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WNT SIGNALING IN MICROGLIA

WNTs as novel regulators of microglia

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Cover: An ICC image of a mouse primary microglia stimulated with 100 ng/ml recombinant WNT-3A for 2h. Red colors represent β -catenin, and blue represent the cell nuclei.

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“Microglia are the best cells ever. Microglia are like soldiers, policemen, chameleons, spiders, housekeepers, gardeners, electricians and garbage collectors. Microglia can be active or resting, branched or blobby, harmful or protective, and they are everywhere and always moving. Are you in love yet?”

-Virginia Hughes *National Geographic*, only human: January 11, 2013

The greatest pleasure in life is doing what people say you cannot do.

-Walter Bagehot

ABSTRACT

Microglia, the immunocompetent cells of the central nervous system (CNS) and the brain's own macrophages are the most motile cells in the CNS and those with highest plasticity, as they rapidly move their projections to actively screen their environment for any type of injury. Upon cell damage or infection, microglia respond quickly: they proliferate, change morphology from ramified to amoeboid state to migrate or invade towards the injury, secrete many types of cytokines and chemokines to communicate with other inflammatory cells, and phagocytose cell debris.

WNTs are secreted lipoglycoproteins, which bind to and act through the Frizzled family of receptors. The Frizzled (FZD) surface receptors belong to a family of seven transmembrane receptors listed as G protein-coupled receptors because of their structural similarities. WNT/FZD-signaling was historically divided into two main branches of pathways, depending whether or not they induce β -catenin stabilization. With increasing knowledge the WNT pathways are mainly named after their protein-induced intracellular events. WNT/FZD-signaling is important during embryonic development, neurogenesis, synaptogenesis, and tissue homeostasis.

Even though WNTs are expressed in the brain and definitely in contact with microglia cells, a link between microglia and WNTs has just recently begun to emerge. The aim of this thesis is to study how microglia cells respond to stimulation with recombinant WNTs with regards to WNT-induced intracellular signaling and physiological outcome. We have investigated this by the use of classical biochemical techniques, such as immunoblotting, immunochemistry, RT/QPCR, GDP/GTP exchange assay and proliferation assay.

The results show that primary microglia cells isolated from mice and a microglia-like cell line (N13) express several receptors for WNTs and respond to recombinant WNT stimulation. Stimulation with WNT-3A induced the WNT/ β -catenin-dependent pathway, and, in parallel, a classical GPCR pathway leading to phosphorylation of the MAPKs ERK1/2. Interestingly, by the use of the $G\alpha_{i/o}$ protein inhibitor, pertussis toxin (PTX), we pinpoint a central role for heterotrimeric G proteins in both WNT-3A-induced pathways. Further, stimulation of microglia with recombinant WNT-5A induced a classical GPCR MAPK signaling pathway recruiting $G\alpha_{i/o}$ -protein, PKC, calcium and MEK1/2 to phosphorylate ERK1/2.

In addition, WNT stimulation of microglia induced a substantial proinflammatory response by increasing the expression of several proinflammatory cytokines, prostaglandin synthase COX2, proliferation and invasion. Notably, some of these WNT-induced inflammatory markers could be inhibited by PTX or by a MEK1/2 inhibitor, pointing towards a WNT-induced G protein-dependent mechanism.

Furthermore, in Alzheimer's disease, a chronic neuroinflammatory condition associated with activated microglia, amoeboid-like microglia cells show high levels of β -catenin, suggesting that WNT/ β -catenin signaling in microglia plays an important role in AD-associated microglia activation.

In addition, WNT-3A and WNT-5A induced the expression of COX2 dose-dependently, but if microglia are preactivated by the proinflammatory bacterial wall derivative lipopolysaccharide (LPS), WNTs counteract LPS-induced COX-2 expression. This suggests a dual regulatory, i. e. pro-and anti-inflammatory effect of WNTs on microglia.

In conclusion, WNTs are expressed in the brain and have impact on microglia's inflammatory activity; this suggests that WNTs may play important roles as modulators of microglia activity in neuroinflammation and tissue homeostasis.

LIST OF PUBLICATIONS

- I. **Halleskog C.**, Mulder J, Dahlström J, Mackie K, Hortobágyi T, Tanila H, Kumar Puli L, Färber K, Harkany T, and Schulte G. (2011) WNT signaling in activated microglia is proinflammatory. *Glia*. 59: 119-131.
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TABLE OF CONTENTS

INTRODUCTION.....	1
Microglia	1
Inflammatory markers of microglia activity.....	3
Neuroinflammation	6
Alzheimer's disease.....	7
WNT/Frizzled background	9
Frizzled	9
WNTs	10
WNT/Frizzled signaling.....	11
WNT/ β -catenin-dependent signaling.....	12
WNT/ β -catenin-independent signaling.....	13
Mitogen-activated protein kinase in microglia.....	15
WNT/MAPK crosstalk.....	16
WNT-signaling pathophysiology.....	16
The GSK3 hypothesis in Alzheimer's disease	17
WNT signaling in microglia	18
AIMS.....	19
MATERIAL AND METHODS.....	20
Methodological considerations.....	20
Cell line culturing and isolation of microglia.....	20
Animal model for AD: the APdE9 mice	21
Stimulation and inhibitor treatment	21
Proliferation assay	22
Gene-expression analysis	22
Antibody-based techniques: Immunohistochemistry, immunocytochemistry and immunoblotting.....	23
G protein activation assays.....	23
Invasion assay.....	24
RESULTS AND DISCUSSION	25
WNT-proteins and receptors in microglia and N13	25
Recombinant WNT-3A effects on microglia	26
WNT-3A-induced intracellular pathway in microglia	26
WNT-3A proinflammatory modulation of microglia activity	29
β -catenin expression in microglia in AD.....	29
Other WNTs affecting microglia in a G protein- dependent manner	32
Effects of recombinant WNT-5A- on microglia	33
WNT-5A-induced signaling pathway in microglia.....	33
WNT-5A-induced proinflammatory transformation of microglia..	35
Differences between WNT-3A- and WNT-5A-regulated ERK1/2-signaling in microglia.....	37
WNT/FZD-receptor selectivity	37
MAPK p38 and NF- κ B	37

ROR/RYK.....	38
Heterogeneous microglia.....	39
WNT signaling counteracts LPS-induced proinflammation in microglia.....	39
GENERAL DISCUSSION AND FUTURE PERSPECTIVES	42
CONCLUSIONS	45
POPULÄRVETENSKAPLIG SAMMANFATTNING	47
ACKNOWLEDGEMENTS	48
REFERENCES	50

LIST OF ABBREVIATIONS

A β	β -amyloid
AD	Alzheimer's disease
APdE9	Swedish mutation of amyloid precursor protein and deletion of exon 9 coding for presenilin 1
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (Ca ²⁺ chelator)
BIS	Bisindolmaleimide VIII(PKC inhibitor)
BSA	Bovine serum albumin
CaMKII	Calmodulin-dependent kinase II
CD11b	Cluster of differentiation molecule 11b
CK1	Casein kinase 1
CCL	Chemokine (C-C motif) ligand
CNS	Central Nervous System
COX2	Cyclooxygenase 2
D4476	4-(4-(2,3-Dihydrobenzol [1,4] dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl) benzamide (CK1 inhibitor)
DMSO	Dimethyl sulfoxide
DVL	Disheveled
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FZD	Frizzled
GFAP	Glial fibrillary acidic protein
GPCR	G protein-coupled receptor
GSK3	Glycogen synthase kinase 3
GSK3 inhibitor IV	2-Chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone (GSK3 inhibitor)
IBA-1	Ionized calcium-binding adapter molecule 1
iNOS	Inducible nitric oxide synthase
IL-6	Interleukin 6
IL-12	Interleukin 12
JNK	c-Jun N-terminal kinase
KN93	2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) (CaMKII inhibitor)
LRP5/6	Low density lipoprotein receptor related protein 5 and 6
LY294002	2-(4-Morpholinyl)-8-phenyl1-(4H)-benzopyran-4-one-hydrochloride (PI3K inhibitor)
M119	NSC119910; 2-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)-cyclohexane-1-carboxylic acid ($\beta\gamma$ inhibitor)
MAPK	Mitogen-activated protein kinase

MEK1/2	MAPK/ERK kinases 1/2
MKP	Mitogen-activated protein kinase phosphatase
MMP	Matrix metalloprotease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B	Nuclear factor <i>kappa</i> -light-chain-enhancer of activated B cells
NFT	Neuro fibrillary tangle
NO	Nitric Oxide
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD98059	2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (MEK1/2 inhibitor)
PG	Prostaglandin
PI3K	Phosphatidylinositol 3'-kinase
PKC	Ca ²⁺ -dependent protein kinase
PLC	Phospholipase C
PS-DVL	Phosphorylated and shifted DVL
PSEN1	Presenilin 1
PTX	Pertussis toxin for <i>Bordetella pertussis</i>
QPCR	Quantitative reverse transcriptase PCR
Ro-318220	3-(3-(4-(1-methyl-1 <i>H</i> -indol-3-yl)-2,5-dioxo-2,5-dihydro-1 <i>H</i> -pyrrol-3-yl)-1 <i>H</i> -indol-1-yl)propyl carbamimidothioate (MKP/PKC inhibitor)
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1 and 2
RT-PCR	Reverse transcriptase PCR
RYK	Related to receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SFRP	secreted Frizzled-related protein
SL327	α -[Amino-(4-aminophenylthio)methylene)-2-(trifluoromethyl)phenylacetonitrile (MEK1/2 inhibitor)
TCF/LEF	T-cell specific transcription factor/Lymphoid enhancer factor
TNF α	Tumor necrosis factor alpha
U73122	1-[6-(((17 β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino)hexyl]-1 <i>H</i> -pyrrole-2,5-dione (PLC inhibitor)
WB	Western blot/immunoblotting
WNT	<i>Wingless/int-1</i> , Wingless-type mouse mammary tumor virus integration site family

INTRODUCTION

The brain and spinal cord are collectively named the central nervous system (CNS), and are made up of different cell types, including neurons and glial cells. Neurons process and transmit information throughout the nervous system. The brain is composed of 90% glial cells, which in turn include astrocytes, ependymal cells, microglia, oligodendrocytes, Schwann cells and satellite cells. These cells serve many functions, such as neuronal support and maintenance, production of cerebrospinal fluid, immune defense, myelin production and, to some extent, synaptic transmission.

Microglia

The immunocompetent microglia, are known as the fastest moving cells of the CNS, which constantly and actively screen their surrounding for any imbalance in tissue homeostasis (Nimmerjahn et al., 2005; Hanisch and Kettenmann, 2007; Polazzi and Monti, 2010; Kettenmann et al., 2011). Microglia account for approximately 20% of the glial cell population and around 5-20% of the total cells of the adult CNS, depending on the species (Lawson et al., 1990; Polazzi and Monti, 2010; Aguzzi et al., 2013).

Microglia, were first named microgliocytes when they were discovered around 1920 by the Spanish neuroscientist Pio del Rio-Hortega, who identified the cells with a silver carbonate staining (Kettenmann et al., 2011; Marin-Teva et al., 2011). The developmental origin of microglia remains a matter of debate either they are derived from invasion of mesodermal or mesenchymal origin, from neuroectodermal matrix cells together with macroglia (astrocytes and oligodendrocytes), from pericytes, from invading circulating monocytes in early development, or, according to later research, are derived from macrophages produced by primitive hematopoiesis in the yolk sac (Ling and Wong, 1993; Alliot et al., 1999; Ginhoux et al., 2010; Ranshoff and Cardona, 2010). Despite their origin, there are not any fundamental or functional differences between microglia and peripheral macrophages; thus microglia are still classified as the CNS's own macrophages, expressing several macrophage-associated markers (Guillemin & Brew, 2004; Saijo and Glass, 2011). Microglia cells have high plasticity and mobility as they can rapidly transform from a ramified phenotype, with small body and multiple branches, into a more active amoeboid-like cell (reactive microglia) (**Figure 1**) with capacity to proliferate or migrate, invade and phagocytose (Lynch, 2009; Kettenmann et al., 2011, 2013; Marin-Teva et al., 2011). Although the ramified shape was long considered as the “resting state” of microglia waiting for pathology, evidence indicates that ramified microglia actively move their fine processes in the healthy brain (Nimmerjahn et al., 2005; Olah et al., 2011; Kettenmann et al., 2013), making the term “surveying microglia” a more accurate description (Hanisch and Kettenmann, 2007; Lynch, 2009). Further, the ramified microglia have shown to play critical roles in determination of neuronal fate, axonal growth and synaptic remodeling during CNS development (Smith et al., 2012; Kettenmann et al., 2013).

Microglia cells are the first line of defense in the CNS and are the key regulators of neuroinflammation (Rivest, 2009; Kettenmann et al., 2011; Smith et al., 2012). To be able to detect and recognize possible dangerous signals that disturb homeostasis, and to respond to injury signals, microglia express a panoply of receptors, including neurotransmitter receptors and pattern recognition receptors (PRRs) (Pocock and Kettenmann, 2007; Kettenmann et al., 2011). Via the receptors for neurotransmitters, neuropeptides, and neuromodulators, microglia have the capacity to sense neuronal activity. These receptors are speculated to suppress microglia during normal conditions and rapidly influence them when pathological processes occur (Pocock and Kettenmann, 2007). PRRs are a group of innate receptors that recognize DAMPs (damage associated molecular patterns) and PAMPs (pathogen associated molecular patterns) (Kettenmann et al., 2011; Jounai et al., 2012). DAMPs include molecules released from damaged cells or tissue, such as high levels of ATP, DNA and RNA outside the nucleus (or mitochondria) and molecules that become modified as a consequence of tissue damage, such as oxidized lipoproteins and fragments from extracellular matrix proteins. PAMPs warn of the presence of foreign molecules such as the bacterial wall component lipopolysaccharide (LPS) or repeats of bacterial and viral nucleotide acids. DAMPs and PAMPs share many receptors, such as Toll-like receptors (TLRs) and induce overlapping sets of genes (Kettenmann et al., 2011). Upon a change in the brain micro-environment, stimulation of microglia's receptors induces signaling cascades that further lead to transcription and expression of new proteins, including cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2) and major histocompatibility complex II (MHCII) (Hanisch, 2002; Rock and Peterson, 2006; Brown and Neher, 2009; Graeber, 2010; Kettenmann et al., 2011). In addition, to clean areas from pathogens, dying cells and their fragments, and to accumulate in areas where neuronal death occurs, microglia migrate or/and invade, proliferate and/or phagocytose cellular debris (Brown and Neher, 2009; Neumann et al., 2011; Marin-Teva et al., 2011).

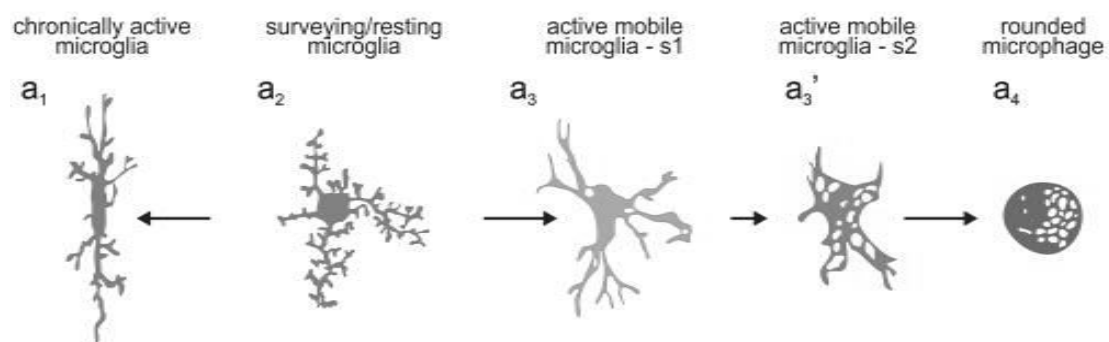


Figure 1: Microglia undergoes phenotypic transformation. A schematic image show how activated microglia change phenotype from a ramified surveying microglia (a_2) towards a more round microphage like (a_4), with migrating/invading capacities or into a chronically active microglia (a_1).

It is well established that neurons, other glia and immune cells exist in several subtypes, and recent evidence indicates that microglia are likewise not a uniform cell type (Lynch, 2009; Scheffell et al., 2013). Not only may they vary in terms of regional densities but also in their functional properties (Lawson et al., 1990; Kim et al., 2000; Hanisch and Kettenmann, 2007; Olah et al., 2011). It has been suggested that microglia achieve this broad range of capabilities by task splitting, which can help explain how activated microglia can simultaneously manage proliferative expansion and executive functions, such as invasion, cytokine secretion and proliferation (Hanisch, 2013; Scheffell et al., 2013).

Inflammatory markers of microglia activity

As described earlier in the text, upon pathological activation, microglia transform into a more active pro-inflammatory state characterized by change of morphology, enhanced proliferation, phagocytosis, invasion/migration, or/and induction of transcription of distinct inflammatory molecules and proteins (**Figure 2**) (Hanisch, 2002; Lynch, 2009; Kettenmann et al., 2011). Some of these classical changes – the hallmarks of microglia activity – are commonly used as readouts for microglia activation. The proteins and molecules studied in this thesis are listed below.

Cytokines

Cytokines are small polypeptides (8-30 kDa), which are tremendously diverse in their potential actions as they signal in an autocrine or paracrine fashion, and when they bind to their specific receptor trigger signal transduction pathways that ultimately alter gene expression in the target cell (Akiyama et al., 2000b; Smith et al., 2012). At low concentrations they can locally modulate cellular activities including survival, growth and differentiation. For example, the maintenance of microglia's immature state under normal conditions in the CNS is probably related to the cytokines present in the microenvironment, and two cytokines – transforming growth factor- β (TGF- β), and interleukin-10 (IL-10) – have been strongly implicated not only in this process, but also in deactivation of proinflammatory microglia (Smith et al., 2012). Further, cytokines released from astrocytes, namely TGF- β , macrophage colony-stimulating factor (M-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) promote the ramified morphology of microglia (John et al., 2003; Smith et al., 2012). Microglia - being the dominant cytokine secretor within the CNS - are experts in promoting and suppressing inflammation (Hanisch, 2002; John et al., 2003; Graeber, 2010).

One of the major proinflammatory cytokines microglia produce and release is interleukin-6 (IL-6). IL-6 is normally expressed in the nervous system during development, but at scarcely detectable levels. Under pathological conditions, IL-6 expression strongly increases due to secretion by microglia, macrophages and T cells to stimulate immune response to inflammatory trauma (Akiyama et al., 2000). IL-6 is capable of crossing the blood-brain barrier and binds to its soluble or membrane bound receptor to form biologically active IL-6 receptor complex, which can regulate cell growth, proliferation, survival and differentiation. IL-6 can for example initiate the synthesis of prostaglandin E2 in the hypothalamus to affect the body temperature set

point (Marais et al., 2011). IL-6 has even been discussed as a target for regulating chronic inflammation and cancer (Scheller et al., 2006; Smith et al., 2012). Another classical proinflammatory cytokine is interleukin IL-1 β . LPS-induced IL-1 β expression in microglia has shown to have negative impact on learning and memory in rats (Oitzl et al., 1993). IL-12 is a heterodimeric cytokine expressed by microglia to enhance phagocytic activity and increase other immune cells' ability to release proinflammatory cytokines, including IL-12, thereby regulating innate immunity and determining the type and duration of an adaptive immune response (Trinchieri, 1998).

