labelled. We used a transgenic Fucci line of HeLa (**He**nrietta **La**cks) cells for our studies.

Our data from HeLa-Fucci cells sorted into different cell cycle phases [250] revealed that Dvl accumulated in the G2/M phase, similarly to the centrosomal linker proteins and NEK2 kinase (**Figure 14**). We thus hypothesized that Dvl in a complex with the centrosomal linker proteins controls configuration of the centrosome during mitosis. Indeed, when we knocked down all Dvl isoforms (Dvl1-3) using siRNA, we observed defects in centrosomal separation, which was not so apparent in the single Dvl knock-down (KD), possibly due to the isoforms redundancy. Dvl KD did not cause defects in localization of the centrosomal linker proteins nor in the centrosome morphology as analyzed by the electron microscopy. These data show that Dvl is not crucial for the centrosomal linker structure, but it is functionally important for the centrosomal separation.

By performing another set of phospho-proteomics, we show that Dvl is not required for the NEK2-dependent phosphorylation of the linker proteins. However, NEK2 was able to remove Dvl from the centrosome in a kinase-activity dependent manner, similarly as it does for C-NAP1 [252]. We thus asked whether NEK2 is in a complex with Dvl during the centrosomal separation. We show that Dvl was able to displace C-NAP1 and CDK5/Rab2 from the centrosome similar to NEK2 itself. When we overexpressed Dvl in higher levels we observed an increase in multinuclear cells which usually occurs when the centrosomal function is disturbed, typically creating a monopolar mitotic spindle. Similar defects were observed when overexpressing dominant negative NEK2 [18]. These observations were confirmed by the Dvl3-ΔDIX which failed to cause such defects. We tested several Dvl phospho-mutants and identified that the formation of the monopolar spindle is dependent on the lack of the sequential Dvl phosphorylation by NEK2, especially at the C1 and C2 phospho-clusters, and at the S697 residue of Dvl3. We thus concluded that Dvl mediates NEK2-triggered displacement of linker proteins from centrosome via phosphorylation of Dvl on its C-terminus.

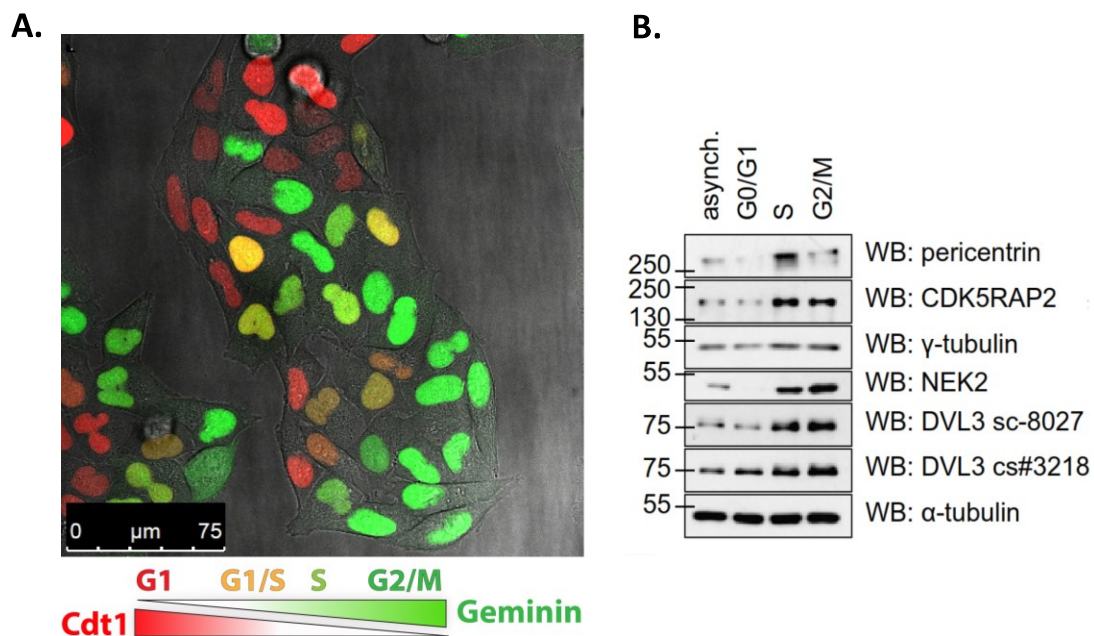


Figure 14: HeLa S. Fucci cell line. A. A photo of HeLa-Fucci by confocal imaging. **B.** Sorted Fucci populations show that Dvl accumulates in the G2/M phase together with Nek2 kinase

Last but not least, we tested whether the NEK2-Dvl-mediated separation of the linker proteins from the centrosome is dependent on Wnt/β-catenin activity. We also asked whether the function of Dvl in the ciliogenesis where it binds to other centrosomal proteins, also requires the phosphorylation by NEK2 [253]. Indeed, we observed that NEK2 affected the interaction of Dvl with Inversin but not with Chibby nor with CEP164, proteins important in ciliogenesis. These data indicate that probably the NEK2-Dvl complex requires the presence of other proteins during ciliogenesis. To evaluate the

involvement of Wnt/β-catenin signaling, we used a TOPFlash assay to measure the activation of TCF/LEF genes. TOPFlash is a dual luciferase assay based on overexpression of plasmid with eight TCF/LEF repeats (Super8x) to measure their Wnt/β-catenin-dependent expression [166]. This signal is then normalized to the luciferase signal of transfected, constitutively active cnidarian protein Renilla, which represents overall translational activity in the cells. Our TOPFlash experiments showed that neither NEK2 alone or in presence of Dvl mediate the Wnt/β signaling. Nevertheless, NEK2 increased Wnt/β-catenin signaling in the presence of CK1ε and Dvl. This finding was further confirmed by the knock-down experiments and exogenous treatment of Wnt3a.

To conclude, we proposed a novel mechanism of how Dvl, upon sequential phosphorylation by NEK2, regulates the centrosomal cycle by displacing the centrosomal linker proteins C-NAP1and CDK5Rab2 from the centrosome during the G2/M phase. We further suggest that phosphorylation of Dvl on multiple sides by NEK2 and CK1ε kinases leads to subsequent activation of Wnt/β-catenin signaling (**Figure 15**). We also propose that the Dvl-NEK2 complex might be of importance in other centrosomal structures, such as the basal body of primary cilia where it probably requires additional protein interaction. As Wnt signaling and the correct position of the centrosome control subcellular polarity, we thus speculate that Dvl, when localized in the centrosome, contributes to such re-organizations, a possibility which should be further investigated.

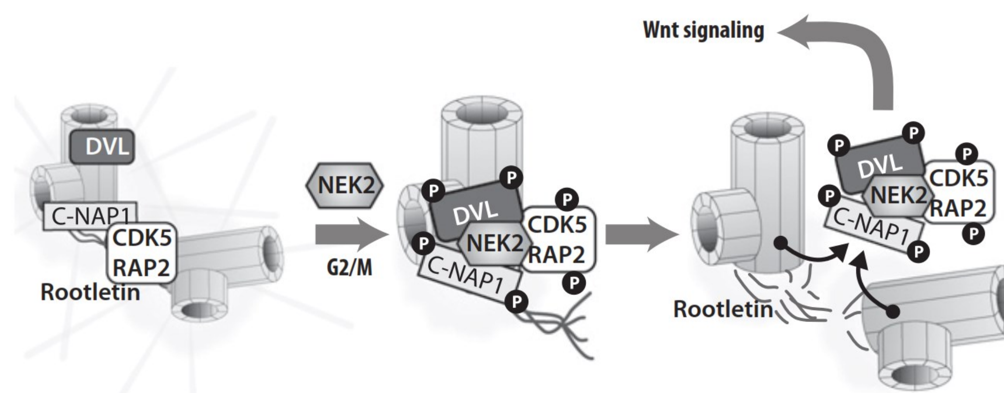


Figure 15: A **scheme of the mechanism by which Dvl upon NEK2 phosphorylation controls the G2/M phase** progression by disassembling the centrosomal linker proteins from centrioles.

3.2 STUDY II: WNT5A IS TRANSPORTED VIA LIPOPROTEIN PARTICLES IN THE CEREBROSPINAL FLUID AND REGULATES PROGENITOR PROLIFERATION

3.2.1 Introduction

Wnts control many aspects of embryogenesis by forming protein concentration gradients within a tissue. Since Wnts are hydrophobic molecules, it is likely that they use a transport mechanism that helps them to diffuse and reach their destinations over longer distances. A few models of Wnt transport have been proposed with some including protein and lipoprotein carriers, or exosomal transport [75, 77, 78, 254], but more investigations is required to determine the Wnt transport mechanism in distinct mammalian tissues. It has been shown that Wnt4a is expressed in the hindbrain choroid plexus [210]. In this study, we investigated the expression and transport of Wnt5a in the developing mouse and human choroid plexus. We used an ultracentrifugation protocol to isolate lipoprotein particles and exosomes, proteomics, IP, western blotting, RT-PCR, in situ hybridization (ISH), IF, confocal microscopy, choroid plexus primary cultures, and *Wnt5a*$^{-/-}$ mice.

3.2.2 Results and discussion

To identify what Wnts are expressed in ChPs, we first analyzed the expression profiles of all Wnt ligands by ISH in mice embryos at E13.5. The Wnt with the strongest expression was Wnt5a, which was specific for the hindbrain ChP (HbChP, 4th ventricle). Using qPCR, Wnt5a expression was found from E12.5 to E17.5 in HbChP. Notably, Wnt5a was not detected in the telencephalic ChP (TelChP, lateral ventricle), a result which was in line with previous findings [186]. On the other hand, Wnt5a was found in the adjacent cortical hem where Wnt2b, 3b, 7a, 7b, 8b and 9b were also expressed.

