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WNT/FZD Signaling:
An Odyssey from Molecular Pharmacology to Brain (Patho)Physiology

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And it is solely by risking life that freedom is obtained.

~ Hegel

ABSTRACT

The 19 members of the lipoglycoprotein family of WNTs interact with the highly conserved cysteine-rich domain (CRD) of ten members of the Frizzled (FZD_{1-10}) family. The seven transmembrane-spanning surface receptors are listed as G protein-coupled receptors and are known to interact with heterotrimeric G proteins as well as the scaffolding protein disheveled. Upon ligand binding, activation of β-catenin-dependent and/or –independent pathways are induced. The WNT/Frizzled signaling pathway is highly conserved across species and plays an essential role in a plethora of physiological processes, such as embryonic stem cell differentiation, adult stem cell renewal, migration, proliferation and cell polarity. In humans, dysregulation of the pathway is related to developmental alterations, as well as numerous diseases, among which various types of cancers.

The general aim of this thesis was to investigate the role of WNT/FZD signaling in physiological and pathophysiological processes of the central nervous system. Additionally, we attempted to dissect the molecular features of the WNT/FZD pathway regarding signaling specification, a contribution aimed at increasing the possibilities of future drug development.

To date, WNT/FZD binding specificity, as well as the downstream signaling trigged by different ligand-receptor combinations, have not been systematically mapped. Furthermore, the involvement and role of heterotrimeric G proteins downstream of FZDs is unclear. In paper I, we demonstrated that WNT-5A is able to activate PTX-sensitive $G\alpha_{i/o}$ proteins in the microglia-like cell line N13. Additionally, in paper II we concluded that WNT-3A, -4, and -5A have a putative functional selectivity for individual downstream signaling pathways depending on the FZD present.

WNT-5A is known to be expressed in the central nervous system, where it plays a role in various neurological processes. However, it had not been fully delineated which cell type expresses and secretes WNT-5A in the brain. In paper IV, we identified a subpopulation of astrocytes that express high levels of WNT-5A in the brain of adult mice. These astrocytes are localized in the subventricular zone, the rostral migratory stream, and in the hippocampus. By studying a $WNT\text{-}5A^{+/-}$ mouse model, we show that some astrocytes release WNT-5A and fulfill a crucial role in guiding the migration of neuronal precursor cells from the subventricular zone to the olfactory bulb. In paper V, we investigated the pathological implications of WNT-5A signaling to the prognostics of human glioblastomas, and described a strong association between WNT-5A expression in in the tumor microenvironment and increased infiltration of microglia.

Besides the role in neuronal precursor migration, WNT-5A induced a proinflammatory response in mouse microglia cells, shown by the expression of several proinflammatory markers (paper III). Additionally, WNT-5A induced proliferation, invasion and calcium signaling in microglia. By employing the $G\alpha_{i/o}$ inhibitor PTX, as well as the MEK1/2 inhibitor, a part of these events could be blocked, suggesting G protein-dependent mechanisms downstream of WNT-5A.

LIST OF SCIENTIFIC PAPERS

I. **WNT-5A stimulates the GDP/GTP exchange at pertussis toxin-sensitive heterotrimeric G proteins**
Kilander MBC, **Dijksterhuis JP**, Ganji RS, Bryja V, Schulte G
*Cellular Signalling (*2011) *23: 550-554*

II. **Systematic mapping of WNT-Frizzled interactions reveals functional selectivity by distinct WNT-Frizzled pairs**
Dijksterhuis JP, Baljinnyam B, Stanger K, Sercan HO, Ji Y, Andres O, Rubin JS, Hannoush RN and Schulte G
The Journal of Biological Chemistry (2015): In press

III. **Heterotrimeric G protein-dependent WNT-5A signaling to ERK1/2 mediates distinct aspects of microglia proinflammatory transformation**
Halleskog C, **Dijksterhuis JP**, Kilander MBC, Becerril-Ortega J, Villaescusa JC, Lindgren E, Arenas E, Schulte G
Journal of Neuroinflammation (2012) 9:111.

IV. **Partial ablation of WNT-5A affects neuronal precursor migration along the rostral migratory stream**
Dijksterhuis JP, Kamuda K, Gyllborg D, Persson A, Uhlén M, Brismar H, Blom H, Arenas E, Mulder J and Schulte G
Manuscript

V. **WNT-5A is upregulated in a subset of human glioblastoma and is associated with a distinct inflammatory phenotype caused by microglia invasion**
Dijksterhuis JP, Arthofer E, Marinescu V, Nelander S, Mathias Uhlén, Pontén F, Mulder J, Schulte G
Manuscript

ADDITIONAL PUBLICATIONS

Related published papers and database entry involving the author that are not included in the thesis:

I. **Sequential activation and inactivation of Dishevelled in the Wnt/beta-catenin pathway by casein kinases**
Bernatik O, Ganji RS, **Dijksterhuis JP**, Konik P, Cervenka I, Polonio T, Krejcin P, Schulte G, Bryja V
The Journal of Biological Chemistry (2011) 25;286(12):10396-410.

II. **The Concise Guide to PHARMACOLOGY 2013/14: overview**
CGTP Collaborator
British Journal of Pharmacology (2013) 170(8):1449-58.

III. **WNT/Frizzled signalling: receptor-ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3**
Dijksterhuis JP[*]**,** Petersen J[*], Schulte G
British Journal of Pharmacology (2014) 171(5):1195-209.

IV. **Frizzled Class GPCRs entry on the IUPHAR database**
Contributor
www.iuphar-db.org

* These authors contributed equally.

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LIST OF ABBREVIATIONS

7TM	seven-transmembrane
AD	Alzheimer's disease
Akt	protein kinase B
APC	adenomatous polyposis coli
CaMKII	calmodulin-dependent kinase II
cAMP	cyclic adenosine monophosphate
CE	convergent extension
CK1	casein kinase 1
CNS	central nervous system
COX2	cyclooxygenase 2
CRD	cystein rich domain
CsA	cyclosporin A
DCX	doublecortin
DVL	disheveled
ERK	extracellular signal-regulated kinase
EVI/WIs	wntless
FZD	frizzled
GBM	glioblastoma multiforme
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GPCR	g protein-coupled receptor
GSEA	gene set enrichment analysis
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
IL-10	interleukin 10
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
IP3	inositol triphosphate
JNK	c-Jun N-terminal kinase
LPS	lipopolysacharide
LRP5/6	low density lipoprotein receptor related protein 5 and 6
MAPK	mitogen-activated protein kinase
MMP	matrix metalloprotease
NFAT	nuclear factor of activated T cells
NO	nitric oxide
OB	olfactory bulb

PCP	planar cell polarity
PDZ	postsynaptic density 95/disc-large/zona occludens-1
PI3K	phospatidylinositol 3’-kinase
PKC	Ca2+-dependent protein kinase
RMS	rostral migratory stream
PS-DVL	phosphorylated and shifted DVL
PTX	pertussis toxin (from Bordetella pertussis)
ROR1/2	receptor tyrosine kinase-like orphan receptor 1 and 2
RYK	related to receptor tyrosine kinase
SFRP	secreted Frizzled-related protein
SVZ	subventricular zone
TCF/LEF	T-cell specific transcription factor/Lymphoid enhancer factor
TCGA	The Cancer Genome Atlas
TNFα	tumor necrosis factor alpha
WB	western blot
Wg	wingless
WLS	wntless
WNT	wingless/int-1

1 INTRODUCTION

The 19 different isoforms of WNT proteins serve as ligands for the seven-transmembrane (7TM) Frizzled (FZD) receptor proteins (FZD_{1-10}) upon which different downstream signaling pathways can be activated. This complex signaling network has been associated with a plethora of developmental processes such as stem cell proliferation, organogenesis as well as adult stem cell renewal. Dysregulation of the pathway is connected to a wide range of diseases among which different types of cancer and neurodegenerative diseases. Precisely because of their abundant role in both physiological and pathophysiological processes, this pathway is highly relevant and rewarding to study. To date around 30 clinical studies are being conducted worldwide within the area of WNTs or FZDs, as registered on the clinical trial website of the National Institutes of Health (NIH, 2014). An example of a promising ongoing phase I clinical trial describes the use of a peptide named Foxy-5, which is able to mimic the effect of WNT-5A to impair migration of epithelial cancer cells. The researchers suggest therefore that this compound can potentially be used as an anti-metastatic cancer drug and thereby increase the survival rates of patients with solid malignant tumors.

In this thesis I focus on the current understanding on WNT/FZD signaling in general, WNT and FZD signal specification and downstream signaling partners, and the role of the pathways in (patho)physiological processes in the brain, with WNT-5A as a common nominator. One could say these subjects seem to be far apart from each other, however I would rather state they cover a broad spectrum within the field of WNT signaling. I believe it is of utmost importance to be able to link pharmacology and physiology in order to make progression in the field of drug discovery.

1.1 WNT/FZD SIGNALING

1.1.1 Historical overview on discoveries in the WNT/FZD signaling field

The lipoglycoprotein family of WNTs was first described over 30 years ago by Nusse and Varmus (Nusse & Varmus, 1982, Nusse *et al.*, 1991)[1], who were at the time

[1] Nusse and Varmus called the gene initially *int1*, to denote the first common integration site of the MMTV provirus. Around 1990, this nomenclature became inadequate and unworkable due to the discovery of several unrelated MMTV target genes that also received the *int* pronounce. To avoid confusion they agreed on naming the original *int-1*-related genes 'Wnt', an acronym combined from Wingless-related integration site (Nusse *et al.,* 1991*)*.

investigating putative proto-oncogenes in mammary tumors of mice. In hindsight, even though they were the first ones to give a full description of the gene, many other groups identified members of the WNT family much earlier through (mostly spontaneous) gene mutation experiments in different species (Bittner, 1936, Korteweg, 1936). After deciphering the sequence and structure of WNT-1 it became clear that the gene starts with a signal sequence, revealing its properties as a secretory protein (van Ooyen & Nusse, 1984, Fung *et al.*, 1985) and thereby generating the idea that it might serve as an extracellular growth factor. Despite the fact that this was of interest for many researchers, production and isolation of WNTs was only possible nearly two decades later after the discovery that detergents are required to keep the soluble lipid-modified structure of WNTs during extraction from cells (Willert *et al.*, 2003, Schulte *et al.*, 2005). Although technically challenging, to date all the 19 members of the WNT-family are commercially available as recombinant proteins[2]. The *Drosophila* int-1 homologue, *Wingless*, had been identified even before the initial experiments on the mammalian counterpart. Mutation in the allele resulted in loss of wing tissue (Sharma & Chopra, 1976), revealing the importance of these genes in developmental processes. In the late 1980s researchers were working on the hypothesis that cancer may origin from cells that have abnormal developmental progression, therefore the link between a protein that is involved in cancer and embryogenesis was well appreciated. Not surprisingly, WNTs became therefore the model protein to study this phenomenon. Later it appeared that it is not in principle mutations in WNTs that cause cancer, but rather the downstream signaling components that are generally altered. This resulted in a more complex and challenging field of research, meanwhile opening new avenues for the understanding and treatment of diseases.

In the quest to find the putative receptors for WNTs, scientist discovered that in fact several groups had been working on one class of receptors for WNTs, the 7-TM FZD proteins, however unaware of their connection to WNTs. The earliest reports date back to 1944 where the receptor group is described phenotypically in the context of a mutant screen (Bridges, 1944). To date, it is generally accepted that FZDs serve as WNT receptors and that this interaction can involve the presence of a growing list of WNT co-

[2] However caution should be taken when using them in experimental designs due to the fact that the different WNTs may exhibit only partial activity after purification.

receptors, e.g. low density lipoprotein receptor-related protein (LRP) 5/6, receptor tyrosine kinase (ROR) 1/2 and related to receptor tyrosine kinase (Ryk) (Schulte, 2010).

1.1.2 Lipoglycoproteins of the WNT family; structure, function and transport

The lipoglycoprotein family of WNTs consists of 19 members in mammals. WNTs are lipid modified and have therefore hydrophobic characteristics (Willert et al., 2003). For many years purification of active WNT proteins was challenging, explaining why relatively little pharmacological information regarding WNT/FZD signaling is published. At the moment all WNTs are commercially available as recombinant proteins, creating new opportunities for pharmacologists to better understand receptor-ligand kinetics, conformational changes of the receptor upon WNT-binding and activation of downstream signaling partners, to name but a few. This information will contribute to the development of drug targets in which this pathway plays a role. The level of purity and the percentage of active protein after purification however has been a matter of concern. For example, Cajanek et al. tested different lots of recombinant WNT-3A and discovered that, even though all tested lots activated the WNT/β-catenin pathway, certain individual lots activated the phospatidylinositol 3'-kinase/protein kinase B/glycogen synthase kinase 3 (PI3/ /GSK3) pathway independent of WNTs (Cajanek *et al.*, 2010), raising the question which activation is caused by WNTs and which by impurities in the WNT preparation.

Recently the first crystal structure of a member of the WNT family, *Xenopus* WNT-8 (XWNT8), was described. The authors crystallized XWNT8 in complex with the cystein rich domain (CRD) of FZD_8. Determination of the WNT8/FZD_8 structure can lead to a better understanding in which manner WNTs activate FZDs and explain the relevance for binding or activation of for example the presence of the CRD on FZDs or the lipid modification of WNTs. The structure of WNT8/FZD_8-CRD showed two important domains of WNT, referred to as the thumb and the index finger that are both connected to a central palm domain, interacting with FZD_8 CRD at two sites (Site1 and Site 2). The complex is formed in such a way that the WNT-lipid is shielded from aqueous solvents (Janda *et al.*, 2012). The structural features of the XWNT8 do not show any obvious resemblances to any other currently known protein fold. Even though clarification of the WNT/FZD-CRD complex provides key information for the development of pharmacological agents to modulate the pathway, it also raises more questions. Are WNT/FZD combinations selective? Does a conformational change of FZD occur upon WNT binding? Where is the place of the coreceptors in the WNT/FZD

complex and how do they influence the binding of WNTs? Future structural biology experiments will possibly shed light on these issues.

In the *Drosophila* wing Wingless (Wg) proteins act as morphogens and exert their effect through concentration-dependent gradients reaching target cells from a short to long range, thereby induce patterning and cell differentiation. Several studies have identified necessary components for the secretion of WNT proteins, such as the 7TM cargoreceptors Wntless (EVI/WIs or WLS)[3] and the acyltransferase Porcupine. The latter is necessary for the addition of a palmitoleic acid to WNT proteins, which is essential for secretion (Takada *et al.*, 2006). Another crucial lipid modification that contributes to the hydrophobicity of the protein is the palmitoylation on a cysteine residue, a modification that is required for the activity of WNTs (Willert et al., 2003, Kurayoshi *et al.*, 2007). In which matter WNTs are released and subsequently travel through the interstitial space has been of interest for many years. Given the fact WNTs are rather hydrophobic, the likelihood that WNTs use cell membranes or carrier proteins for transport is high. Recently the hypothesis that active WNT proteins are secreted on exosomes *in vivo* and *in vitro* has been tested by several research groups. Gross et al. show that WNTs, together with Evi/WIs, are transported through endosomal compartments onto exosomes (Gross *et al.*, 2012). However, the details on this route of transport remain to be elucidated.

1.1.3 Frizzled receptors and their downstream binding partners

FZDs exhibit the typical G protein-coupled receptor (GPCR) features, among which the 7TM subunit structure. Despite a longstanding redundancy in the field that these receptor are indeed coupling to G proteins, several reports from our lab and others have been published to date proofing the ability of FZD to transduce heterotrimeric G protein signaling (Kilander *et al.*, 2014a, Kilander *et al.*, 2014b, Slusarski *et al.*, 1997a, Slusarski *et al.*, 1997b, Katanaev *et al.*, 2005) (**paper I**). Although FZDs may be able to activate G proteins, it is likely that the receptors functions in an atypical manner, and received therefore their own class within the Guide to Pharmacology, composed by the IUPHAR network (Alexander *et al.*, 2013, Foord *et al.*, 2005). Currently over 40% of the medication used in the clinics is targeting G protein-coupled receptor (GPCR) signaling, of which one of the most eminent is probably the group of beta-receptor antagonist used to decrease hypertension (Lundstrom, 2009). Given the previous and the abundant role

[3] In *Drosophila* the gene is called Evi or WIs, the human homologue is called WLS.

of WNT/FZD signaling in disease, the answer on why to study the possible involvement of G proteins in FZD signaling becomes self-evident.

The earliest reports on the involvement of G protein signaling in relationship to FZDs describes the activation of a WNT/FZD/Ca^{2+} pathway after injection of RNA coding for different WNTs and FZDs in embryos of *Xenopus laevis* and *Danio rerio.* This activation could be inhibited by the Gαi/o subunit inhibitors pertussis toxin (PTX) or through the overexpression of Gα sequestering βγ subunits (Slusarski et al., 1997a, Slusarski et al., 1997b). To date, a series of publications show that FZDs indeed couple to heterotrimeric G proteins and that this interacting is PTX-sensitive (Sheldahl *et al.*, 1999, Sheldahl *et al.*, 2003, Kremenevskaja *et al.*, 2005, Dejmek *et al.*, 2006, Ma & Wang, 2006, Halleskog *et al.*, 2012, Halleskog & Schulte, 2013a, Katanaev et al., 2005). It has been proposed that FZDs - after ligand binding - do not activate heterotrimeric G proteins under all circumstances, but that the activation depends on a number of factors such as the type of ligand, presence or perhaps absence of certain co-receptors, cell type and stage of development (Egger-Adam & Katanaev, 2008, Schulte, 2010). Also the presence of the downstream protein disheveled (DVL) could play a role. There are three variants known in humans (DVL1, 2 and 3) and they serve as central scaffold proteins localized at the crossroad of multiple WNT/FZD pathways (Gao & Chen, 2010). So far there is literature showing that the heterotrimeric G proteins could both act upstream as well as downstream of the DVL and that this can lead to either β-catenin-dependent and –independent pathways (Sheldahl et al., 2003, Liu *et al.*, 2005, Bikkavilli & Malbon, 2009). Furthermore, the fact that DVL itself can bind to FZDs as well through their centrally located PDZ (Psd-95/Disc large/ZO-1) domain, expands the variety of possible complex models. In a recent published paper it was demonstrated that two conserved motifs, located at the third intracellular loop and the classic C-terminal motif of FZD, is required for DVL binding through its DEP and C-terminal domains. This binding is crucial for the Wnt-induced β-catenin activation in cultured cells and *Xenopus* embryos (Tauriello *et al.*, 2012). Presumable hypothesis are that DVL and heterotrimeric G proteins either (i) compete (ii) cooperate or (iii) bind to different FZD isoforms upon ligand binding (Kilander et al., 2014b).

