Analysis of the relation between inflammation and conserved signalling pathways in the CNS using the example of *Wnt* signalling in experimental autoimmune encephalomyelitis

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Tag der mündlichen Prüfung:_____

“Science is like a blabbermouth who ruins a movie by telling you how it ends.”

Ned Flanders

Abstract

Disease progression of multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), as one of its animal models, is characterized by demyelination and neuronal damage in white and gray matter structures, including the hippocampus. It is thought that neurological dysfunction in the hippocampus, a primary locus of learning and memory consolidation, may contribute to cognitive impairment in MS patients. Previous data documented an increased generation of hippocampal neuronal progenitors and a dysregulation of *Wnt* target genes in acute stages of EAE, whereas the microenvironmental signals triggering this processes remained unknown. In the present study the molecular mechanisms underlying the activation of the hippocampal neurogenic niche upon autoimmune-mediated neuroinflammation were elucidated by utilizing the *Wnt* signalling reporter mouse Axin2$^{lacZ/+}$. Histological and enzymatic examinations in Axin2$^{lacZ/+}$ mice during the disease course of EAE led to a detailed description of hippocampal *Wnt*/β-catenin activity, the key signaling pathway of adult neurogenesis. *Wnt* signalling was transiently upregulated in the acute stage of disease, along with parallel induction of canonical and non-canonical *Wnt* ligands. This enhancement coincided with hippocampal neuronal damage and local expression of immune cytokines such as TGFβ, TNFα, IL-1β and IFNγ, linking the inflammatory milieu with the activation of the *Wnt* pathway. In line, transient exposure to pro-inflammatory cytokine TNFα and anti-inflammatory cytokine TGFβ triggered *Wnt* signalling in hippocampal organotypic slice cultures, here used as a model to mimic the impact of neuroinflammation on hippocampal processes. Importantly, *Wnt* activity was although reduced in the hippocampus of a CNS-specific TGFβ-receptor 2 knock-out mouse strain, further connecting TGFβ and *Wnt* signalling in hippocampal function. Moreover, inflammation-mediated activation of the *Wnt*/β-catenin pathway was associated with increased neurogenesis *ex vivo* in hippocampal slice cultures and *in vivo* in two EAE models, indicating potential role of *Wnt* signalling in hippocampal tissue regeneration and repair.

In summary, this study raises the possibility indicates that enhancement of *Wnt* signalling is transiently enhanced can support the neurogenic processes to cope with neuronal deficit upon autoimmune-mediated neuroinflammation, possibly contributing to regenerative processes to restore hippocampal functions.

Zusammenfassung

Der Krankheitsverlauf der Multiplen Sklerose (MS) und der experimentellen autoimmunen Enzephalomyelitis (EAE), einem anerkannten MS-Tiermodell, ist charakterisiert durch Entmarkung und neuronale Schäden in der weißen und grauen Substanz, einschließlich des Hippocampus. Die Beteiligung des Hippocampus könnte zudem verantwortlich sein für die bei MS-Patienten bekannten kognitiven Störungen, die mit eingeschränkter Aufmerksamkeit und Gedächtnisleistung einhergehen. Daten aus früheren Studien konnten bereits eine erhöhte hippocampale Neurogenese und eine Dysregulation von *Wnt*-abhängigen Genen in den akuten Stadien einer EAE nachweisen, wobei die hierfür verantwortlichen Faktoren, bislang unbekannt blieben. Angesichts von Hinweisen auf die Beteiligung des *Wnt* Signalweges erfolgte in der vorliegenden Arbeit die Aufklärung der *Wnt*-abhängigen molekularen Mechanismen, die zur Aktivierung der hippocampalen neurogenen Nische während autoimmun-vermittelter Neuroinflammation führen. Als Ausgangspunkt diente die Verwendung des Axin2$^{lacZ/+}$-Stammes, der als Reporter-Maus des *Wnt*-Signalweges eingesetzt wird. Die histologischen und enzymatischen Untersuchungen in der Axin2$^{lacZ/+}$-Maus während des Krankheitsverlaufes einer EAE ermöglichten die detaillierte Beschreibung der Aktivität des hippocampalen *Wnt*/β-catenin-Signalweges. Die *Wnt*-Signalweg-Aktivität zeigte eine transiente Hochregulierung in den akuten Stadien der Krankheit mit einer parallelen Induktion von kanonischen und nicht-kanonischen *Wnt*-Liganden, Aktivatoren dieses Signalweges. Gleichzeitig mit dieser Hochregulierung detektierte man hippocampale neuronale Schäden und eine lokale, erhöhte Expression von Zytokinen, die vor allem im Immunsystem exprimiert werden, wie zum Beispiel TGFβ, TNFα, IL-1β und IFNγ. Diese Ergebnisse setzen das entzündliche Gewebe-Milieu mit der Aktivierung des *Wnt*-Signalweges in einen Zusammenhang. Diese Annahme unterstützend, aktivierte die transiente Behandlung mit dem pro-inflammatorischen Zytokin TNFα und dem anti-inflammatorischen Zytokin TGFβ den *Wnt*-Signalweg in hippocampalen organotypischen Schnittkulturen, die als Model zur Untersuchung des Effektes von Neuroinflammation auf hippocampale Prozesse eingesetzt wuren. Überdies war die *Wnt*-Aktivität im Hippocampus von Mäusen mit einer ZNS-spezifischen Deletion des TGFβR2-Genes signifikant herunterreguliert, was eine weitere Verbindung zwischen den *Wnt*- und TGFβ-Signalwegen im Zusammenhang mit hippocampalen Funktionen aufzeigt. Außerdem war die durch Entzündung erhöhte *Wnt*/β-catenin-Aktivität verbunden mit einem Anstieg der Neurogenese, wie sich durch *ex vivo* in hippocampalen organotypischen Schnittkulturen und *in vivo* in zwei unterschiedlichen EAE Modellen detektieren ließ. Zusammenfassend zeigen diese Resultate, dass der *Wnt*-Signalweg im Rahmen der autoimmunen Neuroinflammation kompensatorisch aktiviert wird und hierdurch möglicherweise zur Regeneration des entzündlich geschädigten Hippocampus beiträgt.

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1. Introduction

1.1 Multiple Sclerosis (MS)

1.1.1 General aspects of MS

Multiple sclerosis (MS) is a chronic demyelinating disease of central nervous system (CNS) with both, inflammatory and degenerative aspects (Trapp, Nave 2008; Aktas et al. 2007b; Sospedra, Martin 2005) affecting mainly young people aged between 20 and 40. The disease takes three main forms: relapsing-remitting (RR-MS), in which unpredictable acute immune attacks on the myelin sheet of neuronal structures are interposed with periods of stability; primary-progressive MS (PP-MS), characterized by a gradual but steady progression of disability and secondary-progressive MS (SP-MS), which begins with a relapsing–remitting course, and then becomes steadily progressive (Vukusic, Confavreux 2007). Relapsing MS strikes twice as many women than men and worldwide, about three millions of MS patients are mainly located in Europe and in countries with Caucasian immigration, such as North America, Australia and Northern Asia (Ebers, Sadovnick 1993; Goris et al. 1999). It is generally known that the pathogenic process of MS is initiated by a break-down of immune tolerance to CNS antigens due to genetic or environmental factors. This leads to activation and proliferation of encephalitogenic-reactive T cells in the peripheral immune system in susceptible individuals (see Fig.I and (Hemmer et al. 2002)). The encephalitogenic-reactive T cells cross the blood-brain-barrier (BBB) and cause white and grey matter destruction in the brain and the spinal cord, which is characterized by myelin damage, local gliosis, and axonal injury, leading to neuronal dysfunction. The latter is also caused by local microglial activation and generation of chemokines and inflammatory cytokines indirectly or directly linked to the immune attack (Fig.I). Subsequent waves of lymphocytic and monocytic cell infiltrations give rise to widespread neuroinflammation to ensue myelin and neuronal damage (Fig.I). The regulatory processes that are able to compensate this neuronal destruction are a specific interest of this work. Up to now, studies relating to MS pathology concentrated on pro-remyelinating therapies, trying to repair the myelin damage by enhancing the differentiation of neural progenitor cells into oligodendrocytes for myelin repair (Kremer et al. 2015; Olsen, Akirav 2015). Only recently, new investigations explored processes like neurogenesis (Chang et al. 2008), a mechanism that has the ability to compensate rather neuronal than myelin loss, in lesions of MS patients.

Diffuse neuronal damage occurs from the very beginning on (Ferguson et al. 1997; Trapp et al. 1998; Kornek et al. 2000) and may comprise motor and sensory deficits as well as cognitive impairment in the majority of cases including deficits in attention, information processing, executive functioning, processing speed, and long-term memory (Chiaravalloti, DeLuca 2008). In line with this, the hippocampus, where mainly cognitive functions like memory and information processing are located, is more and more the focus of novel investigations (Aktas, Hartung 2011; Hulst et al. 2015) (see following 1.1.2).

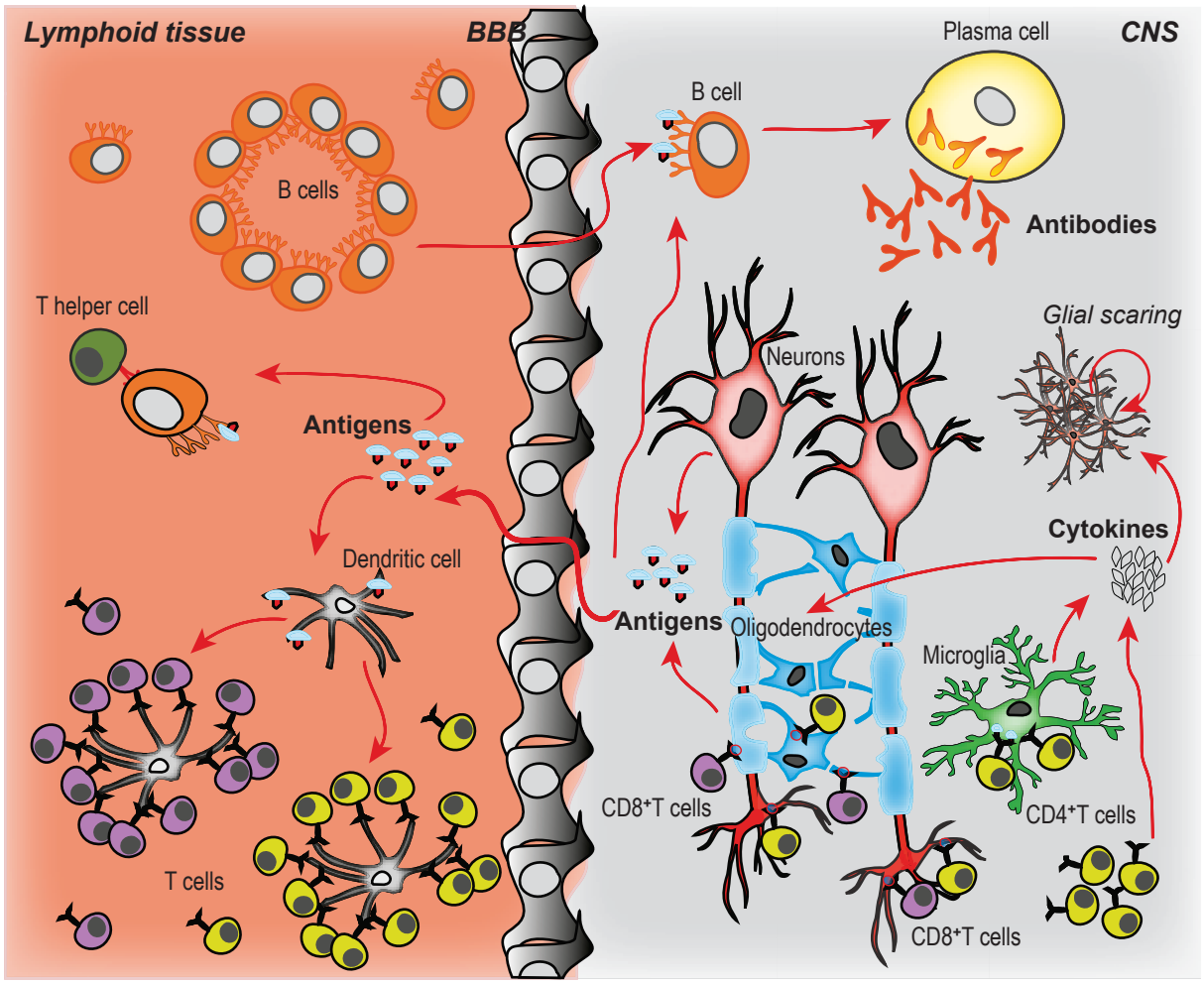

Figure I| **Autoimmune attack mechanisms in multiple sclerosis.**
The schematic was modified from (Hemmer et al. 2002). Glia scaring mechanisms in MS were reviewed in (Fitch et al. 1999; Silver, Miller 2004).

1.1.2 Cognitive dysfunction in MS involving hippocampal function

Recent clinical observations indicate that MS is more than an inflammatory demyelinating CNS disease. Supporting this view, brain magnetic resonance imaging studies in patients with MS have shown structural changes in the hippocampus (Sicotte et al. 2008). This is in agreement with post-mortem studies showing demyelination and neuropathology in the hippocampus of MS patients (Geurts et al. 2007; Roosendaal et al. 2010) with cellular and molecular alterations. These alterations were recently examined in more detail and presented protein alterations in synaptic and axonal structures in post mortem hippocampal tissue from MS patients which were affected by extensive demyelination (Dutta et al. 2011). Further, gene expression analysis in the same post mortem samples revealed reduced levels of neuronal miRNAs and AMPA receptors (Dutta 2013).

As nearly half of all patients diagnosed with multiple sclerosis will develop cognitive dysfunction, a symptom associated with significant decline in activities of daily living (Amato et al. 2006), the general attention has shifted towards the development of neuroprotective strategies to prevent long-term neurological disability. Unfortunately, the pathological mechanisms during hippocampal damage are not fully understood yet which underlines the importance to investigate the related mechanisms and to increase the knowledge about MS pathology.

In experimental autoimmune encephalomyelitis (EAE), an important animal model of MS (see 1.2), many aspects of hippocampal pathology in MS such as demyelination, alteration in synaptic plasticity and transmission (Ziehn et al. 2010; Ziehn et al. 2012; Nisticò et al. 2013), neuronal loss (Diestel et al. 2003; Aktas et al. 2005; Sajad et al. 2009; Vogt et al. 2009; Ziehn et al. 2010) and cognitive deficits (Mandolesi et al. 2010) were also documented. Thus, this model became very important for the investigation of hippocampal neuropathology and MS-related memory deficits.

Previous data identified already adult hippocampal neurogenesis, as a primary target of autoimmune-mediated neuroinflammation (Aharoni 2005; Huehnchen et al. 2011).

The purpose of this work is to further characterize the impact of hippocampal damage on its regular function concentrating on adult neurogenesis as a candidate for neuronal regeneration.

1.1.3 Neuroinflammation in the context of MS

Neuroinflammation in the CNS refers to the response of microglia/macrophges, and to a lesser extent of astrocytes and oligodendrocytes, against diverse insults designed to remove or inactivate the noxious agents and to cope with harmful effects.
In MS, the relationship between systemic inflammation and disease development is detailed described, but little is known regarding the interplay between pro-inflammatory stimuli and the damage to axons and oligodendrocytes (Moreno et al. 2011), the myelin producing cells, in active or chronic plaques. These plaques are defined by their activation status, there are active, chronic active and chronic inactive plaques in MS, indicated by ongoing inflammatory processes and acute neuronal damage (Yu et al. 1999; Ingram et al. 2014). These plaque patterns are often discussed in the context of regeneration in MS. Thus, in plaques a complex matrix of environmental factors act upon oligodendrocyte precursor cells, causing their activation and eventual differentiation into remyelinating oligodendrocytes (Zawadzka, Franklin 2007). These inflammatory mediators, however, have also the capability to block oligodendrocyte precursor cell maturation, keeping cells in a proliferative state (Kuhlmann et al. 2008) and also inhibit axonal outgrowth (Yiu, He 2006). Apart from that, inflammatory cells contribute by providing components of the signalling matrix and by the phagocytic removal of myelin debris. Many factors within the signalling environment have redundant functions, one a feature of regeneration with possible implications for therapeutic solutions.

Investigating these neuro-immune interactions is of great interest, therefore this work tries to clarify the connection between neuroinflammation and neurodegeneration to generate strategies for the repair of structural and functional damage. A focus on specific cytokines, like TNFα (see 1.5) or TGFβ (see 1.6), in context to regeneration and repair mechanisms in the MS disease course could be the first step in understanding the natural damage response.

1.2 Experimental autoimmune encephalomyelitis (EAE)

Experimental autoimmune encephalomyelitis (EAE) is widely used to mimic the human inflammatory demyelinating disease, MS and is one of the most intensively studied and accepted animal model of this autoimmune disease (Gold 2006). The major features in common with MS are: the destruction of myelin sheaths of nerve fibres; the relative sparing of the other elements of the nervous tissue, such as axis cylinders, nerve cells and supporting structures; the presence of multiple CNS lesions distributed in time and position, generally being more pronounced in the brain stem and spinal cord; the predominantly perivascular location of infiltrates; the temporal maturation of lesions from inflammation through demyelination, to gliosis and partial remyelination; and the presence of immunoglobulin in the CNS and cerebrospinal fluid (Adams, Kubik 1952). Since its development, EAE has been conducted in a broad spectrum of species, including mice (Olitsky, Yager 1949), rats (Lipton, Freund 1952), guinea pigs (Freund et al. 1947), hamsters (Tal et al. 1958), rabbits (Morrison 1947), chicken (Ranzenhofer et al. 1958), goats (Innes 1951), sheep (Innes 1951), dogs (Thomas et al. 1950), and marmosets (Genain et al. 1995). Interestingly, the clinical features, including disease progression and histopathology, varied significantly depending on the species used for disease induction. The most common mode of EAE induction, the so called “active EAE”, is based on the injection of an encephalitogenic peptide, myelin oligodendrocyte glycoprotein (MOG_{35-55}) or proteolipid protein ($PLP_{139-151}$), which is emulsified in complete Freund’s adjuvant (CFA) containing mycobacterium tuberculosis strains H37RA, followed by intraperitoneal injections of pertussis toxin to provoke a strong immune system reaction. The resulting clinical manifestation depends mainly on the antigen source and the genetic background of the animal strains used. For example, immunization with $PLP_{139-151}$ induces a relapsing-remitting EAE in SJL/J mice, whereas immunization with MOG_{35-55} leads to a rather chronic-progressive EAE in C57BL/6 mice (Stromnes, Goverman 2006a). Another very common model is the “passive” or adoptive-transfer EAE. It can be induced in recipient animals by transferring pathogenic, myelin-specific CD4 T cells generated in donor animals by active immunization (Stromnes, Goverman 2006b). The latter type of EAE was instrumental in understanding disease pathogenesis (Pettinelli, McFarlin 1981). Adoptive-transfer EAE has enabled researchers to focus on mechanisms associated with the “effector phase” of the disease without having to rely on an induction phase.

Further, it is possible to manipulate encephalitogenic T cells *in vitro* to characterize the role of specific cytokines and other biological agents before the adoptive transfer into recipients.

These cells can also be conveniently labelled to follow their localization, survival and interaction with other cell types in the recipient host. In addition, adoptive transfer of cells made it possible to address the role of a variety of inflammatory molecules in different aspects of disease development and regulation by the use of gene-targeted donor or recipient animal strains (Stromnes, Goverman 2006b). EAE plays a major role in identifying and characterizing aspects of immune surveillance, inflammation and immune-mediated tissue injury, and was the experimental animal model which was used to develop several clinical therapeutics for MS (Steinman, Zamvil 2006) like glatiramer acetate (Teitelbaum et al. 1971), mitoxantrone (Lublin et al. 1987) and natalizumab (Yednock et al. 1992). Notably, even when a treatment shows much potential in the animal model, it can fail to display clinical efficacy in humans (Sriram, Steiner 2005). One reason that EAE requires an external immunization step to develop, whereas in humans, disease develops spontaneously (Trapp, Nave 2008).

This work is using the EAE model as many aspects of hippocampal pathology are similar to the ones seen in MS patients, making EAE a validated system to analyze the possible damage mechanisms.

1.3 *Wnt* pathway

1.3.1 *Wnt* signalling cascade

The *Wnt* signalling pathway is an often discussed pathway emerging from early studies in the simplest multicellular organisms (Ryan et al. 2013). Accumulating evidence suggests that in early metazoa, *Wnt* was an ancestral symmetry-disrupting signal that parted embryos into two halves (anterior versus posterior domain) and by this allowed the evolutionary development of axially patterned organisms (Petersen, Reddien 2009; Holstein et al. 2011). Generally, the ancient role of *Wnt* signalling in the most basic beings was pattern formation and pattern maintenance (Gurley et al. 2008; Petersen, Reddien 2008; Lengfeld et al. 2009). Further, *Wnt* genes and components of *Wnt* signalling pathways have been implicated in a wide spectrum of important biological phenomena, extending from early development to cell behaviour and several diseases.

In general, *Wnt* proteins (*Wnt* is a combination of w*g*: referring to drosophila gene homolog known as *wingless*; and *int*: the Wingless-related integration site) are a family of secreted proteins, which act as ligands to activate distinct *Wnt* signalling pathways (Klaus, Birchmeier 2008; Niehrs 2012). In mammals, there are currently 19 known *Wnt* ligand proteins and 10 different Frizzled-*Wnt* receptors (Niehrs 2012). Since there is no clear ligand-receptor-pathway relationship, mechanisms that discern between activation of distinct pathways are complex (Niehrs 2012; Clevers, Nusse 2012). *Wnt* ligands belong to a conserved family of cysteine-rich glycoproteins, which are fundamental for a variety of biological processes including proliferation (Nusse 2012; Clevers, Nusse 2012), polarity (Habib et al. 2013), mitosis (Moreno-Estellés et al. 2012), apoptosis (Nusse 2012), inflammation (Marchetti, Pluchino 2013), and differentiation (Lange et al. 2006). Until now, little is known about the active conformation of *Wnt* ligands, they might function as monomers, or in homo-, hetero- or oligomeric complexes (Nusse, Varmus 2012). A prominent example of the significance of *Wnt* signalling is the lately documented role of *Wnt* ligands in stem cell homeostasis (Lange et al. 2006; Inestrosa, Arenas 2010). Stem cells fuel tissue development, renewal, and regeneration, and these activities are controlled by the local stem cell microenvironment, their “niche”. *Wnt* signals emanating from the microenvironment of a niche can act as self-renewal factors for stem cells in multiple mammalian tissues. *Wnt* signals, by virtue of their short-range nature, are ideal “niche factors”, controlling immediately adjacent stem cells. The roles of *Wnt* ligands in stem cell self-renewal or lineage specific differentiation in various tissues *in vivo* are manifold, therefore *Wnt* signals are a useful instrument to influence stem cell homeostasis *in vivo* and *in vitro* (Lange et al. 2006; Inestrosa, Arenas 2010).

On the basis of initial studies, *Wnt* has been traditionally classified as an either canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) signalling pathway (Niehrs 2012). This classification, however, only serves as a rough guide, as within canonical and non-canonical *Wnt* signalling various sub-branches are used in different cellular environments (Kikuchi et al. 2011; Clark et al. 2012).

The canonical *Wnt* pathway initiates with the binding of *Wnt* ligands to one member of the Frizzled seven-transmembrane-spanning receptor family. The *Wnt*-Frizzled interaction requires the low-density lipid receptor-related protein 6 (LRP6), which acts as a co-receptor for the *Wnt* ligand. Then, this complex recruits the scaffold protein Disheveled (Dvl). At the intracellular level, the canonical *Wnt* pathway requires the intracellular β-catenin protein, whose levels remain low by the action of a so-called destruction complex formed by several proteins including Axin, adenomatous polyposis coli (APC), casein kinase Iα (CK-1), and the enzyme glycogen synthase kinase 3β (GSK3β). This complex drives β-catenin phosphorylation, stimulating its ubiquitination and proteosomal degradation (Niehrs 2012). Canonical *Wnt* signalling leads to dissociation of the β-catenin destruction complex by a series of phosphorylations, thus inhibiting the activity of GSK3β and resulting in hypophosphorylated β-catenin that accumulates in the cytoplasm and translocates into the nucleus. In the nucleus, nuclear β-catenin binds to the T cell-specific transcription factors (Tcfs) which finally leads to expression of *Wnt* target genes like *Axin2* and *CyclinD1* (Fig.II and Clevers, Nusse 2012).

The non-canonical Wnt signalling can be divided into the planar cell polarity pathway (*Wnt*/PCP) and the *Wnt*/Ca^{2+} pathway. In the *Wnt*/PCP pathway, the *Wnt*-Frizzled-dependent recruitment of Dvl activates small GTPase proteins, such as Rho and Rac, which subsequently trigger the c-Jun N-terminal kinase (JNK). In this case, Rho and Rac are either transported to the nucleus or modify the stability of the cytoskeleton, as they affect the phosphorylation of microtubule-associated proteins (MAPs) and interact with actin-regulator proteins (Rosso et al. 2005).