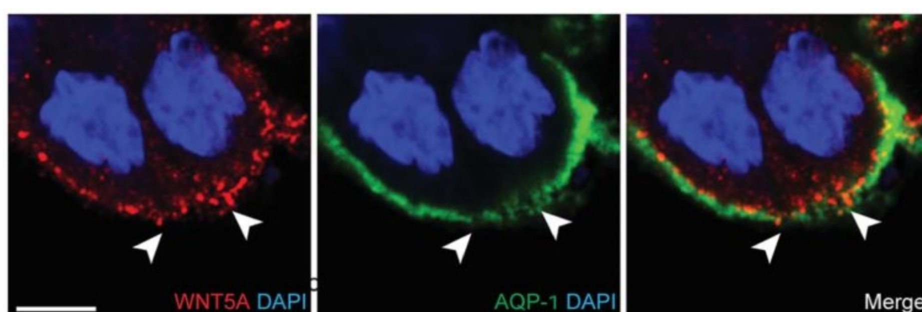


Figure 16: Wnt5a is localized on the apical side of the HbChP epithelial cells in human embryos. IF staining.

Interestingly, within the HbChP, the highest Wnt5a expression was found in the epithelium. These data were further confirmed at the protein level using a specific antibody against Wnt5a, which was validated in the HbChP of Wnt5a$^{-/-}$ mice. At postnatal stages, the expression and protein levels of Wnt5a in the HbChP progressively decreased suggesting that Wnt5a can control the HbChP development during the

embryogenesis. Wnt5a was typically found in the apical part of the cytoplasm of secretory epithelial cells, and sometimes in punctuate structures close to or above the apical cell membrane, which was determined by presence of Aquaporin-1 (AQP-1). These stainings were confirmed when using 9 week old human embryos where Wnt5a was localized at the apical side of the epithelial cells in direct contact with the CSF (**Figure 16**).

We next examined whether the HbChP expresses Gpr177 (Wntless in *Drosophila*), a protein indispensable for Wnt secretion and trans-synaptic transport in *Drosophila* [254, 255]. Indeed, we observed that Gpr177 was highly expressed in the epithelium of the HbChp and not in the TelChP. These data were confirmed by WB and IF in E12.5-E17.5 old embryos. The biological activity of Wnt5a secreted by the epithelial HbChlP cells was further verified by establishing primary cultures from TelChP and HbChP, and collecting the supernatant from these cells. We first analyzed the cell lysates and the supernatant from these primary cultures, and confirmed by WB that Wnt5a was present only in the supernatant of HbChP cells. We next expected that if the epithelial cells secrete biologically active Wnt5a, we could obtain a conditioned medium from them which we subsequently collected. A mouse embryonic fibroblast (MEF) cell line was incubated with conditioned medium of either the TelChP or the HbChP cells in order to examine the activation of Wnt/PCP signaling by Wnt5a. The activity of the secreted Wnt5a was analyzed by its capacity to mediate the phosphorylation of Dvl3, which is identified by WB as the heaviest band [256]. Our data show that the epithelial cells of the HbChP, but not the TelChP, secrete biologically active Wnt5a in mouse and human brain during prenatal development (**Figure 17**).

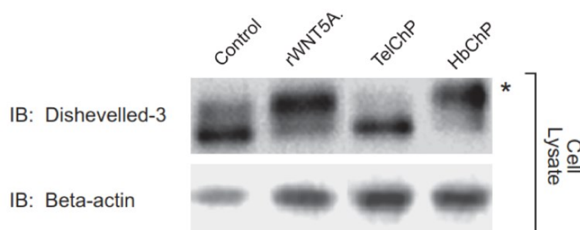


Figure 17: Primary cell cultures of ChP epithelial cells. HbChP but not TelChP cells secrete biologically active Wnt5a as assessed by the increased phosphorylation of Dvl3 in treated MEF cells.

We next investigated the mechanism by which Wnt5a is transported from these cells. We performed an ultracentrifugation of the conditioned media from HbChP primary cells in order to separate exosomes from lipoprotein particles of different sizes. We determined the quality of such fractionation using exosomal markers (CD63, Flotillin-2) and lipoprotein structural components including ApoE, ApoA1, Clusterin and ApoJ. We observed that Wnt5a associated with apolipoproteins in the high density lipoprotein fraction (HDL) and to a lower extent in the low-density lipoprotein fraction (LDL). Wnt5a was absent in the exosomal fraction, which was further confirmed by the IF staining. To confirm whether

Wnt5a physically binds apolipoproteins, the structural units of the lipoprotein particles, we pulled down the exogenous Wnt5a in HEK293 cells and observed that Wnt5a binds to co-expressed ApoE and ApoJ. We also analyzed Wnt5a pulldown by mass spectrometry, and identified an enrichment in additional proteins commonly associated with the HDL-specific proteome, such as ApoA1, ApoA2 and Vitamin D-binding protein.

To further investigate the necessity of the lipoprotein particles for Wnt5a transport, we used a lipid removal agent (LRA) to delipidate the serum which was used in our primary HbChP epithelial cell cultures. Wnt5a was not detected in the supernatant of primary HbChP epithelial cultures upon lipid removal as observed by WB. This effect was rescued when we added mouse HDL into the media after removing the lipids. These data indicated that lipoproteins are at least in part required to restore the presence of Wnt5a in the primary HbChP epithelial cells.

CSF is delivered to the brain parenchyma upon ChP secretion. We thus investigated whether we can detect Wnt5a-lipoprotein particles in the cells of the ventricular zone which are in direct contact with the CSF but localized distally from the HbChP. Our requirement was that the cells cannot express the Wnt5a themselves. Based on these conditions, we selected progenitor cells in contact with the ventricle in the dorsal hindbrain, anterior to the HbChP at E13.5. We first analyzed whether these cells express the core Wnt/PCP receptors, Celsr2 and Vangl2, and/or more general Wnt receptors Fzd3 and Fzd10. Indeed, these progenitor cells were positive for all 4 receptors suggesting that they can bind Wnt5a in the CSF. We next stained these cells with a Wnt5a antibody and observed the presence of Wnt5a in the apical side of these progenitor cells in WT but not in Wnt5a$^{-/-}$ mice. Notably, Wnt5a co-localized with ApoE and ApoJ in the apical surface of the hindbrain progenitors (**Figure 18**), supporting the hypothesis that apolipoproteins may contribute to the transport of Wnt5a towards the receiving cells in the ventricular cavity.

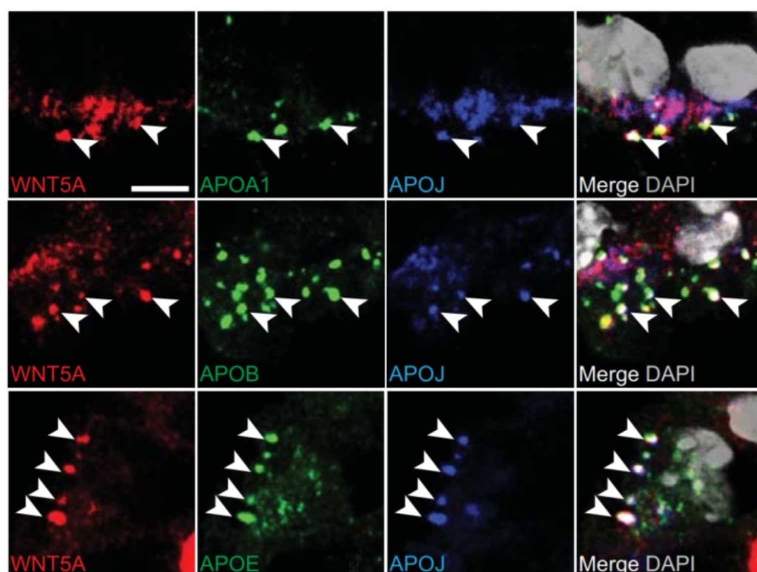


Figure 18: Wnt5a is localized in vesicles at the apical side of the HbChP epithelial cells *in vivo* where it co-localizes with apolipoproteins.

Since the Wnt5a plays a key role in controlling the balance between the cell proliferation and the differentiation during the development of other cell types such as mDA neurons [89], we thus investigated whether Wnt5a regulates the proliferation of the hindbrain progenitors. We quantified a number of proliferating cells (Ki67+) in WT and *Wnt5a*$^{-/-}$ of E16.5 old embryos. This analysis revealed a significant increase in the proliferation of the hindbrain progenitors in *Wnt5a*$^{-/-}$ mice compared to the WT. These data indicated that Wnt5a might be required to inhibit proliferation of the hindbrain progenitor cells in the ventricle, a function that is consistent with previous findings in other cell types.

Overall, our data confirmed that Wnt5a is secreted specifically by the HbChP in the 4th ventricle, and thus support the hypothesis that the expression of different Wnt ligands in the distinct ChPs creates a particular composition of the CSF proteome, which likely contributes to the regionalization of the brain areas during embryogenesis [186]. We also show that Wnt5a can be transported over long distances in complex with HDL particles where it binds to ApoE and ApoJ, and that these protein complexes can reach distant hindbrain progenitor cells in the ventricles. By comparing WT and Wnt5a deficient mouse we further show that the secreted and transported Wnt5a inhibits the proliferation of the hindbrain progenitor cells in the ventricular zone. It was previously reported that the *Drosophila* Wnt ortholog Wingless is transported in exosomes in a complex with its protein carrier Wntless across the synapses in neuromuscular junctions [254]. Based on our data we propose that Wnt5a can also be transported in lipoprotein particles over long distances and create concentration gradients with the highest concentration at HbChP.

3.3 STUDY III: ROR2 AND VANGL2 CONTROL DOPAMINERGIC NEUROGENESIS AND MULTIPLE ASPECTS OF CELL POLARITY IN THE MIDBRAIN FLOOR PLATE

3.3.1 Introduction

As assessed by *in vivo* loss-of-function studies and *in vitro* differentiations protocols, Wnt5a is an essential morphogen for the anterior-posterior patterning of the ventral midbrain (VM), and the propagation and maturation of mDA neurons during embryogenesis [89]. Nevertheless, molecular mechanisms underlying these developmental processes have not been identified. Transgenic mice lacking two core Wnt5a receptors, Vangl2 and Ror2, show an abnormal development of the neural tube which fails to close [31]. We previously found that the loss of Wnt5a affects VM morphogenesis and cause Wnt/PCP defects which include shortening of the anterior-posterior (A-P) axis and lateral expansion of the mDA domain. Moreover, *Wnt5a*$^{-/-}$ mice show decreased levels of mDA neurons and an increased pool of mDA progenitors at E12.5 [89, 211]. In this study, we thus asked whether the Ror2-Vangl2 receptor complex mediates some of the Wnt5a functions and controls different aspects of mDA neuron

development and VM morphogenesis. The expression of Ror2 and Vangl2 was examined by bulk RNA-sequencing in the VM region of TH-GFP+ mice at different developmental stages. We then analyzed the development of cells in the mDA lineage at E12.5 and E14.5, by using immunofluorescence for mDA markers, and several transgenic mice models including *Wnt5a*$^{-/-}$, *Ror2*$^{-/-}$;*Vangl2*$^{-/-}$; *Ror2*$^{-/-}$;*Wnt5a*$^{-/-}$, and conditional overexpression of *Wnt5a* (*Wnt5a OE*), which was induced with doxycycline at E10.