1.1.4 Downstream signaling pathways

Downstream of WNT/FZD stimulation, several different pathways can be activated. Historically, the two main signaling branches were divided into canonical and non-canonical signaling, depending on the involvement of the transcriptional regulator β-catenin. To date, these branches are named β-catenin-dependent and –independent signaling respectively. Most research has been done on the function and regulation of β-catenin-dependent pathway, since the pharmacological readout for this pathway – activation of the luciferase T-cell specific transcription factor/lymphoid enhancer factor (TCF/LEF)-driven reporter gene called TOPflash assay - was already well-established since 1996 (Molenaar *et al.*, 1996, Van de Wetering *et al.*, 1996), and has proven to be a robust assay. Moreover, activation of this pathway can be investigated by several other methods such as embryo body axis duplication in *Xenopus Laevis*, β-catenin stabilization by western blot (WB) or immunocytochemistry and transformation of C57MG cells (Barker, 2008). The β-catenin-independent pathways encompass several complex signaling routes: FZD/planar cell polarity (PCP), WNT/Ca^{2+}, WNT/cyclic adenosine monophosphate (cAMP), WNT/ROR, WNT/RAC, and the WNT/RHO pathway (Semenov *et al.*, 2007, Schulte, 2010). Due to the diverse and complex nature of these pathways, the field suffers from a lack of suitable and robust assays to investigate this branch in more depth.

1.1.4.1 β-catenin-dependent pathway

As mentioned earlier, the β-catenin-dependent pathway is named after its main player, the cadherin-binding and transcriptional regulator β-catenin. Signal initiation happens upon the formation of a ternary complex between WNT, FZD and the co-receptor LRP 5/6, followed by the recruitment of the scaffolding protein DVL. In turn, this complex forms signalosomes and triggers the phosphorylation of LRP6 (Bilic *et al.*, 2007). This causes a cascade of intracellular events leading to the inhibition of the destruction complex consisting of GSK3, axin, adenomatous polyposis coli (APC) among others. In the absence of a functional destruction complex, β-catenin stabilizes, accumulates in the cytosol and subsequently translocate into the nucleus. There it binds to the transcription factors factor TCF and LEF resulting in the regulation of many target genes, such as the proliferative genes c-*myc* and *cyclinD1*, or inflammatory genes cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) (Ramsay *et al.*, 2003, Barker, 2008, Du *et al.*, 2009, MacDonald *et al.*, 2009, Tetsu & McCormick, 1999). The β-catenin pathway

is especially known for its regulatory role in cell growth and differentiation, and is therefore actively studied in cancer research, neurodegenerative diseases and stem cell research (Moon, 2005). A schematic overview of β-catenin-dependent pathways is shown in figure 1.

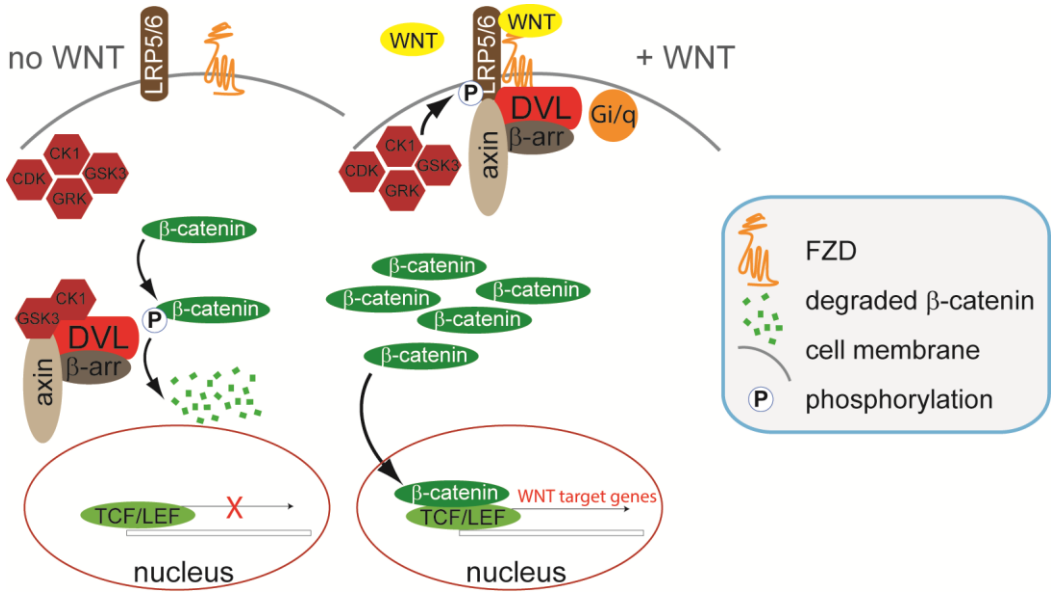


Figure 1: Schematic view of the β-catenin-dependent signaling pathway. For detailed description see text. Abbreviations not mentioned in the text: β-arr, β-arrestin; Gi, inhibitory heterotrimeric G protein; Gq, heterotrimeric G protein; CK1, casein kinase; GRK, G protein-coupled receptor kinase; CDK, cyclin dependent kinase. Image modified from Schulte et al. 2010.

1.1.4.2 β-catenin-independent pathways

The pathways included in this branch all signal independently of β-catenin, therefore they are referred to as the β-catenin-independent pathways. However, because this branch encompasses several complex signaling routes that can either signal independently, cross talk or partially overlap, it would be more appropriate to speak of a network rather than a group of pathways. The cellular responses and the ultimate (patho)physiological effect in organisms induced by these pathways relate to cytoskeletal rearrangements of cells leading to processes such as cell motility, migration, invasion and tissue polarization (Schulte, 2010). The most well described pathways are the PCP and WNT/Ca^{2+} pathway. PCP signaling refers to 2-dimensional cellular orientation processes of which for example the wing of *D. melanogaster* and the insect eye have served as models to study this phenomenon. In vertebrates similar mechanisms are known as for example convergent extension (CE), neural tube closure and hair follicle orientation in the skin

(Fanto & McNeill, 2004). Activation of the WNT/Ca^{2+} pathway results in the increase in intracellular calcium followed by the initiation of a number of different cellular functions. Calcium has several downstream targets such as nuclear factor of activated T cells (NFAT) (Saneyoshi *et al.*, 2002, Dejmek et al., 2006), Ca2+-dependent protein kinase (PKC) and Ca^{2+}-calmodulin dependent protein kinase II (CamKII) (Sheldahl et al., 2003, Kuhl *et al.*, 2000). Pathway activation is most likely occurring through heterotrimeric G proteins. DVL seems to be required for activation as well (Sheldahl et al., 2003), however there is also evidence showing that the WNT-5A induced rise in calcium is functional in cells depleted of DVL by siRNA (Ma & Wang, 2007).

1.1.5 Conceptualizing specification and bias in WNT/FZD signaling

Over history, scientists have attempted to create drugs to improve medical conditions. Most drugs consisted of plant extracts that were used based on empirical knowledge. The relatively recent discovery of membrane receptors allowed the formulation of molecules that targeted specific functions of the cells. This mentality of research has guided discovery over the last century. Recently, it has been discussed the possibility that different ligands may trigger distinct signal pathways, acting through a single receptor. This concept, called "biased agonism", works with the hypothesis that the multidimensional structure of a receptor may be influenced by multiple ligands, greatly affecting the different downstream signaling pathways in a receptor conformation-dependent manner. The WNT/FZD binding specificity, the activation, and the involvement of associated cellular components determining the signal outcome have so far not been systematically addressed and are therefore poorly understood. This is partly due to the fact that only until recently all the existing WNT proteins became commercially available in recombinant form. Table 1 gives an overview of the different receptor/ligand combinations known so far from the literature.

Table 1: WNT/FZD interaction overview. Tabulated the reported WNT/FZD interaction partners. Crosses (x) indicate direct binding based on immunoprecipitation data, circles (o) indicate interactions proven by experimental evidence such as colocalization, internalization, TCF/LEF activity, red crosses (#) indicate interactions based either CRD binding or phosphorylation of β-catenin, DVL or LRP6 as described in **paper II**. (Modified according to (Dijksterhuis *et al.*, 2013).

WNT	1	2	2B	3	3A	4	5A	5B	6	7A	7B	8A	8B	9A	9B	10A	10B	11	16
FZD_1	x	x		o	x#		x#	o#		o	x								
FZD_2		o		o	o#	#	x#	#	o	o	o		o						
FZD_3		x			X		x												
FZD_4		x	x		o#	#	o#	#			x								
FZD_5		o			x#	#	x#	#		x					o		o		
FZD_6					X	x	x	o		o									
FZD_7					x#	#	o#	#		o									
FZD_8					x#	#	#	#							o				
FZD_9		x																	
FZD_{10}											x								

Because of the high complexity of the different signaling pathways that possibly even cross talk, it is not known whether certain WNT/FZD signaling pairs can selectively activate or prefer certain signaling routes or downstream binding partners above others. For future drug discovery studies it is of utmost importance to dissect the signal specification of the WNT/FZD pathway. For example, in some case, one might prefer to activate solely heterotrimeric G proteins through FZD without affecting DVL. This response can be achieved by the development of small molecules that induce a certain conformational change of the receptor, resulting in a receptor conformation-dependent activation of G proteins. Figure 2 envisions both the challenges and opportunities when investigating biased agonism in WNT/FZD signaling.

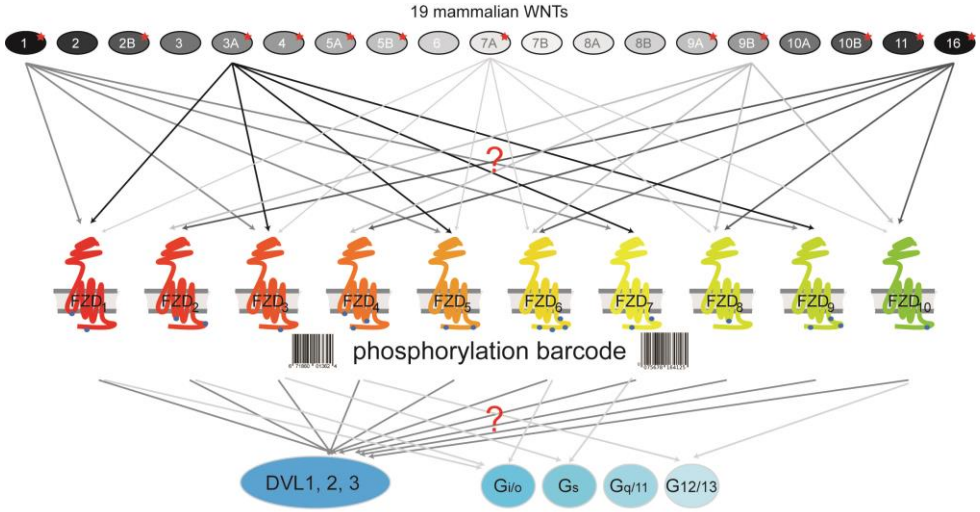


Figure 2: WNT/FZD interaction overview. Schematically visualized the possible WNT/FZD binding combinations, the potential activation or binding of various known downstream signaling proteins – DVL and Gα-subunits - and the various receptor phosphorylation sites (marked with blue dots). The different binding and activation arrangements will determine a so called 'phosphorylation barcode' at the c-terminus of the FZDs. The question mark indicates the uncertainties in pathway continuation.

1.2 ROLE OF WNT-5A IN BRAIN PHYSIOLOGICAL PROCESSES

1.2.1 Function, expression and distribution in the central nervous system

WNT signaling has various functions in the development of the central nervous system (CNS), including synaptogenesis, axon growth and guidance and differentiation (Inestrosa & Varela-Nallar, 2014a, Inestrosa & Varela-Nallar, 2014b, Inestrosa & Arenas, 2010). Members of the WNT-family have different functions in developmental processes within the CNS, depending on the WNT-isoform, region of expression and the receptor repertoire present in the microenvironment. For example, they can serve as pro- or anti-synaptogenic factors in both vertebrates and invertebrates (Inestrosa & Arenas, 2010, Budnik & Salinas, 2011, Sahores & Salinas, 2011). In-depth research has been done on the function of WNT-7A in synapse formation and growth (Hall *et al.*, 2000). FZD_5 appears to be one of the key receptors mediating this response (Sahores *et al.*, 2010). WNT-5A has been a WNT-isoform of interest regarding CNS development as well. After the first experiments in loss-of-function mutation of WNT-5A, the mice showed severe developmental aberrations, among which the inability of the extension of the A-P axis. Additionally, truncated limbs as well as outgrowth defects in the face, ears and genitals led the authors to conclude that WNT-5A regulates pathways important in

extension of structures growing outwards from the primary body axis. This is likely due to the fact that WNT-5A regulates proliferation of progenitor cells in the mesoderm, shown by the incorporation of the nucleotide analogue 5-bromo-2'-deoxyuridine (BrdU) (Yamaguchi *et al.*, 1999). To date, WNT-5A is known to regulate proliferation as well as differentiation in neuronal progenitor cells, and has therefore a function in neurite development (Yu *et al.*, 2006, Paina *et al.*, 2011). Furthermore, it plays a role in dopaminergic axon growth and guidance (Blakely *et al.*, 2011, Castelo-Branco *et al.*, 2006), has shown to regulate postsynaptic assembly through activation of β-catenin-independent WNT signaling in neurons (Farias *et al.*, 2009, Cuitino *et al.*, 2010) and regulates the clustering of the postsynaptic density protein-95, a protein involved in the regulation of AMPA and NMDA receptors (Farias et al., 2009).

Even though WNT-5A shows to have a plethora of different functions within the CNS, little is known about the WNT-5A producing cell and the regional location of the protein. This is probably due to the fact that antibodies raised against WNT proteins have been of questionable quality and immunohistochemistry stainings turned out to be challenging. It has been suggested that glia cells present in the ventral midbrain, but not in the cortical region, serve as a source for WNT-5A protein. The WNT-5A secreted by these glia cells is responsible for the differentiation of Nurr1-expressing neuronal precursor cells into the dopaminergic phenotype (Castelo-Branco et al., 2006).

A closer look at the WNT-5A in gene expression data publicly available through the dataportal of the Allen Developing Brain Atlas (Allen, Developing Mouse Brain Atlas, ©2013) shows us that the expression in the brain is highest during prenatal stage E11.5 and postnatal stage P14 (see Fig 3). Regions of interest are the Telencepahalic vesicle (Tel) and the Medullary hindbrain (MH). Seemingly, during those stages of maturation, WNT-5A is required for the development of the CNS. Although useful data, it does not provide information on the cell type that is expressing WNT-5A.

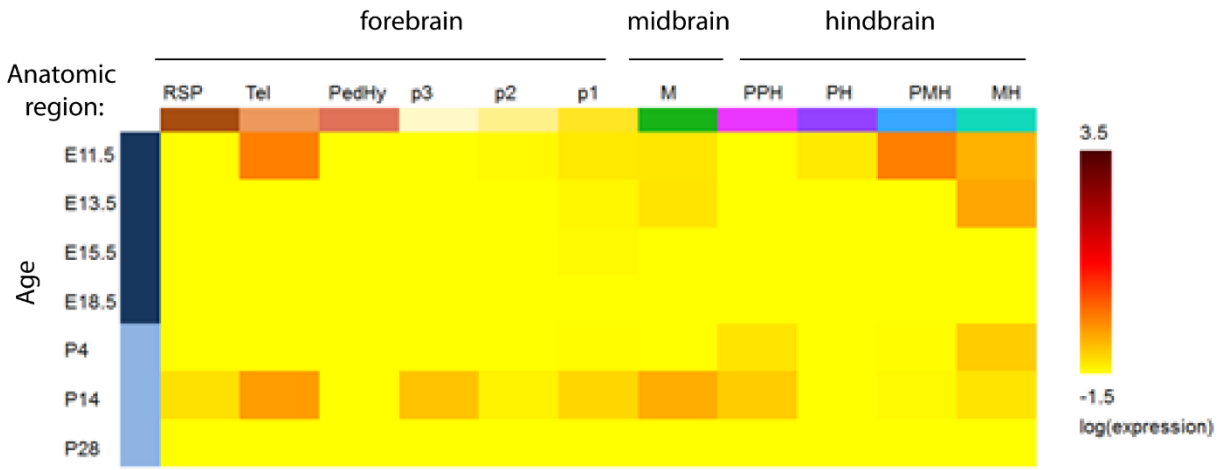


Figure 3: WNT-5A expression in the mouse brain. Heatmap of WNT-5A expression in the mouse brain at different stages of development (E11.5 till p28). The abbreviations stand for Rostral secondary prosencephalon (RSP), Telencephalic vesicle (Tel), Peduncular (PedHy), Prosomere 3,2 and 1 (p3,2,1), Midbrain (M), Prepontine hindbrain (PPH), Pontine hindbrain (PH), Pontomedullary hindbrain (PMH) and Medullary hindbrain (MH). Figure modified from the Developing Mouse Brain Atlas (Allen, Developing Mouse Brain Atlas, ©2013).