Tumor necrosis factor α (TNF α) is another proinflammatory cytokine contributing to both physiological and pathophysiological processes, and is mainly produced by microglia and macrophages (Sriram and O'Callaghan, 2007; Chu, 2013). Because TNF α stimulates the production of other inflammatory cytokines, including its own, TNF α is considered a key mediator of both acute and chronic reactions (Chu, 2013). However, it has both a neurotoxic and a neuroprotective role in the inflammation to maintain tissue homeostasis (Sriram and O'Callaghan, 2007). TNF α has a prominent role in tumor development, i.e., its increased expression and activation is often associated with increased tumorigenesis, tumor progression, invasion and metastasis (Cordero et al., 2010), rendering TNF α a common readout for proinflammatory activity.

Chemokines are a superfamily of small polypeptides, which are basically chemoattractive cytokines that control chemotaxis, adhesion, and activation of many types of immune cells under both physiological and pathophysiological conditions (Woodcock and Morganti-Kossmann, 2013). Some chemokines are constitutively expressed to regulate homeostasis or development, while others are involved in the inflammatory process. The chemokines possess four conserved cysteine residues, and based on the position of two of the four invariant residues, they fall into four subgroups: C, CC, CXC and CX₃C chemokines (X stands for another amino acid separating the conserved cysteine residues). Chemokines exert an effect by binding to their G protein coupled receptor, which is classified in accordance to the ligands (e.g. CCR, CXCR). Pathological activation of microglia may induce expression of cytokines to engage peripheral infiltrating cells to support neuroinflammatory processes (Kettenmann et al., 2011; Woodcock and Morganti-Kossmann, 2013). For example, CCL7 and CCL12, also known as monocyte chemoattractant protein 3 and 5, respectively, induced in activated microglia are important for monocyte and leukocyte recruitment (Opdenakker et al., 1993; Sarafi et al., 1997).

Cluster of Differentiation (CD)

CDs are a group of cell membrane molecules that antibodies bind to, providing suitable targets for immunophenotyping cells. CD11b is expressed by immune cells and commonly used as a microglia marker to distinguish microglia from surrounding cells within the CNS (Kettenmann et al., 2011). CD11b is also known as macrophage-1-antigen (Mac-1), CD18, and complement receptor 3 (CR3) (Akiyama and McGeer, 1990). However, CD11b is a heterodimeric integrin involved in several immune responses in the innate immune system, and its expression levels seem to increase upon microglia activation (Kettenmann et al., 2011). Thus, the macrophage marker ionized

calcium-binding adapter molecule 1 (IBA-1), a protein with a suggested role in calcium homeostasis, is constitutively expressed by microglia and often used as microglia marker (Akiyama and McGeer, 1990).

CD40, is a type I cell surface protein and a member of the TNF receptor family mediating a broad variety of immune and inflammatory responses, where increased expression/stimulation is in line with increased TNF α , IL-6 and NO synthesis (Ponomarev et al., 2006; Kawahara et al., 2009). CD69, is a type II integral membrane glycoprotein, a C type lectin, induced in active immune cells and serves for communication with astrocytes and other peripheral immune cells such as lymphocytes and natural killer cells (Marzio et al., 1999). Thus, an increased expression of CD40 or/and CD69 provides targets for activated microglia.

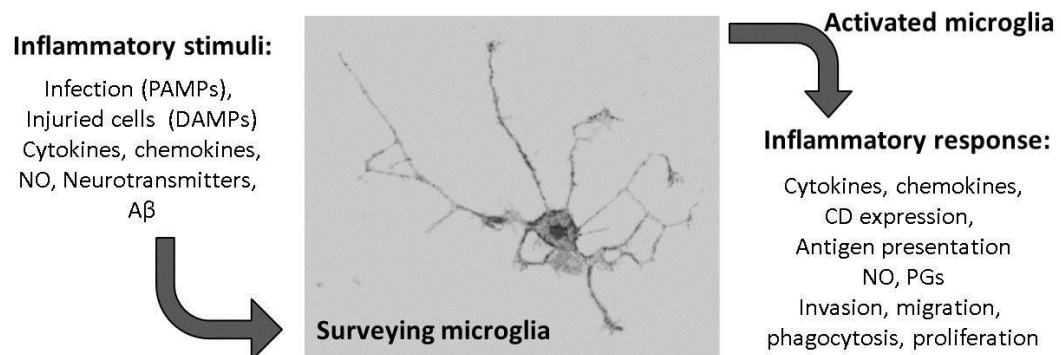


Figure 2: Surveying microglia transform into activated microglia upon inflammatory stimuli. Surveying microglia constantly screen for any imbalance in tissue homeostasis. Upon a change in the brain micro-environment, different inflammatory mediators stimulate microglia to become activated by change of morphology, enhanced proliferation, phagocytosis, invasion/migration, or/and induction of transcription and secretion of distinct inflammatory molecules and proteins. PAMPs, pathogen associated molecular patterns; DAMPs, damage associated molecular patterns, NO, nitric oxide; A β , β -Amyloid; CD, cluster of differentiation; PG; prostaglandin.

Cyclooxygenase

Cyclooxygenase 1 and 2 (COX1 and COX2), also known as prostaglandin-endoperoxide synthase-1 and 2, are enzymes that catalyze the conversion of the prostanoid precursor arachidonic acid to prostaglandin endoperoxide H₂, and further to prostaglandins (PGs) (Smith et al., 2000; Choi et al., 2009). COX1 is constitutively expressed in most tissue and is primarily responsible for homeostatic PG synthesis (Phillis et al., 2006). COX2 is normally weakly expressed in tissues but increases upon inflammation. Thus, COX2 provides a major target for non-steroidal anti-inflammatory drugs (NSAIDs) (Smith et al., 2000). Inhibition of COX2 by NSAID decreases fever and pain, and might also have neuroprotective effects in Alzheimer's disease (Akiyama et al., 2000). Upon stimulation with PAMP or DAMP and cytokines, microglia increase the expression of COX2 to secrete PGs (Choi et al., 2009; Jamieson et al., 2012), thus, increased COX2 expression is a common readout for proinflammatory activated microglia.

Nitric Oxide and Inducible Nitric Oxide Synthase (iNOS)

Nitric oxide (NO) is both a signaling and an effector molecule in diverse biological systems (Garthwaite, 1991). Inducible NO synthase (iNOS), also known as NO synthase 2 (NOS2), is normally expressed in the brain by neurons (Wen et al., 2011). Induced expression of iNOS in microglia is in line with continuous production of high levels of NO, that on one hand have neuroprotective effects by blocking neuronal cell death, but on the other hand, NO can react with superoxide to form peroxynitrite which is toxic to neurons and oligodendrocytes (Garthwaite, 1991; Brown and Neher, 2009; Wen et al., 2011).

Matrix metalloproteinase

Matrix metalloproteinases (MMPs) are proteolytic enzymes, capable of degrading components of the extracellular matrix (ECM), which is a fundamental biological process for normal growth, development and repair of the CNS (Candelario-Jalil et al., 2009). MMPs are divided into subgroups of collagenases, gelatinases, and other MMPs according to their substrate specificity and function (Candelario-Jalil et al., 2009). MMPs are considered to be important effectors of the inflammatory process, to serve during migration and invasion of immune and cancer cells (Ii et al., 2006). MMP-13 for example, is expressed by microglia upon stimulation with IL-1, -6 and TNF α , or LPS (Choi et al., 2010). In addition to ECM-degrading capability, MMPs play also a central role in signalling through modulation of other MMPs (Leeman et al., 2002).

Neuroinflammation

Inflammation is a reaction of living tissues to repair a chemical, biological or physical injury. As mentioned before, a foreign molecule may trigger an inflammatory state in which components of the innate immune response attempt to clear away and/or destroy the invader (Medzhitov and Janeway, 2000; Rivest, 2009). The intrinsic immune capacity of microglia has a crucial point of convergence for the innate immune response in the brain and spinal cord (Rivest, 2009; Smith et al., 2012; Aguzzi et al., 2013). Interestingly, microglia are not the only cell type in the CNS with the immune response capability of secreting cytokines or phagocytosis; astrocytes also play a role in the generation of proinflammatory mediators (von Bernhardi and Eugénin, 2004; Li et al., 2011).

Inflammation is divided into acute and chronic inflammation. Acute inflammation is the immediate response to an injury, defined by four cardinal signs of “heat, pain, redness and swelling” and is usually short-lived. Chronic inflammation, on the other hand, is when inflammatory stimuli persist for a longer time and the inflammation is not completely turned off or extinguished (Streit et al., 2004). Neuroinflammation, is also known as “reactive gliosis”, and is characterized by the accumulation of enlarged glial cells, i.e. active microglia (microgliosis) and astrocytes (astrogliosis) appearing immediately after CNS injury has taken place. After limited acute neuronal damage, involving loss of either afferents or efferents, there is a more subtle response of the brain’s own immune system, i.e. rapid activation of glial cells. In the absence of blood-

brain barrier breakdown and leukocyte infiltration, the microglia and astrocytes can fulfill their programmed repair functions, going through a resolution stage back to ramified stage, to benefit the organism as a whole (Streit, 2002; Streit et al., 2004; von Bernhardi and Eugenin, 2004; Li et al., 2011). However, the term “neuroinflammation” is actually more relevant to the concept of chronic inflammation, when the inflammation persists due to active, transformed glia cells expanding the initial neurodestructive effects, thus maintaining and worsening the disease progress through their actions (Streit et al., 2004; O’Callaghan et al., 2008; Brown and Neher, 2009; Aguzzi et al., 2013). However, the actual underlying cause and the activating trigger of neuroinflammatory diseases remains elusive.

Alzheimer’s disease (AD), Parkinson’s disease (PD), and multiple sclerosis (MS) are some examples of well-characterized neuroinflammatory/degenerative diseases that take place in the CNS where microglia cells are key regulators of the neuroinflammatory processes (Dheen et al., 2007; Farfara et al., 2008; Amor et al., 2010; Heneka et al., 2010; Miller and Streit, 2007; Morales et al., 2010). Microglia exert underlying, molecularly diverse effects on disease pathology, which compromise neuronal survival and function. The microglia response to neuropathology results in initiating production of cytotoxic and neurotrophic factors, such as NO, and is the major source of proinflammatory cytokines, such as IL-1, IL-6 and TNF α (Akiyama et al., 2000; Dheen et al., 2007; Hanisch and Kettenmann, 2007; Walter and Neumann, 2009). The importance of NO in microglia-mediated neurodegeneration is supported by the observation that addition of NO synthase inhibitors to neuron-glia cultures inhibits LPS-induced accumulation of nitrite and reduces neuronal cell loss (Boje and Arora, 1992; Chao et al., 1992). As mentioned before, short-term microglia activity is generally accepted to serve a neuroprotective role, while during chronic inflammation, microglia seem to have a more neurotoxic influence in neurodegenerative disorders (Minghetti and Levi, 1998; Brown and Neher, 2009; Morales et al., 2010). For this reason, proinflammatory microglia are discussed as potential targets for drugs against neuroinflammatory diseases (Rock and Peterson, 2006; Heneka et al., 2010).

Alzheimer’s disease

Alzheimer’s disease (AD) is a chronic neurodegenerative disease and the most common cause of dementia in the elderly. AD is characterized by progressive CNS neuroinflammation and neurodegeneration, deposition of insoluble β -amyloid (A β) peptides forming senile plaques and the formation of intracellular neurofibrillary tangles (NFTs) made of the microtubule-associated protein tau (Heese et al., 2004; Morales et al., 2010). However, these features characterize late stage AD, and the earlier stages are not clear defined.

A β plaque formation

A β is formed from the integral transmembrane glycoprotein amyloid precursor protein (APP), which is abundant in the CNS. APP is sequentially cleaved by two enzymes, β - and γ -secretase to form A β monomers, which then aggregate successively into dimers, oligomers, protofibrils, and are ultimately deposited as A β plaques (De-Paula et al.,

2012). In fact, most cases of familial AD involve a mutation in the presenilin (PS) genes, 1 and 2. These proteins together form the γ -secretase, a catalytic enzyme that cleaves APP (Schellenberg and Montline, 2012). $A\beta_{40-42}$ is associated with AD, and the subscripted number stands for how many amino acids γ -secretase has cleaved off APP. $A\beta_{42}$, in comparison to $A\beta_{40}$, is more likely to form toxic plaques. Mutations in APP, PS1 and PS2 increase $A\beta_{42}$ formation which also has been proved in transgenic mouse models (Duff et al., 1996).

Tau, NFT and Braak stages of AD

Tau is a microtubule-associated protein, and an important component of the cytoskeleton in neurons, to maintain neuronal structure, axonal guidance and neuronal plasticity (De-Paula et al., 2012). Tau activity is regulated by phosphorylation and dephosphorylation at serine threonine and phosphoepitopes. NFT is formed when this phosphorylation balance is interrupted, and tau becomes hyperphosphorylated to an insoluble form. This leads to impaired axonal transport and synaptic metabolism, collapse of the microtubular cytoskeleton, which ultimately lead to neuronal death (De-Paula et al., 2012).

The initial symptom and one of the earliest features of AD is impairment of memory. This slowly worsens and gradually transforms into personality changes, language impairment, and ultimately motor dysfunction (Braak and Braak, 1991, 1995). These clinical symptoms reflect the gradual development of brain destruction and the formation of NFTs, which begins in a few limbic areas of the cerebral cortex (Braak stage I and II) and spreads in a nonrandom manner across hippocampus (Braak stage III and IV), neocortex and a number of subcortical nuclei (Braak stage V and VI) (Braak and Braak, 1991, 1995).

Microglia in AD

There is growing evidence that the chronic immune response may contribute significantly to the damage and degeneration of neurons leading to dementia (Akiyama et al., 2000; Streit et al., 2004). Microglia play a crucial role in inflammation and clearance of destroyed neurons and $A\beta$ (Pocock et al., 2002; Farfara et al., 2008). With a specific stimulatory factor such as anti- $A\beta$ antibodies or growth factor $\beta 1$, or by secretion of degrading enzymes, microglia can phagocytize and/or clear off $A\beta$ (Koenigsknecht-Talboo and Landreth, 2005; Farfara et al., 2008). However, in response to multiple damage signals, $A\beta$ peptides and neurofibrillary tangles, microglia becomes overactive and release neurotoxic products such as reactive oxygen species (superoxide and NO) and proinflammatory cytokines (Akiyama et al., 2000; Morales et al., 2010). Further, $A\beta$ plaques contain IL-6 and IL-1 β , which attract infiltrating reactive microglia and activate mitogen-activated protein kinase (MAPK) signaling, further triggering the release proinflammatory cytokines (TNF α , IL-1 β , IL-6), and other neurotoxic factors such as superoxide (Pocock and Liddle, 2001). This will proceed by increasing the number of proinflammatory, active microglia and infiltrating immune cells. Meanwhile, the AD is still progressing with increased formation of $A\beta$ plaques, while microglia have lost their ability to clear $A\beta$ (Wilkinson and El Khory, 2012).

WNT/Frizzled background

Frizzled

Frizzled surface receptors (FZD) are seven-transmembrane receptors (7TMR), structurally reminiscent of G protein-coupled receptors (GPCRs) (Vinson et al., 1989). FZDs were recently included in the International Union of Basic and Clinical Pharmacology (IUPHAR) GPCR list as a separate family – the Class Frizzled (Foord et al., 2005; Schulte and Bryja, 2007; Schulte, 2010a; Katanaev, 2010). FZDs were first discovered as products of the *frizzled* locus in *Drosophila melanogaster*, where the name FZD refers to the irregularly arranged and tightly curled hairs and bristles on thorax wings of the *frizzled* mutant of *D. melanogaster* (Vinson et al., 1989). Mammals are known to have ten different isoforms of FZDs, all of which contain a large extracellular N terminus with a cysteine-rich domain (CRD) (Schulte, 2010a). Even if FZDs are listed as GPCRs, the absence of biochemical evidence supporting such interaction meant that it was initially believed that FZDs can act independently of G proteins (Schulte, 2010a; Nichols et al., 2013). Ligand binding to a GPCR induces a conformational change that catalyzes guanine diphosphate (GDP) release and guanine triphosphate (GTP) capture by the α -subunits of the heterotrimeric G proteins (Oldham and Hamm, 2008). This leads to a dissociation of the heterotrimer, after which both the $G\alpha$ and $\beta\gamma$ -subunits activate effector proteins. Heterotrimeric G proteins are grouped according to the α subunit's characteristics into four subgroups: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (Gilman, 1987). The $G\alpha_{i/o}$ proteins are ADP ribosylated upon activation and are thereby inhibited by the bacterial toxin from *Bordetella pertussis* (pertussis toxin, PTX) (Birnbaumer et al., 1990). In fact, the first initial study of potential G protein signaling downstream of FZDs demonstrated that *Xenopus* WNT-5A (XWNT-5A) induced calcium release via FZD₂ in a PTX-sensitive manner (Slusarski et al., 1997a-b). This result has been confirmed in another study on *Danio rerio* embryos (Sheldahl et al., 1999) and in a more recent study on mouse embryonic cells (Ma and Wang, 2006). In summary, these data indicate classical GPCR coupling to $G\alpha_{i/o}$ protein signaling through release of $\beta\gamma$ subunits, activation of phospholipase C (PLC) and the release of calcium-dependent protein kinases (Dorsam and Gutkind, 2007) and was named the WNT/ Ca^{2+} pathway (Kühl et al., 2000a). Further, due to lack of purified, active WNTs or drugs acting on FZDs, chimeric receptors have been constructed with the ligand-binding and the transmembrane segment from the β 1- or β 2-adrenergic receptor combined with the cytoplasmic domains from FZD₁ or FZD₂ (Liu et al., 1999; Li et al., 2004). This *in vitro* system supported requirement of PTX-sensitive G proteins upon β -adrenergic receptor agonist binding to the chimeric β -FZD₂, and argues for different induced signaling between different FZDs (Li et al., 2004). Additional studies show involvement and/or interaction of $G\alpha$ protein activation downstream of FZDs, which has impact on several of the WNT-induced signaling pathways (Liu et al., 1999, 2001, 2005; Egger-Adam and Katanaev, 2008; Bikkavilli et al., 2008; Nichols et al., 2013). However, these studies on the role of heterotrimeric G protein for WNT signaling have been employed in overexpression systems such as mammalian cells (Liu et al., 1999; Ahumada et al., 2002; Ma and Wang, 2006; Bikkavilli et al., 2008), or non-

mammalian species (Slusarski et al., 1997; Sheldahl et al., 1999; Katanaev et al., 2005, Katanaev and Buestorf, 2009), except in a recent study on membrane preparations with endogenously expressed FZDs, WNT induced a PTX-sensitive GDP/GTP-exchange on heterotrimeric G proteins (Kilander et al., 2011a). Despite more recent and direct proof of WNT-induced and FZD-mediated activation of heterotrimeric G proteins the direct coupling of FZDs to heterotrimeric G proteins remains to be clarified (Schulte, 2010b, Schulte, 2013).