3.3.2 Results and discussion

We first investigated whether *Wnt5a* overexpression stimulates the differentiation of mDA neurons or whether it causes disturbances due to a signaling imbalance. We observed that *Wnt5a OE* partially phenocopies *Wnt5a*$^{-/-}$ mice with regard to the lateral expansion of the mDA domain and the decreased number of mature mDA neurons [89]. Nevertheless, we did not observe A-P defects, and as the Wnt5a levels change in the *Wnt5a OE* animals over time, we did not detect differences in the mDA neuroblast pool. Interestingly, we found that Wnt5a gain of function leads to an increased invagination of the ventricle and a narrower ventricular space of the FP, which was previously seen in *Wnt1*$^{-/-}$ [211]. These data confirmed the critical role of Wnt5a in mDA neuron development, and suggested that any imbalance in Wnt5a-mediated signaling causes disturbances in VM patterning and the development of the mDA lineage.

Next we explored the expression and levels of Wnt5a receptors, *Ror2* and *Vangl2* in different stages of VM development. We used IF and True-seq RNA sequencing of the mDA domain which was dissected along the GFP-labelled TH+ neurons (**Figure 19**).

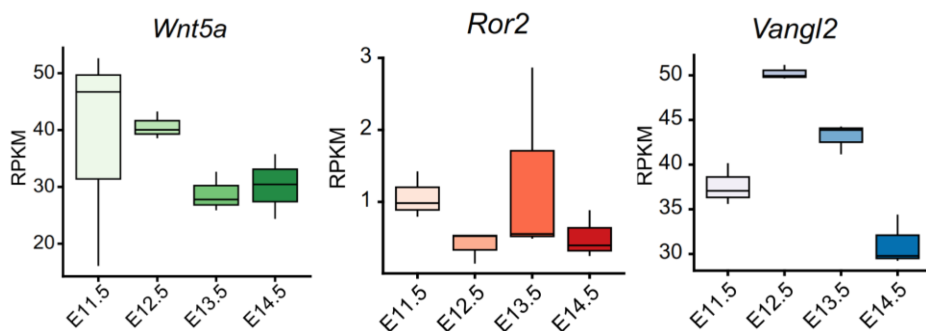


Figure 19: Bulk RNA-sequencing revealed different expression patterns of *Wnt5a, Ror2* and *Vangl2* in TH-GFP+ domain during the mouse VM development.

RNA sequencing showed rather low expression levels of *Ror2* with a decreasing tendency from E12.5 onwards. It has been shown that *Ror1*$^{-/-}$;*Ror2*$^{-/-}$ mice phenocopy *Wnt5a*$^{-/-}$ mutant animals, which suggested that Ror proteins are the main receptors for Wnt5a. Thus, we first analyzed *Ror2*$^{-/-}$ animals. However, we did not observe defects in VM development. In line with this data, we observed only mild worsening of the *Wnt5a*$^{-}$

$^{/-}$ phenotype in our novel *Ror2*$^{-/-}$;*Wnt5a*$^{-/-}$ transgenic line. This data suggested that there might be a functional redundancy between Ror2 and Ror1, or between Wnt5a and other Wnts. However *Ror1* is expressed only laterally in the basal plate, and was not induced in the floor plate of *Ror2*$^{-/-}$ mice.

On the other hand, *Vangl2* was highly expressed in the VM with a distinct expression peak at E12.5 and E13.5, and with the lowest expression at E14.5. The Vangl2 expression pattern correlated with the dynamics of mDA neurogenesis. Analysis of *Ror2*$^{-/-}$;*Vangl2*$^{-/-}$ mice has previously showed severe Wnt/PCP phenotypes, including neural tube closure defects [31]. We thus wanted to elucidate whether this receptor complex also controls VM development. The *Ror2*$^{-/-}$;*Vangl2*$^{-/-}$ mice revealed strong alterations in VM morphogenesis, some of which phenocopied the *Wnt5a*$^{-/-}$ mice, including collapsed ventricles along the dorsal-ventral and lateral axis, A-P shortening, and widening of the floor plate. Strikingly, we also observed a new phenotype involving the left-right asymmetry of the proliferating mDA progenitors and mDA lineage (**Figure 20**). Similarly, it has been shown that Vangl2 controls Wnt5a-stimulated neuronal outgrowth and A-P axonal guidance of commisural neurons, and regulates the bilateral symmetry of the spinal cord by internalization of Fzd3 [41]. We also found that, the total number of postmitotic mDA neuroblasts and mature mDA neurons were decreased by 40% and 50% respectively at E12.5, indicating a defect in mDA neurogenesis. Differences in the differentiation (the ratio between Nurr1+ and Nurr1+; TH+) were also detectable at E14.5.

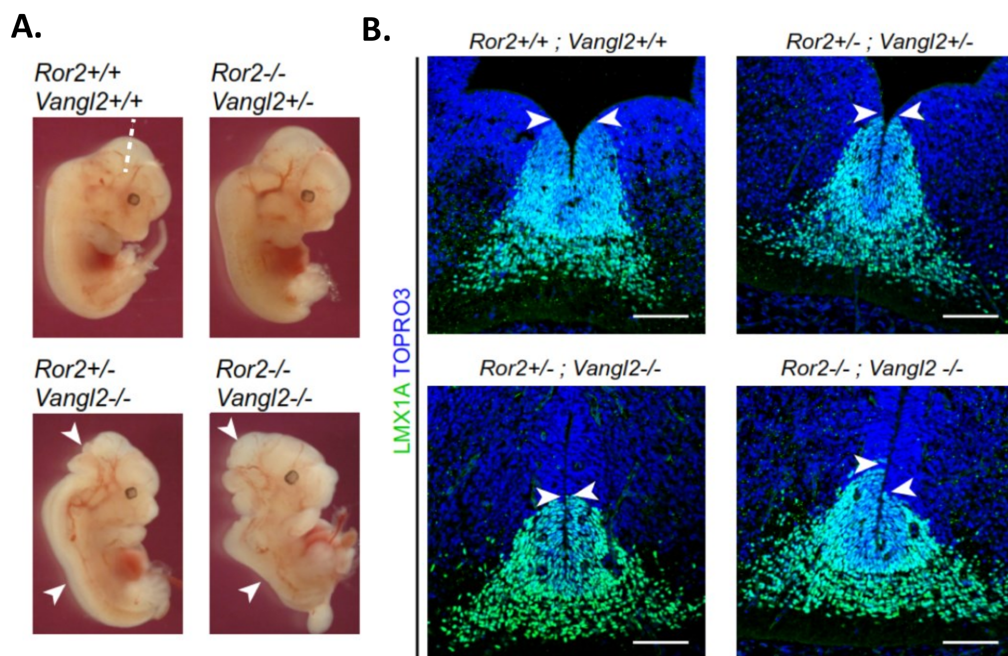


Figure 20: *Ror2*$^{-/-}$;*Vangl2*$^{-/-}$ show severe phenotype during embryogenesis and in mDA lineage development. A. *Ror2*$^{-/-}$;*Vangl2*$^{-/-}$ mice display worsening severity of the Wnt/PCP phenotype at E12.5. **B.** IF of Lmx1a shows the left-right asymmetry of proliferating progenitors and mDA lineage in VM of *Ror2*$^{-/-}$;*Vangl2*$^{-/-}$ embryos. The scale bar is 100µm.

Thus our results suggest that Wnt/PCP signaling through the Wnt5a-Ror2-Vangl2 axis controls VM morphogenesis and bilateral symmetry as well as different aspects of mDA neuron development, such as mDA neurogenesis and the differentiation of mDA neuroblasts into mDA neurons in a sequential manner.

3.4 STUDY IV: THE PRONEUROTROPHIN RECEPTOR SORCS2 IS A NOVEL REGULATOR OF THE WNT/PCP PATHWAY DURING EMBRYOGENESIS

3.4.1 Introduction

It has been shown previously that the Wnt5a-Ror2 signaling axis can recruit additional proteins, such as Ptk7 or Vangl2 to form alternative Wnt/PCP signaling complexes [121]. In addition, the role of this pathway in dopaminergic circuits has not been investigated in full detail. Therefore, we decided to address these issues by performing proteomics on Ror2 binding partners in dopaminergic cells (SN4741). This approach was followed by a detailed biochemical analysis using different antibodies, mutants and treatments. Functionally, we explored the role of novel protein complexes in Wnt/PCP signaling by using genetic manipulations in *X. laevis* and *D. rerio* models in form of microinjections of 1-4 cell stage embryos, and subsequent quantification of the Wnt/PCP phenotype during CE movements, somitogenesis and brain development. We also used WT and transgenic mice in order to investigate novel regulatory mechanisms of the Wnt5a-Ror2 signaling pathway during the VM development.

3.4.2 Results and discussion

Our IP-Ror2-MS/MS analysis uncovered a large number of novel Ror2 interactors that can functionally regulate Wnt signaling, endocytosis or the cell cycle. Our datasets provide a useful resource platform for the future investigations of Ror2 function in dopaminergic neurons but also for a comparison to disease and other tissues. We validated a few interesting candidates from the IP-Ror2-MS/MS data sets on a small scale using specific antibodies and genetically manipulated cell lines including mDA cells, MEF cells, HEK293 cells, and VM lysates of E11.5-E14.5 (**Figure 21**). We confirmed that Ror2 specifically interacts with a) a VPS10-domain containing receptor SorCS2 (SorCS2) from the sortilin receptor family, which function as a proneurotrophin receptor and regulates dopaminergic wiring *in vivo* [257, 258]; b) Ptk7, a transmembrane receptor with an inactive tyrosine-kinase domain and known regulator of Wnt/PCP pathway [62, 148, 259]; and c) Lrp4, a low-density lipoprotein Wnt receptor which is involved in formation and maintenance of neuromuscular junctions and synaptic plasticity in the brain [260-262].