1.2.2 Immunological effects on microglia

Microglia serve as the immunocompetent cells of the brain and account for approximately 10% of the total cell population in the adult CNS (Aguzzi *et al.*, 2013). It is generally acknowledged that microglia are not uniformly distributed throughout the brain and they consist of several subtypes exerting their own function within immunological processes (Lawson *et al.*, 1990). If microglia have a function under normal physiological conditions, or solely comes into play during pathophysiology, remains a matter of debate. So far, it is known that microglia continuously sense their surroundings in the search for disturbed homeostasis. Upon disturbance of the homeostasis within the CNS - due to for example injury or bacterial or viral intrusion - microglia act as immune regulators upon which multiple signaling cascade are activated. Following the initial activation response, release, transcription and expression of several proteins are initiated leading to proliferation and invasion of microglia and phagocytosis of bacteria and debris. Key immune modulators involved in this process are the groups of cytokines and chemokine (-receptors), major histocompatibility complex II, cluster of differentiation proteins, matrix metalloproteinases (MMPs) and iNOS (Kettenmann *et al.*, 2011). Inflammatory responses by microglia are not uniform and can strongly depend on the type of trigger or the receptor repertoire present on the cells. Traditionally, responses were divided into pro- and anti-inflammatory, also referred to as ‘classical’ M1 and ‘alternative’ M2 morphological types. However, to date a more complex model

of several ‘in-between’ inflammatory stages is suggested, which exert and prefer different functions within the scope of immunological responses (Gertig & Hanisch, 2014, Eggen *et al.*, 2013).

A classical way to study activation of microglia in culture is by stimulating the cells with lipopolysaccharide (LPS), a compound normally found in the bacterial wall of gram-negative bacteria. Microglia express the LPS receptor Toll-like receptor 4 on their cell membrane, and are therefore capable to recognize it as a foreign compound, resulting in acquirement of a proinflammatory phenotype. Upon binding, microglia will release or induce several proinflammatory substances such as tumor necrosis factor alpha (TNFα), COX-2), interleukin-6 (Il-6) and nitric oxide (NO) (Hanisch & Kettenmann, 2007). A shortcoming of this method is that it only initiates one type of pro-inflammatory response, which does not reflect the effect in a pathological condition. In the search for a better understanding on how microglia exert their function *in vivo*, studying this cell type in their natural milieu would be preferred.

WNTs have been associated with an array of immunological processes in the body, however their precise role is not fully delineated. For example, WNT-5A and FZD_5 are increased in synoviocytes in rheumatoid arthritis (Sen *et al.*, 2000) and synoviocyte activation is suppressed upon blockage of the WNT-5A/FZD_5 pathway (Sen *et al.*, 2001). Additionally, genetic variations within WNT signaling components play a role in the initiation and maturation of neurodegenerative diseases such as autism, Alzheimer’s disease (AD), schizophrenia and Parkinson (De Ferrari & Moon, 2006). The combination WNT-5A/FZD_5 is upregulated in AD mouse brain as well, suggesting WNT-5A signaling is aberrantly activated during AD pathogenesis (Li *et al.*, 2011). A study from our lab showed β-catenin accumulation in microglia undergoing a proinflammatory morphogenic transformation in human brain material, a phenomenon likely to occur in areas undergoing neuroinflammation such as AD (Halleskog *et al.*, 2011). Furthermore, *in vitro* analysis revealed both WNT-3A and WNT-5A as inducers of a proinflammatory response in primary mouse microglia. This was shown by several experiments including the increase of multiple proinflammatory cytokines, chemokines and innate immune response factors (Halleskog et al., 2012, Halleskog et al., 2011). However, both WNTs did also counteract LPS-induced expression of proinflammatory components COX2, IL-6 and TNF-α, suggesting they serve as homeostatic regulators for microglia regarding immunological functions (Halleskog & Schulte, 2013b).

1.3 GLIOBLASTOMA MULTIFORME

1.3.1 Introduction to glioblastoma pathogenesis

Glioblastoma multiforme (GBM) are the most frequently diagnosed and lethal primary tumors in the CNS, with a median survival of approximately one year (Ohgaki & Kleihues, 2005). GBMs are ranked by the World Health Organization classification scheme as stage IV tumors within the group of gliomas, a classification based on severity (stage I-IV) (Louis *et al.*, 2007). Treatment options are limited and the standard entails currently surgical resection (Rostomily *et al.*, 1996, Chang *et al.*, 2003), followed by radiation therapy (Burton & Prados, 2000, Castro *et al.*, 2003) and the chemotherapeutic drug Temozolomide (Temadol®) (Burton & Prados, 1999, Castro et al., 2003). In essence however, they are incurable. Despite significant efforts to improve GBM models to study this disease, very little progression has been made in the development of new therapeutic agents against this devastating disease. In fact, the median survival has not changed considerably in almost a century (Bailey C, 1926).

Gliomas commonly consist of a heterogeneous cell population derived from a combination of cancer cells with random mutations, as well as a large group of infiltrating brain cells from diverse origin. A popular hypothesis on the propagation of the tumor, supported by a growing body of evidence, entails the presence of a cancer stem cell (CSC) population within the tumor. The CSCs share features with somatic stem cells, showing self-renewing behavior and thereby allowing the tumor to grow (Lathia *et al.*, 2011). This would also explain the ability of tumors to resist and evade both the body-own immune attack on the tumor as well as therapeutic intervention. The acquired knowledge on the presence of CSCs within the tumor adds a challenge to study tumor biology *ex-vivo,* given the fact that employing serum-cultured cell lines derived from clonal expansion simply do not suffice. To map the tumor behavior employing the use of a full repertoire of cells present in the tumor appears to be a necessity. Together with the increased interest in CSCs, the cellular origin of primary malignant gliomas becomes of point of attention.

Based on histological features, gliomas can be classified in three main types: astrocytomas, oligodendrogliomas and mixed oligoastrocytomas (Louis et al., 2007). Within these subtypes further stratification are made upon tumorigenic properties, such as the presence of necrosis, mitosis and a vascular network, which define the grade of the tumor (WHO grade I-IV). Even though this classification system does give insight in the aggressiveness and clinical course of gliomas, the variability in biological behavior

within the different grades cannot be explained by this scheme. Due to the high complexity of these tumors and the inability to predict patient outcomes based on histopathological features, the search for a more accurate classification system is crucial. As a result of the growing field of molecular genetics, Verhaak et al. published recently a hallmark paper introducing a new classification system for GBMs alone, entirely based on gene-expression profiles of tumors. The authors employed sequence data of a large GBM cohort (n=206 patients) generated and provided by The Cancer Genome Atlas (TCGA) Research Network. Gene expression and alterations of EGFR, NF1 and PDGFRA/IDH1 define the subtypes, respectively Classical, Mesenchymal and Proneural (Verhaak *et al.*, 2010). The fourth subtype, Neural, was typified based on the expression of typical neuronal markers such as NEFL and GABRA1. The four subtypes show similarities with various neural lineages and respond differently upon chemotherapy, making it possible to clinically predict patient survival after therapy.

GBM are thought to arise from either preexisting lower-grade tumors (secondary GBM) or as primary tumors, emerging *de novo (Ohgaki & Kleihues, 2005, Scherer, 1940)*. The quest to find the tumor initiating cells has been ongoing for over 150 years already and continues to be a matter of debate. The three mainstream hypothesis are based on: (i) the embryonic displacement theory postulated by Virchow in his book Cellular pathology in 1858 (Virchow, 1858), (ii) the idea that gliomas arise from the subependymal zone (first phrased by (Globus, 1944) (iii) or from astrocytes (Levy *et al.*, 2009). Even though opinions about the cellular origin of gliomas have been going back and forth throughout history, with today's knowledge we know that these three hypothesis do not necessarily contradict. The cancer stem cell hypothesis (Huntly & Gilliland, 2005) would explain the initiation by (i) 'embryonic tissue' (Virchow) due to the presence of stem cells (ii) the origin of gliomas in the subependymal zone due to the observation that most gliomas exist in or close to the SVZ, proven to be a harbor for stem cells in the brain and (iii) the fact that astrocytes arise from the SVZ stem cells (Siebzehnrubl *et al.*, 2011).

Gliomas consist of, besides the tumor cells, multiple other cell types often referred to as 'tumor-associated cells'. For example neuronal precursor cells, vascular cells, microglia and peripheral immune cells. The cellular composition of the glioma plays a crucial role in the course of the pathology. The majority of tumor-associated cells are microglia, the macrophages of the brain (Charles *et al.*, 2011). The stromal cells of gliomas play part in the pathology and are therefore interesting to target by therapeutic agents. For example, recruitment of pericytes and vascular smooth muscle cells are crucial for the establishment of the vascular tree and promoting angiogenesis, processes

essential for the survival and growth of the tumor (De Palma *et al.*, 2005, Song *et al.*, 2005). Targeting HIF-1α, a protein (partially) responsible for the recruitment of pericyte progenitor cells in gliomas (Du *et al.*, 2008), can indirectly prevent the formation of new vessels in and surrounding the tumor and thereby inhibit tumor growth. Furthermore, the ability of astrocytes to promote GBM cell invasion through the activation of pro-MMP2 (Le *et al.*, 2003) and the production of neurotrophic factors such as Transforming growth factor alpha and C-X-C motif chemokine 12 (Hoelzinger *et al.*, 2007) makes them worthwhile to investigate for future therapy. However, due to the fact they do not proliferate, have proven to be inherently resistant to cytotoxic therapies, and serve probably no critical function within the tumor they are not a preferred study object. Additionally, drugs targeting for example PI3K, notch and sonic hedgehog signaling pathways – critical pathways activated in the perivascular niche of the tumor - are already clinically implemented or under development (Charles et al., 2011). However, even though the stromal niche seems to be an interesting target for drug therapy, a cautionary note should be taken concerning resistance to treatment specifically controlled by the tumor microenvironment. New studies demonstrate that microenvironment-derived resistance can alter the effectivity of drugs, and therefore a combination treatments targeting both the cancer cells as well as cells from the tumor microenvironment is probably most beneficial (Ostman, 2012). A more recent and rapidly emerging field regarding treatment focusses on immunotherapy, an approach that has already been proven to demonstrate clinical benefit for other cancers. Examples of such therapies that are FDA approved and currently used in clinics are the humanized monoclonal antibody against cytotoxic T-lymphocyte antigen-4 (Ipilimumab) (Hodi *et al.*, 2010), and the dendritic cell cancer vaccine (sipuleucel-T) (Kantoff *et al.*, 2010). GBMs are capable to avoid endogenous anti-tumor immune response, by generating a local immunosuppressive shield through the secretion of immunosuppressive factors such as tumor growth factor-β (TGF-β) (Constam *et al.*, 1992) and interleukin-10 (IL10) (Huettner *et al.*, 1995). Additionally, the systemic immune attack is also inhibited through impairment of T cell function and an increased T regulatory infiltration (Waziri, 2010). Given the previous, considerable achievements can be made with an immotherapeutic approach against GBMs. However, the current understanding on how GBMs foster immunotolerance is limited and therefore more research on the subject is a precondition for future successes in this type of therapy.

1.3.2 Glioma-associated microglia

Up to 30% of the tumor mass can consist of resident, ramified tumor associated microglia (Hanisch & Kettenmann, 2007), which was initially published already in 1925 (Penfield, 1925). The author, Wilder Penfield, used silver carbonate staining to detect the microglia within human gliomas samples and describes his finding as follow: "(..) these cells (microglia) are found to be plentiful in zones encircling the foci of tumor softening.", recognizing the fact that a large part of the tumor consist of microglia. Furthermore, the author states that microglia cells are 'in active *dendrophagocytosis',* referring to the phagocytic function of microglia (Penfield, 1925).

Microglia can be present in the brain in different immunological states, depending on their environment and the role in (patho)physiological processes they employ. Their morphology ranges from a ramified phenotype, with a small body and multiple branches, to a more amoeboid-like shape, enabling them to migrate and invade throughout the neuronal tissue. The ramified phenotype was historically described as a resting state, but now it is generally accepted that these microglia are however actively surveying their surroundings. They do so by continuous rebuilding their fine processes, allowing scanning of their surroundings for potential disturbed homeostasis. Therefore the term of 'surveying microglia' is more appropriate. The amoeboid-like phenotype is often referred to as the 'active' microglia, exerting functions as migration, phagocytosis, release of chemoattractive factors and antigen presentation to T cells to assist the adaptive immune system. Microglia can be triggered to transform from the surveying state to the activated state by external stimuli caused by for example cellular damage or the presence of foreign molecules. Between the surveying and active state, microglia can display many 'intermediate' and distinct phenotypes depending on the external stimuli and the situational context (Hanisch & Kettenmann, 2007, Eggen et al., 2013, Gertig & Hanisch, 2014, Kettenmann et al., 2011).

Even though the main function of microglia is to induce an immune attack against cells or pathogens disturbing the homeostasis in the brain, they are not able to perform this task within gliomas. On the contrary, several reports have suggested that microglia promote gliomas migration and tumor growth, favoring the tumor rather than a healthy condition (Bettinger *et al.*, 2002, Zhai *et al.*, 2011, Markovic *et al.*, 2005). Nonetheless, major histocompatibility class II (MHC Class II) molecule expression is seen on microglia within glioma tissues (Proescholdt *et al.*, 2001, Tran *et al.*, 1998), suggesting immunoreactivity against the tumor. However, proper antigen presentation for cytotoxic

and helper T cell seems to be deficient, probably due to a lack of expression of co-stimulatory factors necessary for the activation of T-cells (Flugel *et al.*, 1999). Attempts to interfere with the glioma-microglia interaction by using cyclosporine A (CsA) showed encouraging results *in vitro* and *in vivo.* CsA impaired the protumorigenic phenotype of microglia and thereby reduced the activation and invasion of glioma cells (Sliwa *et al.*, 2007, Gabrusiewicz *et al.*, 2011).

1.3.3 WNT signaling in glioblastoma

To date, little research has been done on the role of the WNT signaling pathway in the course of glioma pathology. Initial reports show a relationship between typical β-catenin-dependent signaling pathway activation and GBMs. For example, inhibiting GSK3β by lithium chloride, induces cell differentiation and inhibits neurosphere formation in human GBM *ex vivo* cell cultures (Korur *et al.*, 2009). Furthermore, DVL2 is overexpressed and is responsible for proliferation and differentiation of both cultured human glioma cell lines as well as patient-derived glioma cells. In the same study the authors show that by depleting DVL2 in GBM cells injected intracranial in immunodeficient mice, the tumor formation is inhibited compared to control (Pulvirenti *et al.*, 2011). Recently, more studies start to focus on the role of WNT-5A different processes of the tumor and its microenvironment. Initial studies showed that WNT-5A induces glioma cell proliferation (Pulvirenti et al., 2011) and is associated with infiltrative activity through the expression of MMP-2 in human gliomas (Kamino *et al.*, 2011). Furthermore, the co-receptor Ryk showed to be an essential protein for the WNT-5A-dependent invasiveness in human gliomas (Habu *et al.*, 2014). Although recently several studies were published showing a potential role of WNT-5A in GBM, the link between WNT-5A, GBMs and the relationship to immunological processes has so far not been investigated in detail.

2 SPECIFIC AIMS

The general aim of this thesis is to address the role of WNT/FZD signaling in brain physiology and disease and to dissect the molecular features of the pathway important for future use in drug development. The specific aims following the general aim were based on the reflections described in the introduction and are as follow:

1. To determine whether WNT-5A can induce activation of heterotrimeric G protein.

2. To assess functional selectivity of WNT-FZD signaling by characterizing binding selectivity and activitation of specific downstream signaling pathways.

3. To characterize the WNT-5A mediated proinflammatory response in microglia and to further dissect the WNT-5A induced intracellular signaling network.

4. To map WNT-5A distribution and define the WNT-5A-expressing cell type in the mouse CNS

5. To identify the role of WNT-5A on the migration and the integration of neuronal precursor cells from the subventricular zone, via the rostral migratory stream, towards the olfactory bulb.

6. To validate the presence of WNT-5A in GBM and further elucidate the effect of WNT-5A in the glioma cellular milieu with focus on immunological processes.

3 MATERIAL & METHODS

For this thesis a range of well-established techniques have been used which are listed in table 2. A description of these standard methods is displayed in the material and method sections of each paper and will therefore not be further discussed here. Solely methodological considerations relevant for the studies will be described in this section.

Table 2: Techniques employed in this thesis.

Method	Paper
Cell culture propagation	I-IV
Isolation and culturing of primary microglia from mice	III
Cell membrane isolation	I
[γ-^{35}S]-GTP assay	I
DNA (stable) transfection methods of cells	I, II
Native-PAGE immunoblotting	I
SDS-PAGE immunoblotting	I-IV
RNA extraction and cDNA synthesis	I-IV
Reverse transcriptase and quantitative PCR	I-IV
WNT/ FZD CRD-Fc binding assays with Biolayer Interferometry (BLI)	II
Immunoprecipitation	I
Immunocyto- and histochemistry	III-V
Inhibitor treatment/WNT-stimulation	I-III
Mesoscale for TNFα	III
$[Ca^{2+}]_i$ imaging	III
cAMP measurements	III
Collagen invasion assay	III
MTT-assay	I, III
Cell counting	I, III
EdU IP injections in mice	IV
WNT-5A heterozygous mouse model	IV
Super resolution/Confocal/fluorescent microscopy	III-V
The Cancer Genome Atlas investigation on GBM dataset	V
Bioinformatical tools: DAVID, Cytoscape, MIMi, MCode, C-bio portal, GSEA and Oncomine	V
Affimetrix gene array	V

3.1 ASSAYS TO ADDRESS G PROTEIN ACTIVATION

In paper I we employed the GTPγS assay to quantify G protein activation. In this method the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) measured through the accumulation of non-hydrolysable gamma-labeled GTP, occurring after stimulation of an appropriate ligand (Harrison & Traynor, 2003). This effect is believed to be

the first receptor mediated-event after receptor activation. The characteristics of the GTPγS assay are schematically visualized in the figure below.

