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DOCTORAATSPROEFSCHRIFT

The role of TNF-α, ADAM17 and IL-13 in CNS injury and repair

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LIST OF ABBREVIATIONS

ADAM17: A Disintegrin And Metalloprotease-17

Bax: bcl-2-associated X protein
Bcl-2:B-cell lymphoma 2
BMS: Basso mouse scale

CNS: central nervous system
CST: corticospinal tract

EAE: experimental autoimmune encephalomyelitis
EC: entorhinal cortex
EGFs: epidermal growth factors
EGFR: epidermal growth factor receptor
ERK: extracellular signal-regulated kinase

FADD: fas-associated death domain protein
Fas: tumor necrosis factor receptor superfamily

IL-1β: interleukin-1β
IL-4: interleukin-4
IL-6: interleukin-6
IL-10: interleukin-10
IL-13: interleukin-13
IRS-1: insulin receptor substrate 1
IRS-2: insulin receptor substrate 2

JAK/STAT: janus kinase/Signal Transducer and Activator of Transcription
JAK: janus kinase
JNK: c-jun N-terminal kinase

MAG: myelin-associated glycoprotein
MAPK: mitogen activated protein kinase
MMPs: matrix metalloproteases

NF-κB: nuclear factor κ B

PI3K: phosphoinositide 3-kinase
p38: p38 mitogen-activated protein kinase
p75NTR: p75-neurotrophin receptor

RIP1: receptor interacting protein kinase 1
ROS: reactive oxygen species

SCI: spinal cord injury
STAT3: signal Transducer and Activator of Transcription 3
STAT6: signal Transducer and Activator of Transcription 6

TACE: TNF converting enzyme
TGF-α: transforming growth factor-α
TGF-β: transforming growth factor-β
Th1: T helper type 1
Th2: T helper type 2
TIMPs: tissue inhibitor of metalloproteases
TNF-α: tumor necrosis factor-alpha
TNFRs: tumor necrosis factor receptors
TNFR-1: tumor necrosis factor receptor type 1
TNFR-2: tumor necrosis factor receptor type 2
TRADD: TNFR-associated death domain
TRAF: tumor necrosis factor receptor associated factor
TRAIL: TNF-related apoptosis-inducing ligand

CHAPTER 1

General introduction and aims

Parts of this chapter are based on: Vidal PM., Lemmens E., Dooley D., and Hendrix S. The role of "anti-inflammatory" cytokines in axon regeneration. Cytokine Growth Factor Rev. 2013 Feb; 24(1):1-12. Epub 2012 Sep 15.

1.1 SPINAL CORD

For a long time the spinal cord was just considered as a prolongation of the brain (Bartanusz et al., 2011); today we know that it is a very important component of the central nervous system (CNS). It is protected by the vertebral column and surrounded by the cerebral spinal fluid. Anatomically, it is a cylindrical structure of millions of nerve fibers which transmits electrical information from the brain to different organs and body parts, and *vice versa*, enabling the proper functioning of these organs, and controlled movement of these body parts (Byrne, 1997, Nógrádi and Vrbová, 2000).

1.1.1 Motor and sensory organization: spinal tracts and propriospinal connections

Sensory information from receptors throughout the body is transmitted to the brain via ascending tracts. When the brain needs to direct motor responses as a reaction to sensory inputs, nerve impulses travel down the spinal cord via axons of the descending tracts.

The main ascending tracts of the human spinal cord are the dorsal columns, important for the appreciation of touch, and the spinothalamic tracts, which conduct information about pain and temperature. The descending spinal tracts, carrying information from the brain downwards, can be divided into two major pathways, i.e. (I) the lateral pathways which are under direct control of the cortex, and are involved in voluntary movement, and (II) the ventromedial pathways that are controlled by the brain stem and involved in the control of posture. The main component of the lateral pathways is the corticospinal tract (CST). The CST originates in the motor cortex, passes through the internal capsule and the midbrain, to decussate in the pyramid, and to end in the ventral horn of the contralateral spinal cord where it makes contact with motor neurons and interneurons controlling muscle movement. Since the CST is the longest and one of the largest axonal tracts in the CNS, with CST axons descending in several tracts, lesions to this tract results in severe motor deficits. In most of the species, corticospinal axons originate mainly from neurons in layer V in the sensorimotor cortex (Tuszynski and

Steward, 2012). In contrast to the human spinal cord, in which the CST is located laterally, the CST in the mouse spinal cord is situated dorsally, in the dorsal column (Treuting and Dintzis, 2011).

The spinal cord has also its own intrinsic pathways, known as the comma tract, the Lissauer's tract, the septomarginal tract, the cornucomissural tract and the anterior and lateral ground bundles, which are also known as propriospinal connections. They are responsible to establish connections between different neuronal populations and segments of the spinal cord, as well as proper communication between spinal neurons and descending pathways (Nógrádi and Vrbová, 2000).

1.1.2 Spinal cord injury

1.1.2.1 Epidemiology and symptomatology

Spinal cord injury (SCI) is defined as "an insult to the spinal cord resulting in a change, either temporary or permanent, in its normal motor, sensory, or autonomic function" (Dawodu et al., 2011). The incidence of SCI in Europe is reported to be around 11000 new cases each year ((Europe, 2002) Parliamentary assembly, council Europe, 2002On the basis of reports to the National Spinal Cord Injury Statistical Center (NSCISC, 2004) males are more susceptible than females to have SCI. The major causes of SCI are motor vehicle crashes and other traffic accidents (50.4%), falls (23.8%), acts of violence (11.2%) and sport activities (9%). Also age is arising as a very important parameter, with an increasing number of SCI patients among the elderly. This is not surprising as they frequently experience falls due to age-related muscle weakening and instability of the vertebral column, for example due to osteoporosis. With increasing age, the regenerative capacity is decreased, and it is more likely to develop other health problems such as arthritis, heart diseases, diabetes and obesity, thus dramatically reducing their life expectancy (DeVivo et al., 1990).

There are mainly two types of SCI: contusions and transection injuries (Young, 2002, Talac et al., 2004), with contusion being the most common type of injury in humans. The symptomatology differs depending on the

location and the severity of the injury (complete or incomplete); The American Spinal Injury Association (ASIA) has defined a severity classification for a person's impairment following SCI, ranging from complete (category A; both sensory and motor function are lost), over incomplete (category B, C, or D; either sensory or motor function is preserved), to normal (category E). Besides severity of the injury, also location influences impairment after SCI; however injuries at any level (cervical, thoracic, lumbar) may cause impairment in bowel, bladder and sexual functions, pain, muscle weakness, as well as complete loss of motor and sensory function (paralysis) (Oudega, 2012).

1.1.2.2 Treatment options

The annual costs for patients living with SCI vary according to the severity of the lesion. Unfortunately, there is no current treatment available that can reverse damage to the spinal cord. The existing therapies are mainly focused on immune suppression, by using anti-inflammatory drugs such as methylprednisolone, in combination with rehabilitation. In fact, the latter is the only therapeutic option that can improve the patient's outcome in the long-term. Therefore, nowadays, the best hope for SCI patients is basic research that is focused on the understanding of how the environment around the lesion site influences regeneration for the better or the worse.
This strategy has facilitated the development of new promising therapeutic strategies, of which some are in clinical trials at the moment (i.e. transplantation of Schwann cells, Miami Project to Cure Paralysis (Scott, 2013)). More innovative alternatives include the development of biodegradable materials as scaffold to provide support to the spinal cord, and directionality to regenerating axons (Straley et al., 2010, Silva et al., 2012), as well as prostheses controlled by a neural interface system (Ajiboye et al., 2012, Hochberg et al., 2012).

1.1.2.3 Distinguishing between regenerated and spared axons

Before going into more detail in the regenerative processes after SCI, it is important to define the terms ***regeneration*** and ***sprouting***. In axons, ***regeneration*** refers to regrowth and extension beyond the lesion site of a transected axon. Regenerative axons can either end abortively, form ectopic connections, or form connections with their normal cell targets. In contrast, ***sprouting*** has been defined as the growth arising from axons that were not themselves damaged. Subcategories of sprouting have been defined based on the distance over which axons grow (Steward et al., 2003, Tuszynski and Steward, 2012)

In humans, regeneration after injury to the CNS normally fails. Loss of function after CNS injury frequently occurs through the interruption of axonal connectivity. The reestablishing of neuronal innervation after injury is one of most important processes to achieve functional recovery after injury to the CNS. For example, after injury to the peripheral nervous system, sensory and motor axons can regenerate over long distances, supporting axonal regeneration and functional recovery. In the CNS the situation is quite dissimilar, mainly due to the non-permissive environment generated after injury (Donnelly and Popovich, 2008, Boulenguez and Vinay, 2009, Giger et al., 2010, Tuszynski and Steward, 2012). A correlation between axons growing long distances and functional recovery has been previously observed with other factors, such as EpHA4 blockers, inhibition of SHP-1 and chondroitinase ABC combined with NT-3 treatment, that promote both regeneration and functional recovery after SCI (Garcia-Alias et al., 2011, Goldshmit et al., 2011, Tanaka et al., 2013). For research purposes, contusion injury is mostly used to understand the acute physiological responses occurring after SCI, and the development of secondary injury. Contusion injuries generated by impact can destroy the dorsal CST but, it can spare both the dorsolateral and ventral CST. This makes difficult to determine whether CST axons caudal to the injury are the result of sprouting from spared axons or regeneration (Tuszynski and Steward, 2012). On the other hand, T-cut or hemisection injury models (Fig. 1.1) are used to evaluated possible strategies that may be involved in axonal regeneration after injury, as is the purpose of this thesis since regenerative processes can

only be studied when all of the axons of a projecting system are lesioned. This type of lesion destroys the dorsal, dorsolateral and ventral CST axons (Fig. 1.1).

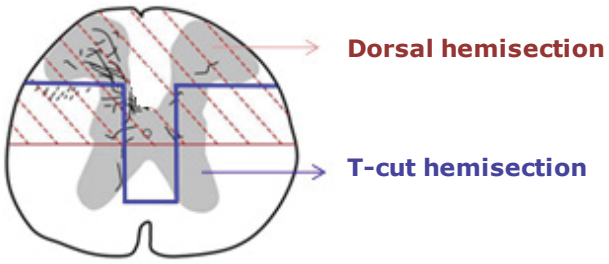

Fig. 1.1: Schematic representation of a coronal view of a T-cut hemisection injury in rodents. The red lines denote a common dorsal hemisection injury, while the blue region represents a T-cut lesion, which completely interrupts the CST in rodents. Image modified from (Tuszynski and Steward, 2012). For more details refer to materials and methods section, chapter III.

1.1.2.4 Pathophysiology

The pathophysiological events occurring after SCI can be divided into two phases, i.e. the primary injury phase, and the secondary injury phase. The first one is, in principal, defined as the initial mechanical damage, causing hemorrhage, loss of microcirculation and cellular death at the lesion site, followed by the expansion of the lesion and the promotion of secondary damage. The secondary injury phase is characterized by further impairment of the microcirculation, rupture of the blood-spinal cord barrier, demyelination, activation of glial cells resulting in the production of inhibitory molecules (i.e. myelin-associated glycoprotein, neurite outgrowth inhibitor Nogo), the formation of a glial scar, apoptosis, and immune cell migration and infiltration, among others (Silver and Miller, 2004, Oyinbo, 2011) (Fig. 1.2). Together, these processes trigger the secondary loss of neighboring intact nerve fibers, neurons, and oligodendrocytes (Springer, 2002, Keane et al., 2006).

1.1.2.5 Apoptosis after injury

Cell death has been theoretically divided into apoptosis (programmed cell death) and necrosis (traumatic cell death). Apoptosis is characterized by cellular shrinkage and breakdown via caspase-dependent mechanisms, whereas necrosis involves swelling or bursting of the cell with organelle degeneration and loss of plasma membrane integrity (Raff, 1998).

As mentioned above, after SCI, there is a high percentage of cells that undergo apoptosis, with different susceptibilities between different cell populations (Crowe et al., 1997, Shuman et al., 1997). Oligodendrocytes and motor neurons have been identified as two of the most susceptible cell populations to undergo apoptosis. It has been reported that SCI alters the expression (at mRNA and protein levels) of proteins associated with apoptosis, such as cytochrome C, poly (ADP-ribose) polymerase, caspase-3, and Bcl-2-associated X protein (Bax). Cytokines such as interleukin- (IL-) 1, IL-6, and tumor necrosis factor alpha (TNF-α) are also known to induce apoptosis *in vitro* in neurons, astrocytes and oligodendrocytes. Their expression is upregulated rapidly at the lesion site after injury (Ahn et al., 2006, Pineau and Lacroix, 2007), and modulation of their level affects outcome after SCI. For example, inhibition of TNF-α after SCI leads to a decrease in neuron and oligodendrocyte apoptosis and significant improvement after injury in a mouse model of SCI (Genovese et al., 2006) (Cantarella et al., 2010, Chen et al., 2011). Other processes known to be involved post-traumatic apoptosis are, increasing intracellular levels of Ca^2 (Mills et al., 2004, Colak et al., 2009), as well as extracellular glutamate and the surface expression of AMPA receptors (Beattie et al., 2010).

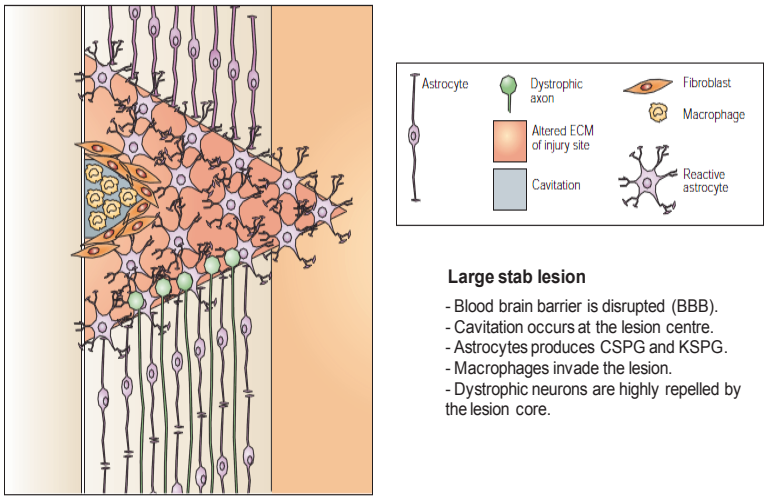

Fig. 1.2: Schematic representation of the CNS environment after a stab lesion (e.g. hemisection SCI). This type of lesion causes penetration of the meninges and allows fibroblast and macrophages/microglia activation and invasion. Astrocyte alignment is altered at the lesion site and the production of chondroitin sulfate proteoglycan (CSPG) and keratin sulfate proteoglycans (KSPGs), transforming growth factor (TGF), ephrin-B2 and Slit protein is increased. This inhibits axonal regeneration. ECM= extracellular matrix. Picture modified from (Silver and Miller, 2004).

1.1.2.6 Distinct inflammatory phases after injury

Inflammation is part of the initial response of tissue against injury and is characterized by increased blood flow and vascular permeability, edema formation, and activation and infiltration of leukocytes producing inflammatory mediators, such as cytokines. The inflammatory response is critical for the clearance of cellular debris, which can leave a hostile environment for axonal regeneration (Oyinbo, 2011). However, over-activation of the inflammatory response can also exacerbate damage. The time-course of the injury and the inflammatory response, are thus of vital importance to achieve reparative processes. Phase-specific immune responses occurring after CNS injuries, are starting to be recognized (Donnelly and Popovich, 2008). At least four main stages can be distinguished after SCI: acute, sub-acute, early chronic and late chronic

phase. The acute phase, which typically lasts for a few hours, is characterized by an upregulation of pro-inflammatory cytokines, such as IL-1β and TNF-α (Bethea et al., 1999, Pineau and Lacroix, 2007). It is defined by an augmentation in damage, i.e. neuronal and axonal destruction, as well as demyelination close to the injury site (Schwab et al., 2006). It is further characterized by an infiltration of neutrophils, reaching the highest level one day after injury (Beck et al., 2010), and by an increase in activated B and T cells in the spleen and bone marrow (Ankeny et al., 2006). In the sub-acute phase, between 2 to 7 days after injury, the levels of some pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) start to decrease (Pineau and Lacroix, 2007, Donnelly and Popovich, 2008). At this stage, the inflammatory response is mainly controlled by macrophages, neutrophils, T cells, activated astrocytes and microglia (Keane et al., 2006). Meanwhile, there is an increase in anti- and pro-inflammatory factors (Fig. 1.3). Besides the above mentioned cytokines, also other molecules are involved in the acute inflammatory phase, such as the cytokines IL-8 and IL-11, and the chemokines granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor. Together, these activate macrophages, neutrophils, as well as natural killer cells. Interestingly, already during this sub-acute phase, recovery of locomotor skills can be observed in rodents.

Later on, during the early and late chronic phases, which can last for weeks or even months, specific humoral and cellular immune responses develop to facilitate cleaning of the injury site (Donnelly and Popovich, 2008, Vidal et al., 2012). During the early chronic phase, the levels of T cells, macrophages and neutrophils start to increase again showing a second peak of these immune cells and their secreted factors. It is not clear whether this event is related to the changes in functional recovery. It has been suggested that inflammation may support regeneration in the chronic phase because a reduction of macrophages/microglial infiltration led to a decrease in functional recovery following SCI (Ankeny et al., 2006, Beck et al., 2010).

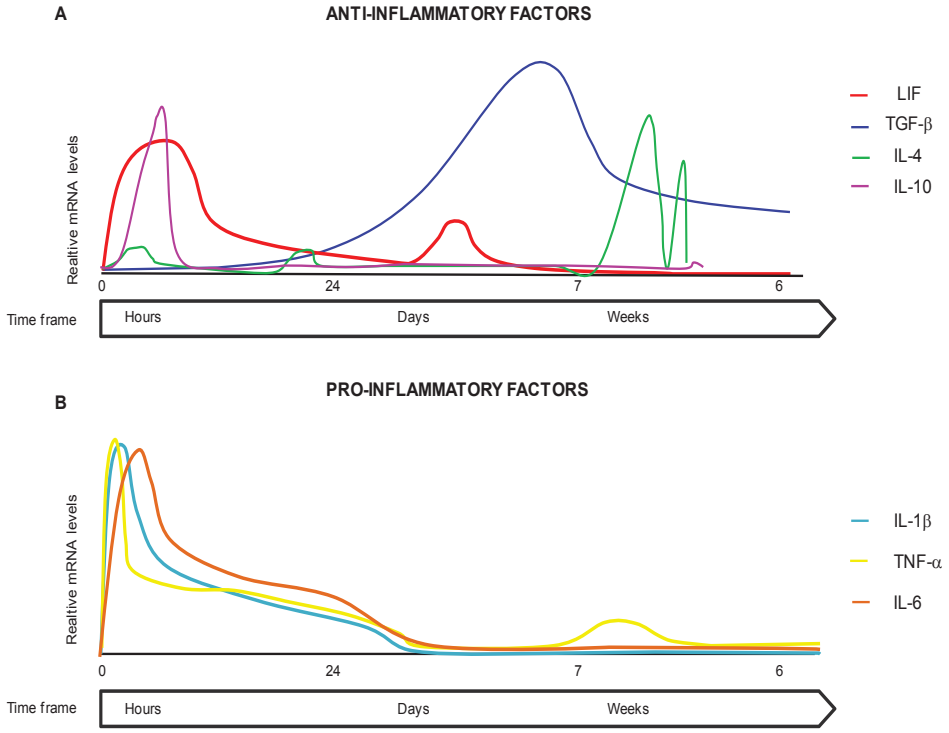

Fig. 1.3: Temporal expression patterns of anti- and pro-inflammatory cytokines after injury. **A**. Curves represent mRNA levels after SCI for IL-4, IL-10, Leukemia inhibitory factor (LIF) and TGF-β. During the first hours after injury there is an upregulation of the anti-inflammatory factors LIF and IL-10, while during the early chronic phase there is an increase of TGF-β levels **B.** Curves represent mRNA levels after SCI for IL-1β, TNF–α and IL-6. Pro-inflammatory factors are immediately upregulated after SCI. TNF-α has a second peak during the early chronic phase. Image modified from (Donnelly and Popovich, 2008, Vidal et al., 2012).

1.2 CYTOKINES

1.2.1 General definitions

The activity of cytokines was first recognized in the mid-1960s, when supernatants derived from *in vitro* cultures of lymphocytes were found to contain factors that could regulate proliferation, differentiation, and maturation of allogenic immune cells, induced by activation with antigen or with nonspecific mitogens (Goldsby et al., 2000). Cytokines can thus be defined as proteins with pleiotropic, redundant, synergetic and/or antagonistic effects mediated via several signaling cascades, which permit them to regulate cellular activity (such as proliferation, differentiation and maturation) in a coordinated and interactive way over extensive networks (Vidal et al., 2012). They are mediators of the immune system, which can basically be divided into two components. The first one is called innate immunity, which is non-specific and consists of cells and proteins that are always present and ready to be mobilized against pathogenic agents. The second, specific component is called adaptive immunity, which is recruited into action against pathogens that have not been cleared by the innate immune defenses (Fisher, 2011).

Functionally, cytokines and the cells that secrete them have been classified as either "pro-inflammatory" (stimulatory, or T helper cell type 1 [Th1], or type 1) or "anti-inflammatory" (inhibitory, or T helper cell type 2 [Th2], or type 2). In most publications, the terms "pro-inflammatory", stimulatory, T helper cell type 1 [Th1] or type 1, are used interchangeably, although it is semantically not correct to use them as synonyms. The same criticism applies to the terms, "anti-inflammatory", inhibitory, T helper cell type 2 [Th2] or type 2. Type 1 cells mainly activate macrophages and control infections; meanwhile, type 2 cells activate B cells and aid in eradication of extracellular parasites (reviewed in (Hendrix and Nitsch, 2007)). However, most of the cytokines have an overlap in function, exerting both "pro- and anti-inflammatory" effects depending on the tissue milieu, which often makes it difficult to understand the actual effect they induce as mediators of the immune response. For this reason, along the text we will be using quotation marks to make reference to both types of cytokines. For example,

it has been suggested that some so-called ‘‘anti-inflammatory’’ cytokines, such as IL-4, IL-10 and TGF-β may present pro-inflammatory properties under certain experimental conditions (Bethea et al., 1998). However, it has been suggested that the T cell subpopulation T helper type 2 cells, might be particularly beneficial following CNS and peripheral nervous system lesions and after neuropathic pain following peripheral nerve injury, especially by producing the “anti-inflammatory” cytokines IL-4 and IL-10 (Maini and Taylor, 2000). In addition, inhibition of “pro-inflammatory” cytokines, such as TNF-α and IL-1β , has been shown to have beneficial effects after CNS injury (Genovese et al., 2006, Kimbler et al., 2012). The ‘‘anti-inflammatory’’ cytokines are not only secreted by activated Th2 lymphocytes, but also by other immune cells such as macrophages, microglia and mast cells at or near the site of injury, thus acting mostly locally. The focus of this thesis is on the “anti-inflammatory” cytokine IL-13, and on the “pro-inflammatory” cytokine TNF-α, which is modulated by the enzyme A Disintegrin And Metalloprotease 17 (ADAM17).