We further proceeded with Ptk7 and SorCS2, and demonstrated that the levels of these proteins are downregulated in MEF-Ror1$^{-/-}$;Ror2$^{-/-}$ cell line [67] pointing out that Ror proteins are required for their presence at correct levels. Overexpression studies *in vitro* and in *X. laevis in vivo* have previously shown that Ptk7 binds to Ror2, and that they control neural crest cells migration [121, 122]. Our results suggest that the interaction between Ptk7 and Ror2 may also serve a function in the VM, a possibility which remains to be elucidated. Since we observed several receptors binding to Ror2, we might further speculate whether large, highly organized receptor complexes are required for Wnt5 signal transmission and the tissue specificity - a hypothesis which meets big experimental challenges.

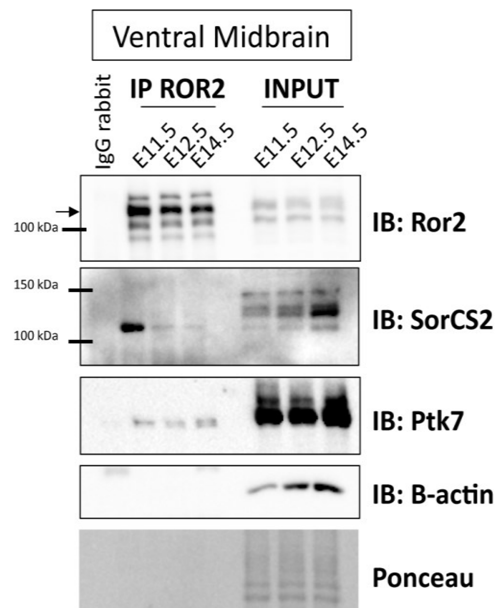


Figure 21: Ror2 physically binds SorCS2 and Ptk7 in VM tissue during mDA neurons development. Ror2-SorCS2 interaction is strongest at E11.5 whereas Ror2-Ptk7 does not change during the VM development.

In this study we mostly focus on SorCS2, a receptor that is highly expressed in mouse VM floor plate at E11.5, and its expression spreads caudally into the hindbrain floor plate at E13.5 [263]. Interestingly, SorCS2 has been involved in protein trafficking, growth cone collapse of mDA neurons, and in synaptic plasticity in the adult brain [257, 258, 264]. However, the function of the Ror2-SorCS2 receptor complex and its possible role in Wnt/PCP signaling, embryogenesis and mDA lineage development have not been investigated.

First, we explored the biochemistry behind the Ror2-SorCS2 complex, and used a panel of Ror2 and SorCS2 mutants to define the protein-protein interaction. We showed that the CRD domain of Ror2 is crucial for the Ror2-SorCS2 binding, thus the same domain where Wnt5a [103, 120] and Fzd2 [119] bind to Ror2. Different proteolytic processing of SorCS2 is believed to be used by glial cells and neurons for distinct cellular responses towards proneurotrophins such as pro-BDNF or pro-NGF [257, 265]. We observed that Ror2 preferably binds to the 2-chain variant of SorCS2 in dopaminergic cells and in ventral midbrain. Moreover, we show that overexpressed SorCS2 binds to Wnt5a, which suggests that SorCS2 may control the Wnt5a-Ror2 signaling axis. We also found that overexpressed SorCS2 also mediates internalization of Ror2 and its binding partners Wnt5a, Vangl2 and Ptk7 *in vitro*. Notably, the internalization of these Ror2

interactors has been previously shown to be important for creation and maintenance of the planar cell polarity [34, 36, 41]. The Vps10-domain containing receptor family has the capacity of sorting proteins by triggering the lysosomal degradation, a pathway impaired in a number of neurodegenerative diseases, including Parkinson's disease [264, 266-268].

We next examined the possible role of SorCS2 *in vivo* in the context of Wnt/PCP signaling. 4-8 cell stage *Xenopus* embryos were single or double injected with mouse Ror2 and human SorCS2. Increased levels of exogenous Ror2 alone induced a shorter A-P axis and sharper angle between the head and tail, both of which are Wnt/PCP phenotypes. Strikingly, this phenotype was partially rescued in the presence of SorCS2. Interestingly, the overexpression of SorCS2 alone showed morphological defects in the head, which were repressed in the presence of Ror2 (**Figure 22**). These data thus show that SorCS2 is a novel Wnt/PCP regulator, and suggest that it can control the PCP signaling via internalization and/or receptor sorting of Ror2. We are still working on the biochemical analysis of this mechanism. We want to particularly investigate endocytosis because proteins involved in this process appear in our MS/MS data, and because it participates in Wnt/PCP pathway regulation *in vivo* as mentioned above.

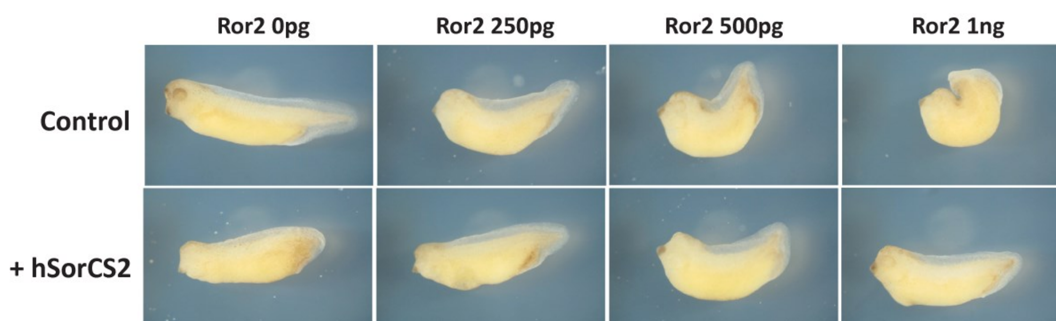
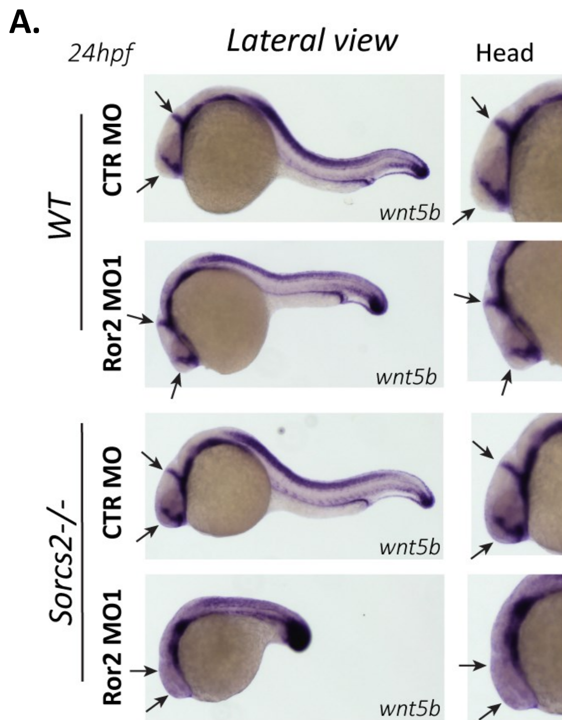


Figure 22: SorCS2 regulates Wnt/PCP signaling *in vivo*. Overexpression of SorCS2 mRNA resulted in a mild Wnt/PCP phenotype and its co-expression with Ror2 mRNA lead to a partial rescue of the Ror2-mediated Wnt/PCP phenotype (shorter axis, sharper angle between the head and the tail).

The expression of *SorCS2* during early embryogenesis has not been examined much. We thus explored the expression of *SorCS2* in zebrafish embryos during the first 24 hours post fertilization (hpf), including gastrulation and somitogenesis, by whole mount *in situ* hybridization (WISH) and real-time PCR. Both methods revealed that SorCS2 is very weakly or not at all expressed at 3.5hpf, but appeared to be gradually expressed at 50% of epiboly till older stages. Interestingly, we observed slightly stronger WISH staining in the head and in the tail compared to the rest of the body at bud stage and 8 somites stage which might suggest SorCS2 polarization. Expression of *SorCS2* at 24hpf was quite dispersed in the embryo, labeling mostly the eye, the floor plate in the hindbrain, and the midbrain. We observed higher *SorCS2* expression in telencephalon and diencephalon after the eyes removal. The expression of *SorCS2* during gastrulation thus

corresponded with that previously described for *Ror2* in these developmental stages. The *Ror2* expression at 24hpf was mostly located in the head, labelling telencephalon, diencephalon, midbrain, and weakly the hindbrain as seen previously [269, 270]. These findings showed that *SorCS2* and *Ror2* are expressed in the same areas during early embryogenesis.



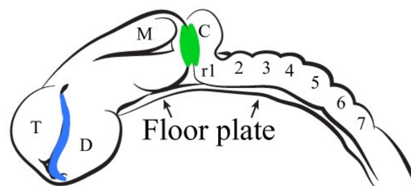


Figure 23: Ror2-SorCS2 complex controls brain development in the Wnt5b-dependent manner. A. WISH staining of *Wnt5b* in WT and *SorCS2*$^{-/-}$ embryos injected with Ror2 MO reveals that the expression of *Wnt5b* in the MHB and Telencephalon-Diencephalon boundary is lost in the double mutant embryos. **B.** A scheme of fish brain anatomy at 24hpf (according to [7]).