WNTs

WNTs are a family of secreted lipoglycoproteins, whose name originates from the names of two genes: *wingless* and *int* (Nusse et al., 1991). The *D. melanogaster* gene *wingless* (*wg*) was first identified for its function in wing, where it halts wing formation during embryogenesis (Cabrera et al., 1987). The *Int-1* gene was identified as a mammary carcinoma promotor, due to an insertion of the mammary tumor virus (MMTV) into the *int-1* locus on chromosome 15 (Nusse and Varmus, 1982). With a 54% protein sequence homology (Rijsewijk et al., 1987), the name WNT (wingless-related MMTV integration site) designates a novel family of signaling molecules (Nusse et al., 1991).

There are 19 different WNTs in mammals, which were subdivided into two main categories. Those with ability to morphologically transform C57MG mammary cells (Wong et al., 1994) through β -catenin stabilization were called “canonical/WNT1” class (WNT-1,-2,-3/A, -7A, -8A/B) (Shimizu et al., 1997; Willert et al., 2003). The rest of the WNTs were grouped into the “non-canonical class” (originally including WNT-4, -5A, and -11) that triggered signaling independently of β -catenin, the non-transforming WNTs. For example, WNT-5A regulates convergence and extension movements during gastrulation and axis duplication in *Xenopus* embryos (Moon et al., 1993; Kühl et al., 2000a; Park et al., 2006). However, the ability of WNTs to induce particular downstream signaling pathways highly dependent on context (e.g. receptor repertoire and subcellular distribution) (Cadigan and Liu, 2006), and various reports have shown activation of the WNT/ β -catenin dependent signaling pathway by WNTs from the “non-canonical class” (He et al., 1997; Mikels and Nusse, 2006b) and vice versa (Habas et al., 2003). Although, the nomenclature “canonical” and “non-canonical” WNT signals is used to refer to the WNT/ β -catenin dependent signaling which was discovered first, new discoveries have increasingly revealed the complexity of the WNT signaling networks, and the WNT-induced pathways should be named after the main components involved (Schulte, 2010a).

Secreted WNTs are hydrophobic molecules with poor water solubility, carrying several posttranslational modifications such as glycosylation, palmitoylation and palmitoleoylation, and require a membrane protein Wntless/Evi/Sprinter for secretion (Ching and Nusse, 2006). These modifications are necessary not only for WNT secretion but also for their signaling capabilities (Willert et al., 2003; Takada et al., 2006; Ching et al., 2008). Consequently, only a few purified and recombinant WNTs are available on the market and most studies are done on WNT-3A and WNT-5A, the

first two WNT ligands purified (Willert et al., 2003; Schulte et al., 2005; Mikels and Nusse, 2006a).

WNT/Frizzled signaling

WNT/FZD-signaling plays a critical role in a vast array of biological processes, such as cell proliferation, migration, polarity establishment and stem cell self-renewal. Dysfunction of WNT-signaling is associated with cancer and developmental deficits (Logan and Nusse, 2004; Moon et al., 2004; Clevers, 2006). WNTs interact with the highly conserved cysteine-rich domain (CRD) of FZDs, or with accessory proteins, or co-receptors, to define specific downstream signaling events and exert physiological effects (Xu and Nusse, 1998; Schulte and Bryja, 2007; Janda et al., 2012). Due to the lack of recombinant purified WNTs (Willert et al., 2003; Willert and Nusse, 2012) and despite the recent advances on structural aspects of WNT-FZD interaction (Janda et al., 2012) it is still not known which WNT binds to which FZD (or co-receptor) to exert an effect (except for a few cases). As mentioned, the β -catenin signaling was the first one described, and was referred to as the canonical pathway (Shimizu et al., 1997; Chien et al., 2009), the increasing knowledge about the complex signaling of the “non-canonical” network induced by WNTs, makes it better to designate the WNT-induced pathway in terms of the main components involved (He, 2003; Schulte, 2010a; Marchetti and Pluchino, 2013; Schulte, 2013). In fact, several studies have emphasized the importance of G proteins in the WNT/ β -catenin signaling pathway (Liu et al., 2001; Malbon et al., 2001; Bikkavilli et al., 2008; Jernigan et al., 2010).

Disheveled

A central player in most of the WNT/FZD signaling pathways, is the cytosolic scaffold protein disheveled (DVL) (Shan et al., 2005; Gao and Chen, 2010). DVL is highly conserved during evolution and three DVL homologs were identified in mammals, DVL1, 2 and 3. The DVL protein is composed three conserved domains: the N-terminal DVL-Axin (DIX) domain, a central Postsynaptic density 95-Disc Large-Zonula occludens (PDZ) motif and a C-terminal DVL-Egl-10-Plectsrin (DEP) domain (Gao and Chen, 2010). The PDZ motif plays an important role in WNT-induced pathways, where it binds to the KTxxxW (x= any amino acid) conserved motif on FZDs (Wong et al., 2003). Upon WNT binding, the DIX domain of DVL interacts with the homologous DIX domain of Axin, which brings DVL to the destruction complex. In this way DVL inhibits the function of Axin in the β -catenin destruction complex and plays a crucial role in the WNT/ β -catenin dependent pathway (Julius et al., 2000). The DEP domain is important for DVL interactions with other proteins (Gao and Chen, 2010). WNT stimulation induces DVL phosphorylation (and possible ubiquitinylation) which can be visualized by the western blotting (WB) technique when DVL appears as a slow-migrating band, also known as the formation of PS-DVL (phosphorylated and shifted). However, this WNT-induced phosphorylation of DVL, possible through a casein kinase 1 (CK1) δ and CK1 ϵ dependent mechanism, is not necessarily related to its functional degree of activity (Bryja et al., 2007a-b; Bernatik et al., 2011; Gonzalez-Sancho et al., 2013).

WNT/ β -catenin-dependent signaling

In absence of WNTs, a constitutively active destruction complex consisting of glycogen synthase 3 β (GSK3 β), casein kinase 1 α (CK1 α), the scaffold protein Axin and adenomatous polyposis coli (APC), phosphorylates cytosolic β -catenin (CK1 at Ser45 and GSK3 β at Ser33/37/Thr41). This sequential phosphorylation primes β -catenin for ubiquitination by β -transducin repeats-containing protein-1 and thereby for proteasomal degradation to keep cytosolic β -catenin levels low (Clevers., 2006; MacDonald et al., 2009; Marin-Teva et al., 2011; Li et al., 2012). In the presence of WNT-1-like ligands (including WNT-1, -3A, -8), WNTs form a ternary complex together with FZD and the single-pass co-receptor low density lipoprotein receptor related protein 5 or 6 (LRP5/6). This leads to a rapid recruitment of CK1 γ and GSK3 β to phosphorylate LRP5/6, which in turn redistributes the destruction complex, and forms an LRP5/6 signalosome consisting of a WNT-FZD-LRP5/6-DVL-axin platform (Bilic et al., 2007). This redistribution and displacement of proteins in the destruction complex, leads to its inhibition allowing β -catenin accumulation, stabilization and further translocation to the cell nucleus. Once in the nucleus, β -catenin binds and activates the transcription factors T-cell factor/lymphoid enhancer binding factor (TCF/LEF). Without nuclear β -catenin TCF/LEF represses gene transcription of target gene promoters (Malbon et al., 2001; MacDonald et al., 2009) such as c-myc and cyclin D1 (He et al., 1998; Tetsu and McCormick, 1999), **Figure 3**.

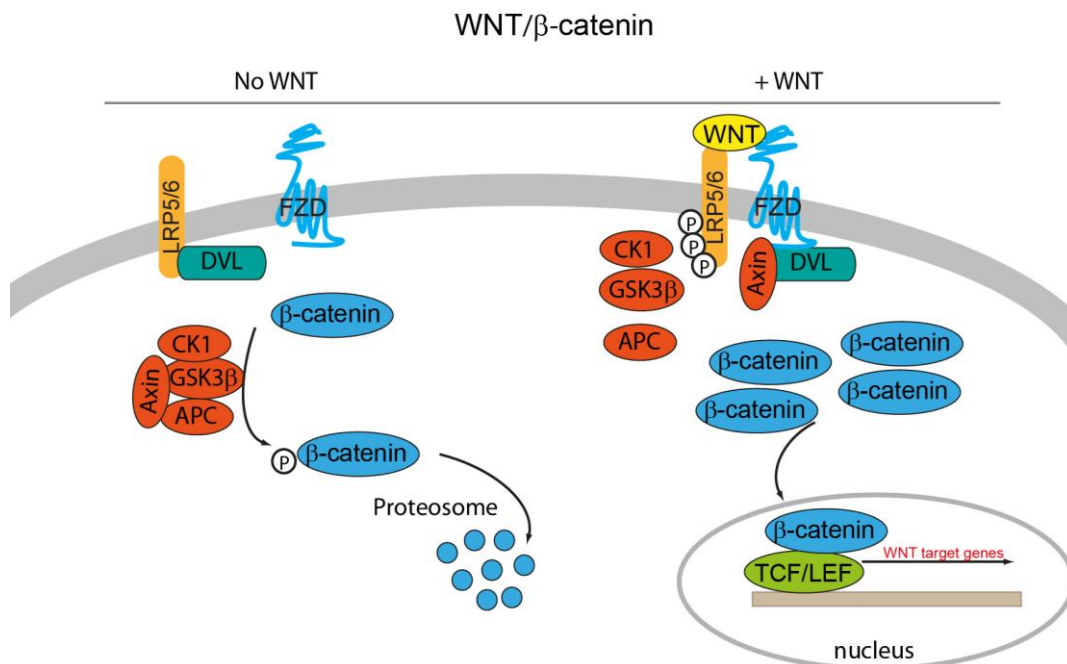


Figure 3: The WNT/ β -catenin-dependent pathway

In absence of WNTs, the continuously active destruction complex consisting of Axin, APC, CK1 α , and GSK3 β , phosphorylates β -catenin for proteasomal degradation. In presence of WNTs, WNT binds to FZD and the co-receptor LRP5/6 leads to inhibition of the destruction complex. In turn, β -catenin accumulates and stabilizes for its translocation into the cell nuclei, where it binds the transcription factors TCF/LEF to activate transcription of WNT target genes.

WNT/ β -catenin-independent signaling

The WNT signaling that occurs independently of β -catenin stabilization, consists of a network of signaling pathways, such as the planar cell polarity (PCP) pathway (including WNT/RHO and WNT/Ras-related C3 botulinum toxin substrate (RAC) signaling axis), the WNT/Calcium (Ca^{2+})-signaling or WNT/cAMP signaling routes (Moon et al., 1993; Kühl et al., 2000b; Semenov et al., 2007; Schulte, 2010a; Schellenberg and Montline, 2012). However, as mentioned earlier, some of these signaling events are cross-linked or even identical, depending on cell type, receptor repertoire and their subcellular distribution (Dejmek et al., 2006; Mikels and Nusse, 2006a; van Amerongen et al., 2008; Chien et al., 2009). This indicates that WNT-signaling works through what might better be described as a signaling network, rather than individual signaling pathways.

WNT/PCP-signaling

WNT/PCP-signaling, governs the orientation of cells within an epithelial plane, a layered sheet a single cell thick. The best described samples are the uniform array of hairs on the wing of *Drosophila* (Seifert and Mlodzik, 2007) and the orientation of stereocilia in the inner ear of mammals (Montcouquiol et al., 2006). In *Drosophila* the PCP pathway acts independently of WNTs; however, the “PCP core proteins” FZD and DVL are still present, and signaling occurs through relocation of the core proteins, including Strabismus and Prickle (Seifert and Mlodzik, 2007).

In vertebrates, WNT/PCP signaling involves activation of several small Rho GTPases, which have different intracellular targets and appear constitute separate pathways (Dejmek et al., 2006; Seifert and Mlodzik, 2007). Upon WNT binding to FZDs, DVL can associate with the small GTPase Rho via the Formin homology adaptor protein Daam 1 (DVL associated activator morphogenesis 1) (Habas et al., 2001). Daam1 is a cytoplasmic auto-inhibited protein, which goes through a conformational change and activation upon DVL-binding, enabling its interaction with RhoA and the formation of a Rho-GTP complex which in turn activates Rho-associated kinases (ROCK) and remodels the cytoskeleton, eliciting changes in cell morphology (Habas et al., 2001; Kishida et al., 2004).

Another small GTPase of the Rho family that can be activated upon WNT-FZD binding and activation of DVL is RAC1. Activation of RAC stimulates the downstream effector c-Jun N-terminal kinase (JNK), to further activate JNK targeting transcription factors, such as c-Jun. This pathway for example regulates convergent extension movements during *Xenopus* gastrulation (Habas et al., 2001, 2003). JNK activation leads subsequently to activation of transcription factors such as c-Jun (Rosso et al., 2005).

ROR1/2 and RYK belongs to the family of receptor tyrosine kinases (RTK). ROR is characterized by extracellular FZD-like CRDs and intracellular tyrosine kinase (Trk) domains, resembling those of the Trk-family, while RYK has a homology to WNT inhibitory factor (WIF) to enable interaction with WNT ligands (Forrester, 2002; Fradkin et al., 2010). The lack of specific ROR/RYK ligands makes it is somewhat unclear under which circumstances WNT/ROR and WNT/RYK signaling involves

cooperation with FZDs, or as autonomous WNT receptors (Cadigan and Liu, 2006; Li et al., 2008; Fradkin et al., 2010). Although, it has been shown that WNT-5A binding to ROR2, independently of FZD, triggers the WNT-JNK pathway and/or inhibit the β -catenin-dependent pathway (Oishi et al., 2003; Mikels and Nusse, 2006a). The WNT/ROR2-induced JNK activation involves PI3K, the GTPase Cdc42, protein kinase C (PKC) and the transcription factors of JNK target genes, such as c-Jun, where its activation regulates convergence and extension movements in *Xenopus* (Oishi et al., 2003; Schambony and Wedlich, 2007). This pathway is activated downstream of both FZD and ROR2, suggesting crosstalk between WNT/PCP and WNT/ Ca^{2+} pathways (Choi and Han, 2002). In cooperation with FZD and DVL, RYK support WNT/ β -catenin-dependent pathway. However, WNT-5A can recruit RYK in a β -catenin independent manner, to increase the release of intracellular Ca^{2+} (Li et al., 2009), **Figure 4.**

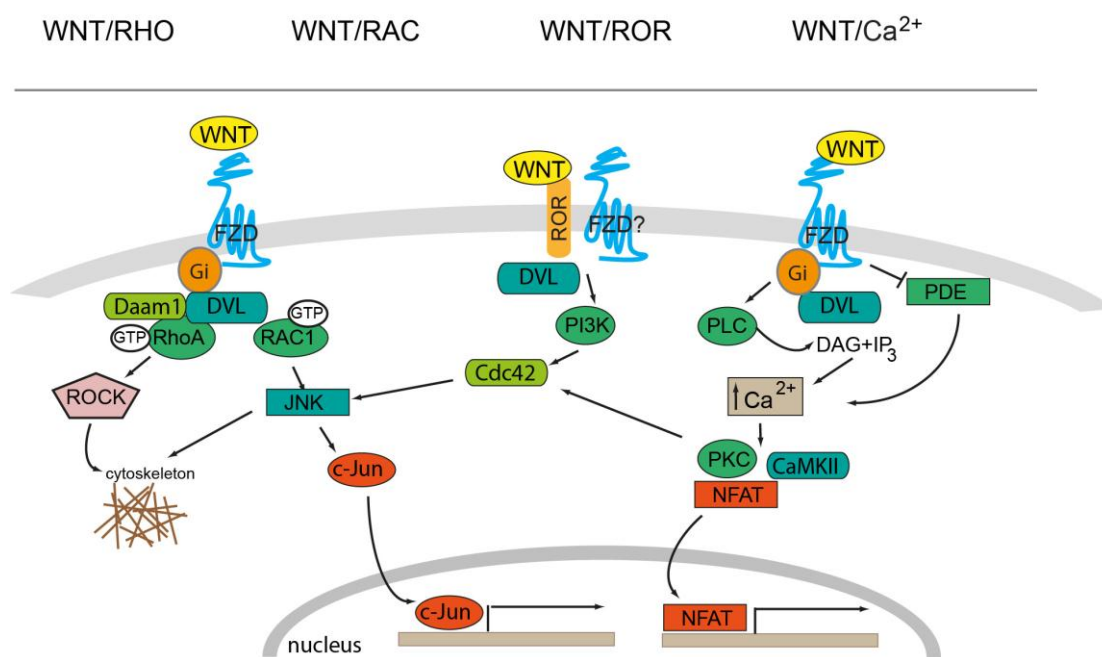


Figure 4: WNT / β -catenin-independent signaling network

WNT/RHO: WNT can activate RhoA, which requires DVLS' recruitment of Daam1. RhoA activates RhoKinase which regulates actin cytoskeleton rearrangements. WNT/RAC: WNTs binds to FZD to activate JNK via DVL and RAC1. JNK in turn activates transcription of JNK target genes via c-Jun. WNT/ROR: WNT binds ROR2 (or/and FZD) to activate JNK via PI3K and Cdc42. WNT/ Ca^{2+} : WNT binding to FZD activates PLC directly or via DVL which elevates intracellular Ca^{2+} levels via DAG and IP₃, which in turn activates PKC and CaMKII. CaMKII activates the transcription factor NFAT, while PKC may regulate Cdc42.

WNT/ Ca^{2+} signaling

WNT/FZD binding activates phospholipase C (PLC) and/or phosphodiesterase (PDE) via heterotrimeric G proteins leading to the generation of diacylglycerol (DAG) and inositol triphosphate (IP₃) which in turn leads to generation of Ca^{2+} fluxes (Ahumada et al., 2002). Ca^{2+} is a second messenger i.e. a central regulator of cell function and controls the activity of multiple intracellular proteins, including PKC, Ca^{2+} /calmodulin-dependent kinase (CamKII) (Kühl et al., 2000a; Choi and Han, 2002), calcineurin and

the transcription nuclear factor of activated T-cells (NFAT) (Cook et al., 1996; Saneyoshi et al., 2002; Dejmek et al., 2006). The WNT-induced Ca^{2+} release is dependent on signaling through the $\text{G}_{i/o}$ and G_q family of proteins, as demonstrated by the fact that PTX blocks this pathway (Slusarski et al., 1997a; Sheldahl et al., 1999, 2003; Kühl et al., 2000b). Recombinant WNT-5A has further been shown to induce a dose-dependent Ca^{2+} -signaling in mammary epithelial cells expressing low levels of endogenous WNT-5A (Dejmek et al., 2006).