In the *Wnt*/Ca^{2+} pathway, Dvl signalling downstream stimulates G proteins and the enzyme phospho-lipase C (PLC), which increases the production of inositol triphosphate (IP3), thus triggering an increase in intracellular Ca^{2+}. Consequently, Ca^{2+}-dependent proteins, like protein kinase C (PKC), calcium-calmodulin-dependent protein kinase II (CaMKII) and the phosphatase calcineurin, are activated (Fig.II and Varela-Nallar et al. 2010; Nusse, Varmus 2012; Niehrs 2012). The *Wnt*/Ca^{2+} pathway is known as a mediator in development and also regulates actin polymerisation to stabilize cell cytoskeleton structure (Fig.II and Kühl et al. 2000).

Given the manifold roles of the *Wnt* pathway in numerous cell biological aspects, this study plans to explore novel *Wnt*-dependent neurobiologicalmechanisms in the autoimmune disease MS.

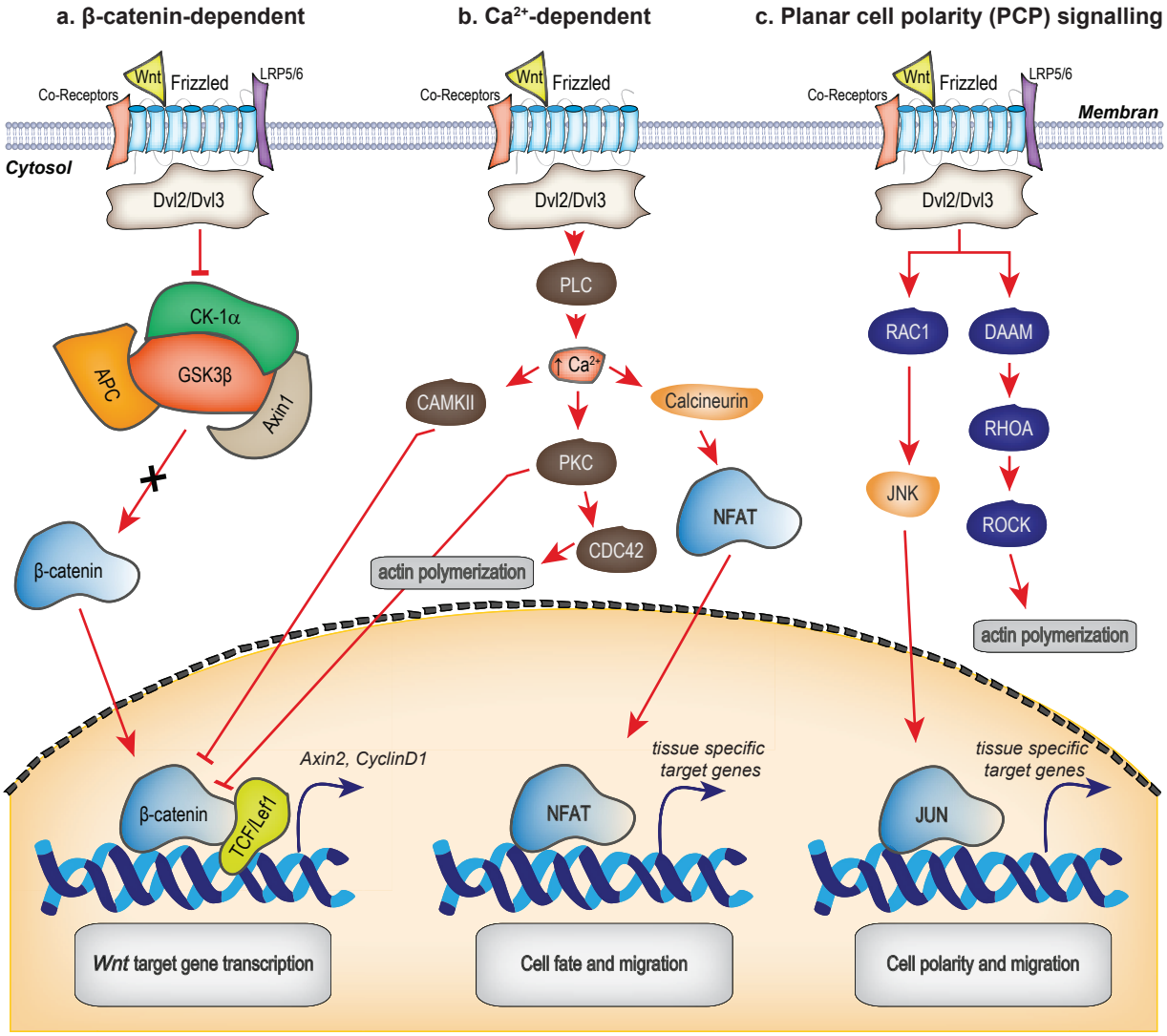

Figure II| **The Wnt signalling cascade. Schematic of the main Wnt pathways directed by specific Wnt, Frizzled and Wnt co-receptor interactions.**

(a) Under steady-state conditions, glycogen synthase kinase 3 (GSK3) phosphorylates β-catenin, which triggers its degradation. However, in the presence of specific Wnt ligands, the destruction complex (comprising GSK3, CKIα, Axin and APC) is recruited to the Wnt-receptor complex and inactivated. This allows β-catenin to accumulate and translocate to the nucleus, where it activates the transcription of target genes under the control of TCF, among others. (b) The Wnt-Ca^{2+} pathway activates Ca^{2+}-and CAMKII, PKC and calcineurin. Calcineurin activates nuclear factor of activated T cells (NFAT), which regulates the transcription of genes controlling cell fate and cell migration. PCP and Ca^{2+} pathways antagonize β-catenin signalling at various levels. (c) PCP signalling triggers activation of the small GTPases RHOA and RAC1, which in turn activate RHO kinase (ROCK) and JNK, respectively, leading to actin polymerization and microtubule stabilization. This pathway is prominently involved in the regulation of cell polarity, cell motility and morphogenetic movements.

1.3.2 *Wnt* signalling in MS

In recent years, involvement of the *Wnt* signalling pathway in the pathogenesis of MS was described in the literature. In MS lesions the expression of multiple *Wnt* signalling genes was described using gene microarrays (*Wnt2*, *Wnt7a*, *β-catenin*, *Tcf4*, and *GSK3β*) (Lock et al. 2002) and proteomic analysis (Wnt3a, APC, and β-catenin) (Han et al. 2008), showing that their expression levels were higher in chronic active plaques than in chronic silent plaques and normal-appearing white matter. Additionally, data collected by a blood RNA profiling study in a large cohort of MS patients provided additional proof for an involvement of the *Wnt* pathway in MS pathogenesis after a global pathway analyses (Nickles et al. 2013). Further, *GSK3β* was examined by an allelic discrimination analysis for four common *GSK3β* gene variants in MS patients, resulting in *GSK3β* being a susceptibility factor for MS (Galimberti et al. 2011). Interestingly, the expression of Axin2, the famous negative feedback regulator of *Wnt* signalling (Lustig et al. 2002), was only detected in active MS lesions, but not in normal white matter or chronic silent plaques, where myelin repair seem to be delayed (Fancy et al. 2011). This data implies *Wnt* signalling as a negative factor in the myelination process. Further, Tcf4, a transcriptional target of canonical *Wnt* signalling, expression was re-expressed in human adult tissue from MS patients, and is specifically expressed in oligodendrocyte progenitor cells (OPCs) in active plaques. This expression pattern suggests a potential role for Tcf4-mediated *Wnt* signalling in the remyelination process (He et al. 2007).

In EAE, a similar involvement of the *Wnt* signalling pathway has been documented and this provides an opportunity to increase the understanding of *Wnt*-related mechanisms in MS. Indeed similar to findings in MS patients (Galimberti et al. 2011), lithium administration ameliorated EAE, depending on the inhibition of GSK3β (Sarno et al. 2008). Additionally, *Wnt3a*, *Wnt5a* and β-catenin were upregulated in the spinal cord of EAE mice, and this increased expression may contribute to the occurrence of chronic pain (Yuan et al. 2012). Interestingly, alone the inhibition of *Wnt5a* by the antagonist Box5 could attenuate this symptoms (Yuan et al. 2012). On the other hand, beneficial effects of *Wnt* signalling in EAE have also been proposed in several studies.

In context to the immune system, the regulatory effect of the *Wnt*/β-catenin signalling in peripheral T cell differentiation makes *Wnt* an important factor in regulating the development of EAE, knowing the essential role of Th1/Th17 cells in its pathogenesis (Mix et al. 2010). In agreement with this, Tcf1, another transcriptional downstream *Wnt* target, has been described to influence T cell differentiation by promoting Th2 but repressing Th1 cell differentiation (Yu et al. 2009). Thus suppressing EAE and in line with this $Tcf1^{-/-}$ T cells induced more severe clinical symptoms, CNS inflammation, and demyelination in a passive EAE model (Ma et al. 2011).

Furthermore, Tcf7$^{-/-}$ EAE mice developed a significantly more severe disease that is associated with an increase in Th17 cell numbers in secondary lymphoid organs (Yu et al. 2011).

Overall, these data anticipate a relevant role for the *Wnt*/β-catenin signalling members in MS pathogenesis, myelination and its animal model EAE. The involvement of *Wnt* signalling, however, in the cognitive decline and hippocampal pathology (see 1.1.2) is not discussed yet, therefore this study will focus on this aspect by applying a *Wnt* reporter mouse strain in the EAE animal model.

1.3.3 *Wnt* signalling reporter mouse systems

There are various "reporter transgenes" that respond to *Wnt* signals in intact animals and therefore, reveal endogenous *Wnt* signalling activity (reviewed in (Barolo 2006)). These reporters are mainly based on the TCF binding site, driving expression of β-galactosidase or GFP. For β-galactosidase two mice have been described, both are under the control of an inducible TCF/β-catenin-dependent promoter, one called *TOPGAL* (generated by DasGupta, Fuchs 1999), an engineered mouse harbouring a *lacZ* gene and the other is called *BAT-gal* (generated by Maretto et al. 2003), expressing the *lacZ* gene together with a nuclear transportation sequence. For GFP also two transgenic mouse lines were created under similar TCF/β-catenin gene control, the *ins-TOPEGFP* that expresses EGFP for visualization (generated by Moriyama et al. 2007) and one construct with a LEF-EGFP fusion protein (generated by Currier et al. 2010). The GFP expression allows for a simpler localisation of cell types where *Wnt* signalling is active by applying histochemistry.
An additional approach to visualize *Wnt* activity was done by involving the *LGR5* gene (also called *GPR49*), another target of the *Wnt* pathway, detected in stem cells of various tissues (Barker et al. 2007). This gene was used to create a *lacZ* reporter mouse (generated by Barker et al. 2007) and provides another *in vivo* read-out of the *Wnt* pathway.
In this study, a *Wnt* reporter mouse was used that depends on the expression of Axin2. Axin2 is under the control of *Wnt* activity (Fig.II) and it may even be a universal *Wnt* target, expressed and induced in many different tissues (Whyte et al. 2012). In general, there are two transgenic mouse lines, based on the *Axin2/Conductin* gene promoter, one using GFP (generated by Jho et al. 2002), another using β-galactosidase (generated by Lustig et al. 2002) as a read-out.
In response to the often-discussed issue of the “optimal” *Wnt* reporter system, there is no easy answer. Interestingly, a study investigating murine lung development and repair compared three *Wnt* reporter mice: *TOPGAL*, *BAT-gal* and *Axin2-lacZ* and described differences in their expression patterns. In lung tissue, there seem to be cells that are positive for the *TOPGAL* reporter and negative for Axin2/Conductin-based reporter (Al Alam et al. 2011). Unfortunately, there is no comparable data for CNS tissue available yet. Over the past several years, many laboratories have used transgenic TCF or Axin2 reporters to analyse *Wnt* or β-catenin-mediated signalling *in vivo*, trying to solve the problem of reliably detecting its activity. The challenging detection is compounded by difficulties in assessing *Wnt* gene expression itself which is best done by *in situ* hybridization as antibodies are rarely good enough. Barolo and colleagues proposed three limitations occurring by the usage of *Wnt* reporters:
(1) In cases where a TCF reporter is activated but a requirement for *Wnt* signalling has not yet been established in that context, additional data with direct and independent supporting evidence should be collected before claiming a novel role for the *Wnt* pathway;

(2) A lack of reporter activity should not be interpreted as substantial evidence that *Wnt* ligands, β-catenin and/or TCFs play no role in a certain context;
(3) Artificial *Wnt* reporters should not be considered a representative for natural *Wnt* target genes (Barolo 2006).
The three cautions display the limitations of reporter systems, but as the tools for analysing *in vivo Wnt* activity are limited, investigations concentrating on the pathway have to rely on reporter animals for gathering data for possible mechanisms.

In case of this study, the *Axin2-lacZ* mouse was chosen as this mouse was already used for damage-related studies in muscle and skin tissue (Whyte et al. 2013), Axin2 is well detectable in CNS tissue (Kalani et al. 2008) and the *lacZ* gene allows for a reliable quantification of the Axin2 signal via an enzymatic assay using β-galactosidase.

1.3.4 *Wnt* signalling in tissue repair

Acute injury triggers the endogenous, β-catenin-dependent *Wnt* pathway leading to an essential and subsequent healing. This activation is typically rapid, transient and spatially restricted to the site of damage. The canonical *Wnt* pathway participates in the healing process from control of inflammation and programmed cell death to the mobilization of stem cells at the edges of the wound site which differentiate to generate new cells. Accordingly, *Wnt* signalling is presented as one of the initial molecular responses to injury, activating two mechanisms: Tissue repair and regeneration (Whyte et al. 2012; Lambert et al. 2015).

In general, *Wnt*-induced tissue repair can be characterized by three main phases: The first, the inflammatory phase is characterized by an increase in local *Wnt* signalling; during the second, the maturation and remodelling phase, where an extracellular matrix is developed to alter the damaged area; and the third, the regeneration phase, is characterized by an increase of *Wnt* signalling leading to reconstruction of the original tissue architecture by reducing the inflammatory response (Di Liddo et al. 2015) and increasing angiogenesis (Dejana 2010) and cellular proliferation (Inestrosa, Arenas 2010). Moreover, accruing evidence demonstrates that *Wnt* signalling blockade disrupts the recruitment of stem cells to the injury site (Denayer et al. 2008; Ramachandran et al. 2011) and adversely affects the proliferative phase of the healing process (Stoick-Cooper et al. 2007; Qyang et al. 2007). In the Xenopus eye, at postembryonic stages, the canonical *Wnt* signalling pathway continued to promote proliferation and maintaining of retinal stem cells and transcriptional repression by a genetically manipulated Xenopus of the pathway inhibited proliferation of these cells (Denayer et al. 2008). Further, genetic and pharmacological inhibition by GSK3β activators of *Wnt* signalling suppressed injury-dependent proliferation of Müller glia-derived progenitor cells and the *Wnt* pathway was essential for successful retina regeneration in the zebrafish (Ramachandran et al. 2011). Likewise, activation of *Wnt*/β-catenin signalling by overexpression of *Wnt8* increases proliferation of progenitor cells during regeneration of the zebrafish tail (Stoick-Cooper et al. 2007). In cardiac mesenchymal cells, it was shown that the *Wnt*/β-catenin pathway is a major component by which these cells modulate the pre-specification, renewal, and differentiation, their mitosis rate was even reduced significantly following β-catenin deletion (Qyang et al. 2007).

In line with this, a number of studies have shown that the exogenous addition of *Wnt* signalling stimulates healing in a number of different injuries, including bone fractures (Chen et al. 2007), retinal damage (Ramachandran et al. 2011), skin wounding (Whyte et al. 2013), and myocardial infarction (Aisagbonhi et al. 2011).

The CNS is one of the most vulnerable systems regarding external damage, as it exhibits limited recover capacity, especially regarding cognitive function reestablishment after injuries.

The *Wnt* signalling pathway is known to be important in the regenerative response after injury in the CNS, activating diverse mechanisms including the stimulation of adult neurogenesis (Lie et al. 2005), synaptogenesis (Inestrosa, Arenas 2010) and the recovery of cognitive brain functions (Inestrosa, Arenas 2010; Varela-Nallar, Inestrosa 2013; Rosso, Inestrosa 2013).
Further, *Wnt* signalling is discussed in various CNS diseases: In an Amyotrophic lateral sclerosis (ALS) transgenic mouse model, recent evidence displayed the upregulation of some *Wnt* ligands in the spinal cord (Pinto et al. 2013) promoting cell proliferation through the regulation of *Wnt* target genes (Chen et al. 2012b). Blockage of GSK3β activity by lithium was followed by a decrease in neuronal death in the hippocampus post-traumatic brain injury (TBI), leading to the improvement of cognitive function (Zhao et al. 2012). In several Alzheimer´s disease (AD) animal models the relationship between *Wnt* signalling and the disease were documented (Inestrosa et al. 2012), showing destabilization of endogenous levels of β-catenin, increased GSK3β activity and decreased *Wnt* target gene transcription in context to AD pathology (Inestrosa et al. 2012). The Parkinson´s disease (PD) is a neurodegenerative disease characterized by the degeneration of the midbrain nigro-striatal dopaminergic system and accumulation of aggregated α-synuclein in the CNS (Beitz 2014). In PD, *Wnt* signalling is of interest, since several aspects of midbrain dopaminergic neuron differentiation are depending on the *Wnt*/β-catenin pathway through the canonical ligands *Wnt1* or *Wnt3a* (Inestrosa, Arenas 2010) and the *Wnt*/PCP pathway through the non-canonical ligand *Wnt5a* (Inestrosa, Arenas 2010). Recent data even indicate that appropriate levels of *Wnt* signalling are necessary to improve the quantity and quality of stem cell- or reprogrammed cell-derived DA neurons which can be used in drug discovery and cell replacement therapy for PD (reviewed in Arenas 2014).

The presented data regarding the critical role of *Wnt* signalling in injury repair, especially in the CNS, suggests that the manipulation of *Wnt* pathway activity represents an interesting target for therapeutic intervention.

1.4 Adult neurogenesis

1.4.1 Adult neurogenesis

In the adult brain, a continuous generation of new neurons, namely adult neurogenesis, has been reported in a number of mammalians including humans (Ming, Song 2011). Adult neurogenesis is considered as an active biological process encompassing the genesis, migration, differentiation, and maintenance of new neurons, which can be functionally integrated into the existing neuronal circuitry (Ming, Song 2011; Kempermann et al. 2015). In the intact adult mammalian CNS, this process occurs mainly in two discrete 'neurogenic' regions: the subventricular zone (SVZ) of the lateral ventricles in the forebrain (producing neurons destined for the olfactory bulb) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (producing for example dentate granule cells) (Gage 2000; Alvarez-Buylla, Garcia-Verdugo 2002; Zhao et al. 2008; Kempermann et al. 2015). The adult SGZ of the DG contains glial fibrillary acidic protein (GFAP)-, Nestin-, and SRY (sex determining region Y)-box 2 (Sox2)-expressing radial glia-like cells (RGLs) representing the quiescent neural stem cell (NSC) pool, known as a type 1 cells. Clonal analysis of individual RGLs has revealed the multipotential capacities of this population that can generate proliferating progenitor cells with the ability to differentiate into neurons, astroyctes and oligodendrocytes (Ming, Song 2011; Kempermann et al. 2015). The so called asymmetric cell division of RGLs is activated by cognitive functions like learning or acute damage and give rise to neuronal lineage restricted progenitor daughter cells (type 2 cells) that expresses Nestin and Sox2, but not GFAP (Seri et al. 2001). Type 2 cells in turn give rise to neuroblasts expressing doublecortin (Dcx) and PSA-NCAM which then differentiate into dentate granule cells (Alvarez-Buylla, Lim 2004). In general, adult neurogenesis in the DG of the hippocampus undergoes five developmental stages described in figure IV.

In the adult SVZ, proliferating RGLs give rise to transient amplifying cells, which in turn generate Dcx and PSA-NCAM-positive neuroblasts. These neuroblasts form a chain and migrate towards the olfactory bulb in the rostral migratory stream through a channel like structure formed by astrocytes (Ming, Song 2011).

Once reaching the core of the olfactory bulb, immature neurons detach from the rostral migratory stream and migrate radially towards glomeruli where they differentiate into different subtypes of interneurons (reviewed by (Lledo et al. 2006)).

SGZ and SVZ neurogenesis differ in many ways, but both proceed via a remarkable process to generate new neurons by activating neural stem cells and direct them through all necessary stages for the production (Ming, Song 2011; Kempermann et al. 2015).

Accumulating evidence suggests that these new neurons are essential for brain functions, such as learning, memory and olfaction (Deng et al. 2010). Since the discovery of neurogenic niches in the adult brain, many groups have investigated the molecular mechanisms that regulate this process. Several cell intrinsic and cell extrinsic factors that regulate this balance have been identified, including *Wnt* signalling (Ming, Song 2011).

They are thought to originate from multipotent adult neural stem cells, but their exact identity is still subject to debate and their multipotency at the clonal level *in vivo* has not been universally demonstrated.

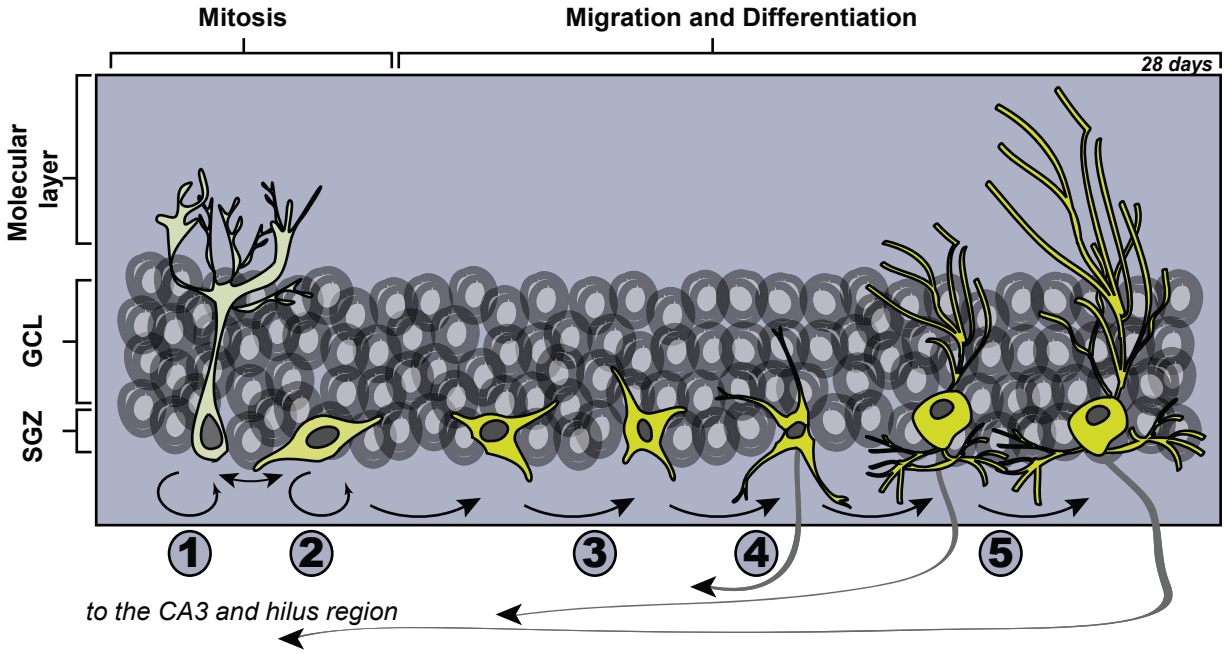

Figure III| **Adult neurogenesis in the dentate gyrus in five stages.**
In the first stage stem cells proliferate with their cell bodies still located within the SGZ and their radial processes are projected through the granular cell layer. In the second stage the differentiation starts as some progenitor cells differentiate into immature neurons and proliferating progenitors form with astrocytes a neurogenic niche. In the next stage immature neurons migrate over a short distance into the granule cell layer to start the integration in existing networks. The fourth stage illustrates the axon or dendrite ”targeting” where immature neurons widen their axonal projections to the CA3 pyramidal cell layer and send their dendrites in the opposite direction toward the molecular layer to establish the connections to the adult neuronal network. In the last stage synaptic integration completes the entire process. New granule neurons receive inputs from the cortex and send their processes to the CA3 and hilus regions.

1.4.2 Adult neurogenesis and *Wnt* signalling

Neurogenesis in adults is regulated by a number of intrinsic as well as extrinsic factors (Kempermann et al. 2015) and controlled by many signalling pathways including Notch, Shh, BMPs, and *Wnt* (Suh et al. 2009; Ming, Song 2011; Kempermann et al. 2015). Endogenous extrinsic factors in the local microenvironment include cell-to-cell interactions, secreted factors, neurotransmitters and oxygen availability (Alvarez-Buylla, Lim 2004; Prozorovski et al. 2015). Microenvironments of the SVZ and SGZ are thought to have specific factors that are permissive for the differentiation and integration of new neurons (Ming, Song 2011; Kempermann et al. 2015), but after injury also other brain areas are capable of neurogenesis (see 1.4.3).