Ror2 regulates Wnt/PCP signaling during gastrulation and somitogenesis [270]. We performed loss-of-function (LOF) experiments by injecting single or double Morpholino oligomers (MOs), which inhibit translational machinery of *Ror2* and/or *SorCS2*. We examined whether deletion of *SorCS2* can worsen the *Ror2*-LOF-mediated Wnt/PCP phenotype. We thus either injected WT with Ror2 and SorCS2 MOs, or we injected SorCS2 KO embryos with *Ror2* MOs, and compared them to WT injected with Ror2 MO. In both models we observed that *Ror2;SorCS2* double LOF worsens the Wnt/PCP phenotype compared to the single *Ror2* KD embryos which display short A-P axis, smaller heads at 24hpf, and shorter A-P axis and somite area at 7-somite stage. These data confirmed that Ror2 is in a functional complex with SorCS2 during convergent extension and somitogenesis. Moreover, these embryos lacked expression of *Wnt5b*, the fish orthologue of *Wnt5a*, in specific brain areas labeling the MHB and telencephalon-diencephalon (TD) boundary (**Figure 23**), whereas it did not affect *Wnt5b* expression in the rest of the trunk at the 7-somites stage. The TD boundary was not described before we thus speculate that it can be a novel signaling center. We are currently working on the structural determination of these signaling centers to uncover whether the Ror2-SorCS2 controls the expression of *Wnt5b* in the brain or if it regulates the morphogenesis of

these structures which fail to express *Wnt5b* in absence of *Ror2* and *SorCS2*. We are also currently exploring the possible molecular mechanism by which the Ror2-SorCS2 receptor complex regulate Wnt5a-dependent signaling in *Ror2*$^{-/-}$;*SorCS2*$^{-/-}$ dopaminergic cells, and *Ror1*$^{-/-}$;*Ror2*$^{-/-}$;*SorCS2*$^{-/-}$ MEF cells.

Our results show that the Ror2-SorCS2 receptor complex controls embryogenesis by regulating the Wnt/PCP pathway in fish and frog. These observations are also supported by the fact that *SorCS2* KO mice show A-P shortage and a decreased in weight. Moreover, a colony of deaf mice with *SorCS2* gene mutations was found to exhibit shorter and disorganized stereocilia in the cochlea of the inner ear, a typical Wnt/PCP phenotype [271]. *SorCS2* KO animals are also known to display decreased dopamine levels, and dopaminergic hyperinnervation in the prefrontal cortex [257]. Moreover, *SorCS2* expression changes were observed in the subthalamic nucleus after deep brain stimulation in PD mice [268]. Our results suggest that the Wnt5-Ror2-SorCS2 signaling axis controls brain development and might regulate DA neuron development in the diencephalon and hindbrain, near the midbrain–hindbrain and telencephalon-diencephalon boundaries in zebrafish [272, 273].

SorCS2 is expressed spatiotemporally in various places in mice, with high levels in the midbrain floor plate, spinal cord and in adult hippocampus [257, 258, 263].There are no specific Ror2 antibodies available for IF methods [274]. To track the expression of Ror2 and SorCS2 in the VM tissue, we used RNA-scope *in situ* hybridization (RNA-ISH) which is a novel, commercially available, highly sensitive and selective ISH assay which detects single molecules of RNA in an intact, fresh frozen tissue. It uses carefully designed double Z probes which have to hybridize to the target sequence simultaneously in order to amplify the signal [275]. By using RNA-scope *in situ* hybridization, IF and single cell RNA-sequencing data of mouse and human midbrain, we show that the Ror2-SorCS2 interaction occur in the mouse VM *in vivo*. These data were confirmed by IP-Ror2 from WT VM of E11.5-E14.5 stages, where the Ror2 binding to SorCS2 2-chain variant was the strongest at E11.5 (**Figure 20**). Ror2 and SorCS2 were localized the same cells in the VM floor plate at this stage. From E12.5, SorCS2 is expressed in Sox2+, Glast+ and BLBP+ positive radial glia in the floor plate (**Figure 24**), and laterally in the ventricular zone, and in radial glia which are Sox2 negative, Glast+, BLBP+ in the intermediate zone. Moreover, SorCS2 is expressed by mDA neuroblasts which are Nurr1+ positive and by the TH+ dopaminergic neurons in marginal zone, as previously shown [257]. *Ror2* displays partial expression separation from *SorCS2* at E12.5 stage onwards. We are currently collecting *Ror2*$^{-/-}$;*SorCS2*$^{-/-}$ embryos to investigate the precise function of this receptor complex in the mDA lineage, radial glia populations and VM morphogenesis *in vivo*. We will also examine other cell types possibly expressing Ror2 and SorCS2, such as motor neurons in the basal plate.

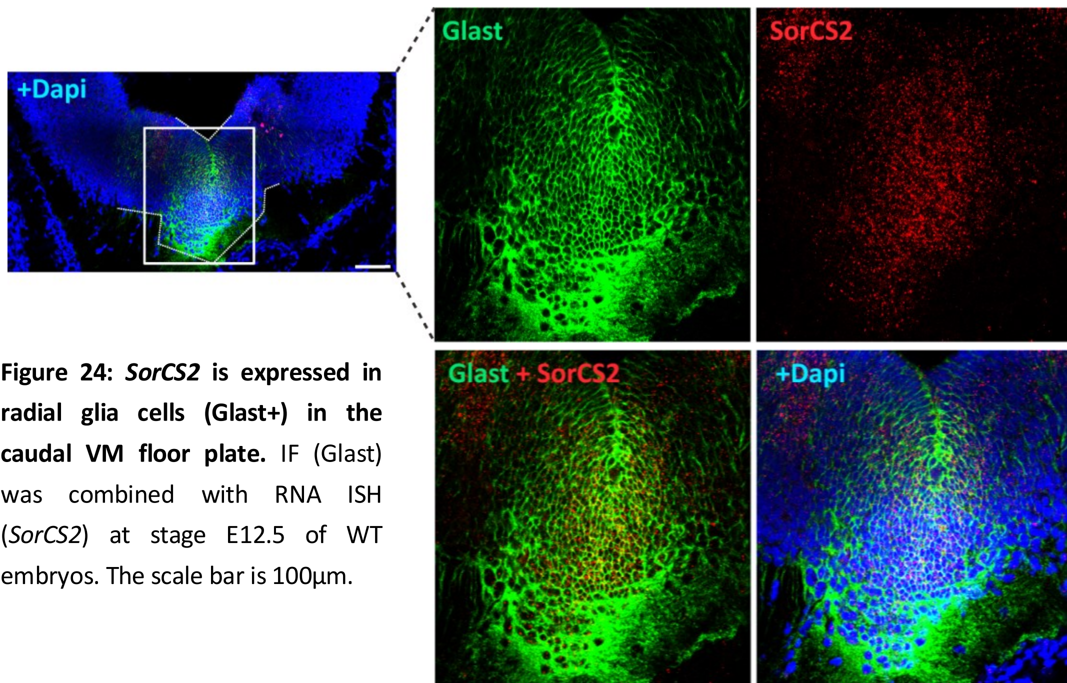


Figure 24: *SorCS2* is expressed in radial glia cells (Glast+) in the caudal VM floor plate. IF (Glast) was combined with RNA ISH (*SorCS2*) at stage E12.5 of WT embryos. The scale bar is 100µm.

To conclude, our study shows that SorCS2 is a novel Wnt/PCP regulator and that the Ror2-SorCS2 receptor complex controls a number of processes during convergent extension, and brain development. Ror2 and its co-receptors were shown to regulate synaptogenesis and synaptic plasticity [105, 106, 218, 261]. We thus propose that the correct understanding of Ror2-SorCS2 signaling may be of importance not only for the wiring of the mDA system, but also for its generation during early development and its demise in Parkinson's disease.

3.5 STUDY V: A PROTEOMIC ANALYSIS OF LRRK2 BINDING PARTNERS REVEALS INTERACTIONS WITH MULTIPLE SIGNALING COMPONENTS OF THE WNT/PCP PATHWAY

3.5.1 Introduction

Autosomal-dominant mutations in Leucine-rich repeat kinase 2 (Lrrk2) appear in 40% of the patients with inherited PD. Lrrk2 is a large, multi-domain protein composed of 2527 amino acids, and as such it regulates not only several different proteins in a number of cellular compartments, but also its own activity. The most common Lrrk2 mutations lead to excessive or persistent activation of Lrrk2, suggesting that the pathogenesis of PD involves a gain-of-function, rather than loss-of-function, as shown by comparison to Lrrk2 knock-out models [276-278]. It has been suggested that overexpressed Lrrk2 remains mostly monomeric in the cytoplasm, while it oligomerizes once relocated to the plasma membrane [279]. Although many Lrrk2 substrates have been suggested, the identity of true endogenous substrates at physiological levels of Lrrk2 protein remains to be determined.

It has been reported that Lrrk2 is involved in Wnt/β-catenin signaling [243, 244]. Since Wnt/β-catenin and Wnt/PCP signaling pathways maintain their balance by inhibiting each other, we thus asked whether Lrrk2 also interacts with regulatory components of Wnt/PCP pathway in mDA cells.

We used several biochemical methods in this study. We again took advantage of an unbiased approach and used IP-MS/MS with a specific antibody to pulldown Lrrk2 in a mouse *Substantia nigra* cell line (SN4741), which in contrast to the majority of cell lines available, exhibits endogenously detectable physiological levels of Lrrk2 protein. By using CRISPR/Cas9 technology, we generated SN4741 cell line with Lrrk2 mutations in exon1 which shows decreased protein levels of Lrrk2. We also used human embryonic kidney 293 cell line (HEK293) to overexpress human Lrrk2. We performed a number of endogenous and overexpression experiments followed by IP, WB, and IF in order to identify and validate the novel Lrrk2 binding partners. We tested a spectrum of different proteins, either using specific antibodies or panel of plasmids. We used SN4741 cells, HEK293T cells, and lysates from the VM of E18.5 embryos. The functional importance of Lrrk2 in Wnt/PCP signaling was determined by the TOPFlash assay to examine the capacity of Lrrk2 to inhibit Wnt/β-catenin signaling. We also examined the importance of Lrrk2 domains by using truncated Lrrk2 mutants. Last but not least, *X. laevis* was used to investigate the functional involvement of Lrrk2 in the inhibition of Wnt/β-catenin pathway, and the regulation of Wnt/PCP-dependent functions *in vivo*.

3.5.2 Results and discussion

Since the preservation of protein-protein interactions highly depends on the sample preparation, we tested 3 different protocols in our IP-Lrrk2-MS/MS analysis. Lists of candidate interactors were manually analyzed using published literature, and selected for their involvement in Wnt signaling. These included the PDZ-domain containing protein Gipc1, the Integrin-linked protein kinase ILK, and the Lipoma-preferred partner homolog LPP.