Mitogen-activated protein kinase in microglia

The family of mitogen activated protein kinases (MAPK) is highly conserved during evolution. MAPKs represents one of the most important kinase families in inflammatory cells, whose activity is regulated in response to variety of stimuli, including growth factors, cytokines or even environmental stress (Turjanski et al., 2007; Kaminska et al., 2009). The MAPK family includes the extracellular signal-regulated kinases (ERK1/2; also known as p44/42), the c-jun N-terminal kinases (JNKs; also known as stress-activated protein kinases (SAPKs)), the p38 MAPKs, and the ERK5/big MAP kinase1 (BMK1) (Koistinaho and Koistinaho, 2002; Turjanski et al., 2007; Keshet and Seger, 2010). Microglia express several receptors that exert physiological effects after activation through MAPKs. For example, stimulation of calcium/calmodulin-activated K^+ channels are linked to activation of p38 MAPK, which in turn induce iNOS expression leading to neurotoxic effects (Kaushal et al., 2007); stimulation of the ionotropic ATP receptor P2X_4 induce p38 signaling and may contribute to neuropathic pain (Ji & Suter, 2007; Gong et al., 2009); norepinephrine acting through $\beta_{1/2}$ receptors and via p38/ERK signaling inhibits ATP-induced release of $\text{TNF}\alpha$ (Morioka et al., 2009); and the $\text{G}_{i/o}$ - and G_q -coupled cannabinoid CB_1 and CB_2 receptors reduce microglia neurotoxicity but increase microglia proliferation through MAPK signaling (Stella, 2009). Further, the cellular effects of chemokines are mediated through GPCRs and are linked to several intracellular cascades such as adenylate cyclase, PLC, GTPases (Rho, RAC, and Cdc42) and several MAPKs (Pierce et al., 2000; Kielian, 2004).

ERK1/2, which are the MAPKs studied in this thesis, are connected to the regulation of cell growth, differentiation and proliferation, and in the brain ERK1/2 is also connected to cellular responses, including stress stimuli, such as oxidative stress and increased intracellular calcium levels (Koistinaho and Koistinaho, 2002; Keshet and Seger, 2010). ERK1/2 is located in the cytoplasm and is upon phosphorylation also found in the cell nucleus which appears to phosphorylate transcription factors (Ahn et al., 2004). ERK1/2 is encoded by two genes, ERK1 and ERK2, which encode two main proteins, p44 and p42, respectively (Keshet and Seger, 2010). ERK1/2 is a serine threonine kinase, whose activation is regulated through phosphorylation of both of the Tyr and Thr residues (Koistinaho and Koistinaho, 2002; Turjanski et al., 2007). Upstream of ERK1/2 we find the MAPK kinase (MAPKK) MEK1/2, which phosphorylates ERK1/2, which in turn is phosphorylated by the MAPKK kinase (MAPKKK) RAF. This MAPK cascade is regulated by many kinds of receptors and pathways, especially receptor tyrosine kinases and GPCRs (Keshet and Seger, 2010).

Activation of ERK1/2 in microglia leads to activation of several nuclear transcription factors, cytoskeletal, nuclear and signaling proteins, that in turn leads to microglia secretion and release of numerous of cytokines and inflammatory/neurotoxic mediators (Koistinaho and Koistinaho, 2002; Kaminska et al., 2009).

WNT/MAPK crosstalk

Stimulation with WNT-3A on mouse fibroblasts has been shown to induce proliferation both via the activation of WNT/ β -catenin pathway and via a Ras-Raf-1-MEK-ERK cascade independently (Yun et al., 2005). Further, findings suggests that proline-targeted-kinases from the MAPK family can phosphorylate an intracellular motifs on the LRP6, which is required in LRP6's regulation of the β -catenin destruction complex, thus suggesting a sufficient role of MAPK activation in LRP6-initiated downstream signaling (Wolf et al., 2011). In addition, receptor tyrosine kinases have been shown to crosstalk via ERK1/2 to potentiate LRP6 and regulate β -catenin phosphorylation, thus enhancing the WNT/ β -catenin signaling (Krejci et al., 2012). In a breast cancer cell-line, transactivation of the epidermal growth factor receptor (EGFR) and the WNT/ β -catenin pathway induces proliferation via ERK1/2 activation in a WNT- and DVL-dependent manner, independent of β -catenin stabilization (Schlange et al., 2007). In summary, the MAPKs have been assigned an important, yet poorly defined role in the regulation of and crosstalk with the WNT/ β -catenin pathway on different levels (Bikkavilli and Malbon, 2009).

WNT-signaling pathophysiology

WNTs are important in mediating cell-cell communication, and are therefore crucial for the development of the CNS and stem cell differentiation. Given the wide range of processes affected by WNT-signaling in the developing and adult brain, such as neuronal induction and patterning, cell proliferation, cell fate specification, cell polarization and migration, axon guidance, synaptogenesis, adult neurogenesis and neuron maintenance and regeneration, it comes as no surprise that defects in WNT-induced pathways lead to disease and cancer (Alvarez et al., 2004; Clevers, 2006; Kurayoshi et al., 2006; Malaterre et al., 2007; Inestrosa and Arenas, 2010; Salinas, 2012; Marchetti and Pluchino, 2013). Several studies illustrate the emerging role of WNT and/or WNT/ β -catenin signaling in postnatal brain plasticity (Inestrosa and Arenas, 2010). Neuronal expression of components of the WNT signaling, such as GSK3 β and β -catenin, and several FZDs has been described in the adult animal brain (Inestrosa and Arenas, 2010). Studies in humans indicate that WNT signaling involved in the pathophysiology of AD, especially due to its role in the regulation of GSK3 β activity (Anderton et al., 2000; Ghanevati and Miller, 2005). Ever since the loss of WNT signaling was shown to associated with A β -induced neurotoxicity, studies have been demonstrating numerous WNT components to be altered in AD, and drugs capable of modulating WNT signaling have been discussed as potential tools against diseases associated with neuronal loss (Inestrosa and Toledo, 2008; Toledo et al., 2008; Inestrosa and Arenas, 2010). However, WNT's involvement in the development, homeostasis and diseases of the CNS is mainly based on studies focused on neurons,

and it was not until recently that the WNT signaling components were identified in cells of the immune system (Staal et al., 2008; Marchetti and Pluchino, 2013).

The GSK3 hypothesis in Alzheimer's disease

GSK3 is a serine/threonine inhibitory protein kinase, encoded by GSK3 α and β in vertebrates. GSK3 participates in numerous cell signaling cascades and in contrast to other kinases, GSK3 is highly active in resting cells, and its activity is reduced upon stimulation (Cohen & Goedert, 2004; Kockeritz, et al., 2006). Both isoforms are regulated by phosphorylation: GSK3 α at tyrosine 279 and GSK3 β at tyrosine 216, which increases their overall catalytic activity (Cohen & Goedert, 2004; Kockeritz, et al., 2006). Both isoforms are also inhibited by phosphorylation: GSK3 α at the amino-terminal domain serine 21, while GSK3 β has the equivalent residue serine 9 (Cohen & Goedert, 2004; Kockeritz, et al., 2006). However, little is known about isoform-specific functions. Several protein kinases have been identified as capable of phosphorylating and inactivating GSK3, such as phosphatidylinositol 3' kinase (IP3K) activation of protein kinase B in response to insulin stimuli, or cyclic AMP-dependent protein kinase (PKA) and atypical protein kinase C (PKC) (Cohen & Goedert, 2004; Kockeritz, et al., 2006). In WNT/ β -catenin signaling GSK3 β , as mentioned, belongs to the β -catenin destruction complex, where GSK3 β plays a central role to increase the complex stability by phosphorylation of the other proteins (Axin, APC) at multiple sites (Jope et al., 2007)).

GSK3 overactivity is associated with several neuropathological diseases (Kockeritz et al., 2006; Hooper et al., 2008), diabetes 2 (Kockeritz et al., 2006) cancer (Jope et al., 2007) and even schizophrenia (Lovestone et al., 2007). In AD, several agents associated with neuronal death are affected by GSK3 activity, such as tau, the APP fragment, and A β (Hooper et al., 2008). Tau filaments are hyperphosphorylated at more than 20 sites, at both primed and non-primed phosphorylation sites by activation of GSK3 β and - α (Hooper et al., 2008). Further, GSK3 α has been shown to regulate APP cleavage, which results in an increased production of A β (Phiel et al., 2003).

The progressive neuronal dysfunction in AD and in transgenic AD-mouse models is associated with decreased WNT/ β -catenin signaling in neurons (Pei et al., 1999; De Ferrari and Moon, 2006; Inestrosa and Arenas, 2010), where APP seems to be a major factor in the abnormal down-regulation of β -catenin in neurons (Chen and Bodles, 2007). In addition, restoring WNT/ β -catenin signaling through GSK3 inhibition seems to have a neuroprotective potential by diminishing A β neurotoxicity and by reducing tau hyperphosphorylation (De Ferrari et al., 2003; Alvarez et al., 2004; Inestrosa et al., 2007; Chacón et al., 2008). This was confirmed in a mouse model of AD where LiCl (a GSK3 inhibitor (O'Brien and Klein, 2009)) improved memory performance, which suggests that it alleviated the underlying neuronal deficits (Hooper et al., 2008; Toledo and Inestrosa, 2009). In addition, cortical neurons exposed to A β -peptides have increased expression of the WNT/ β -catenin signaling inhibitor Dickkopf 1 (DKK1), and are associated with neuronal degeneration (Caricasole et al., 2004). DKK1 antagonizes WNT/ β -catenin signaling through interaction of LRP5/6, and inhibits the formation of WNT/LRP5/6/FZD complex that would block the continuously active

GSK3 β in the β -catenin destruction complex (MacDonald et al., 2009). Further, GSK3 promotes inflammation through induction of proinflammatory cytokines and their receptors, and reduction of anti-inflammatory cytokines in monocytes and peripheral blood mononuclear cells (Joep et al., 2007).

In summary, “the GSK3 hypothesis of AD” refers to the central role GSK3 plays in AD development and the observation that GSK3 deregulation accounts for many of the pathological hallmarks of the disease: inflammation, APP, A β and tau phosphorylation (Joep et al., 2007; Hooper et al., 2008; Koistinaho et al., 2011). This GSK3 over-activity drives drug industry currently towards development of GSK3 inhibitors (Joep et al., 2007; Hooper et al., 2008; Palmer, 2011).

WNT signaling in microglia

Despite the important influence WNTs have on adult neurogenesis, neuron maintenance and regeneration, etc. (Malaterre et al., 2007; Inestrosa and Arenas, 2010) and the crucial role microglia have on CNS homeostasis and neuroinflammation (Rock and Peterson, 2006; Miller and Streit, 2007; Amor et al., 2010), it was not until recently that the link between WNTs and microglia began to emerge. For example, overexpression of WNT-5A signaling in microglia/macrophages has been associated with increased invasiveness of breast cancer cells, and especially their metastasis in the brain (Pukrop et al., 2006, 2010).

This thesis reports how recombinant WNTs induce WNT/ β -catenin-dependent and -independent signaling pathways through endogenously expressed FZDs in mouse microglia. The data shows that stimulation with recombinant WNT-3A on microglia induce β -catenin-dependent signaling and, in parallel, a β -catenin independent pathway resembling a classical GPCR cascade, resulting in the phosphorylation of the MAPK ERK1/2 (Halleskog and Schulte, 2013a). Interestingly, the data suggest a central role of G $\alpha_{i/o}$ proteins in the WNT/ β -catenin pathway since the G $\alpha_{i/o}$ protein inhibitor pertussis toxin blocks both WNT-3A/ β -catenin and WNT-3A/ERK1/2 signaling (Halleskog & Schulte, 2013a). In addition, WNT-3A stimulation induces a substantial proinflammatory fingerprint in microglia and together with the increased β -catenin stabilization found in amoeboid-like microglia cells in *postmortem* AD brains, we suggest that WNT are involved in the regulation of microglia in neuroinflammation (Halleskog et al., 2010).

Further, stimulation of microglia with recombinant WNT-5A induces a classical GPCR cascade in microglia involving G $\alpha_{i/o}$ protein, $\beta\gamma$, PLC, PKC, Ca²⁺, and MEK1/2 to phosphorylate ERK1/2. This pathway is responsible for WNT-5A-induced expression of some cytokines, MMPs, proliferation and invasion of microglia (Halleskog et al., 2011).

Finally, in the last study, the data show that both WNT-3A and WNT-5A counteracts on LPS-induced COX2, IL-6 and TNF α in microglia suggesting WNTs as homeostatic regulators of microglia (Halleskog and Schulte, 2013b). However, more detailed studies are required to elucidate underlying mechanism.

AIMS

The general aim of my studies was to investigate how microglia cells respond to stimulation with recombinant WNT. Based on the facts and considerations described in the introduction, the following specific aims were established for this thesis:

1. To investigate if microglia cells express WNT receptors
2. To identify specific intracellular signaling pathways induced by stimulation with recombinant WNT-3A and WNT-5A
3. To characterize WNT-mediated G protein activation in microglia, and to further dissect the WNT-induced intracellular signaling network
4. To validate WNT-3A- and -5A-induced activation of microglia with regard to proinflammatory markers, cytokine expression, proliferation, and invasion
5. To investigate WNT/ β -catenin-induced signaling in activated microglia in a human neuroinflammatory condition (AD) and in a corresponding mouse model of AD
6. To determine whether WNTs would promote or attenuate LPS-induced proinflammatory changes in microglia

MATERIAL AND METHODS

The material and methods that were used in this thesis are all described in the individual papers, and the descriptions will therefore not be repeated here. Most of the techniques are standard procedures and are listed in **table 1**, and the remainder of this section will bring up different methodological considerations of importance for the study.

Table 1: Techniques used in this thesis

Methods	Paper
Cell line culturing	I, III
Isolation and culture of primary microglia from mice	I, II, IV, V
Inhibitor treatment/ WNT-stimulation	I-V
SDS-PAGE/ Western blotting (immunoblotting)	I, II, III, IV, V
Immunocytochemistry	I, IV
Immunohistochemistry	I
RNA extraction and cDNA synthesis	I, IV, V
Reverse transcription polymerase chain reaction (RT-PCR)	I
Real-time/quantitative RT-PCR (QPCR)	I, IV, V
Affymetrix Expression Analysis	I
Mesoscale for TNF α	IV
Enzyme-linked immunosorbent assay (ELISA)	I
[Ca ²⁺] _i imaging	IV
cAMP measurements	IV
Invasion assay	IV
[γ - ³⁵ S]-GTP assay	III, IV
MTT-assay	III, IV
Cell counting	I, IV

Methodological considerations

Cell line culturing and isolation of microglia

Cultured cell lines are often used as *in vitro* models for the study of specific cellular mechanisms. N13 is a cell line which manifests microglia-like features because it is obtained by immortalization of primary microglia cultures isolated from the ventral mesencephalon and cerebral cortex of CD1 mice at embryonic day 12-13 (Righi et al., 1989; Ferrari et al., 1996). However, after prolonged time in culture, cell lines have the drawback that they become more homogenous and adapt to growing in culture, which in turn leads to loss of physiological features. In order to obtain more realistic and physiological readout closer to *in vivo* situations, we have isolated microglia cells and astrocytes from fresh tissue, so-called primary cultures. In Paper I-II, IV and V we obtained cells from newly decapitated C57BL/6 wild-type (wt) mouse pups (postnatal

day 1-3; P1-3), according to our ethical permits (N26/05, N144/08, and N436/10, approved by Stockholms Norra Djurförsksetiska Nämnd). The animals were bred at the Department of Physiology and Pharmacology, Karolinska Institute, and housed at a constant room temperature (22°C; 12h light/dark cycle) and the handling of the animals was in the accordance with the NIH *Guide for the Care and Use of Laboratory Animals*. The isolation protocol follows a standard procedure described by Giulian and Baker (1986) (Giulian & Baker, 1968)), with modifications, that are described in greater detail in Paper IV (Halleskog et al., 2012). After microglia cells were harvested from the astrocytic layer, their purity was checked, mainly by immunocytochemistry with the antibodies against microglia marker CD11b (Mac-1) or IBA-1 (Akiyama and McGeer, 1990) in combination with antibodies against the astrocytic marker GFAP (Glia fibrillary acidic protein) (Cahoy et al., 2008).

Animal model for AD: the APdE9 mice

Rodents do not develop AD as humans, with formation of plaque or tangles; but creation of transgenic mice allows us to mimic various aspects of the disease. A few mouse models of human AD are available, all of which involve mutation of the gene encoding APP to form A β plaques. The APdE9 mouse is a double transgenic mouse model where AP stands for the Swedish mutation (K595N and M596L) in the gene coding for amyloid precursor protein (APP) and dE9 for the deletion of the presenilin 1 (PSEN1) gene at exon 9. Combining these two mutations, APP and PSEN1, increases the likelihood of early disease onset and an alteration of A β ₄₂ formation. The APdE9 mice have been examined both biochemically for A β plaque formation followed by chronic inflammation, astrogliosis and microgliosis, and behaviorally for memory deficits (Jankowsky et al., 2004; Garzia-Alloza et al., 2006). PSEN1 has been shown to interact with β -catenin and its stability (Sato and Kuroda, 2000), i.e., loss of PSEN1 function results in increased stability of cytosolic and this association has been implicated in modulating the WNT/ β -catenin signaling pathway (Kang et al., 1999). However, the PSEN1 mutation used in Paper I does not affect β -catenin steady-state levels in cells (Soriano et al., 2001).

The tissue from transgenic mice was purchased from the University of Eastern Finland, Kuopio, and kept at the Department of Medical Biochemistry and Biophysics, Karolinska Institute. In accordance with ethical permit N26/05, it was only used for immunological *postmortem* studies.

Stimulation and inhibitor treatment

Pharmacological inhibitors are useful tools for *in vitro* studies to dissect signaling pathways. To note, it is quite common that inhibitors are rather unspecific for their actual target protein. To overcome this and to confirm blockade or non-blockade, several inhibitors against the same protein were used in this thesis, and if possible, structurally different inhibitors were selected, e.g., wortmannin and LY294002 inhibiting PI3K, and BIS and RO318220 inhibiting PKC. Additionally, when it comes to experimentation on immune cells like microglia cells, it is difficult to use inhibitors dissolved in certain substances (such as DMSO), or to attach carrier proteins that can

affect their activity, such as BSA (Hopper et al., 2009). This was overcome by the use of sham-stimulated cells. Therefore, for longer treatment, inhibitors were preferentially chosen based on their solubility in ethanol (SL327 blocking MEK1/2 instead of PD98059), and only carrier-free WNTs dissolved in PBS were used (except in Paper I). In order to block WNT-induced signaling, we have used endogenous inhibitors in recombinant form, such as Dickkopf 1 (DKK1) and a soluble form of FZD, secreted Frizzled-Related Proteins (SFRP1). DKKs (DKK1-4), is a family of secreted glycoproteins that inhibit WNT-ligands interaction with LRP5/6-FZD complexes and prevents the WNT/FZD/LRP5/6 formation. DKKs are therefore seen as negative regulators of the WNT/ β -catenin signaling pathway (Bourhis et al., 2010; Krupnik et al., 1999; Mao et al., 2001; MacDonald et al., 2009). Expression of DKK1 is required for proper neuronal development during the embryonic period, regulating normal formation of the midbrain structure (Glinka et al., 1998). The family of SFRP1-5 is structurally related to the WNT-binding CRD of the FZDs. The SFRPs are suggested to interact with WNTs and thereby inhibit WNT-ligands interaction with FZDs and induced signaling (Kawano and Kypta, 2003; Rattner et al., 1997). In addition, increased WNT signaling due to decreased expression of SFRPs is related to human breast tumors and associated with poor prognosis (Schlange et al., 2007). However, the CRD of SFRP can interact with other CRDs, including FZDs (Bafico et al., 1999), and this results in biphasic effects, i.e. high concentrations of SFRP1 decrease WNT/ β -catenin signaling, whereas low SFRP1 concentrations induce β -catenin stabilization (Uren et al., 2000).