1.2.2 The anti-inflammatory cytokine interleukin-13

IL-13 is a 10kDa protein which mediates its effects via the IL-13 receptor, expressed on human B cells, innate type 2 cells, basophils, eosinophils, mast cells, endothelial cells, fibroblasts, monocytes, macrophages, respiratory epithelial cells, and smooth muscle cells. It is a major effector molecule for T helper type 2 inflammation and is pathogenic in allergic diseases such as asthma. Execution of type 2 immune responses highly relies on the production of type 2 cytokines such as IL-4, IL-5 and IL-13. Besides Th2 cells, also innate immune cells such as macrophages, basophils and mast cells secrete these cytokines. Recently, a new population of innate type 2 immune effector cells have been identified, designated ‘innate type 2 helper cells (Ih2)’ by some (Price et al., 2010), or called ‘nuocytes’ by others (Neill et al., 2010). These cells are characterized by the production of high levels of IL-5 and IL-13 in response to IL-25, IL-33, or *N. brasiliensis* infection, and

were found to promote Th2 cytokine-dependent responses by mechanisms yet to be defined (Saenz et al., 2010).

Two types of IL-13 receptors exist, one being a heterodimer of the subunits IL-13Rα1 and IL-4Rα, to which IL-4 can bind as well, and a second IL-13 specific receptor consisting of an IL-13Rα2 chain (Hershey, 2003, Hallett et al., 2012) (Fig. 1.4). Although IL-13 binds to the IL-13Rα2 chain with high affinity (0.25-1.2nM) (Caput et al., 1996), it is considered to be primarily a non-signaling "decoy" receptor because its cytoplasmic tail is short and does not contain any obvious signaling motif (Bernard et al., 2001). However, under some circumstances, IL-13 binding to IL-13Rα2 can promote fibrosis by induction of TGF-β, through a signal transducer and activator of transcription 6 (STAT6)-independent activator protein 1 pathway (Fichtner-Feigl et al., 2006). *In vivo*, expression of the IL-13Rα1 and IL-13Rα2 chains is highly regulated by IL-13 itself, but also by IL-4, IL-10, and Interferon-gamma (Zheng et al., 2003). IL-13 binds to IL-13Rα1 first with low affinity (Kd= 2-10nM) and then recruits IL-4Rα to the complex, generating a high affinity receptor (Kd= 0.03-0.4nM) (Aman et al., 1996). This results in the activation of the Janus kinase (JAK) TYK2 and JAK1, followed by activation of STAT6, and to a lesser degree to STAT3 (Hecker et al., 2010), causing expression of various IL-13 inducible genes (Izuhara and Arima, 2004).

Thus, signal transduction via IL-4Rα is thought to be responsible for most of the functional characteristics of IL-4 and IL-13. Both IL-4 and IL-13 are involved in downregulating the production of pro-inflammatory cytokines such as IL-1, TNF-α, IL-1Rα, IL-12, by monocytes/macrophages (Cash et al., 1994, Gordon, 2003), human monocytes (Folcik et al., 1997), and microglia (Paludan et al., 1997, Szczepanik et al., 2001), and are involved in increasing the secretion of IL-10 (Doherty et al., 1993). However, additional studies demonstrated that IL-13 possesses several unique effector functions that differentiate it from IL-4; in activated monocytes/macrophages, for example, it was found that IL-4 and IL-13 both activate STAT3 and STAT6, while STAT1 is activated by IL-13 only (Bhattacharjee et al., 2013).

Since there is evidence that IL-4 and STAT6 are involved in promotion of axonal regeneration (Deboy et al., 2006), it is therefore probable that IL-13, via JAK/STAT signaling, may also contribute to axonal regeneration.

In the CNS, IL-13 mRNA levels are upregulated within hours after CNS trauma (our own unpublished data). The effect of IL-13 on neuronal survival however is not clear, and both positive (Yang et al., 2002, Shin et al., 2004) and negative effects (Park et al., 2009) have been reported. In the brain, neurons and microglia act cooperatively to diminish brain inflammation by inducing IL-13 expression in microglia and enhancing prostaglandin-endoperoxide synthase expression (Yang et al., 2002); the latter has been previously associated with cytotoxicity in brain diseases (Iadecola et al., 2001), thereby causing death of activated microglia and leading to an increase in neuronal survival (Yang et al., 2002, Shin et al., 2004). Activation of microglia, using thrombin, leads to an upregulation of IL-13 and reactive oxygen species (ROS) levels, resulting in a decrease in neuronal survival. On the other hand, the blockade of IL-13 reduces inflammatory cytokine expression, thus increasing neuronal survival in the hippocampus *in vivo* (Park et al., 2009). In the CNS, activated microglia or macrophages seem to create a favorable environment for regeneration by degrading inhibitory molecules which prevent axonal growth and reactive neurite sprouting (Avellino et al., 1995). It is thus possible that modulating this microglial response after CNS injury by factors such as IL-13 could be helpful to either prevent or degrade depositions of these regeneration-inhibiting factors. In summary, IL-13 has been implicated in cellular survival, with both detrimental and beneficial effects reported. However, no studies have been performed yet to investigate any direct effect of this cytokine on axonal regeneration.

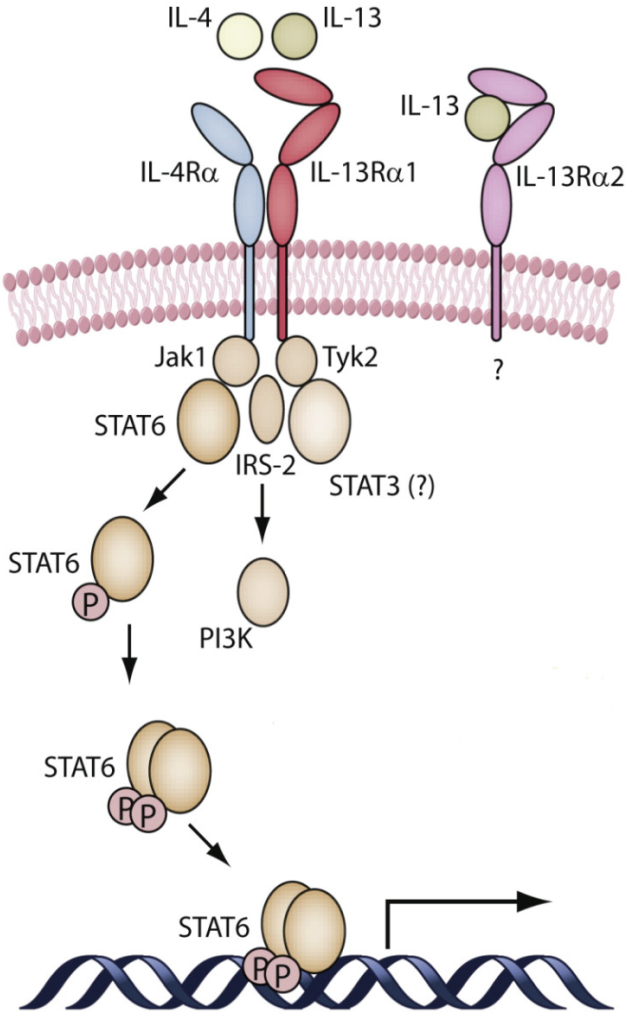

Fig. 1.4: IL-13 signaling. IL-13 and IL-4 can both bind to the same receptor; IL-4 binds to the IL-4Rα chain thereby recruiting IL-13Rα1, while IL-13 binds to IL-13Rα1 followed by recruitment of IL-4Rα; both processes result in the activation of JAK/STAT and IRS-1/IRS-2 pathways, leading to the transcription of genes involved in proliferation and apoptosis. IL-13 can also bind to the IL-13Rα2 chain as either the transmembrane form (TM) without noticeable transduction of signal, or as a soluble form acting as a decoy receptor for IL-13. Figure modified from (Gordon and Martinez, 2010).

1.2.3 The pro-inflammatory cytokine TNF-α

TNF-α is a pleiotropic proinflammatory cytokine that can elicit a wide spectrum of cellular responses by interacting with high affinity receptors. TNF-α has been involved in the pathogenesis of various immune mediated diseases, such as septic shock (Tracey and Lowry, 1990), rheumatoid arthritis (Maini and Taylor, 2000) and inflammatory bowel disease (Crohn's disease) (Wajant et al., 2003). In addition, TNF-α also causes fever, sickness behavior, anorexia, sympathetic discharge and stimulation of pituitary hormones (Rothwell and Hopkins, 1995). TNF-α is produced by many cell types including activated macrophages, T cells, astrocytes, glial cells, Schwann cells and mast cells. It is synthesized as a monomeric transmembrane protein (26kDa, also called membrane-bound TNF-α or pro-TNF-α) that is inserted into the membrane as an homotrimer protein. This membrane-bound TNF-α is cleaved by the TNF converting enzyme (TACE), also known as ADAM17, to produce the soluble form (17kDa) (Black et al., 1997, Moss et al., 1997). Both the membrane-bound and soluble TNF-α forms are biologically active (Idriss and Naismith, 2000, MacEwan, 2002), and can be synthesized in the CNS by microglia, astrocytes, and some populations of neurons (Lieberman et al., 1989, Morganti-Kossman et al., 1997). TNF-α exerts its biological effects via interaction with two high-affinity TNF receptors (TNFRs), i.e. TNFR-1 (p55, 55-60kDa) and TNFR-2 (p75, 75-80kDa), both membrane glycoprotein receptors. TNFRs can also be cleaved by ADAM17 to generate soluble TNFRs which can bind soluble TNF-α in the circulation, thereby functioning as decoy receptors for TNF-α (Novick et al., 1989, Engelmann et al., 1990).

TNFRs are an increasing superfamily of proteins consisting of at least 41 members characterized by their extracellular sequence homology, such as TNFR-1, TNFR-2, Fas, CD40, the low-affinity nerve growth factor receptor, TRAIL receptor, RANK and death and decoy receptors (Aggarwal, 2000, Locksley et al., 2001). However, TNF-α only has the ability to bind two of these receptors, namely TNFR-1 and TNFR-2 (MacEwan, 2002) (Fig. 1.5).

TNFR-1 is expressed in most tissues, and can be fully activated by both the membrane-bound and soluble forms of TNF-α; in contrast, TNFR-2 is found only in cells of the immune system (including microglia), and preferentially

responds to the membrane-bound form of TNF-α (Grell, 1995, Sato et al., 1997). TNFR-1 has a 10-fold higher affinity and slower association/dissociation kinetics than TNFR-2 for TNF-α (Grell et al., 1998), and is known to mediate most of the biological functions of TNF-α. Another difference between TNFR-1 and TNFR-2 is their role in the biological activities of TNF-α after binding to its receptors. Depending on the microenvironmental conditions, TNFR-1 activation can lead to the induction of proliferation, apoptosis or necrosis (MacEwan, 2002, Cabal-Hierro and Lazo, 2012), while TNFR-2 plays a role in angiogenesis, migration of intestinal and Langerhans cells, and proliferation of myofibroblasts (Takayama et al., 1999, Corredor et al., 2003, Theiss et al., 2005, Goto et al., 2006). Under certain conditions, TNFR-2 is able to trigger cell death independently of TNFR-1 activation, an effect that is enhanced after the inhibition of caspases (Biragyn et al., 2008). However, in general, one can say that TNFR-1 is mainly related to apoptotic responses, while TNFR-2 is mainly associated with anti-apoptotic responses (Reinhard et al., 1997, MacEwan, 2002).

Receptors of the TNFR superfamily are unable to trigger a biological response by themselves. They depend on the binding of adaptor proteins to activate intracellular pathways. Among them, two different types can be distinguished: adaptor proteins with a death domain, such as TNFR-associated death domain (TRADD) or Fas-associated death domain protein (FADD), and a group of adaptor proteins that do not have a death domain, i.e. TNFR-associated factors (TRAF) (Tartaglia et al., 1993, Locksley et al., 2001, Dempsey et al., 2003). On one hand, the recruitment of TRADD can promote the association of the TNFR-1 complex with the death domain of FADD, which induces caspase activation and cell death; on the other hand, TRADD can also recruit receptor-interacting protein kinase 1 (RIP1) and TNFR-associated factor-2 (TRAF2), which activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) c-Jun N-terminal kinase (JNK) pathway, leading to cell survival and pro-inflammatory responses (Hsu et al., 1996, Locksley et al., 2001, Aggarwal, 2003) (Fig. 1.5). In cells from TRADD-deficient mice, apoptosis, NF-κB signaling and extracellular signal regulated kinase (ERK) activation are defective (Chen et al., 2008). TRAFs

are a major group of intracellular adaptors consisting of six members (TRAF1-TRAF6); they have yet undefined enzymatic activities, but are known to induce the activation of several kinase cascades (NF-κB, JNK, ERK, p38 and phosphatidylinositide 3-kinase (PI3K)), which ultimately regulate cellular processes such as proliferation, differentiation and apoptosis (Dempsey et al., 2003). TRAF2 is the main adaptor protein responsible for TNFR-2 signaling, but several studies indicate the existence of a crosstalk between TNFR-1 and TNFR-2, in which TRAF2 is recruited to TNFR-1 through TRADD, enhancing the cytotoxic effects of TNFR-1 (Shu et al., 1996).

In the CNS, TNF-α mRNA levels are upregulated immediately after injury (Pineau and Lacroix, 2007). Several studies indicate that it is important to regulate increased TNF-α levels immediately after injury to control the excitotoxic effect of TNF-α (Stellwagen et al., 2005, Zhao et al., 2010), and to decrease TNF-α-induced apoptosis after injury (Genovese et al., 2006). Consistently, it was shown in an SCI model that a single dose of a TNF-α blocker, etanercept, immediately after injury appears to be sufficient to improve hind limb locomotor function and to reduce apoptosis of neurons and oligodendrocytes in the rat spinal cord (Chen et al., 2011). However, the TNF-α inhibitors used do not selectively block one of the two TNF-α forms (membrane-bound or soluble). In different SCI models, it has been suggested that soluble TNF-α may reduce inflammation and pain-related behaviors, as well as promotion of cell death after injury (Peng et al., 2006, Ferguson et al., 2008). In experimental autoimmune encephalomyelitis (EAE) models, blocking of soluble TNF-α using XPro1595, improved functional recovery by increasing myelin and axon preservation as well as decreasing inflammation (Brambilla et al., 2011, Taoufik et al., 2011). On the other hand, membrane-bound TNF-α has been shown to control pathogen infection and to promote cell migration (Saunders et al., 2005, Allenbach et al., 2008). These data suggest a distinctive role of both TNF-α forms that could in part explain why beneficial and detrimental effects have been attributed to this cytokine. So far there is no clear information about the role of the two TNF-α forms in SCI.

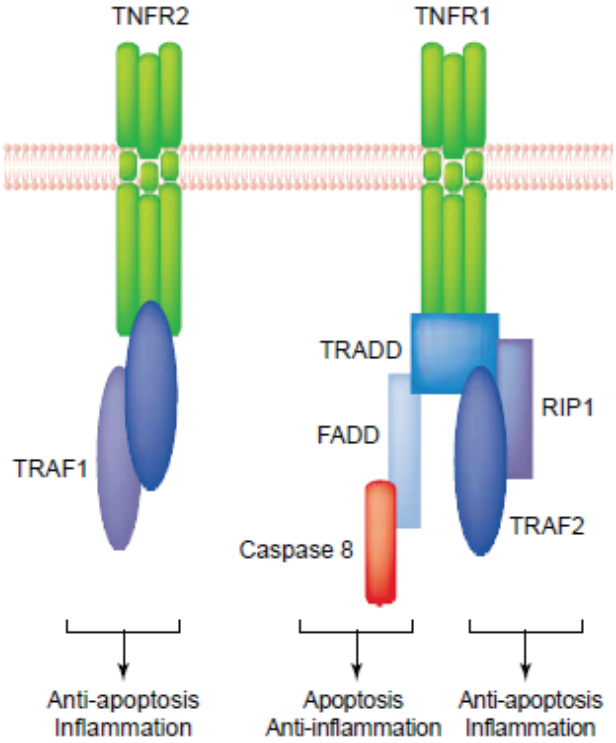

Fig. 1.5: TNF-α signaling via TNFR-1 and TNFR-2: Binding of TNF-α to TNFR-1 triggers activation of apoptotic and anti-inflammatory signals through the recruitment of TRADD and subsequent recruitment of FADD and caspases. TNFR-1 can also mediate anti-apoptotic signaling via the recruitment of RIP1 and TRAF2. Activation of TNFR-2 leads to anti-apoptotic signals via the recruitment of TRAF2 and TRAF1. Figure modified from (Baud and Karin, 2001).

1.3 ADAM17/TACE

The ADAMs family of transmembrane proteins belongs to the zinc protease superfamily. Their structure is composed by a pro-domain, a metalloprotease catalytic region, a disintegrin domain, a cysteine-rich segment, an epidermal growth factor like (EGF-like) domain, a transmembrane domain, and a cytoplasmic tail (Seals and Courtneidge, 2003) (Fig. 1.6). One of the ADAM family members, ADAM17, or also called TACE, is a proteolytic sheddase synthesized as a zymogen (pro-enzyme) with the pro-domain acting as its inhibitor. During its maturation, the pro-domain is removed by a furin-like

convertase, generating the biologically active mature ADAM17 (Peiretti et al., 2003). Mature ADAM17 can exit the late Golgi to reach the cell surface (Schlondorff et al., 2000), where it is responsible for the cleavage of several membrane-bound molecules, such as neuregulin-1 type III (La Marca et al., 2011), TNF-α (Black et al., 1997, Moss et al., 1997), p75-neurotrophin receptor (p75NTR), epidermal growth factor receptor (EFR) and several ligands (e.g. TGF-α, extracellular Notch1, IL-6R, L-selectin, and TNFRs) (Brou et al., 2000, Condon et al., 2001, Marin et al., 2002, Sunnarborg et al., 2002). However, despite intensive study, no common recognizable pattern around the cleavage site of the substrates of ADAM17 has been found yet. Although ADAM17 is a very important sheddase with multiple biological functions and substrates (Peschon et al., 1998), the molecular regulation of its activation remains poorly understood. Some studies suggest that mitogen-activated protein kinase (MAPK) p44 (also called ERK) or p38 signaling, are necessary for ADAM17 activation or trafficking (Diaz-Rodriguez et al., 2002, Soond et al., 2005), but at the same time ADAM17 has the control of EGFR and ERK activation through the cleavage of EGFRs substrates (Murthy et al., 2010). Furthermore, tissue inhibitor of metaloprotease-3 (TIMP-3) an endogenous regulator of matrix metalloproteases (MMPs) (Brew et al., 2000), inhibits ADAM17 ectodomain shedding (Amour et al., 2000, Xu et al., 2012), controlling ADAM17 levels.

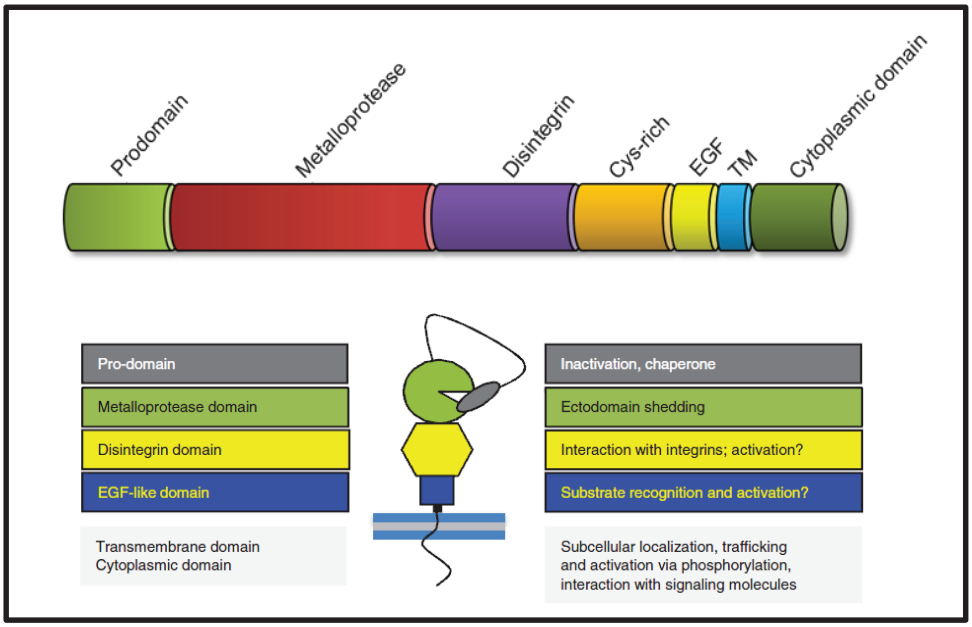

Fig. 1.6: Schematic representation of the ADAM17 domain structure. ADAM17 is synthesized as a zymogen with 7 different domain structures; upon removal of the pro domain by a mechanism involving cleavage by furin, the metalloprotease domain (catalytic domain) is activated. Picture modified from (Evers and Reiss, 2011, Scheller et al., 2011).

1.4 AIMS OF THIS STUDY

To date, the role of some molecules that are up or downregulated immediately after SCI, such as TNF-α and IL-13, is still not clear. Delineation of the precise functions of these "anti- and pro-inflammatory" cytokines may provide insights on how to modulate the inflammatory reaction after SCI to promote regeneration.

For a long time, "anti-inflammatory" cytokines have been considered to play a beneficial role in trauma-induced inflammation, while "pro-inflammatory" cytokines are thought to have a more detrimental role. As an example, the "anti-inflammatory" cytokine IL-4 has been considered to play a positive role in cell survival *in vitro* (Sholl-Franco et al., 2001, Deboy et al., 2006) and reduction of cavity formation and by regulating the activation of macrophages *in vivo (Lee et al., 2010).* Nevertheless, the role of IL-13, its closest family member and one of the main players in Th2 immune responses, has not been thoroughly investigated in the context of nervous system trauma.

On the other hand, the "pro-inflammatory" cytokine TNF-α has been considered a harmful molecule, the levels of which need to be reduced to promote recovery. Indeed, several, studies exist which show that inhibiting TNF-α leads to a better outcome after SCI (Genovese et al., 2006). The "anti-inflammatory" cytokine IL-13 suppresses TNF-α production at the translational level in monocytes (Minty et al., 1993) and macrophages (Mijatovic et al., 1997). This cytokine can also inhibit TNF-α-induced activation of nuclear transcription factors (NF-κB and activation protein-1) and apoptosis (Manna and Aggarwal, 1998). Another way to reduce circulating TNF-α levels is to use ADAM17 inhibitors (Kim et al., 2008). Treatments with ADAM17 inhibitors have been tested in preclinical trials in a mouse model of arthritis. Clinical trials, however, failed to confirm the promising results. Moreover, signs of liver toxicity of ADAM17 inhibitors were detected (Moss et al., 2008, Rose-John, 2013). So far, there is no information available on the role of this enzyme and its inhibitors in recovery after SCI.