Several studies have shown that canonical *Wnt*/β-catenin signalling can be one of the factors in the microenvironment which regulate adult neurogenesis (Lie et al. 2005; Varela-Nallar, Inestrosa 2013). *Wnt* ligands and other mediators of *Wnt* signalling, like β-catenin, are upregulated when neurogenesis is increased *in vitro*, implying a connection between them (Wexler et al. 2009). Blocking *Wnt* signalling suppressed, while stimulating *Wnt* signalling enhanced proliferation of neuroblast cells detected by a $Dcx^+/BrdU^+$ analysis, as BrdU labelling is marking only dividing cells (Jessberger et al. 2009). *Wnt* ligands can induce different effects on neuronal precursor cells. *Wnt1*, *Wnt3a*, *Wnt7a* are reported as inducers of proliferation as well as enhancer of *in vitro* neurogenesis (Wexler et al. 2009). Non canonical *Wnt5a* acts as differentiating factor (Parish et al. 2008) and chemical inhibition of GSK3β was able to stimulate neuronal differentiation (Dastjerdi et al. 2012). In addition, *Wnt* ligands secreted by adult hippocampal progenitors can stimulate canonical *Wnt* signalling, and inhibition of this autocrine *Wnt* pathway leads to a loss of multipotency of these progenitor cells (Inestrosa, Arenas 2010). *In vitro* experiments revealed that *Wnt* ligands derived from astrocytes activate canonical *Wnt* signalling in cultured adult hippocampal progenitor cells and increase the number of neurons (Lie et al. 2005). The mechanism behind this was further elucidated as the *Wnt3a* treatment increased the total number of neural progenitor cells, including the numbers of $Ki67^+$ proliferating cells and $Tuj1^+$ differentiated neurons. This result verified that Wnt3a promoted neural progenitor cell proliferation, without affecting the neuronal differentiation process itself (Yoshinaga et al. 2010).

The same effect was documented in neonatal progenitor cells where canonical *Wnt* activation increased their proliferation and led to more neurogenesis (Hirsch et al. 2007). *In vivo*, stereotaxic injection of lentiviral vectors expressing *Wnt3a* or a mutant *Wnt1* that blocks the activation of canonical *Wnt* signalling, showed an decrease in neurogenesis, while stimulating canonical *Wnt* pathway induces a strong increase in adult hippocampal neurogenesis (Lie et al. 2005).

Recently, Neurogenic differentiation 1 (NeuroD1), a transcription factor important for the differentiation of granule cells and olfactory neurons in the embryonic and adult brain (Gao et al. 2009) was identified as a downstream effector of *Wnt* ligands in adult neurogenesis (Kuwabara et al. 2009). NeuroD1 is selectively expressed in dividing neural progenitor cells in the DG. *Wnt3a* treatment induced the expression of NeuroD1 in adult neural progenitors *in vitro*, and β-catenin was directly associated with the *NeuroD1* gene promoter during the course of neurogenesis (Kuwabara et al. 2009). The *NeuroD1* promoter contains overlapping DNA-binding sites for Sox2 and Tcf/Lef, therefore, activation of the *Wnt* pathway induces expression of NeuroD1 by removal of Sox2-repression. Deletion of *NeuroD1* in stem cells prevented adult neurogenesis *in vivo*, and SVZ neurospheres with the same deletion had impaired neuronal differentiation capacity (Gao et al. 2009).
Further, it was shown that the *Wnt* inhibitors such as Dkk1 and sFRP3 negatively regulate neurogenesis. Inducible deletion of Dkk1 in adult CNS caused an increase in self-renewal and the number of neuronal progenitor cells (Seib et al. 2013), and sFRP3 knockdown in the dentate gyrus through a lentiviral approach increased neural progenitor cell proliferation (Jang et al. 2013).

These results indicate that *Wnt*/β-catenin signalling enhances the proliferation of neuronal progenitors during adult neurogenesis and by this increases the number of new-born neurons. This connection is central for this work and will be analysed in the context of MS by using a *Wnt* reporter mouse in the EAE model.

1.4.3 Adult neurogenesis in CNS regeneration and repair

According to the classical textbook view, the mammalian central nervous system (CNS) has very limited capacity for renewing cells and is not capable of compensating for cells lost after injury or disease. Although constitutive neurogenesis normally occurs in only two areas of the adult mammalian brain (SVZ and DG), recent studies propose that it is possible to manipulate endogenous neural precursors *in situ* to undergo neurogenesis in other regions of the adult brain as well. Interestingly, it has long been thought that neurons generally are not replaced in the mammalian brain after injury or during the course of disease, but lately many investigations have reported compensatory proliferation and neurogenesis in response to injury or disease in the mammalian brain (reviewed in Goldman 2005; Zhang et al. 2005; Emsley et al. 2005).
Compensatory or regenerative neurogenesis from inherent progenitor cells was first reported in the neocortex after selective ablation and apoptosis of cortical interneurons (Magavi et al. 2000) and later in long projecting corticospinal neurons (Chen et al. 2004). This work has been extended towards other brain areas like the striatum (Arvidsson et al. 2002) and the hippocampus (Nakatomi et al. 2002; Parent et al. 2002). In a stroke model, induced by transient middle cerebral artery occlusion in adult rats, an increase of cell proliferation was detected in the SVZ. The stroke-generated new neurons migrated into the damaged area of the striatum, where they expressed markers of developing and mature, striatal neurons (Arvidsson et al. 2002). In response to ischemia, endogenous progenitors proliferated and subsequently migrated into the hippocampus to regenerate new neurons and Intraventricular infusion of growth factors augmented these natural response, thereby increasing the number of new-born neurons (Nakatomi et al. 2002). After focal ischemic injury, an increase of SVZ neurogenesis was observed and newly generated neuroblasts migrated to sites of damage and differentiated into a region-appropriate phenotype (Parent et al. 2002). In line with this, it was shown that injury-induced newly generated granular cells integrate into the existing hippocampal circuitry (Emery et al. 2005; Sun et al. 2007; Sun 2014), and that endogenous neurogenesis is associated with innate cognitive recovery following TBI (Sun et al. 2007; Sun 2014). In human brains, a recent study has even found an increased number of cells expressing stem cell markers, including DCX, PSA-NCAM, SOX2 and NEUROD1, in the cortex after traumatic brain injury (TBI), indicating induced neurogenesis (Zheng et al. 2013).
Taken together, these results clearly indicate that compensatory neurogenesis can take place in the adult mammalian brain and, more essentially, that it may be possible to evoke endogenous neuronal repair in normally non-neurogenic brain regions. Moreover, the data indicated the inherent attempts of the brain to repair and regenerate following injury by activating neurogenesis, providing a basis for therapeutic purposes, in particular in neurodegenerative diseases.

Stimulating neurogenesis and/or activating stem cells are research topics in which *Wnt* signalling is known to be a crucial participant. *Wnt* signalling was shown to be essential for cortical neurogenesis after focal ischemic injury (Shruster et al. 2012) and for neuronal progenitors to undergo neurogenesis *in vitro* and *in vivo* (Inestrosa, Arenas 2010). After TBI, recent observations demonstrated an increase of *Wnt*/β-catenin activity and adult neurogenesis in the mouse dentate gyrus (Zhang et al. 2013). Interestingly, compensatory neurogenesis and *Wnt* signalling seem to have the same activity pattern after acute damage, indicating a potential interaction between them.

To advance the knowledge of compensatory neurogenesis connected to *Wnt* signalling activity specifically in MS pathology, this study wants to examine in detail these two aspects in the hippocampus of mice in acute stages of EAE.

1.5 TNFα signalling

Tumor necrosis factor alpha (TNFα) is a member of the TNF superfamily, which is composed of 19 ligands and 29 receptors, all playing a contrasting role in biological systems. The interest in TNF research has increased more and more over the years as indicated by the large body of literature, documenting beneficial and potentially harmful effects of the TNF superfamily members (Aggarwal 2003).

One of the most discussed superfamily members, TNFα, was first identified as a factor with an antitumor activity (Balkwill 2006). TNFα is generated as a precursor form called transmembrane TNFα that is expressed as a cell surface type II polypeptide consisting of 233 amino acid residues (26 kDa) on activated macrophages and lymphocytes as well as other cell types (Horiuchi et al. 2010). It has been connected to proliferation and differentiation of various cell types (Arnett et al. 2001; Balkwill 2006; Peng et al. 2008) and it has been linked to a wide range of diseases, including cancer (Balkwill 2006), cardiovascular (Ferrari 1999), metabolic (Chen et al. 2009), pulmonary (Mukhopadhyay et al. 2006), autoimmune (Bradley 2008), and neurological disorders (Su-Yin Lim and Cris S. Constantinescu 2010). The function of TNFα is relayed by two structurally distinct receptors, TNF receptor (TNFR) 1 and TNFR2. Both belong to the TNFα receptor superfamily, a group of type I transmembrane glycoproteins, characterized by a conserved homologous cysteine-rich domain in their extracellular region and non-covalently bound homotrimers at cell surfaces (MacEwan 2002). TNFR1, is highly promiscuous and is expressed by every mammalian cell type in the body (Aggarwal 2003). This may reflect the receptor's diverse functions in different cell types. The expression of TNFR2, in comparison, is limited to cells of the immune system, endothelial cells, and nerve cells (Faustman, Davis 2010). TNFα signalling cascade is highly conserved and illustrated in figure III. Moreover, TNFα is a key player in innate and adaptive immunity (Su-Yin Lim and Cris S. Constantinescu 2010) and a pro-inflammatory cytokine that exerts both homeostatic and pathophysiological roles in the CNS. This pro-inflammatory effect of TNFα is mediated through NF-κB-regulated molecules, such as IL-6, IL-8, IL-18 and various chemokines, all major mediators of inflammation. Additionally, some of these are also associated with cell survival and cell proliferation (Lawrence 2009). In the healthy CNS, TNFα has regulatory functions on crucial physiological processes such as synaptic plasticity (Beattie et al. 2002), learning and memory (Baune et al. 2008), sleep (Krueger 2008), food and water intake (Plata-Salamán 2001), and astrocyte-induced synaptic strengthening (Santello et al. 2011). In case of neurogenesis, TNFα-TNFR2 signalling was implicated in hippocampal neurogenesis in healthy mice (Chen, Palmer 2013a) and treatment with 1 ng of TNFα in SVZ-derived cultures of neural progenitors triggered proliferation (Bernardino et al. 2008).

TNFa influence in various CNS diseases was also described. Thus, defficiency for TNFR2 abrogated the number of newly generated neurons in the hippocampus (Iosif 2006), but not in the SVZ (Iosif et al. 2008) in a model of status epilepticus. Complementary to these data, infusion of neutrilizing antibodies against TNFα had an anti-proliferative effect on rat hippocampal progenitors in the rat model of stroke (Heldmann et al. 2005).

In pathological conditions, microglia are considered as major source of TNFα, leading to the activation and recruitment of inflammatory cells to the site of injury. This production of TNFα is an important component of the neuro-inflammatory response that is associated with several neurological disorders, like depression (Dowlati et al. 2010), bipolar disorder (Brietzke, Kapczinski 2008), epilepsy (Murashima et al. 2008), AD (Swardfager et al. 2010; Perry et al. 2001), PD (Çomoğlu et al. 2013; Nagatsu, Sawada 2005), and MS (Su-Yin Lim and Cris S. Constantinescu 2010). Taking a particular interest in the auto-immune disease MS and in murine models of chronic inflammation, a diminished expression of TNFα has been associated with the emergence of autoimmune diseases (Kollias 2005). For example, in EAE TNFα-deficient mice develop more severe disease symptoms than wild-type or $TNF\alpha^{+/-}$ animals (Liu et al. 1998). Additionally, administration of recombinant human TNFα in TNFα-deficient mice prevents the occurrence of the disease or reverses symptoms when administered 10 days after EAE induction (Liu et al. 1998). As TNFα was believed to have harmful effects in autoimmune diseases, a controlled clinical trial of TNFα blockade in MS was planned. Surprisingly, the clinical trial has shown a worsening of the symptoms in many patients receiving the treatment, associated with a trend towards increased demyelinated lesions visualized by magnetic resonance imaging (van Oosten et al. 1996). This data underscores the complexity of TNFα biology in autoimmune diseases, particularly MS, and emphasizes the necessity to enlighten the neurobiological role of TNFα signalling in inflammatory conditions.

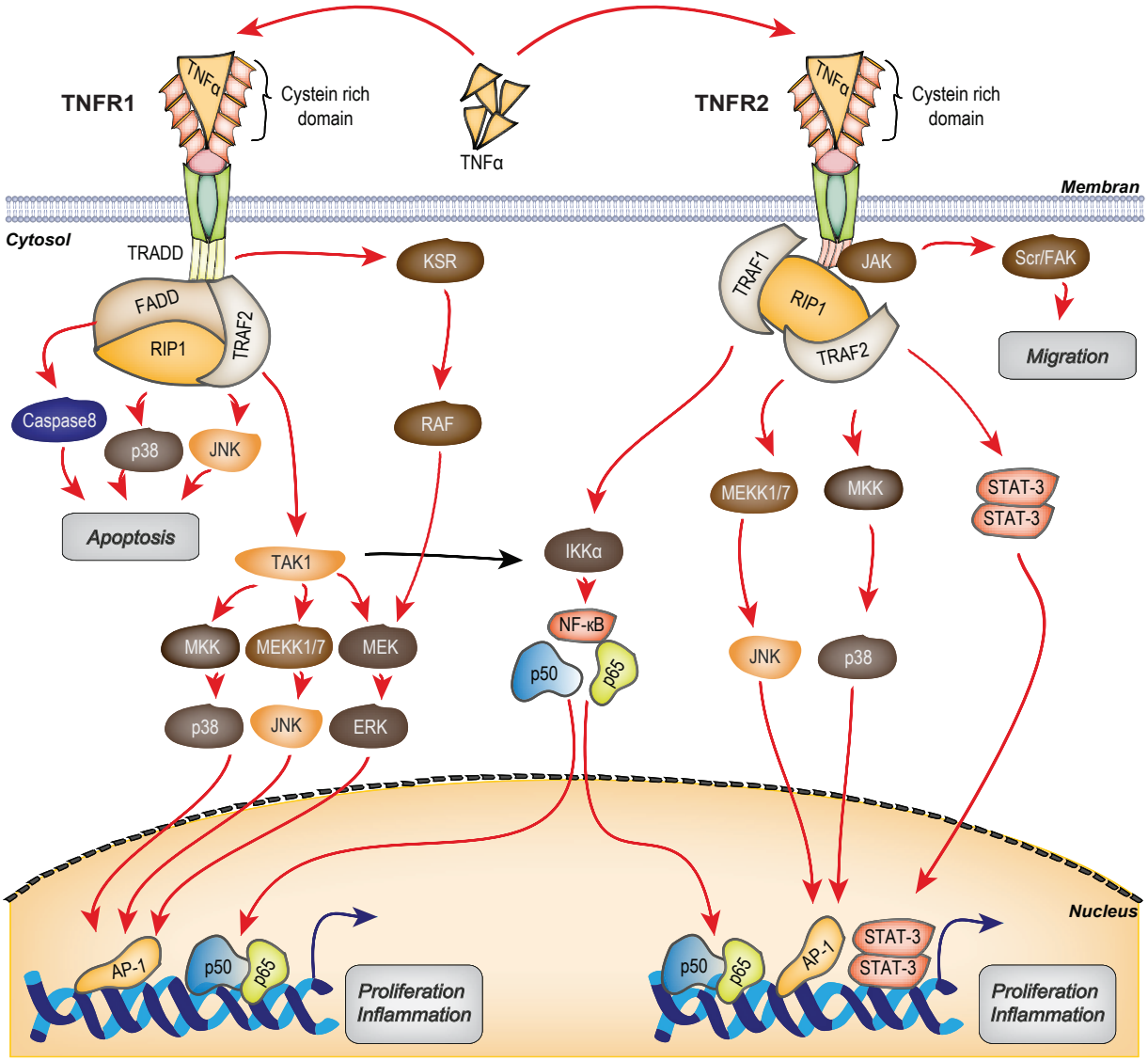

Figure IV| **TNFα signalling cascade** (modified from (Palladino et al. 2003; Moelants et al. 2013).

1.6 TGFβ pathway

1.6.1 TGFβ signalling cascade

The transforming growth factor beta (TGFβ) family of growth factors control a various number of cellular responses and prominently regulates the development and homeostasis of many biological processes essential in invertebrates as well as in vertebrates. Research over the past years has revealed significant insights into the TGFβ signal transduction network, presenting its involvement in cell growth, development, tissue homeostasis and regulation of the immune system (Massagué 2012; Gordon, Blobe 2008). Genetic evidence indicates that TGFβ family members regulate embryonic and neonatal development. Most knock out animals for one TGFβ superfamily ligand, receptor, protein or signalling protein fail in either gastrulation or mesoderm differentiation (Gordon, Blobe 2008).

There are three known mammalian isoforms of TGFβ (TGFβ1, TGFβ2 and TGFβ3) expressed with highly conserved regions. All of them function through the same receptor signalling pathways (Cheifetz et al. 1987; Mittl et al. 1996). They are synthesized as pro-proteins with large amino-terminal pro-domains, which are required for proper folding and dimerization (Gray, Mason 1990). In most cells, the TGFβ isoforms signal through three types of cell surface proteins: TGFβ receptor I (TGFβRI), II (TGFβRII) and III (TGFβRIII) (Cheifetz et al. 1987; Massagué 2012). Most important are TGFβRI and TGFβRII, which mediate the TGFβ signal transduction. Both receptors are transmembrane serine/threonine kinases, which act as tetramers and consist of an N-terminal extracellular ligand-binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain (Massagué 1992). Canonical TGFβ signalling is the classical pathway involved in almost all functions of the TGFβs. It is initiated by ligand binding to its receptor TGFβRII, which subsequently phosphorylates the TGFβRI (Shi, Massagué 2003). Afterwards, the large ligand-receptor complex consisting of dimeric TGFβ ligand and two pairs of TGFβRI and TGFβRII (Shi, Massagué 2003) leads to phosphorylation of Smad2 and Smad3 proteins, which form a heteromeric complex with Smad4 and translocate into the nucleus. There, they regulate the transcription of TGFβ target genes (Shi, Massagué 2003), subjected to both positive and negative regulation (Fig.V). TGFβs can also activate other signalling cascades in a context-dependent manner, such as mitogen-activated protein kinase (MAPK), JNK and PKC pathways (Feng, Derynck 2005). In the developing CNS, TGFβ signalling is involved in regulating self-renewal of neural stem cells in the developing midbrain (Falk et al. 2008), promoting the sprouting and elongation of neurites in hippocampal cultures (Ishihara et al. 1994), mediating axon specification during brain development (Yi et al. 2010) and regulating synaptic growth (Ng 2008).

In the adult CNS, TGFβ2, TGFβ3, and TGFβ receptors are widely distributed (Pratt, McPherson 1997), whereas TGFβ1 is expressed mostly in response to injury and/or aging (Finch et al. 1993; Tesseur, Wyss-Coray 2006). TGFβ family proteins are present in the brain immediately after injury as they are carried into the wound by the blood stream (Lenzlinger et al. 2001). Additionally, extracellular TGFβ proteins are activated and released from their latent protein complexes in the brain parenchyma (Annes et al. 2003). Following acute injury, TGFβ protein levels are elevated in astrocytes (Doyle et al. 2010), microglia, macrophages (Stoll et al. 2004), neurons (Krieglstein et al. 2002), ependymal cells (Maharaj et al. 2008) and choroid plexus cells (Maharaj et al. 2008) with peak expression around three days after the damage (Logan et al. 1992). Under these conditions, it has been shown that TGFβ1 can be both: Beneficial and harmful, displaying neuroprotection (Krieglstein et al. 2002; Tesseur, Wyss-Coray 2006), gliosis (Buckwalter, Wyss-Coray 2004), and vascular fibrosis (Wyss-Coray et al. 2000). Consistent with its role in regulating developmental neurogenic processes, TGFβ1 is also able to reduce adult neurogenesis by inhibiting the proliferation of neural progenitor cells and keeping stem cells in a quiescent state (Buckwalter et al. 2006; Wachs et al. 2006).
The available data indicate that TGFβ1 signalling may be active in the neurogenic regions after injury, and it is, however, currently unclear in which manner TGFβ1 can affect the behaviour of neural stem cells. Given that TGFβ1 signalling evidently effects adult neurogenesis in the healthy adult brain, more research in this area is necessary to fully elucidate the effect of brain injury on this pathway and all mechanisms through which these changes modulate post-injury neurogenesis.

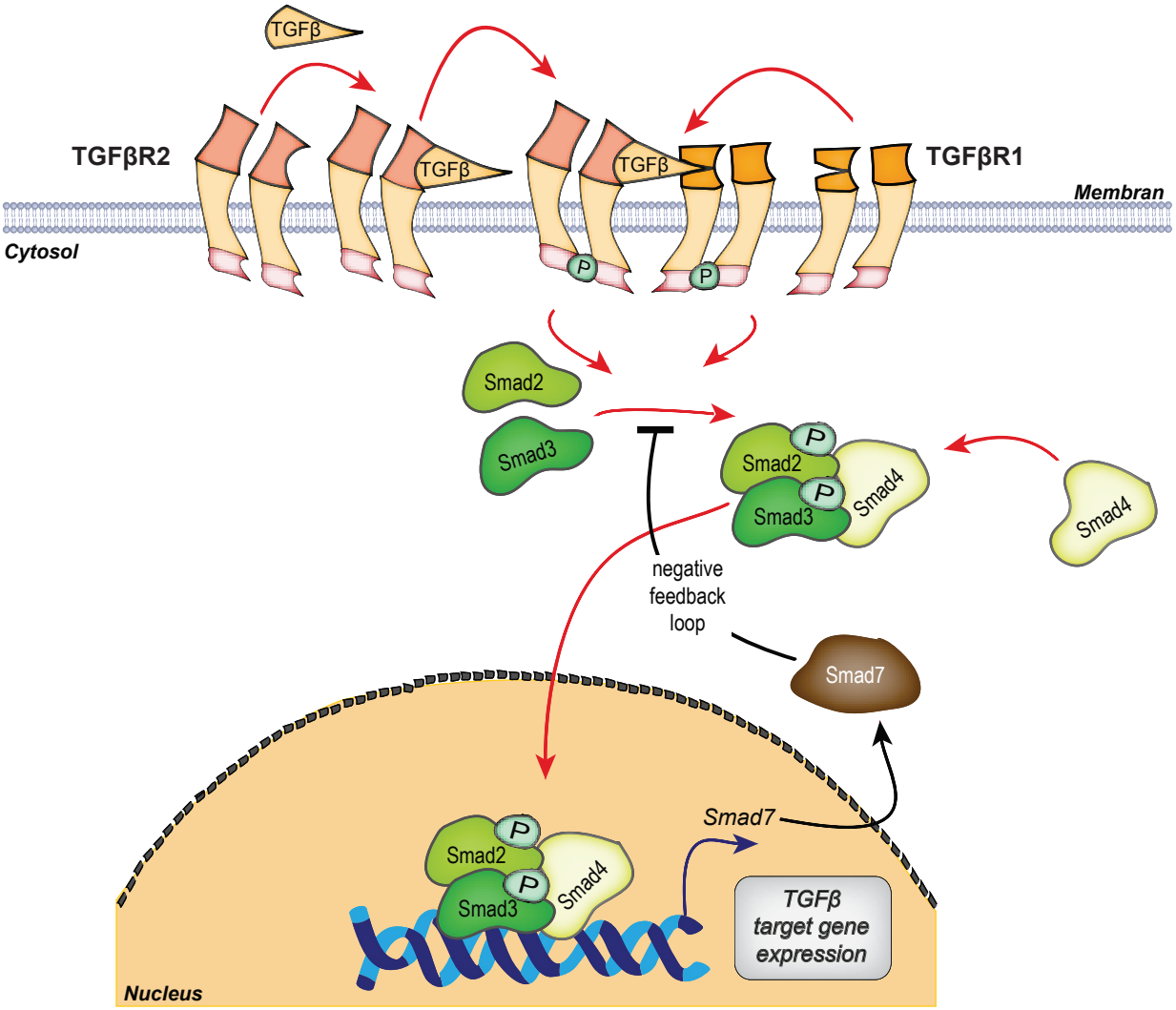

Figure V| **TGFβ signalling cascade.**
The canonical TGFβ signalling is the classical pathway involved in almost all functions of the TGFβs. It is initiated by ligand binding to its receptor TGFβRII, which subsequently phosphorylates the TGFβRI. Then, the large ligand-receptor complex consisting of dimeric TGFβ ligand and two pairs of TGFβRI and TGFβRII leads to phosphorylation of Smad2 and Smad3 proteins, which form a heteromeric complex with Smad4 and translocate into the nucleus where they regulate the transcription of TGFβ target genes, subjected to both positive and negative regulation.

1.6.2 TGFβ signalling in MS

In many years of research, TGFβ was associated with beneficial disease remission, as it can be found in local tissue components, mainly glia cells, during recovery from inflammatory diseases within the CNS. TGFβ was considered an immunosuppressive cytokine and its local production in demyelinated lesions suggested a prominent role in downregulation of the inflammatory response in brain inflammation, together with other cytokines and neurotrophins (Trapp, Nave 2008).