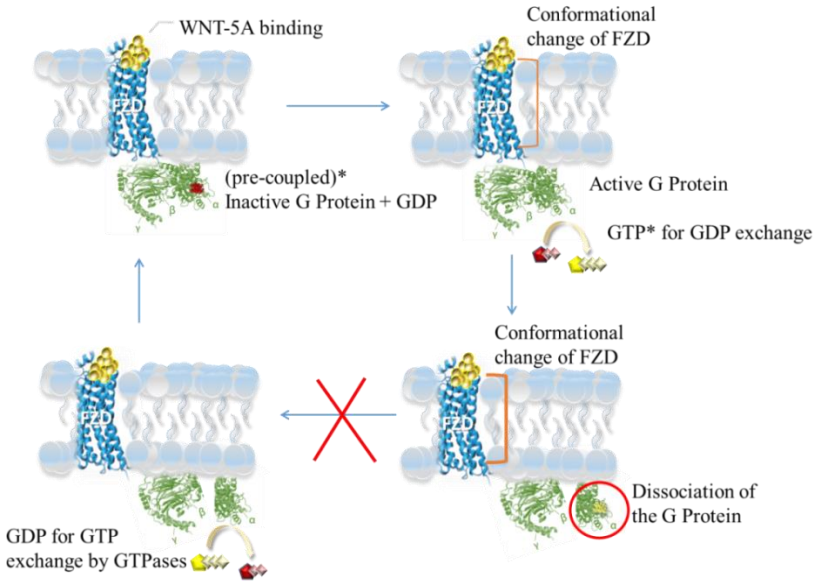


Figure 4: Schematic overview on GTPyS assay for WNT/FZD interaction. See text for the details.

A description of the assay, presuming WNTs are inducing the exchange through FZDs, is as follow: (I) The 7TM units of FZD are imbedded in the lipid membranes of cells and are believed to be either precoupled to inactive heterotrimeric G proteins bound to GDP (Kilander et al., 2014b, Kilander et al., 2014a) or in close proximity to. After WNT-5A binding to FZDs, a conformational change of the receptor occurs resulting in (II) the activation of the intracellular G proteins through the exchange of GDP for GTP at the Gα-subunit. (III) After activation of the G protein, dissociation of the Gα and Gβγ subunit follows upon which they can interact with effector systems. FZD is thought to return to its previous conformational state, making it more conceivable for the binding of inactive G proteins. (IV) GTPases reform the Gα-GDP binding, allowing Gα and Gβγ to form a heterotrimer. In a GTPyS assay, a non-hydrolysable and radioactive form of GTP binds to the active Gα subunit, allowing accumulation of the complex in the system. The accumulation of Gα-GTPyS can be measured with a beta counter (Harrison & Traynor, 2003).

An alternative method to measure G protein activation used in study I entails antibody-recognition of GTP-Gαi with immunoblotting under native conditions. The antibody recognizes solely the activated form of Gαi – the form that is bound to GTP - and became recently commercially available. Native conditions are required since denaturation of the complex will disrupt the binding between Gαi and GTP (Lane *et al.*, 2008).

3.2 INVASION ASSAYS

There are several established methods to measure cellular invasion and migration *in vitro*. This type of assays are clinically most relevant in cancer research determining motility of cancer and stromal cells. MMPs belong to a group of proteins known to break down the extracellular matrix (ECM), a key process of for example primary tumor cells initiating a metastasis (Hadler-Olsen *et al.*, 2011). Migration assays occur on 2D surfaces, as the goal is to show directional cell movement mimicking the *in vivo* situation where cells migrate within or between different organs. The invasion assays take place on obstructive services such as collagen networks or matrigel, representing the *in vivo* situation in which cells have to modify and interact with the ECM to be able to show directional movement (Kramer *et al.*, 2013). Within the term of invasion are therefore several processes enclosed, such as adhesion, morphological changes, migration and proteolysis of the ECM (Friedl & Wolf, 2010). In paper III, we performed a collagen I invasion assay with primary mouse microglia cells. Since we were interested in the migratory effects of WNT-5A on microglia – in relationship to the pro-inflammatory and proliferative features shown in other experiments - we decided upon an invasion assay rather than migration assay. After solidification of the collagen matrix on a glass bottom plate, fluorescently labeled microglia are seeded on top of the matrix and WNT-5A is subsequently added to the growth medium. The labeling of cells was done with cell tracker red CMTPX dye. This dye passes freely through the cell membrane and then becomes cell-impermeant due to transformation processes within the cell. It is proven to have low cytotoxicity and will therefore not affect cell viability or proliferation. Furthermore it allows for multigenerational tracking of cellular movement and has signal retention for more than 72 hours. After allowing the microglia to invade for 24 hours, a 3D image stack with the confocal was taken to visualize the location of the microglia within the collagen matrix. Analysis was done with the spot-function in the Imaris software, defining every fluorescent signal within a certain range as one cell. To improve the representation of this assay with the *in vivo* situation, one could think about mechanisms to generate a WNT gradient within the matrix. This however is challenging due the fact that WNTs move freely in liquids. Furthermore, co-culture experimentation with other cell types present in the brain would give a better overview on the interaction interplay between different cell types in the brain. However, this would also complicate the study set-up.

3.3 USE OF MOUSE PRIMARY MICROGLIA

In paper III we used primary mouse microglia to study the inflammatory potential of WNT-5A. Even though the use of cultured cell lines showing microglia-like features, such as N13, would be more practical, it is also less physiologically relevant to study. For the preparation of

the primary microglia newly born (P1-3) C57BL6 mouse pups were used housed in our animal facility at the Department of Physiology and Pharmacology, Karolinska Institute. Experimentation was done according to ethical permit N144/08 and N436/10; local ethical committee Stockholms Norra Djurförsöksetiska Nämd. A detailed description of the procedure is given in Paper III. Microglia were harvested through gentle agitation of the bottle, allowing the cells to separate from the astrocytic monolayer for a maximum of three times per batch. To determine the contamination of astrocytes in our microglia preparation, we employed an immunocytochemistry experiment using fluorescein isothiocyanate (FITC)-conjugated Griffonia simplicifolia isolectin B4 or anti-CD11b for microglia staining combined with the astrocytic marker anti-GFAP (glial fibrillary acidic protein). The purity of the preparation was validated >95%.

3.4 THE CANCER GENOME ATLAS SEARCH AND ASSOCIATED DATABASES

The cancer genome atlas is a community research project providing genomic data from at least 200 different types of cancer. This freely available data is accessible through the TCGA Data Portal and the Cancer Genomics Hub and was founded in 2006 by National Cancer Institute and National Human Genome Research Institute. Biospecimens are collected after asking patients to donate part of their tumor tissue that had been removed in accordance to their treatment schedule, as well as samples from normal tissue such as blood. Subsequently the biospecimens have to meet a stringent set of criteria concerning the quality of the DNA and RNA before they undergo a complete genomic characterization and analysis. All the data is coded, meaning the sample information cannot be connected to the patient's private information. The overarching goal of the atlas is to improve the ability to diagnose, treat and prevent cancer. By gaining a better understanding on the genomic alterations in cancers, new treatment options related to personalized medicine can be in reach.

Following the release of the data portal of the TCGA, different platforms launched websites and software that can be used to interpret the genomic information. For example, in paper V we used a set of platforms including: Gene set enrichment analysis (GSEA) (Subramanian *et al.*, 2005, Mootha *et al.*, 2003), DAVID (Huang da *et al.*, 2009b, Huang da *et al.*, 2009a), Oncomine (Rhodes *et al.*, 2004), Cytoscape (Saito *et al.*, 2012) with the plugins Mimi (Jayapandian *et al.*, 2007, Tarcea *et al.*, 2009) and MCode (Bader & Hogue, 2003) and c-Bio portal (Cerami *et al.*, 2012, Gao *et al.*, 2013). The GSEA focuses on sets of genes that share common biological functions, chromosomal location or regulation. The C-bio portal provides the opportunity to explore the genomics across different cancer types through visualizing and analyzing genes, samples and data types. The portal also provides exploration

in biological pathways, mutual exclusivity or co-occurrence analysis between genes of interest and survival curves. Cytoscape allows network data integration, analysis and visualization of complex networks.

3.5 THE USE OF WNT-5A HETEROZYGOUS MOUSE MODEL

The heterozygous mouse model used in paper IV, was first described in Yamaguchi et al., 1999. It was created as follow: A targeting vector with neomycin positive (PGK-*neo*) and a thymidine kinase negative (MC1*tk*) selection markers was used to disrupt exon 2 at codon 31. The null mice are created by electroporation of the construct into AB1 embryonic stem cells. Resistant colonies were isolated, expanded and injected into day 3.5 blastocytes of the C57BL/6 strain (Yamaguchi et al., 1999). Even though studying a homozygous adult phenotype would give us better understanding on the role of WNT-5A on neuronal migration, the mice are perinatal lethal due to respiratory failure at birth. Furthermore, the homozygous *WNT-5A*-null embryos show severe shortening of the body and limbs, truncated facial features and loss distal structures such as the tail. There is not much literature published on the heterozygous phenotype, except some reports on the double heterozygous Wnt5a+/− combined with other mutations, such as for example LPR6 (Bryja *et al.*, 2009). Another possibility to study the function of WNT-5A in the brain would be by generating a doxycycline-inducible GFAP-CreERT/WNT-5A-loxP mouse. This animal model allows to dynamically regulate astrocyte expression of WNT-5A in adult mice. Because our results show that WNT-5A is mostly secreted by $GFAP^+$ cells, it would be interesting to see what the function is of WNT-5A produced by this astrocyte population.

4 RESULTS & DISCUSSION

4.1 WNT/FZD BINDING, SELECTIVITY AND ACTIVATION OF DOWNSTREAM SIGNALING PARTNERS

4.1.1 Activation of heterotrimeric Gαi2/3 proteins by WNT-5A in the microglia like cell line N13 (Paper I)

FZDs have recently been grouped in a separate class within the superfamily of GPCRs by the IUPHAR and The British Pharmacological Society in The Guide to Pharmacology (Alexander et al., 2013, Foord et al., 2005). For many years however, the coupling of heterotrimeric G proteins to FZDs has been a matter of debate. Even though the general opinion is shifting at present towards FZDs signaling as bonafide GPCRs (Schulte & Bryja, 2007, Schulte, 2010), there are opponents arguing the importance of this coupling for the activation of downstream signaling components. In this article we investigated if WNT-5A is able to induce G protein activation in cell membranes of the microglia like cell line N13 at endogenous expression levels of relevant signaling components. We employed a [γ-^{35}]GTP assay, established over three decades ago for β-adrenergic receptors (Asano *et al.*, 1984) and muscarinic receptors (Kurose *et al.*, 1986). In this assay the accumulation of Gα-GTP is measured, which is induced by agonist occupation of GPCRs followed by the exchange of GDP for GTP at the α-subunit (Harrison & Traynor, 2003). This response is seen as the first event after stimulation of a GPCR, and therefore used as a method to study GPCRs activity. In this article we show a WNT-5A mediated exchange of GDP for GTP in a microglia-like cell line. To our knowledge, this was the first report to describe such an event using physiological G protein and FZD levels.

First we investigated WNT-5A induced proliferation in the microglia-like cell line N13. Proliferation is a physiological relevant event in microglia, occurring after microglia undergo the transition from the surveying towards the activated state. In case of an inflammatory response, microglia migrate towards the site of action and proliferate to be able to perform their intrinsic function properly. We showed that N13 cells proliferate upon WNT-5A stimulation and that this stimulation is sensitive to the $G_{i/o}$ inhibitor PTX, revealing a role for $G_{i/o}$ proteins in this response. Even though one could question the relevance of this assay with the use of an immortalized cell line, a later study from our lab showed a similar effect in primary mouse microglia after WNT-5A stimulation (**Paper IV**).

Even though we now had initial proof that WNT-5A can induce G protein related effects in our cell line, we were further interested if we could detect a direct interaction between FZDs and G proteins. Therefore we employed a [γ-^{35}]GTP assay, resulting in the discovery of dose-dependent and PTX sensitive WNT-5A activation of the GDP for GTP exchange at the

α-subunit (see figure 5 A and B). It is therefore likely to assume that WNT-5A can activate G proteins through FZDs, and that this response is dependent on the $G\alpha_i$ subunit.

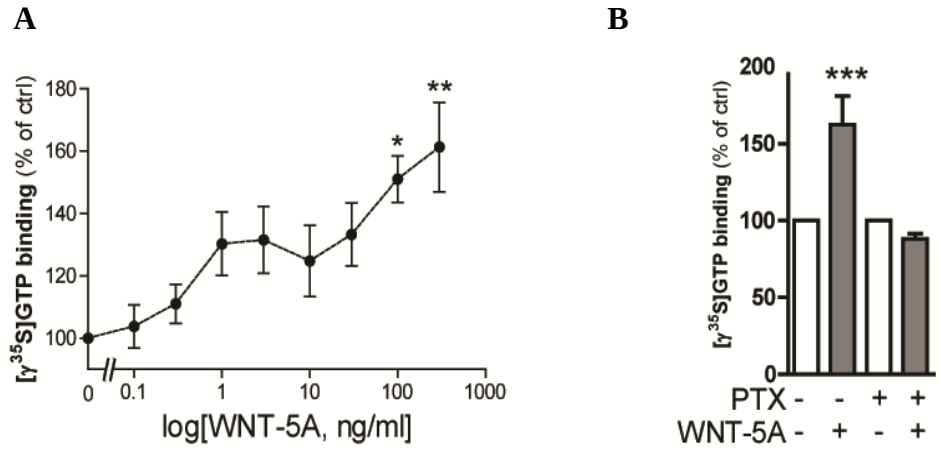


Figure 5: WNT-5A evokes a GDP/GTP exchange in Gi/o α-subunits in membranes of N13 cells.
(A) A dose dependent activation of G proteins by WNT-5A (300 ng/ml) measured by the incorporation of [γ-^{35}]GTP. (B) The WNT-5A induced G protein activation is PTX sensitive (100 ng/ml, incubation overnight).

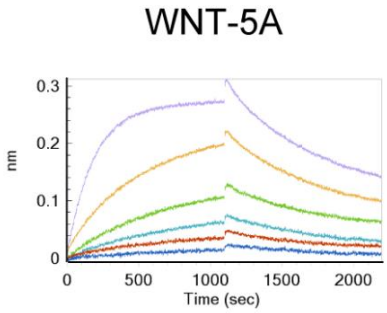
The biphasic dose-response curve (figure 5A) differs from a sigmoidal dose-response curve as one expects from a classical ligand/receptor response. It is known that WNT-5A can bind to several FZDs (Dijksterhuis et al., 2013), which could lead to opposing responses resulting in the biphasic effect. Additionally, WNT binding to Heparan sulfate proteoglycans, proven to be necessary for the maintenance of the solubility and thereby the stability and activity of WNTs in mammalian tissues (Fuerer *et al.*, 2010), might distort the classical sigmoid relationship.

To further substantiate the finding that WNT-5A can induce G protein activation from the $G_{i/o}$ family, we performed immunoblotting experiments with an antibody selectively recognizing the active form of $G_{i/o}$ (G_i-GTP antibody) and tested this in both N13 cell membranes and permeabilized cells. The positive result of this experiment was in agreement with the earlier findings from [γ-^{35}]GTP assay, strengthening the hypothesis that WNT-5A can induce G protein activation. qPCR experiments revealed the FZD expression profile in N13 cells as follows: FZD_5>FZD_7>>FZD_2>FZD_4>=FZD_9>FZD_8. The FZD_5 expression is 3 times higher than the FZD_7 and it is therefore likely to assume that the G protein activation is transduced through FZD_5. Further investigation by western blot analysis of WNT-5A stimulated FZD_5 showed a mobility shift on the SDS page, suggesting that a portion of the receptors get phosphorylated after activation. If and how this specific interacting induces G protein activation remains to be elucidated.

4.1.2 Analysis of WNT/FZD interactions and their functional selectivity (Paper II)

The puzzling thought that mammalians have 19 different WNTs, 10 different FZDs, as well as several associated downstream signaling networks inspired us to investigate WNT/FZD binding pairs and explicit downstream activity systematically. In order to do so, we employed the myeloid progenitor cell line 32D, which has the unique feature of a complete lack of endogenous FZD expression. This enabled us to introduce specific FZD genes and thereby to study WNT-induced activation of FZDs individually.

Here we used Biolayer Interferometry (BLI) to assess WNT/FZD-CRD binding partners of WNTs. For this experiment, recombinant CRD proteins of the related FZDs were used. Although measurement of binding between WNT and the full length FZD protein - not just the CRD domain - would be more biologically relevant, it is so far experimentally not possible. In most GPCRs binding assays to full length receptors are straight-forward. However for FZDs the method is not established yet, leaving us to rely on the biochemical analysis of the WNT/FZD-CRD interaction. WNT binding experiments have been challenging to perform, partially due to the fact that most cellular systems express several isoforms of FZDs and a suitable systems to study is lacking so far. Furthermore, WNTs have a very high unspecific binding to the extracellular matrix of the cell membrane because of their lipid modifications, making it difficult to measure binding affinities. The advantage of the biochemical WNT/FZD-CRD interaction measurement is that with certainty the direct binding affinity between WNTs to CRDs can be determined, excluding interference from other receptors. Furthermore, it provides accurate affinity measurement outcomes, enabling quantitative comparison between the different WNT/FZD pairs.



[WNT] 200 nM 100 nM 50 nM 25 nM 12.5 nM 6.25 nM

Ligand \ CRD	FZD_1	FZD_2	FZD_4	FZD_5	FZD_7	FZD_8
mWNT-3A	+++	+++	++++	++++	++++	++++
mWNT-4	NQ	+	++	+++	++	++++
h/mWNT-5A	+++	+++	+++	++++	++	++++
mWNT-5B	++	++	++	+++	++	++++

Figure 6. WNT/FZD-CRD bioloayer interferometry. (A) Representative binding affinity curve for WNT-5A to FZD_1-CRD (B) Tabulated the summary of WNT/FZD-CRD binding data shown as: - no binding, + very weak binding (> 100 nM), ++ weak binding (40-100 nM), +++ intermediate binding (10-40 nM), ++++ strong binding (< 10 nM). NQ stand for weak binding, not quantifiable due to narrow response signal window in the binding curves. WNT-7A, -9B, - 10B, -11 and FZD_{10} show no detectable binding to respectively any of the chosen FZD-CRD's or WNTs in this study (data not shown).