In most of the studies on the modulation of TNF-α in SCI, TNF-α blockers were administered immediately after injury. Even though it is clinically more

relevant, the effects of TNF-α antagonist administration in later phases after SCI have not yet been investigated. Interestingly, there is also evidence that TNF-α contributes to axonal sprouting *in vitro* and *in vivo* (Oshima et al., 2009, Schmitt et al., 2010), while others demonstrated that the addition of TNF-α reduces neurite outgrowth and branching of primary hippocampal neurons (Neumann et al., 2002) as well as induction of "classically activated" macrophages (Kigerl et al., 2009). It is thus tempting to speculate that TNF-α may have differential effects depending on the context and immunological phase in which it is present. For this reason, further studies need to be performed to clarify the specific time-related role of TNF-α in SCI.

In addition, after injury to the CNS there is a strong inflammatory response characterized by the release of "pro-inflammatory" cytokines mainly by microglia/macrophages (Donnelly and Popovich, 2008). These immune cells seem to play a key role in promoting reparative or harmful processes. However, the mechanisms behind these opposing functions are yet poorly understood. Besides modulation of TNF-α, IL-13 can also induce an M2 microglia/macrophage phenotype. Under physiological conditions (non-injured), microglia/macrophages possess an M2 phenotype in the CNS (Ponomarev et al., 2007). It has been suggested that M1 microglia/macrophages are neurotoxic while M2 macrophages can promote long distance axon growth and reduction of inflammation as well as wound healing after CNS injury (Gordon, 2003, Kigerl et al., 2009). Temporal blocking of IL-6 receptor signaling with the monoclonal antibody MR16-1 after SCI induced a change in the cytokines profile present at the injured site, reducing interferon-gamma and TNF-α, while increasing IL-4 and IL-13 levels, and promoting functional recovery after injury (Guerrero et al., 2012). All these results suggest a strong relation between IL-13 and TNF-α. Based on these data in this thesis we have started to characterize IL-13 and TNF-α associated pathways in order to investigate interactions of these pathways in the future.

The aims investigated in this thesis are:

1. To study the potential function of the "anti-inflammatory" cytokine IL-13, in neurite regeneration and cell survival using an *in vitro* model of central nervous system regeneration.

2. To investigate the effects of the TNF-α antagonist etanercept in the chronic phase after SCI, in order to explore if inhibiting TNF-α can be used as a possible phase specific immunomodulatory therapy for SCI. This will be addressed by measuring TNF-α mRNA and protein levels before and after a T-cut hemisection SCI *in vivo*. Functional recovery after administration of soluble TNF-α or the TNF-α antagonist etanercept was assayed using the BMS score.

3. To investigate the physiological and cellular roles of ADAM17, and the therapeutic potential of a specific ADAM17 inhibitor for SCI. Measurements of cell viability *in vitro* were performed using an MTT assay, while *in vivo* immunofluorescence analysis was used to determine the expression of different immunological markers after SCI

CHAPTER 2

IL-13 promotes neurite outgrowth *in vitro* via a JAK-dependent mechanism

This chapter is based on: Lemmens E.*, Vidal PM.*, Nelissen S., Vangansewinkel T., Boato F., Lemmens S., Dooley D., Hendrix S. Interleukin-13 dose-dependently promotes neurite outgrowth *in vitro* via a Janus kinase-dependent mechanism, and promotes functional recovery *in vivo*. (Manuscript under preparation) (*equally contributing)

2.1 ABSTRACT

Increasing evidence suggests that cytokines associated with Th2 cells, contribute to regeneration after trauma to the CNS. We therefore tested in the present study the effects of IL-13, a canonical Th2 cytokine, on neurite growth and cell survival *in vitro* using primary neurons and organotypic brain slices from BALB/c mice. We show that the IL-13 receptor (IL-13R) is expressed on the cell body and neurites of primary neurons. We found IL-13 to be highly potent in promoting neurite growth in primary neurons and organotypic brain slices. While survival of primary neurons was not influenced, in the organotypic model a low dose of IL-13 (5ng/ml) protected from NMDA-induced toxicity, while a high dose (500ng/ml) was toxic in control conditions, but interestingly also significantly stimulated outgrowth from brain slices and the reinnervation of a denervated hippocampus. Furthermore, using specific inhibitors for selected IL-13 signaling pathway members, we demonstrated that the effect on outgrowth was mediated via Janus kinase signaling. In conclusion, the known anti-inflammatory properties of IL-13 as well as its dose-dependent influence on neurite growth and survival *in vitro*, make IL-13 a promising candidate for regenerative therapies after CNS trauma.

2.2 INTRODUCTION

A mounting body of evidence suggests that Th2 cells exert beneficial effects in the context of injury to the CNS (Hendrix and Nitsch, 2007). Therefore, during the last two decades it became commonly accepted that Th2-associated cytokines such as IL-4, IL-10 and IL-13, can be potent promoters of neuroprotection and regeneration after CNS trauma. Most of this evidence is indirect (reviewed in (Hendrix and Nitsch, 2007)). For example, glatiramer acetate, a potent inducer of a Th2 shift, was shown to suppress degeneration of motor neurons after nerve axotomy (Angelov et al., 2003); furthermore, Alum or Incomplete Freund's Adjuvant used for immunization, both promoters of a Th2 directed immune response, increased neuronal survival and axon regeneration (Huang et al., 1999, Sicotte et al., 2003), compared

to Complete Freund's Adjuvant which is a Th1 inducer (Shibaki and Katz, 2002). However, some studies showed a more direct link between the anti-inflammatory cytokine IL-10 and neuroprotection in the CNS (Bethea et al., 1999, Brewer et al., 1999, Zhou et al., 2009b), promoting functional outcome thus suggesting regeneration. In the present study, we investigated the hypothesis that selected Th2 factors exert beneficial effects on neurite outgrowth and cell survival *in vitro*. More specifically, we tested whether the canonical Th2 cytokine IL-13 influences key processes of CNS repair *in vitro*.

IL-13 is secreted predominantly by Th2 cells, but also by other T cell subsets, mast cells, dendritic cells, microglia, and macrophages (Hershey, 2003, Shin et al., 2004, Shirey et al., 2008). The IL-13R is known to be expressed on many different cell types, including B cells, mast cells, endothelial cells, fibroblasts, monocytes, and macrophages, but is absent on T cells (Hershey, 2003). To the best of our knowledge, whereas IL-13R RNA was detected using *in situ* hybridization in dopaminergic neurons (Morrison et al., 2012), the expression of IL-13R protein on neurons has not yet been reported. The biological functions of IL-13 are pleiotropic, and IL-13 may exert either immunostimulatory or immunosuppressive effects. For example, IL-13 inhibits the expression of pro-inflammatory cytokines (e.g. IL-6, IL-1β, and TNF-α) and other inflammation-associated factors (e.g. NO, ROS). IL-13 also plays an important role in the body's challenge against parasites and cancer; however, by promoting immunoglobulin E production, it contributes to the pathophysiology of allergic diseases such as asthma (reviewed in (Wynn, 2003)). Furthermore, in the EAE rodent model of multiple sclerosis, IL-13 has been shown to exert neuroprotective effects, decreasing infiltration of inflammatory cells and diminishing axonal loss, and to suppress clinical symptoms (Cash et al., 1994, Offner et al., 2005, Ochoa-Reparaz et al., 2008).

Here, we have determined the expression pattern of the IL-13R protein on primary neurons and have investigated whether IL-13 modulates neuronal cell survival and neurite regeneration *in vitro* using primary neuronal cell cultures and organotypic brain slices. We demonstrate for the first time that the IL-13R protein is expressed in both the cytoplasm of neurons as well as on neurites. In addition, we show that IL-13 dose-dependently promotes cell

survival and neurite growth, especially in brain slices, an effect mediated via JAK signaling.

2.3 MATERIALS AND METHODS

2.3.1 Primary neurons

2.3.1.1 Isolation and culture protocol

Primary neuronal cells were prepared from embryonic day 15 (E15) BALB/c mouse cortices by enzymatic dissociation using 0.5% trypsin and DNase treatment (0.1mg/ml) in Hank's balanced salt solution (HBSS), followed by mechanical dissociation by trituration to obtain a single cell suspension. Cells were seeded on poly-D-lysine coated cell culture plates directly (96-well plate) or on coated coverslips placed inside the wells of a 24-well plate, and cultured at 37 C and 5% CO_2 in Neurobasal medium containing 2% B-27, 1% L-glutamine and 1% penicillin/streptomycin (PS) (hereafter named 'neuron medium') for 2h prior to the start of the experiments. In this way, we obtained a mixed culture containing 80% neurons (80% beta-III tubulin positive cells; around 1% were $CD11b^+$ or $Iba1^+$ cells, while the other 19% are more likely NCAM+ progenitor cells; data not shown). All cell culture reagents were from Gibco® (Invitrogen, Belgium).

2.3.1.2 Viability assays

To measure the effect of IL-13 on cell viability (cell death and survival), two different tests were used; first, a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, for which primary neuronal cells were seeded on a 96-well plate at $1x10^5$ cells per well, and secondly, a lactate dehydrogenase (LDH) assay for which cells were seeded in a 24-well plate at $3x10^5$ cells per well; cells were incubated for 72h in neuron medium deprived of B-27 (deprivation model) with selected concentrations of mouse recombinant IL-13 (5, 50, or 500ng/ml; PeproTech, Rocky Hill, NJ) added simultaneously or after pretreatment with IL-13 for 48h. Cells treated with

10% DMSO were used as positive control of cell death in the LDH assay. To measure viability with the MTT assay, 1mg/ml MTT (Sigma, Belgium) in neuron medium was added to the cells and incubated with for 4h, after which the cells were lysed and the formazan crystals dissolved in a mixture of DMSO and glycine (0.1M); finally the absorption was measured at a wavelength of 550nm. To more specifically determine neuronal cytotoxicity after IL-13 treatment, an LDH assay was performed according to the manufacturer's instructions (LDH cytotoxicity assay kit II; Abcam, Cambridge, UK).

2.3.1.3 Neurite outgrowth assay

To measure neurite outgrowth from primary neurons, cortical neuronal cells were plated on coated coverslips in a 24-well plate at a density of $7.5x10^4$ cells/well, and IL-13 (5, 50, or 500ng/ml) was added to the neuron medium. After 72h, neurons were fixed with 4% paraformaldehyde (PFA) in 0.1M PBS for 15 min, permeabilized using PBS containing 0.2% Triton X-100 for 10 min, and incubated with 10% normal goat serum (NGS) in PBS for 60 min to block nonspecific binding. Cells were stained subsequently with primary monoclonal mouse anti-beta-III-tubulin antibody (Millipore, Belgium) diluted 1:500 in PBS containing 1% NGS (overnight at 4°C), and peroxidase-(HRP) conjugated goat anti-mouse secondary antibody (DakoCytomation, Belgium) diluted 1:400 in PBS for 60 min at room temperature (RT). Staining was visualized using 3,3'-diaminobenzidine (SigmaFast tablets; Sigma-Aldrich, Belgium) and finally, nuclear counterstaining with hematoxylin was performed before mounting the coverslips on a glass slide with Aquatex mounting medium (Merck, Belgium). Pictures of 6 randomly chosen fields per coverslip were taken with a digital camera connected to a Nikon Eclipse 80i microscope and analyzed using ImageJ software (NIH) as described before (Holtje et al., 2009). Average length of the longest neurite was measured by manual tracing for a total number of 50 neuronal cells per condition per experiment; the following exclusion criteria were used: neurites making contact with itself or with other neurites or cell soma,

neurites with a length smaller than twice the diameter of the cell soma, and neurites running outside the borders of the picture.

2.3.1.4 Immunofluorescence for the IL-13 receptor

A double immunofluorescence staining was performed to determine the expression of IL-13Rs on cortical neurons. In brief, after 72 hours, neurons were fixed with 4% PFA and permeabilized as described for the neurite outgrowth assay. Neurons were detected using an anti-beta-III-tubulin antibody (Millipore, Belgium), using a polyclonal rabbit antibody against IL-13Rα1 or a monoclonal rat antibody against IL-13Rα2 overnight (1:250; Abcam, UK). Glass coverslip were washed with PBS and then incubated with the corresponding secondary antibodies (goat anti-mouse Alexa 568, combined with goat anti-rabbit Alexa 488, or goat anti-rat Alexa 488 (1:250; Invitrogen, Belgium). DAPI was used to visualize the cells nuclei, and coverslips were mounted onto glass slides using immu-mount (ThermoFisher Scientific, Belgium). Cells were analyzed for beta-III-tubulin and IL-13R staining using a Nikon Eclipse 80i fluorescence microscope.

2.3.2 Organotypic brain slices

All slice culture reagents used in the two models described below were obtained from Gibco® (Invitrogen, Belgium) unless stated otherwise.

2.3.2.1 Cytotoxicity assay with propidium iodide staining

Hippocampal entorhinal cortex slices were isolated from 8 day old (P8) BALB/c mice, with some modifications. Briefly, brains were carefully dissected out in ice-cold dissection medium (MEM, containing 2mM L-glutamine and distilled water), and 350µm thick slices containing hippocampus and entorhinal cortex were prepared using a tissue chopper (McILWAIN, H. Saur, Germany). The slices were cultured on a culture insert (0.4µm porous; Millipore, Belgium) with different concentrations of IL-13 (5, 50, 500ng/mL) for 72 hours in incubation medium (50% MEM, 25% HBSS,

25% heat-inactivated horse serum, 4mM L-glutamine, 4ng/mL insulin, 800ng/mL ascorbic acid, 2.4mg/mL glucose and 1% P/S) (Stoppini et al., 1991). To induce cell death, the IL-13 pre-treated slices were incubated with 100µM N-methyl-D-aspartic acid (NMDA; Sigma, Belgium) for 4 hours. Afterwards, the slices were washed with incubation medium, and propidium iodide (PI 5µg/mL; Invitrogen, Belgium) was added during 10 minutes. Pictures were taken with a fluorescence microscope (Nikon Eclipse 80i fluorescence microscope), and the uptake of PI from the cells of the cornu ammonis region (CA1-CA3) of the hippocampus was analyzed. Analysis of fluorescent intensity was performed using ImageJ software.

2.3.2.2 Neurite outgrowth from collagen-embedded entorhinal cortex slices

Entorhinal cortex (EC) slices were prepared from P2 mice as described before (Schmitt et al., 2010). Briefly, brains were removed and placed in ice-cold dissection medium. Slices containing the EC region were prepared using a McIwain tissue chopper (350µm thickness). Each EC slice was embedded in a single drop of collagen (type I, 2mg/mL; Sigma, Belgium), and cultured for 48 hours in the presence or absence of IL-13 (5, 50, 500ng/mL), in combination with different inhibitors for the IL-13 signaling cascade. The following inhibitors were used, directed against JAK1 (100nM); JAK2 (10µM); JAK3 (100µM); Akt (LY294002, 100µM); MAPK (U0126, 100µM), (all from Calbiochem, Merck, Belgium) and STAT6, (lefluromide, 250µM, Sigma-Aldrich, Belgium). The inhibitors were all dissolved in different concentrations of DMSO. As a control 0.1% and 1% DMSO were used for JAK1 and JAK2, and for JAK3, Akt, MAPK, and STAT6 inhibitors, respectively. Neurite outgrowth was measured as described before (Schmitt et al., 2010). Picture processing was performed based on the Sobel algoritm after which the mean intensity was calculated in a standardized area of each slice (Holtje et al., 2009, Hechler et al., 2010, Schmitt et al., 2010, Boato et al., 2011)

2.3.2.3 Reinnervation assay in brain slices

To analyze reinnervation from the hippocampus, EC slices were prepared from P2 β-actin-eGFP$^+$ mice and co-cultured with hippocampus of P2 wild type mice (Hechler et al., 2010). In brief, 350µm thick sections were cut using a tissue chopper and placed on porous membranes (Millicell-CM; Millipore, Belgium) in such a way that the correct anatomical relation between hippocampus and EC was restored. Slices were cultured at 37 °C with 5% CO_2, on a medium with pH 7.2 containing 50% MEM, 25% Basal Medium Eagle (BME) medium, 25% heat-inactivated NHS, 2mM glutamax, and 0.65% glucose; slices were then treated with 500ng/ml of IL-13 for 48h, after which they were fixed with 4% PFA and 5% glutaraldehyde, mounted on slides, and photodocumented. using a Photometrics CoolSNAPTMES camera (VisitronSystems GmbH, Munich, Germany) connected to a fluorescence microscope (Olympus BX50). Growth of eGFP+ fibers from the EC into the GFP-negative hippocampus was determined by intensity analysis of a standardized area in the hippocampus using MetaMorph Image Software (VisitronSystems GmbH).

2.3.3 Statistical analysis

For all the experiments comparing two groups, differences were calculated with a t-test or the non-parametric Mann-Whitney U test. For experiments comparing more than two groups, ANOVA or Kruskal Wallis tests were used.
Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad software Inc., CA, USA) and PASW statistics 18 (SPSS) software. Differences were considered significant at $p<0.05$.

2.4 RESULTS

2.4.1 The IL-13Rα1 subunit is present on primary cortical neurons

Immunohistochemical analysis showed that the IL-13Rα1 subunit is present in primary cortical neurons, while the IL-13Rα2 subunit was not detected (not shown). The staining pattern of IL-13Rα1 was mainly concentrated in the cell cytoplasm as well as staining of the neurite distal end (Fig. 2.1).

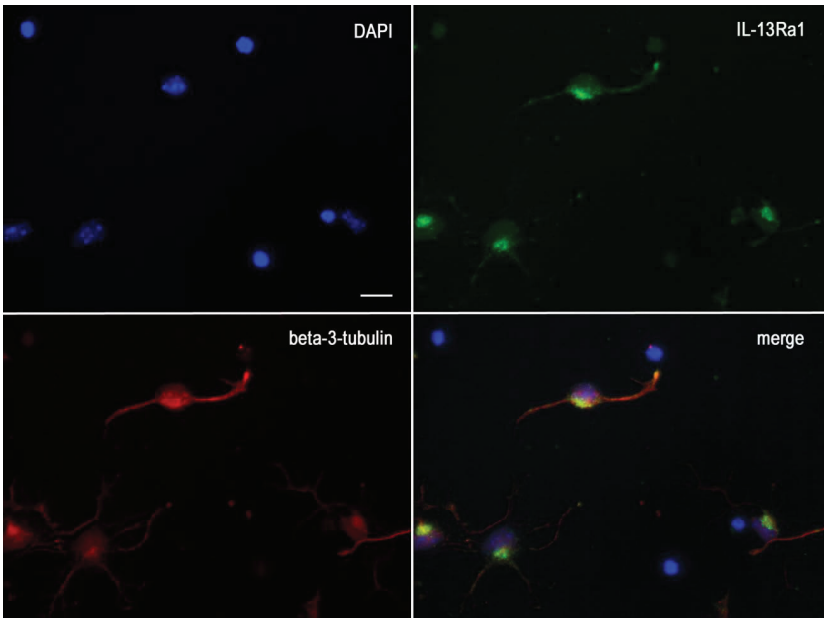

Fig. 2.1: Primary cortical neurons express the alpha-1 subunit of the IL-13 receptor. Immunofluorescent staining of primary cortical neurons showing the cell nucleus (DAPI, blue), the neurites (beta-III-tubulin, red), and the alpha-1 subunit of the IL-13 receptor (IL-13Rα1, green). A homogenous IL-13Rα1 immunoreactivity pattern is detected in the cell cytoplasm as well as on the neurites. Scale bar= 10µm.

2.4.2 IL-13 does not influence the survival of primary neurons *in vitro*

To study the effect of IL-13 on the survival of primary neurons, cortical neuronal cells were isolated from embryonic mice and cultured in the presence or absence of B-27, with or without treatment with selected concentrations of IL-13. Cell survival, as measured with the MTT assay, significantly decreases to 75-80% approximately (**p<0.01) after B-27 withdrawal. However, IL-13 treatment does not promote or impair cell survival. No difference was seen between cells that are simultaneously deprived of B-27 and treated with IL-13 for 72h (Fig. 2.2A), and cells that are first pretreated with IL-13 for 48h and then deprived of B-27 for 72h (Fig. 2.2B). Similar results were found using the LDH assay for cytotoxicity (data not shown).

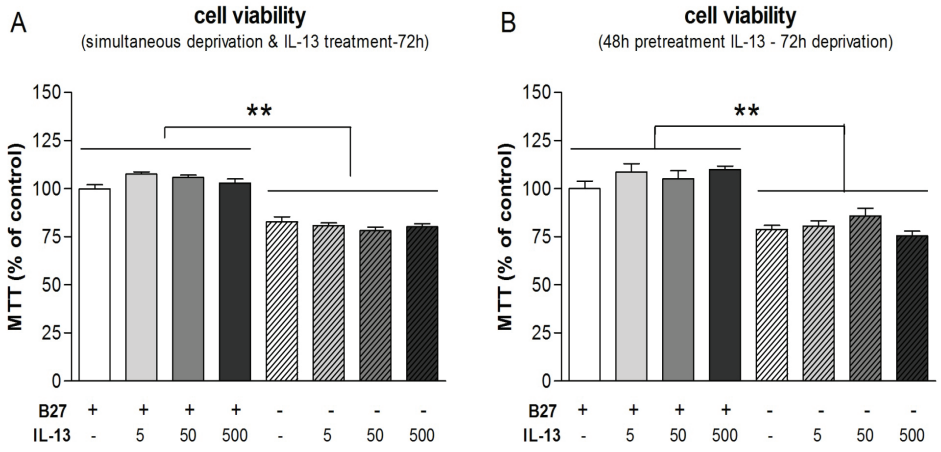

Fig. 2.2. Treatment with IL-13 does not influence cell survival of primary cortical neurons. **A**. Primary neuronal cells were incubated for 72h in neuron medium with or without B-27 and treated with different concentrations (ng/ml) of IL-13; cell viability was measured by an MTT assay and values expressed as percentage of control. B-27 withdrawal induced a decreased cell viability of about 20 to 25% but IL-13 treatment in either concentration had no effect on this. **B**. Pretreatment with different concentrations of IL-13 (ng/mL) for 48h, followed by B-27 withdrawal for 72h also had no effect on cell survival. Data are presented as mean + SEM of four separate experiments, (** $p < 0.01$), (n=15 wells per condition).

2.4.3 IL-13 exerts a concentration-dependent effect on survival of neurons in organotypic brain slices *in vitro*

Since IL-13 may indirectly affect survival of hippocampal neurons via cells expressing the IL-13R such as brain microglia (Shin et al., 2004, Park et al., 2009), organotypic brain slices containing all major brain cells (including neurons, glial cells and local immune cells) were treated with NMDA, as a model for cytotoxicity, to study possible protective effects of selected concentrations of IL-13. Intensity analysis of PI staining, as a measurement of cell death, in the hippocampal CA area (Fig. 2.3A) revealed that a high concentration of IL-13 (500ng/mL) is toxic to hippocampal neurons, while a low concentration of IL-13 (5ng/ml) protects these neurons from NMDA-induced cytotoxicity. Representative photomicrographs show a decreased intensity of PI staining in the hippocampal CA area of these slices (NMDA+ IL-13 5ng/ml) compared to slices treated with NMDA alone (Fig. 2.3B).