Our first interactor, Gipc1, was shown to bind the Wnt/PCP receptor Vangl2, and regulate its removal from the plasma membrane. Disruption of Gipc1 activity affects hair polarity in the mammalian inner ear and in *Drosophila* wing where it regulates the hair cell maturation and the hair bundle orientation, a function that identifies Gipc1 as a Wnt/PCP regulator. Importantly, Gipc1 also interacts with the D2 and D3 dopamine receptors [280-282]. Our second candidate, ILK, is known to control cell adhesion and cell motility, it binds to Dvl and activates the Wnt/PCP pathway [283]. Interestingly, constitutively active ILK also activates the Wnt/β-catenin signaling [284], indicating a more complex function. Lastly, LPP is related to members of the Zyxin family (also identified in our IPLrrk2-MS/MS) and is localized in cell-cell contacts. It has been shown

that LPP binds to the PCP protein Scrib, which mediates convergent extension movements in zebrafish early development [285]. To validate these interactions, we pulled-down Lrrk2 from WT and Lrrk2 KD SN4741 cells, and used specific antibodies against Gipc1, ILK and LPP for WB detection. We observed an enriched interaction of Lrrk2 with Gipc1 and ILK in WT compared to the KD cells. We did not see the enrichment for Lrrk2-LPP binding so we were not convinced of the specificity of this interaction.

Lrrk2 is gradually expressed during the late prenatal development in different tissue. In the adult brain, Lrrk2 is highly expressed in the striatum, olfactory bulb and cerebral cortex, and is present at low levels in *SNpc* [286-290]. We thus investigated whether Lrrk2 interacts with Gipc1, ILK and LPP in developing midbrain *in vivo*. We used lysates of ventral midbrain tissue of WT mice at E18.5 stage, and confirmed that Lrrk2 does interact with Gipc1, ILK and LPP in developing VM.

Since Lrrk2 activity and its localization is greatly affected by Lrrk2 protein levels, we decided to validate whether Lrrk2 binds to several selected Wnt/PCP regulators once overexpressed in HEK293 cells. We selected candidates in our MS/MS data set such as Flotillin-2, which is known to regulate Wnt secretion [291]; and Cullin-3 that inhibits Wnt/β-catenin signaling [292] and is downregulated by Lrrk2 KD [293].C-Jun-amino-terminal kinase-interacting protein 3 (JIP3), a Lrrk2 binding partner [294] was used as positive control; Additionally, we tested core mediators of Wnt/PCP signaling, such as Celsr1, Prickle1, Ror2, and Vangl2. Our results show that Lrrk2 binds to Flotilin-2 and Cullin-3, as well as to Prickle1 and Celsr1, but not to Vangl2 or Ror2. We further found that Prickle1 triggers re-localization of Lrrk2 into punctate cytoplasmic structures (**Figure 25**) similarly to the ones formed by the Lrrk2-Dvl complex [244].

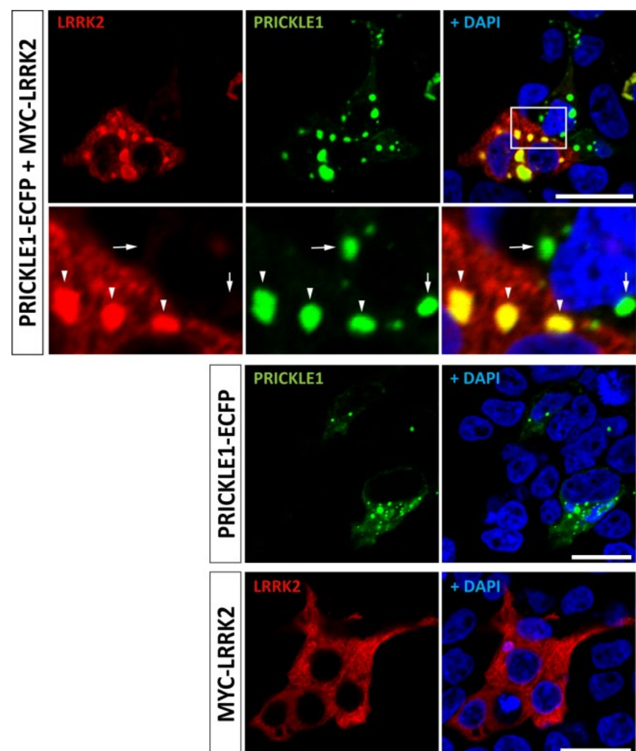


Figure 25: Co-expression of Lrrk2 together with Prickle1 results in their translocation into puncta structures where they co-localize. These structures were not endocytic vesicles. The scale bar is 20µm.

Lrrk2 co-localized with the other partners in cell-cell contacts (Celsr1), cytoplasm (Cullin3, JIP3), and in lamellipodia (Flotillin-2). We also observed that overexpression of Lrrk2 alone inhibits Wnt/β-catenin signaling, which is dependent on its Roc-COR domains. We further showed by IF and TOPFlash assay that Prickle1-Lrrk2 complex forms signalosomes which can either activate or inhibit the Wnt/β-catenin signaling, and thus act as a dual regulators of Wnt/PCP and Wnt/β-catenin signaling. The activity of Prickle1-Lrrk2 complex was modulated by the presence of Dvl2, which seems to compete with Prickle1 for the binding to Lrrk2. These data were confirmed by the functional experiments in *X. laevis* where Lrrk2 overexpression not only inhibited the Wnt/β-catenin pathway, but also induced a shortening of the A-P body axis (**Figure 26**), which identified Lrrk2 is a novel regulator of the Wnt/PCP signaling *in vivo*.

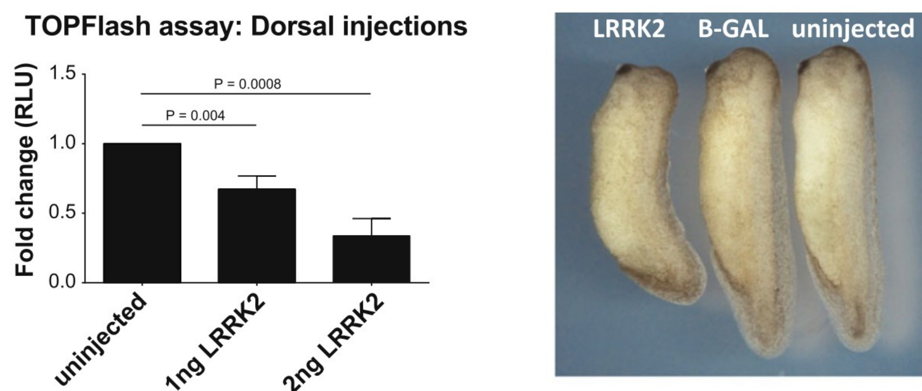


Figure 26: Overexpression of Lrrk2 inhibits Wnt/β-catenin signaling and causes Wnt/PCP defects in *X. laevis* development.

Altered protein levels and localization of Lrrk2 within a cell may be an important determinant for the function and regulation of Lrrk2 activity. It is currently thought that different temporal and spatial events might greatly affect Lrrk2 signaling, and may result in apparently contradictory biochemical assays or read-outs [295]. Our data show that Lrrk2 inhibits the Wnt/β-catenin signaling and activates Wnt/PCP signaling pathway during development, as supported by its interaction with multiple Wnt/PCP regulatory components. We show that the composition of Lrrk2 complexes greatly affects its activity and localization. Moreover, we found that Lrrk2 and its binding partner Prickle1 can act as dual regulators of Wnt/PCP and Wnt/β-catenin signaling pathways, in a fashion that can be modulated by Dvl2. We hypothesize that the vulnerability of mDA neurons in patients carrying Lrrk2 mutations might be caused by the defective Lrrk2 regulation of the Wnt signaling pathways. Taking together, our observations identify multiple novel Wnt/PCP interactors of Lrrk2, and suggest that a deregulation of distinct Wnt signaling pathways may contribute to the pathogenesis of PD.

4 CONCLUDING REMARKS AND PERSPECTIVES

In this thesis we discovered a few novel regulators of Wnt/Planar cell polarity pathway such as Lrrk2 and SorCS2, and explored some of the possible molecular mechanisms by which they control vertebrate embryogenesis, the development of dopaminergic neurons and their function. We used several methodological approaches, including RNA sequencing, proteomics, CRISPR/Cas9 technology, imagining techniques, genetic manipulations of zebrafish and *Xenopus* embryos, transgenic mice, and analysis of human tissue in order to obtain a broad perspective of the molecular mechanisms and functions controlled by the Wnt/PCP signaling. Our discoveries thus contribute to better understanding of the Wnt signaling pathways in multiple cellular processes during embryogenesis, brain and mDA neuron development as well as neuronal degeneration in PD.

I would like to finish this thesis with speculative, but not less important thoughts:

Parkinson's disease seemed simple but turned out complicated. "An essay on shaking palsy", a classification of the motoric PD symptoms as a disease, was written by James Parkinson in 1817 [296]. More than 200 years later, in 2018, we still do not know the cause of the disease, and whether the dopaminergic neurons degenerate because they are dysfunctional or because their microenvironment gives them false or toxic inputs. Likely? Both. But similarly, we do not know the onset of the mDA neurons degeneration. Some Parkinson's disease patients have been diagnosed with the motoric symptoms in their thirties and thus have more than 50% of their *SNpc* DA populations already lost. Should we thus exclude the possibility that an impaired development of mDA neurons might contribute to the increased vulnerability of mDA neurons in the adulthood or to their decreased ability to deal with stressful conditions such as oxidative stress or protein misfolding? Additionally, we also face the lack of reliable diagnostic screening. Nowadays, Parkinson's disease is confirmed only in the postmortem brains. Will the onset of the PD pathology be detected in the brains of young people if we develop more sensitive diagnostic systems? This remains to be seen.