In EAE mice, TGFβ1 synthesis in glial cells and TGFβ-induced signalling in the CNS were activated several days before onset of the disease. Production of TGFβ1 was observed early in glial cells and TGFβ signalling activation was described in neurons, in infiltrating T cells located in inflammatory lesions, but also in meningeal and perivascular infiltrates (Constantinescu et al. 2011). In the active EAE model, double labelling studies revealed that subpopulations of T cells are the main source of TGFβ1 in an early disease phase, followed by macrophages at the peak of disease and microglial cells during the recovery phase (Kiefer et al. 1998). The expression of TGFβ1 by T cells early in the disease may contribute to inflammatory lesion development and expression of immunosuppressive TGFβ1 during remission by microglial cells, which can contribute to recovery (Kiefer et al. 1998). Of note, administration of TGFβ1 for more than a week protected mice completely against relapsing EAE and administration during the induction phase delayed its onset (Kuruvilla et al. 1991). In line with this, administration during the remission phase prevented the occurrence of relapses (Kuruvilla et al. 1991). These effects of TGFβ1 may be based on its ability to selectively suppress autoantigen-induced upregulation of pro-inflammatory cytokines (Link et al. 1995). Additionally, TGFβ is a major mediator associated with EAE and the early production of TGFβ in the CNS may create a favourable environment for the initiation of inflammation (Dobolyi et al. 2012).

Apart from experimental animal models for MS, TGFβ1 was also found to be elevated in MS patients. Additionally, an increased TGFβ1 activity was detected in the supernatants of blood cell cultures from MS patients. In addition, MS was also associated with increased TGFβ mRNA expressing cells in the blood (Mirshafiey, Mohsenzadegan 2009).

Interestingly, in perivascular and parenchymal macrophages and in hypertrophic astrocytes a strong immunoreactivity was apparent for all three TGFβ isoforms and their receptors in active demyelinating lesions (Mirshafiey, Mohsenzadegan 2009). Biologically active TGFβ1 in cerebrospinal fluid correlated positively with the duration of the acute relapse in patients with relapsing MS. The more relapses the patients had the higher the biologically active TGFβ1 was in their cerebrospinal fluid (Söderström et al. 1995).

Genome screenings in MS have identified multiple susceptibility regions supporting a polygenic model for this disease. Evidence, in form of a comprehensive evaluation of common polymorphisms within the TGFβ1 gene was performed in MS families and proved an association between a TGFβ1 haplotype and a mild MS disease course. This data raises the possibility that TGFβ1 may influence disease expression and plays a role in susceptibility to MS (Green et al. 2001; Mirshafiey, Mohsenzadegan 2009).

In the neurobiology of MS, TGFβ is inhibiting astrocyte reactivity and proliferation, increasing the availability of molecules promoting neurite outgrowth, limiting the formation of reactive astrocytes and decreasing microglia and macrophage infiltration.

The important role of TGFβ as an important pleiotropic cytokine, with both stimulatory and inhibitory effects on cell differentiation and growth, makes it an interesting target for this investigation. This work will analyse TGFβ signalling in context of *Wnt* signalling modulation in the hippocampus of healthy and EAE mice, trying to characterize a possible connection of both pathways.

1.7 Aims of the study

The *Wnt* pathway is known to be activated in tissue repair and to be an initial response mechanism after acute injury, but distinct stimuli or modulators leading to the activation are still unknown. Further, damage induced neurogenesis is recognised in various neurological diseases and became of great interest for CNS tissue repair. In case of MS, however, the data concerning *Wnt* signalling and autoimmune damage induced neurogenesis should be extended. Therefore, this study aims to:

(1) characterize *Wnt* signalling activity in the course of EAE,
(2) elucidate the impact of neuroinflammation on *Wnt* signalling and adult neurogenesis in the hippocampal brain area during the disease and
(3) identify distinct *Wnt* activity modulators that are also involved in EAE pathogenesis.

2. Materials and Methods

2.1 Animals

All experimental procedures were conducted following to guidelines and protocols approved by the local animal welfare committee (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV); under protocol number G388-11 (active EAE in Axin2$^{lacZ/+}$ mice; G197-09 (BrdU injections in EAE mice); G363-09 (passive EAE in SJL/J)) and follow the *ARRIVE* criteria and EAE induction guideline (Baker, Amor 2012). All mice were housed in the animal research facility of the University of Düsseldorf under specific pathogen free conditions, at a dark/light cycle of 12 hours, stable temperature of 22-24°C and had unlimited access to food and water.

2.1.1 Axin2$^{lacZ/+}$ mice

Axin2$^{lacZ/+}$ mice with a C57BL/6 background were received from the European Mouse Mutant Archive (EMMA). In Axin2$^{lacZ/+}$ mice the *lacZ* gene containing a nuclear localization signal was introduced in frame to the endogenous *Axin2* (also known as *conductin* or *Axil*; encodes an inhibitor of the *Wnt* signalling pathway and is a direct target of Tcf/Lef1-mediated transcriptional activation) start codon by homologous recombination, thereby replacing exon2 (Lustig et al. 2002).

2.1.2 Nestin^{eGFP} mice

Nestin^{eGFP+} mice with a C57BL/6 background were provided by the animal facility of the University of Duesseldorf. In these transgenic mice the second intron enhancer of the rat *Nestin* gene was placed upstream of the minimum promoter of *heat shock protein 68* (*HSP68*) fused to eGFP cDNA and a polyadenylation signal (Kawaguchi et al. 2001).

2.1.3 TGFβR2$^{loxP/ loxP}$ mice

TGFβR2$^{loxP/loxP}$ mice were received from the European Mouse Mutant Archive (EMMA) and back-crossed to a genetic background of C57BL/6 mice by breeding TGFβR2$^{loxP/+}$ with a wild-type C57BL/6 mouse over more than 10 generations.
Then, TGFβR2$^{loxP/loxP}$ mice, that harbor *loxP* sites surrounding exon 2 of the *TGFβR2* gene (Chytil et al. 2002), were used to create TGFβR2 brain specific conditional knockout mice.

2.1.4 Nestin^{cre} mice

Nestin^{cre} mice with a C57BL/6 background are expressing *cre* recombinase under the control of the promoter and the nervous system-specific enhancer present in the second intron of the rat *Nestin* gene together with hGH poly(A), a human growth hormone polyadenylation signal (Tronche et al. 1999). The *Nestin* gene is expressed around embryonic day 10 (Kawaguchi et al. 2001; Fukuda et al. 2003; Tronche et al. 1999) and is specific for all CNS cell types, providing the possibility to create conditional gene knock-outs specific for the brain.

2.1.5 TGFβR2 conditional knockout mice

TGFβR2^{cKO} mice were generated by crossing TGFβR2$^{loxP/loxP}$ mice with Nestin^{cre+} mice. The generated mice were named TGFβR2^{cKO}, meaning TGFβR2$^{loxP/loxP}$/Nestin^{cre+} mice and compared to control mice with TGFβR2$^{+/+}$/Nestin^{cre+}. These mice have a TGFβR2 conditional knockout specific for the brain, leading to CNS cells which lack the functional TGFβ-receptor 2 and TGFβ signalling.

2.2 EAE

2.2.1 Active EAE induced by immunization with MOG

To induce active EAE in the Axin2$^{lacZ/+}$ mouse strain, female animals were immunized by subcutaneous injection with 200 µg of recombinant myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide (*Pepceuticals*) in complete Freund's adjuvant (CFA, *Sigma-Aldrich*), supplemented with 800 µg heat-inactivated *Mycobacterium tuberculosis* (strain H37RA, *Difco*). Intraperitoneal injections of 200 ng pertussis toxin (PTX, *Sigma-Aldrich*) were performed on the day of immunization and on day 2. The control group was treated only with CFA and 200 ng pertussis toxin on day 0 and day 2. Animals were weighed and scored daily according to following clinical scale: 0 (no clinical signs), 1 (tail plegia), 2 (abnormal gait), 3 (hind limb paralysis), 4 (complete paralysis) to 5 (death or euthanasia) with intervals of 0.5. Mice were euthanized either on day 20, 30 or 50 post immunization (p.i.).

2.2.2 Adoptive Transfer EAE induced by transfer of myelin specific T cells

For passive EAE, female SJL/J mice were subcutaneously immunized with 200 µg of recombinant proteolipid protein peptide ($PLP_{139–151}$) (*Pepceuticals*) supplemented with 800 µg heat-inactivated *Mycobacterium tuberculosis* (strain H37RA, *Difco*) emulsified in 200 µl CFA (*Sigma-Aldrich*) per mouse. On day 10 p.i., spleens and lymph nodes were harvested, and the resulting single-cell suspension was restimulated with $PLP_{139–151}$ (10 µg/mL) in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin, glutamate, and 2-mercaptoethanol (all from *Invitrogen Life Technologies*). After four days in culture, cells were harvested and injected intraperitoneal (i.p.) into naive female SJL/J mice ($3x10^7$ cells per mouse). Control mice received a single i.p. injection of PBS. Animals were weighed and scored daily according to following clinical scale: 0 (no clinical signs), 1 (tail plegia), 2 (abnormal gait), 3 (hind limb paralysis), 4 (complete paralysis) to 5 (death or euthanasia) with intervals of 0.5. Mice were euthanized either on day 6, 20 or 50 post transfer. Hippocampi were dissected and stored at -80°C.

2.3 Histology

2.3.1 Methodological procedure

Mice were anaesthetized with isoflurane (*Piramal Healthcare*) and perfused with PBS. Brain and spinal cord were dissected and either directly frozen for the X-gal staining or postfixed with 4% (v/v) paraformaldehyde (PFA, *Roth*) for 16 hours, followed by dehydration in a 25-30% (v/v) sucrose solution. Tissue samples were cryopreserved in “TissueTek” (*Sakura Fintek Europe*) at -80°C. For histological examination brains and spinal cords were cut with a cryostat (*Leica*). 20 µm slices were permeabilized with 0.5% (v/v) Triton X-100 and blocked using 5% (v/v) horse serum (*Invitrogen Life Technologies*) and 1% (v/v) bovine serum albumin in PBS for two hours. OSCs were washed two times in pre-warmed PBS, fixed for one hour in 4% PFA and permeabilized for one hour with 1% (v/v) Triton X-100 (*Sigma Aldrich*) in PBS. Primary antibodies were diluted in PBS containing 2.5% (v/v) horse serum, 0.25% (v/v) Triton X-100 and incubated with tissue samples overnight (or 48 hours for OSCs) at 4°C. Visualization was performed by incubation with fluorescent Cy2-, Cy3- and Cy5-conjugated secondary antibodies (*Millipore*) for 1-2 hours at RT. Hoechst dye 33258 (*Molecular Probes*) was used to counterstain nuclei followed by mounting on glass slides with “Immuno Mount” (*Thermo Scientific*). Immunostainings were analyzed on an *Olympus* BX51 microscope and overlaid using *Photoshop 13.0* software (*Adobe*).

2.3.2 Bromodesoxyuridine pulse-and-trace experiments

For labeling of proliferating cells, 5-bromo-2-desoxyuridine (BrdU; *Sigma Aldrich*) was injected daily i.p. (50 mg/kg body weight) at the peak of disease (14 d.p.i.) over five days. EAE animals and healthy controls were euthanized and perfused two weeks after the first BrdU-injection. Mouse hippocampi were collected and cryopreserved (see histology part) and then processed for the staining. Sections were incubated with 2N HCl for 30 minutes at 37°C and rinsed shortly in 100 mM tetraborate buffer and washed two times with PBS.
Then, sections were incubated with primary rat anti-BrdU antibody (1:400; *Upstate*) overnight at 4°C, washed two times with PBS (0.1% Triton) and incubated for one hour at RT with an anti-rat secondary antibody and five minutes with Hoechst 33342 dye (*Molecular Probes*) for nuclei counterstains. To finish and conserve, slights were mounted with “Immuno Mount” (*Thermo Scientific*).

2.3.4 Antibodies

Table 1: Primary antibodies used for histology

Primary antibody	**Host**	**Company**	**Dilution**
anti-NF-M	mouse	*Sigma Aldrich*	1:1000
anti-Iba1	rabbit	*Wako*	1:500
anti-GFP	rabbit	*Abcam*	1:1000
anti-Dcx	goat	*Santa Cruz*	1:250
anti-BrdU	rat	*Upstate*	1:400
Anti-MBP	rat	*Millipore*	1:500

Table 2: Secondary antibodies used for histology

Secondary antibody	**Host**	**Company**	**Dilution**
anti-rabbit-Cy2	goat	*Millipore*	1:500
anti-rat-Cy2	goat	*Millipore*	1:500
anti-goat-Cy3	donkey	*Millipore*	1:500
anti-rabbit-Cy3	goat	*Millipore*	1:500
anti-mouse-Cy3	goat	*Millipore*	1:500

2.4 Western blotting

2.4.1 Methodological procedure

Hippocampal tissue isolated from mice was lysed on ice for 15 min in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% (v/v) NP40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS) with protease and phosphatase inhibitors (*Roche*). Lysates were cleared by centrifugation at 14,000 *g* for 20 minutes at 4°C and protein concentration was determined with a BCA protein assay Kit (*Interchim*). Protein samples were loaded on 8-16% gradient "Mini-Protean TGX" gel (*Biorad*). Blotting onto a nitrocellulose membrane was performed using the "Trans-Blot Turbo System" (*Biorad*) for seven minutes at 25 Volts. Afterwards, membranes were incubated with blocking buffer (5% (v/v) skimmed milk in 0.05% (v/v) PBS/Tween) for one hour at RT and incubated overnight at 4°C with under 2.3.2 mentioned antibodies**:** Primary antibodies were detected and quantified by incubation with IR-Dye secondary antibodies (*Li-COR)* for one hour at RT and the Odyssey infrared imaging system (*LI-COR*). Optical density analysis was performed with *ImageJ* software (created by *Rasband, WS*).

2.4.2 Antibodies

Table 3: Primary antibodies used for Western Blotting

Primary antibody	**Host**	**Company**	**Dilution**
anti-β-catenin	rabbit	*Cell Signalling*	1:1000
anti-ABC	mouse	*Cell Signalling*	1:1000
anti-β-actin	mouse	*Sigma Aldrich*	1:2500
anti-NF-H	mouse	*Sigma Aldrich*	1:2000
anti-PSD95	mouse	*Abcam*	1:500
anti-β-III-tubulin	mouse	*Convergence*	1:2000

Table 4: Secondary antibodies used for Western Blotting

Primary antibody	**Host**	**Company**	**Dilution**
anti-rabbit-IgG 800	donkey	*LI-Cor*	1:20000
anti-mouse-IgG 680	donkey	*LI-Cor*	1:20000

2.5 Cell culture

2.5.1 Organotypic slice cultures

Organotypic slice cultures (OSCs) were generated from 10 days old Axin2$^{lacZ/+}$/Nestin^{eGFP+} pups as described before (Aktas et al. 2007a; Kocur et al. 2015). Briefly, the hippocampus was cut into 350 µm thick slices and cerebellum into 400 µm thick slices using a McIllwain tissue chopper (*GaLa Instrumente*). OSCs were dissociated in ice-cold dissecting medium (Hank's Balanced Salt Solution (HBSS), *Invitrogen Life Technologies*) complemented with penicillin/streptomycin (100 U/ml, *Invitrogen Life Technologies*), 2.5 mg/ml glucose (*Sigma Aldrich*) and 10 mM kynurenic acid (*Sigma Aldrich*). OSCs were cultured on Millicell-CM culture plate inserts (*Millipore*) in culture medium (50% (v/v) MEM, 25% (v/v) HBSS, 25% (v/v) heat-inactivated horse serum, 2mM glutamine, penicillin/streptomycin (100 U/ml) (all from *Invitrogen Life Technologies*) and 2.5 mg/ml glucose (*Sigma Aldrich*) for seven days at 37°C in a humidified atmosphere with 5% CO_2. At this point OSCs were used for further experiments. Demyelination was induced by treatment of cerebellar OSCs with lysolecithin (LPC, 0.5 mg/ml; *Sigma Aldrich*). After incubation for 16 hours the LPC containing medium was replaced with fresh medium for additional 24 hours.

For analysis of neurogenesis on hippocampal OSCs, cultures were first pretreated for 24 hours with BrdU (0.5 µM, Sigma) to allow detection of proliferating cells. Next, recombinant cytokines: TNFα (1 ng/ml), IFNγ (100 U/ml) (both from *ImmunoTools*) or *Wnt3a* (20 ng/ml, *R&D Systems*) were added to culture medium for six hours, followed by washing and replacement with cytokine-free media for additional 24 hours. After this period, slice cultures were directly used to analyse β-galactosidase activity (see above) or fixed with aldehydes for histological examination.

2.6 Quantitative real time PCR

2.6.1 RNA isolation

RNA was isolated with a RNA isolation Kit (*Macherey-Nagel*) or Trizol (*Invitrogen Life Technologies*) according to manufacturer's instructions. The quality and amount of isolated RNA was measured with the *Nanodrop* 2000 (*Thermo Scientific*). The isolated RNA was directly used for first-strand cDNA synthesis and rest of RNA was stored at -80°C.

2.6.2 Quantitative rt-PCR

1 µg of purified RNA was used for first-strand cDNA synthesis using "Superscript III Reverse Transcriptase" and oligo(dT) in a final volume of 20 µl according to the manufacturer's instruction (*Invitrogen Life Technologies*). Real-time quantification of gene expression was performed using a SYBR Green qrt-PCR assay (*Applied Biosystems*) or TaqMan 5'(FAM)- and 3'(TAMRA)-labelled fluorescent probes (*Eurofins MWG*). PCR was performed using an "ABI 7500" real-time PCR system (*Applied Biosystems*) with the standard cycling conditions: 40 cycles of 10 sec at 95°C, 60 sec at 60°C. Specificity of the PCR product was confirmed by examination of the dissociation reaction plot and PCR product detection on 2% agarose gel. The samples were run in duplicates and the level of expression of each gene was normalized to the expression of the housekeeping gene glycerinaldehyde-3-phosphate-dehydrogenase (*GAPDH*).

2.6.3 Primer sequences

Table 5: Primer sequences used for quantitative real-time PCR

Target gene	Primer ID	Sequence (5'→3')
Axin2	forward	**CGCCAACGACAGCGAGTTAT**
	reverse	**TACTCCCCATGCGGTAAGGA**
	probe (FAM/TAMRA)	**CAGCGACGCACTGACCGACGA**
Wnt2	forward	**ACACGGAGTCTGACCTGATGTAGA**
	reverse	**CCTGGAGGAGCCACCTGTA**
Wnt3	forward	**CTACTCGGCCTCCTGCTCAGT**
	reverse	**GCTGGGAGGCCAGAGATGT**
Wnt3a	forward	**TGGCTCCTCTCGGATACCTCTTA**
	reverse	**CACAGAGAATGGGCTGAGTGCT**
Wnt4	forward	**CTTTGGGAAGGTGGTGACACA**
	reverse	**CACTGCTGCATGCCCTTGT**
Wnt5a	forward	**TCAAGGAATGCCAGTACCAGTTC**
	reverse	**CGTACGTGAAGGCCGTCTCT**
	probe (FAM/TAMRA)	**TCGGAGATGGAACTGCAGCACAGTG**
Wnt5b	forward	**GGGCACAGCTGCTGACTGA**
	reverse	**GAGCGCCAATGATGAACATCT**
Wnt8b	forward	**TGTGCGTTCTTCTAGTCACTTGTGT**
	reverse	**GCGCTTCGAAGTCCACCAT**
	probe (FAM/TAMRA)	**CCGTGTGCGTTCTTCTAGTCACTTGTGTCC**
Wnt9a	forward	**TGGGCCGGCGCTCTA**
	reverse	**CCTTGAGCGAGGTCTCATATTTGT**
Wnt16	forward	**GGTCGCCACTACCACTTCCA**
	reverse	**GCCATGATGGCATAAATGAATG**
TGFβ1	forward	**CAATCAGGACCACTGCAATAAAAT**
	reverse	**GCAGACTGGACCAGCAATGA**
TNFα	forward	**GGGCCACCACGCTCTTC**
	reverse	**GGCTTGTCACTCGAATTTTGAGA**
IFNγ	forward	**TGCATTCATGAGTATTGCCAAGT**
	reverse	**GCTGGATTCCGGCAACAG**

Target gene	Primer ID	Sequence (5'→3')
IL-1β	forward	**TGCAGCAAGACTCTGGTACCTACA**
	reverse	**CATGAGGCAGAGATGCTTCAGT**
	probe (FAM/TAMRA)	**TTGCACATTCAGAAACGCATCCCACT**
ND1	forward	**CCACGCAGAAGGCAAGGT**
	reverse	**GCTGTATGATTTGGTCATGTTTCC**
	probe (FAM/TAMRA)	**TCCCGAGGCTCCAGGGTTATG**
Prox1	forward	**TCAACATGCACTACAACAAAGCA**
	reverse	**CGCGATGATGGCATTGAA**
Dlx2	forward	**CGTCTCCGGTCAACAACGA**
	reverse	**CTTGGATCGGCGGTTCTG**
Lef1	forward	**TCCTGAAATCCCCACCTTCTAC**
	reverse	**CACCCGTGATGGGATAAACAG**
	probe (FAM/TAMRA)	**ACCCCACCCATTGGCTGGCAA**
Smad7	forward	**GGCTGTCCAGATGCTGTACCTT**
	reverse	**GATCCCCAGGCTCCAGAAG**
Tcf4	forward	**GCTGGTCTGCACGGGATAAC**
	reverse	**TCAGGTCTGTGATCGGAGGAA**
GAPDH	forward	**CTCAACTACATGGTCTACATGTTCCA**
	reverse	**CCATTCTCGGCCTTGACTGT**
	probe (FAM/TAMRA)	**CGGATTTGGCCGTATTGGGCG**

2.7 β-galactosidase detection

2.7.1 Chemiluminescence assay

Tissue or organotypic slices isolated from Axin2$^{lacZ/+}$ mice were lysed on ice for 15 min in triton-containing lysis buffer (*Applied Biosystems*) and cleared by centrifugation at 10,000 g for 10 min at 4°C. Then protein concentration was determined with a BCA protein assay kit (*Interchim*). 50 µg of protein were applied for measuring β-galactosidase activity in triplicates with the "Galactostar Chemiluminescence Assay Kit" (*Applied Biosystems*) in a white 96-well NUNC plate (*Thermo Scientific*) at room temperature (RT) for two hours using a microplate reader (*TECAN*). The maximum of luminescence intensity for each individual sample was used to calculate mean of triplicate measurements. Lysates from β-galactosidase-negative tissue were used to calculate background intensity.

2.7.2 X-gal staining

For X-gal stainings, 20 µm thick cryoslices or OSCs, prepared from Axin2$^{lacZ/+}$ mice, were fixed with PBS containing 2% (v/v) formaldehyde, 0.2% (v/v) glutaraldehyde and 0.02% (v/v) NP40 (all from *Sigma Aldrich*) for 5 min. After washing, slices were incubated for two hours at 37°C in X-gal staining solution, containing 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 0.02% (v/v) NP40, 2 mM $MgCl_2$ and X-gal (0.5 mg/ml) (all from *Sigma Aldrich*) dissolved in PBS. Samples were fixed with 4% (v/v) PFA for 15 min and mounted on glass slides with "Immuno Mount" (*Thermo Scientific*).

2.8 Materials

2.8.1 Chemicals/Supplements

Table 6: List of used chemicals and supplements

Chemicals and Supplements	**Company**
Albumin fraction V, biotin-free	*Sigma Aldrich*
5-Bromo-2-desoxyuridine	*Sigma Aldrich*
Complete mini protease inhibitor	*Roche*
Complete mini phosphatase inhibitor	*Roche*
Dulbecco's Phosphate Buffered Saline	*Invitrogen Life Technologies*
Dulbecco's Modified Eagle's Medium	*Invitrogen Life Technologies*
Formaldehyde (4%)	*Sigma Aldrich*
Glutamax	*Invitrogen Life Technologies*
Glutaraldehyde (25%)	*Sigma Aldrich*
Glucose	*Sigma Aldrich*
Horse serum	*Invitrogen Life Technologies*
IFNγ	*ImmunoTools*
ImmuMount	*Thermo Scientific*
$K_3Fe(CN)_6$ solution	*Sigma Aldrich*
$K_4Fe(CN)_6$ solution	*Sigma Aldrich*
$MgCl_2$ solution	*Sigma Aldrich*
Mini-PROTEAN TGX precast gels	*BioRad*
Mitrocellulose membrane	*BioRad*
Modified Eagle's Medium	*Invitrogen Life Technologies*
NP40	*Sigma Aldrich*
Lysolecithin	*Sigma Aldrich*
Penicillin/Streptomycin	*Invitrogen Life Technologies*
Pierce RIPA buffer	*Thermo Scientific*
Power SYBR Green PCR master mix	*Applied Biosystems*
Protein marker V	*PeqLab*
SDS loading buffer	*BioRad*
TaqMan PCR master mix	*Applied Biosystems*

Chemicals and Supplements	Company
TGFβ1	*ImmunoTools*
TNFα	*ImmunoTools*
Tris/Glycerin running buffer	*BioRad*
Trizol reagent	*Invitrogen Life Technologies*
Triton-X-100	*Sigma Aldrich*
Tween-20	*Sigma Aldrich*
Wnt3a	*R&D Systems*
X-gal substrat	*Sigma Aldrich*

2.8.2 Kits

Table 7: List of used kits

Kit	Company
BC Assay Protein Quantification	*Interchim*
Galactostar Chemiluminescence Assay	*Applied Biosystems*
Multiscribe Reverse Transkriptase	*Invitrogen*
RNA Isolation	*Macherey-Nagel*

2.8.3 Laboratory equipment

Table 8: List of laboratory equipment

Laboratory equipment	**Company**
510 META Laser Scanning Microscope	*Carl Zeiss Microscopy*
6-well Millicell-CM culture plate inserts	*Millipore*
7500 Pro Real-Time PCR Systems	*Applied Biosystems*
96-well optical reaction plate	*Applied Biosystems*
Centrifuge 5417R	*Eppendorf*
Centrifuge Minispin	*Eppendorf*
CO_2 cell culture incubator	*Thermo Scientific*
F-View fluorescence camera	*Soft Imaging System*
Horizontal laminar Flow	*Heraeus*
Leica CM1900 UV	*Leica*
Microplate Reader	*TECAN*
Nanodrop 2000 Spectrophotometer	*Thermo Scientific*
Olympus BX51	*Olympus*
Olympus U-RFL-T Burner	*Olympus*
Standard Power Pack P25	*Biometra*
Sonicator, UW 2070	*Bandelin Electronic*
Thermocycler T Gradient	*Biometra*

2.9 Software

Table 9: List of used software

Software	**Company**
Adobe Creative Suite 6	*Adobe System Inc.*
Adobe Acrobat Reader 12.0	*Adobe System Inc.*
Cell A	*Soft Imaging System*
GraphPad Prism 5	*GraphPad Software*
ImageJ	*Rasband, W.S.*
Magellan Data Analysis	*Tecan Group Ltd.*
Odyssey Imaging System	*LI-Cor*
Office 2013	*Microsoft Corporation*
Primer Express	*Applied Biosystems*

2.10 Statistical Analysis

The values in the figures are shown as mean + SEM. Statistical analysis was performed with GraphPad Prism 5 (*GraphPad Software*). The *p* values of $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$ were determined to be statistically significant by using Student´s *t* test.