2.4.4 IL-13 concentration-dependently increases neurite outgrowth from primary cortical neurons and EC slices

Administration of IL-13 to cultures of primary cortical neurons stimulates neurite outgrowth concentration-dependently. A statistically significant increase is induced with a concentration of 5ng/ml (**$p < 0.01$) and 50ng/ml (*$p < 0.05$). Interestingly, a higher concentration of 500ng/ml ($p= 0.11$) does not influence neurite outgrowth (Fig. 2.4). In contrast, in collagen-embedded EC slice cultures only a non-significant trend towards increased neurite outgrowth is found after administration of 5 or 50ng/ml IL-13, while neurite outgrowth is significantly increased by 25% when EC slices are treated with a high concentration of IL-13 (500ng/ml), about the maximum than can be reached in this model (Fig. 2.5).

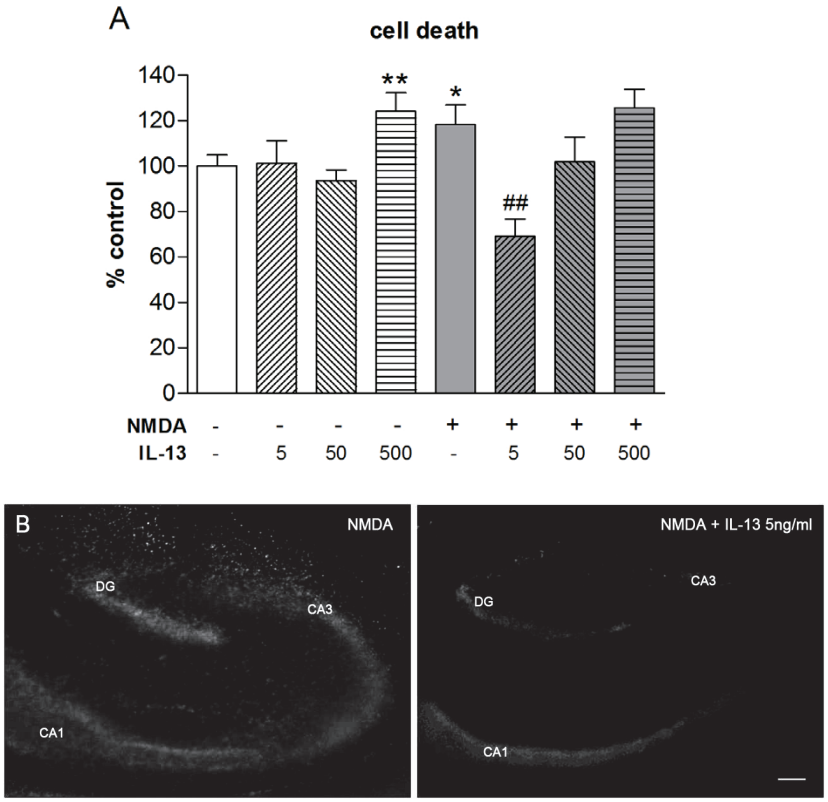

Fig. 2.3. Treatment with IL-13 concentration-dependently affects cell survival in organotypic hippocampal-entorhinal cortex slices. **A**. Hippocampal-entorhinal cortex slices were incubated for 72h with selected concentrations (ng/ml) of IL-13, with or without NMDA (100µM) to induce cell death. While a low concentration of 5ng/ml IL-13 protected hippocampal neurons from NMDA-induced toxicity, a high concentration of 500ng/ml IL-13 solely induced cell death. Data are presented as mean (percentage of control) + SEM, n=18-38 slices per condition, * $p < 0.05$ and ** $p < 0.01$ compared to the untreated control (no NMDA, no IL-13), $^{\#\#}$ $p < 0.01$ compared to the NMDA control (no IL-13; grey bar). **B**. Representative pictures of the PI staining, depicting the hippocampal cornu ammonis (CA) area 1 and 3, showing a decrease in fluorescence intensity back to control values when slices were treated with NMDA + IL-13 (5ng/ml) compared to NMDA treatment alone. DG= dentate gyrus. Scale bar= 250µm

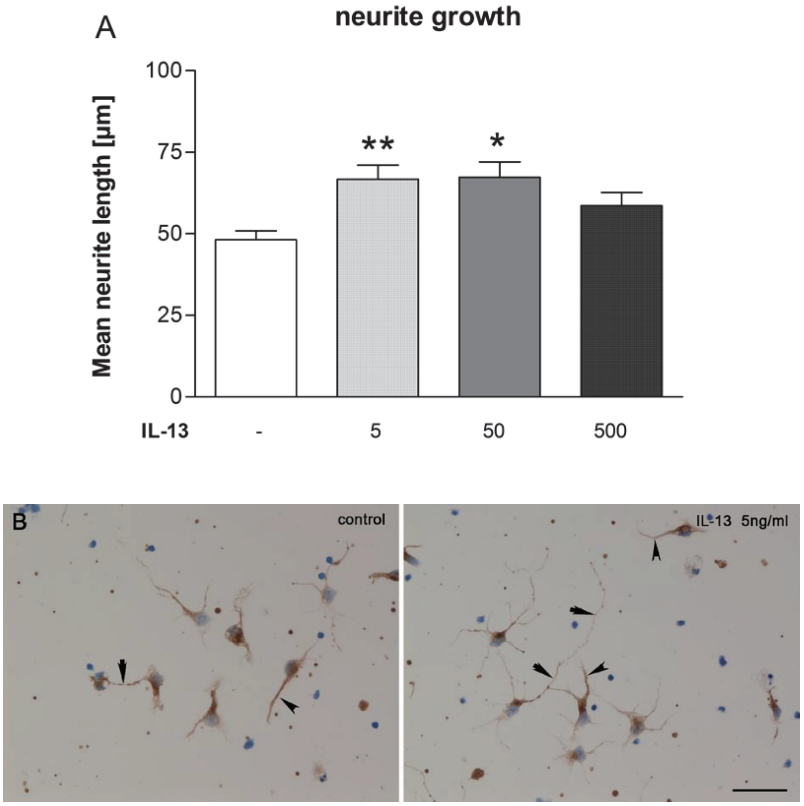

Fig. 2.4. Treatment with IL-13 concentration-dependently increases neurite length in primary neurons. **A.** Primary neuronal cells were incubated for 72h with selected concentrations (ng/ml) of IL-13 and neurite length was measured by freehand tracing of the neurites using ImageJ. Treatment with both 5 and 50ng/ml of IL-13 significantly increased neurite length. Data are presented as mean neurite length + SEM, n=100 neurites analyzed/group, * $p < 0.05$, ** $p < 0.01$ compared to the untreated control (no IL-13 added). **B.** Representative pictures showing beta-III-tubulin staining (brown) of the primary neuron culture in control condition or treated with 5ng/ml IL-13, with arrows pointing towards the longest neurite of the corresponding cell. Scale bar= 100µm.

2.4.5 IL-13 stimulates reinnervation of hippocampal tissue

In order to investigate whether IL-13 may influence not only neurite outgrowth but also growth of axons into the corresponding target tissue, we made use of a complex reinnervation assay adding the high concentration of IL-13 (500ng/ml) which was effective in the outgrowth assay using EC slices. Within this co-culture system, IL-13 was a potent stimulator of reinnervation (Fig. 2.6A), as visualized by increased eGFP intensity in the hippocampus of treated slices (Fig. 2.6B), suggesting increased perforant path fiber growth compared to untreated controls.

2.4.6 The effects of IL-13 treatment on neurite growth in EC slices are mediated via JAK signaling

Collagen-embedded EC slices were treated with 500ng/ml IL-13, as before, and inhibitors of IL-4Rα and IL-13Rα1 signaling pathways were added. Treatment with IL-13 results in increased neurite outgrowth compared to 0.1% DMSO alone (Fig. 2.7A) while IL-13 does not increase outgrowth compared to 1% DMSO (Fig. 2.7B); thus, the following results must be interpreted with caution since adding 1% DMSO (as a control for the inhibitors' dilutions) abolished the IL-13 effect. When adding the JAK inhibitor only, values do not differ from the DMSO control, but after treatment with IL-13+JAK inhibitor, neurite outgrowth is significantly decreased compared to slices treated with IL-13+0.1% DMSO ($p < 0.05$). JAK2 inhibition does not result in significant changes in neurite growth (Fig. 2.7A). Inhibitors of JAK3, MAPK and Akt significantly decrease outgrowth compared to 1% DMSO; while adding IL-13 does not turn outgrowth back to control levels (IL-13+1% DMSO), IL-13 significantly ($p < 0.01$) increases outgrowth in the presence of the JAK3 inhibitor (Fig. 2.7B).

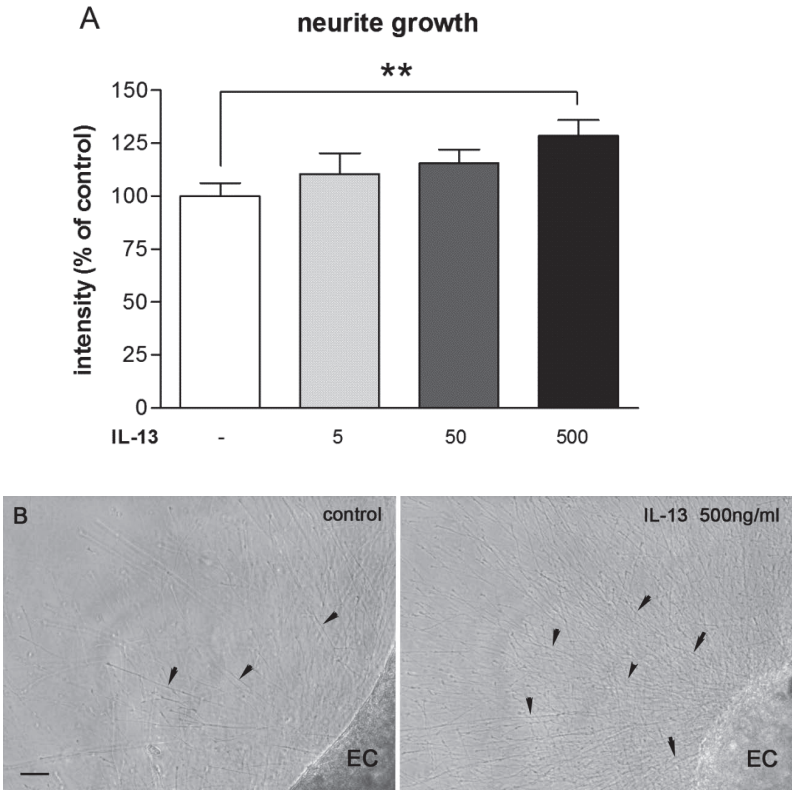

Fig. 2.5. High concentration IL-13 treatment induces increased neurite outgrowth in collagen-embedded entorhinal cortex slices. **A**. Entorhinal cortex (EC) slices were incubated for 48h with selected concentrations (ng/ml) of IL-13, after which pictures were taken and axonal density was analyzed using ImageJ. Only a high dose of 500ng/ml significantly increases outgrowth. Data are presented as mean (% of control) + SEM, n=15-24 slices per condition (** $p < 0.01$). **B**. Representative pictures of EC slices, showing an increased density of neurites (arrow heads) growing out of the slices treated with 500ng/ml IL-13 compared to control slices. Scale bar = 100µm.

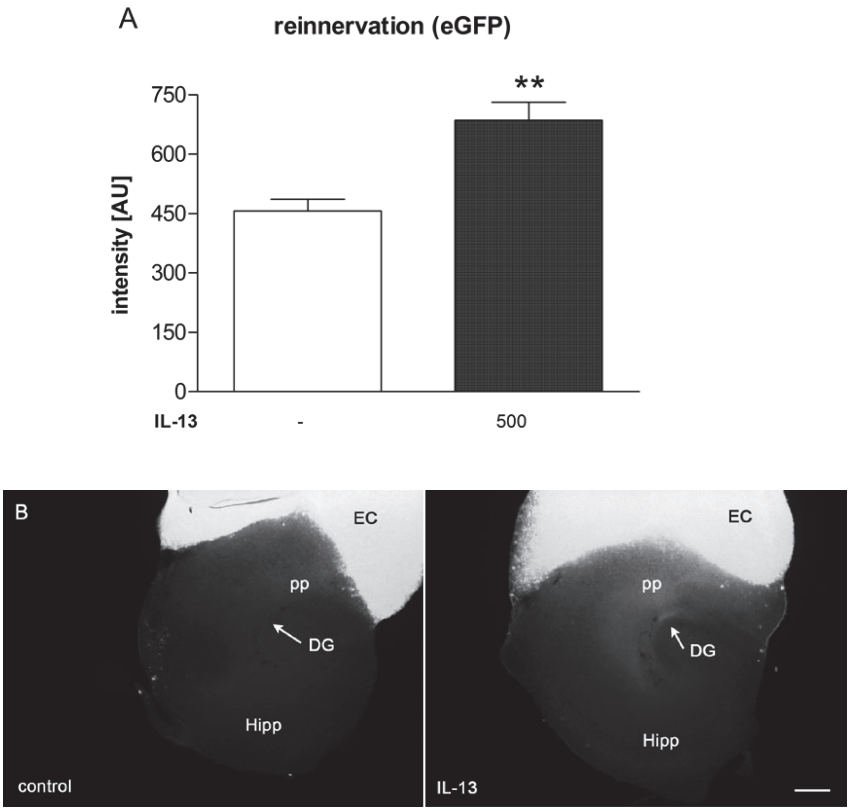

Fig. 2.6. IL-13 stimulates ingrowth of axons from the entorhinal cortex (EC) to the hippocampus. A. Quantification of the intensity of the eGFP positive area in the hippocampus shows a significant stimulation of axonal growth from the $eGFP^+$ EC into the hippocampus when treated with IL-13 (500ng/ml). Data are presented as mean intensity + SEM, n=16-17 slices per condition (** $p < 0.01$). **B**. Representative images of the co-culture model in which an eGFP+ EC is co-cultured with a wild type (eGFP-) hippocampus (Hipp) and axons, of the perforant path (pp) presumably, then reinnervate the dentate gyrus (DG) of the hippocampus. Note the more intense perforant path in the IL-13 treated slice compared to the control slice (arrow). Scale bar= 100µm.

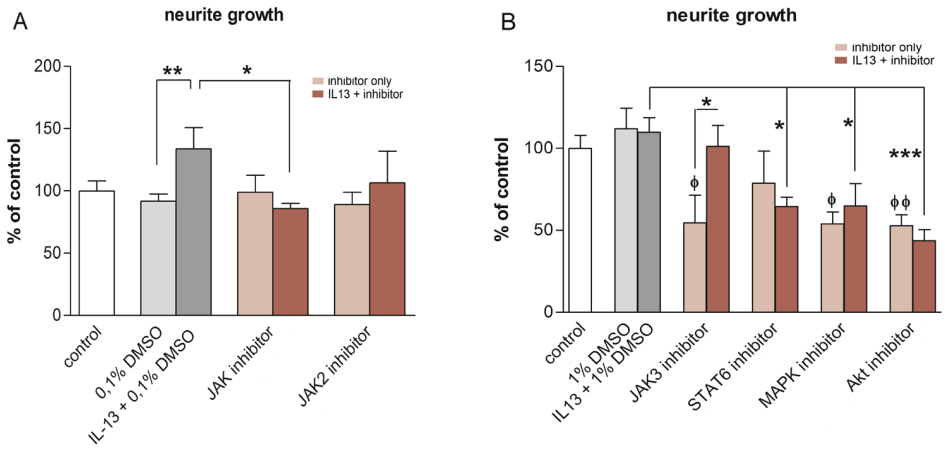

Fig. 2.7. IL-13 induced outgrowth in entorhinal cortex slices can be blocked with JAK inhibitor. **A**. Entorhinal cortex (EC) slices were incubated for 48h with 500ng/ml of IL-13, in the absence or presence of several IL-13 signaling pathway inhibitors (JAK1 and JAK2) in a final concentration of 0.1% DMSO. **B**. Inhibitors for JAK3, STAT6, MAPK, and Akt were diluted to a final concentration of 1% DMSO, which abrogates the IL-13 induced effect on outgrowth. Data are presented as mean (percentage of control) outgrowth + SEM, n=3-20 slices per condition; compared to 1% DMSO: $^{\Phi}$ $p < 0.05$ or $^{\Phi\Phi}$ $p < 0.01$, and compared to rIL-13 + 1% DMSO: * $p < 0.05$ or *** $p < 0.001$.

2.5 DISCUSSION

It is a long held belief that Th2 cytokines, such as IL-4, IL-10, or IL-13, may exert beneficial effects in the context of CNS injury. There is increasing evidence *in vitro* and *in vivo* that Th2 cells or a systemic Th2 shift may increase neuroprotection and axon regeneration after damage to the CNS (Hendrix and Nitsch, 2007, Vidal et al., 2012). A beneficial effect of IL-4 and IL-10 on neuronal survival and neurite outgrowth has been already shown *in vitro* (Butovsky et al., 2006b, Golz et al., 2006, Zhou et al., 2009b) and *in vivo* (Bethea et al., 1999, Brewer et al., 1999, Koeberle et al., 2004, Deboy et al., 2006, Zhou et al., 2009a). For IL-13, only indirect evidence exists, suggesting a regenerative effect after SCI (Hu et al., 2012, Nakajima et al., 2012).

Here we show for the first time that the IL-13Rα1 subunit is expressed in cortical neurons; on the contrary, the IL-13Rα2 subunit is not present in these neurons. The explicit presence of IL-13Rα1 on neurites may suggest a role of IL-13 signaling in neurite growth. It is known that expression of mouse IL-13Rα1 induces activation of STAT3, in addition to STAT6 (Orchansky et al., 1999, Umeshita-Suyama et al., 2000). Furthermore, it was previously shown that expression of IL-13Rα1 is immediately upregulated in brain tissue after mild traumatic brain injury, coinciding with apoptosis (Pun et al., 2011), suggesting an important role for IL-13 signaling in cell survival after CNS injury.

To investigate this, we determined the effects of IL-13 treatment on neuronal survival of primary neurons and organotypic brain slices; IL-13 rescued neurons from NMDA-induced cytotoxic damage in slices. Interestingly, only the lower concentration of IL-13 (5ng/mL) was protective while a high concentration had toxic effects. Indirect effects on survival have been reported before, being either detrimental (Park et al., 2009, Nam et al., 2012) or protective (Shin et al., 2004) and mediated via microglia cells. Complementary studies will need to identify the neuronal population(s) affected by IL-13 treatment, using confocal microscopy to also allow cell counting. Furthermore, we showed that IL-13 concentration-dependently increased neurite outgrowth from primary cortical neurons and organotypic brain slices. Surprisingly, the higher concentration of IL-13 (500ng/mL) did

not significantly increase neurite outgrowth. This phenomenon can be in part explained by the IL-13's equilibrium constant. IL-13 binds to its ligand-binding chains with low affinity, and therefore the saturation point of binding chains is not achieved at low IL-13 concentrations (5-50ng/mL). It is probable then, that the lack of effect observed using the higher concentrations of IL-13 (500ng/mL) is due to saturation of the IL-13R. Another important factor to consider is the number of IL-13Rα1 chains, since the number of signaling chains that can be formed in response to IL-13 correlates with the number of IL-13Rα1 chains (Junttila et al., 2008). IL-13 also promoted reinnervation of hippocampal slices by perforant path fibers. Interestingly, a high concentration of IL-13 results in both increased cell death and increased neurite outgrowth in brain slices. A similar finding was previously reported, where the authors described outgrowth as a mechanism to compensate for the loss of dying cells in a network (Finkelstein et al., 2000). On the contrary, others have shown that axon growth and survival are not necessarily linked (Lu et al., 2001, Goldberg, 2003). Our results using primary neurons suggest that IL-13–induced cell death and neurite growth occur independently in our well-controlled *in vitro* model, since IL-13 did not influence cell survival on primary cortical neurons under optimal culturing conditions and using B-27-deprivation.

Lastly, we demonstrated that the effects of IL-13 on neurite outgrowth are mediated, at least in part, via JAK1 signaling, which is coupled to the IL-4Rα chain of the IL-13R (Hershey, 2003). This was demonstrated by decreased outgrowth when slices were incubated with JAK inhibitor, an effect that could not be reversed when adding IL-13 together with the inhibitor. Our data also suggest the involvement of STAT6, MAPK, and Akt in IL-13 induced outgrowth; furthermore, it is clear that JAK3 signaling is not involved in IL-13 effects on outgrowth, since adding IL-13 together with the JAK3 inhibitor significantly increased outgrowth back to control levels. It is important to mention, that 1% DMSO slightly, but not significantly, increased outgrowth in EC slices. This phenomenon may in part be explained by the known property of 1-2% of DMSO to induce differentiation and morphological changes in diverse cell types (Miller et al., 1991, Pal et al., 2012).

To summarize, the IL-13 receptor is expressed on neurons and IL-13 concentration-dependently promotes survival and neurite growth *in vitro*. These findings, together with the known anti-inflammatory properties of IL-13, make this cytokine a promising candidate for the *in vivo* treatment of CNS injuries.

CHAPTER 3

Late blocking of peripheral TNF-α is ineffective after spinal cord injury in mice

Part of this chapter is based on: Vidal PM.*, Lemmens E*., Geboes L., Vangansewinkel T., Nelissen S., and Hendrix S. Late blocking of peripheral TNF-α is ineffective after spinal cord injury in mice. Immunobiology. 2013 Feb, 218(2):281-4. (*equally contributing)

3.1 ABSTRACT

SCI is characterized by different phases of inflammatory responses. Increasing evidence indicates that the early chronic phase (two to three weeks after SCI) is characterized by dramatic invasion of immune cells and peak of pro-inflammatory cytokines levels, such as TNF-α derived from the injured spinal cord as well as from injured skin, muscles and bones. However, there is substantial controversy whether these inflammatory processes in later phases lead to pro-regenerative or detrimental effects. In the present study, we firstly studied the TNF-α mRNA and protein levels at different time points after a T-cut hemisection injury. We found TNF-α mRNA levels to increase in the first 7 days after SCI, however, TNF-α protein levels were downregulated during this (sub)-acute phase. Reestablishing the decrease in systemic TNF–α levels during the acute phase by a single injection of TNF-α immediately after SCI, did not affect locomotor recovery after injury. Secondly, we investigated whether the inhibition of peripheral TNF-α, which tended to increase in the early chronic phase after injury, promotes functional recovery. Three different approaches were used to continuously block peripheral TNF-α *in vivo*, starting 14 days after injury. We administered the TNF-α blocker etanercept intraperitoneally (i.p; every second day or daily) as well as continuously via osmotic minipumps. None of these administration routes for the TNF-α inhibitor influenced locomotor restoration as assessed by the Basso mouse scale (BMS), nor did they affect coordination and strength as evaluated by the Rotarod test. These data suggest that peripheral TNF-α inhibition may not be an effective therapeutic strategy in the early chronic phase after SCI.