Even though it is possible to define binding affinities between WNTs and FZDs, one cannot assume this binding transduces an effect downstream and induces subsequently a physiological relevant response. Therefore, we investigated several relevant downstream events including the phosphorylation of the signaling components DVL2/3 and LRP-6 and the accumulation of β-catenin.

DVLs stand on the cross road between various WNT/FZD activation pathways and their stimulation requires phosphorylation. This can be visualized on a western blot as a electrophoretic mobility shift and is referred to as phosphorylated and shifted DVL (PS-DVL). It is known that the isoforms of DVL (1,2, and 3) have considerable overlap in function, however there is evidence for functional specification as well (Dillman *et al.*, 2013). In this study we show that 32D/$FZD_{2/4/5}$ cells stimulated with various WNTs show differential effects on PS-DVL2 and PS-DVL3, depending on the WNT/FZD pair present. This result points towards the hypothesis that the signaling route exhibits functional selectivity.

We hypothesize that certain biological relevant WNT-receptor complexes could transduce β-catenin-independent signaling in the absence of LRP5/6, but on the other hand may feed into the β-catenin pathway by forming a complex with LRP5/6 (Kilander 2013, 2014). These hypothetical complexes may exist in parallel, opening the possibility that certain WNTs, for example WNT-3A, can activate both the heterotrimeric G protein-dependent ERK1/2 phosphorylation independent of β-catenin as well as accumulation of β-catenin (Halleskog & Schulte, 2013a). It is likely to assume that WNT/FZD binding affinity as well as functional selectivity plays a role in this.

In agreement with the literature, WNT-3A induced phosphorylation of LRP6 through $FZD_{2,4,5}$ in this study. The possibility exists that a given WNT will interact with FZD but does not activate β-catenin signaling. On the contrary, it might inhibit β-catenin signaling or transduce a signal through FZDs that are independent of β-catenin. Thus, with the presence of LRP5/6 in the 32D system, one could identify specific FZD complexes that require LRP5/6 for signaling. Interestingly, WNT-9B did not show any activation of the assays tested in this study. However, previous reports have shown that WNT-9B can bind LRP5/6 and can either activate the β-catenin-dependent signaling (through TOPflash) (Bourhis *et al.*, 2010, Gong *et al.*, 2010) or the β-catenin-independent signaling pathway (Kilander *et al.*, 2011b), depending on the cell type. Therefore, the role of WNT-9B interacting with LRP5/6 for signal specification remains to be elucidated.

This paper contributes to the understanding of the underlying mechanisms that determine signal specification by WNTs in FZDs. The beauty of the cellular system of choice

entails the presence LRP5/6, but the lack of detectable endogenous expression of Class Frizzled receptors, enabling to distinguish between WNT/FZD pairs activation the β-catenin pathway versus those that do not. Furthermore, characterization of the 32D parental line revealed that the cells contain little expression of WNTs, secreted frizzled-related proteins (sFRP) and dickkopf-related proteins (DKK). Additionally, the 32D cells do not express heparan sulfate proteoglycans (Richard *et al.*, 1995), which is beneficial in our system because it excludes the confounding factor of WNT stickiness to the proteoglycans.

In summary, this cellular system opens the avenue to study functionality of single FZD isoforms in a mammalian cell system, given the fact it shows functional β-catenin accumulation upon stimulation and holds biological relevant endogenous LRP5/6 and DVL expression.

4.2 WNT-5A IN BRAIN (PATHO)PHYSIOLOGICAL PROCESSES

4.2.1 WNT-5A induces a proinflammatory transformation in primary mouse microglia through ERK1/2 mediated signaling (Paper III)

Brain inflammatory processes are a rewarding subject of study due to the fact that several CNS pathologies are accompanied by microglia activation, which can either be detrimental or beneficial (Querfurth & LaFerla, 2010). Previous work from our lab revealed a strong link between the activation of pro-inflammatory processes in microglia and WNT-3A. Furthermore, β-catenin levels are increased in microglia in post mortem brain from patients with AD as well as from the APdE9 (Swedish mutation of amyloid precursor protein and exon 9 deletion coding for presenilin 1) AD mouse model (Halleskog et al., 2011). These results indicate a role for the β-catenin pathway in CNS inflammatory processes. Other reports from our lab already showed activation of heterotrimeric G proteins, phosphorylated and shifted DVL (PS-DVL) and proliferation of the microglia-like cell line N13 (Kilander *et al.*, 2011a, Kilander et al., 2011b). In paper III, we sought to investigate if the β-catenin-independent WNT-5A protein had physiological relevant effects on microglia and through which intracellular pathway this effect is exerted.

To determine whether WNT-5A has physiological relevance in the brain we first investigated if and which cell type produces and secretes the protein in the CNS. Through immunohistochemistry, immunoblotting and qPCR experiments we pinpointed astrocytes as the main source of WNT-5A in the mouse adult brain, while microglia themselves show considerably less expression. WNT-5A signaling in microglia could thus be paracrine or to a lesser extend autocrine. In the search of dissecting the signaling cascade downstream of WNT-5A, we employed several molecular pharmacological relevant assays. In agreement with

previous data, immunoblotting results revealed that WNT-5A does neither affect β-catenin stabilization nor phosphorylation of LRP6. However, it induced a dose- and time-dependent increase in the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and DVL3. ERK1/2 belongs to the family of mitogen-activated protein kinases (MAPK), known for regulating various cellular processes such as proliferation, differentiation, apoptosis and embryogenesis. Other genes having distinct regulation and function within MAPKs family are for example the c-Jun N-terminal kinase (JNK(1-3)) and p38(alpha, beta, gamma and delta) families as well as the ERK5 branch (Raman *et al.*, 2007). In this study we focused on ERK1/2 activation, involved in various cellular processes such as cell motility and proliferation. Activation occurs for example after stimulation by growth factors and ligands for GPCRs or during cellular stress (Lewis *et al.*, 1998, Chen *et al.*, 2001). After applying the Gαi subunit inhibitor PTX, the P-ERK1/2 increase by WNT-5A was abolished. This implies that the WNT-5A-induced MAPK signaling is activated by heterotrimeric G proteins, most likely transduced through FZDs. PS-DVL3 formation was not blocked by PTX, suggesting an alternative signaling branch being activated independent of G proteins. To support the finding of heterotrimeric G protein-activation, we performed GTPγS, cAMP and Ca^{2+} assays, processes typically involved in GPCR signaling. The results confirmed the functional activation of Gαi/o proteins by WNT-5A. By employing a set of inhibitors targeting different molecules important within MAPK signaling pathway, we were able to dissect the pathway activated downstream of WNT-5A. A schematic summary of those results are given in figure 7.

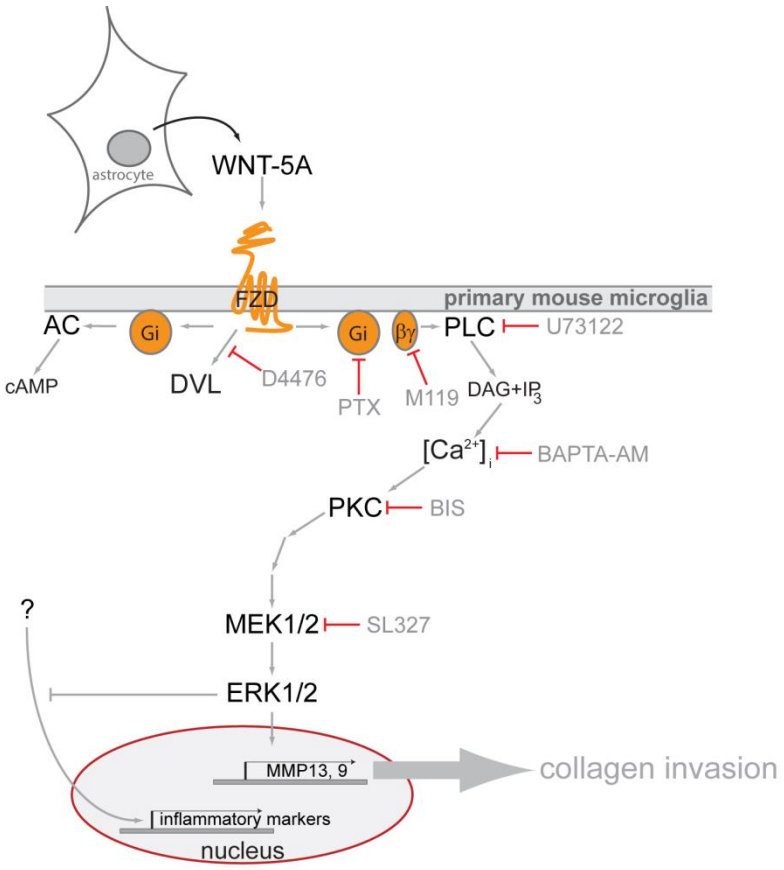


Figure 7: schematic overview of proposed signaling route in microglia downstream of WNT-5A. The pharmacological inhibitors employed are marked in gray. The question mark indicates unknown signaling route. Figure modified from Halleskog et al., 2012.

We then returned to the question whether WNT-5A is physiologically relevant for microglia. As mentioned earlier, there is a strong link between inflammatory processes and WNT signaling in the brain. By stimulating microglia with WNT-5A, a strong pro-inflammatory (shown by increased expression of various pro-inflammatory genes), pro-invasive (figure 8B) and proliferative response was observed. It is known that MEK1 and -2 are upstream of ERK1/2, phosphorylating the tyrosine and threonine residues in the ERK1/2 activation loop (Roskoski, 2012). By employing the MEK inhibitor SL327, we revealed that activation of MEK1 and -2 are part of these processes, shown by a total abrogation of the invasion (figure 8), proliferation and a partial blockade of the increased MMP 9 and 13 gene expression. Several other pro-inflammatory genes are not inhibited by SL327, suggesting that WNT-5A signaling is not solely transduced through the MAPK pathway. Possibly, the pathway branch involving the phosphorylation of DVL3 is part of this activation, since PS-DVL3 formation was not

affected by the Gαi protein inhibitor PTX. This could either occur through the activation of different FZDs – with their own distinct signaling route – or in parallel at the same receptor.

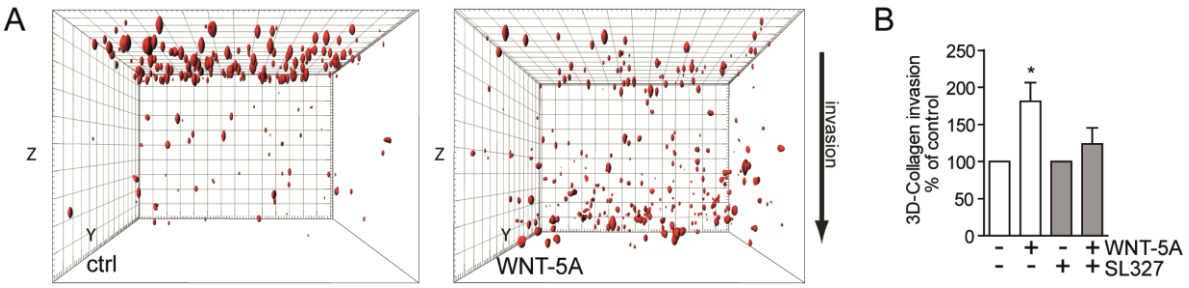


Figure 8: Collagen invasion assay in primary microglia stimulated with WNT-5A. Visualized in (A): fluorescently labeled microglia are seeded on top of a 3D collagen IV layer and stimulated with either WNT-5A or Ctrl (PBS). After 24 hours, microglia are located within the collagen layer with confocal microscopy. The graph in (B) represents the percentage of invasion under different conditions. Figure modified from Halleskog et al., 2012.

4.2.2 WNT-5A is expressed by astrocytes in the rostral migratory stream and affects the neuronal precursor migration towards the olfactory bulb (Paper IV)

Motivated by the results gathered from paper III, we continued our investigation on the location of WNT-5A expression in the brain and the role WNT-5A on physiological processes in the CNS. During brain developmental processes, WNT-5A is expressed by radial glia cells and functions to aid neuronal specification and positioning (Castelo-Branco et al., 2006). However, little is known about the role of WNT-5A in adulthood. Employing classical immunohistochemistry, we were able to map the distribution of WNT-5A in the mouse adult brain. During the initial characterization, we identified several regions of the brain clearly showing either subpopulations of neurons or non-defined subpopulation of astrocytes expressing high levels of WNT-5A. The WNT-5A-expressing neuronal population is predominantly present in the cortex. In the neurogenic regions including the hippocampus and subventricular zone (SVZ) and in the olfactory bulb (OB) and rostral migratory stream (RMS), especially the astrocytic population showed high expression of WNT-5A. This is in agreement with earlier reports, showing that astrocytic expression of WNT-5A has a role in the OB and controls neurite outgrowth during development and adulthood (Halleskog et al., 2012, Pino *et al.*, 2011). In addition WNT-5A regulates the morphology and outgrowth of olfactory sensory neurons *in vitro* (Rodriguez-Gil & Greer, 2008). Careful investigation of the immunohistochemistry stainings of WNT-5A revealed, besides expression within the glial cells, a vesicular appearance of the protein distribution in certain areas of the brain. It is still a matter of debate how WNTs travel through the interstitial space but there are indications that

this transport is facilitated through exosome structures (Gross et al., 2012). Future research will shed light on the detailed structure of these vesicles, where they originate, and if they are dynamic or static.

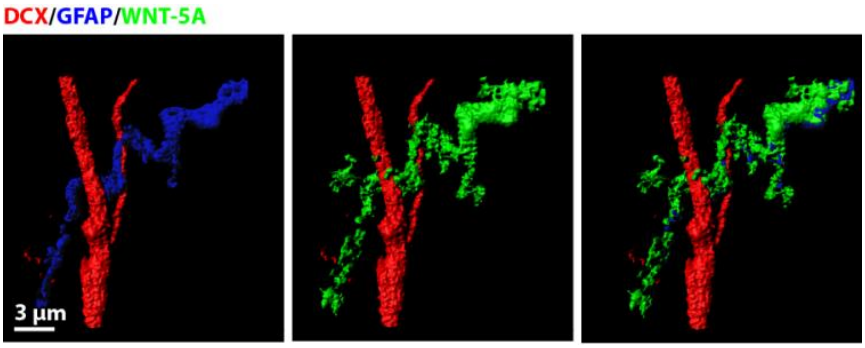


Figure 9: 3D rendering of a Z-stack imaged usinga superresolution microscopy from adult mouse brain stained with doublecortin (DCX; neuronal precursor), glial fibrillary acidic protein (GFAP; astrocyte) and WNT-5A. Note the GFAP/WNT-5A overlap and the close proximity of astrocyte and neuronal precursor.

After showing the distribution of the WNT-5A in the adult mouse brain and revealing the SVZ-RMS-OB region as regions of interest, we were interested in the role of WNT-5A on neuronal processes ongoing in this area. In order to do so, we employed immunohistochemistry on EdU labeled WNT-5A$^{+/-}$ and $^{+/+}$ mouse brain material. EdU incorporates into DNA of dividing cells, thereby labeling cells that undergo division e.g. neuronal precursor cells. The WNT-5A^{+} astrocytes do not co-localize with the EdU labeling, suggesting they do not present a stem cell population or are a source for neuronal precursors. However, WNT-5A^{+} astrocytes were distinctly marking the RMS and seemed to be in close proximity of the doublecortin^{+} (DCX) neuronal precursors. Employing super-resolution microscopy and 3D image analysis revealed that neuronal precursors in the RMS are indeed closely interacting with WNT-5A-positive astrocytes (see figure 9), suggesting intricate communication between the cell types.

Furthermore, in the heterozygous WNT-5A$^{+/-}$ mice an altered distribution pattern and increased number of EdU+ cells could be observed along the RMS. This suggests that the gross anatomy of the OB is altered compared to wild type mice, also illustrated by the difference in size of the OB glomeruli.

In summary, based on this preliminary data, we hypothesize that a subpopulation of astrocytes present in the rostral migratory stream secrete WNT-5A as regulatory key to signal neuronal precursor migration. This process is of importance for the formation and maintenance of olfactory bulb integrity and function. Thus, astrocyte-secreted WNT-5A offers a cue for maintaining neuronal precursor migration along the RMS and thus a more general function

necessary for support of precursor migration resulting in the aid of stem-cell-dependent tissue maintenance and repair. The findings of study IV are schematically summarized in paper IV.

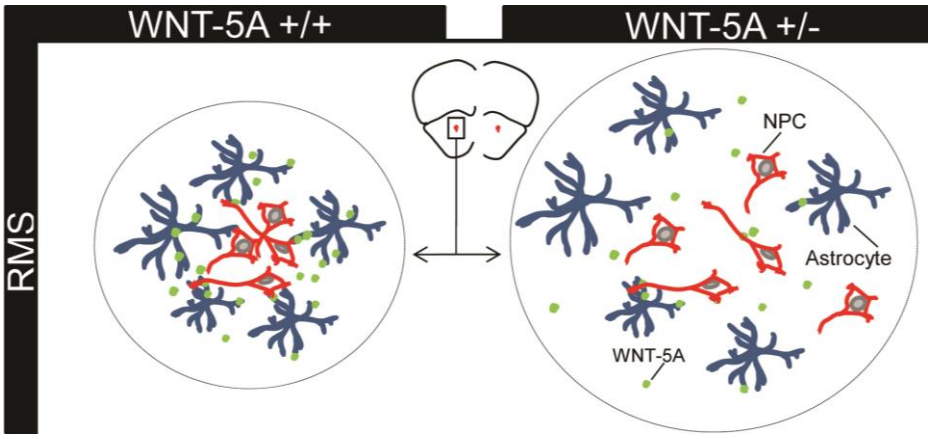


Figure 10: Schematic presentation of paper IV. Astrocytes in the rostral migratory stream express high levels of WNT-5A in close proximity to migrating neuronal precursor cells on their way towards the olfactory bulb. Partial ablation of WNT-5A expression in adult WNT-5A$^{+/-}$ heterozygous mice results in widening of the astroglial tube in the RMS leading to a disturbed neuronal integration in the OB. NPC stands for neuronal precursor cell.