Proliferation assay

Since proliferation is one of the hallmarks of microglia proinflammatory transformation, we have measured cell number by two techniques: MTT assay and direct cell counting. This use of the MTT assay is somewhat indirect, since it actually measures cell viability (Gerlier & Thomasset, 1986). This non-radioactive colorimetric assay uses MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and is based on the capacity of mitochondrial enzymes of active cells to transform and cleave the MTT tetrazolium salt into MTT formazan. The readout is a measure of cell viability, which is proportional to the number of living cells (Mosmann, 1983). We have additionally confirmed the proliferation by counting trypsinized cells in a Bürker-chamber.

Gene-expression analysis

There are various methods for detecting expression of genes. In this thesis, polymerase chain reaction (PCR) techniques have been used to detect and quantify mRNA levels of FZD receptor expression and inflammatory molecules. PCR is a fast and straight forward method which does not require much material. Reverse transcriptase-PCR (RT-PCR) is an end-point technique to analyze the presence of genes, such as receptors. For a more quantitative method, where it is possible to track every cycle, and to measure differences between gene expression in different samples we have used Real-Time-PCR (QPCR). Primer efficiency has been tested by the manufacturer

(Applied Biosystems) and shown to be close to 100% for all the primers we used. In Paper IV, the same primer efficiency was additionally confirmed on the FZD probes by the C_T slope method over six serial cDNA dilutions. Thus, direct comparison and quantitative statements about relative FZD expression levels are justified. QPCR is an easy-to-use technique that does not require a postreaction analysis as for the RT-PCR (end-product is loaded onto a gel to analyze). However, the mRNA levels do not always correspond to the actual protein levels expressed, and in this thesis, some of the data are additionally confirmed by combining expression analysis with a protein detection technique: immunoblotting, ELISA or mesoscale. In order to perform protein detection, it is essentially that functional and reliable antibodies are available. Microarray mRNA technique is a useful method for comparing many genes at the same time in different stimulated cells, and was used in Paper I: the Affymetrix Expression Analysis on 6 h 300 ng/ml WNT-3A vs. control stimulated primary microglia cells. To trace any DNA contamination, the mRNA was first quality controlled with the sensitive Agilent Bioanalyzer and further analyzed on an Affymetrix Mouse Gene 1.0 ST Array at the Bioinformatics and Expression Analysis Core Facility, Department of Biosciences and Nutrition, Karolinska Institute. This technique allowed us to analyze the expression of 28 000 genes at the same time.

Antibody-based techniques: Immunohistochemistry, immunocytochemistry and immunoblotting

Upon activation, many proteins become phosphorylated, such as several of the MAPKs e.g. ERK1/2 and the scaffold protein DVL, or the protein expression is regulated, as in the case of β -catenin. These changes can be visualized by many antibody-based techniques because today, many companies sell antibodies against both the normal and the phosphorylated form of a protein. To test antibody specificity, the antibodies were used on whole cell lysates from unstimulated control cells vs. stimulated cells, separated according to size and charge by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to protein binding membranes (immobilon-P membranes (Millipore)). After incubation with primary antibody, proteins could be visualized by enhanced chemiluminescence with the secondary antibodies conjugated to horseradish peroxidase. By loading a known protein-ladder next to the sample, the size of visualized proteins can be determined. If only one band appears at the right size, the antibody has high specificity and can be considered reliable for immunohistochemistry or immunocytochemistry, when the secondary antibody is labeled to a fluorophore. What needs to be considered is that in immunoblotting, proteins are denatured and elongated which can lead to exposure of epitopes that are normally hidden in the 3-D-structure. This can be overcome by the use of polyclonal antibodies, i.e., antibodies against several epitopes on the same protein.

G protein activation assays

Heterotrimeric G proteins represent the immediate cytoplasmic transducers of GPCRs. GPCR-mediated signaling is a fast response: upon ligand-GPCR-interaction the receptor undergoes a conformational change which enhances its guanine nucleotide

exchange factor activity towards the α -subunit. This results in a guanine nucleotide exchange of GDP for GTP at the heterotrimeric G_α protein and a dissociation of the $\beta\gamma$ subunit (Cabrera-Vera et al., 2003; Oldham and Hamm, 2008). Depending on the G protein family that is involved, both α and $\beta\gamma$ subunits activate diverse effectors (Gilman, 1987; Dorsam and Gutkind, 2007; Oldham and Hamm, 2008). In the [γ - ^{35}S]GTP assay, which involves preparing cell membranes and stimulating them with WNTs in the presence of GDP and the non-hydrolyzable GTP analogue [γ - ^{35}S]GTP, it is possible to measure the increase in the rate of GDP/GTP exchange of membrane-associated G proteins (Harrison and Traynor, 2003).

Adenosine 3'5'-cyclic monophosphate (cAMP) is a second messenger important in many biological processes. cAMP is synthesized from ATP by adenylyl cyclase which is located on the inner side of the plasma membrane and is in turn activated by the G_{α_s} subunit of a G protein, and inhibited by the $G_{\alpha_{i/o}}$ subunit. The response is compared with that elicited by forskolin, a direct adenylyl cyclase stimulator: if cAMP levels decrease in a dose-dependent manner, this would functionally confirm activation of $G_{\alpha_{i/o}}$ proteins. The method that has been used is a sensitive semiautomatic modification of a protein binding assay, in which cAMP-dependent protein kinase from bovine adrenal cortices is used for binding protein and [^3H]cAMP is used as a tracer (Nordstedt and Fredholm, 1990). Before incubation with drugs, the cells are washed in the presence of a phosphodiesterase inhibitor, and cAMP can be extracted with perchloric acid. Defined concentrations of cAMP and [^3H]cAMP are added in combination with the binding protein, and later transferred onto filter plates which can be measured by a Ria-Gamma counter.

Changes in intracellular Ca^{2+} are regulated via GPCR activation through the release of $\beta\gamma$ and the subsequent PLC-dependent production of inositoltrisphosphate (IP_3). The WNT-induced Ca^{2+} release is dependent on $G_{i/o}$ and G_q family proteins and was the first WNT-induced β -catenin independent pathway described (Moon et al., 1993; Kühl et al., 2000b). In this thesis Ca^{2+} influx has been measured in Fluo-3 stained, unfixed microglia cells. Fluo-3 is a single wavelength dye and one of the most popular and widely used Ca^{2+} indicators (Paredes et al., 2008), and has been used for Ca^{2+} influx in microglia (Nolte et al., 1996).

Invasion assay

Enhanced capability to invade into surroundings is a hallmark of proinflammatory activation of microglia. We have implemented a three-dimensional collagen assay to trace fluorescently labeled invading microglia into a collagen matrix. The microglia are seeded on top of the collagen matrix layer, and at the same time stimulated with WNTs. WNTs are small lipoglycoproteins, and can diffuse freely in the collagen, therefore, this is not considered as a chemotactic assay. Microglia invasion actually requires both migration and degradation of the basement gel component, collagen 1.

RESULTS AND DISCUSSION

WNT-proteins and receptors in microglia and N13

WNTs are widely expressed in the human and mouse brain (Inestrosa and Arenas, 2010; Malaterre et al., 2007), and today it is known that WNTs are important for regeneration and for homeostatic maintenance of neurons (Chien et al., 2009; Inestrosa and Arenas, 2010). It is not known what type of influence WNTs have on microglia functions. The first thing to investigate, is if microglia express WNT-receptors at all, since such receptors are required if the cells are able to respond to WNT-stimulation, and second, to evaluate the microglia-like cell line N13 is a good model by comparison with cultured primary microglia cells isolated from mice. By using RT-PCR in paper I we found out that N13 expresses FZD_{2,4,5,7,8,9} and the co-receptors LRP5/6, thus being similar to primary microglia that have a receptor repertoire consisting of FZD_{4,5,7,8} (paper I) and the co-receptors LRP5/6. At the same time, neither of the cell types expressed the co-receptor ROR1/2 or RYK. Using affimetrix gene microarray and QPCR (paper IV) we confirmed our findings in primary microglia, however, according to the microarray, a low expression of ROR2 and high expression of RYK was detected (**Figure 5**). With the microarray we also profiled the WNTs that microglia cells express themselves (**Figure 6**). In paper I, we also used RT-PCR on whole mouse brain cDNA to show that WNT-1,-2,-3 and 7A are expressed in CNS and can putatively induce the WNT/ β -catenin signaling pathways in microglia (Shimizu et al., 1997; Nusse, 2003; Hwang et al., 2004; Malaterre et al., 2007; Schulte, 2010a).

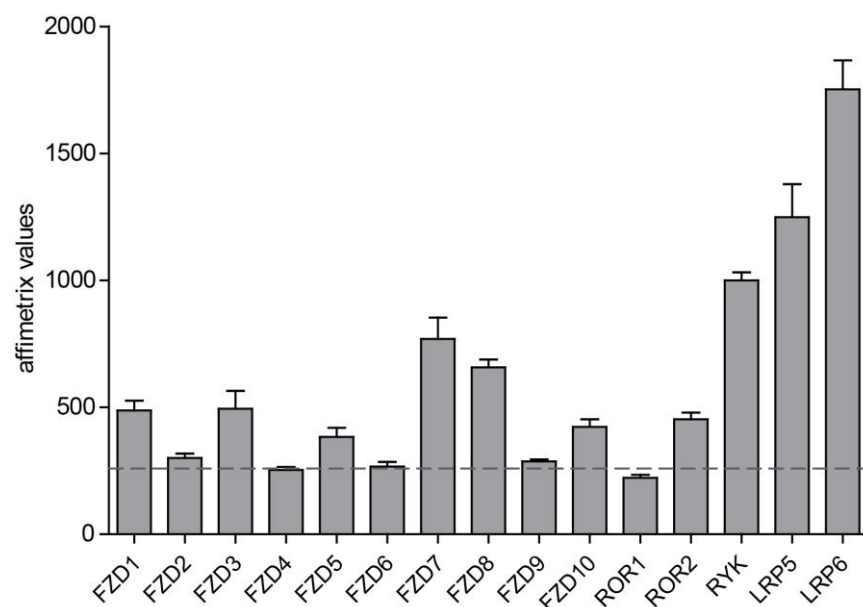


Figure 5: The WNT-receptor expression profile of mouse primary microglia according to affimetrix microarray. The dotted line shows the baseline of expressed genes. Mouse microglia have the receptor repertoire consisting of FZD_{1,3,5,7,8,10} and the co-receptors LRP5/6. To note, according to the microarray, microglia also express ROR2 and RYK.

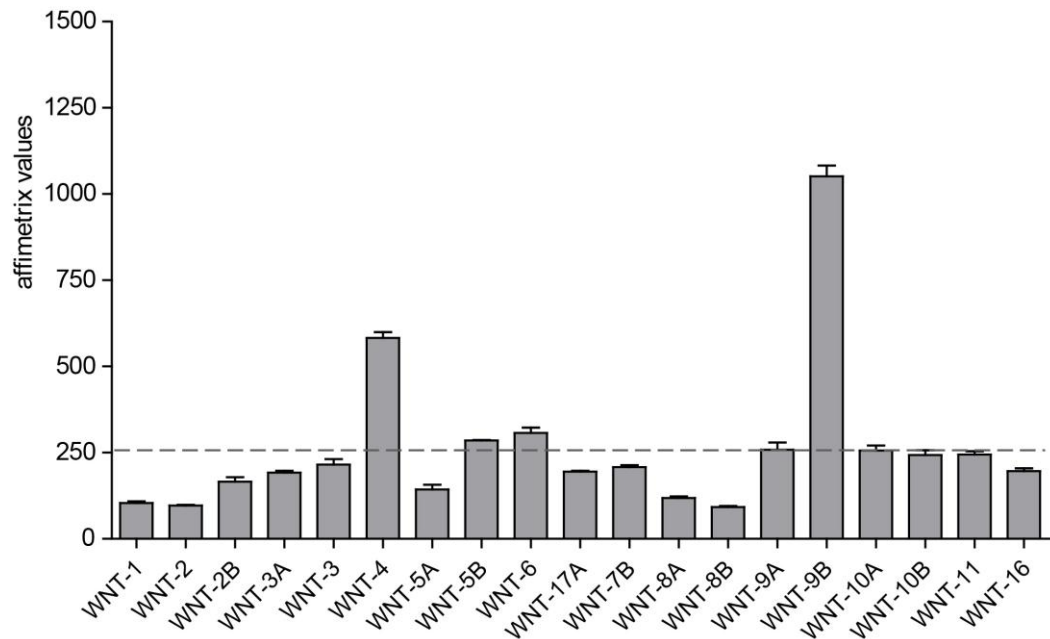


Figure 5: The WNT-expression profile of mouse primary microglia. According to the affimatrix expression analysis primary microglia express WNT-4, -5B, -6, and -9A. The dotted line shows the baseline of expressed genes.

Recombinant WNT-3A effects on microglia

WNT-3A-induced intracellular pathway in microglia

It is widely accepted that WNT-3A belongs to the WNT family that activates the β -catenin-dependent pathway (Shimizu et al., 1997; Nusse, 2003; Bryja et al., 2007a). In paper I, we show by immunoblotting and immunocytochemistry that microglia cells respond to recombinant and purified WNT-3A with a dose- and time-dependent accumulation of β -catenin and, furthermore, that β -catenin translocates into the cells' nuclei. We also show that WNT-3A stimulation induces classical hallmarks of the β -catenin-dependent pathway such as phosphorylation of the co-receptor LRP6 and phosphorylation and shifting of the scaffold protein DVL (MacDonald et al., 2009).

In paper II, we show by immunoblotting that in parallel to the β -catenin pathway, but with slightly different kinetics, WNT-3A stimulation of microglia also induces a dose- and time-dependent phosphorylation of a classical downstream target of GPCRs, the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK1/2) (Gutkind, 2000). ERK1/2 activation is a well-known modulator of microglia activity (Koistinaho and Koistinaho, 2002) and different MAPK have been assigned important but yet poorly defined roles in the regulation of and crosstalk with the WNT/ β -catenin pathway (Bikkavilli and Malbon, 2009; Wolf et al., 2011; Krejci et al., 2012). The WNT-3A-induced P-ERK1/2 has an earlier onset, with a maximum at 60 min, in comparison to WNT-3A-induced- β -catenin stimulation which has an onset at 30-60 min and a long-lasting response with maximal levels even after 24 h. By pre-treating the cells with 1 mg/ml Dickkopf 1 (DKK1), a LRP5/6 inhibitor known to block

the WNT/ β -catenin pathway (Mao et al., 2001), we could block WNT-3A-induced β -catenin stabilization but not the formation of PS-DVL or P-ERK1/2. This indicates that the signaling routes are separate and that WNT-3A induction of P-ERK1/2 might be accomplished through a WNT/FZD complex independent of the LRP5/6 co-receptor. This finding was confirmed when by the use of casein kinase 1 inhibitor D4476 (10 μ M), which is known to block the formation of PS-DVL and β -catenin stabilization (Bryja et al., 2007b). When D4476 was given prior to WNT-3A stimulation, ERK1/2 was phosphorylated even though the upstream LRP6 phosphorylation remained unaffected. To further support our hypothesis that WNT-3A-induced P-ERK1/2 is a distinct signaling route not downstream of β -catenin, we used a pharmacological GSK3 β inhibitor (GSK3 β inhibitor 1V). In these experiments, the GSK3 β inhibitor (20 μ M) induced comparable levels of β -catenin accumulation, but did not affect upstream events, i.e. the formation of PS-DVL or P-LRP6. Moreover, the GSK3 β inhibitor could not induce P-ERK1/2.

To dissect the signaling cascade induced by WNT-3A from the FZD receptor to phosphorylation of ERK1/2, we used a battery of well-characterized pharmacological inhibitors against different cascade proteins. It happens that recombinant proteins on the market are contaminated and impure, and may thus induce unspecific events (Cajane et al., 2010). So to confirm the purity of the recombinant WNT-3A sample, we mixed WNT-3A with a soluble Frizzled-related protein 1 (SFRP1), that binds to the WNT protein and would inhibit any WNT-induced signaling pathway (Kawano and Kypta, 2003), before adding it on the microglia. After this treatment, addition of recombinant WNT-3A did not lead to LRP6 phosphorylation, PS-DVL formation, β -catenin stabilization or phosphorylation of ERK1/2, proving that it is only WNT-3A in the sample that induces this cascade and not a contaminant. ERK1/2 phosphorylation is known to be induced by seven-transmembrane receptors, and our inhibitory battery was selected based on that knowledge. The first inhibitor we used was the well-known $G\alpha_{i/o}$ -protein inhibitor PTX (Birnbaumer et al., 1990), which ADP-ribosylates the α subunit of $G\alpha_{i/o}$ family of proteins. We also used a $\beta\gamma$ -effector interaction inhibitor (M119) (Bonacci et al., 2006), a phospholipase C (PLC) inhibitor (U73122) (Bleasdale et al., 1989), a calcium chelator (BAPTA-AM) (Nq et al., 1988), an two MEK 1/2 inhibitors (SL327 (Selcher et al., 1999) and PD098059) (Marchetti and Pluchino, 2013), which all blocked the ERK1/2 phosphorylation induced by 100ng/mL WNT-3A, at 30 min and 2h. Since the calcium chelator blunted WNT-3A-induced P-ERK1/2 we continued by using two different Ca^{2+} -dependent protein kinase (PKC) inhibitors: BIS (VIII) and Ro 318220 (Davis et al., 1992), and two different Phosphatidylinositol 3'-kinase (PI3K) inhibitors: Wortmannin and LY94002 (Wymann and Schultz, 2012), which did not block WNT-3A-induced P-ERK1/2. **Figure 6** shows a schematic diagram summarize the WNT-3A induced signaling pathway investigated in this study.

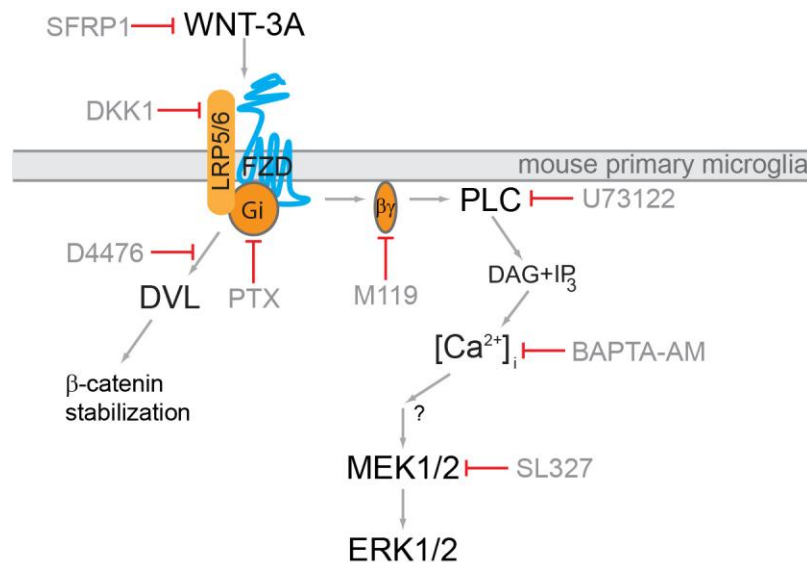


Figure 6: Schematic diagram figure summarize the WNT-3A-induced WNT/ β -catenin and WNT/ERK1/2 signaling in microglia. The WNT-3A-induced signaling pathway indicates the central role of the PTX sensitive $G\alpha_{i/o}$ proteins in both branches. Pharmacological inhibitors used in this study are labeled in light gray. The question mark indicates insecurity in the pathway continuation.