3.2 INTRODUCTION

The pluripotent pro-inflammatory cytokine TNF-α is synthesized by many cell types including neurons, glia, activated macrophages, T cells, astrocytes, Schwann cells and mast cells (Hopkins and Rothwell, 1995). It is immediately upregulated in response to central nervous system (CNS) trauma such as SCI (Tracey and Lowry, 1990, Wajant et al., 2003, Pineau and Lacroix, 2007, Oshima et al., 2009) deriving from the injured CNS itself as well as from injured skin, muscles and bones. Besides the first peak of TNF-α mRNA levels in the acute and subacute phases after SCI (Bartholdi and Schwab, 1997, Streit et al., 1998, Bethea et al., 1999, Pineau and Lacroix, 2007, Koopmans et al., 2009), some authors have also reported a second TNF-α mRNA peak around 14-28 days after injury (Pineau and Lacroix, 2007) .

There is a lot of controversy about the *in vitro* and *in vivo* effects of TNF-α after CNS trauma, reported to be either detrimental (Lee et al., 2000, Clarke and Branton, 2002, Neumann et al., 2002, Chertoff et al., 2011) or neuroprotective (D'Souza et al., 1995, Sullivan et al., 1999, Oshima et al., 2009, Chertoff et al., 2011) and pro-regenerative (Schmitt et al., 2010), depending on the animal model used. These contradicting results may be in part the result of TNF-α interaction with two types of receptors, namely TNFR-1 and TNFR-2, which are expressed on different cell types and are associated with different cellular effects (Wajant et al., 2003). In different models of SCI, TNF-α appears to have detrimental effects on spinal cord recovery, since treatment with TNF-α blockers, such as etanercept and rolipram (Genovese et al., 2006, Genovese et al., 2007, Koopmans et al., 2009), and antibodies to the TNFR-1 (Lee et al., 2000, Clarke and Branton, 2002, Neumann et al., 2002) improved spinal cord recovery substantially in different rodent models. In contrast, the life-long absence of both TNFR-1 and TNFR-2 in knockout mice reduces NF-κB activation and functional recovery after SCI (Kim et al., 2001). However, these TNF-α inhibitors do not selectively block one of the two TNF-α forms (membrane-bound or soluble). In general, it seems that membrane-bound TNF-α has distinct beneficial functions while soluble TNF-α is more associated to detrimental

ones. However, so far there is not clear information about the role of the two TNF-α forms in SCI.

Increased TNF-α levels shortly after CNS trauma have been linked to a sequence of cellular dysregulations, accompanied by enhanced vascular permeability, impaired glutamate metabolism and clearance (Takahashi et al., 2003), and in some settings an excessive inflammatory reaction . Several studies indicate that it is important to regulate increased TNF-α levels immediately after injury to control the excitotoxic effect of TNF-α on AMPA and GABA receptors after injury (Stellwagen et al., 2005, Zhao et al., 2010), and to decrease TNF-α-induced apoptosis (Genovese et al., 2006). Consistently, a single dose of etanercept immediately after injury appears to be sufficient to improve hind limb locomotor function and reduce apoptosis of neurons and oligodendrocytes in the rat spinal cord (Chen et al., 2011).

However, in all functional studies TNF-α antagonists were administered immediately or 6h after injury (Genovese et al., 2006, Genovese et al., 2007, Chen et al., 2011), ignoring the second peak of TNF-α in the later phases after injury. Furthermore, different rodent species (mice and rats) were used, as well as different SCI models (compression and contusion). There is still much controversy whether the inflammatory response in the chronic phase after CNS trauma promotes or inhibits regeneration (Hendrix and Nitsch, 2007). In addition, a modulation of peripheral inflammation may influence substantially the CNS immune status even if the blood brain barrier (BBB) is intact (Teeling et al., 2010). Thus, a modulation of peripheral TNF-α with inhibitors such as etanercept that do not cross the BBB may be a promising approach during the chronic phases after CNS trauma when the BBB is already repaired. In this study, we firstly investigated mRNA and protein levels of TNF-α at different time points after a T-cut hemisection SCI. Secondly, we studied the effect of administration of soluble TNF-α in the acute phase, and the inhibition of TNF-α by etanercept in the early chronic phase, using different doses and administration routes. Our data, however, indicated that all therapeutic approaches were ineffective in modulating the functional outcome after SCI.

3.3 MATERIALS AND METHODS

3.3.1 Spinal cord T-cut hemisection injury

A T-cut hemisection injury was performed as described previously (Boato et al., 2010, Loske et al., 2012, Tuszynski and Steward, 2012). Briefly, 10-week old female C57BL/6 mice (Harlan, the Netherlands) were anesthetized and underwent a partial laminectomy at thoracic level T8. Next, the mice were subjected to a bilateral dorsal T-cut hemisection using iridectomy scissors to transect the left and right dorsal funiculus, the dorsal horns and the ventral funiculus. This procedure leads to a complete transection of the dorsomedial and ventral CST and induces impairment of several other descending and ascending tracts, resulting in impaired movement of the hind limbs, loss of bladder function, etc. The muscles were sutured and the back skin closed with wound clips. After surgery, the mice were placed in a recovery chamber until they were well awake and could be returned to their home cage. The animals' bladders were manually voided daily until the animals were able to urinate independently. Sham operated animals only received laminectomy, without hemisection injury. Control animals were left untouched.

3.3.2 Experimental groups

3.3.2.1 TNF-α reconstitution

To investigate the effect of increasing TNF-α protein levels (which were decreased both, locally and systemically immediately, after SCI) to basal of untouched control animal levels, mice were randomly assigned to one of the following treatment groups immediately after injury: PBS treated mice (control group) and soluble TNF-α treated mice (reconstituted in PBS + 0.1% BSA). For this, one single i.p. injection of 360pg/0.1mL of soluble TNF-α (R&D systems, UK), or vehicle solution (PBS + 0.1% BSA) was immediately applied after injury.

3.3.2.2 Late blocking of TNF-α by etanercept

Since it has been suggested that TNF-α mRNA levels are increased around day 14-28 after SCI (Pineau and Lacroix, 2007), the effect of blocking this late-phase rise in TNF-α was investigated. Before the start of the treatment at day 14 post SCI, mice were equally distributed among two groups according to their mean BMS score during the first two weeks after SCI. For the first experiment, mice were injected i.p once every two days, for twelve days in total, with etanercept (Enbrel®, Amgen, Pfizer; 125µg/mouse) (Genovese et al., 2006), or with saline solution (0.9% w/v NaCl) or IgG from human serum (Sigma; 125µg/mouse) as a control. For the second experiment, animals were injected daily i.p. with saline solution or etanercept (125µg/mouse) for one week. Finally, for the third experiment, the mice were deeply anesthetized with isoflurane, a small incision was made in the back skin to implant s.c. an osmotic minipump (Alzet 2004; 0.25µl/hr) filled with etanercept or saline, and after implantation the back skin was closed with wound clips.

3.3.3 Locomotion tests

Animals were scored daily for functional recovery after SCI using the BMS (Basso et al., 2006) starting at day 1 after injury or with the rotarod performance test starting at day 7. The BMS is a 10-point scale (9=normal locomotion; 0=complete hind limb paralysis) which is based on hind limb movements made in an open field during a 4 min interval, provided the mouse is moving for at least three body lengths using a consistent speed. In this study, the mean score of the left and right hindlimb for each animal was used for BMS analysis.

After allowing mice to recover for 6 days, also rotarod performance (Sheng et al., 2004) was determined once daily up to the end of the observation periods. Hereto, the mice were placed on an accelerated rolling rod (Ugo Basile, Comeris VA, Italy) and the latency to jump off from the rod was automatically recorded by the action of the mouse dropping onto a trigger plate.

3.3.4 TNF-α mRNA levels in the spinal cord by real-time PCR

TNF-α mRNA levels from spinal cord samples were measured at selected time points (1 and 6 hours, and 2, 4, 7, 14 and 21 days) after SCI. Briefly, RNA was isolated from spinal cords of uninjured (control), sham operated (laminectomy only), and mice with a T-cut lesion. Animals were transcardially perfused with ringer solution; this procedure was done at selected time points (1 and 6 hours, and 2, 4, 7, 14 and 21 days) after injury. After perfusion, a standardized area (0.5cm proximal and 0.5cm distal from the lesion site) of spinal cord tissue was dissected out, and homogenized using the RNeasy Mini Plus Kit (Qiagen, the Netherlands), according to the manufacturer's instructions. After reverse transcription (Promega, the Netherlands), cDNAs were amplified (Taqman Gene Expression Assays) on an ABI PRISM 7500 sequence detection system (Applied Biosystems, USA). Amplification conditions consisted of an initial denaturing/activation step at 95°C for 20 seconds, followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. A threshold cycle was calculated and relative quantification was obtained compared to the reference housekeeping genes β-actin, hypoxanthine guanine phosphoribosyl transferase 1, and β-glucuronidase.

3.3.5 TNF-α protein expression in serum and spinal cord

To determine TNF-α protein levels in serum and spinal cord samples, uninjured mice (control), sham operated mice (laminectomy only) and mice with a T-cut dorsal hemisection, were used. After performing a cardiac puncture to obtain blood for serum samples, animals were transcardially perfused with ringer solution; this procedure was done at selected time points (1 and 6 hours, and 2, 4, 7, 14 and 21 days) after injury. After perfusion, a standardized area (0.5cm proximal and 0.5cm distal from the lesion site) of spinal cord tissue was dissected out, and homogenized using Procarta lysis buffer (Panomics, Italy); homogenized tissue was centrifuged at 10000 rpm for 10 minutes at 4°C, after which the supernatant was collected. Protein levels were determined by flow cytometry using (i) the

cytometric bead array (CBA) mouse flex set system (BD Biosciences) for serum and spinal cord samples, according to manufacturer's instructions, or (ii) by western blotting for tissue samples only (data not shonw). CBA analysis was performed using FACSArray Bioanalyzer and FCAP software (BD Biosciences). Data represent mean values + SEM.

3.3.6 Statistical analysis

Behavioral data (rotarod and BMS) were analyzed using a two way ANOVA as previously described (Basso et al., 2006) and graphs represent mean values for all the animals of each experimental group (+ SEM). Real-time PCR, western blotting, and CBA data were performed using a Mann-Whitney U test for comparison between two groups. The analyses were performed using GraphPad Prism 5.0 software (GraphPad software Inc., CA, USA). Every experimental approach was performed three times.

3.4 RESULTS

3.4.1 TNF-α mRNA levels increase in the acute phase after injury, but not in the chronic phase

Several studies have shown increased TNF-α mRNA levels in the acute phase after SCI (Bartholdi and Schwab, 1997, Pineau and Lacroix, 2007). However, most of these data did not include basal TNF-α levels from untouched controls or were performed using a different injury model, or a different rodent species. To investigate the relevance of these findings in our mouse T-cut hemisection injury model, local (spinal cord homogenate) measurement of mRNA TNF-α levels was performed at different time points after SCI. Real-time PCR data confirmed a local increase in TNF-α mRNA levels also in our injury model during the acute phase (1 and 6 hours, 2 days), with the highest peak expression seen 1 hour post injury (Fig. 3.1A). We also detected an increase in TNF-α mRNA levels in the sub-acute phase (4 and 7 days) (Fig. 3.1B). At later time points (chronic phase; days 14 and 21), TNF-α mRNA levels were slightly, but not statistically significantly, higher than control condition (Fig. 3.1B).

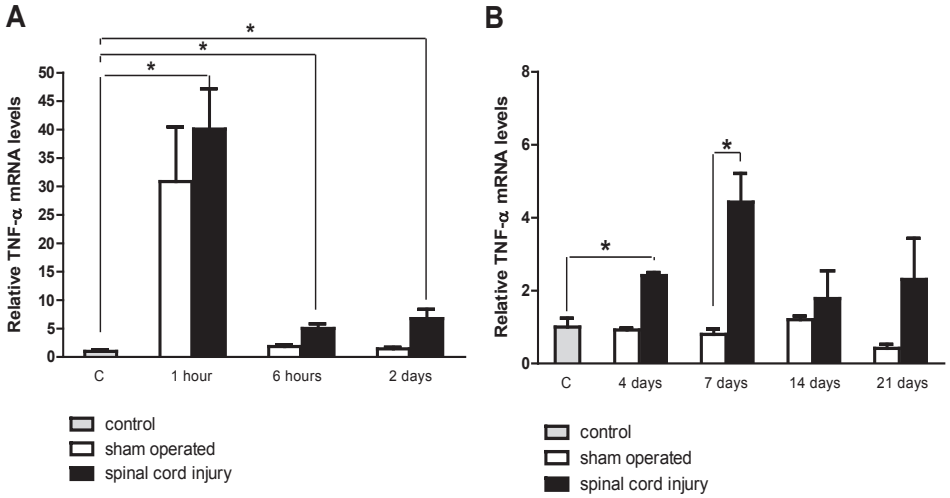

Fig. 3.1: TNF-α mRNA levels are upregulated in the acute phase after injury. **A**. Acute TNF-α mRNA levels were increased compared to the control at all the time points studied, reaching the highest peak 1h after injury. **B.** Chronic TNF-α mRNA levels reached the highest peak 7 days after injury, however, these data were not

significantly different from the control condition only between sham operated and SCI animals (* $p<0.05$; n=3 to 5 mice per time point).

3.4.2 Systemic and local TNF-α protein levels decrease during the acute phase after injury

At the protein level several studies have shown increased TNF-α levels (mice compression SCI and rat contusion SCI, respectively (Genovese et al., 2006, Koopmans et al., 2009). However, as for the mRNA data, different SCI animal models or rodent species were used, and it did not include data of untouched controls. Our results show a local (spinal cord) decrease of TNF-α levels at 6 hours after injury (Fig. 3.2A), TNF-α levels remained lower than in controls until day 4 (Fig. 3.2B). Systemic (serum) TNF-α levels in the SCI injured group were significantly lower than the control condition at 1 and 6 hours, and 2 and 7 days after SCI; the lowest protein levels were found 1 hour and 7 days after injury (Fig. 3.2C). However, no significant differences were seen between the sham and SCI injured groups, in both serum and spinal cord samples, suggesting that the change in TNF-α levels is attributable to the laminectomy itself, and not caused by the injury done to the spinal cord. In the chronic phase, there were slightly increasing average levels of ~200-300 pg/ml TNF-α at days 7, 14 and 21 in injured mice, compared to sham operated mice, however, these differences were not statistically significant (Fig. 3.2C). In serum samples there was a slight, but not significant increase of TNF-α protein levels 21 days after injury when comparing injured to sham operated animals (Fig. 3.2D).

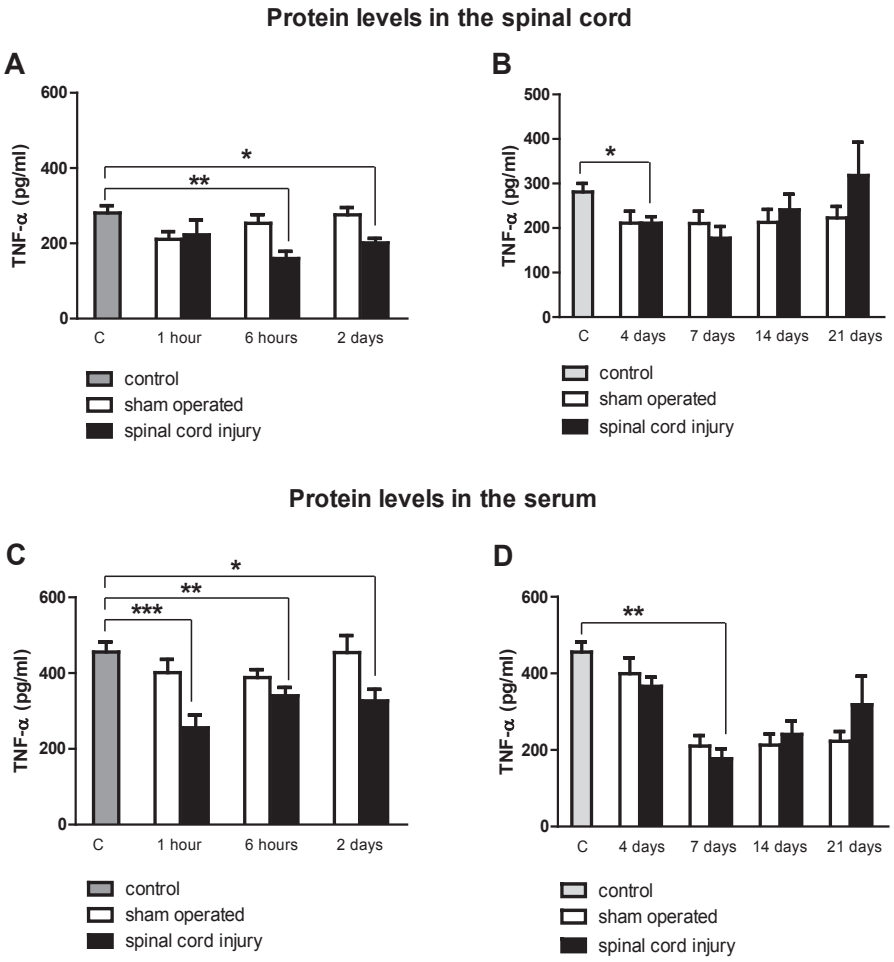

Fig. 3.2: Systemic and local TNF-α protein levels are decreased in the sub-acute phase after injury. A. Decreased (local) TNF-α levels in the spinal cord were detected using cytometric bead array (CBA) analysis in SCI animals compared to untouched controls. **B.** Increasing local TNF-α levels reach a peak 21 days after injury. **C.** Decreased systemic TNF-α protein levels in serum samples of SCI animals compared to basal levels (control group). **D**. While at day 7, TNF-α levels are still decreased in SCI animals, from day 14, TNF-α levels start to increase again (n=5 to 12 mice per time point), (*<p 0.05; **<p0.01; ***<p0.001).

3.4.3 Soluble TNF-α does not influence locomotor recovery after spinal cord injury in mice

There are a few studies that suggested either a beneficial (Peng et al., 2006) or detrimental effect (Ferguson et al., 2008) of the soluble form of TNF-α. As it has been previously shown in this chapter, TNF-α levels seem to decrease in our SCI model. In an attempt to investigate the effect of increasing TNF-α back to basal levels, on functional outcome, we performed SCI in mice, and one single systemic (i.p.) application of soluble TNF-α was administered immediately after injury, to reach a final concentration of ~400pg/mL in the mouse blood (basal levels of TNF-α in untouched controls, Fig. 3.2C-D).
Locomotion analysis using the BMS score, did not significantly differ between mice treated with PBS (control group) and mice treated with soluble TNF-α (Fig. 3.3A). A similar result was observed using the rotarod test (Fig. 3.3B).

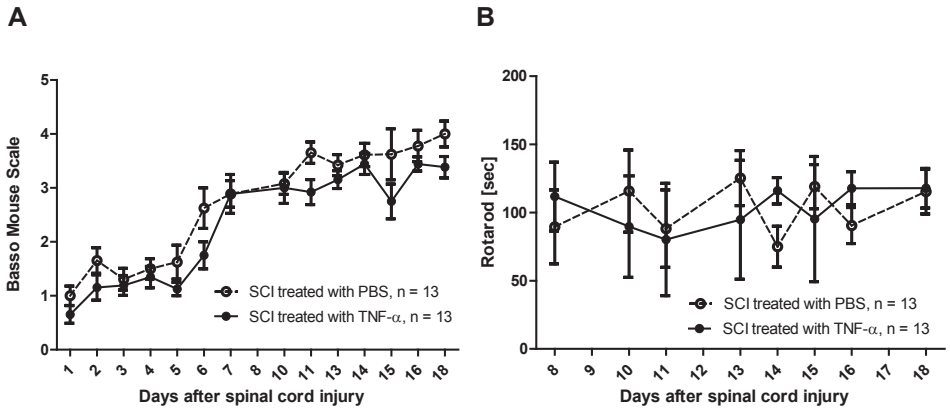

Fig. 3.3: Restoration of TNF-α levels does not achieve locomotor improvement after SCI. A. A single dose of soluble TNF-α, which was administered i.p. to animals immediately after SCI, did not promote locomotion recovery, i.e. no statistical differences were observed between the controls (SCI treated with PBS) and mice treated with TNF-α. **B**. Coordination is not affected by TNF-α levels, as shown using the rotarod test.

3.4.4 Late blocking of peripheral TNF-α is ineffective after spinal cord injury in mice

Etanercept is an FDA-approved TNF-α inhibitor for treating rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis, which has successfully been tested preclinically in rodents as a therapy for SCI (Genovese et al., 2006, Chen et al., 2011).

Using in situ hybridization, it has been reported that TNF-α mRNA levels in the injured mouse spinal cord, show a second peak of TNF-α during the early chronic phase (14-28 days after injury) (Pineau and Lacroix, 2007). In our T-cut hemisection injury model, a slight increase in the average TNF-α mRNA levels was seen at days 7, 14 and 21 after injury in spinal cord samples; however, only at day 7, the difference was statistically significant (Fig. 3.1). In addition, protein levels of TNF-α in spinal cord and in serum samples showed a slight, but not significant increase 21 days after injury compared to sham animals (Fig. 3.2).

Based on these data, and the published findings (Pineau and Lacroix, 2007), we started treatment with the TNF-α inhibitor etanercept at day 14 (early chronic phase after SCI, Fig. 3.4A). Since etanercept is a fusion protein of the soluble TNFR-2 and the Fc component of human IgG1, human IgG was used as additional control. We analyzed the locomotor function in these animals using the BMS score starting immediately after injury (Fig. 3.4B-D), and after 6 days of recovery, the mice were assessed on the rotarod (data not shown). Surprisingly, administration of etanercept every two days for one week did not lead to any difference in motor performance of the treated animals, compared to mice treated with vehicle or control IgG (Fig. 3.4B; rotarod: not shown). In a second approach, we injected etanercept i.p daily for one week (instead of every second day) in order to double the administered dose of etanercept over time. Again, no significant differences in the BMS score were observed between the two control groups and the etanercept-treated group (Fig. 3.4C; rotarod: not shown). Since daily injections may not provide uninterrupted TNF-α inhibition, we used a third approach to obtain continuous levels of etanercept. We implanted osmotic minipumps to release a constant amount (125 µg/day) of etanercept during

the early chronic phase starting at day 14 after SCI. Again, continuous release of etanercept during the early chronic phase did not influence the functional outcome after SCI (Fig. 3.4D; rotarod: not shown).