4.2.3 Analysis of the presence and function of WNT-5A in human glioblastoma regarding inflammatory components (Paper V)

Based on our previous work on WNT-5A signaling in relation to inflammatory pathway activation in microglia and a couple of reports linking WNT-5A signaling to glioma, we were inspired to investigate the involvement of WNT-5A in GBM. Yu et al. reported earlier the upregulation of WNT-5A in human glioma as well as in cultured glioma cell lines (Yu *et al.*, 2007). After initial immunohistochemistry experiments it became clear that indeed WNT-5A is highly expressed in GBMs on protein level. This finding was supported by a TCGA analysis in Oncomine, showing that the WNT-5A gene is 4.1 times upregulated in GBM samples compared to control brain tissue. The other members of the WNT family did not show a significant increased expression. The expression level of WNT-5A did not appear to have an effect on median survival of the patients. However, patient groups with higher expression of two members of the FZD family, that is FZD_4 and FZD_7, do show a difference in median survival of 2.1 and -4.2 months respectively (see figure 11), indicating potential pathway specificity in the response.

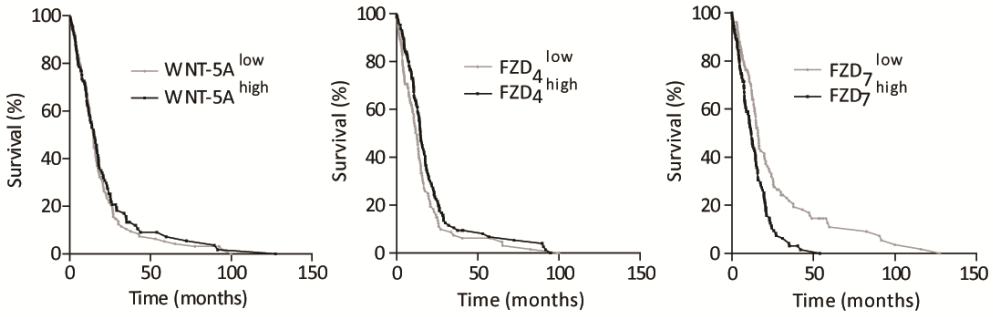


Figure 11: Kaplan-Meier curves between the patient groups expressing 25% low versus 25% high levels of WNT-5A, FZD_4 and FZD_7. The median survival in months between the low versus high expressing groups of WNT-5A, FZD_4 and FZD_7 are respectively: 14.7 - 15.1 (p = 0.423), 12.6 – 14.7 (p = 0.042) and 16.1 - 11.8 (p = <0.0001) months.

In depth investigation of the group of genes higher expressed in the patient group with 25% highest level of WNT-5A (WNT-$5A^{high}$ group) revealed an association to processes involved in immunology, such as the Gene Ontology terms immune response (GO:0006955) and antigen processing and presentation of peptide or polysaccharide antigen via MHC class II (GO:0002504). To perform this analysis we employed several open source bioinformatics platforms such as GSEA, DAVID and Cytoscape plus the additional plug-ins MiMI and MCode). We were puzzled by the outcome, acknowledging the fact that GBMs generally create a local immunosuppressive microenvironment thereby avoiding potential anti-tumor immune response (Waziri, 2010). The activation of the antigen presentation pathway would suggest activated immune attack. Additional investigation led to the discovery that in the WNT-$5A^{high}$ patient group known microglia markers (as described in kettenman *et al*. 2011) are enriched compared to the WNT-$5A^{low}$ group. This indicates that increased WNT-5A levels in the GBM microenvironment correspond to higher presence of microglia within the tumor. Additionally, co-occurrence analysis revealed positive correlation between several microglia markers and the WNT-5A gene. The activated immunological processes in the WNT-$5A^{high}$ group can possibly be explained by the higher incidence of microglia within the GBMs. We hypothesized that the increase in MHC class II components within the WNT-$5A^{high}$ group can be due to the presence of microglia as well. Microglia are antigen presenting cells, hence capable of expressing MHC class II components on their cell surface. Indeed,

immunohistochemistry experiments done in tissue micro arrays of GBM samples, showed a strong correlation between the microglia marker IBA-1 (ionized calcium-binding adapter molecule 1) and HLA-DMA/DPB1. It remains questionable if the MHC class II on the surface of microglia is functional. Reports have shown that MHC Class II expression in TAM is either decreased, impaired or both. In agreement with that, we found that several essential components necessary for a fully functional antigen presentation response, such as CD40 and B7-1, are not enriched in the WNT-5A group and their overall expression is low. The findings of study V are summarized in a schematic overview as shown in the figure below.

Collectively, even though one cannot draw direct conclusions on the effect of WNT-5A on the progression of the tumor growth, the results of paper V pinpoints towards a novel role of WNT-5A on the presence of glioma-associated microglia infiltration. An investigation of a detailed phenotypical state of the microglia present in the WNT-5A^{high} GBM patient group would give us a better understanding if and how we can utilize this increased microglia invasion as a target for drug development.

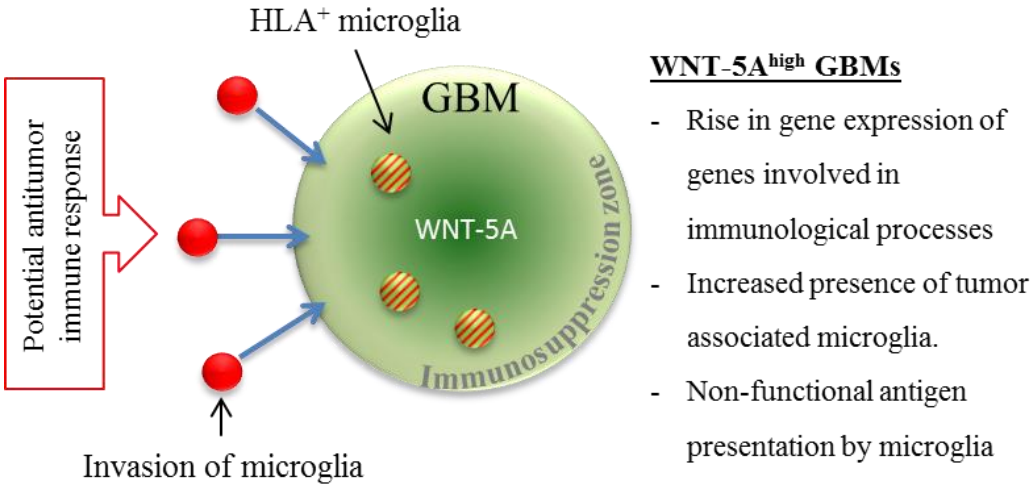


Figure 12: Schematic overview on the findings of paper V. The green circle represents the GBM and its microenvironment.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In this thesis I aimed to create a better understanding of WNT/FZD signaling on different levels of medical research. With this intent, classic studies were combined to recent discoveries, pharmacological data compared to clinical studies, and physiological functions were related to pathophysiological alterations. The present chapter encloses scientific considerations regarding this aim. It is in no way intended to serve as a complete and round story, but rather a collection of thoughts that could help guiding future research.

The ultimate goal of performing fundamental medical research is to develop treatment options against diseases. There is still, however, much to gain in bridging the gap between fundamental findings and the applied sciences. The field of translational sciences aims to apply results acquired in basic research into clinical use. This is, of course, a vital step ensuring basic research will at last improve public health. Currently, significant improvements have been made towards increased multidisciplinarity in medical sciences. They include genomic screenings of patient groups, the use of bioinformatical tools to comprehend the often vast amount of data, and the intent to develop personalized medicine in order to employ this knowledge with higher effectivity. To achieve these goals, future challenges include the establishment of better communication between scientist from different research areas, as well as clinicians, patients and the population as a whole. In my opinion, it would be immensely beneficial to integrate opinions and knowledge from various disciplines to strengthen medical research.

There are interesting questions raised by the research presented in this thesis that deserve additional attention. The first is, naturally, the potential use of WNT/FZD signaling as target for therapy. To define the druggability of the WNT/FZD signaling pathway, numerous aspects should be taken into consideration. From a broader perspective, one should first determine the specific link of the pathway to different diseases. Are we dealing with mutations, alteration of gene expression of specific molecules, or rather disturbance of parts or the whole regulatory pathway affecting the disease progression? For instance, the supposed role that FZD_4, FZD_6 and APC play in, respectively, familial exudative vitreoretinopathy (FEVR) (Wang *et al.*, 2012), nail dysplasia (Frojmark *et al.*, 2011) and colorectal cancer (Clevers, 2004), are caused by specific mutations within the protein codes in the DNA. The follow up question that logically can be asked is what the effect of this mutation is on the functionality of

the protein. In the case of FZD_6 in nail dysplasia, it is known that the receptor is still produced, but is not translocated efficiently to the membrane due to a missense mutation. This results in non-functional WNT/FZD signaling. The development of drugs for this disease would require a different approach than, e.g., colorectal cancer, where a premature stop codon leads to a complete dysfunctional formation of the APC protein.

The contribution of WNT-5A in the glioma disease progression is not entirely clear. In paper V, we do conclude that an increase in WNT-5A gene expression results in higher protein levels in the tumor microenvironment. Furthermore, in patients whose gliomas express high levels of WNT-5A, a larger number of microglia could be observed. However, this positive association does not seem to affect survival, as indicated by the comparison of the survival curves of low- versus high-expressing patient groups. Thus, the link between WNT-5A and the disease can be strong, but the question remains whether WNT-5A could be used as a therapeutic target for drugs. Likewise, will it be beneficial for the patient? Additionally, a body of literature, as well as the findings reported in paper III and IV, provide evidence that WNT-5A does have a crucial function in brain development and maintenance processes. If one would simply antagonize WNT-5A, other essential processes could be inhibited, possibly leading to undesirable side effects. The presence of microglia surrounding the tumor is often associated with poor disease prognostic for several reasons (Yang *et al.*, 2010). It would be interesting to explore the possibility to pharmacologically activate the immune-competent cells to trigger immune attack against the cancer cells, thus halting the disease progression. Recent developments in cancer immunotherapy indicate that body's own immune cells can be used to eliminate the tumor (Hodi et al., 2010). This can be done through different approaches, for instance by a non-specific boost of the immune system or the development of a cancer vaccine. The fact that WNT-5A and MHC Class II components show high correlation is promising, even though the MHC Class II does not seem to be functional. Future studies could investigate the reason why the antigen presentation is not functional within the tumor, and investigate ways to stimulate the immune cells in a way it can efficiently function as proper defense system against cancer. An example of a drug that is currently commercially available and uses a similar approach is the Ipilimumab (Yervoy®), a monoclonal antibody that blocks CTLA-4 receptors. This blocking "lifts" the immune checkpoint that usually prevents T-cells to attack cells belonging to its own body (Shin & Ribas, 2015). This same checkpoint prevents immune cell to attack the cancer cells. Therefore, WNT-5A could be considered in glioma therapy, knowing it (i) induces proinvasive behavior in microglia (paper III) (ii) initiates a proinflammatory response and is positively correlated with parts of the MHC Class II component genes in GBM (paper III and IV), and could therefore help inducing an anti-tumor immune attack.

Recombinant WNT-5A or small molecules mimicking WNT-5A function may serve as an adjuvant therapy in combination with other drugs boosting the general immune attack or lifting the immune check points in microglia. Although tempting to speculate on, the serious side effects that this type of treatments might induce (e.g. life threatening whole body immune attack) should not be neglected.

Downstream signaling components or parts of the pathways can also serve as possible candidates for drug therapy. In paper I we identify the WNT-5A-induced activation of heterotrimeric G proteins and in paper III the importance of ERK1/2 phosphorylation for the WNT-5A-induced expression of MMP 9 and 13, invasion and proliferation in microglia. The disadvantage of targeting for example heterotrimeric G proteins or ERK1/2 is that these components are essential in many more cellular processes of the body. Inhibition or blocking will therefore be physiologically highly unspecific and likely to cause multiple side effects. However, stipulating the activation of heterotrimeric G proteins causing the disease, development of specific cell permeable nanobodies targeting the potential G protein-binding site of the FZDs would be an interesting approach. These nanobodies can serve as activators, inhibitors or biased signaling molecules for G proteins on the intracellular domains of FZDs. The development of such drugs requires excellent screening methods for G protein activation transduced by FZDs, as proposed by Koval & Katanaev (Koval & Katanaev, 2012).

Important to note is that, before even considering applying drugs to target FZDs intra- or extracellular, the receptor expression repertoire present in the area of interest and the interplay between the various activated downstream signaling components should be taken into account. This because, by simply blocking one receptor, an alternative signaling pathway may be over-activated resulting in either beneficial or detrimental effects. For instance, antagonizing solely FZD_7 due its role in different cancer types can lead to augmented receptor occupancy of for example FZD_2 which in turn might lead to for example increased Ca^{2+} signaling. The consequences of the overrepresentation of this pathway are difficult to oversee. Furthermore, by recognizing that certain WNT/FZD pairs exhibit functional selectivity (paper II), one should keep in mind that a specific isoform of WNT does not solely activate one specific pathway but that it depends on the receptors present. The same accounts for the specific FZDs, where the presence of WNT repertoire defines the activation of downstream signaling components. Although this knowledge creates a more complex general picture, it may prove to be useful as now the characteristics of the functional selectivity can be employed. In the event that a certain disease is solely induced by the β-catenin-dependent signaling, the possibility to skew pathway activation towards β-catenin-independent signaling can be achieved by either blocking specific FZDs, WNTs or co-receptors.

Another important factor to consider regarding the use WNT/FZD pathway in future therapy is to question if and how suitable targets can be reached. For example, to augment the existing pathway, the use of recombinant WNT as therapeutic proteins could be difficult to apply systemically. Given the fact rWNTs will only be able to bind FZDs and subsequently exert their function when the protein structure is unaffected, a tablet or capsule as administration route is out of the question. Furthermore, systemic administration through for example an intravenous injection can be challenging due to the lipid modified structure of WNTs, affecting the distribution of the drug throughout the body and making the pharmacokinetic properties hard to predict. If however a high local concentration at a specific target site would be desirable, injection with rWNTs might be realistic and effective. As an alternative to recombinant whole proteins, small molecules mimicking or antagonizing WNTs at the site of the receptor can be used. The distribution of small molecules or peptides throughout the body is certainly more feasible. Furthermore, reaching the target site can be improved by applying biochemical design and modifications to the molecules. There are several potential avenues to pursue regarding the design of pharmacological agents in WNT/FZD pathway. With the current finding of the orthosteric binding site of FZD_8 and the acknowledgment that there is a potential bias in the signaling pathway, the possibilities of the development of various pharmacological agents such as agonists, inverse agonists, allosteric modulators, biased ligand or antagonist come within reach. The development of good screening methods however, is critical for success.

Additionally, in paper IV we identify astrocytes as one of the main sources of WNT-5A production in the CNS and discovered it serves as a migratory stimulant in neuronal precursor migration from the SVZ to the OB. This feature may be of use in therapies where integration of new neurons is required e.g. brain damage, stroke or Parkinson's disease. Although highly speculative, stimulating astrocytes to produce and secrete WNT-5A resulting in an induction of neuronal precursor migration to the injured site could therefore be beneficial.

In summary, the results encompassed in this thesis contribute to the understanding of WNT/FZD signaling in general. It comprises several levels of investigation, ranging from basic pharmacological properties of ligand binding to physiological and pathophysiological relevance in the CNS. The aim of this work is to contribute on the foundations of future therapeutic approaches based on either altering dysfunctional components or by employing physiological functional components of the pathway.

6 CONCLUSIONS

Paper I

1. WNT-5A can induce the exchange of GDP for GTP at heterotrimeric G proteins in the microglia-like cell line N13.
2. The exchange is PTX sensitive, pinpointing $G_{\alpha i/o}$ family proteins as the responsible subunit for the G protein activitation
3. Assesment of the FZD and G protein repetoire in N13 cells indicates FZD_5 and the $G_{\alpha i2/3}$-subunit as signaling components most likely to transduce the WNT-5A-induced activation of heterotrimeric G proteins.

Paper II

1. The myeloid progenitor cell line 32D does not express endogenous FZDs. It is however possible to overexpress FZD in these cells allowing us to investigate individual functional WNT/FZD pairs and downstream signaling specificity.
2. WNT-3A, -4, -5A are functional binding partners to $FZD_{2,4,5}$ with different affinities to the CRD domains of the FZDs and have a putative functional selectivity for individual downstream signaling pathways.

Paper III

1. Recombinant WNT-5A stimulation induces proliferation and causes a proinflammatory and pro-invasive response in mouse primary microglia *in vitro*.
2. WNT-5A causes phosphorylation of ERK1/2, activation of the subunits $G\alpha_{i/o}$ and β/γ and phospholipase C, PKC and MEK1/2 in primary microglia. Additionally, it induces invasion, proliferation and increase in Ca^{2+} signaling.
3. Inhibition of the MEK1/2 pathway by SL327 blocks invasion, proliferation and the gene upregulation of metalloproteases 9/13, indicating that WNT-induced G protein signaling is biologically relevant.

Paper IV

1. WNT-5A is highly expressed by astrocytes in the mouse rostral migratory stream and olfactory bulb
2. Partial ablation of WNT-5A causes (i) an increase in the proliferation of neuronal precursors and (ii) an altered cellular organization of the RMS/OB arguing for a crucial role of astrocytic WNT-5A to guide neuronal progenitor cells along the RMS.

Paper V

1. WNT-5A is upregulated in human glioma tissue shown by immunhistochemistry.
2. High WNT-5A expression is associated with pro-inflammatory effects in the glioma microenvironment and with an increased microglia infiltration.