Interestingly, in paper II, for the first time on endogenously expressed FZDs, we show by immunoblotting that pre-treatment with PTX, but not with the $\beta\gamma$ -blocker M119, completely abolished the WNT-3A-induced LRP6-phosphorylation, PS-DVL formation and β -catenin stabilization. This indicates that WNT-3A-induced phosphorylation of LRP6 requires $G\alpha_{i/o}$ subunits, subsequent PS-DVL3 and β -catenin stabilization. This is in line with what has been shown on L929 cells, a mouse fibroblast cell line, where PTX blocks WNT-3A-induced disruption of the GSK3 β /Axin complex (Katanaev et al., 2005; Liu et al., 2005). In addition, the use of the $\beta\gamma$ inhibitor M119 clarifies that WNT-3A-dependent communication with PLC but not with LRP6/ β -catenin requires the release of $\beta\gamma$ subunits from the $G\alpha_{i/o}$. Thus these data indicate that WNT-3A-induced signaling in microglia regulates and mediates crosstalk between β -catenin-dependent and -independent pathways through the PTX-sensitive heterotrimeric $G\alpha_{i/o}$ proteins upstream of the FZD/LRP6 receptor complex (**Figure 6**). In paper III, by use of the [γ - 35 S] GTP-assay, we showed WNT-3A-induced GDP/GTP exchange on N13 membrane preparations, which in addition confirms WNT-3A-induced activation of G proteins in microglia, and that endogenous FZDs are capable of working as conventional GPCRs. However, it remains unclear if the WNT-3A-induced signaling axes depend on identical or different FZD-isoforms. Hypothetically, WNT-3A stimulation could recruit LRP6-FZD $_x$ for the WNT/ β -catenin pathway, whereas ERK1/2-induced signaling could be mediated by FZD $_y/z$ in the absence of LRP6 recruitment, or by another FZD $_y/z$. To date, owing to the lack of pharmacological tools selective for the FZD isoforms, this hypothesis remains untested.

WNT-3A proinflammatory modulation of microglia activity

Hallmarks for microglia proinflammatory activation can be proliferation, morphological changes, phagocytosis, increased cytokine and chemokine expression etc. (Kühl et al., 2000b; Lynch, 2009; Kettenmann et al., 2011). In addition, β -catenin-dependent signaling can evoke proliferative effects in cell culture systems (Castelo-Branco et al., 2003; Boland et al., 2004; Yun et al., 2005). We studied whether WNT-3A effects microglia proliferation. To do this, N13 cells were seeded in 24-well plates and starved for 24 h before stimulation with 300 ng/mL WNT-3A, corresponding control (1% BSA), and 10% FBS. After 24 h the cells were detached with trypsin and counted in a Bürker-chamber. WNT-3A does not induce microglia proliferation, in comparison to cells with serum-starved media and the positive control FBS-stimulated cells whose numbers increased by 10%. These data are additionally confirmed in paper III by a MTT assay. The second step was to address another hallmark of proinflammatory microglia, namely cytokine expression and release (Lynch, 2009; Kettenmann et al., 2011). After 24 h of stimulation with 300 ng/mL of WNT-3A, medium was collected for ELISA and mRNA was isolated and synthesized to cDNA for QPCR analysis of the proinflammatory cytokines IL-6, IL-12 and TNF α . The increased mRNA levels were related to the released cytokines in the media. Furthermore, we continued the readout of these data by an affimetrix genome-wide expression profiling. After 6 h of stimulation with 300ng/mL WNT-3A, a whole range of proinflammatory genes was induced, such as soluble proinflammatory factors (IL-6, IL-1 α and chemokines), iNOS, members of the TNF superfamily, the crucial player of prostanoid synthesis COX2 (Minghetti and Levi, 1998), matrix metalloproteases etc. (see figure 7, paper I). These data show that WNT-3A induces proinflammatory (fingerprint) transformation of microglia.

In paper II, where we dissected the WNT-3A induced signaling pathway, we also looked at COX2 expression by immunoblotting, and showed that the MEK1/2 inhibitor (SL327), completely blocked the WNT-3A-induced COX2 expression in microglia. These data are surprising because COX2 expression has been identified as a downstream target for WNT/ β -catenin signaling (Haertel-Wiesmann et al., 2000; Pishvaian and Byers, 2007; Yun and Im, 2007). In another recent study, WNT-3A stimulation of primary rat microglia leads to the secretion of exosomes independently of GSK3 (Hooper et al., 2012). This further suggests an important role of the WNT-3A-induced β -catenin-independent signaling pathway in microglia.

β -catenin expression in microglia in AD

AD is a serious neurodegenerative condition with chronic inflammation owing to a high presence of activated microglia (Pocock et al., 2002; Rivest, 2009; Morales et al., 2010; Wilkinson and El Khory, 2012). The role of microglia in AD appears to be double-edged: on one hand microglia cells are supposed to diminish the disease by clearing amyloid-beta (A β) aggregates and dead neurons (Hanisch and Kettenmann, 2007; Farfara et al., 2008), but on the other hand, when the neuroinflammatory condition persists, microglia seem to have detrimental effects (Farfara et al., 2008). In

AD, GSK3 plays a central role in the development of the disease with regard to inflammation, A β -formation, APP cleavage and the hyperphosphorylating of tau to form intracellular tangle (Pei et al., 1999; Hooper et al., 2008). Restoring WNT/ β -catenin signaling through GSK3 inhibition seems to have a neuroprotective potential both by diminishing A β neurotoxicity and by reducing tau hyperphosphorylation (Alvarez et al., 2004). In addition, Dickkopf-1 (DKK1) treatment reinforces beneficial effects of WNT signaling in neuronal survival (Caricasole et al., 2004) suggesting a beneficial effect of maintaining neuronal β -catenin by pharmacological GSK3 inhibitors or enhanced WNT signaling (Alvarez et al., 2004; De Ferrari and Moon, 2006; Dinamarca et al., 2008; Inestrosa and Arenas, 2010). Furthermore, GSK3 blockade by LiCl has improved memory performance in the AD mouse model APdE9 (Toledo and Inestrosa, 2009). Interestingly, no one has studied β -catenin levels in microglia cells before, and it is unknown whether the treatment with a GSK3 inhibitor would be able to exacerbate microglia-mediated inflammatory reaction.

With protein-specific antibodies we could do immunohistochemistry on *postmortem* brain tissue from patients with AD vs. age-matched controls to evaluate the β -catenin levels in microglia cells. We found a low to moderate staining of β -catenin in perikarya and intensely labeled nuclei of cells with multipolar and fine-caliber processes, likely representing micro- or astroglia cells. By combining the β -catenin staining with the microglia marker Ionized calcium-binding adapter molecule-1 (IBA-1) (Akiyama and McGeer, 1990) we found closely associated immunoreactivities in brain from patients with Braak stage VI. Cells that stained positively for IBA-1, i.e. activated microglia (or macrophages) which, were surrounded by dystrophic neurons that were AT8-positive (i.e. hyperphosphorylated tau AT8 (Porzig et al., 2007). This was additionally confirmed by the co-expression of IBA-1 and the cannabinoid receptor: CB₂R, which has enhanced expression on glia in neuritic plaques (Walter et al., 2003). Comparison of these data with data obtained in postmortem brain tissue from healthy subjects, suggests a shift of β -catenin expression from neurons towards (micro-) glia in patients with AD-related neuroinflammation. In addition, co-staining with β -catenin and the astrocytic marker glial fibrillary acidic protein (GFAP) showed that β -catenin levels are generally low in astrocytes, irrespective of the AD stage.

Microglia cells have the capacity of phenotypic transformation, from a ramified stage to a more actively moving amoeboid/macrophage-like stage with phagocytic capability (Kettenmann et al., 2011; Marin-Teva et al., 2011). Therefore, we wanted to see if β -catenin stabilization in microglia cells can be connected particularly to any of the different phenotypes. From our histochemistry images we concluded that ramified microglia expressed less β -catenin, whilst active mobile microglia progressing towards an amoeboid structure were β -catenin positive. The β -catenin in these round microglia, appeared to be located in the cytosol, submembranously or in the cell nuclei though the precise location could not be determined because of the limited resolution of the microscope (see Fig 2, paper I). To provide biochemical support for our hypothesis that microglia have higher expression of β -catenin in AD subjects, we did immunoblotting and densitometric quantification of the bands and normalization of β -catenin, GFAP, CB₂R intensities to β -actin (as a loading marker). We plotted β -catenin-stained cells and CB₂R-stained cells, for individual control, moderate AD and severe AD, and

compared it with the astrocytic marker GFAP together with β -catenin in a regression analysis. In comparison to aged-matched controls or subjects with moderate AD, we found significantly increased levels of CB₂R in subjects with severe AD. Furthermore, we found a close relationship between β -catenin and CB₂R in severe AD cases but only a quasi-random relationship in control and moderate AD cases. These neuroanatomical and biochemical findings, together, confirm that β -catenin expression is significantly increased in microglia, or invaded peripheral macrophages, in AD.

To support our findings in human AD, we moved to an AD-like mouse model, the APdE9 mice. The APdE9 mice show pathology comparable with the human AD, such as chronic inflammation, microgliosis and astrogliosis, progressive β -amyloid production, increased production of inflammatory cytokines, and an increased activity of the complement system. TNF α is an example of a proinflammatory cytokine increased in neuroinflammatory processes (Jankowsky et al., 2004; Sriram and O'Callaghan, 2007). Thus, using QPCR to measure mRNA levels of TNF α in the brain, we could confirm the inflammation with a significantly increased TNF α expression in the APdE9 mouse vs. wild-type aged-matched controls, 7 and 12 months of age. Furthermore, immunoblotting showed that total β -catenin levels were increased, by $29 \pm 13\%$, in APdE9 mice at 14 months of age. Interestingly, in the polymorph layer of the dentate gyrus, which is considered to play a crucial role in associative memory (Ohm, 2007), β -catenin was increased in microglia in the elderly wild-type mice (**Figure 7**), which can be explained by aging also being associated with a progressive inflammatory reaction with a transformation of microglia morphology and function (Akiyama et al., 2000; Pocock and Liddle, 2001). Additionally, β -catenin was accumulated in microglia in the brain of the APdE9 mice (**Figure 7**). Taken together, these facts show that β -catenin, is stabilized in activated and proinflammatory microglia in AD.

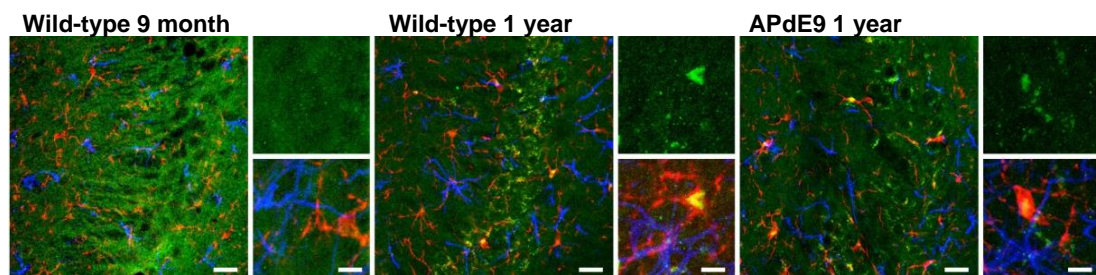


Figure 7: Increased β -catenin levels in microglia in elderly and APdE9 mice. Fluorescent immunohistochemistry was performed on brain sections from wild-type and APdE9 mice. The image show cellular distribution of β -catenin (green), IBA-1 (red, microglia marker) and GFAP (blue, astrocyte marker) in the hippocampus. Co-localization of β -catenin and IBA-1 identify increased levels of β -catenin in microglia in the ageing and in the APdE9 mice. Scale bars 15 μ M and insets 5 μ M.

To exclude other soluble molecules affecting microglia and/or GSK3 activity from being the cause of the β -catenin stabilization in microglia in AD and in the aging brain, we exposed N13 to several microglia activators for 1h: LPS, interferon α/β , ATP, insulin, TNF α , UTP, NECA, isoproterenol, apomorphine, thrombin, glutamate (Jin et

al., 2008; Amor et al., 2010). Displayed by immunoblotting, only the stimulation with WNT-3A and the GSK3 inhibitor LiCl (O'Brien and Klein, 2009) induced β -catenin stabilization, in microglia. Furthermore, considering that A β has been shown to bind and activate FZD (Magdesian et al., 2008), A β -treatment in N13 for 24h induced another hallmark of microglia proinflammatory activation: iNOS expression (Hanisch and Kettenmann, 2007; Brown and Neher, 2009). Nevertheless, the β -catenin levels remained unaffected. In paper III, in the screen of all the commercially available recombinant WNTs on the market, it was only WNT-3A that induced β -catenin accumulation in N13.

Other WNTs affecting microglia in a G protein- dependent manner

Because of difficulties purifying active WNTs only a subset of the 19 mammalian WNT isoforms have ever been available in recombinant form for use in experimental studies *in vitro* i.e., WNT-3A, -4, -5A, -5B, -7A and -9B (Willert, 2008). In paper I, we show on mRNA levels that the microglia-like cell line N13 has the FZD receptor repertoire of FZD_{2,4,5,7,8,9}. These data are confirmed in another study by Kilander et al., (2011), where QPCR analysis revealed the following expression pattern: FZD₅>FZD₇>FZD₂, and low levels of FZD₄ and FZD₂ in N13 (Kilander et al., 2011a). In paper III, we compared the efficacy of the different recombinant WNTs on N13, with regard to LRP6 phosphorylation, β -catenin stabilization, ability to form PS-DVL, G protein activation and a physiological outcome: proliferation. By immunoblotting on lysates of stimulated cells, we could see that all the WNTs induce phosphorylation and shift of DVL3, after 2 h of stimulation with 200 ng/ml WNT, and WNT-3A and -4 elicited the strongest activation (**Table 2**). We evaluate the purity of the recombinant WNT samples, by using SFRP1 and found that all the WNT-induced PS-DVL3 formation was completely blocked, except for WNT-9B. This can be explained by existence of other SFRPs that could be more specific for certain WNTs, for example SFRP4 has low affinity to WNT-3A, but high affinity to WNT-7A (Carmon and Loose, 2010). Continuing with immunoblotting we also could see that only WNT-3A induced the hallmarks of WNT/ β -catenin signaling, i.e. P-LRP6 and β -catenin stabilization. To measure WNTs' ability to activate G proteins, we measured WNT-induced GDP/GTP-exchange in N13-membrane preparations, using an assay based on the hydrolysis-resistant γ -³⁵S-labeled GTP (Milligan, 2003). All the WNTs induced the exchange, which corroborates the findings of other recent studies showing that WNTs are capable of activating heterotrimeric G proteins (Liu et al., 2005; Koval and Katanaev, 2011; Kilander et al., 2011a). FZD₅ as the dominant receptor expressed by N13, is also an established WNT-5A receptor (He et al., 1997; Säfholm et al., 2006; Kurayoshi et al., 2007; Kilander et al., 2011a). The WNTs with highest efficacy to induce G protein (WNT-5A and -9B) also induced proliferation, measured by MTT-assay, where the cells' measured viability correlates with cell number (Gerlier and Thomasset, 1986), after 40 h of stimulation.

Table 2: Results of the WNTs used in paper III. The WNT preparations are commercially available from R&D Systems. The WNT's capacity to transform C57MG cells and their expression in the brain was summarized from the literature (for references see text in paper III). Further, data from the present study are summarized. - no activation; + weak activation; ++ intermediate activation; +++ strong activation.

WNT	Transformation of C57MG cells	Expression in the brain	P-LRP6	β -catenin stabilization	PS-DVL3	G protein activation	Proliferation
WNT-3A	+++	-	+++	+++	+++	+	-
WNT-4	-	+	-	-	+++	+	-
WNT-5A	-	+	-	-	++	+++	+
WNT-5B	-	+	-	-	+	++	-
WNT-7A	+	+	-	-	+	+	-
WNT-9B	+	+	-	-	+	+++	+

Effects of recombinant WNT-5A- on microglia

WNT-5A-induced signaling pathway in microglia

WNT-5A has a huge impact on morphogenesis, neurogenesis and tissue homeostasis (Castelo-Branco et al., 2006; Pukrop and Binder, 2008) and there is evidence that WNT-5A is crucial for macrophage-induced invasion of breast cancer cells (Pukrop et al., 2006). These observations, in combination with the fact that the N13 microglia-like cell line responds to recombinant WNT-5A signaling by activation of heterotrimeric G proteins, PS-DVL formation and proliferation (Kilander et al., 2011a) made us interested to investigate what intracellular pathways are induced by WNT-5A and what physiological outcome they can have on primary microglia. First of all, we searched for an endogenous source of WNT-5A, and using immunohistochemistry, immunoblotting and QPCR, we found that astrocytes express high levels of WNT-5A, and therefore can be a possible paracrine WNT-5A-based communication between astrocytes and microglia. In agreement with earlier findings (Bryja et al., 2007b, 2008), stimulation with 300 ng/ml WNT-5A induced a β -catenin-independent pathway in primary microglia. WNT-5A stimulation did not affect β -catenin stabilization or LRP6 phosphorylation as WNT-3A, but induced a dose- and time-dependent phosphorylation of ERK1/2 and PS-DVL3 formation. The amount of WNT-5A-induced P-ERK1/2 reached a maximum at 30 minutes ($198 \pm 6.2\%$), and returned to basal levels after 2 h. When SFRP1 was used, the effect of WNT-5A on microglia was abolished, confirming the purity of the recombinant WNT-5A batches that have been used throughout this study.

In order to dissect and characterize the WNT-5A-induced signaling route from FZD to ERK1/2 phosphorylation, we employed the same series of pharmacological inhibitors as in the study on WNT-3A, and continued by using several biochemical techniques to measure WNT-5A induced signaling activity. To start with, by immunoblotting we found that PTX, the inhibitor of $G\alpha_{i/o}$ proteins, abrogated the WNT-5A induced P-ERK1/2 but not the PS-DVL3 formation. PTX also blocked WNT-5A-induced P-ERK1/2 immunoreactivity as shown by immunocytochemistry.