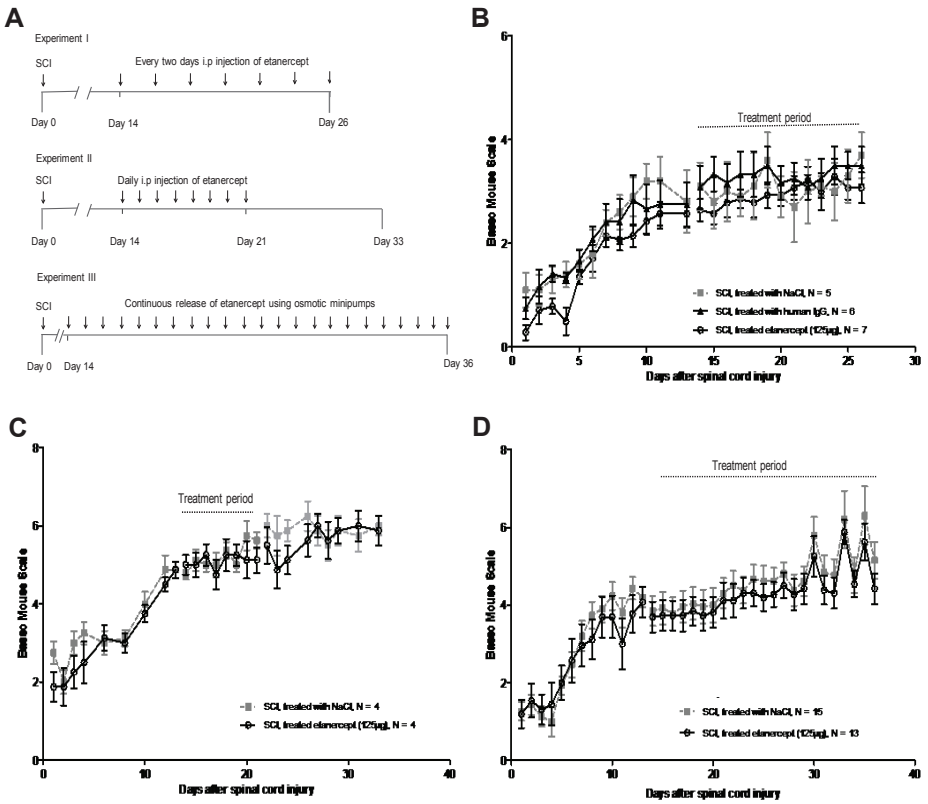

Fig. 3.4: TNF-α inhibition in the early chronic phase is ineffective after SCI independently of administration route. A. Scheme of the three different treatment approaches used. For each experiment, treatment was started at day 14 after SCI. **B.** Mice received every second day i.p injections with etanercept (125µg) or, as controls, human IgG (125µg) or NaCl. **C.** Mice received daily i.p injections with etanercept (125µg) or NaCl, as control for 1 week. **D.** Osmotic minipumps were implanted s.c into mice in order to reach continuous daily release of etanercept (125µg, released at 0.25µl/hr) for three weeks or NaCl as a control.

3.5 DISCUSSION

The pro-inflammatory cytokine TNF-α has been extensively studied in animal models of SCI, due to its rising expression immediately after injury. Its role in CNS diseases is however still controversial, given that many *in vitro* and *in vivo* effects have been reported, being either detrimental (Lee et al., 2000, Clarke and Branton, 2002, Neumann et al., 2002), neuroprotective (D'Souza et al., 1995, Sullivan et al., 1999, Oshima et al., 2009, Schmitt et al., 2010) or neutral, depending on the pathological setting (Vidal et al., 2012). In this chapter, new insights are given on the pathophysiological significance of TNF-α after SCI.

Published results have shown an increased TNF-α mRNA levels (Pineau and Lacroix, 2007) in the early chronic phase (14-28 days after injury). We have found significantly increased TNF-α mRNA levels 7 days after SCI; later on, the levels remain higher than the sham operated group, but these differences were not statistically significant. Interestingly, in our T-cut hemisection SCI model in mice there is a systemic and local reduction of TNF-α protein levels immediately after injury (acute phase). These results are in contrast to earlier report (Bethea et al., 1999) in which a rise of TNF-α was seen in the acute phase. However, they are in agreement with a recently published study, where TNF-α levels started to decrease from 6 hours after contusion injury in rats (Stammers et al., 2012). The mechanism behind this decrease it still unknown; but, our data on restoration of TNF-α levels indicate that a decrease in TNF-α does not play a pathophysiological role after SCI. It is important to consider that TNF-α mRNA has a short half-life and its stability and translation is highly controlled by several factors, such as NF-kB, p38γ/δ and AU-rich elements (ARE) (Espel et al., 1996, Kontoyiannis et al., 1999, Gonzalez-Teran et al., 2013). However, similar discrepancies between mRNA and protein levels have been reported before. For example, in genetically engineered synovial fibroblast-like cells carrying a high copy number of the TNF transgene, high mRNA levels but no protein production could be detected. This discrepancy between mRNA and protein levels was attributed to ARE elements (Kontoyiannis et al., 1999). A similar situation was also reported in myeloid cells, where p38γ/δ promoted protein

production of TNF-α without changes in TNF-α mRNA levels in a LPS-induced hepatitis model (Gonzalez-Teran et al., 2013). To our knowledge this is the first time that this discrepancy is reported after SCI.

One may speculate, on the basis of our results, that the differences seen are related to the diverse roles of membrane-bound and soluble TNF-α *in vivo*. Indeed, under pathological conditions, such as chronic inflammation and EAE, soluble TNF-α has shown a more detrimental role, while the membrane-bound form has a more protective one (Alexopoulou et al., 2006, Brambilla et al., 2011, Taoufik et al., 2011). Thus, studies in depth are needed to distinguish the "beneficial" and "detrimental" effects elicited by different forms of TNF-α.

Blocking TNF-α signaling by etanercept significantly reduces TNF-α production and confers improved functional recovery after SCI, if it is administered immediately after injury (Genovese et al., 2006, Chen et al., 2011). This TNF-α blocker has effectively shown to inhibit murine TNF-α under other pathological conditions (infection after diabetes, ulcerative colitis, collagen induced arthritis, etc) (Liu et al., 2006, Popivanova et al., 2008, Wang et al., 2013). However, large molecules, such as etanercept, cannot cross the BBB when delivered systemically (Zhou et al., 2011). These findings suggest that either the damage to the BBB must be sufficient to reach an adequate concentration of etanercept at the lesion site or that a modulation of the peripheral TNF-α levels results in beneficial outcome, or both. Based on these data we started treatment with etanercept at day 14 after SCI, using different doses as well as different administration routes of the TNF-α inhibitor. Locomotor recovery was assayed using the BMS score. Intraperitoneal etanercept injections every second day or daily as well as continuous etanercept release through osmotic minipumps, were ineffective as treatment in the early chronic phase after SCI. Although a lot of inflammatory processes have also been described in the early chronic phase (Trivedi et al., 2006, Beck et al., 2010), our data indicate that an inhibition of TNF-α signaling is ineffective in influencing the clinical outcome after SCI in the time period investigated here. However, intrathecal administration of large TNF inhibitors (with all potential side effects) may be still an option as well as specifically targeted molecules which pass the BBB. Nevertheless, our

data suggest that inhibition of peripheral TNF-α may not be effective in the early chronic phase after SCI.

CHAPTER 4

ADAM17/TACE is a survival factor after SCI

Part of this chapter is reported in Vidal PM., Lemmens E., Avila A., Vangansewinkel T., Chalaris A., Rose-John S., and Hendrix S. ADAM17 is a survival factor of microglial cells after spinal cord injury in mice.
(manuscript under preparation)

4.1 ABSTRACT

ADAM17 is a metalloprotease with important substrates such as TNF-α and its receptors, the p75 neurotrophic receptor (p75NTR), and members of the epidermal growth factor family. The rationale of this study was to inhibit ADAM17-induced shedding of the pro-inflammatory soluble form of TNF-α in order to reduce detrimental inflammation after SCI. However, using the specific ADAM17 blocker BMS-561392 in cultures of neuronal and glial cells *in vitro* we show that proper functioning of ADAM17 is vital for the survival of cultured immature and mature oligodendrocytes, microglia and astrocytes, in a mitogen-activated protein kinase (MAPK) p44 dependent manner. Surprisingly, blocking ADAM17 *in vivo* after SCI does not change the levels of membrane-bound or soluble TNF-α forms substantially, but increases the expression of the pro-apoptotic marker Bax after SCI. Blocking ADAM17 also increases lesion size, astrogliosis, and microglial apoptosis and impairs functional recovery after SCI in line with these findings, cell survival of cultured microglial cells from ADAM17 knockout mice (ADAM17ex/ex) showed a nearly 40% reduction of cell survival compared to microglia from wildtype littermates, while the viability of ADAM17-deficient oligodendrocytes was unchanged. Our data suggest that ADAM17 is a key survival factor for microglial cells after CNS injury *in vitro* and *in vivo*.

4.2 INTRODUCTION

Central nervous system trauma, either in the form of traumatic brain injury or SCI, is characterized by marked neuropathology and limited functional recovery. An excessive post-traumatic inflammatory response plays an important role in secondary injury processes, which develop after SCI.

Several studies have demonstrated an upregulation of pro-inflammatory cytokines, such as TNF-α and IL-1β within the first few hours post-injury (Bethea et al., 1999, Pineau and Lacroix, 2007). The pluripotent TNF-α is synthesized as a type II transmembrane protein (membrane-bound TNF-α) arranged in stable homotrimers. The soluble TNF-α form is produced after the proteolytic shedding of the membrane-bound form of TNF-α by ADAM17

(Black et al., 1997). ADAM17 belongs to the superfamily of Zn-dependent metalloproteinases, and is involved in proteolytic shedding of a variety of cell-surface molecules (Seals and Courtneidge, 2003, Rose-John, 2013). Its first discovered biological function was the shedding of the membrane-bound form of TNF-α (Black et al., 1997, Moss et al., 1997), and since then, many other factors have been reported as its substrates. Due to its broad spectrum of activities, ADAM17 plays a role in many basic processes such as shedding of TNFRs, members of the EGF family, p75NTR and IL-6, among others (Black et al., 1997, Weskamp et al., 2004, Yang et al., 2006). It also plays an important role in acute and chronic neurodegenerative diseases such as ischemia (Wang et al., 2004) and Alzheimer's disease (Kim et al., 2008).

The two forms of TNF-α, membrane-bound and soluble TNF-α, show different binding affinities for TNFR-1 and TNFR-2. TNFR-1 has been primarily associated with apoptosis through the recruitment of TRADD, whereas TNFR-2 lacks a death domain and is associated with the anti-apoptotic effects of TNF-α (Wajant et al., 2003, McCoy and Tansey, 2008). There are a few studies that have clearly defined the role of both TNF-α forms. For example, the membrane-bound TNF-α form has a higher affinity for TNFR-2 and may therefore play a more important role in the regulation of cell survival (Wajant et al., 2003), remyelination after experimental autoimmune encephalomyelitis (Brambilla et al., 2011), reduction of the inflammatory response after atherosclerosis (Canault et al., 2004), and anti-bacterial response *in vivo* (Pasparakis et al., 1996). On the contrary, the soluble form has a higher affinity for TNFR-1 and may therefore be more important for apoptosis and inflammation (Grell, 1995, Holtmann and Neurath, 2004).

In SCI models, TNF-α is a well-known "pro-inflammatory" cytokine with detrimental effects on recovery after lesion, since treatment with TNF-α blockers (etanercept and rolipram) (Genovese et al., 2006, Koopmans et al., 2009) improved functional recovery after injury (Lee et al., 2000). Another alternative to reduce TNF-α, has been demonstrated in rheumatoid arthritis (Moss et al., 2008) by using ADAM17 blockers to reduce the production of soluble TNF-α., which has been related to an increased inflammatory response and demyelination (Brambilla et al., 2011, Taoufik et al., 2011). To

date there is a lack of available information about the expression and role(s) of ADAM17 after SCI.

In this chapter we have studied the role of ADAM17 using recombinant mouse ADAM17 (rADAM17), the specific ADAM17 blocker BMS-561392, and the non-specific inhibitors TAPI-0 and TAPI-1, in cultures of neuronal and glial cells *in vitro*. We show that proper functioning of ADAM17 is vital for the survival of cultured immature and mature oligodendrocytes, microglia and astrocytes in a MAPK p44 dependent manner. In cultured primary neuronal cultures, ADAM17 seems to be directly involved in the promotion of neurite outgrowth. In addition, in a mouse model of T-cut hemisection SCI, *in vivo* blocking of ADAM17 by BMS-561392 impairs functional recovery. The latter was also confirmed using ADAM17 knockout mice, which is accompanied by an increase in lesion size, astrogliosis as well as microglial apoptosis. Our data thus suggest that ADAM17 is a key survival factor for microglial cells after CNS injury *in vitro* and *in vivo* as well as a possible promoter of astrocytes survival in vitro (MTT assay) and *in vivo* (GFAP expression).

4.3 MATERIALS AND METHODS

4.3.1 ADAM17 inhibitor

The ADAM17 inhibitor, BMS-561392, was kindly provided by Bristol-Myers Squibb. The drug was prepared as described before (Kim et al., 2008), by reconstituting the drug in freshly prepared phosphate buffered saline (PBS) pH 3.5. For the *in vitro* study, different concentrations of the ADAM17 inhibitor were used (between 0.08mM to 2.7mM), while the *in vivo* experiments were performed using 210µg/day (2.2mM) (Kim et al., 2008). For the *in vitro* and *in vivo* studies a broad range of different concentrations of rADAM17 (R&D Systems, UK) were used (between 1.6µM to 3.7mM).

4.3.2 *In vitro* experiments

4.3.2.1 ADAM17 activity assay

To determine the enzymatic activity of ADAM17 in microglia and oligodendrocyte cell lines, as well as the efficacy of the ADAM17 inhibitor BMS-561392, a fluorimetric assay (SensoLyte® 520 TACE Activity Assay kit; Tebu-bio, Belgium) was used according to manufacurer's instructions. Briefly, microglial (BV-2 cells) and immature oligodendrocyte (human oligodendroglioma cell line (HOG)) cell lines were grown to semiconfluence (70-80%). The medium was then removed and cells were washed with PBS, and lysed on ice using assay buffer (component C of the assay) containing 0.1% triton X-100). The lysed cells were centrifuged at 2500g for 10 minutes at 4°C. The supernatant was aliquoted and total protein levels were determined using the BCA protein assay kit (Thermo Scientific, Belgium). The samples were stored at -80°C until further use afterwards, the lysed product was pre-incubated for 10 minutes in a 96-well plate with different doses of the ADAM17 inhibitor, and TAPI-1 (rADAM17 was used as a positive control). Assay buffer and ADAM17 substrates were after added to the plate to start the fluorescent reaction. Fluorescent emission (excitation 490nm/emission 520nm) upon cleavage of the quenched fluorogenic peptide

(ADAM17 substrate) was monitored with a FluoStar Optima plate reader. The results are displayed as relative fluorescence units (RFU) for the pure enzyme and RFU/total concentration of proteins. The fluorescence from each sample was corrected by subtracting the fluorescence of the blank (assay buffer).

4.3.2.2 Cell cytotoxicity assay

Primary cortical neuronal cells were prepared from E15 C57BL/6 mice and cultured on poly-D-lysine-coated 96-wells with 5 x 10^4 cells/well in neuronal media (Neurobasal media, containing 2% B-27, 1% glutamine and 1% penicillin/streptomycin) and selected concentrations of BMS-561392 (0.3mM, 1.3mM, 2.7mM), TAPI-1 (10µM, 100µM; Merck, Belgium), or rADAM17 (1µM, 10µM) for 48 hours. To measure the effect of ADAM17 inhibition on neuronal survival, 1 hour after isolation, cells were incubated in B-27-deficient media with or without BMS-561392, TAPI-1, or rADAM17 for 48 hours. MTT solution (1mg/mL) was added for 4 hours after which the cells were lysed in a mixture of DMSO and glycine 0.1M. The absorption was measured at 550nm using a microplate reader (Bio-Rad).

Primary oligodendrocytes and microglia cultures were prepared from P3 ADAM17 wildtype (wt/wt) and ADAM17-deficient (ADAM17 ex/ex) mice (Chalaris et al., 2010). The cells were kept in a mixed culture for 14 days in OA2 medium (DMEM media, containing 1% penicillin/streptomycin and 10% FCS) and then the cells were separated by the shake off procedure (Ni and Aschner, 2010). Microglia and oligodendrocytes were cultured on poly-D-lysine-coated 96-wells seeded with 5 x 10^4 cells/well for 72 hours. To measure the effect of ADAM17 depletion in these two primary cell cultures, the MTT assay was again used.

BV-2 murine microglial cells were seeded on poly-D-lysine-coated 96-wells at a density of 5 x 10^4 cells/well in microglia media (DMEM media, containing 1% penicillin/streptomycin and 10% FCS). To study astrocyte survival, a human astrocytoma cell line (CCF) was plated at a density of 5 x 10^4 cells/well and cultured in DMEM/F-12 medium containing 10% FCS and 1% penicillin/streptomycin. To study the effect of ADAM17 on oligodendrocyte survival, two undifferentiated oligodendrocyte cell lines were used (HOG and

human glial (oligodendrocytic) hybrid cell line (MO3.13)). These cells were cultured in RPMI medium or MEM medium with 10% FCS and 1% penicillin/streptomycin, respectively. MO3.13 cells were differentiated by removing FCS from the culture medium and adding Phorbol 12-myristate 13-acetate (PMA) to the cells for 72 hours (Buntinx et al., 2004). All of these cells (BV-2, CCF, HOG, MO3.13) were grown under optimal conditions and treated with different concentrations of BMS-561392 or rADAM17 to measure cell viability using the MTT assay.

4.3.2.3 Western blot analysis

The above described cell lines for microglia (BV-2), astrocytes (CCF) and oligodendrocytes (HOG) were used to perform western blotting against different protein targets. Briefly, when the cells reached 80-90% confluence, different concentrations of BMS-561392 or TAPI-1 were added to the cells for 3 hours. The cells were then washed with PBS and lysed on ice using Procarta lysis byffer. The lysis product was centrifuged for 30 minutes at 14000rpm at 4°C. The supernatant was aliquoted and stored at -20°C until measurement. To determine membranous TNFR-1 and TNFR-2 expression, the Pierce cell surface protein isolation kit (Thermo Scientific, Belgium) was used according to manufacturer's instructions. Total protein levels were determined using the BCA Protein Assay Kit. Between 5 and 20µg of total protein was electrophoretically separated and transferred to PVDF membranes. The primary antibodies were incubated 1 hour at RT or overnight at 4°C (table 4.1). Afterwards, the membranes were washed with PBS and then incubated for 1 hour at RT with the corresponding secondary antibody (table 4.1). The membranes were re-blotted using an antibody against β-actin as a loading control. The signal was detected using the ECL Plus Western blotting detection (GE Healthcare, The Netherlands). Densitometric analysis was performed using ImageJ. The results are expressed as a percentage of the control (set as 100%).

4.3.2.4 Neurite outgrowth assay

Neurite outgrowth was measured from primary cortical neuronal cells from E15 C57BL/6 mice, and plated on coated coverslips ($7.5x10^4$ cells/well) in a 24-well plate in neuronal media. Selected concentrations of rADAM17 and BMS-561392 were added to the neuronal medium for 72 hours. Afterwards, the neurons were fixed using 4% PFA for 15 minutes and permeabilized (0.2% Triton X-100 in PBS) for 10 minutes. The cells were incubated in blocking solution for 1 hour (10% normal serum in PBS), and then incubated overnight with beta-III-tubulin antibody (1% normal serum in PBS) (table 4.2). The cells were washed with PBS and the secondary antibody was incubated in PBS for 1 hour at RT. Neurons were visualized using 3,3′-diaminobenzidine (Sigma Fast tablets; Sigma-Aldrich, Belgium) and nuclear counterstaining with hematoxylin. The coverslips were finally mounted (Aquatex mounting medium; Merck, Belgium) on a glass slide.
A set of 50 randomized pictures per experiment, were taken from each condition. The pictures were manually analyzed with ImageJ as it has been described before (Holtje et al., 2009). The data represent the average length of the longest neurite per condition as a mean of three experiments.

Table 4.1: List of antibodies used for western blotting

Primary antibody	Source	Dilution
Mouse anti-Bax (6D150)	Santa Cruz Biotechnology, Inc	1:1000
Mouse anti-Bcl-2 (C-2)	Santa Cruz Biotechnology, Inc	1:1000
Mouse anti-β-actin (C4)	Santa Cruz Biotechnology, Inc	1:5000
Rabbit anti-TNF-α	Abcam, UK	1:1000
Rabbit anti-TNFR-1	Abcam, UK	1:250
Goat anti-TNFR-2	R&D Systems	1:500
Rabbit anti-TRADD	Santa Cruz Biotechnology, Inc	1:125
Rabbit anti-NGF	Santa Cruz Biotechnology, Inc	1:125
Mouse anti-TRAF2	Santa Cruz Biotechnology, Inc	1:125
Rabbit anti-p75NTR	Santa Cruz Biotechnology, Inc	1:250
Rabbit anti-p44/p42 MAPK (ERK 1/2)	Cell Signaling Technology, United States	1:1000
Mouse anti-phospho-p44/p42 MAPK (ERK 1/2)	Cell Signaling Technology, United States	1:1000
Secondary antibody	**Source**	**Dilution**
Anti-mouse immunoglobulins/HRP	Wako, Germany	1:5000
Rabbit anti-IgG-H&L	Abcam, UK	1:5000
Anti-goat immunoglubulins/HRP	Wako, Germany	1:5000

Abbreviations: Bcl-2–associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), tumor necrosis factor-alpha (TNF-α), tumor necrosis factor (TNF) receptor type 1 (TNFR-1), receptor type 2 (TNFR-2), TNF receptor associated death domain (TRADD), nerve growth factor (NGF), TNF receptor associated factor 2 (TRAF2), p75 neurotrophic receptor (p75NTR), mitogen-activated protein (MAP) kinase (MAPK), extracellular-signal-regulated kinase (ERK), horse radish peroxidase (HRP), immunoglobulin (Ig) G heavy & light chain (H&L).

Table 4.2: List of antibodies used for immunostaining

Primary Antibody	Target	Source	Dilution
Rat anti-CD4	T cells	BD Biosciences, Belgium	1:500
Mouse anti-CD34	Endothelial cells	Abcam, UK	1:50
Mouse anti-GFAP	Astrocytes	Sigma-Aldrich, Belgium	1:500
Rabbit anti-MBP	Mature oligodendrocytes	Millipore, Belgium	1:100
Mouse anti-CC-1	Mature oligodendrocytes	Merck, Belgium	1:20
Rabbit anti-iba1	Microglia/macrophages	Wako, Germany	1:350
Rat anti-CD11b	Microglia	AbSerotec, Uk	1:200
Rabbit anti-active caspase-3	Caspase-3	Cell Signaling Technology, United States	1:300
Goat anti-GFP	CFP protein	Rockland Immunochemicals, United States	1:20
Rat anti-F4/F80	Microglia/macrophages	AbD Serotec, Germany	1:100
Mouse anti-beta-III-tubulin	Neurons	Millipore, Belgium	1: 500
Secondary antibody	**Source**		**Dilution**
Anti-rat Alexa 568	Invitrogen, Belgium		1:250
Anti-mouse Alexa 568	Invitrogen, Belgium		1:250
Anti-goat Alexa 647	Invitrogen, Belgium		1:250
Anti-rabbit Alexa 488 or Alexa 555	Invitrogen, Belgium		1:250
Anti-rabbit Alexa 568	Invitrogen, Belgium		1:250
Anti-mouse immunoglobulins HRP	DakoCytomation, Belgium		1:400

Abbreviations: glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), ionized calcium binding adaptor molecule 1 (Iba1), cluster of differentiation (CD), green fluorescent protein (GFP), cyan fluorescent protein (CFP).