3. Results

3.1 EAE characterization in Axin2$^{lacZ/+}$ mice

To fulfil the proposed aims of the study, the first step was the induction of active EAE by immunization of Axin2$^{lacZ/+}$ reporter mice with a CNS self-antigen, MOG_{35-55} peptide (see 2.2.1). There is no EAE study in Axin2$^{lacZ/+}$ reporter mice yet, so a characterization of the disease progression and a comparison with the classical disease course was necessary to plan further analyses.

3.1.1 Quantification of cell infiltrates in the spinal cord and cerebellum

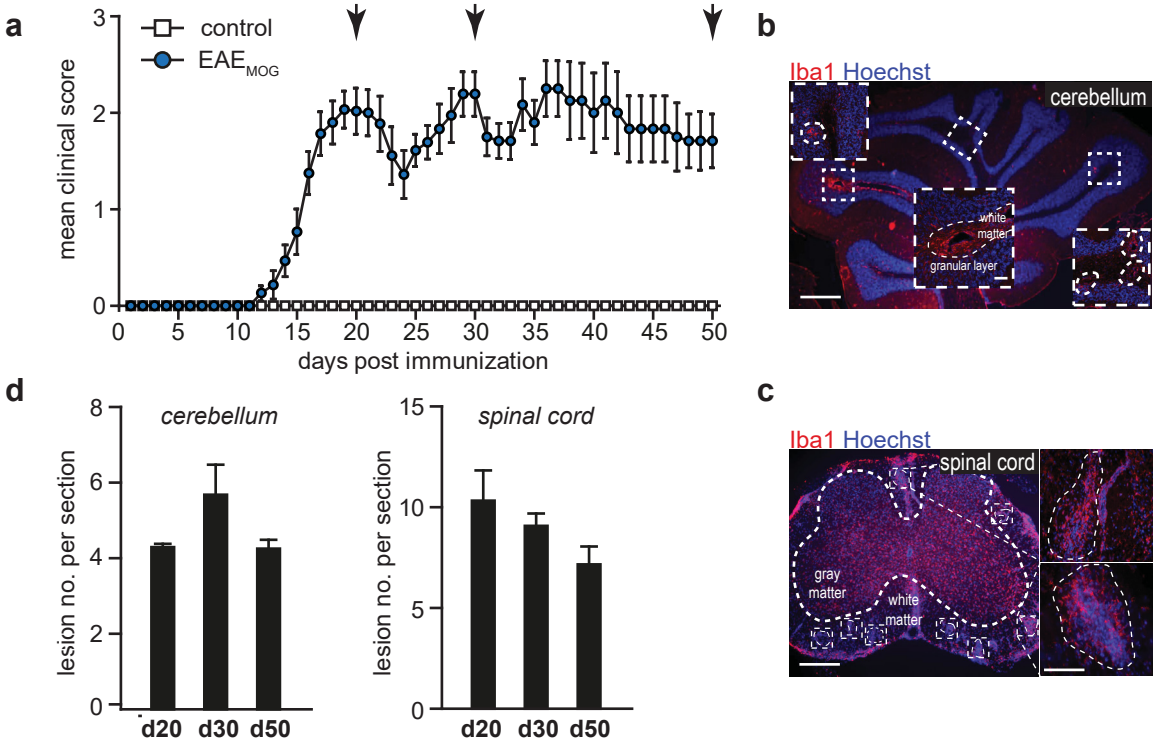

Figure 1| **Quantification of inflammatory foci in the spinal cord and cerebellum of EAE mice.**
(a) Disease course in Axin2$^{lacZ/+}$ mice. EAE was induced in female reporter mice on the C57BL/6 genetic background by immunization with MOG_{35-55} peptide emulsified in complete freund's adjuvant (CFA), followed by the PTX injections. Control group were injected with CFA and PTX. Data are shown as a mean clinical score ± standard error (SEM). Arrows indicate the time points when mice were sacrificed for further analysis: day 20, day 30 and day 50 post immunization. (b) Iba1 immunostaining of a cerebellar (left panel) and spinal cord (right panel) sections from mice at day 20 of Axin2$^{lacZ/+}$ EAE, revealing perivascular lesions (highlighted by the white dashed line) mainly in white matter tissue. Nuclei were counterstained with Hoechst. Scale bar, 100 µm in insets and 1000 µm in the overview images. (c)+(d) Quantification of lesion number characterized by perivascular infiltrates and Iba1 immunoreactivity in the cerebellum and spinal cord. Histogram represents the mean of lesions per section. For spinal cord: Day 20 (n = 4; 15-35 sections per mouse), day 30 (n = 3; 10-23 sections per mouse), day 50 (n = 3; 10-17 sections per mouse). For cerebellum: Day 20 (n = 4; 7-11 sections per mouse), day 30 (n = 4; 16-35 sections per mouse), day 50 (n = 3; 7-15 sections per mouse).

Upon immunization, Axin2$^{lacZ/+}$ reporter mice developed a non-remitting chronic EAE, with an onset of disease at 11-12 days post immunization (d.p.i.). (Fig.1a). Histological examination revealed the occurrence of typical perivascular infiltrates predominantly in the white matter of the spinal cord and the cerebellum, which are characterized by increased Iba1 immunoreactivity, the marker for activated microglia and macrophages (Fig.1b+c).

Analyzing the infiltrates of macrophages and the activation of microglia is a common approach to describe the severity of neuroinflammation in different animal models. The quantifications of the number of lesions per section in the cerebellum and spinal cord of EAE in Axin2$^{lacZ/+}$ mice during the disease course showed a persistent lesion number in both CNS compartments. In the cerebellum the lesion number did not change from day 20 towards day 50; showing four lesions per section at day 20, six lesions per section at day 30 and four lesions per section at day 50 (Fig.1d).

In the spinal cord the lesion number was also not significantly changed during active EAE course, indicated by ten lesions per section at day 20, nine lesions per section at day 30 and eight lesions per section at day 50 (Fig.1d). In general, the active EAE in Axin2$^{lacZ/+}$ reporter mice showed persistent number of inflammatory foci in the CNS, which should lead to neuroinflammatory processes during the entire examined disease period in areas affected by autoimmune attacks. As the EAE in Axin2$^{lacZ/+}$ mice showed the classical phenotype of the MOG_{35-55} peptide-induced disease course (Constantinescu et al. 2011), it provides the study with a useful model to analyze the *Wnt* activity pattern in context to neuroinflammation and disease progression. The induction of EAE in Axin2$^{lacZ/+}$ mice offers the possibility for examining different CNS tissues in different stages of the disease with histological and biochemical methods with focusing on the Axin2 expression pattern, which is simply assessable by β-galactosidase analyses.

3.1.2 Microglia activation in the hippocampus in the active EAE model

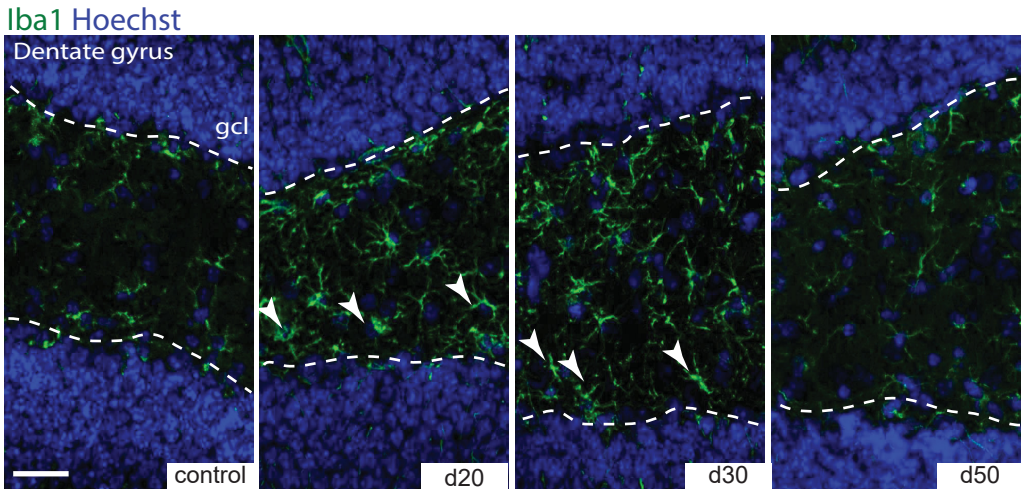

Figure 2| **Microglia activation in the hippocampus in the active EAE model.**
Representative images of Iba1 immunostaining in hippocampal section of EAE mice at days 20, 30 and 50, compared to control mice. White arrow heads indicate reactive microglia (green) located in close proximity to the granular cell layer (gcl). Nuclei were counterstained with Hoechst (blue). Scale bar: 100 µm.

In the adult brain active *Wnt* signalling is strongly associated with the hippocampus, defining *Wnt* activity as key regulator of adult neurogenesis in this brain region (Lie et al. 2005; Varela-Nallar, Inestrosa 2013). To analyze the effect of neuroinflammation on *Wnt* activity, the expression of Iba1, the microglia/macrophage marker, was examined in the dentate gyrus of EAE mice in comparison to control animals. Accordingly, hippocampal tissue was stained throughout the disease course and a higher number of Iba1-positive cells was found at days 20, 30 in the dentate gyrus of EAE mice (Fig.2). At day 50, the Iba1-activation returned to the level of control animals. Only recently EAE-induced damage in the hippocampus moved into focus, as more and more MS patients were diagnosed with cognitive impairment demanding more research in this area. The enhanced Iba1 expression is associated with more microglia and the expression of pro-inflammatory cytokines (Smith et al. 2012). By this, *Wnt* activity can be studied in the inflamed hippocampus, as stated in the aim of the study.

3.2 *Wnt* activity in Axin2$^{lacZ/+}$ mice with EAE

Wnt activity serves as an early response to inflammatory-mediated tissue injury in various organs (Whyte et al. 2012), including the CNS (Lambert et al. 2015). To study the *Wnt* activity pattern throughout the EAE course, the *Axin2-lacZ* transgenic mouse was used.

3.2.1 *Wnt* activity in the spinal cord during the active EAE disease course

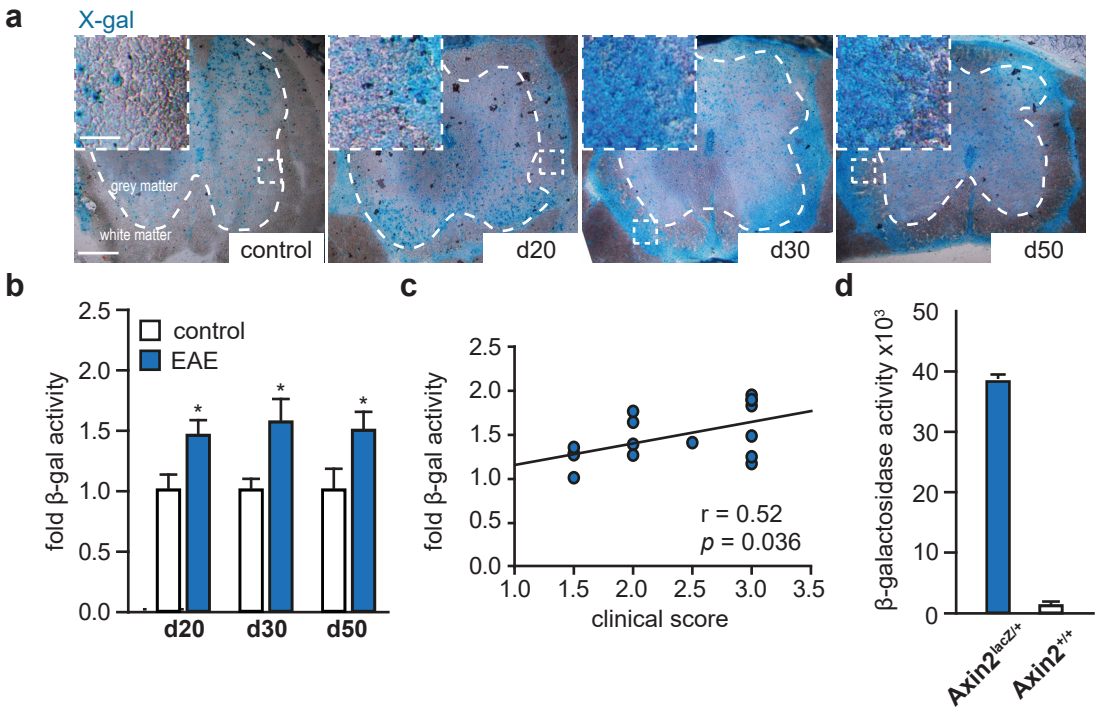

Figure 3| **Wnt activity in the spinal cord during the active EAE disease course.**
(a) Expression of lacZ and thus activation of the Axin2 promoter were detected by X-gal staining in spinal cord sections from EAE mice at days 20, 30 and 50 and respective controls. Representative images are shown. Top insets show magnified cells of the white or gray matter area. Scale bar, 100 µm in insets and 1000 µm in the overview images. (b) β-galactosidase assay of spinal cord tissue. Histogram represents mean (+ SEM) of fold-changes of β-galactosidase activity in EAE mice relative to respective controls (CFA) set as 1. Day 20 (EAE, n = 6; CFA, n = 3); day 30 (EAE, n = 4; CFA, n = 3); day 50 (EAE, n = 8; CFA, n = 4). Two-tailed, unpaired Student's t-test. *p < 0.05. (c) A regression analysis shows a Pearson correlation (r = 0.52, p = 0.036) between the β-galactosidase activity examined in the spinal cord and clinical disease score of individual EAE animals. (d) β-galactosidase assay of spinal cord tissue isolated from Axin2$^{LacZ/+}$ and Axin2$^{+/+}$ (wt) mice. Histogram represents β-galactosidase activity in arbitrary units (+ SEM) and shows the minimal signal of lacZ negative tissue.

The spinal cord is the primary location of inflammation in mice with EAE (Constantinescu et al. 2011), pointed out by the paralysis of the mouse tail, as an early clinical sign of the disease. Inflammatory foci are found in every stage of EAE (Fig.1d). The X-gal staining of the spinal cord presented a strong upregulation of Axin2 in the white matter of EAE animals (Fig.3a) and almost no expression in the white matter of control mice. X-gal staining of the grey matter showed no differences between control and EAE animals and was specifically located in regions of motor neurons (spinal cord structure reviewed in ((Harrison et al. 2013)).

Interestingly, the X-gal staining showed a similar pattern in the white matter (Fig.3a) as already seen for Iba^{+}-infiltration sites (Fig.1b), indicating a local *Wnt* activation in these inflamed spinal cord areas. The β-galactosidase activity assay demonstrated a significant upregulation of the *Wnt* pathway in all examined disease stages (Fig. 3b). The *Wnt* activity increased at day 20 to 1.45±0.10 fold, at day 30 to 1.56±0.15 fold and to 1.49±0.12 fold at day 50 (Fig. 3b). These quantitative measurements confirmed the results of the X-gal staining and also showed that β-galactosidase activity in the spinal cord tissue positively correlates with the clinical disease score of individual EAE mice (Fig.3c, Pearson correlation $r = 0.52$; $p = 0.036$). Of note, only trace amounts of β-galactosidase activity were observed in EAE tissue from wt ($Axin2^{+/+}$) mice (Fig.3d), confirming the accuracy of the β-galactosidase measurements. In sum, a sustained increase in *Wnt* activity in the spinal cord through all examined EAE stages (Fig.3a+b) can be correlated with a large number of inflammatory foci (Fig.1d). Upregulation of *Wnt* activity in the spinal cord evaluated by the use of the Axin2-lacZ-reporter mouse extended previous findings demonstrating the induction of β-catenin and *Wnt3a* in this tissue in EAE mice (Yuan et al. 2012).

3.2.2 *Wnt* activity in the cortex during the active EAE disease course

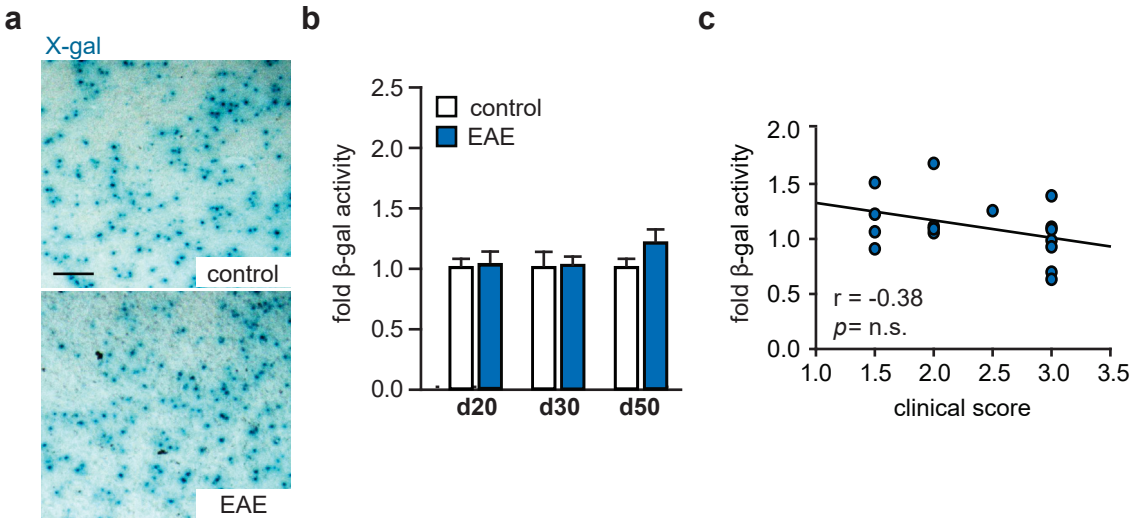

Figure 4| **Wnt activity in the cortex during the active EAE disease course.**
(a) Expression of lacZ and thus activation of the Axin2 promoter were detected by X-gal staining in cortex sections from EAE mice at day 20 and respective controls. Representative images are shown. Scale bar, 100 µm. (b) β-galactosidase assay of brain tissue isolated from frontal/motor cortex area revealed no significant changes. Histogram represent mean (+ SEM) of fold-changes of β-galactosidase activity in EAE mice relative to respective controls (CFA) set as 1. Day 20 (EAE, n = 6; CFA, n = 3); day 30 (EAE, n = 4; CFA, n = 3); day 50 (EAE, n = 8; CFA, n = 4). (c) A regression analysis of cortical tissue shows no correlation (r = -0.38, p = n.s.) between the β-galactosidase activity and clinical disease score in individual EAE animals.

The investigation of the motor cortex of EAE animals, a brain area normally not affected by the autoimmune attack (Constantinescu et al. 2011), was used to assess the *Wnt* activity pattern in the disease course. Indeed, no visible changes in the X-gal staining were detected between EAE and control mice in cortical brain regions (Fig.4a) and the β-galactosidase activity assay likewise did not show significant differences in *Wnt* activity during the disease (Fig.4b). Conclusively, there was no correlation between clinical disease scores and cortical *Wnt* activity (Fig.4c). Measurements in the cortex, serving our investigation as an internal "negative control", displayed that unharmed brain tissue has no detectable changes in *Wnt* signalling activity during EAE.

3.2.3 *Wnt* activity in the hippocampus during the active EAE disease course

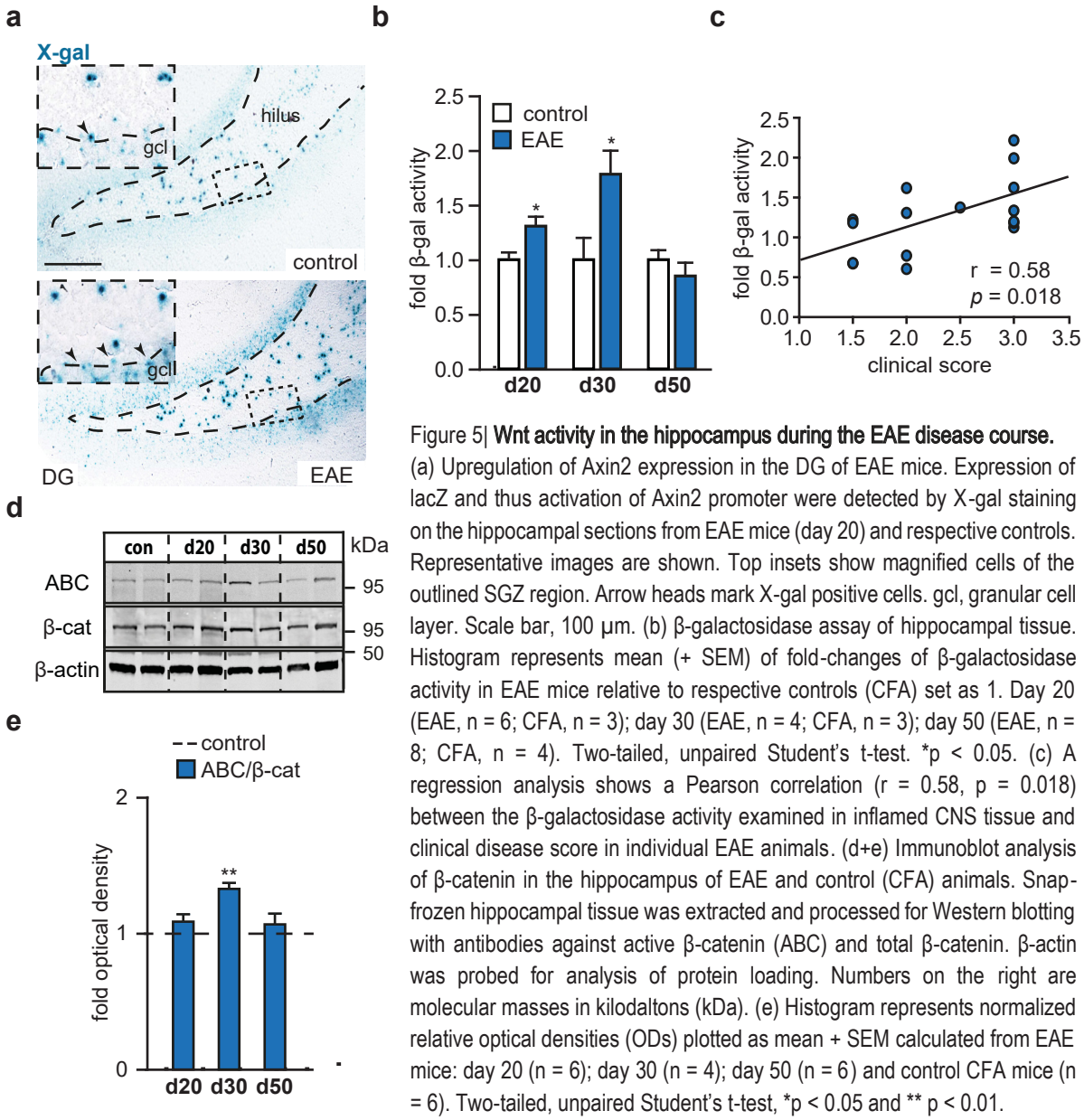

Figure 5| **Wnt activity in the hippocampus during the EAE disease course.** (a) Upregulation of Axin2 expression in the DG of EAE mice. Expression of lacZ and thus activation of Axin2 promoter were detected by X-gal staining on the hippocampal sections from EAE mice (day 20) and respective controls. Representative images are shown. Top insets show magnified cells of the outlined SGZ region. Arrow heads mark X-gal positive cells. gcl, granular cell layer. Scale bar, 100 µm. (b) β-galactosidase assay of hippocampal tissue. Histogram represents mean (+ SEM) of fold-changes of β-galactosidase activity in EAE mice relative to respective controls (CFA) set as 1. Day 20 (EAE, n = 6; CFA, n = 3); day 30 (EAE, n = 4; CFA, n = 3); day 50 (EAE, n = 8; CFA, n = 4). Two-tailed, unpaired Student's t-test. *p < 0.05. (c) A regression analysis shows a Pearson correlation (r = 0.58, p = 0.018) between the β-galactosidase activity examined in inflamed CNS tissue and clinical disease score in individual EAE animals. (d+e) Immunoblot analysis of β-catenin in the hippocampus of EAE and control (CFA) animals. Snap-frozen hippocampal tissue was extracted and processed for Western blotting with antibodies against active β-catenin (ABC) and total β-catenin. β-actin was probed for analysis of protein loading. Numbers on the right are molecular masses in kilodaltons (kDa). (e) Histogram represents normalized relative optical densities (ODs) plotted as mean + SEM calculated from EAE mice: day 20 (n = 6); day 30 (n = 4); day 50 (n = 6) and control CFA mice (n = 6). Two-tailed, unpaired Student's t-test, *p < 0.05 and ** p < 0.01.