Parkinson's disease affects about 1.5% of the population over 65 years, and thus aging seems to be a major factor contributing to the disease onset and progression. So you might ask: "Why shall we care about the development?" During a disease, the expression of particular genes or the function of proteins is altered, but these events should not be seen as irreversible. Upon an injury, cells often attempt to respond but cannot do that in the exactly same way as they were capable during development. Interestingly, the first detectable α-synuclein aggregates and the simultaneous worsening of the smell are localized in highly neurogenic brain area, in olfactory bulbs [297]. It has been shown that this dopaminergic pool can be functionally restored in PD mouse model by induction of

adult neurogenesis [297]. The rejuvenation and reactivation of developmental programs in somatic cells, as e.g. performed during iPS reprogramming by Yamanaka's protocol [298], may endow cells the capacity to "come-back" and participate in tissue repair or regeneration by activating specific developmental events. I think that a detailed understanding of cell signaling during developmental processes is thus crucial for advances in translational research and regenerative medicine [299].

And last but not least, the importance of Wnts in the adult CNS is currently coming to the light with the growing evidence of Wnt regulation of various processes that are involved in modulation of brain circuits [13, 300]. Wnt signaling is a family of very complex, tissue specific pathways which actively crosstalk with other signaling pathways such as BMP or Notch. The "Wnt combinatorics" helps cells to continuously determine various intrinsic and extracellular signals, to evaluate them and to trigger specific responses. I believe that Wnts do regulate nigrostriatal circuits since they are key players in their development and their expression is maintained during the adulthood. The problem is how to achieve this in a selective manner, as a systemic modulation of Wnt signaling may be deleterious. We therefore have to design the right tools to address this challenge.

In my opinion, these are all questions of high importance which should be investigated, as well as how the healthy mDA neurons keep their homeostasis before and after their integration into the brain circuits. I honestly cannot wait to see the advances of the Wnt signaling and Parkinson's disease research in the future.

5 ACKNOWLEDGEMENTS

My doctoral studies at Karolinska Institute have been a thrilling journey filled with excitement of discovery, prestigious scientific talks, countless laboratorial up- and downs, and dynamic interaction with many excellent junior and senior researchers without whom I certainly wouldn't be able to reach this great milestone.

It goes without a doubt, that the person who is to acknowledge the most is my main supervisor **prof. Ernest Arenas**. Even though my beginning was a bit shaky, you have never given up on me, and let me do the research that I've always wanted – the biology behind the Parkinson's disease. I have always enjoyed your strong analytical and critical thinking, but also the amazing ability to get out of the box and come up with completely crazy, but truly fun ideas. Your constant support gave me self-confidence to trust my scientific instincts and to follow the creativity and sense for adventure within me, which allowed me to fully explore my own thoughts. Indeed, this working style enabled me to grow not only as a scientist, but also as a person. I wish that you will mentor many more young researchers because you are so good in planting the seeds of curiosity and enthusiasm in anybody you are talking with. I also hope that we will go skiing again, because I still need to improve my jumping skills :).

The second most important person to acknowledge is my co-supervisor **ass. prof. Víťa Bryja**. I still remember my interview with you as a bachelor student. I was only twenty and incredibly nervous. I didn't want to order a beer in front of you, which now, retrospectively, seems like a miracle that you gave me the chance anyway ;). I hope you know that you are a great supervisor who creates fantastic working environment where people are happy, helpful and fully productive. Without your continuous support, I wouldn't be where I am now. Thank you for your friendship, and I hope we will continue our (Wnt) interactions in the future.

The last year of my PhD was a roller coaster when I developed a small project into an exciting story. The person to acknowledge the most is **prof. Anders Nykjær**. Anders, thank you so much for the great support, but also for all the funny stories, and the happy evenings out with the lab. You have a great gift to make everybody laugh and to feel comfortable. I not only appreciate your bright mind, kindness and equality by which you treat everybody, but also the fact that for you the person comes always first before the serious business. Indeed, it has been a fun ride!:)

It was a great pleasure to have **Dr. Emma Andersson** as my co-supervisor. Even though we didn't interact so much, I want you to know that you have been always an outstanding example of smart, strong and highly competent young woman scientist who literally manages everything she aims for (at least in my eyes :)). I hope that your enthusiasm and hard-work will guide you to the great future.

I have spent the majority of my research at the division of Molecular Neurobiology (**MolNeuro**) which is a pretty atypical place, with a dynamic development and a high concentration of broad knowledge. In particular, I would like to thank all the group leaders, professors **Ernest Arenas**, **Patrik Ernfors**, **Sten Linnarsson, Per Uhlén,** and **Tibor Harkany,** as well as to the junior PIs **Gonçalo Castello-Branco**, **Jens Hjerling-Leffler**, and **Ulrika Marklund**, for leading MolNeuro through the tough challenges towards the ground-breaking research. I also thank people responsible for running MolNeuro, **Alessandra Nanni** for all the great administrative work, kindness and your open heart; **Johny Söderlund** for being a fantastic colleague, for the jokes , and for always solving the problems before they even occurred; and **Ahmed Moshref** for keeping up the great work. Next, I'd like to thank all present and past MolNeuro members, you are truly awesome! In particular, I'd like to thank:

EA group: **Spyros Theofilopoulos**, for the great discussions, beer nights and football games, for all the jokes, and the lunches at 10am. I hope that our paths will meet again. **Karol Kaiser**, for your bottomless energy, curiosity, and the humor by which you lifted the stress levels at MolNeuro. Also thanks for your incredible ineptitude which always turns into great party stories. **Shanzheng Yang** for being such a sweet and kind colleague and friend, for your bright mind, discussions and great laughs. **Chika Yokota**, for your kind friendship, all the nice chats, and for your important contribution to my PhD projects which really made the difference! **Geeta Ravindran**, who was like my lab mum, always taking care of me; I loved our office-time together! **Carmen Salto**, who is the true heart of the lab, for your kindness and help with everything. **Daniel Gyllborg**, for your 200% productivity and making me tolerate dance music :). **Kaneyasu Nishimura** for your enthusiasm and careful work, as well as your kindness. **Willy Oliveira** for the fun days and your friendship. **Lottie Jansson Sjöstrand** for being such a great lab-mate and for all the interesting discussions. **Pia Rivetti di Val Cervo** for giving new standards to the term hard-work and for being always on top of your research. **Enrique Toledo** for your love for computing, and the patience with my IT anti-skills. **Dawei Zhang** for always being in a good mood. **Isabel Martin Caballero** for our scientific discussions and chats. **Mark Denham** and **Fabia Febbraro** for re-connecting in Aarhus. **Carlos J. Villeascusa** for your help, scientific discussions and feedback during the seminars.

PE group: Prof. **Patrik Ernfors** for your sharp mind and fun lunch discussions. **Daohua Lou** for your honest friendship, all the up and downs we went through together, all the laughs and exciting exchanges about our cultures. **Lili Li** for your big heart, your kindness and for your friendship. **Changgeng Peng** for being a great friend, and for all the interesting discussions during the weekend lab-lunches. **Mitya Usoskin** for keeping the Slavic contribution at MolNeuro, for your interesting perspectives, enthusiasm, and the passion for skiing. **Boris Eleuteri**, my favorite Italian office-mate, whose knowledge and skills in biochemistry has been exemplary to me. Thanks for creating a sweet little Italy in the office.

Anneke Navis for your great help with CRISPR/Cas9 and your interest in skating and horn playing. **Moritz Lübke** for creating the core of MolNeuro, it's not the same without you anymore! **Dongok Kwak** for your clear mind, helpfulness and kindness. **Hind Abdo** for being a sweet colleague and for the awesome hat you gave me! **Albert "Blanchi" Blanchart** for your lively character, cool discussions and great work. **Mingdong Zhang** for exchanging our PhD experiences through-out these years. **Puneet Rinwa** for your funny jokes and interesting inputs. **Jana Sontheimer** for fantastic manager skills and helpfulness. **Alessandro Furlan** for interesting discussions and the structured complains. **Martin Häring** for your calmness and the love for chocolate.

PU group: Prof. **Per Uhlén** for your kindness and the great CLICK facility. **Ivar Dehnisch** for the fun stories and jokes, and for your open-mindedness. **Manuel Varas** for your contribution to my project, pure heart, longing friendship, and the crazy fun we had together. **Erik Smedler** for being a nice office-mate and a good colleague. **Paola Rebellato** for your friendship and for having a great time sharing the office. **Shigeaki** and **Sachie Kanatani**, for your bright insights, your kindness and helpfulness. **Songbai Zhang** for our western blotting time together, and for your awesome jokes. **Göran Månsson** for all the great chats about sports, adventures or simply anything else. **Nicholas Fritz** for organizing a great career course, and for all the nice discussions. **Dagmara Kaczynska** for your Slavic sense of humor and all the fun we had in Japan. **Lauri Louhivuori** for all the interesting discussions and nice time in the lab. **Connla Edwards** for doing a great job with the CLICK facility and for your rowing passion.

SL group: Prof. **Sten Linnarsson** for your stoicism and advances in RNA sequencing. **Lars Borm** for being an awesome and fun friend, for your kindness and for your shared passion for skating and hockey. **Simone Codeluppi** for being a great colleague who is always ready to help others or discuss anything over a coffee. I still don't understand how you can perform on such high levels all the time! **Hannah Hochgerner** for having a sweet nature, being helpful and have always smile to spare. **Amit Zeisel** for being yourself at all times and for having solid opinions. **Peter Lönnerberg** for the chats about the Nordic skating. **Anna Johnsson** for all the interesting discussions and for standing behind your opinions. **Kasper Karlsson** for having you around and for the nice chats. **Job van der Zwan** for all the fun discussions and parties. **Gioele La Manno** for the midbrain sequencing and all the discussions.

JHS group: **Jens Hjerling-Leffler** for all the great courses your organized. **Ana** Mariíí **Munóz Manchado** for your great friendship, dinners and simply all the years we shared together. It's amazing to watch you climbing the career ladder because you truly deserve a big success for all your ideas and hard work! **Hermany Munguba** for your friendship, and all the fun time we spent together. **Carolina Bengtsson Gonzales** for your dedication to MolNeuro and

your hug times. **Kasra Nikouei** for our chats and party times. **Nathan Skene** for your bright opinions and enthusiasm about science.

GCB group: **Gonçalo Castello-Branco** for the high quality courses and symposiums you organized as well as for the cool discussions and social activities at MolNeuro. **Ana Mendanha Falçao** for your friendship, your pureness of heart, as well as all the good and bad times that we went through. **Sueli Marques Spencer** for being a great office-mate and a friend, and for your incredible cooking skills. **Marek Bartošovič** for maintaining the 1% Czechoslovakian presence at MolNeuro, and for your love to climb. **David van Bruggen** for all the nice chats and great parties. **Mandy Meijer** for being a sweet office-mate and for taking care of Daniel! **Samudyata** for your sharp focus and the love for comics. **Elisa Floriddia** for fighting the postdoc rights, for all your activities and hard work.