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8 REFERENCES

Aguzzi, A., B. A. Barres and M. L. Bennett, 2013: Microglia: scapegoat, saboteur, or something else? *Science,* **339,** 156-161.

Alexander, S. P., H. E. Benson, E. Faccenda, A. J. Pawson, J. L. Sharman, J. C. McGrath, W. A. Catterall, M. Spedding, J. A. Peters, A. J. Harmar, C. Collaborators, N. Abul-Hasn, C. M. Anderson, C. M. Anderson, M. S. Araiksinen, M. Arita, E. Arthofer, E. L. Barker, C. Barratt, N. M. Barnes, R. Bathgate, P. M. Beart, D. Belelli, A. J. Bennett, N. J. Birdsall, D. Boison, T. I. Bonner, L. Brailsford, S. Broer, P. Brown, G. Calo, W. G. Carter, W. A. Catterall, S. L. Chan, M. V. Chao, N. Chiang, A. Christopoulos, J. J. Chun, J. Cidlowski, D. E. Clapham, S. Cockcroft, M. A. Connor, H. M. Cox, A. Cuthbert, F. M. Dautzenberg, A. P. Davenport, P. A. Dawson, G. Dent, J. P. Dijksterhuis, C. T. Dollery, A. C. Dolphin, M. Donowitz, M. L. Dubocovich, L. Eiden, K. Eidne, B. A. Evans, D. Fabbro, C. Fahlke, R. Farndale, G. A. Fitzgerald, T. M. Fong, C. J. Fowler, J. R. Fry, C. D. Funk, A. H. Futerman, V. Ganapathy, B. Gaisnier, M. A. Gershengorn, A. Goldin, I. D. Goldman, A. L. Gundlach, B. Hagenbuch, T. G. Hales, J. R. Hammond, M. Hamon, J. C. Hancox, R. L. Hauger, D. L. Hay, A. J. Hobbs, M. D. Hollenberg, N. D. Holliday, D. Hoyer, N. A. Hynes, K. I. Inui, S. Ishii, K. A. Jacobson, G. E. Jarvis, M. F. Jarvis, R. Jensen, C. E. Jones, R. L. Jones, K. Kaibuchi, Y. Kanai, C. Kennedy, I. D. Kerr, A. A. Khan, M. J. Klienz, J. P. Kukkonen, J. Y. Lapoint, R. Leurs, et al., 2013: The Concise Guide to PHARMACOLOGY 2013/14: overview. *Br J Pharmacol,* **170,** 1449-1458.

Allen, Developing Mouse Brain Atlas, ©2013 Allen Institute for Brain Science [Internet]. Available from: http://developingmouse.brain-map.org.

Asano, T., S. E. Pedersen, C. W. Scott and E. M. Ross, 1984: Reconstitution of catecholamine-stimulated binding of guanosine 5'-O-(3-thiotriphosphate) to the stimulatory GTP-binding protein of adenylate cyclase. *Biochemistry,* **23,** 5460-5467.

Bader, G. D. and C. W. Hogue, 2003: An automated method for finding molecular complexes in large protein interaction networks. *BMC bioinformatics,* **4,** 2.

Bailey C, C. H., 1926: Classification of Tumors of the Glioma Group on a Histogenic Basis with a Correlated Study of Prognosis. . *Philadelphia: JB Lippincott Company*.

Barker, N., 2008: The canonical Wnt/beta-catenin signalling pathway. *Methods Mol Biol,* **468,** 5-15.

Bettinger, I., S. Thanos and W. Paulus, 2002: Microglia promote glioma migration. *Acta neuropathologica,* **103,** 351-355.

Bikkavilli, R. K. and C. C. Malbon, 2009: Mitogen-activated protein kinases and Wnt/beta-catenin signaling: Molecular conversations among signaling pathways. *Commun Integr Biol,* **2,** 46-49.

Bilic, J., Y. L. Huang, G. Davidson, T. Zimmermann, C. M. Cruciat, M. Bienz and C. Niehrs, 2007: Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science,* **316,** 1619-1622.

Bittner, J. J., 1936: Some Possible Effects of Nursing on the Mammary Gland Tumor Incidence in Mice. *Science,* **84,** 162.

Blakely, B. D., C. R. Bye, C. V. Fernando, M. K. Horne, M. L. Macheda, S. A. Stacker, E. Arenas and C. L. Parish, 2011: Wnt5a regulates midbrain dopaminergic axon growth and guidance. *PLoS One,* **6,** e18373.

Bourhis, E., C. Tam, Y. Franke, J. F. Bazan, J. Ernst, J. Hwang, M. Costa, A. G. Cochran and R. N. Hannoush, 2010: Reconstitution of a frizzled8.Wnt3a.LRP6 signaling complex reveals multiple Wnt and Dkk1 binding sites on LRP6. *J Biol Chem,* **285,** 9172-9179.

Bridges, C. B., Brehme, K.S. , 1944: *The mutants of Drosophila melanogaster*. . Carnegie Institute, Washington DC

Bryja, V., E. R. Andersson, A. Schambony, M. Esner, L. Bryjova, K. K. Biris, A. C. Hall, B. Kraft, L. Cajanek, T. P. Yamaguchi, M. Buckingham and E. Arenas, 2009: The extracellular domain of Lrp5/6 inhibits noncanonical Wnt signaling in vivo. *Mol Biol Cell,* **20,** 924-936.

Budnik, V. and P. C. Salinas, 2011: Wnt signaling during synaptic development and plasticity. *Current opinion in neurobiology,* **21,** 151-159.

Burton, E. and M. Prados, 1999: New chemotherapy options for the treatment of malignant gliomas. *Current opinion in oncology,* **11,** 157-161.

Burton, E. C. and M. D. Prados, 2000: Malignant gliomas. *Current treatment options in oncology,* **1,** 459-468.

Cajanek, L., L. Adlerz, V. Bryja and E. Arenas, 2010: WNT unrelated activities in commercially available preparations of recombinant WNT3a. *Journal of cellular biochemistry,* **111,** 1077-1079.

Castelo-Branco, G., K. M. Sousa, V. Bryja, L. Pinto, J. Wagner and E. Arenas, 2006: Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. *Mol Cell Neurosci,* **31,** 251-262.

Castro, M. G., R. Cowen, I. K. Williamson, A. David, M. J. Jimenez-Dalmaroni, X. Yuan, A. Bigliari, J. C. Williams, J. Hu and P. R. Lowenstein, 2003: Current and future strategies for the treatment of malignant brain tumors. *Pharmacology & therapeutics,* **98,** 71-108.

Cerami, E., J. Gao, U. Dogrusoz, B. E. Gross, S. O. Sumer, B. A. Aksoy, A. Jacobsen, C. J. Byrne, M. L. Heuer, E. Larsson, Y. Antipin, B. Reva, A. P. Goldberg, C. Sander and N. Schultz, 2012: The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer discovery,* **2,** 401-404.

Chang, S. M., I. F. Parney, M. McDermott, F. G. Barker, 2nd, M. H. Schmidt, W. Huang, E. R. Laws, Jr., K. O. Lillehei, M. Bernstein, H. Brem, A. E. Sloan, M. Berger and I. Glioma Outcomes, 2003: Perioperative complications and neurological outcomes of first and second craniotomies among patients enrolled in the Glioma Outcome Project. *Journal of neurosurgery,* **98,** 1175-1181.

Charles, N. A., E. C. Holland, R. Gilbertson, R. Glass and H. Kettenmann, 2011: The brain tumor microenvironment. *Glia,* **59,** 1169-1180.

Chen, Z., T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, B. Xu, A. Wright, C. Vanderbilt and M. H. Cobb, 2001: MAP kinases. *Chemical reviews,* **101,** 2449-2476.

Clevers, H., 2004: Wnt breakers in colon cancer. *Cancer cell,* **5,** 5-6.

Constam, D. B., J. Philipp, U. V. Malipiero, P. ten Dijke, M. Schachner and A. Fontana, 1992: Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. *J Immunol,* **148,** 1404-1410.

Cuitino, L., J. A. Godoy, G. G. Farias, A. Couve, C. Bonansco, M. Fuenzalida and N. C. Inestrosa, 2010: Wnt-5a modulates recycling of functional GABAA receptors on hippocampal neurons. *J Neurosci,* **30,** 8411-8420.

De Ferrari, G. V. and R. T. Moon, 2006: The ups and downs of Wnt signaling in prevalent neurological disorders. *Oncogene,* **25,** 7545-7553.

De Palma, M., M. A. Venneri, R. Galli, L. Sergi Sergi, L. S. Politi, M. Sampaolesi and L. Naldini, 2005: Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer cell,* **8,** 211-226.

Dejmek, J., A. Safholm, C. Kamp Nielsen, T. Andersson and K. Leandersson, 2006: Wnt-5a/Ca2+-induced NFAT activity is counteracted by Wnt-5a/Yes-Cdc42-casein kinase 1alpha signaling in human mammary epithelial cells. *Mol Cell Biol,* **26,** 6024-6036.

Dijksterhuis, J. P., J. Petersen and G. Schulte, 2013: WNT/Frizzled signaling: receptor-ligand selectivity with focus on FZD-G protein signaling and its physiological relevance. *Br J Pharmacol*.

Dillman, A. R., P. J. Minor and P. W. Sternberg, 2013: Origin and evolution of dishevelled. *G3,* **3,** 251-262.

Du, Q., X. Zhang, J. Cardinal, Z. Cao, Z. Guo, L. Shao and D. A. Geller, 2009: Wnt/beta-catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression by inhibiting nuclear factor-kappaB activation in cancer cells. *Cancer Res,* **69,** 3764-3771.

Du, R., K. V. Lu, C. Petritsch, P. Liu, R. Ganss, E. Passegue, H. Song, S. Vandenberg, R. S. Johnson, Z. Werb and G. Bergers, 2008: HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer cell,* **13,** 206-220.

Eggen, B. J., D. Raj, U. K. Hanisch and H. W. Boddeke, 2013: Microglial phenotype and adaptation. *J Neuroimmune Pharmacol,* **8,** 807-823.

Egger-Adam, D. and V. L. Katanaev, 2008: Trimeric G protein-dependent signaling by Frizzled receptors in animal development. *Front Biosci,* **13,** 4740-4755.

Fanto, M. and H. McNeill, 2004: Planar polarity from flies to vertebrates. *J Cell Sci,* **117,** 527-533.

Farias, G. G., I. E. Alfaro, W. Cerpa, C. P. Grabowski, J. A. Godoy, C. Bonansco and N. C. Inestrosa, 2009: Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons. *J Biol Chem,* **284,** 15857-15866.

Flugel, A., M. S. Labeur, E. M. Grasbon-Frodl, G. W. Kreutzberg and M. B. Graeber, 1999: Microglia only weakly present glioma antigen to cytotoxic T cells. *Int J Dev Neurosci,* **17,** 547-556.

Foord, S. M., T. I. Bonner, R. R. Neubig, E. M. Rosser, J. P. Pin, A. P. Davenport, M. Spedding and A. J. Harmar, 2005: International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacol Rev,* **57,** 279-288.

Friedl, P. and K. Wolf, 2010: Plasticity of cell migration: a multiscale tuning model. *J Cell Biol,* **188,** 11-19.

Frojmark, A. S., J. Schuster, M. Sobol, M. Entesarian, M. B. Kilander, D. Gabrikova, S. Nawaz, S. M. Baig, G. Schulte, J. Klar and N. Dahl, 2011: Mutations in Frizzled 6 cause isolated autosomal-recessive nail dysplasia. *Am J Hum Genet,* **88,** 852-860.

Fuerer, C., S. J. Habib and R. Nusse, 2010: A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. *Dev Dyn,* **239,** 184-190.

Fung, Y. K., G. M. Shackleford, A. M. Brown, G. S. Sanders and H. E. Varmus, 1985: Nucleotide sequence and expression in vitro of cDNA derived from mRNA of int-1, a provirally activated mouse mammary oncogene. *Mol Cell Biol,* **5,** 3337-3344.

Gabrusiewicz, K., A. Ellert-Miklaszewska, M. Lipko, M. Sielska, M. Frankowska and B. Kaminska, 2011: Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas. *PLoS One,* **6,** e23902.

Gao, C. and Y. G. Chen, 2010: Dishevelled: The hub of Wnt signaling. *Cell Signal,* **22,** 717-727.

Gao, J., B. A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S. O. Sumer, Y. Sun, A. Jacobsen, R. Sinha, E. Larsson, E. Cerami, C. Sander and N. Schultz, 2013: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science signaling,* **6,** pl1.

Gertig, U. and U. K. Hanisch, 2014: Microglial diversity by responses and responders. *Frontiers in cellular neuroscience,* **8,** 101.

Globus, J. H. a. K., H., 1944: The subependymal plate (matrix) and its relationship to brain tumors of the ependymal type. *J. Neuropathol., 3, 1*.

Gong, Y., E. Bourhis, C. Chiu, S. Stawicki, V. I. DeAlmeida, B. Y. Liu, K. Phamluong, T. C. Cao, R. A. Carano, J. A. Ernst, M. Solloway, B. Rubinfeld, R. N. Hannoush, Y. Wu, P. Polakis and M.

Costa, 2010: Wnt isoform-specific interactions with coreceptor specify inhibition or potentiation of signaling by LRP6 antibodies. *PLoS One,* **5,** e12682.

Gross, J. C., V. Chaudhary, K. Bartscherer and M. Boutros, 2012: Active Wnt proteins are secreted on exosomes. *Nature cell biology,* **14,** 1036-1045.

Habu, M., H. Koyama, M. Kishida, M. Kamino, M. Iijima, T. Fuchigami, H. Tokimura, M. Ueda, M. Tokudome, C. Koriyama, H. Hirano, K. Arita and S. Kishida, 2014: Ryk is essential for Wnt-5a-dependent invasiveness in human glioma. *J Biochem,* **156,** 29-38.

Hadler-Olsen, E., B. Fadnes, I. Sylte, L. Uhlin-Hansen and J. O. Winberg, 2011: Regulation of matrix metalloproteinase activity in health and disease. *The FEBS journal,* **278,** 28-45.

Hall, A. C., F. R. Lucas and P. C. Salinas, 2000: Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell,* **100,** 525-535.

Halleskog, C., J. P. Dijksterhuis, M. B. Kilander, J. Becerril-Ortega, J. C. Villaescusa, E. Lindgren, E. Arenas and G. Schulte, 2012: Heterotrimeric G protein-dependent WNT-5A signaling to ERK1/2 mediates distinct aspects of microglia proinflammatory transformation. *J Neuroinflammation,* **9,** 111.

Halleskog, C., J. Mulder, J. Dahlstrom, K. Mackie, T. Hortobagyi, H. Tanila, L. Kumar Puli, K. Farber, T. Harkany and G. Schulte, 2011: WNT signaling in activated microglia is proinflammatory. *Glia,* **59,** 119-131.

Halleskog, C. and G. Schulte, 2013a: Pertussis toxin-sensitive heterotrimeric G(alphai/o) proteins mediate WNT/beta-catenin and WNT/ERK1/2 signaling in mouse primary microglia stimulated with purified WNT-3A. *Cell Signal,* **25,** 822-828.

Halleskog, C. and G. Schulte, 2013b: WNT-3A and WNT-5A counteract lipopolysaccharide-induced pro-inflammatory changes in mouse primary microglia. *J Neurochem*.

Hanisch, U. K. and H. Kettenmann, 2007: Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci,* **10,** 1387-1394.

Harrison, C. and J. R. Traynor, 2003: The [35S]GTPgammaS binding assay: approaches and applications in pharmacology. *Life Sci,* **74,** 489-508.

Hodi, F. S., S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbe, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos and W. J. Urba, 2010: Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine,* **363,** 711-723.

Hoelzinger, D. B., T. Demuth and M. E. Berens, 2007: Autocrine factors that sustain glioma invasion and paracrine biology in the brain microenvironment. *Journal of the National Cancer Institute,* **99,** 1583-1593.

Huang da, W., B. T. Sherman and R. A. Lempicki, 2009a: Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research,* **37,** 1-13.

Huang da, W., B. T. Sherman and R. A. Lempicki, 2009b: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols,* **4,** 44-57.

Huettner, C., W. Paulus and W. Roggendorf, 1995: Messenger RNA expression of the immunosuppressive cytokine IL-10 in human gliomas. *Am J Pathol,* **146,** 317-322.

Huntly, B. J. and D. G. Gilliland, 2005: Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer,* **5,** 311-321.

Inestrosa, N. C. and E. Arenas, 2010: Emerging roles of Wnts in the adult nervous system. *Nature reviews. Neuroscience,* **11,** 77-86.

Inestrosa, N. C. and L. Varela-Nallar, 2014a: Wnt signaling in the nervous system and in Alzheimer's disease. *Journal of molecular cell biology,* **6,** 64-74.

Inestrosa, N. C. and L. Varela-Nallar, 2014b: Wnt signalling in neuronal differentiation and development. *Cell and tissue research.*

Janda, C. Y., D. Waghray, A. M. Levin, C. Thomas and K. C. Garcia, 2012: Structural basis of Wnt recognition by Frizzled. *Science,* **337,** 59-64.

Jayapandian, M., A. Chapman, V. G. Tarcea, C. Yu, A. Elkiss, A. Ianni, B. Liu, A. Nandi, C. Santos, P. Andrews, B. Athey, D. States and H. V. Jagadish, 2007: Michigan Molecular Interactions (MiMI): putting the jigsaw puzzle together. *Nucleic acids research,* **35,** D566-571.

Kamino, M., M. Kishida, T. Kibe, K. Ikoma, M. Iijima, H. Hirano, M. Tokudome, L. Chen, C. Koriyama, K. Yamada, K. Arita and S. Kishida, 2011: Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. *Cancer science,* **102,** 540-548.