The activation of heterotrimeric G proteins was measured by [γ - 35 S]-GTP assay where the WNT-5A-induced GDP/GTP exchange was measured on primary microglia membrane preparations, and showed an activity of $157.7 \pm 4.3\%$. We went on by using RT-PCR to characterize the PTX-sensitive $G\alpha_{i/o}$ proteins expressed in microglia: $G\alpha_{i1}$, $G\alpha_{i3}$, $G\alpha_o$. All these can typically reduce cAMP levels and induce changes in intracellular calcium concentrations ($[Ca^{2+}]_i$) through $G\alpha_{i/o}$ proteins with the release of $\beta\gamma$, and PLC-dependent production of inositoltrisphosphate (Dorsam and Gutkind, 2007). Indeed, WNT-5A dose-dependently reduced the adenylyl cyclase inhibitor (forskolin)-induced cAMP levels, measured in a competitive protein binding assay with [3 H]-labeled-cAMP (Nordstedt and Fredholm, 1990). Further, changes in $[Ca^{2+}]_i$ induced by WNT-5A in a PTX-sensitive manner was measured through live cell imaging of Fluo-3-loaded primary microglia. Based on these findings we hypothesized that WNT-5A-induced P-ERK1/2 involves a classical MAPK cascade consisting of G protein activation, $\beta\gamma$ release, recruitment of PLC, Ca^{2+} and Ca^{2+} -dependent PKC (Dorsam and Gutkind, 2007), and independently of PS-DVL formation. This pathway was confirmed by immunoblotting when we employed all the previously described inhibitors where M119 ($\beta\gamma$ -inhibitor), U73122 (PLC inhibitor), BIS (PKC-inhibitor), BAPTA-AM (Ca^{2+} chelator), and SL327 (MEK1/2 inhibitor) blocked the WNT-5A-induced P-ERK1/2, but not wortmannin or LY294002 (PI3L inhibitor), or D4476 (CK1 inhibitor). In summary, for the first time, we showed that WNT-5A can, besides activating the PS-DVL-dependent pathway, recruits a separate signaling axis consisting of $G\alpha_i$ protein, PLC, PKC, and MEK1/2 to regulate P-ERK1/2, see **Figure 8**.

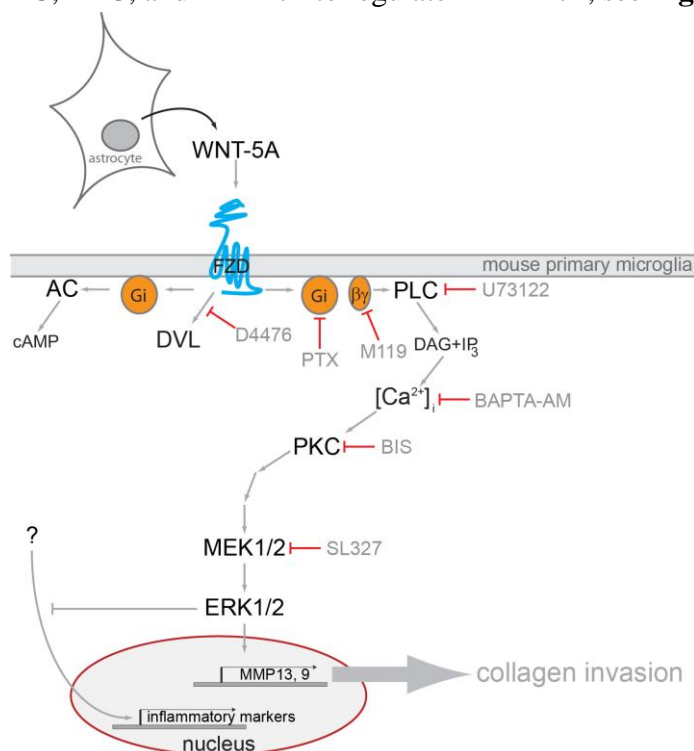


Figure 8: Schematic overview summarizes the WNT-5A-induced WNT/ERK1/2 signaling in microglia. WNT-5A is produced by astrocytes, received by microglia, where it triggers a $G\alpha_i$ -protein, PLC, PKC, and MEK1/2 axis to regulate P-ERK1/2. The pharmacological inhibitors that exerted an inhibitory effect in this study are labeled in light gray. The question mark indicates insecurity in the pathway continuation.

WNT-5A-induced proinflammatory transformation of microglia

The heterogeneity of microglia cells renders them capable of responding to an injury in different ways (Scheffel et al., 2013). An injury *in vivo* would induce the release of activators that would make microglia able to; proliferate, communicate (secreting cytokines), migrate towards the injury (through chemotaxis), or, if necessary, invade (by inducing the expression of metalloproteases) (Choi et al., 2010; Kettenmann et al., 2011). Since WNT-5A-induced P-ERK1/2 and the ERK1/2 cascade is an established proliferative pathway with proinflammatory functions, it is likely that stimulation by WNT-5A induces a proinflammatory transformation of microglia. Indeed, WNT-5A stimulation for 6 h induces expression of iNOS, COX2 and TNF α in microglia, measured by immunoblotting. Release of TNF α was further confirmed by mesoscale measurements on medium after 24 h control versus WNT-5A-stimulation. Further, WNT-5A stimulation led to proliferation and invasion of microglia, in a G protein, ERK1/2-activation sensitive manner. The proliferation was both measured by the MTT assay and confirmed by counting in cell number. By employing 100 ng/ml PTX or 10 μ M SL327 (the MEK1/2 inhibitor) in the MTT assay, we inhibited WNT-5A-induced proliferation and supported our hypothesis that the G α i-PLC-PKC-MEK1/2-ERK1/2 signaling axis is involved in WNT-5A-induced proliferation of microglia. Additionally, in a three-dimensional collagen invasion assay, where microglia cells are seeded on top of a collagen-matrix gel and stained with a cell-tracker dye (measured by confocal microscope Z-stack scanning), 24 h stimulation with WNT-5A induced microglia invasion into the gel, which was completely blocked by SL327. To continue, we characterized the WNT-5A-induced inflammatory fingerprint on microglia by gene expression analysis. RNA was isolated after 6h stimulation with WNT-5A, then transcribed into cDNA and analyzed by QPCR for several proinflammatory microglia markers: All of the following were induced (**Table 3**).

- i) IL-1 β , IL-6, IL-12, and TNF α . These cytokines are known to be secreted by proinflammatory microglia for communication with surrounding microglia, macroglia and neurons, and infiltrating immune cells in neurodegenerative diseases, trauma or infection (Lynch, 2009; Kettenmann et al., 2011).
- ii) CC motif chemokines CCL7 and CCL12. These chemokines are important for recruitment of peripheral infiltrating immune cells, specifically monocytes and leukocytes (Opdenakker et al., 1993; Sarafi et al., 1997). Whereas,
- iii) Cluster of differentiation CD40 and CD69. CD40 and CD69 are expressed to support neuroinflammatory processes by mediating communication with astrocytes, infiltrating lymphocytes and natural killer cells (Marzio et al., 1999; Hanisch, 2002).
- iiii) Matrix metalloproteases MM9 and MMP13. MMPs play a role in extracellular matrix remodeling and contribute to the processing, activation and release of growth factors, cytokines, integrins and additional MMPS (Candelario-Jalil et al., 2009; Choi et al., 2010).

Since the ERK1/2 activation is important for WNT-5A-induced proliferation and invasion, we continued to use the same MEK1/2 inhibitor (SL327) to assess the role of ERK1/2 activation in WNT-5A-induced regulation of gene expression. Our results showed regulation in a bi-directional manner: WNT-5A-induced MMP9 and MMP13 induction was blocked, which can explain the inhibited invasion, whereas WNT-5A-induced TNF α , CCL7, CCL12, COX2 and CD40 were instead amplified (**Table 3**). An explanation for these findings may be that the WNT-5A/ERK1/2 pathway has a bi-directional regulative role in gene expression to integrate inflammatory input, and that a separate PS-DVL formation pathway or other candidate that was not further investigated, might provide crucial crosstalk on the incoming WNT-5A stimulus.

Table 3: Summary of the gene expression analysis by QPCR of microglia stimulated 6 h with 300 ng/ml WNT-5A, with and without 10 μ M of the MEK1/2 inhibitor SL327. The numbers provide the percentage of WNT-5A-induced increase over control or SL327 treatment alone, converted from arbitrary units (fold change of unstimulated control microglia, $2^{-\Delta\Delta C_t}$). The ratio provides a relative measure of decrease (value <1) or increase (>1). Rows in light grey indicate statistically significant increase in WNT-5A-induced gene expression upon SL327, whereas dark grey underlines the efficient block of WNT-5A-induced changes in gene expression by SL327, n=4. *, P<0.05; **, P<0.01

Gene	WNT-5A	WNT-5A/SL327	Ratio
IL1 β	209.8 \pm 78.2	88.1 \pm 16.3	0.4
IL6	947.0 \pm 554.4	541.0 \pm 175.8	0.6
IL12	33622.0 \pm 26855.0	31504.0 \pm 14202.0	0.9
TNF α	43.0 \pm 5.7	366.3 \pm 84.5**	8.5
CCL7	29.1 \pm 5.1	574.7 \pm 348.2*	19.7
CCL12	26.7 \pm 7.0	354.7 \pm 206.3**	12.9
COX2	93.3 \pm 30.4	448.3 \pm 169.9*	4.8
CD40	558.0 \pm 133.0	1989.0 \pm 398.0**	3.6
CD69	456.0 \pm 155.0	1723.0 \pm 606.0	3.8
MMP9	5.1 \pm 1.2	1.0 \pm 0.1*	0.2
MMP13	28.3 \pm 8.0	6.7 \pm 0.9**	0.2

Differences between WNT-3A- and WNT-5A-regulated ERK1/2-signaling in microglia

The thesis data show that both WNT-3A and WNT-5A stimulation of primary microglia induce ERK1/2 phosphorylation, via a similar intracellular signaling route: WNT-5A via a $G\alpha_{i/o}$ protein, $\beta\gamma$, PLC, Ca^{2+} , PKC and MEK1/2 pathway, while WNT-3A via the same pathway but apparently PKC-independent and some as yet unidentified steps are connecting Ca^{2+} with the MAPK. Interestingly, WNT-3A-induced COX2 expression is dependent on activation of ERK1/2 (paper II) while WNT-5A-induced COX2 was instead amplified when the MEK1/2 inhibitor SL327 was used (10 μ M) (paper IV). This outcome might have several explanations: i) different receptor binding and efficacy; ii) WNT-3A and WNT-5A activate parallel intracellular proteins that are important in inflammatory activation of microglia, but which we have not investigated, e.g. phosphorylation and activation of the MAPK p38, and NF- κ B; iii) the low expression of ROR2 and RYK (according to the affimetrix analysis) plays a role in WNT-5A-induced cytokine expression; iiii) differing degrees of activity of the heterogeneous microglia.

WNT/FZD-receptor selectivity

In paper III, we study the efficacy various WNTs on activation of FZDs in N13. Some FZDs are accepted as specific receptors of specific WNTs. For example WNT-5A binds FZD₅ (He et al., 1997; Blumenthal et al., 2006; Kikuchi et al., 2007) and WNT-3A binds FZD₂ (Mikels and Nusse, 2006a). Nonetheless, different WNTs can act on the same FZD (Caricasole et al., 2003; Karner et al., 2009). The QPCR data from paper IV revealed the FZD receptor expression pattern in primary microglia: FZD₇>FZD₃>FZD₈>FZD₅, and in N13 cells FZD₅ is more abundant (Kilander et al., 2011a). Further, in paper III, we show that WNT-5A amplifies WNT-3A-induced PS-DVL formation in N13, and in paper II and IV, that the $G\alpha_{i/o}$ blocker PTX inhibits WNT-3A-induced but not WNT-5A-induced PS-DVL formation in primary microglia. In summary, these data suggest that WNT-3A and -5A binds to and act via different FZDs; however, we still do not know which WNT binds and activates which FZDs.

MAPK p38 and NF- κ B

The p38 MAPK cascade is associated with signaling pathways activated in response to cellular stress and inflammation (Koistinaho and Koistinaho, 2002; Keshet and Seger, 2010). Phosphorylation of the MAPK p38 is involved in the regulation of several cytokines expressed by microglia (Koistinaho and Koistinaho, 2002). For example, p38 activation is increased in spinal cord microglia cells in neuropathic and inflammatory pain (Ji and Suter, 2007; Gong et al., 2009), and since p38 regulates release of PG and NO, it may contribute to the pain and inflammation (Matsui et al., 2010). In one study done on murine macrophages, both WNT-5A and WNT-3A stimulation led to phosphorylation of p38, and WNT-5A seemed to have a bit higher potential for p38 phosphorylation (Ji and Suter, 2007; Neumann et al., 2010). In

microglia, only WNT-5A stimulation (300 ng/ml) for 30 min induces phosphorylation of p38 (*unpublished data*), (**Figure 9**).

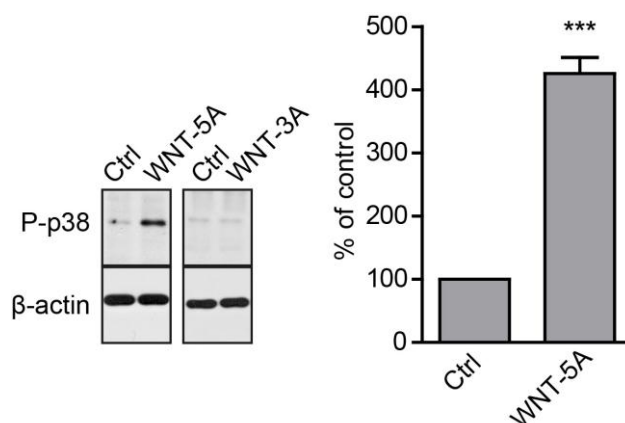


Figure 9: WNT-5A stimulation induces p-p38 in microglia. Stimulation for 30 min with 300 ng/ml recombinant WNT-5A but not 300 ng/ml WNT-3A, induces phosphorylation of the MAPK p38 in microglia (*unpublished data*).

NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that becomes activated in cells in response to different inflammatory stimuli, such as LPS and cytokines. In microglia, NF- κ B controls transcription of many important inflammatory molecules, such as TNF α , IL-6, and IL-1 β (Morales et al., 2010). NF- κ B has been shown to be activated in bone marrow macrophages by both WNT-3A and WNT-5A stimulation – with somewhat different onsets – but only WNT-5A drives the NF- κ B reporter gene expression (Schaale et al., 2011). NF- κ B activation in microglia cells upon WNT stimulation has not been investigated in this thesis.

ROR/RYK

The ROR family of RTKs does not appear to be strongly expressed in human tissues, but abnormal expression of individual receptors has been reported in different kinds of human cancers (Ford et al., 2012). Evidence indicates at WNT-5A as the ligand for ROR2 and that binding leads to antagonism of WNT/ β -catenin-dependent signaling and an activation of the WNT-JNK and calcium pathway (Oishi et al., 2003; Mikels and Nusse, 2006a; Schulte, 2010a). Nothing has so far been published on WNT/ROR signaling in microglia, although there is one study describing WNT/ROR signaling in bone marrow macrophages being involved in the enhancement of osteoclastogenesis (Maeda et al., 2012). RYK is expressed by various cell types in the spinal cord of rats, suggesting a role of RYK in the spinal cord under normal physiological conditions. Further, after spinal cord injury, the RYK expression increased in several glia cells, notable in activated microglia/macrophages, suggesting a biological relevance for RYK activity during spinal cord injury (González et al., 2013).

Heterogeneous microglia

The existence of subtypes of neurons, certain glia cells and immune cells is well established. Recent evidence suggests that microglia may not be a single, uniform cell type (Olah et al., 2011; Scheffel et al., 2013). The multitude of tasks microglia manage to perform upon activation, such as proliferation and executive functions, may be explained by subgroups of microglia performing different tasks (Hanisch, 2013; Scheffel et al., 2013). Further, microglia are heterogeneous and can as a group of cells co-exist in different active states (Lynch, 2009). The diverse WNT-induced genes in microglia might reflect different activation states and also performance of task splitting. The heterogeneity of microglia is clearly visible in mouse primary microglia when live cell imaging assay performed, such as the Fluo-3-loaded microglia for $[Ca^{2+}]_i$ imaging, where only a minority of cells is responsive to WNT-5A stimulation (Figure 10). Thus, WNT-3A and WNT-5A seem to induce different activation states of microglia, and probably influence differently depending on microglia's state of activation.

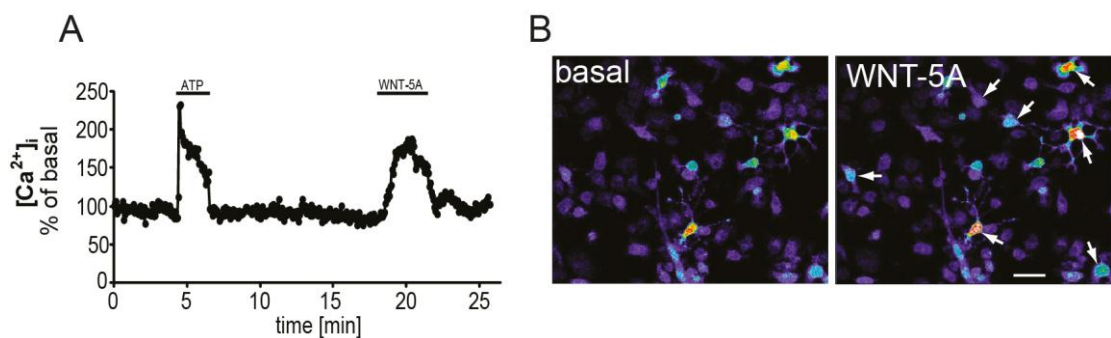


Figure 10: WNT-5A-induced mobilization of $[Ca^{2+}]_i$ shows the heterogeneity of microglia. Stimulation of Fluo-3-loaded primary microglia with WNT-5A induced fast and transient elevation of $[Ca^{2+}]_i$. ATP was used as a positive control. The $[Ca^{2+}]_i$ trace shown in A originates from a single cell. B: Shows a representative view of Fluo-3-loaded cells at baseline and upon WNT-5A (300 ng/ml) exposure. The images are pseudocolored with warm colors representing high $[Ca^{2+}]_i$ and cold colors low $[Ca^{2+}]_i$. Size bar 20 μ M. Typically 15-30% of the cultured microglia responded to WNT-5A, with mobilization of $[Ca^{2+}]_i$, which can be explained by their heterogeneity, i.e. either they exist in different stages of activity and/or they perform task splitting.

WNT signaling counteracts LPS-induced proinflammation in microglia

Studies on peripheral macrophages indicate that WNTs can counteract or contribute to an ongoing inflammation: WNT-3A acting on the FZD₁ receptor exerts anti-inflammatory effects via the WNT/ β -catenin pathway to reduce mycobacterium-induced TNF α (Neumann et al., 2010) and WNT-5A plays a critical proinflammatory role in patients with severe sepsis, by becoming upregulated upon LPS- and interferon γ treatment (Blumenthal et al., 2006; Pereira et al., 2008). Since microglia are considered as the macrophages of CNS (van Rossum and Hanisch, 2004) and share

many similarities with peripheral macrophages (Guillemin and Brew, 2005; Saijo and Glass, 2011), we wanted to investigate the WNT's potential to attenuate or impair already activated microglia, by combining stimulation with WNT-3A or WNT-5A the bacterial cell wall component LPS. LPS is well known to induce proinflammatory activation of microglia via Toll-like receptor 4 (TLR4) and induction of the MAPKs ERK and p38 (Prinz et al., 1999; Lehnardt, 2010).