The neurobiology of the hippocampus is intensely connected to *Wnt* activity, as *Wnt* signalling is an important regulator of adult neurogenesis (Lie et al. 2005; Varela-Nallar, Inestrosa 2013), which is one main function of this brain area (van Praag et al. 2002). The impact of EAE on this *Wnt*-dependent hippocampal function was the central focus of this thesis.

First, to characterize the Axin2 expression pattern, a X-gal substrate solution was applied, that revealed increased signal in cells of the hilus, granular layer and SGZ of active immunized mice (Fig. 5a). More Axin2-positve cells in the SGZ of EAE mice were mainly located in areas with neural progenitor cells (Fig. 5a). Next, quantification of *Wnt* activity by applying β-galactosidase substrate assay showed upregulation of Axin2 only at days 20 and at day 30 after disease induction to 1.31±0.06 fold and 1.78±0.17 fold, respectively (Fig.5b).

In the chronic stage of disease (50 d.p.i.), no change in *Wnt* activity has been observed in the hippocampus, indicating that the pathway activation occurs transiently and mainly in acute state (Fig.5b) despite persistent Iba1 expression (Fig.2). As seen for the spinal cord (Fig.3c), hippocampal β-galactosidase activity also positively correlated with clinical disease scores in individual mice, indicating a solid connection between *Wnt* activity and disease severity (Fig.5c, Pearson correlation, $r = 0.58$; $p = 0.018$). To confirm the results of the β-galactosidase analysis, activation state of the *Wnt* signalling mediator, β-catenin (Fig. II and Niehrs 2012), was examined in the hippocampal tissue of naïve and EAE mice by Western blotting. The ligation of *Wnt* ligands, involved in the canonical pathway, induces stabilization (dephosphorylation) of β-catenin via inactivation (phosphorylation at Ser9) of GSK3β, one member of the β-catenin destructive complex (Fig. II and Niehrs 2012). The protein analysis supported the results of the enzymatic assay, because a significant increase at day 30 in the ratio of active β-catenin (ABC)/total β-catenin to 1.32±0.04 fold was detected in EAE mice as compared to their respective controls (Fig.5d+e).

Taking together, these results of the chemiluminescent and protein analysis indicate the transient upregulation of hippocampal *Wnt* signalling in acute stages of EAE (20-30 d.p.i.) with a significant correlation to the disease severity.

3.3 *Wnt* signalling ligands and its activity in passive EAE

The first part of the study described the *Wnt* activity pattern in the active model of EAE, giving new insights in *Wnt*-dependent mechanisms effected by autoimmune-induced neuroinflammation in different brain areas. To exclude the possible confounders associated with the immunization process, the next examination was performed in the passive model of EAE, which is induced by adoptive transfer of myelin specific T cells without the use of CFA and pertussis toxin (see 2.2.2 and (Stromnes, Goverman 2006b)).

3.3.1 *Axin2* gene expression in the course of the passive EAE model

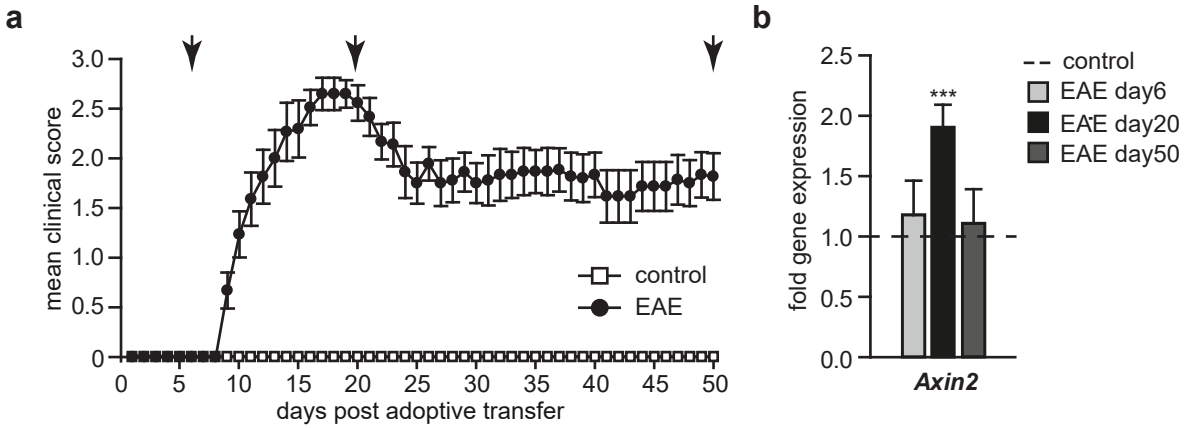

Figure 6| **Axin2 gene expression in the course of the passive EAE model.**
(a) Disease course in passively transferred EAE in mice on SJL/J background. Female SJL/J mice received activated encephalitogenic T lymphocytes derived from immunized donors and re-stimulated with $PLP_{139–151}$ peptide. Control mice were injected with PBS. Data are shown as mean clinical score ± SEM. Arrows indicate the time points when mice were sacrificed for further analysis: days 6, 20 and 50 after adoptive transfer. (b) Quantitative PCR analysis (qPCR) shows transient upregulation of Axin2 gene expression in the hippocampus of mice with passively transferred EAE. Data represent mean +SEM of fold-changes of gene expression in EAE mice relative to respective controls set as 1. Transcription was normalized to GAPDH. Day 6 (EAE, n = 5; control, n = 4); day 20 (EAE, n = 6; control, n = 8); day 50 (EAE, n = 12; control, n = 11). Two-tailed, unpaired Student's t-test, *** $p < 0.001$.

To confirm previous findings of the $Axin2^{lacZ/+}$ EAE in the active model, the messenger RNA (mRNA) expression of *Axin2* at similar time points were analyzed in the adoptive transfer EAE model, adding day 6, a disease stage without any visible clinical signs, and sparing day 30, as in this passive EAE no relapses were evident (Fig.6a). Indeed, again the transient upregulation of hippocampal *Axin2* on transcriptional level was observed (Fig.6), supporting our previous findings and indicative for active *Wnt* signaling only in the early stage of disease (20 d.p.i.). At day 6 and day 50, the *Axin2* gene expression did not show any difference compared to control animals (injected only with PBS) (Fig.6b).

3.3.2 Hippocampal gene expression analysis of *Wnt* ligands in passive EAE

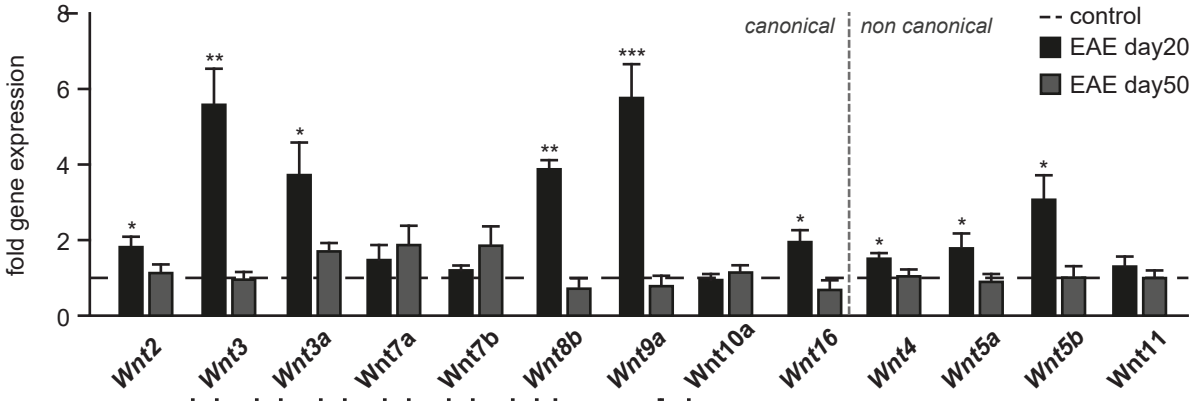

Figure 7| **Gene expression analysis of Wnt ligands in passive EAE.** qPCR analysis of selected Wnt ligand genes in the hippocampus at early (day 20) and chronic (day 50) stage of passive EAE. Histogram represents mean +SEM of fold-changes relative to control group set as 1. Transcription was normalized to GAPDH. Day 20 (EAE, n = 4 - 6; control, n = 4); day 50 (EAE, n = 8 - 13; control, n = 7-11). Two-tailed, unpaired Student's t-test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The *Wnt* gene family encodes for 19 cysteine-rich secreted signaling molecules orchestrating diverse cellular processes in the adult brain including self-renewal and cell fate determination of stem/progenitor cells, synaptic plasticity and tissue homeostasis. β-catenin stability and context-dependent transcriptional activity of *Axin2* and other direct target genes is regulated by certain *Wnt* ligands (Inestrosa, Arenas 2010). The expression of *Wnt* ligands is known to be upregulated upon inflammation and may play a role in neuroimmune interactions (Marchetti, Pluchino 2013). To determine the particular *Wnt* ligands activated in the hippocampus of passive immunized EAE mice, a gene expression profiling analysis was performed (Fig.7). At day 20, EAE mice exhibited quantitative differences in expression levels of a number of *Wnt* ligand genes relevant for the canonical β-catenin-dependent pathway (*Wnt2*, *Wnt3*, *Wnt3a*, *Wnt8b*, *Wnt9a* and *Wnt16*; fold inductions ranging from 2 to 6) and ligands acting through β-catenin-independent mechanisms (*Wnt4*, *Wnt5a*, *Wnt5b*; fold inductions ranging from 1.5 to 3.5) (Fig.7). Other *Wnt* ligands found to be expressed in the hippocampus exhibited no significant differences as compared to controls (Fig.7). Comparable to the transient profile of enhanced *Wnt* signalling evaluated in immunized mice (Fig.5b+e), the expression level of *Wnt* ligands in chronic state of passive EAE returned to the one observed in control animals (Fig.7). Consistent with previous reports focusing on other CNS regions like the spinal cord, in the hippocampus, acute states of EAE are associated with the induction of several *Wnt* ligands that are known to trigger *Wnt*/β-catenin activity (Inestrosa, Arenas 2010).

3.4 Hippocampal damage and neuroinflammation in EAE

Wnt signalling regulates the first response to inflammatory-mediated tissue injury in different organs (Whyte et al. 2012) and is activated in various CNS diseases (Lambert et al. 2015). The hippocampal pathology in MS is associated with demyelination, decreased synaptic density, neuronal loss and microglia activation (Aktas, Hartung 2011; Dutta et al. 2011). To correlate former results on *Wnt* signalling, the grade of hippocampal damage and neuroinflammation in both EAE models was investigated. In the active model, Western blot analysis for specific neuronal markers were performed and in the passive model, microglia activation and the expression profile of distinct cytokines defining the neuroinflammatory milieu were studied.

3.4.1 Hippocampal synaptic and neuronal damage in the active EAE model

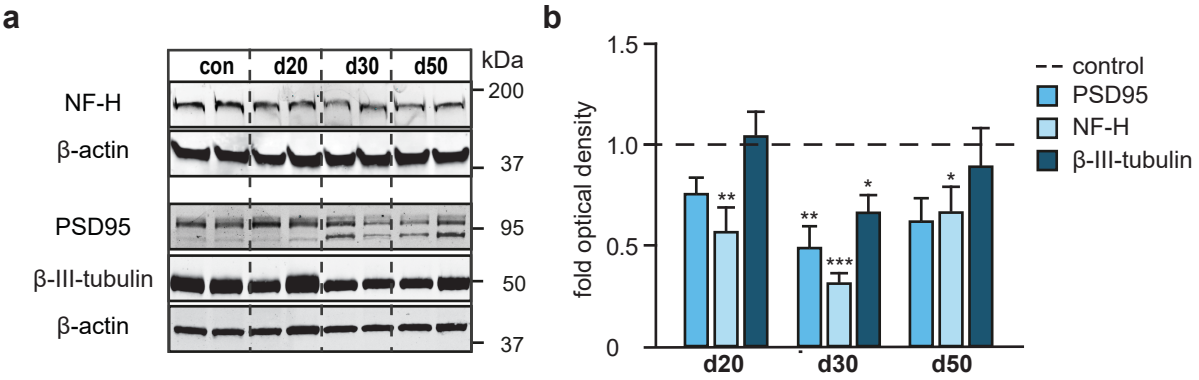

Figure 8| **Hippocampal synaptic and neuronal damage in the active EAE model.**
(a+b) Immunoblot analysis of neuronal proteins in the hippocampus of Axin2$^{lacZ/+}$ EAE and control (CFA) animals. Snap-frozen hippocampal tissues were extracted and processed for Western blotting with antibodies against postsynaptic protein 95 (PSD-95), neurofilament-H (NF-H) and β-III-tubulin. β-actin was probed for analysis of protein loading. Numbers at right are molecular masses in kilodaltons (kDa). (b) Histogram represents normalized relative optical densities (ODs) plotted as mean + SEM calculated from EAE mice: day 20 (n = 6); day 30 (n = 4); day 50 (n = 6) and control CFA mice (n = 6). Two-tailed, unpaired Student's t-test, *p < 0.05 and ** p < 0.01

To take a closer look at hippocampal neuronal injury in active EAE, the analysis of postsynaptic PSD95, axonal marker neurofilament H (NF-H) and neuronal marker β-III-tubulin protein levels were performed (Fig.8). In line with findings observed in MS patients with hippocampal demyelination (Dutta et al. 2011), the protein contents of PSD95 were reduced throughout all stages of disease, significantly at day 30 to 0.48±010 fold (Fig.8a+b). Similarly, a persistent decreased level of NF-H (Fig.8a+b) was found, indicating an early occurring neuronal injury that is not compensated in chronic state of non-remitting EAE (Fig.1a). The β-III-tubulin protein levels were significantly downregulated only at day 30, the disease stage with the most damage of neuronal and synaptic structures.

This Western blot analysis extends previous reports, documenting hippocampal damage of neuronal and synaptic structures in the CA1 region of EAE animals by using TUNEL stainings co-labeled with the neuronal marker NeuN and PSD95 immunohistochemistry (Ziehn et al. 2010; Ziehn et al. 2012).

3.4.2 Neuroinflammation in the hippocampus of the passive EAE model

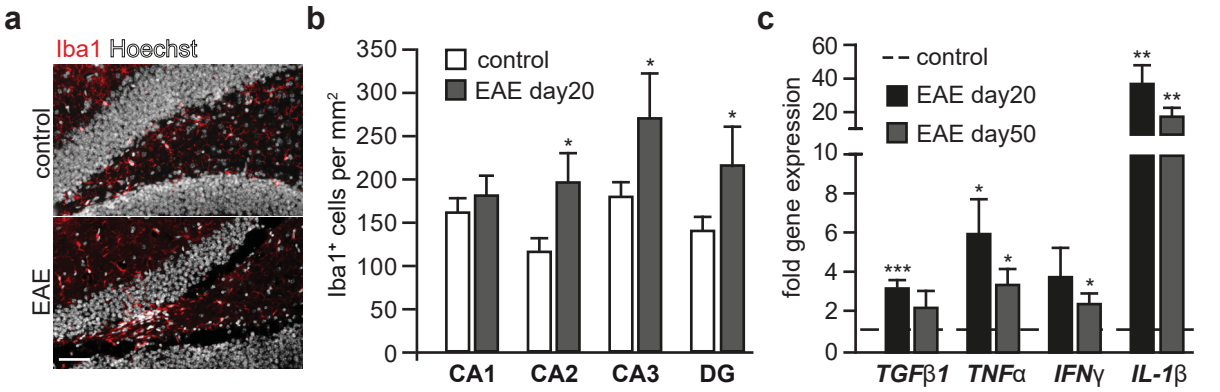

Figure 9| **Neuroinflammation in the hippocampus of the passive EAE model.**
(a) Representative image of perivascular lesion in the DG. Iba1 (red) immunostaining in hippocampal section from EAE mice at the day 20. Nuclei were counterstained with Hoechst (grey). Scale bar, 50 µm. (b) Quantification of microglial cells stained for Iba1 in the cornu ammonis 1-3 (CA1-3) and dentate gyrus (DG) of EAE and control (CFA) animals at day 20. Data represents mean +SEM of Iba1-positive cells per mm². EAE (n = 3) and control (n = 3); Two-tailed, unpaired Student's t-test, * $p < 0.05$. (c) qPCR analysis of gene expression for inflammatory cytokines in the hippocampus at early (day 20) and chronic (day 50) stage of passive EAE. Histogram represents mean +SEM of fold-changes relative to control (PBS) group set as 1. Expression was normalized to GAPDH. Day 20 (EAE, n = 3 - 6; control, n = 3 - 8); day 50 (EAE, n = 9 - 10; control, n = 6 - 7). Two-tailed, unpaired Student's t-test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In the passive EAE model, the lack of hippocampal damage was also evident in the chronic disease state, defined by an increased number of Iba1-positive cells in the hippocampus (Fig.9a+b). Despite its rare occurrence, hippocampal tissue stainings showed perivascular lesions enriched for Iba1-immunoreactive cells located in close proximity to the SGZ, the germinative zone of the DG at day 20 (Fig.9a+b). Quantification of Iba1-positive cells per mm² hippocampus in different areas showed an overall increase in microglia activation in the acute disease state at day 20 (Fig.9b).

The increased number of Iba1⁺ microglia/macrophages were observed in three areas of the hippocampus: in cornu ammonis 2 (CA2) Iba1⁺ cell number extended to 196±19 cells per mm² compared to 111±6 cells in control animals; in CA3 Iba1⁺ cell number increased to 308±29 cells per mm² compared to 180±32 cells in control animals and in DG Iba1⁺ cell number increased to 215±26 cells per mm² compared to 141±4 cells in control animals (Fig.9b).

Furthermore, mRNA gene expression analysis of animals suffering from adoptive transfer EAE presented a significant upregulation of inflammatory mediators (TNFα, IFNγ and interleukin-1 beta (IL-1β)) at day 20 and day 50 of the disease in the hippocampus (Fig.6a+9c). Additionally, the mRNA levels of TGFβ1, an anti-inflammatory cytokine, were significantly induced in early stages of EAE (Fig.9c) consistent with its CNS specific permissive role for initiation of the disease (Luo et al. 2007).

In summary, these data indicate that upregulation of *Wnt* activity coincides with microglia activation, occurring throughout all examined EAE stages, neuronal/synaptic protein damage and increased neuroinflammatory cytokine expression in the early and late state of EAE.

3.5 Adult neurogenesis in EAE

In the adult brain the *Wnt*/β-catenin pathway regulates proliferation and self-renewal of hippocampal stem/progenitor cells implicating its role in the neurogenic response to various physiological and pathological conditions (Lambert et al. 2015). Hence, the next investigation focused on whether an increase in *Wnt* activity would also trigger proliferation and generation of hippocampal neuronal precursor cells and subsequently regulate the pool of new-born neurons in the hippocampus of EAE mice.

3.5.1 Hippocampal gene expression analysis of neurogenic factors in EAE

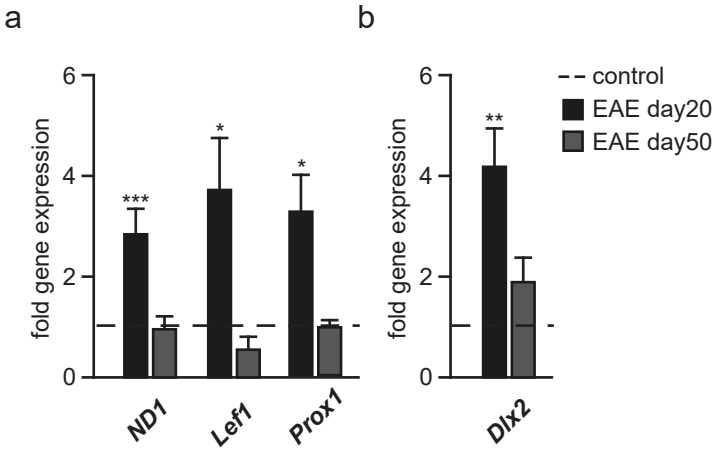

Figure 10| **Hippocampal gene expression analysis of neurogenic factors in passive EAE.** (a+b) qPCR analysis shows upregulated transcription of Wnt-dependent genes (NeuroD1 (ND1), Prox1, Lef1) (a) and Dlx2, a marker of transient amplifying neuroblasts (b). Histograms represent mean +SEM of fold-changes in gene expression of EAE mice relative to control (PBS) group set as 1. GAPDH was used as an endogenous control. Day 20 (EAE, n = 4 - 8; control, n = 4 - 8); day 50 (EAE, n = 4 - 10; control, n = 4 - 11). Two-tailed, unpaired Student's t-test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

First, the gene expression of known downstream targets of β-catenin/Tcf transcriptional activity (Fig.10a) and pro-neurogenic factors (Fig.10a+b) were examined in the adoptive transfer EAE model (Fig.6a). Indeed, at an early stage of disease (20 d.p.i) corresponding to the enhancement of *Wnt* activity a significant upregulation of β-catenin-dependent genes was detected with NeuroD1/ND1, induced to 2.84+0.46-fold, Lef1, induced to 3.72+0.99 fold and prospero homeobox 1 (Prox1), induced to 3.24+0.69-fold (Fig.10a). In line, the mRNA level of the pro-neurogenic factor distal-less homeobox 2 (Dlx2) was also significantly upregulated to 4.18+0.72 fold in the acute EAE phase (Fig.10b), indicating again an increase of neuronal processes in the hippocampus. In general, the hippocampal gene expression profile during EAE showed an activation of neurogenesis in the same time frame of elevated *Wnt* signalling.

3.5.2 Proliferation analysis of progenitor cells in the acute EAE stage

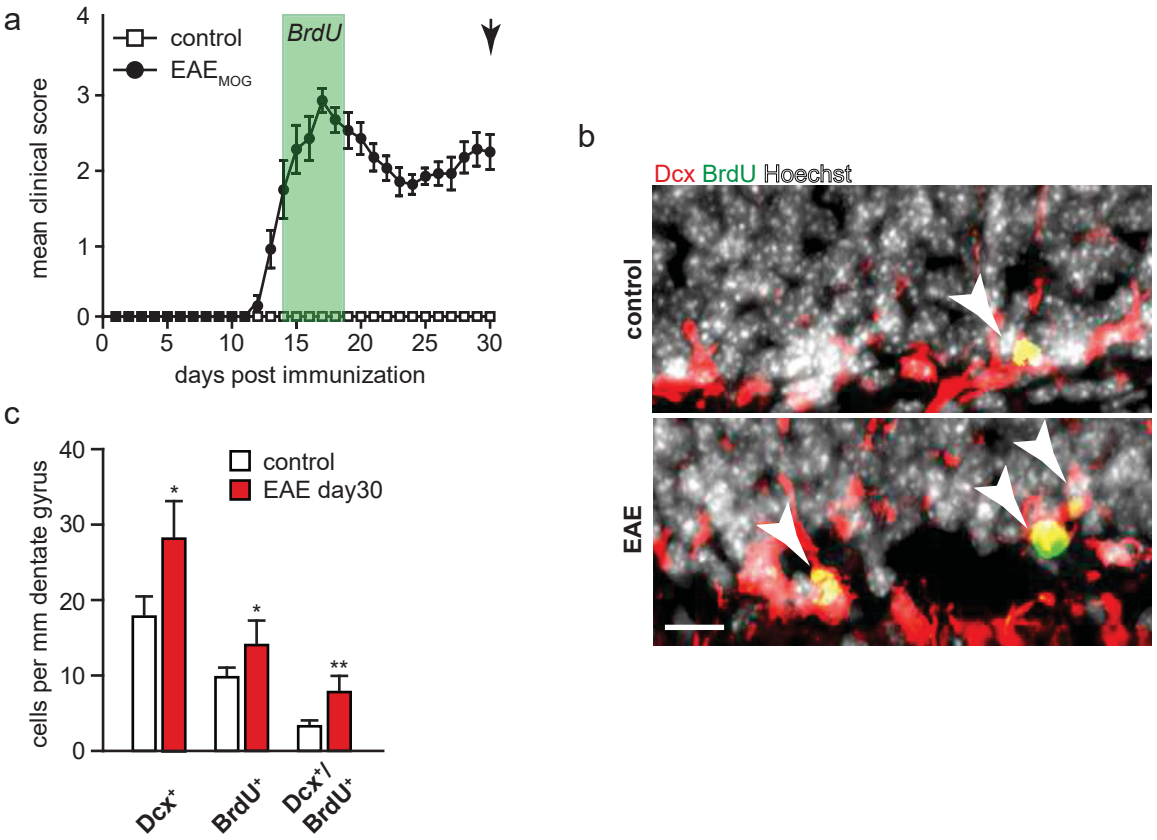

Figure 11| **Analysis of BrdU⁺/Dcx⁺ progenitor cells in the active EAE model.**
(a) Disease course of C57/Bl6 mice with active induced EAE by immunization with encephalitogenic MOG_{35-55}. Control group was injected with CFA and PTX. Data are shown as a mean clinical score ± SEM. In acute phase (days 15 – 20; highlighted by green area) EAE and control mice received daily i.p. injections of BrdU (50 mg/kg body weight). After a 2-weeks period without administration of BrdU, mice were sacrificed for histological analysis (day 30, marked by black arrow). (b) Histological analysis of proliferating cells in the DG of EAE mice. Immunostaining for BrdU (green) and Dcx (red) in hippocampal sections. Arrow heads indicate BrdU⁺/Dcx⁺ co-labeled neuronal progenitors. Nuclei were counterstained with Hoechst (grey). Scale bar, 50 µm. (c) The frequency of BrdU label-retaining cells in the DG significantly increased in mice with EAE (day 30; n = 3 mice; 18 - 28 sections per mouse) as compared to control mice (n = 3 mice; 17 - 28 sections per mouse). Data is shown as mean + SEM of positive cells per mm of DG. Two-tailed, unpaired Student's t-test,* $p < 0.05$ and ** $p < 0.01$.