UM group: **Ulrika Marklund** for your calm nature and kindness. An awesome office-mate and friend, **Fatima Memic**, who always makes my day brighter - not mentioning the incredible cakes! My sweet Austrian friend **Viktoria Knoflach**, thank you for the fun parties, big laughs, shared worrying about Karol, and the love for mountains and skiing.

Next, I would like to thank **Gunnar Schulte**, who was in my half-time committee and gave me a through-out feedback about my Wnt research. I also want to thank the other two members of my half-time board, **Eva Hedlund** and prof. **András Simon** for their valuable questions, inputs and enthusiasm by which they supported me. I also thank **Michael Andäng** for the work on the Fucci project, and scientific advices, and prof. **Ola Hermanson** for having him as my mentor and for simply being cool.

During my PhD studies, I spent 3-months in Víťa Bryja's lab in Czech Republic and 11-months in Anders Nykjær lab in Denmark. In both labs, I received incredibly warm welcome, big experimental help, and had indeed a memorable time. In particular, I would like to thank:

Masaryk University, Czech republic: **Ondra Bernatík** for your sarcasm, experimental help and for your realistic thinking. **Zankruti Dave** for your great work and curiosity about science and the world around you. **Jakub "James" Harnoš** for being an incredibly funny and sweet bíťák, for all our gaming and inappropriate jokes! **Tomek Radaszkiewicz** for your help with CRISPRs, for your awesome beers and funny jokes. **Kasia Radaszkiewicz** for your friendship, your sweetness, and for taking care of Tomek. **Pája Janovská** for your hard-work and enthusiasm. **Katka Straková** for fighting your way through the Frizzled project. **Lucka Smyčková** and **Bára Valnohová** for being very helpful and sweet at any time of the day. **Honza Kučera** for being awesome and for having you in Stockholm for a little bit. **Jožka Večeřa** for all our discussions, climbing time and beers on the boat. **Zuzanka Šrámková** for your friendship and for being always yourself. **Pavel Hyršl** for being such a good friend and for organizing stuff when you are in Stockholm. And the Wnt master of all times, **Lukáš**

Čajánek :) Thank you for replying my million emails when I joined the EA lab, your sharp thinking, your friendship, and for the fun times we had together.

Aarhus University, Denmark: **Susanne Schousboe Sjøgaard** for being 200% productive, for fixing any problem that I come up with, and for being a great office-mate. **Pernille Thomasen**, **Karen Marie Sørensen**, **Niels Kjærgaard Madsen** and **Peter Ovessen** for all the experimental help, shared PhD stories and the fun evenings out! **Anja Aagaard Danneskjold Pedersen**, **Benedicte Vestergaard**, and **Anne Kerstin Thomassen** for being awesome technicians. **Kasper Kjær-Sørensen** for introducing me to zebrafish. **Karen-Marie Pedersen**, **Hande Login**, **Mikhail Paveliev** and **Anne Kathrine Sørenssen** for being great lab-mates. **Olav Andersson** for your friendship, for your critical questions about my data, and for all the fun times ahead of us. **Kerstin Imrell** for your friendship and all the great discussions we had together. **Mariam Mahmoud**, **Sergío Almeida, Sara Ferreira,** and **Giulia Monti** for your friendship. And to the rest of Biomedicine and Dandrite.

When you live abroad without a family, it feels very lonely at first. But once you meet the right people, you gain an extra family instead. Thus, I would like to thank all my friends from Stockholm who supported me during these years:

Our tiny ***Czech community***, namely **Simča Hankeová** for being a great example of a talented young scientist with good senses for partying. **Igor Červenka** and **Janča Valnohová** for all the possible aliquots you got me, your long friendship, and the maintenance of Bryja lab powers at KI. **Katarina Tiklová** for being like an older sister to me, always sparing the time for me and for all the beautiful shared memories. **Petra Sekyrová** for all the struggles, discussions and nice times we had together. **Maciej Szeszula** for the climbing lessons and for your "bad" jokes. **Tom Drápal**, for our won and lost battles, but importantly, for the fun and the adventure that came with it. **Tom Matras** for having a true friend in you and for all the past and future trips. **Jarda Zaoral** for your adventure spirit.

Our formal ***floorball team*****,** **Pawel Modewski**, **Robko Hovorka**, **Vivek Sharma**, **Voravit Tanyingyong**, **Abi Singh**, and **Yasar Al-Mosawi** who filled my days with joy, and made me feel good in Sweden. I miss you guys a lot, we should start playing again!

My many ***awesome KI friends*** - I own you a lot for being on the same boat with me. You made my time in Stockholm unforgettable: **Teresa Fernandez Zafra**, for your pure friendship, big heart, and all the strong and never ending encouragements when I struggled the most. Thank you for everything and mostly for being you! **Yildiz Kelahmetoglu** for the beautifulness you carry within everywhere you go, for your bright head, for your creativity, enthusiasm and the drive that you handle things. **Jorge Correia**, the man I know since I moved to Stockholm :) Thanks for all the beers, board-gaming and other fun stuff we did together. **Kuba Lewicki**, my Biomedicum pub mate, an Adobe Illustrator freak, a hipster, but

most importantly, a totally awesome friend. Thank you for all the fun times we had together, I'm awaiting for some more! **Jesse Coleman** for spending only few weeks with you but gaining a life-longing friend! Thanks for all the awesomeness! **Susie Neumann** for your indescribable jokes, for your bright mind, perfection and your loving heart. **Joanne Bakker**, the one of the three people who have ever read my thesis :D, for your kind heart, your big dedication, and your energy which can move rocks. **Gonçalo Brito** for your coolness, a kind heart and great organization skills. **Erik Keimpema** for your friendship, the beer nights, and the fun discussions. **Manideep Gupta** for your rare, sensible character, for the true friendship, caring, and for all the fun events. **Paula Valente de Silva** for your friendship, and the ability to see unicorns all around you. **Sandra Petru Reuer** for your dedication, craziness and your bright head. **Luismi Nino** for all the good times! **Ilgar Abdullayev** for your kind heart, the big strength within, and the cool discussions. **Junwei Zhang** for the great jokes and your cooking skills! **Jonathan Mudry** for your adventure spirit and sport personality which brought us together. **Erik Müllers** for never given up on me and inviting me for events. Thank you for all the fun discussions and the great old times! **Helena Silva Cascales** for your amazing enthusiasm, your energy and the happiness you spread around. **Kasia Maleńczyk** for all the fun in the good times! **Igor Adameyko** for being so inspiring. **Theresa Madler** and **Tom Reichenbach** for your sweet friendship and nice times together. **Mauricio Barrientos** for your positive attitude and fun events. **Alex Bersellini Farinotti** for being very capable, and the most chick man I know. **Anas Kamleh**, **Marin Jukic**, **Javier Avila Cariño**, **Carmen Fou**, and **Olivia Miossec** for your friendship, good work and the fun times.

To my ***best friends in Czech Republic*** who have always stood besides me, and kept me connected with my home:

Také děkuji svým nejlepším kamarádům z Česka, kteří při mě stáli po celou dobu studia a představovali pro mě to nejdůležitější – spojení s domovem. Děkuji **Martince Fajmonové**, **Tadeáškovi**, **Magdalénce**, **Zdeňkovi Fajmonovi**, a **Terezce Černé** za lásku a všechny super výlety, na které jsme spolu vyrazili a ještě vyrazíme! Děkuji **Míši Píchové** za moji Pražskou spojku, za všechny věci minulé i budoucí. Děkuji **Renatce Svorové** za neutuchající podporu a za to, že ať se děje co se děje, nic se mezi námi nezmění. Děkuji **Pavlince Čapkové** za celoživotní přátelství a za všechny milé noční návštěvy. Děkuji **Janči Daňkové** za kopec srandy, kterou si vždycky spolu užijem. Děkuji **Davídkovi Bednářovi** za to, že jsi :). Děkuji všem **Vajzarům** na **Tisovce**, doma u **Jandů** v **Prachatisích** a u **Drápalů** v **Třebíčí**, za všechnu lásku, podporu a přátelství, které ste mi za ty roky projevili. A taky za ten rum a víno, co se vypilo :).

From all my heart, I also want to thank my boyfriend **Giuseppe Santopolo**, for an incredible support, encouragement, care and love that you are constantly doping me with. Thank you for always being besides me, in good and bad times, with laughter or tears. Thank you for our countless, stimulating scientific and non-scientific discussions. You are an amazing human being, and a great scientist (trust me, I'm a biologist ;))!

Vorrei anche ringraziare **la famiglia e gli amici di Giuseppe**, che mi hanno accolta a braccia aperte e coi quali mi sento come a casa. Voglio ringraziare in particolare i suoi genitori, **Anna Colosimo** e **Vincenzo Santopolo** per la loro gentilezza e le loro battute, che a volte possono essere capite anche senza bisogno di tradurle :); **Daniela Santopolo** per la sua natura esuberante e viaggiatrice; e **Lina Colosimo** e **Marianna Commisso** per la loro dolcezza ed il loro supporto.

Last but not least, I would like to thank all ***my family***, particularly my parents, who have been incredibly understanding, and supported me all these years when I was abroad:

A to nejdůležitější nakonec. Ráda bych na tomto místě z celého srdce poděkovala své nejbližší rodině, především taťuldovi **Petru Salaši** a mamince **Aleně Salašové**, ale také svým prarodičům **Eduardu Salaši, Karlu Prokopovi** a **Jarmilce Prokopové,** a bráškovi **Kubovi Salaši**, za všechnu jejich lásku, podporu, trpělivost a sebeodříkání za posledních 7 let mého pobytu v zahraničí. Ste nejlepší a bez vás by to nešlo! Dále bych ráda poděkovala ujkovi **Lukášovi "Honzovi" Prokopovi**, tetě **Ivě Kochové**, **Monči Kochové** a **Jurovi Laborovi** za to, že k sobě patříme, a že si vždy užijem spoustu srandy.

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