In paper V, we show irrespective of the induced signaling pathway and the different proinflammatory effects that WNT-3A and WNT-5A exert on microglia, both WNT-3A and WNT-5A act anti-inflammatory by counteracting LPS-induced COX2, IL-6 and TNF α . Our earlier findings were confirmed by immunoblotting of lysates of whole microglia cells stimulated with WNT-3A or WNT-5A for 6 h (0, 30, 100, 300, 1000 ng/ml). This treatment dose-dependently induced expression of COX2, a generic marker of proinflammatory transformation (Mitchell et al., 1995). The effect was statistically significant at doses of 300 ng/ml and 1000 ng/ml. Strikingly, a co-stimulation with 100 ng/ml LPS together with increasing doses of WNT-3A or WNT-5A, dose-dependently reduced the LPS-induced COX2 expression after 6 h stimulation. WNT-3A reduced LPS-induced COX2 expression to 66% (300 ng/ml) and 82% (1000 ng/ml), and WNT-5A decreased the LPS-response to 62% (300 ng/ml) and 67% (1000 ng/ml). This result was confirmed at the gene levels by QPCR on mRNA, where the combination of 100 ng/ml LPS with 300 ng/ml WNT-3A or WNT-5A for 6 h significantly reduced LPS-induced COX2 (WNT-3A to 25%, WNT-5A to 36%). In addition, WNT-3A and WNT-5A significantly diminished LPS-induced mRNA expression of two other important proinflammatory markers (IL-6 and TNF α), WNT-3A to 35% and 35%, respectively, and WNT-5A to 46% and 57%, respectively.

The dual effect WNTs exert on microglia, both the pro- and anti-inflammatory properties, mirrors the dual role microglia have in health and diseases, providing both supportive and inflammatory cues depending on the physiological context (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Zhang et al., 2011). In this way, WNTs resemble cytokines such as TNF α , acting both anti- and pro-inflammatory on surveying microglia, i.e. simultaneously being anti-inflammatory on pre-activated microglia and maintaining tissue homeostasis (**Figure 11**) (Lawson et al., 1990). The findings in paper I, where β -catenin is dramatically increased in amoeboid microglia in *postmortem* brains of patients with AD, in the AD mouse model (APdE9) and in elderly mice, and the fact that WNT-3A was the only factor that induced β -catenin in microglia and induced a substantial proinflammatory fingerprint and transformation, suggests that WNT promotes proinflammatory effects on microglia. However, based on these new findings, that WNTs' are capable of attenuate a LPS-induced proinflammation, instead suggests that WNTs could be there to attenuate the ongoing inflammation through microglia. Although, we do not know which WNT-3A-induced signaling pathway that are responsible for the anti-inflammatory effect, and despite the ability of both WNT-3A and WNT-5A to induce the WNT/ β -catenin pathway and their opposing signaling profiles (Topol et al., 2003; Nemeth et al., 2007), both WNT-3A and WNT-5A reduced LPS-induced COX2, IL-6 and TNF α in a similar manner. These data suggest that the WNTs' anti-inflammatory effect on microglia is mediated via common signaling pathways, such as heterotrimeric G α protein-dependent ERK1/2

activation, to crosstalk between LPS receptor TLR4 and WNT signaling at receptor level, on intermediate steps or at transcription level. The commonly induced pathway leading to the ERK1/2 phosphorylation and activation provides mechanistic crosstalk downstream of G proteins. The TLR4 membrane expression, functionality and signaling has shown to be reduced upon costimulation with diverse pharmacological compounds (Lin et al., 2007; Park et al., 2011; Jung et al., 2013). Further, the LPS-induced gene expression inhibited by WNTs, suggests that the WNT regulation is at transcriptional level. Thus, more detailed studies are required to elucidate underlying mechanisms.

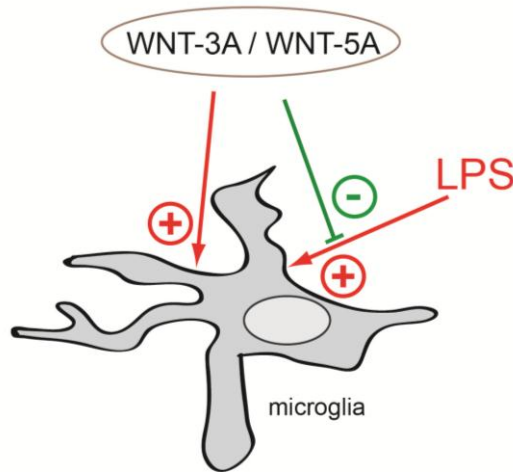


Figure 11: WNTs act as homeostatic regulators. Both WNT-3A and WNT-5A induce proinflammation in primary microglia. However, in presence of LPS, both WNTs counteract LPS-induced COX, IL-6 and TNF α .

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Chronic neuroinflammatory diseases are prolonged and devastating conditions with poor prognosis. In the last few decades, the CNS' own macrophages – microglia – have emerged as an essential cellular component involved in brain homeostasis and disease development. That inflammation persists is mainly because of activated immune cells over-producing proinflammatory mediators, such as microglia secreting proinflammatory cytokines and chemokines. Thus, understanding and eventually being able to treat the underlying causes of the deregulation of cytokines and other factors involved in neuroinflammatory diseases, requires increased knowledge through examination of specific signaling pathways responsible for regulating microglia's inflammatory activity.

The pathophysiological significance of WNT/ β -catenin-dependent and -independent signaling in neuronal differentiation, growth and dysfunction is broadly accepted (Malaterre et al., 2007; Inestrosa and Arenas, 2010; Salinas, 2012; Marchetti and Pluchino, 2013). In fact, several studies have shown numerous WNT components to be altered in neurodegenerative diseases, and drugs capable of modulating WNT signaling have been discussed as potential tools against diseases associated with neuronal loss (Alvarez et al., 2004; Inestrosa and Arenas, 2010). The ongoing research studying the link between WNT-signaling and a growing number of disease conditions will uncover details that further contribute to our understanding of these complex signaling pathways (Chien et al., 2009). However, to date, many questions about WNT signaling remain unanswered, particularly with regard to its role in human disease.

Microglia reside and act in an environment where members of the WNT family are expressed (Malaterre et al., 2007; Inestrosa and Arenas, 2010). A plethora of factors modulate microglia activity ranging from neurotransmitters, cytokines, chemokines, and cellular debris to pathogens, to name but a few (Kettenmann et al., 2011). Further, given the important role microglia play in CNS homeostasis and neuroinflammation in combination with the important influence WNTs have on adult neurogenesis and neuron maintenance, it is likely that also WNTs interact and influence microglia activity. Indeed, a link between microglia and WNTs has just recently begun to emerge.

This thesis shows that stimulation with recombinant WNT-3A in cultured mouse microglia induces a WNT/ β -catenin-dependent pathway, and release of proinflammatory cytokines. The observation that β -catenin stabilization in microglia is related to the activated amoeboid phenotype in AD, both in human *postmortem* brain slices, in the mouse model with AD-like pathology (APdE9), and in elderly WT mouse suggests that β -catenin stabilization is regulated, and – at one or more levels – participates in microglia-dependent inflammatory processes. In addition, in a multiple screening assay with compounds shown to affect microglia or β -catenin levels in other cell types, only WNT-3A and the GSK3 β inhibitor LiCl induced β -catenin stabilization in microglia. Taken together, these data suggest that WNTs are responsible for the induced β -catenin stabilization in microglia. Further, stimulation with recombinant

WNT-3A induced a wide range of proinflammatory mediators in cultured microglia, which suggests that WNTs are involved in mediating the proinflammatory transformation of microglia during neuroinflammation. However, with regard to WNT-3A's attenuation of LPS-induced proinflammation in microglia, is it a bit unclear if WNTs enhance the inflammatory process or participate in the attenuation of ongoing neuroinflammation. To clarify the role of WNTs and the downstream signaling branches involved in the regulation of microglia more research will be required.

"The GSK3 hypothesis of AD" brings up the diverse roles GSK3 plays in different aspects of promoting the disease. Due to the relation between overactive GSK3 and the formation of toxic A β ₄₂ and neurofibrillary tangles, as well as the inflammation progression, the pharmaceutical industries are evaluating GSK3 inhibitors as possible treatment for AD (Joje et al., 2007; Hooper et al., 2008; Palmer, 2011). The potential usefulness of this approach is supported by several studies where restoring the WNT/ β -catenin signaling via GSK3 inhibition seems to have neuroprotective potential, diminishing A β neurotoxicity and reducing tau hyperphosphorylation (De Ferrari et al., 2003; Alvarez et al., 2004; Chacón et al., 2008; Toledo et al., 2008). Inhibition of GSK3 with LiCl has improved memory performance in a mouse model of AD, by alleviating the underlying neuronal deficits (Toledo and Inestrosa, 2009). Further, GSK3 β has been shown to become insoluble early in the disease, suggesting that WNT pathway activation may be an initial step in the neurodegenerative process (Wiedau-Pazos *et al.*, 2009). However, these studies explicitly looked at neurons, disregarding the presence of non-neuronal cells. The given increase of β -catenin in the proinflammatory phenotype of microglia (or invading macrophages), and treatment with GSK3 inhibitors might increase β -catenin stabilization in microglia and thereby exacerbate the inflammatory response.

Research on WNT signaling is a fast growing field initiated by the discovery of the WNT/ β -catenin pathway and continuing with the discovery of more and more β -catenin-independent pathways (He, 2003; Nichols et al., 2013; Schulte, 2010a). For example, stimulation with recombinant WNT-5A induced formation of PS-DVL, and did not affect LRP6 phosphorylation or β -catenin levels. Instead, stimulation of microglia with recombinant WNT-5A induced a classical G protein-dependent axis: WNT-5A-induced GDP/GTP exchange at PTX-sensitive G proteins led to attenuation of forskolin-induced cAMP levels, and increases in Ca²⁺ influxes, and phosphorylation of the MAPKs ERK1/2. The central role ERK1/2 activation plays in microglia proinflammatory activity was also confirmed here, where the WNT-5A-induced invasion and proliferation were regulated in an ERK1/2-dependent manner. Interestingly, WNT-5A induced expression of several proinflammatory markers of microglia with bidirectional sensitivity to the MEK1/2 inhibitor, i.e. some markers increased in expression in presence of the MEK1/2 inhibitor. Hypothetically, this result can be explained by WNT-5A-induced gene expression being downstream of other signaling components, that have not been identified here, and that ERK1/2 activation provides a negative crosstalk with those other proinflammatory mediators limiting the inflammatory transformation. A proposed crosstalk could take place at different levels, such as the receptor level, intermediate signaling components or at the transcription level. To map the underlying mechanisms would require further investigations.

A major finding that provides the WNT field with new insight is that WNT-3A, a classical WNT/ β -catenin pathway inducer, is capable of inducing ERK1/2 activation in parallel with β -catenin stabilization, to mediate a distinct, physiologically relevant response in microglia. In addition, we show that WNT-3A-induced LRP6 phosphorylation, PS-DVL3 formation, and β -catenin stabilization are sensitive to PTX treatment, indicating that the WNT-3A-induced β -catenin-dependent pathway in microglia requires activation of heterotrimeric $G_{\alpha_{i/o}}$ proteins. Thus, the crosstalk between the β -catenin-dependent and -independent pathways induced by WNT-3A lies upstream of the FZD/LRP6 receptor complex, i.e. at the level of PTX-sensitive heterotrimeric $G_{\alpha_{i/o}}$ proteins.

Microglia are present in large numbers within the CNS; however, these are multifaceted cells, with uneven distribution density, and variable morphology and activity state (Lawson et al., 1990; Lynch, 2009; Olah et al., 2011). In addition, recent discoveries indicate that microglia can perform task splitting, i.e. they can exist not only in surveying ramified or active amoeboid form, but also in several intermediate states, which explains how stimulated cultured microglia manage proliferative expansion and executive functions at the same time (Lynch, 2009; Scheffell et al., 2013). This statement could be visualized in the live-cell imaging Ca^{2+} experiment, where not all the microglia responded to the WNT-5A treatment by mobilization of Ca^{2+} . WNT-5A stimulation regulates gene expression, proliferation, and invasion of microglia, i.e. multiple tasks; it is likely that these tasks are split. Further, microglia can co-exist in different activation states, thus some microglia in the culture might already exist in an activated state, and thus respond to the WNT-5A stimuli by reduction of the inflammation, which might involve signaling pathways independent of Ca^{2+} .

Cytokines, such as $TNF\alpha$, are known to act in a dual manner, being both cytotoxic/pro-inflammatory on surveying microglia as well as protective/anti-inflammatory on pre-activated microglia to maintain and promote tissue homeostasis (Sriram and O'Callaghan, 2007). Interestingly, the last study in this thesis suggests that WNTs act on microglia to accomplish homeostatic functions and thus protect tissue in the CNS. The data shows that both WNT-3A and WNT-5A, in a similar manner, attenuate LPS-induced expression of the proinflammatory mediators COX2, $TNF\alpha$ and IL-6. These bidirectional effects of WNTs as both pro- and anti-inflammatory regulators mirror the dual role of microglia in health and disease, providing both supportive and inflammatory cues depending on the physiological context (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011).

In summary, these data laid the foundation for an understanding of WNT signaling in neuroinflammation where microglia are involved. In addition, microglia have served as a model for mechanistic studies of molecular aspects of WNT signaling in non-transfected, genuine cells. Such models will undoubtedly be of value in the future, as additional studies under *in vivo* conditions will be required to clarify the mechanisms and intracellular crosstalk involved in WNT signaling. Nevertheless, this thesis shows that WNTs modify microglia activation – pro- and anti-inflammatory – to accomplish homeostatic functions that could serve of importance in the CNS.

CONCLUSIONS

1. Primary microglia cells isolated from C57Bl/6 mice and the microglia-like cell line N13 express several WNT receptors, namely FZDs and the co-receptors LRP5/6. Microglia respond to recombinant WNT-3A and WNT-5A stimulation by inducing β -catenin-dependent and -independent pathways.
2. In primary microglia, WNT-3A stimulation leads to activation of the β -catenin-dependent pathway and, in parallel, phosphorylation of the mitogen activated protein kinase ERK1/2, where both pathways are dependent on $G\alpha_{i/o}$ -protein activation.
3. WNT-3A regulates ERK1/2 phosphorylation through a $G\alpha_{i/o}$, $\beta\gamma$, PLC, Ca^{2+} , and MEK1/2-dependent mechanism, which also is involved in WNT-3A-induced expression of the pro-inflammatory protein COX2.
4. WNT-3A stimulation modulates microglia activity with a strong proinflammatory response by inducing microglia's expression of several proinflammatory cytokines, chemokines and innate immune response factors.
5. β -catenin is increased in activated microglia cells in human AD, in the AD-mouse model APdE9, and elderly WT mice.
6. All the commercially available recombinant WNTs induce PS-DVL formation in N13 and activation of heterotrimeric G proteins, with different efficacy. The WNTs with higher efficacy for G protein activation also induce microglia proliferation.
7. WNT-5A does not inhibit WNT-3A-mediated β -catenin signaling in N13, but rather increases the PS-DVL formation, which most likely has to do with N13s FZD receptor repertoire, and WNT/FZD-binding combinations.
8. WNT-5A is highly expressed by astrocytes in the adult mouse brain, suggesting a paracrine WNT-5A-based communication from astrocytes to microglia.
9. Stimulation of microglia with recombinant WNT-5A leads to an activation of β -catenin-independent signaling resulting in phosphorylation of ERK1/2 through the activation of $G\alpha_{i/o}$ protein, $\beta\gamma$, PLC, PKC, Ca^{2+} , and MEK1/2 axis.
10. WNT-5A stimulation induces a proinflammatory transformation of microglia, where the WNT-5A-induced ERK1/2 phosphorylation is responsible for expression of matrix metalloproteases 9 and 13, invasion and proliferation.

11. Both WNT-3A and WNT-5A counteracts LPS-induced expression of COX2, IL-6 and TNF α , which suggests that WNTs have homeostatic functions on microglia, i.e. acting both in a pro- and anti-inflammatory manner dependent on the microenvironment.
12. In conclusion, WNTs are expressed in the adult brain and have impact on microglia's inflammatory activity; this suggests that WNTs may play important roles as modulators of microglia activity in neuroinflammation and tissue homeostasis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärnan består av flera olika typer av celler. Den största delen av dessa celler är så kallade gliaceller. Gliaceller trodde man förr i tiden bara fungerade som stödjeceller för nervcellerna, medan man i dagsläget vet man att de kan göra mycket mer än så. 20% av alla gliaceller är nervsystemets egna immunförsvarsceller och kallas för mikroglia. Mikroglia är små celler med massor av små utskott som ständigt är i rörelse för att känna av eventuella förändringar i hjärnan, så som tecken på infektion eller inflammation. Om mikroglia får en signal om infektion eller skada kan de ändra utseende, föröka sig, förflytta sig, äta upp cellrester och bakterier och kommunicera med omkringliggande celler genom att skicka ut signalerande molekyler. Hos människor med neurodegenerativa sjukdomar såsom Alzheimers eller Multipel skleros, pågår en ständig inflammation i hjärnan, som i stor del beror på aktiverade mikroglia. Mikroglia ska hjälpa till att skydda friska nervceller och städa bort redan döda, dock har det visat sig att mikroglia kan bli överaktiva vilket leder till att de attackerar friska nervceller och på så sätt förvärrar inflammationen och vidare förvärrar sjukdomen.

Cellerna i kroppen kommunicerar med varandra genom att skicka ut olika typer av signalmolekyler som kan binda till olika proteinstrukturer på cellytan, så kallade receptorer. När en specifik molekyl binder till sin receptor, ändrar receptorn konformation, som i sin tur leder vidare till en kaskad av händelser inuti cellen som leder vidare till en fysiologisk cellförändring, t ex förflyttning och utsöndring av andra molekyler. Ett exempel på sådana molekyler är WNTs och receptorerna för dessa kallas Frizzled. I tidigare studier har man visat att WNTs i hjärnan har en viktig funktion i nervcellernas utveckling, förbindelser och regeneration, men inget i hur WNTs påverkar mikroglia.

Avhandlingen sammanfattar hur en mikroglia-lik cellinje (N13) och mikroglia cellkulturer isolerade från möss reagerar på stimulering av WNTs. Med hjälp av olika biokemiska metoder visar avhandlingen vilka Frizzled och andra WNT-receptorer som mikroglia uttrycker, vilka typer av intracellulära händelser som WNTs inducerar och några av mikroglias olika fysiologiska förändringar i svar på WNT-stimuleringen. Det visar sig att WNTs kan få mikroglia att bli proinflammatoriska genom att få dem att föröka sig och uttrycka flera proinflammatoriska signalmolekyler. Intressant nog så kan WNT stimulering även reducera en redan bakterie-inducerad inflammation i mikroglia, vilket tyder på att WNTs även har en antiinflammatorisk egenskap. Avhandlingen visar också att ett specifikt intracellulärt protein för WNT/Frizzled signaleringen; β -catenin, har ett förhöjt uttryck i aktiverade amöbaliknande mikroglia hos patienter med Alzheimers. Denna förhöjda β -catenin nivå visas även i mikroglia hos en Alzheimers musmodell och hos äldre möss. Detta indikerar på att WNTs är involverat i mikroglias inflammatoriska aktivitet i hjärnan och kanske är ett möjligt mål för framtida farmaceutiska behandlingar av neurodegenerativa sjukdomar.

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