Kantoff, P. W., C. S. Higano, N. D. Shore, E. R. Berger, E. J. Small, D. F. Penson, C. H. Redfern, A. C. Ferrari, R. Dreicer, R. B. Sims, Y. Xu, M. W. Frohlich, P. F. Schellhammer and I. S. Investigators, 2010: Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *The New England journal of medicine,* **363,** 411-422.

Katanaev, V. L., R. Ponzielli, M. Semeriva and A. Tomlinson, 2005: Trimeric G protein-dependent frizzled signaling in Drosophila. *Cell,* **120,** 111-122.

Kettenmann, H., U. K. Hanisch, M. Noda and A. Verkhratsky, 2011: Physiology of microglia. *Physiological reviews,* **91,** 461-553.

Kilander, M. B., J. Dahlstrom and G. Schulte, 2014a: Assessment of Frizzled 6 membrane mobility by FRAP supports G protein coupling and reveals WNT-Frizzled selectivity. *Cell Signal,* **26,** 1943-1949.

Kilander, M. B., J. P. Dijksterhuis, R. S. Ganji, V. Bryja and G. Schulte, 2011a: WNT-5A stimulates the GDP/GTP exchange at pertussis toxin-sensitive heterotrimeric G proteins. *Cell Signal,* **23,** 550-554.

Kilander, M. B., C. Halleskog and G. Schulte, 2011b: Recombinant WNTs differentially activate beta-catenin-dependent and -independent signalling in mouse microglia-like cells. *Acta Physiol (Oxf),* **203,** 363-372.

Kilander, M. B., J. Petersen, K. W. Andressen, R. S. Ganji, F. O. Levy, J. Schuster, N. Dahl, V. Bryja and G. Schulte, 2014b: Disheveled regulates precoupling of heterotrimeric G proteins to Frizzled 6. *FASEB J,* **28,** 2293-2305.

Korteweg, R., 1936: On the manner in which the disposition to carcinoma of the mammary gland is inherited in mice. *Genetica* **18,** 350-371.

Korur, S., R. M. Huber, B. Sivasankaran, M. Petrich, P. Morin, Jr., B. A. Hemmings, A. Merlo and M. M. Lino, 2009: GSK3beta regulates differentiation and growth arrest in glioblastoma. *PLoS One,* **4,** e7443.

Koval, A. and V. L. Katanaev, 2012: Platforms for high-throughput screening of Wnt/Frizzled antagonists. *Drug Discov Today,* **17,** 1316-1322.

Kramer, N., A. Walzl, C. Unger, M. Rosner, G. Krupitza, M. Hengstschlager and H. Dolznig, 2013: In vitro cell migration and invasion assays. *Mutation research,* **752,** 10-24.

Kremenevskaja, N., R. von Wasielewski, A. S. Rao, C. Schofl, T. Andersson and G. Brabant, 2005: Wnt-5a has tumor suppressor activity in thyroid carcinoma. *Oncogene,* **24,** 2144-2154.

Kuhl, M., L. C. Sheldahl, M. Park, J. R. Miller and R. T. Moon, 2000: The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet,* **16,** 279-283.

Kurayoshi, M., H. Yamamoto, S. Izumi and A. Kikuchi, 2007: Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem J,* **402,** 515-523.

Kurose, H., T. Katada, T. Haga, K. Haga, A. Ichiyama and M. Ui, 1986: Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J Biol Chem,* **261,** 6423-6428.

Lane, J. R., D. Henderson, B. Powney, A. Wise, S. Rees, D. Daniels, C. Plumpton, I. Kinghorn and G. Milligan, 2008: Antibodies that identify only the active conformation of G(i) family G protein alpha subunits. *FASEB J,* **22,** 1924-1932.

Lathia, J. D., J. Gallagher, J. T. Myers, M. Li, A. Vasanji, R. E. McLendon, A. B. Hjelmeland, A. Y. Huang and J. N. Rich, 2011: Direct in vivo evidence for tumor propagation by glioblastoma cancer stem cells. *PLoS One,* **6,** e24807.

Lawson, L. J., V. H. Perry, P. Dri and S. Gordon, 1990: Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience,* **39,** 151-170.

Le, D. M., A. Besson, D. K. Fogg, K. S. Choi, D. M. Waisman, C. G. Goodyer, B. Rewcastle and V. W. Yong, 2003: Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. *J Neurosci,* **23,** 4034-4043.

Levy, M. L., A. L. Ho, S. Hughes, J. Menon and R. Jandial, 2009: Stem cells and the origin of gliomas: A historical reappraisal with molecular advancements. *Stem cells and cloning : advances and applications,* **1,** 41-47.

Lewis, T. S., P. S. Shapiro and N. G. Ahn, 1998: Signal transduction through MAP kinase cascades. *Advances in cancer research,* **74,** 49-139.

Li, B., L. Zhong, X. Yang, T. Andersson, M. Huang and S. J. Tang, 2011: WNT5A signaling contributes to Abeta-induced neuroinflammation and neurotoxicity. *PLoS One,* **6,** e22920.

Liu, X., J. S. Rubin and A. R. Kimmel, 2005: Rapid, Wnt-induced changes in GSK3beta associations that regulate beta-catenin stabilization are mediated by Galpha proteins. *Current biology : CB,* **15,** 1989-1997.

Louis, D. N., H. Ohgaki, O. D. Wiestler, W. K. Cavenee, P. C. Burger, A. Jouvet, B. W. Scheithauer and P. Kleihues, 2007: The 2007 WHO classification of tumours of the central nervous system. *Acta neuropathologica,* **114,** 97-109.

Lundstrom, K., 2009: An overview on GPCRs and drug discovery: structure-based drug design and structural biology on GPCRs. *Methods Mol Biol,* **552,** 51-66.

Ma, L. and H. Y. Wang, 2006: Suppression of cyclic GMP-dependent protein kinase is essential to the Wnt/cGMP/Ca2+ pathway. *J Biol Chem,* **281,** 30990-31001.

Ma, L. and H. Y. Wang, 2007: Mitogen-activated protein kinase p38 regulates the Wnt/cyclic GMP/Ca2+ non-canonical pathway. *J Biol Chem,* **282,** 28980-28990.

MacDonald, B. T., K. Tamai and X. He, 2009: Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Developmental cell,* **17,** 9-26.

Markovic, D. S., R. Glass, M. Synowitz, N. Rooijen and H. Kettenmann, 2005: Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloprotease-2. *Journal of neuropathology and experimental neurology,* **64,** 754-762.

Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree and H. Clevers, 1996: XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell,* **86,** 391-399.

Moon, R. T., 2005: Wnt/beta-catenin pathway. *Sci STKE,* **2005,** cm1.

Mootha, V. K., C. M. Lindgren, K. F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, P. Puigserver, E. Carlsson, M. Ridderstrale, E. Laurila, N. Houstis, M. J. Daly, N. Patterson, J. P. Mesirov, T. R. Golub, P. Tamayo, B. Spiegelman, E. S. Lander, J. N. Hirschhorn, D. Altshuler and L. C. Groop, 2003: PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet,* **34,** 267-273.

NIH, 2014: Clinical trial website of the National Institutes of Health. www.clinicaltrial.gov.

Nusse, R., A. Brown, J. Papkoff, P. Scambler, G. Shackleford, A. McMahon, R. Moon and H. Varmus, 1991: A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell,* **64,** 231.

Nusse, R. and H. E. Varmus, 1982: Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell,* **31,** 99-109.

Ohgaki, H. and P. Kleihues, 2005: Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. *Journal of neuropathology and experimental neurology,* **64,** 479-489.

Ostman, A., 2012: The tumor microenvironment controls drug sensitivity. *Nature medicine,* **18,** 1332-1334.

Paina, S., D. Garzotto, S. DeMarchis, M. Marino, A. Moiana, L. Conti, E. Cattaneo, M. Perera, G. Corte, E. Calautti and G. R. Merlo, 2011: Wnt5a is a transcriptional target of Dlx homeogenes and promotes differentiation of interneuron progenitors in vitro and in vivo. *J Neurosci,* **31,** 2675-2687.

Penfield, W., 1925: Microglia and the Process of Phagocytosis in Gliomas. *Am J Pathol,* **1,** 77-90 15.

Pino, D., Y. Choe and S. J. Pleasure, 2011: Wnt5a controls neurite development in olfactory bulb interneurons. *ASN Neuro,* **3,** e00059.

Proescholdt, M. A., M. J. Merrill, B. Ikejiri, S. Walbridge, A. Akbasak, S. Jacobson and E. H. Oldfield, 2001: Site-specific immune response to implanted gliomas. *Journal of neurosurgery,* **95,** 1012-1019.

Pulvirenti, T., M. Van Der Heijden, L. A. Droms, J. T. Huse, V. Tabar and A. Hall, 2011: Dishevelled 2 signaling promotes self-renewal and tumorigenicity in human gliomas. *Cancer Res,* **71,** 7280-7290.

Querfurth, H. W. and F. M. LaFerla, 2010: Alzheimer's disease. *The New England journal of medicine,* **362,** 329-344.

Raman, M., W. Chen and M. H. Cobb, 2007: Differential regulation and properties of MAPKs. *Oncogene,* **26,** 3100-3112.

Ramsay, R. G., D. Ciznadija, M. Vanevski and T. Mantamadiotis, 2003: Transcriptional regulation of cyclo-oxygenase expression: three pillars of control. *International journal of immunopathology and pharmacology,* **16,** 59-67.

Rhodes, D. R., J. Yu, K. Shanker, N. Deshpande, R. Varambally, D. Ghosh, T. Barrette, A. Pandey and A. M. Chinnaiyan, 2004: ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia,* **6,** 1-6.

Richard, C., J. P. Liuzzo and D. Moscatelli, 1995: Fibroblast growth factor-2 can mediate cell attachment by linking receptors and heparan sulfate proteoglycans on neighboring cells. *J Biol Chem,* **270,** 24188-24196.

Rodriguez-Gil, D. J. and C. A. Greer, 2008: Wnt/Frizzled family members mediate olfactory sensory neuron axon extension. *J Comp Neurol,* **511,** 301-317.

Roskoski, R., Jr., 2012: ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacological research : the official journal of the Italian Pharmacological Society,* **66,** 105-143.

Rostomily, R. C., G. E. Keles and M. S. Berger, 1996: Radical surgery in the management of low-grade and high-grade gliomas. *Bailliere's clinical neurology,* **5,** 345-369.

Sahores, M., A. Gibb and P. C. Salinas, 2010: Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis. *Development,* **137,** 2215-2225.

Sahores, M. and P. C. Salinas, 2011: Activity-mediated synapse formation a role for Wnt-Fz signaling. *Current topics in developmental biology,* **97,** 119-136.

Saito, R., M. E. Smoot, K. Ono, J. Ruscheinski, P. L. Wang, S. Lotia, A. R. Pico, G. D. Bader and T. Ideker, 2012: A travel guide to Cytoscape plugins. *Nature methods,* **9,** 1069-1076.

Saneyoshi, T., S. Kume, Y. Amasaki and K. Mikoshiba, 2002: The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. *Nature,* **417,** 295-299.

Scherer, H. J., 1940: A Critical Review: The Pathology of Cerebral Gliomas. *Journal of neurology and psychiatry,* **3,** 147-177.

Schulte, G., 2010: International Union of Basic and Clinical Pharmacology. LXXX. The class Frizzled receptors. *Pharmacol Rev,* **62,** 632-667.

Schulte, G. and V. Bryja, 2007: The Frizzled family of unconventional G-protein-coupled receptors. *Trends Pharmacol Sci,* **28,** 518-525.

Schulte, G., V. Bryja, N. Rawal, G. Castelo-Branco, K. M. Sousa and E. Arenas, 2005: Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. *J Neurochem,* **92,** 1550-1553.

Semenov, M. V., R. Habas, B. T. Macdonald and X. He, 2007: SnapShot: Noncanonical Wnt Signaling Pathways. *Cell,* **131,** 1378.

Sen, M., M. Chamorro, J. Reifert, M. Corr and D. A. Carson, 2001: Blockade of Wnt-5A/frizzled 5 signaling inhibits rheumatoid synoviocyte activation. *Arthritis and rheumatism,* **44,** 772-781.

Sen, M., K. Lauterbach, H. El-Gabalawy, G. S. Firestein, M. Corr and D. A. Carson, 2000: Expression and function of wingless and frizzled homologs in rheumatoid arthritis. *Proc Natl Acad Sci U S A,* **97,** 2791-2796.

Sharma, R. P. and V. L. Chopra, 1976: Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Developmental biology,* **48,** 461-465.

Sheldahl, L. C., M. Park, C. C. Malbon and R. T. Moon, 1999: Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Current biology : CB,* **9,** 695-698.

Sheldahl, L. C., D. C. Slusarski, P. Pandur, J. R. Miller, M. Kuhl and R. T. Moon, 2003: Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. *J Cell Biol,* **161,** 769-777.

Shin, D. S. and A. Ribas, 2015: The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? *Current opinion in immunology,* **33C,** 23-35.

Siebzehnrubl, F. A., B. A. Reynolds, A. Vescovi, D. A. Steindler and L. P. Deleyrolle, 2011: The origins of glioma: E Pluribus Unum? *Glia,* **59,** 1135-1147.

Sliwa, M., D. Markovic, K. Gabrusiewicz, M. Synowitz, R. Glass, M. Zawadzka, A. Wesolowska, H. Kettenmann and B. Kaminska, 2007: The invasion promoting effect of microglia on glioblastoma cells is inhibited by cyclosporin A. *Brain,* **130,** 476-489.

Slusarski, D. C., V. G. Corces and R. T. Moon, 1997a: Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature,* **390,** 410-413.

Slusarski, D. C., J. Yang-Snyder, W. B. Busa and R. T. Moon, 1997b: Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. *Developmental biology,* **182,** 114-120.

Song, S., A. J. Ewald, W. Stallcup, Z. Werb and G. Bergers, 2005: PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nature cell biology,* **7,** 870-879.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander and J. P. Mesirov, 2005: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A,* **102,** 15545-15550.

Takada, R., Y. Satomi, T. Kurata, N. Ueno, S. Norioka, H. Kondoh, T. Takao and S. Takada, 2006: Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Developmental cell,* **11,** 791-801.

Tarcea, V. G., T. Weymouth, A. Ade, A. Bookvich, J. Gao, V. Mahavisno, Z. Wright, A. Chapman, M. Jayapandian, A. Ozgur, Y. Tian, J. Cavalcoli, B. Mirel, J. Patel, D. Radev, B. Athey, D. States and H. V. Jagadish, 2009: Michigan molecular interactions r2: from interacting proteins to pathways. *Nucleic acids research,* **37,** D642-646.

Tauriello, D. V., I. Jordens, K. Kirchner, J. W. Slootstra, T. Kruitwagen, B. A. Bouwman, M. Noutsou, S. G. Rudiger, K. Schwamborn, A. Schambony and M. M. Maurice, 2012: Wnt/beta-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proc Natl Acad Sci U S A,* **109,** E812-820.

Tetsu, O. and F. McCormick, 1999: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature,* **398,** 422-426.

Tran, C. T., P. Wolz, R. Egensperger, S. Kosel, Y. Imai, K. Bise, S. Kohsaka, P. Mehraein and M. B. Graeber, 1998: Differential expression of MHC class II molecules by microglia and neoplastic astroglia: relevance for the escape of astrocytoma cells from immune surveillance. *Neuropathology and applied neurobiology,* **24,** 293-301.

Van de Wetering, M., J. Castrop, V. Korinek and H. Clevers, 1996: Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol Cell Biol,* **16,** 745-752.

van Ooyen, A. and R. Nusse, 1984: Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact. *Cell,* **39,** 233-240.

Verhaak, R. G., K. A. Hoadley, E. Purdom, V. Wang, Y. Qi, M. D. Wilkerson, C. R. Miller, L. Ding, T. Golub, J. P. Mesirov, G. Alexe, M. Lawrence, M. O'Kelly, P. Tamayo, B. A. Weir, S. Gabriel, W. Winckler, S. Gupta, L. Jakkula, H. S. Feiler, J. G. Hodgson, C. D. James, J. N. Sarkaria, C. Brennan, A. Kahn, P. T. Spellman, R. K. Wilson, T. P. Speed, J. W. Gray, M. Meyerson, G. Getz, C. M. Perou, D. N. Hayes and N. Cancer Genome Atlas Research, 2010: Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell,* **17,** 98-110.

Virchow, R., 1858: Die Cellularpathologie in ihrer Begründung auf physiologische und pathologische Gewebelehre. *Berlin.*

Wang, Y., A. Rattner, Y. Zhou, J. Williams, P. M. Smallwood and J. Nathans, 2012: Norrin/Frizzled4 signaling in retinal vascular development and blood brain barrier plasticity. *Cell,* **151,** 1332-1344.

Waziri, A., 2010: Glioblastoma-derived mechanisms of systemic immunosuppression. *Neurosurg Clin N Am,* **21,** 31-42.

Willert, K., J. D. Brown, E. Danenberg, A. W. Duncan, I. L. Weissman, T. Reya, J. R. Yates, 3rd and R. Nusse, 2003: Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature,* **423,** 448-452.

Yamaguchi, T. P., A. Bradley, A. P. McMahon and S. Jones, 1999: A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development,* **126,** 1211-1223.

Yang, I., S. J. Han, G. Kaur, C. Crane and A. T. Parsa, 2010: The role of microglia in central nervous system immunity and glioma immunology. *J Clin Neurosci,* **17,** 6-10.

Yu, J. M., E. S. Jun, J. S. Jung, S. Y. Suh, J. Y. Han, J. Y. Kim and K. W. Kim, 2007: Role of Wnt5a in the proliferation of human glioblastoma cells. *Cancer Lett,* **257,** 172-181.

Yu, J. M., J. H. Kim, G. S. Song and J. S. Jung, 2006: Increase in proliferation and differentiation of neural progenitor cells isolated from postnatal and adult mice brain by Wnt-3a and Wnt-5a. *Molecular and cellular biochemistry,* **288,** 17-28.

Zhai, H., F. L. Heppner and S. E. Tsirka, 2011: Microglia/macrophages promote glioma progression. *Glia,* **59,** 472-485.