4.3.3 *In vivo* experiments

4.3.3.1 Animals

All experiments were performed using either 10-week old C57BL/6 mice (Harlan, The Netherlands), ADAM17ex/ex and ADAM17wt/wt mice, generated from ADAM17 wt/ex mice (kindly provided by Dr. S. Rose-John, Christian Albrechts University, Kiel, Germany) (Chalaris et al., 2010), or transgenic PLP-eCFP mice (to visualize oligodendrocytes (Steffens et al., 2012); kindly provided by Dr. F. Kirchhoff, University of Saarland, Germany). Mice were housed in a conventional animal facility at Hasselt University under standardized conditions (i.e. temperature-controlled room (20±3°C), a 12h light-dark cycle, food and water *ad libitum)*; all evaluations were performed by blinded investigators. All experiments were approved by the local ethical committee of Hasselt University and were performed according to the guidelines described in Directive 2010/63/EU on the protection of animals used for scientific purposes.

4.3.3.2 Spinal cord injury: T-cut hemisection injury

The T-cut hemisection injury was performed as described in the previous chapter (see section 3.3.1).

4.3.3.2.1 Experimental groups

The treatment protocols are summarized in Fig. 4.8 and Fig. 4.9.

4.3.3.2.1.1 Systemic application of rADAM17

In a first approach, immediately after SCI mice were randomly distributed in three groups and injected i.p. with one single dose of vehicle solution (ultra pure water), or with 1.6µM of rADAM17, or 16µM of rADAM17. In a second approach, immediately after injury mice were randomly distributed in three groups: rADAM17 (0.7µM, or 7µM) or vehicle solution (ultra pure water), and injected i.p. daily for 7 days.

4.3.3.2.1.2 Local application of rADAM17

To investigate the effect of rADAM17 in the spinal cord, after injury the lesion site was covered with a piece of gelfoam soaked with 0.3mM or 3.7mM of rADAM17, or vehicle solution (ultrapure water) as control.

4.3.3.2.1.3 Inhibition of ADAM17

SCI was performed on C57BL/6 mice or using transgenic PLP-eCFP. Mice were distributed over two groups; one group was subcutaneously injected daily for 15 days with PBS pH 3.5, and the other group with BMS-561392 (2.2mM/day; Bristol- Myers Squibb).

4.3.3.3 Locomotion tests

Animals were scored daily for functional recovery after SCI using the BMS (Basso et al., 2006) and the Rotarod *(*Sheng et al., 2004*)* as it was described in the previous chapter.

4.3.3.4 Immunohistochemical analysis of spinal cord tissue

Animals treated with the ADAM17 inhibitor BMS-561392, were transcardially perfused with 4% PFA, 16 days after surgery. Spinal cords were isolated and used to prepare cryosections (10µm thickness), which were incubated for 60 minutes in PBS with 10% normal goat serum and 0.5% Triton X-100. All the primary antibodies were incubated overnight (4°C) (Table 4.2). Spinal cord cryosections were washed and the corresponding secondary antibodies were applied for 1 hour at RT (Table 4.2). To visualize the cell nuclei, DAPI counterstaining was performed for 10 minutes (1:100; Invitrogen, Belgium), and sections were mounted.

For measurement of lesion size, astrogliosis, and inflammatory infiltrate, 5 to 9 sections per mouse were used (control mice: n = 4, ADAM17 inhibitor treated mice: n= 7). To determine the number of active caspase-3/CC-1 (apoptotic oligodendrocytes) and active caspase-3/CD11b (apoptotic

microglia) double positive cells, 4 to 7 sections per mouse were used (n = 4 per group). To quantify the amount of gliosis (glial fibrillary acidic protein (GFAP) staining) and microglia/macrophage infiltration (Iba1 staining), an intensity analysis was performed using ImageJ software (NIH). For the analysis of gliosis, a squared area of 100µm X 100µm cranial and caudal to the lesion site was analyzed (Fig. 4.10E), while for Iba1 expression an area extending from 500µm cranial to 500µm caudal to the lesion site was analyzed (Fig. 4.10G, 4.10J). Lesion size (via GFAP immunoreactivity) and demyelinated area (via MBP immunoreactivity) were evaluated by manually delineating the lesion site (Fig. 4.10A, 4.10B). T cell numbers were determined in the entire perilesional area, in squares of 225µmX225µm (cranial, lesion itself and caudal). Apoptotic oligodendrocytes and microglia were counted in a squared area of 225µmX225µm cranial and caudal to the lesion site. Images were obtained with a Zeiss LSM510 meta confocal microscope or with a Nikon 80i fluorescence microscope.

4.3.3.5 Western blotting of spinal cord tissue

Animals were transcardially perfused with Ringer's solution 16 days post-surgery. Spinal cord tissue was dissected out (from 0.5cm cranial to 0.5cm caudal from the lesion site) and homogenized using Procarta lysis buffer (Panomics, Italy). Homogenized tissue was centrifuged at 10000rpm for 10 minutes at 4°C. The supernatants were collected and stored at -80°C until further use. Total protein levels were determinate using the BCA Protein Assay Kit (Thermo Scientific, US) according to manufacturer's instructions. A total amount of 75µg (for measurement of Bax and Bcl-2 levels) or 40µg of total protein (to measure TNF-α expression) were separated electrophoretically and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in PBS-Tween 0.05% for 1 hour. Primary antibodies were incubated overnight (4°C) or for 1 hour at RT (table 4.1). The membranes were washed in PBS-Tween 0.05% and incubated with secondary antibody for 1 hour at RT (table 4.1). The membranes were re-blotted using an antibody against β-actin as a loading control. The signal was detected using the ECL Plus Western blotting detection (GE Healthcare, The

Netherlands). Densitometric analysis was performed using ImageJ. The results are expressed as a percentage of the PBS or control treated group (set as 100%).

4.3.3.6 Statistical analysis

Statistical analyses were performed with Prism 5.0 (GraphPad Software) and PASW statistics 18 (SPSS) software.
Behavioral data (BMS and rotarod) were analyzed using a two-way ANOVA as previously described (Basso et al., 2006). Immunohistochemical and western blotting analyses were performed using a Mann-Whitney U test for comparison between two groups. For semi-quantitative protein detection (western blotting), ADAM17 activity, cell cytotoxicity assay, and neurite outgrowth values were compared using one-way ANOVA followed by Bonferroni post-test. All data are given as mean ± SEM.

4.4 RESULTS

4.4.1 *In vitro* experiments

4.4.1.1 BMS-561392 is a potent inhibitor of ADAM17

To understand the cellular effects of ADAM17 inhibition, we firstly evaluated the ability of the specific inhibitor of ADAM17 (BMS-561392) to block ADAM17 activity in lysed microglial and oligodendrocytic cell lines. In a first approach, the kinetic of the pure enzyme alone, or in the presence of a non-specific inhibitor of ADAM17 (TAPI), or the specific inhibitor (BMS-561392), was measured during 1.5 hours (5400 sec). Both TAPI and BMS-561392 completely inhibited the intrinsic activity of ADAM17 (1.6µM and 16µM), (Fig.4.1A, 4.1B). Lysed microglial cells (BV-2 cell line; Fig. 4.1C) and undifferentiated oligodendrocytic cells (HOG cell line; Fig 4.1D) revealed the presence of active ADAM17. Microglial ADAM17 had an average activity of 19429 RFU/total protein (µg/µl), while ADAM17 from oligodendrocytes had

6494 RFU/total protein (µg/µL), almost a 3 fold decreased activity. In both cell types, the two highest concentrations of the specific ADAM17 inhibitor (2.1mM and 4.4mM respectively) effectively decreased the metalloprotease activity. The non-specific inhibitors of ADAM17, TAPI-0 (provided with the enzymatic assay) and TAPI-1 (purchased from Merck), also decreased the intrinsic activity of the enzyme but to a lesser degree than BMS-561392. The lowest concentration (0.4mM) did not decrease ADAM17 activity, showing a kinetic curve similar to the average lysed cells to which no inhibitor was added (Fig 4.1C, 4.1D).

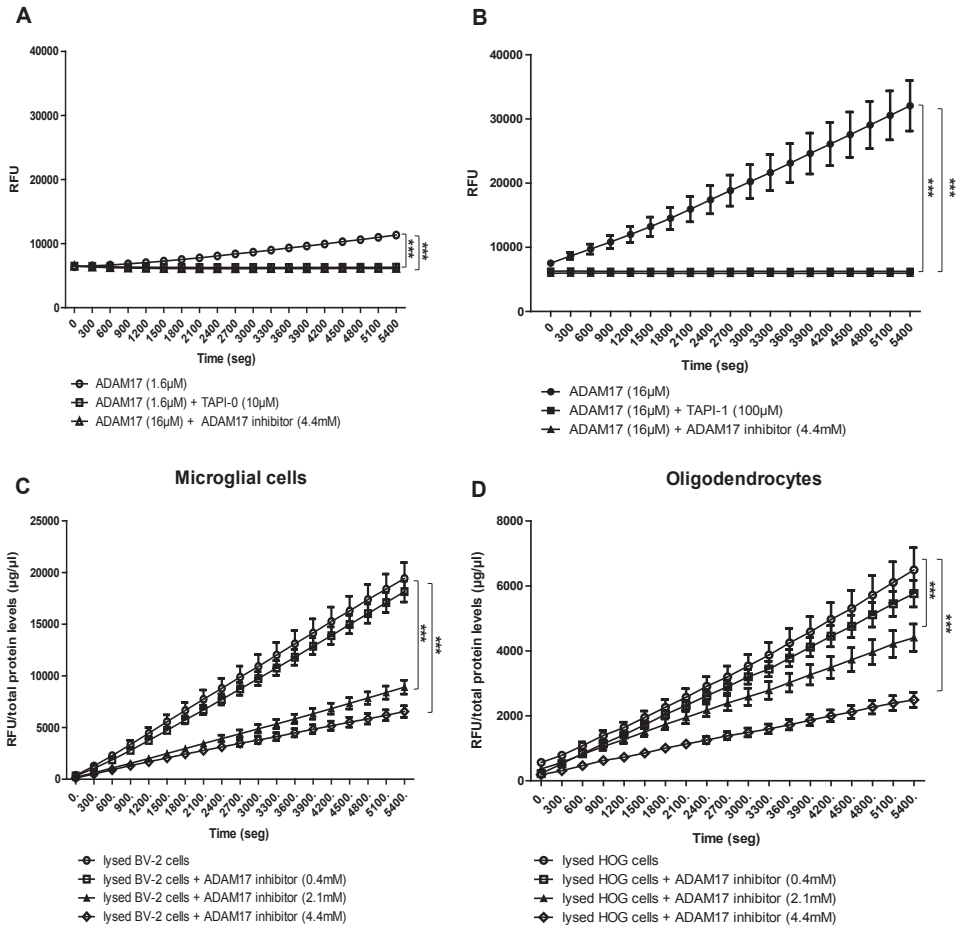

Fig. 4.1: BMS-561392 effectively decreases ADAM17 activity. A, B. TAPI-0, TAPI-1, and BMS-561392 completely inhibited the activity of the pure ADAM17 enzyme. **C, D.** Lysed microglia and oligodendrocytic cell lines showed a concentration-

dependent inhibition of the enzymatic activity of ADAM17. The ADAM17 inhibitor (4.4mM) showed an average inhibition of 66.3% and 62% of ADAM17 derived from microglial and oligodendrocytic cell lines (n=19 wells for each condition).

4.4.1.2 ADAM17 inhibition by BMS-561392 decreases viability of microglia and oligodendrocytic cell lines *in vitro*

To understand the cellular effects of ADAM17 inhibition, we used the main cellular types present in the central nervous system, being oligodendrocytes, neurons, astrocytes and microglia. Firstly we evaluated the effects of ADAM17 on cell survival during 48 hours in two different cell lines (HOG and MO3.13) of immature (Fig. 4.2A-D) and mature oligodendrocytes (Fig. 4.2E-F). The rADAM17 did not influence survival. In contrast, both differentiated and undifferentiated oligodendrocytic cell lines were strongly affected by BMS-561392; undifferentiated, or also called immature oligodendrocytes, were more susceptible to ADAM17 inhibition (Fig. 4.2B, 4.2D), showing a concentration-dependent reduction in survival ranging from 10 to 45% with the low (0.3mM) and mid concentration (1.3mM), respectively, up to 89.5-93.5% with the highest concentration of the inhibitor (2.7mM). A similar effect was seen using TAPI-1 (100μM), where viability was decreased with 10 to 20%; no significant changes were observed with a lower dose of TAPI-1 (10μM) (Fig. 4.2B, 4.2D). However, survival of mature oligodendrocytic cell lines was significantly affected only by the highest concentration of the ADAM17 inhibitor (2.7mM) and TAPI-1 (100μM), leading to a reduction of 80% or 25%, respectively, of oligodendrocyte survival (Fig. 4.2F). Secondly, survival of neurons was determined. Primary cortical neurons were treated with the ADAM17 inhibitor (BMS-561392) the non-specific inhibitor (TAPI-1) or rADAM17 in the presence or absence of B-27. While a low concentration of rADAM17 (1μM) promotes cell survival in the presence of B-27, a high concentration of rADAM17 (10μM) decreases cell survival (Fig. 4.3A). Deprivation of B-27 induced a reduction in cell survival of 30-35%; however, rADAM17 did not have any effect on neuronal survival under this condition (Fig. 4.3C). Similarly, inhibition of ADAM17 with different concentrations of BMS-561392 did not influence neuronal survival, neither in the presence or

absence of B-27 (Fig. 4.3, 4.3D). In parallel, TAPI-1 had a concentration-dependent effect on survival, inducing an increase (10μM) or decrease (100μM) in presence or absence of B-27 (Fig. 4.3B, 4.3D). On the contrary, using an astrocytic cell line, both rADAM17 (10μM) and BMS-561392 (2.7mM) increased viability, whilst TAPI-1 significantly reduced it (Fig. 4.4A, 4.4B). Finally, using a microglial cell line, inhibition of ADAM17 using the mid (1.3mM) and high concentration (2.7mM) decreased viability by 50 to 94% respectively (Fig. 4.4D). In contrast, the low dose of the specific inhibitor (1.3mM) significantly increased viability compared to the control condition; meanwhile, TAPI-1 did not influence microglial survival (Fig. 4.3D), while rADAM17 promoted survival in a dose dependent manner (Fig. 4.3C).

Fig. 4.2. ADAM17 inhibition decreases viability of undifferentiated and differentiated oligodendrocytic cell lines. A and C. Undifferentiated HOG and MO3.13 were treated for 48 hours with selected concentrations of rADAM17 (μM). Cell survival was measured using the MTT assay (HOG, n=22-27; MO3.13, n=17 wells per condition). **B and D**. Undifferentiated HOG and MO3.13 were incubated for 48 hours with the specific ADAM17 inhibitor (BMS 561392) and the non-specific inhibitor TAPI-1. Significant decrease in cell viability was seen after ADAM17 inhibition. The values are expressed as percentage of the control. The results are the mean of three independent experiments (*p<0.05; **p<0.01; ***p<0.001) (HOG, n=12-18; MO3.13, n=17 wells per condition). **E and F.** Undifferentiated MO3.13 were differentiated using PMA for 72 hours; afterwards, cells were treated with rADAM17 for 48 hours, or with BMS-561392 or TAPI-1. Cell viability was measured using an MTT assay. There was no significant effect using rADAM17, meanwhile a significant decrease in cell viability was observed after ADAM17 inhibition (BMS-561392 (2.7mM) and TAPI-1 (100μM)). The results are the mean of three independent experiments and are represented as a percentage of the control condition (*p<0.05; **p<0.01; ***p<0.001) (MO3.13 treated with rADAM17, n=27 wells per condition; MO3.13 treated with ADAM17 inhibitor n=15-19 wells per condition).

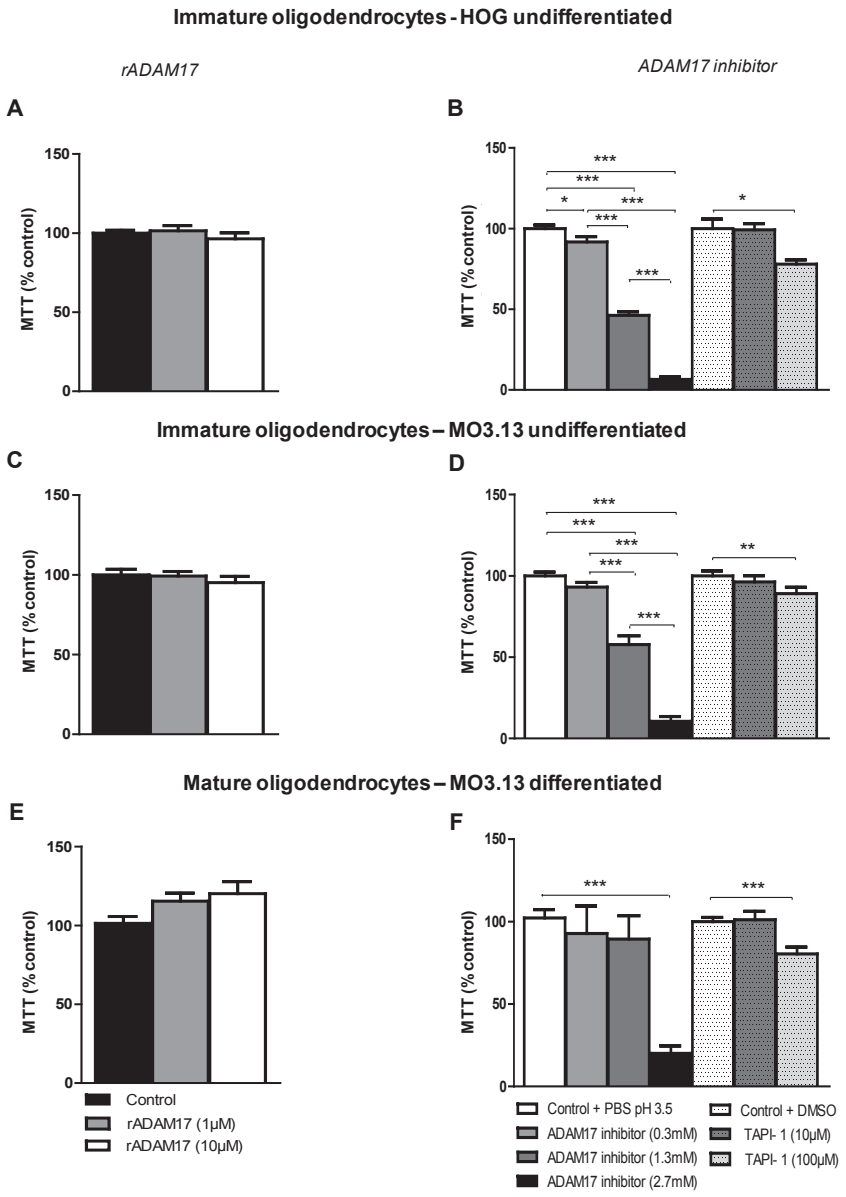
Immature oligodendrocytes - HOG undifferentiated
rADAM17
ADAM17 inhibitor
A
B
MTT (% control)
Immature oligodendrocytes – MO3.13 undifferentiated
C
D
Mature oligodendrocytes – MO3.13 differentiated
E
F
Control
rADAM17 (1µM)
rADAM17 (10µM)
Control + PBS pH 3.5
ADAM17 inhibitor (0.3mM)
ADAM17 inhibitor (1.3mM)
ADAM17 inhibitor (2.7mM)
Control + DMSO
TAPI- 1 (10µM)
TAPI- 1 (100µM)

Fig. 4.2.

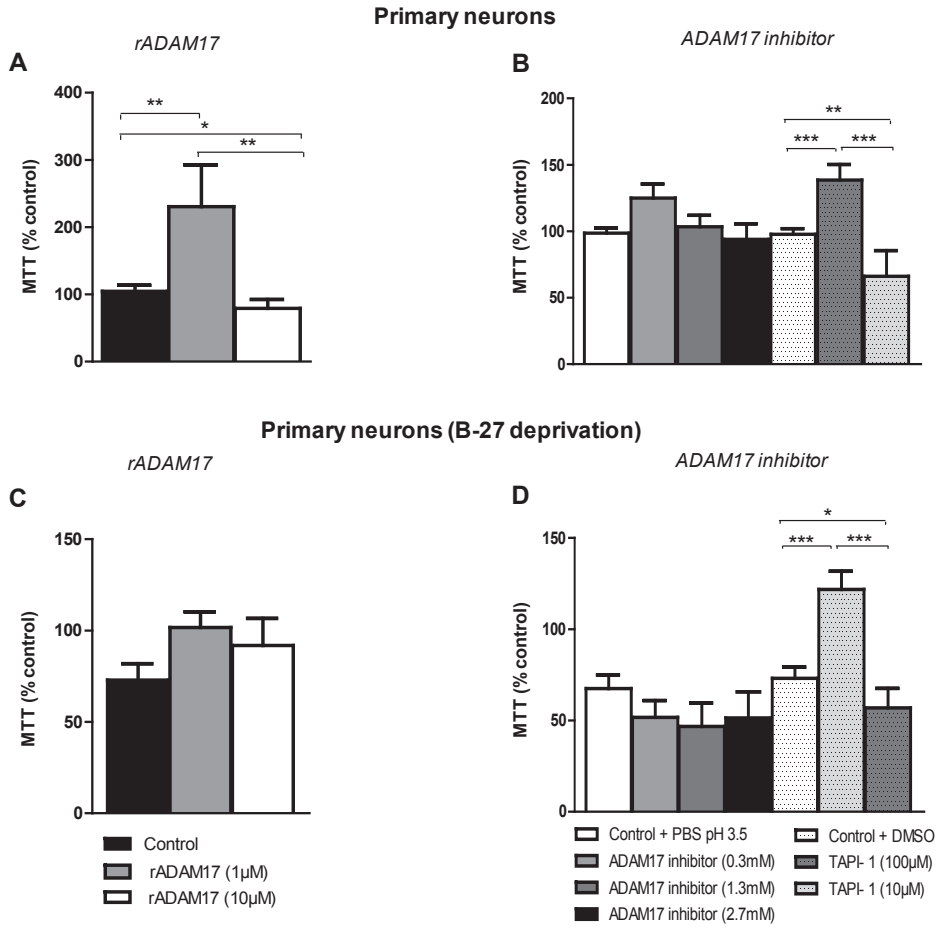

Fig. 4.3. ADAM17 has a concentration-dependent effect on the survival of cortical neurons. A. Cortical neurons were incubated with rADAM17 for 48 hours in presence of B-27. The lower concentration (1µM) significantly promotes cell survival while the higher concentration (10µM) decreases it. **B**. Cortical neurons were treated with three concentrations of the ADAM17 inhibitor BMS-561392 (mM) and two concentrations of the non-specific inhibitor of ADAM17 TAPI-1 (µM). While BMS-561392 did not have an effect on survival, TAPI-1 concentration-dependently increased (10µM) or decreased (100µM) survival. The values are expressed as percentage of control with B-27 and represent 3 independent experiments (*p<0.05; **p<0.01) (n=19-21 wells per condition). **C.** Cortical neurons were incubated with rADAM17 (1-10µM) for 48 hours under deprivation of B-27. No significant changes were seen between the control condition and the cells treated with the recombinant protein. **D**. Cortical neurons were treated with three concentrations of the ADAM17 inhibitor (mM) and two concentrations of the non-specific inhibitor of ADAM17 TAPI-1 (µM) under depletion of B-27. The non-specific inhibitor of ADAM17, TAPI-1 influenced cell viability in a concentration-dependent manner. The values are expressed as percentage of the control condition with B-27 and represent three independent experiments (*p<0.05; **p<0.01; ***p<0.001) (n=13-19 wells per condition).