The standard technique to analyse adult neurogenesis *in vivo*, is the administration of BrdU with i.p. injections. By this it is possible to trace and label proliferating cells (Sahay et al. 2011). To mark the pool of neuronal progenitor cells in the hippocampus the neurogenic marker doublecortin, expressed specifically in progenitors destined to the neuronal fate (Ming, Song 2011; Kempermann et al. 2015), was used. According to early activation of the *Wnt* signalling (Fig.5-7), BrdU was administered at 15 d.p.i., the peak of disease, for five consecutive days, followed by a 2-weeks period without BrdU (Fig.11a). A significant increase in the total number of label-retaining cells in the dentate gyrus (14 ± 2 cells/mm in EAE mice; 10 ± 1 cells/mm in control mice) was found. Similarly, the frequency of newly generated neuroblasts, measured by co-labelling of BrdU⁺-retaining cells with Dcx, were significantly enhanced in EAE mice (8 ± 2 cells/mm of DG in EAE mice; 4 ± 1 cells/mm of DG in control mice) (Fig.11b+c).

Collectively, these results support the previous findings in MOG_{35-55}-induced EAE mice, showing enhancement of neurogenesis during the acute phase (Huehnchen et al. 2011) and are consistent with the idea that increased hippocampal *Wnt* activity is relevant for activation of adult neurogenesis in acute disease states.

3.6 Analysis of *Wnt* activity in organotypic slice cultures (OSCs)

Although the induction of *Wnt* signalling has been previously reported in context to various tissue damaging paradigms (Lambert et al. 2015; Whyte et al. 2012), the precise mechanisms triggering *Wnt* activity still remain unknown. Here, using Axin2$^{lacZ/+}$ reporter mice, the possible contribution of various inflammatory factors promoting *Wnt* activity in neural tissue was characterized. Furthermore the results were connected to inflammation-induced *Wnt* activity with hippocampal function by using Nestin^{eGFP} mice.

3.6.1 *Wnt* activity in cerebellar OSCs after LPC-demyelination

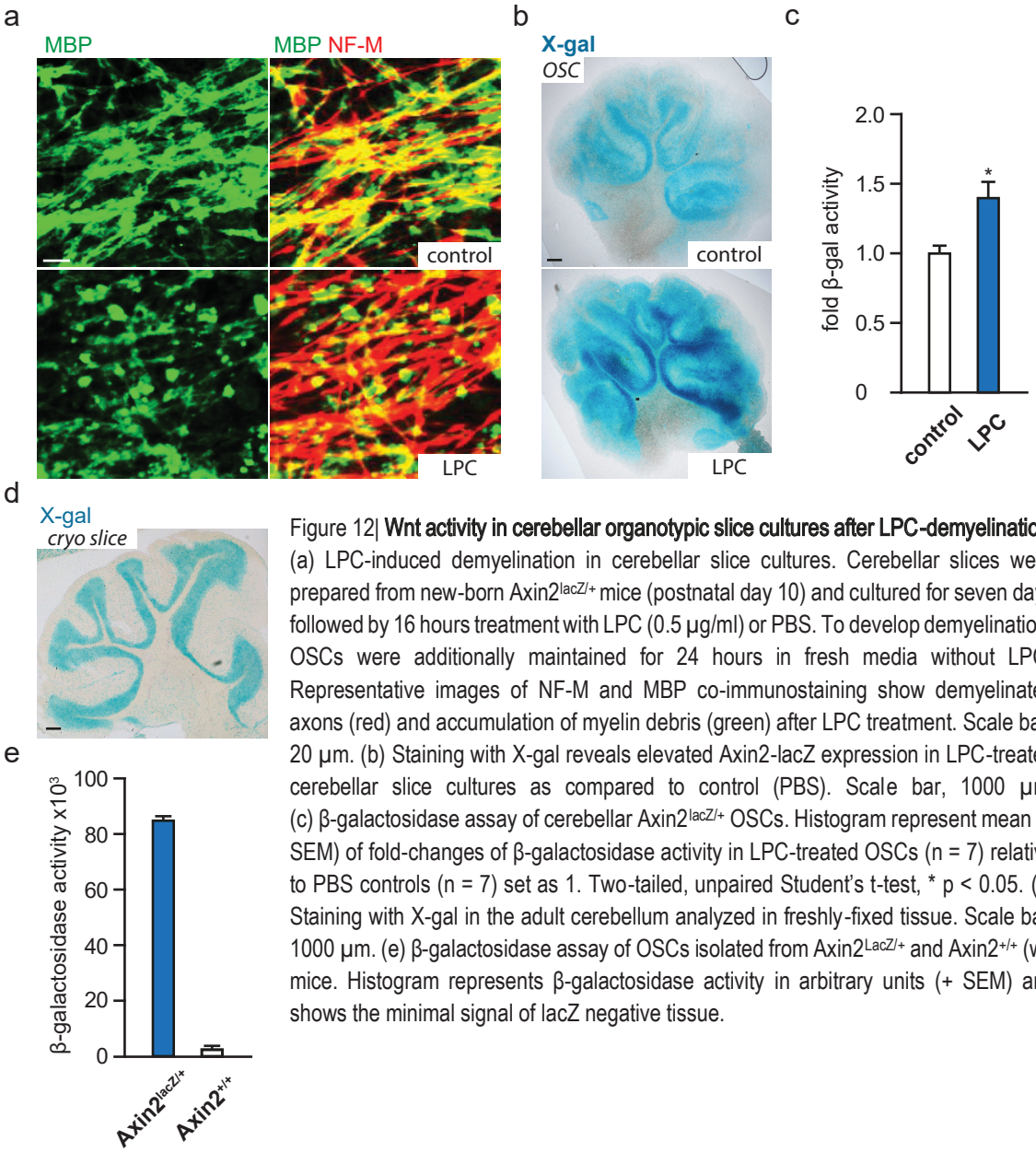

Figure 12| **Wnt activity in cerebellar organotypic slice cultures after LPC-demyelination.** (a) LPC-induced demyelination in cerebellar slice cultures. Cerebellar slices were prepared from new-born Axin2$^{lacZ/+}$ mice (postnatal day 10) and cultured for seven days followed by 16 hours treatment with LPC (0.5 µg/ml) or PBS. To develop demyelination, OSCs were additionally maintained for 24 hours in fresh media without LPC. Representative images of NF-M and MBP co-immunostaining show demyelinated axons (red) and accumulation of myelin debris (green) after LPC treatment. Scale bar, 20 µm. (b) Staining with X-gal reveals elevated Axin2-lacZ expression in LPC-treated cerebellar slice cultures as compared to control (PBS). Scale bar, 1000 µm. (c) β-galactosidase assay of cerebellar Axin2$^{lacZ/+}$ OSCs. Histogram represent mean (+ SEM) of fold-changes of β-galactosidase activity in LPC-treated OSCs (n = 7) relative to PBS controls (n = 7) set as 1. Two-tailed, unpaired Student's t-test, * $p < 0.05$. (d) Staining with X-gal in the adult cerebellum analyzed in freshly-fixed tissue. Scale bar: 1000 µm. (e) β-galactosidase assay of OSCs isolated from Axin2$^{LacZ/+}$ and Axin2$^{+/+}$ (wt) mice. Histogram represents β-galactosidase activity in arbitrary units (+ SEM) and shows the minimal signal of lacZ negative tissue.

To determine if acute demyelination has an effect on *Wnt* signalling, cerebellar OSCs, isolated from Axin2$^{lacZ/+}$ pups, were established and exposed to lysolecithine (LPC). Treatment with 0.5 mg/ml LPC for 16 hours induced axonal demyelination (indicated by destroyed myelin basic protein (MBP)) without visible neuronal damage (observed by neurofilament (NF-M)) when compared to control slices (Fig.12a) and previously documented microglia activation (Birgbauer et al. 2004). The examined X-gal staining of cerebellar OSCs (Fig.12b) was similar to freshly-fixed cerebellar tissue, presenting a pattern of Axin2-positve cells mostly confined to the neuronal compartment of the cerebellar granular layer (Fig.12d). In demyelinated cerebellar OSCs the X-gal staining showed a strong upregulation of Axin2 compared to healthy OSCs (Fig.12b). Analysis of β-galactosidase activity 24 hours following LPC removal confirmed the histological observation and revealed a significant increase in *Wnt* signalling activity as compared to control slice cultures (1.41 ± 0.11-fold) (Fig.12c). Of note, only trace amounts of β-galactosidase activity were observed in OSCs isolated from wt (Axin2$^{+/+}$) mice (Fig.12e), confirming the accuracy of the β-galactosidase measurements. These results indicate that conditions associated with demyelination and microglia activation are sufficient to induce *Wnt*/β-catenin activity in responsive cells, showing a possible trigger for *Wnt* signalling stimulation.

3.6.2 *Wnt* activity in hippocampal OSCs after neuroinflammatory stimuli

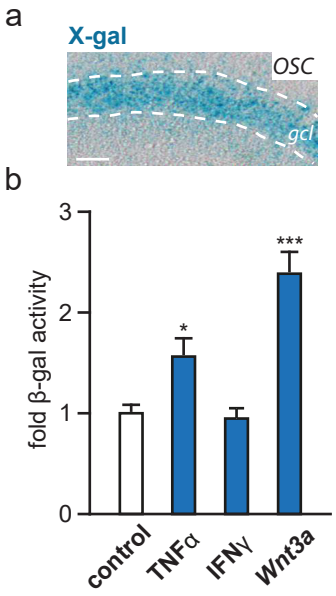

Figure 13| **Wnt activity in hippocampal organotypic slices after neuroinflammatory stimuli.** (a) X-gal staining of 7-days cultured untreated hippocampal slice culture shows Axin2 expression predominantly in the granular cell layer (gcl). The representative image of the DG is shown. Scale bar, 50 µm. (b) β-galactosidase assay of hippocampal OSCs. Hippocampal slices were prepared from Axin2$^{lacZ/+}$ mice (postnatal day 10) and cultured for seven days followed by treatment with Wnt3a (20 ng/ml) or inflammatory cytokines: TNFα (1 ng/ml) or IFNγ (100 U/ml). After six hours of treatment the medium was replaced with fresh cytokine-free media and OSCs were cultured for additional 24 hours. Histogram represents mean (+ SEM) of fold-changes of β-galactosidase activity after treatment with Wnt3a (n= 15), TNFα (n = 14) or IFNγ (n = 14) relative to PBS controls (n = 20) set as 1. Two-tailed, unpaired Student's t-test, * $p < 0.05$ and *** $p < 0.001$.

Next, to elucidate the impact of particular cytokines, known to be secreted by activated microglia and macrophages (Moreno et al. 2011), the experimental setup switched to hippocampal slice cultures isolated from Axin2$^{lacZ/+}$ pups. The cultured hippocampal slices comprise the adult germinative zone (Fig.13a), offering a useful model to study neurogenic processes *ex vivo*. In addition to the well-characterized effect of *Wnt3a*, in those experiments, the focus laid on the role of TNFα and IFNγ knowing their beneficial role in demyelinating pathologies (Arnett et al. 2001; Lees, Cross 2007). Treatment with TNFα or *Wnt3a* for six hours and subsequent incubation of hippocampal slices in cytokine-free media for 24 hours led to a significant upregulation of *Wnt* activity (1.56 ± 0.20 fold and 2.39 ± 0.18 fold, respectively) (Fig.13b). Interestingly, *Wnt* pathway activity was not enhanced after the treatment with the other pro-inflammatory cytokine, IFNγ (Fig.13b), suggesting a specific link between TNFα and *Wnt* and providing an additional trigger for *Wnt* signalling stimulation in EAE.

3.6.3 Neurogenesis in hippocampal OSCs after inflammatory stimuli

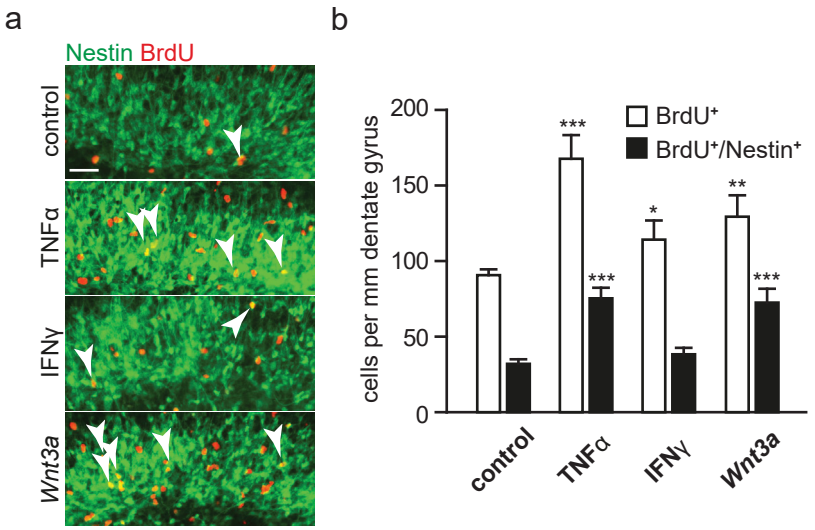

Figure 14| **Neurogenesis in hippocampal organotypic slices after neuroinflammatory stimuli.**
(a) Histological analysis of proliferating cells in the DG of hippocampal Nestin^{eGFP} OSCs. BrdU was administered to slice culture for 24 hours prior to treatments with cytokines (performed as described for Fig. 13). Representative images show the immunostaining for BrdU (red) and eGFP (green). Arrow heads indicate BrdU$^+$/eGFP$^+$ co-labeled newly generated hippocampal progenitors. Scale bar, 50 µm. (b) The frequency of BrdU label-retaining cells in the DG of hippocampal Nestin^{eGFP} OSCs significantly increased after treatment with TNFα (1 ng/ml) or Wnt3a (20 ng/ml) as compared to control cultures. BrdU was administered to slice culture for 24 hours prior to treatments with cytokines. After six hours of treatment the medium was replaced with fresh cytokine-free media and OSCs were cultured for additional 24 hours. Data are shown as mean (+SEM) of BrdU$^+$ or BrdU$^+$/eGFP$^+$ cells per mm of DG. Control (n = 15 – 20 slices); TNFα (n = 3 – 9 slices), IFNγ (n = 4 – 11 slices) and Wnt3a (n = 3 – 9 slices); Two-tailed, unpaired Student's t-test,* * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

To investigate the link between TNFα and *Wnt* activity, further experiments focused on whether TNFα-dependent *Wnt* stimulation could also impact hippocampal progenitor proliferation in the slice culture model isolated from Nestin^{eGFP} pups (Fukuda et al. 2003). To assess the proliferation of stem/progenitor cells in one-week cultured slices, slices were pretreated with BrdU for 24 hours prior to six hour exposure to the individual cytokine or the positive control *Wnt3a*. After 24 hours incubation in cytokine-free media, histological analysis was applied to evaluate the number of BrdU-retaining neural progenitor cells (eGFP$^+$) (Fig.14a). Treatment with TNFα or *Wnt3a*, but not IFNγ, led to a significant increase in frequency of BrdU$^+$/eGFP$^+$ neural progenitor cells (75±6 and 73±7 cells per mm DG, respectively, compared to 26±4 cells per mm of DG in control OSCs) and correspondingly in the number of BrdU$^+$ cells (175±36 and 137±27 cells per mm of DG, respectively, compared to 96±5 cells per mm of DG in control OSCs, Fig.14b). IFNγ treatment seemed to influence only cells without neural origin (eGFP$^-$) and is therefore not effecting neurogenesis (Fig.14b). In conclusion, the *ex vivo* results are consistent with the idea of TNFα stimulating *Wnt* signalling to enhance initial steps in the hippocampal neurogenic program.

3.7 Involvement of TGFβ signalling in hippocampal *Wnt* activity

TGFβ signalling is involved in various mechanisms including CNS development (Falk et al. 2008) and pathology (Tesseur, Wyss-Coray 2006). The crosstalk between the *Wnt* and the TGFβ pathway is well known, however, still not completely understood in certain neuronal processes. By using EAE, organotypic slice cultures and CNS-specific knockout mice, TGFβ´s link to hippocampal *Wnt* activity was investigated by clarifying their interaction in the established experimental models.

3.7.1 TGFβ signalling in the passive EAE model

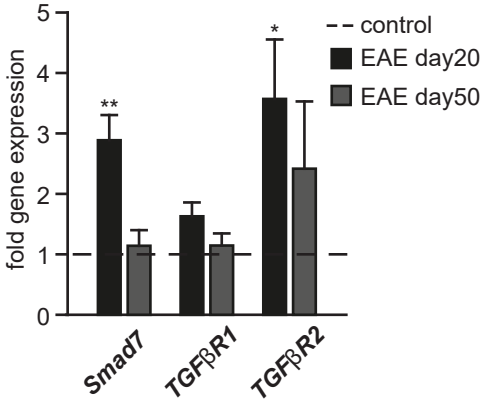

Figure 15| **Analysis of TGFβ signalling members in the passive EAE model.** qPCR analysis of gene expression for TGFβ signalling members in the hippocampus at early (day 20) and chronic (day 50) stage of passive EAE. Histogram represents mean +SEM of fold-changes relative to control (PBS) group set as 1. Transcription was normalized to GAPDH. Day 20 (EAE, n = 4; control, n = 4); day 50 (EAE, n = 4; control, n = 4). Two-tailed, unpaired Student's t-test, * $p < 0.05$ and ** $p < 0.01$.

The role of TGFβ in MS and EAE is intensively discussed (Mirshafiey, Mohsenzadegan 2009; Luo et al. 2007). To verify the role of TGFβ in the passive EAE system (Fig.6a), the mRNA expression of *Smad7*, a negative feedback regulator the of the TGFβ pathway, and the two transmembrane receptors (TGFβ-R1 and -R2) were measured in the disease course of mice with adoptive transfer EAE (Fig. 15). Indeed, at day 20 a significant upregulation of two TGFβ pathway members, *Smad7* (2.89±0.41 fold) and TGFβR*2* (3.47±1.11 fold) was detected, whereas at day 50 no expression changes compared to control animals were visible (Fig.15). Thus, these results indicate an impact of TGFβ1 in our disease model with the same activation pattern as seen for the *Wnt* pathway (Fig.5) and its members (Fig.7).

3.7.2 Impact of TGFβ on hippocampal *Wnt* activity in OSCs

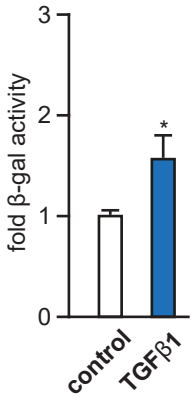

Figure 16| **Impact of TGFβ on hippocampal Wnt activity in organotypic slice cultures.** β-galactosidase assay of hippocampal OSCs. Hippocampal slices were prepared from Axin2$^{lacZ/+}$ mice (postnatal day 10) and cultured for seven days followed by treatment with TGFβ1 (10 ng/ml). After six hours of treatment the medium was replaced with fresh cytokine-free media and OSCs were cultured for additional 24 hours. Histogram represents mean (+ SEM) of fold-changes of β-galactosidase activity after treatment with Wnt3a (n= 15), TNFα (n = 14) or IFNγ (n = 14) relative to PBS controls (n = 20) set as 1. Two-tailed, unpaired Student's t-test, * $p < 0.05$.

To investigate the above mentioned connection between TGFβ and *Wnt* signalling in more detail, hippocampal slice cultures, prepared from Axin2$^{lacZ/+}$ pups, were used. After applying TGFβ for six hours and subsequent incubation of hippocampal slices in cytokine-free medium for 24 hours, a significant upregulation to 1.56+0.21 fold of the *Wnt* activity was measured via the β-galactosidase assay (Fig.16). The induced *Axin2* expression after a short TGFβ-treatment in hippocampal slices revealed its regulatory role of *Wnt* activity and led to further *in vivo* investigations.

3.7.3 The TGFβR2 conditional knockout mouse

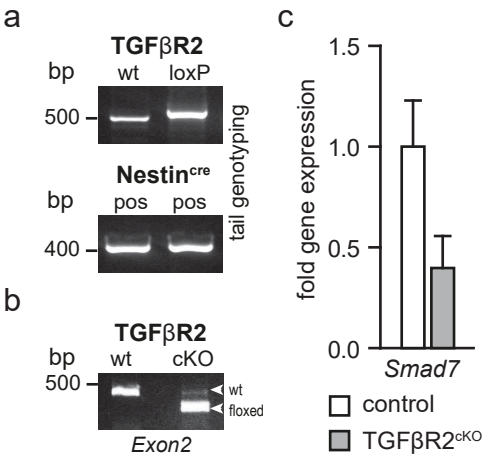

Figure 17| **The TGFβR2 CNS specific conditional knockout mouse system.** (a) Agarose gel images of the genotyping analysis for detection of the TGFbR2$^{loxP/loxP}$ and Nestin^{cre} genes in TGFβR2^{cKO} and control mice. (b) Agarose gel image of a qPCR analysis for floxed TGFβR2 mRNA in TGFβR2^{cKO} mice. (c) qPCR analysis of Smad7 gene expression in the hippocampus of TGFβR2^{cKO} and control (Nestin^{cre}) animals. Histogram represents mean +SEM of fold-change relative to control (Nestin^{cre}) group set as 1. Transcription was normalized to GAPDH. TGFβR2^{cKO}, n = 11; control, n = 5).

To take a closer look at the impact of TGFβ on *Wnt* signalling, TGFβR2$^{loxP/loxP}$ mice were breeded with Nestin^{cre} mice (Fig.17a), leding to a CNS specific conditional knockout mouse strain lacking TGFβR2 and consequently TGFβ signalling activity. TGFβ-signalling initiates with the binding of one TGFβ ligand to TGFβR2 and this complex fuses with TGFβR1 to create an intracellular signal for the signalling transduction (Fig.V). Therefore, without the expression of TGFβR2 the canonical signal transduced by TGFβ can not be functionally processed. Hereinafter, this mouse is called TGFβR2^{cKO}. TGFβR2^{cKO} mice are born healthy and develop normally as compared to their wt littermates, which are positive for the *Nestin^{cre}* transgen and were always used as a control. The novel mouse strain showed a floxed *TGFβR2* gene product, indicated by a smaller mRNA in the brain of conditional knockouts compared with control mice (Fig.17b). In the TGFβR2^{cKO} mice there were still two different products detected as a complete CNS-knockout of the receptor is not possible by the usage of Nestin^{cre} mice. Microglia cells, resident in the brain, are also expressing TGFβR2, but not the NESTIN protein. *Smad7* transcription was used as readout to confirm the functional knockdown of TGFβ-signalling. Indeed, the number of *Smad7* transcripts were downregulated to 0.40±0.16 fold in the hippocampus (Fig.17c).

In conclusion, this specific CNS-knockout for TGFβR2 was effective and can be used for the investigation of the *Wnt*/TGFβ connection.

3.7.4 Hippocampal *Wnt* signalling activity in naïve TGFβR2^{cKO} mice

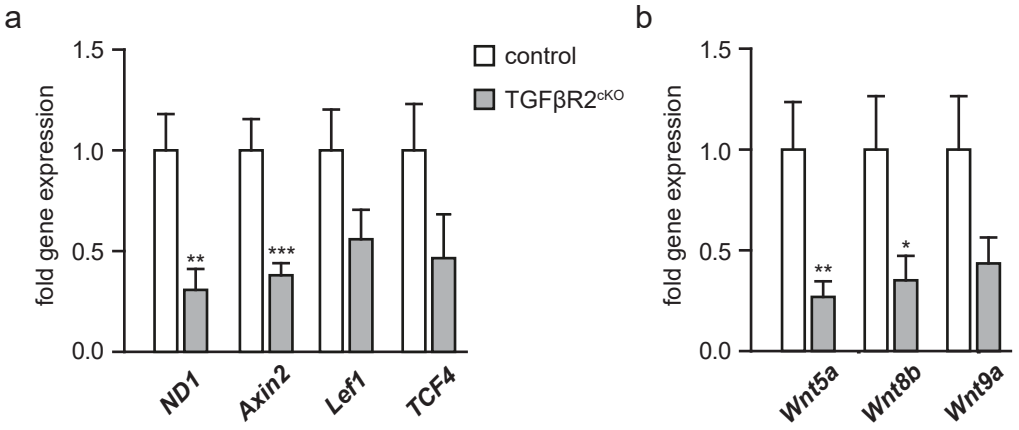

Figure 18| **Transcriptional analysis of hippocampal Wnt signalling activity in naïve TGFβR2 CNS specific knockout mice.** (a+b) qPCR analysis of gene expression for Wnt-dependent transcription factors (a) and ligands (b) in the hippocampus of TGFβR2^{cKO} and control (Nestin^{cre}) animals. Histograms represents mean +SEM of fold-change relative to control (Nestin^{cre}) group set as 1. Transcription was normalized to GAPDH. TGFβR2^{cKO}, n = 11; control, n = 5). Two-tailed, unpaired Student's t-test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Confirming the *ex vivo* results from hippocampal Axin2$^{lacZ/+}$ OSCs (Fig.15b), *Axin2* mRNA expression in the hippocampus of the CNS specific knockout mice was examined, which displayed a significant downregulation to 0.38±0.06 fold as compared to Nestin^{cre+} mice (Fig.17a). Additionally, expression of *Wnt*-dependent transcription factors were also downregulated: *ND1* to 0.31±0.10 fold, *Lef1* to 0.31±0.15 fold and *Tcf4* to 0.47±0.22 fold (Fig.17a), representing a lower basal hippocampal *Wnt* activity in these animals. Furthermore, in TGFβR2-defficient animals lower levels of *Wnt* ligands were observed: expression of non-canonical *Wnt5a*, canonical *Wnt8b* and *Wnt9a* were downregulated to 0.27±0.08 fold, 0.35±0.12 fold and 0.58±0.09 fold, respectively (Fig.17b). In contrast, all these *Wnt* ligand coding genes were upregulated in the peak of EAE (20 d.p.i.) (Fig.6+7). At the same stage of disease, also transcription of TGFβ was enhanced (Fig.9d), which further demonstrated the connection between *Wnt* and TGFβ signalling. In summary, the presented data imply that endogenous levels of TGFβ activity serve as support of *Wnt* activity, supporting the observation in organotypic hippocampal slice cultures.

4. Discussion

4.1 *Wnt* signalling and autoimmune-induced damage

The discovery of *Wnt* pathway activity in the adult CNS in physiological and pathological conditions initiated new investigations. Many studies focused on the mechanism of its activation and whether the unique involvement of *Wnt* in developmental processes (such as adult neurogenesis, oligodendrogenesis, synaptogenesis and axonal guidance (Clevers 2006; Inestrosa, Arenas 2010; Clevers, Nusse 2012) may contribute to tissue renewal and repair.
In the present study Axin2$^{lacZ/+}$ reporter mice were used to monitor the dynamic changes in hippocampal *Wnt* activity in acute and chronic stages of EAE, an important animal model of MS. The results show, that CNS pathology, associated with tissue injury and inflammation (Fig.8+9), leads to a transient increase in hippocampal *Wnt* activity (Fig.5+6). Additionally, the activation of *Wnt* signalling was connected with inflammatory processes (Fig.8+9), which is consistent with previous reports of MS pathology in context to remyelination in lesions (reviewed in (Xie et al. 2014)). Using different biochemical techniques, upregulation of *Wnt* pathway members such as Axin2 (histological examination (Fancy et al. 2009)), GSK3β, β-catenin and Tcf4 (proteomic analysis (Han et al. 2008)) was already demonstrated in active, acute inflammatory plaques of the white matter and was absent in chronic silent plaques or normal-appearing white matter. These observations detected possible *Wnt* activation usually in close proximity to damaged areas followed by neuroinflammation. Interestingly, other inflammatory CNS diseases, like ALS (Chen et al. 2012b) and TBI (Zhang et al. 2013) also displayed upregulation of *Wnt* signalling in injured areas.
In the spinal cord of Axin2$^{lacZ/+}$ mice, *Wnt*/β-catenin signalling was enhanced throughout all examined EAE stages (Fig.3) and correlated with a large number of inflammatory foci (Fig.1), representing ongoing active inflammatory processes. Thus, it is feasible that new waves of inflammation-mediated damage trigger a persistent activation of *Wnt* signalling in the spinal cord. Increased Wnt activity in the injured spinal cord tissue supports previous reports demonstrating the upregulation of different components of the canonical *Wnt* pathway in various models of acute spinal cord pathology (such as EAE (Yuan et al. 2012), spinal cord injury (González-Fernández et al. 2014) and LPC-induced demyelination (Fancy et al. 2009)).

This activity pattern of the *Wnt* pathway was already observed in many other tissues (reviewed in Whyte et al. 2012) and leads to the hypothesis that *Wnt* signalling acts as a support apparatus in the repair program after damage.

4.2 *Wnt* ligands in neurogenesis and CNS pathology

The enhanced hippocampal *Wnt*/β-catenin activity in acute stages of passive and active EAE (Fig.6+7) was correlated with a transient upregulation of canonical (*Wnt2*, *Wnt3*, *Wnt3a*, *Wnt8b*, *Wnt9a* and *Wnt16*) and non-canonical (*Wnt4*, *Wnt5a* and *Wnt5b*) *Wnt* ligand mRNA levels (Fig.7), indicating a role of *Wnt* ligands in the pathway activation in EAE. *Wnt* ligands are known to regulate self-renewal and cell fate determination of stem/progenitor cells, synaptic plasticity and tissue homeostasis (Clevers 2006; Inestrosa, Arenas 2010; Clevers, Nusse 2012). Moreover, recent data showed a distinct pattern of *Wnt* ligand expression after acute CNS damage and a new role of *Wnt* ligands in neuroimmune interactions was proposed (reviewed in (Marchetti, Pluchino 2013)). The involvement of *Wnt* ligands in neurogenesis, gives certain *Wnt* ligands a two possible purposes in the hippocampus of EAE animals: first as a response to damage and second as an enhancer of adult neurogenesis by activating the *Wnt* pathway.

Accordingly, *Wnt3* and *Wnt3a* are potent activators of self-renewal and neuronal fate (Lee et al. 2000; Lie et al. 2005) in the adult hippocampus and cultured neural progenitor cells (Wexler et al. 2009). Regarding this, it was shown that lentiviral-based overexpression of *Wnt3a* in the striatum enhances neurogenesis and neuronal differentiation, leading to an improved neuronal function in the model of focal ischemic injury (Shruster et al. 2012). *Wnt2* enhances neurogenesis in the developing brain (Sousa et al. 2010) and its induction in the dentate gyrus after electroconvulsive seizures (Madsen et al. 2003) was reported to be associated with increased hippocampal neurogenesis (Madsen TM, Treschow A, Bengzon J, Bolwig TG, Lindvall O, Tingström A 2000; Malberg et al. 2000). For *Wnt8b*, two neurogenic functions are known: its regulatory role for dorsal thalamic neurogenesis during zebrafish development (Lee et al. 2006) and the enhancement of proliferation in cultured rat adult hippocampal progenitor cells (Wexler et al. 2009).

Wnt9 is one of the least studied members of the *Wnt* family and at present there is no data for its function in the CNS. *Wnt9*, however, in tissue repair was previously discussed: *Wnt9a* was found to be essential for normal liver morphogenesis (Matsumoto et al. 2008), and development of iris and corneal epithelium (Fokina, Frolova 2006).

Further, in sea cucumber was shown that *Wnt9* transcripts are extensively expressed during gut regeneration and hypothesized that the protein products contribute to the injury-triggered plasticity of adult tissue seen in echinoderms (Mashanov et al. 2012). These data suggests that the induction of *Wnt9a* in the hippocampus during EAE may has a regenerative function. Expression of *WNT16A* (human analogue of mouse *Wnt16*) was found in the human brain (Fear et al. 2000), nonetheless its role in the CNS remains to be examined.

Considering the transient induction of *Wnt16* and activation of *Wnt*/β-catenin pathway at the early stage of osteoarthritis (Dell'Accio et al. 2008), it is a plausible hypothesis that *Wnt16* can mediate the response to neural tissue injury via promotion of *Wnt*/β-catenin signalling.

For the non-canonical *Wnt* system, literature is only recently increasing in context to neuronal functions. Current data already showed that *Wnt4* acts predominantly as paracrine factor in the neurogenic niche (Elizalde et al. 2011). *Wnt4* and *Wnt5a* are expressed in proliferating cells and are increased during differentiation, arguing for a distinct spatially regulated role of these molecules in proliferation and early differentiation (Lange et al. 2006). Further, in the spinal cord, *Wnt4*, where it acts as a guidance cue to induce growth of commissural axons (Lyuksyutova et al. 2003), is induced in lesion areas after unilateral hemisection (Liu et al. 2008). Consistent with this, *Wnt4* is constantly expressed by neuronal progenitors in the dorsal regions of the spinal cord (Daneman et al. 2009). *Wnt4* may also play a role in the hippocampus, as it is expressed by hippocampal neurons (Cerpa et al. 2008). In case of *Wnt5a*, current knowledge supports its involvement in the inflammatory response, as some evidence proofs the upregulation of *Wnt5a* by pathogens leading to activation of non-canonical signalling in macrophages (George 2008). In the spinal cord, *Wnt5a* was robustly and acutely induced broadly in the gray matter following unilateral hemisection (Liu et al. 2008). In general, a distinct expression pattern of *Wnt* ligands was already observed after spinal cord injury, presenting an early induction of *Wnt3* and a late induction of *Wnt2* and *Wnt9*, ligands activating specifically the *Wnt*/β-catenin-dependent pathway, in close proximity to the lesion site (González-Fernández et al. 2014).

In MS, *Wnt* ligands (*Wnt2*, *Wnt7a*: microarray data (Lock et al. 2002)); *Wnt3a*: proteomic analysis (Han et al. 2008)) were detected in active, acute inflammatory plaques of the white matter and in EAE, transient induction of *Wnt1* was demonstrated in the SVZ of the lateral ventricle (Pluchino et al. 2008), indicating a possible role of *Wnt* ligands in this autoimmune disease.

The simultaneous induction of canonical and non-canonical ligands provides the study with possible candidates which are able to trigger *Wnt* activity in EAE and with new interesting elements involved in tissue repair.

These findings and data from other research groups should raise an interest in *Wnt* ligands regarding the development of novel therapeutic approaches to treat neuronal injury.

4.3 *Wnt*-dependent activation of neurogenesis in pathological conditions

The essential involvement of *Wnt* signalling in oligodendrogenesis (Guo et al. 2015) makes it a valuable target to investigate remyelination processes. In contrast to this, studies focusing on adult neurogenesis, another *Wnt* dependent process (Lie et al. 2005; Kuwabara et al. 2009), in relation to MS, only recently became of particular interest.

In this thesis the induction of *Wnt* activity (Fig.5+6) in the hippocampus of EAE mice was associated with gene induction of neurogenic factors (Fig.10) and enhanced proliferation of neuronal progenitors and generation of Dcx^+ neuronal precursor cells (Fig.11). Increased proliferation and enhanced generation of immature neurons in the acute stage of EAE was previously reported by our (Huehnchen et al. 2011) and other groups (Aharoni 2005; Giannakopoulou et al. 2013). Enhancement of neurogenesis in EAE is transient (Aharoni 2005; Huehnchen et al. 2011) and seems to be specific to the hippocampal neurogenic niche, whereas in the SVZ, the generation of neuroblasts is reduced (Pluchino et al. 2008). In MS brains, immature neurons were found in a subpopulation of subcortical white matter lesions, which may indicate an activation of neurogenic processes serving to compensate neuronal deficits (Chang et al. 2008). However, the origin of these cells is unknown. In contrast, specification of stem/progenitor cells seems to be shifted towards the glial lineage in the SVZ of MS patients compared to the healthy brain, leading to a diminished generation of Dcx^+ neuroblasts (Tepavčević et al. 2011).

In general, activation of neurogenesis to improve regneration was already reported in models of CNS injury. In TBI pharmacological enhancement of adult neurogenesis is already discussed for therapy (Sun 2014) and cellular replacement techniques are planned to initiate neurogenesis with injections of neural stem cells in damaged brain areas (Quadrato et al. 2014).

Therefore, related literature and this investigation prompt the possibility that enhanced *Wnt* activity may trigger hippocampal neurogenesis in order to replenish damaged neurons in MS and other neurodegenerative diseases.

4.4 TNFα and *Wnt* signalling under pathological conditions

The validation of different factors, occurring after CNS damage, identified TNFα, an inflammatory cytokine upregulated in the hippocampus of EAE mice (Fig.9), as a potent factor contributing to *Wnt* signalling activation. Consistent with the idea that acute inflammation triggers *Wnt* activity, acutely LPC-demyelinated cerebellar slice cultures showed an upregulation of *Wnt* signalling (Fig.12). Using this model, it was previously demonstrated that microglia activation occurs consequently to demyelination induced by treatment with LPC (Birgbauer et al. 2004; Miron et al. 2010). Thus, a plausible hypothesis is that the inflammatory response to myelin damage initiated by microglia mediates *Wnt* signalling activity. In this regard, TNFα, a microglial autocrine mediator, induced hippocampal *Wnt* activity in organotypic slice cultures (Fig. 13). In line, previous reports demonstrated the interplay between those two signalling pathways in another cellular context: in the adipocyte cell line 3T3-L1, TNFα inhibits the proteasomal degradation of β-catenin and blocks adipogenesis (Cawthorn et al. 2007); in gastric cancer cells macrophage-secreted TNFα increased GSK3β phosphorylation and β-catenin stabilization to promote cell growth (Oguma et al. 2008) and in bone marrow-derived cells TNFα indirectly enhanced *Wnt* signalling by β-catenin nuclear translocation (Popivanova et al. 2008). Notably, increased proliferation and induction of *Wnt* genes (such as of *Wnt3*, *Wnt5a*, *β-catenin*) were observed upon TNFα-induced trans-differentiation of human mesenchymal stem cells towards the neural lineage (Egea et al. 2010). Further, in cultured progenitor cells, TNFα potentially triggers the proliferation of neural stem/progenitor cells, a mechanism involving activation of CyclinD1, a classical *Wnt* target gene (Widera et al. 2006; Peng et al. 2008; Bernardino et al. 2008). In nucleus pulposus cells, a new interaction between *Wnt* signalling and TNFα was documented, in which *Wnt* signalling regulates TNFα expression and both form a positive-feedback loop to cause degeneration of these cells (Hiyama et al. 2013). This represents the possibility that the *Wnt* pathway is able to induce TNFα.

Taking advantage of hippocampal slice cultures, a useful model to study neurogenic processes *ex vivo* (Raineteau et al. 2004), the impact of certain cytokines on *Wnt* activity and neurogenesis was analysed.

Results of this work demonstrated that TNFα-dependent activation of the *Wnt* pathway was associated with an increase in the proliferation of Nestin^{+}-progenitor cells in the dentate gyrus of hippocampal slices. While the mechanism remains to be elucidated, these data strengthened the hypothesis in which mainly TNFα-TNFR2 signalling is contributing to hippocampal neurogenesis in healthy mice (Chen, Palmer 2013) and upon various CNS pathologies.

In the model of status epilepticus, deficiency for TNFR2 drastically abrogates the number of newly generated neurons in the hippocampus (Iosif 2006), though not in the SVZ (Iosif et al. 2008). Complementary to these findings, infusion of neutralizing antibodies against TNFα had an anti-proliferative effect on hippocampal progenitors in a rat model of stroke (Heldmann et al. 2005) and in hippocampal progenitors, isolated from mice defficient for TNFR2, a decreased cell division was observed (Chen, Palmer 2013).

Understanding the role of TNFα in MS is a complex task and has been re-considered in the last years (Finsen et al. 2002; Su-Yin Lim and Cris S. Constantinescu 2010; Wood 2012). While anti-TNFα therapy has been used successfully to treat various autoimmune pathologies, it was proven to be deleterious in MS patients (van Oosten et al. 1996). The aggravation of EAE in TNFR2-deficient mice, particularly in the chronic phase (Eugster HP et al., 1999), may indicate the principal involvement of the TNFα-TNFR2 axis in processes of regeneration and tissue repair. Supporting this idea, the beneficial effect of TNFα and TNFR2 on expansion of the endogenous pool of oligodendrocyte progenitors and related remyelination in a toxin-induced demyelinating model was demonstrated (Arnett et al. 2001). Oligodendrocyte progenitors and remyelination are also studied in context to *Wnt* signalling (Xie et al. 2014), suggesting that TNFα and *Wnt* signalling are working hand in hand in this CNS repair mechanism.

4.5 TGFβ and *Wnt* signalling

TGFβ and *Wnt* signalling are related by a large body of research. Both pathways are linked in various biological processes, e.g. development (Guo, Wang 2009), cancer (Xu, Pasche 2007) and importantly, neurogenesis (Falk et al. 2008).

In vitro, this study revealed TGFβ as a potent *Wnt* activator in hippocampal organotypic slice cultures (Fig.16). In line, in the hippocampus of naïve TGFβR2^{cKO} mice, a downregulation of several *Wnt* target genes (*ND1*, *Axin2*, *Lef1* and *Tcf4*) and *Wnt* ligand transcripts (*Wnt5a*,*Wnt8b* and *Wnt9a*) was detected (Fig. 17), arguing for a reduced basal hippocampal *Wnt* activity in these mice. Moreover, the expression pattern of *Wnt* and TGFβ members is similar in the hippocampus of EAE animals (Fig.7+15), showing a possible relationship in this disease model.

These results display a novel interaction between TGFβ and *Wnt* signalling in neural stem cells, linking these pathways in inflammatory conditions in the context of adult neurogenesis.

The synergic liaison of TGFβ and *Wnt* is already known in literature, offering examples for an effective interaction of both pathways in different tissues. The best-defined venue of the *Wnt*/TGFβ cross-talk is shown in the cell nucleus, where the Smad/β-catenin/Lef1 protein complex regulates a host of common target genes, usually in a synergistic manner (Guo, Wang 2009). Further, in precartilaginous stem cells, TGFβ promotes β-catenin nuclear accumulation, regulating *CyclinD1/c-myc* gene transcription to eventually enhance cell proliferation (Cheng et al. 2014). In human mesenchymal stem cells, TGFβ cooperates with the *Wnt* pathway by promoting osteoblast differentiation and inducing β-catenin stabilization with increasing *TCF/LEF*-dependent gene expression (Chen et al. 2012a). In cells derived from developing orofacial tissue, binding assays showed that Smad proteins and Dvl2/3 can physically interact (Warner et al. 2003) and demonstrated a functional cross-talk between the TGFβ and *Wnt* signalling pathways in embryonic maxillary mesenchymal cells (Warner et al. 2005). In chondrocytes, TGFβ activated a luciferase-based *Wnt* reporter system, regulated by *Tcf4/Lef1* transcriptional binding sites, demonstrating that TGFβ is capable of activating the *Wnt*/β-catenin system (Tuli 2003; Sato 2006).

TGFβ can also stimulate *Wnt* signalling by other mechanisms such as inhibition of GSK3β aside decreasing Dkk1 expression, this was observed in two different cell types: mesenchymal stem cells (Amini Nik et al. 2007) and fibroblasts (Cheon et al. 2004). Recent studies have identified a cross-talk between the TGFβ and β-catenin signalling pathways in embryonic stem cells (Gadue et al. 2006) and mesenchymal stem cells (Jian 2006), in which TGFβ promoted β-catenin nuclear translocation in a complex with Smad3 without affecting β-catenin stability or phosphorylation.

The data on the cross-talk of *Wnt* and TGFβ in various tissues and cell types indicate a possible collaborative mechanism. Projecting this knowledge onto the background of autoimmune-mediated neuroinflammation, the interaction of TGFβ and *Wnt* signalling in neural stem cell regulation raises the hypothesis that TGFβ has the potential to direct neural stem cell differentiation in the inflamed CNS. The functional outcome of this interaction, however, has to be further examined in the context of healthy and various pathological conditions.

4.6 Conclusion

For the first time, this work identified an enhancement of the *Wnt* signalling pathway in the hippocampus of EAE mice and linked this observation to an increased proliferation of neural hippocampal progenitor cells. Such an enhancement of *Wnt* signalling may provide a beneficial strategy for treatment of neuronal injuries and should be further examined in autoimmune neuroinflammation.

The here described positive impact of TNFα and TGFβ on hippocampal *Wnt* signalling activity, may mechanistically explain the activation of *Wnt* in the inflamed CNS and indicate their contribution to neurogenic processes in the adult brain.

The results confirm and extend previous findings of different research groups indicating that early stages of EAE are associated with the enhancement of hippocampal neurogenesis in the hippocampus.

Overall, this thesis describes the impact of adult neurogenesis on regeneration of neuronal damage and cognitive deficits specifically in the context of MS pathology. This important topic should be further investigated to discover new therapeutic approaches helping patients with their various symptoms.

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5. Appendix

5.1 Contribution

Data of this thesis have been presented:

As a poster contribution:

Glia 2012 (Berlin)

"*Differential regulation of the Wnt signalling pathway in experimental autoimmune encephalomyelitis*"

R. Schneider, B. Bosch, F. Schröter, J. Ingwersen, C. Berndt, HP. Hartung, T. Prozorovski and O. Aktas

9th FENS (Forum of Neuroscience) 2014 (Milano)

"*Up-regulation of the Wnt signalling pathway in the hippocampus of mice with experimental autoimmune encephalomyelitis*"

R. Schneider, B. Kopp, F. Schröter, J. Ingwersen, H.-P. Hartung, O. Aktas and T. Prozorovski

Glia 2015 (Bilbao)

"*Characterization of the Wnt signalling pathway in the hippocampus of mice with experimental autoimmune encephalomyelitis*"

R. Schneider, B. Kopp, F. Schröter, J. Ingwersen, H.-P. Hartung, O. Aktas and T. Prozorovski

In an original publication:

"Activation of *Wnt* signalling promotes hippocampal neurogenesis in experimental autoimmune encephalomyelitis" Schneider R. *et al.,* submitted

5.2 List of abbreviations

5-Bromo-2-desoxyuridine	BrdU
Adenomatous polyposis coli	APC
Alzheimer´s disease	AD
Amyotrophic lateral sclerosis	ALS
Animal research: reporting of *in vivo* experiments	*ARRIVE*
Blood-brain-barrier	BBB
Calcium-calmodulin-dependent protein kinase II	CaMKII
Casein kinase-Iα	CK-1α
Complete Freund's adjuvant	CFA
Central nervous system	CNS
c-Jun N-terminal kinase	JNK
Cornu ammonis 1-3	CA1-3
Days post immunization	d.p.i.
Dentate gyrus	DG
Disheveled	Dvl
Distal-less homeobox 2	Dlx2
Doublecortin	Dcx
European Mouse Mutant Archive	EMMA
Experimental autoimmune encephalomyelitis	EAE
Fetal calf serum	FCS
Glycerinaldehyd-3-phosphat-dehydrogenase	GAPDH
Glycogen synthase kinase 3β	GSK3β
Hank's Balanced Salt Solution	HBSS
Heat shock protein 68	HSP68
Inositol triphosphate	IP3
Interferon gamma	INFγ
Interleukin-1 beta	IL-1β
Intraperitoneal	i.p.
Landesamt für Natur, Umwelt und Verbraucherschutz	LANUV
Lymphoid enhancer-binding factor 1	Lef1
Lysolecithin	LPC
Mitogen-activated protein kinase	MAPK

Multiple sclerosis	MS
Myelin myelin basic protein	MBP
Myelin oligodendrocyte glycoprotein	MOG
nuclear factor of activated T cells	NFAT
Neurofilament	NF
Neurogenic differentiation 1	NeuroD1/ND1
Olfactory bulb	OB
Oligodendrocyte progenitor cell	OPC
Organotypic slice culture	OSC
Paraformaldehyde	PFA
Parkinson´s disease	PD
Pertussis toxin	PTX
Phospho-lipase C	PLC
Postsynaptic density protein	PSD95
Primary-progressive MS	PP-MS
Prospero homeobox 1	Prox1
Protein kinase C	PKC
Proteolipid protein	PLP
Radial glia-like	RGL
Receptor-related protein 6	LRP6
Secondary-progressive MS	SP-MS
Secreted frizzled-related protein	sFRP
Subgranular zone	SGZ
Subventricular zone	SVZ
T cell-specific transcription factor	Tcf
TNF receptor	TNFR
Transforming growth factor beta	TGFβ
Traumatic brain injury	TBI
Tumor necrosis factor alpha	TNFα
wild-type	wt

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Declaration

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Die Dissertation wurde weder in dieser noch in einer abgewandelten Form bereits einer anderen Fakultät vorgelegt.

Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 5. November 2015

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(Reiner Schneider)