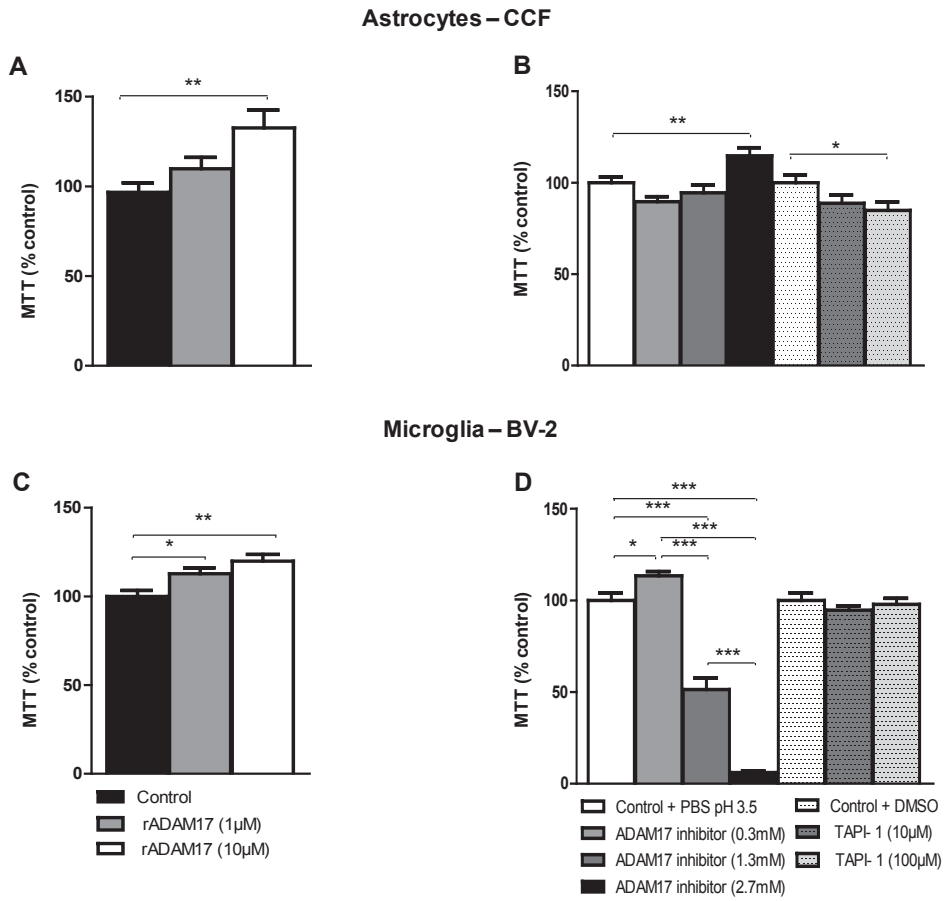

Fig. 4.4 ADAM17 promotes survival of astrocytic and microglial cell lines. A. The astrocytic cell line, CCF, was treated for 48 hours with rADAM17. Afterwards cell survival was measured using an MTT assay. There was an increase in viability using 10μM of rADAM17 (**p<0.01). **B.** CCF cells were treated for 48 hours with BMS-561392 or TAPI-1, and an increase in cell survival was seen using BMS-561392 (2.7mM) while TAPI-1 (100μM) decreased survival (*p<0.05; **p<0.01) (n=15 wells per condition). **C.** The microglial cell line BV-2 was treated for 48 hours with rADAM17. Cell survival was measured using an MTT assay. A significant increase in microglial survival was seen after rADAM17 treatment (1 and 10μM; *p<0.05; **p<0.01). **D.** Inhibition of ADAM17 by BMS-561392 for 48 hours in BV-2 cells decreases survival of these cells. The results are the mean of three independent experiments and are represented as a percentage of the control condition (*p<0.05; **p<0.01; ***p<0.001) (n=15 wells per condition).

4.4.1.3 ADAM17 inhibition increases TNFR-1 expression on the microglia membrane

To investigate the signaling pathway(s) affected by ADAM17 inhibition that are involved in microglia and oligodendrocyte apoptosis, we studied three well-known pathways that are related to apoptosis and are modulated by ADAM17. The first one involves TNF-α signaling via TNFR-1 and TNFR-2, the second one involves unprocessed NGF precursor (pro-NGF), that binds to p75NTR, and the last one implicates MAPK activation/inhibition (Ware et al., 1996, Beattie et al., 2002a, Zhan et al., 2012). Firstly, we analyzed the expression of TNFR-1 and TNFR-2 in the membrane of microglia and oligodendrocytes using the highest concentration of the ADAM17 inhibitor studied in the MTT assay. A significant increase was seen in the expression of TNFR-1 in the membrane of microglial BV-2 cells in the presence of BMS-561392 compared to the control condition (Fig. 4.5C), while no changes were seen in the immature oligodendrocytic cell line treated with the specific ADAM17 inhibitor, or oligodendrocytes treated with TAPI-1 (100µM) (Fig. 4.5A). For TNFR-2 no significant changes were seen in both cell types using the ADAM17 inhibitor, however, a significant reduction in TNFR-2 expression was seen in the presence of TAPI-1 (100µM) in microglia cells (Fig. 4.5D). TRADD is a specific mediator of TNFR-1 activation inducing recruitment of other adaptor proteins, such as TRAF2 in a first step (Kim et al., 2011) and RIP1 and associated protein with FADD in a second step (Micheau and Tschopp, 2003), leading to apoptosis in the end (Hsu et al., 1996). Surprisingly, none of the concentrations of ADAM17 inhibitor or TAPI-1 used did significantly influence TRADD expression on oligodendrocytes or microglia (Fig. 4.5E, 4.5G).

Secondly, we analyzed the expression of p75NTR in the oligodendrocytic cell line HOG only, since the microglial cell line BV-2 does not express p75NTR (Zhang et al., 2003); no significant changes were seen in p75NTR expression (Fig. 4.5F). We also analyzed the expression of pro-NGF and NGF in oligodendrocytes and microglia after 3 hours of treatment with different concentrations of the ADAM17 inhibitor BMS-561392 or with TAPI-1; as for p75NTR no significant changes were seen in pro-NGF expression in both cell

types using the mid (1.3mM) and high dose (2.7mM) of the specific ADAM17 inhibitor and TAPI-1 (100μM); however, a significant increase in pro-NGF expression was seen in the presence of the low concentration (0.3mM) of the ADAM17 inhibitor in microglial cells (Fig 4.5H, 4.5I). The levels of NGF were below detection limit under the experimental conditions used in this study.

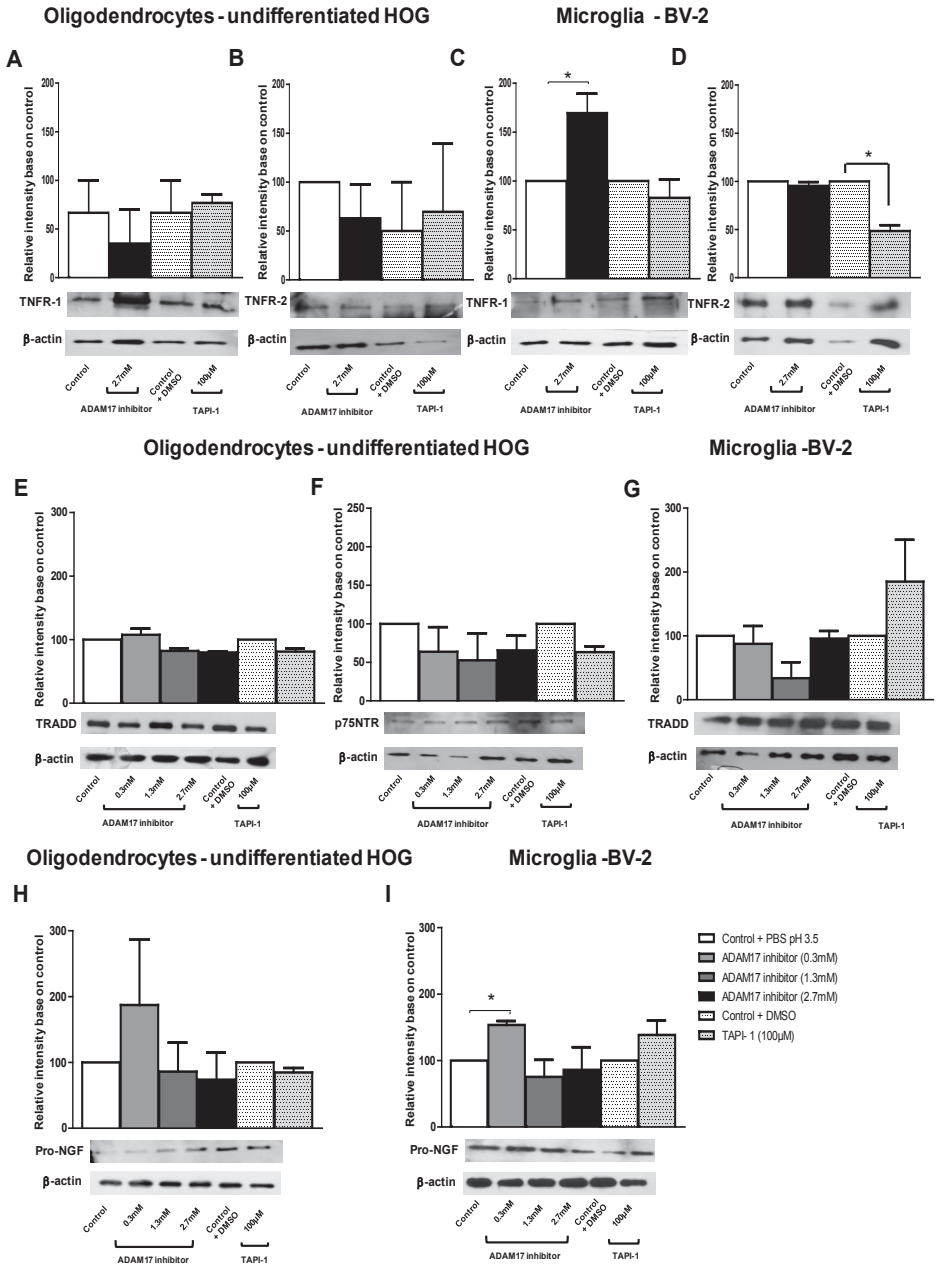

Fig. 4.5. Blocking of ADAM17 using a specific inhibitor increases TNFR-1 expression on microglial membranes. A and B. Undifferentiated HOG cells were treated for 3 hours with TAPI-1 or BMS-561392, after which the cells were lysed and membrane fractions were collected. No significant differences were seen in TNFR-1 and TNFR-2 expression. **C and D.** Membranes of BV-2 cells were isolated after ADAM17 inhibition. Electrophoretic analysis of collected membranes showed an

increase of 50% in TNFR-1 expression in the cell membrane after ADAM17 inhibition (2.7mM) but no significant changes in TNFR-2 expression. **E and G.** Densitometric analysis showed no significant changes in TRADD expression in both oligodendrocytes and microglia cells. **F.** p75NTR expression was also not affected in oligodendrocytes after ADAM17 inhibition. **H and I.** Oligodendrocytes did not show differences in pro-NGF levels after inhibition of ADAM17 either with the specific and non-specific inhibitor (mean of 3 experiments), while low inhibition of ADAM17 (0.3mM) significantly increased pro-NGF expression of cultured BV-2 cells (mean of 4 independent experiments). Data are represented as a mean percentage of the control condition (*$p<0.05$) (n=2-4 per condition).

4.4.1.4 Blocking of ADAM17 decreases phosphorylation of p44 MAPK

MAPKs are serine/threonine protein kinases involved in diverse cellular signaling pathways in many different cell types. Among these, ERK is one of the most important MAPKs regulating cell death and survival (Lee et al., 2012, Zhan et al., 2012). We therefore investigated the modulation of phosphorylation of ERK1/2 (also called p44 and p42 MAPK), in oligodendrocytes and microglia, after treatment with different doses of the ADAM17 inhibitor BMS-561392 and TAPI-1. No effects were seen using TAPI-1. In oligodendrocytes levels of p44 MAPK phosphorylation were decreased with the highest concentration of the specific inhibitor (Fig. 4.6A). Phosphorylation of p44 MAPK was increased with the lower concentration of BMS-561392 (0.3mM), but reduced with increasing concentrations of the ADAM17 inhibitor in microglial cells (Fig. 4.6B).

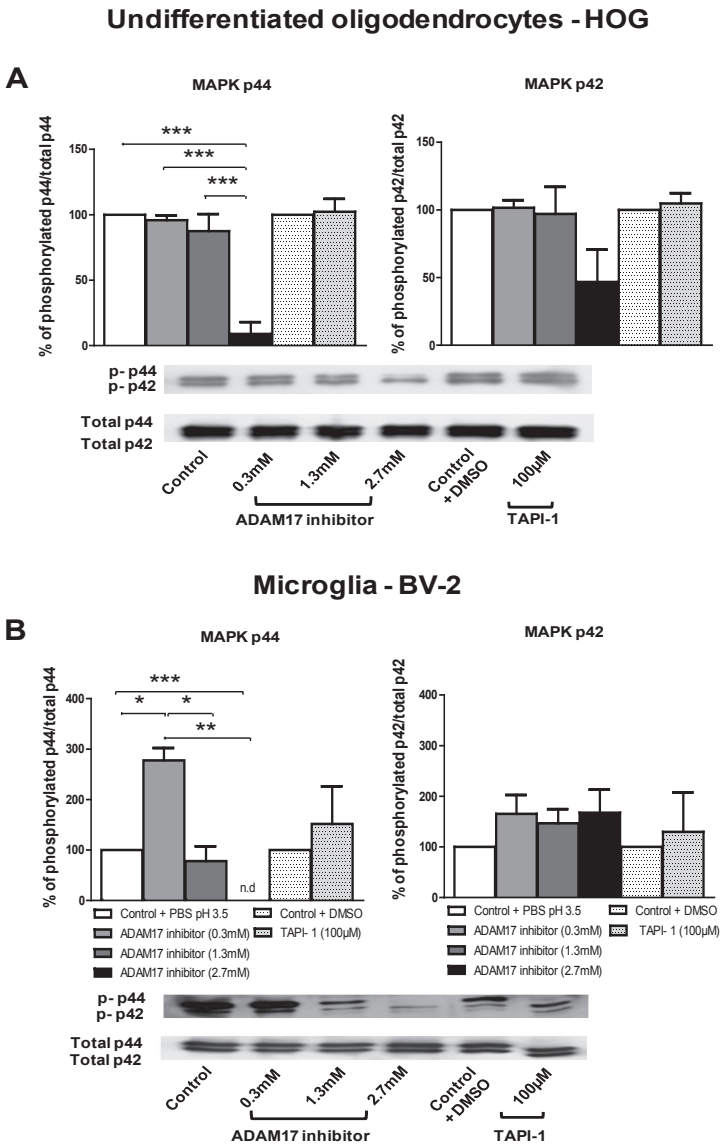

Fig. 4.6. Inhibition of ADAM17 modulates phosphorylation of p44 MAPK. **A**. Levels of p44 MAPK phosphorylation were decreased after ADAM17 inhibition with mid and high concentrations of BMS-561392, while p42 MAPK phosphorylation was not affected in immature HOG oligodendrocytes. **B.** Microglial cells (BV-2) were treated with different concentrations of BMS-561392 and TAPI-1. A significant increase was seen in p44 MAPK phosphorylation using 0.3mM of the inhibitor, while higher concentrations decreased or ever abolished p44 phosphorylation. p42 MAPK phosphorylation was not affected by BMS-561392 or TAPI-1 treatment. The results are the mean of three independent experiments and are represented as a percentage of the control condition (*p<0.05; **p<0.01; ***p<0.001) (n=3-4 wells per condition).

4.4.1.5 ADAM17 increases neurite outgrowth *in vitro*

Since ADAM17 has been involved in the shedding of different proteins, it is not surprising that ADAM17 has been implicated in neurite outgrowth. It has been shown that in dorsal root ganglion cultures, ADAM17 induces the cleavage of Nogo-66 receptor (NgR) and p75NTR, promoting outgrowth and branching of neurites *in vitro* (Ahmed et al., 2006). In hippocampal neurons, ADAM17 also controls neurite outgrowth by the cleavage of neural cell adhesion molecules, a molecule involved in neurite outgrowth (Kalus et al., 2006). It was also suggested that ADAM17 may play a role in modulating the sensitivity of neurons to the repulsive guidance molecule by cleavage of neogenin in cortical neurons co-cultured with Chinese Hamster Ovary cells (Okamura et al., 2011).

To determine the role of rADAM17 and its specific inhibitor BMS-561392, neurite outgrowth was measured from treated primary cortical neuronal cultures (Fig. 4.7A). The specific ADAM17 inhibitor reduces neurite length in a concentration-dependent manner, reaching 60% of inhibition using the highest concentration (0.8mM). TAPI-1 reduced neurite outgrowth by approximately 10%, compared to the control condition (control + DMSO) (Fig. 4.7B). Primary neurons were also cultured in the presence of rADAM17; the lower concentration (0.8mM) did not stimulated neurite outgrowth, while the higher concentration (3.7mM) significantly increased neurite outgrowth by ~25% (Fig. 4.7C).

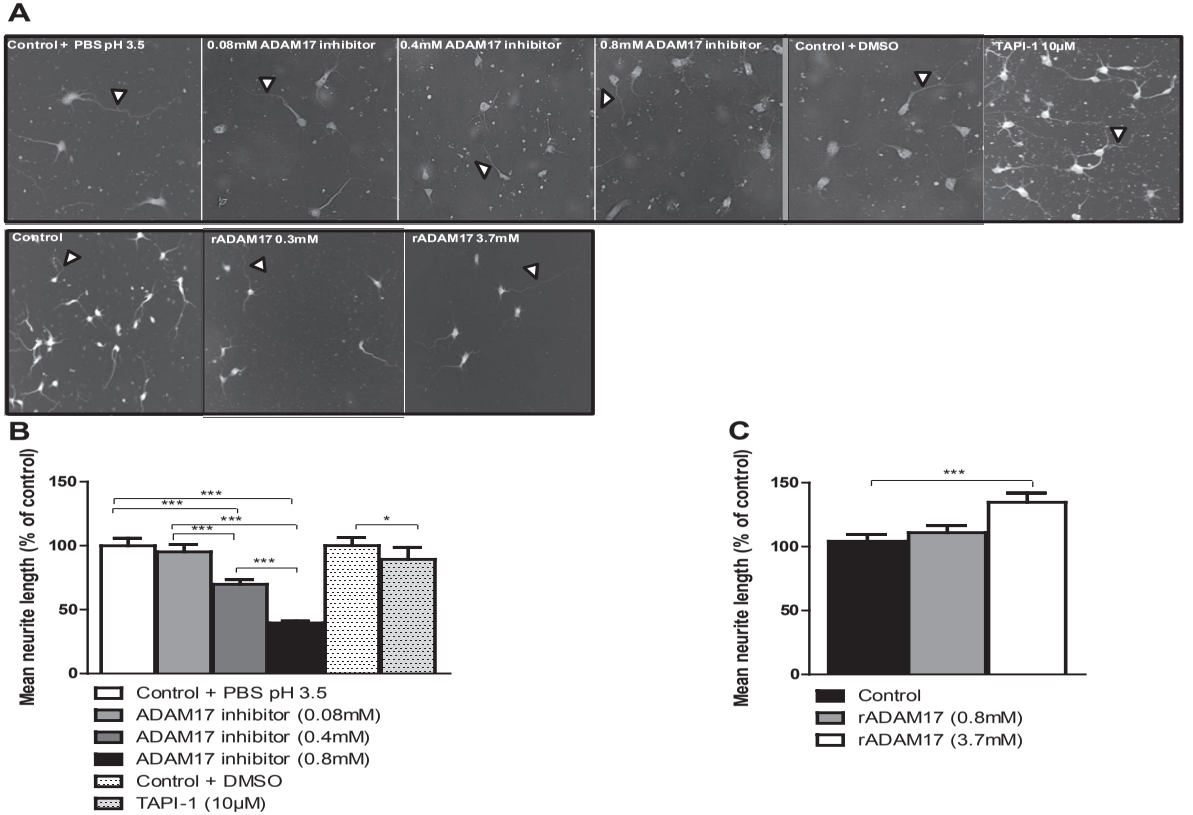
A
Control + PBS pH 3.5
0.08mM ADAM17 inhibitor
0.4mM ADAM17 inhibitor
0.8mM ADAM17 inhibitor
Control + DMSO
TAPI-1 10µM
Control
rADAM17 0.3mM
rADAM17 3.7mM
B
Mean neurite length (% of control)
150
100
50
0
***
***
***
***
***
*
Control + PBS pH 3.5
ADAM17 inhibitor (0.08mM)
ADAM17 inhibitor (0.4mM)
ADAM17 inhibitor (0.8mM)
Control + DMSO
TAPI-1 (10µM)
C
Mean neurite length (% of control)
150
100
50
0
***
Control
rADAM17 (0.8mM)
rADAM17 (3.7mM)

Fig. 4.7

Fig. 4.7: Treatment with rADAM17 increases neurite length in primary neurons while inhibition of ADAM17 decreases outgrowth. A. Representative pictures of primary neuronal cells incubated with different concentrations of ADAM17 inhibitors and rADAM17. The arrows are pointing towards some of the longest neurites. **B.** Quantification of neurite length of primary neurons cultured in the presence of the specific or non-specific ADAM17 inhibitor. Both ADAM17 inhibitors concentration-dependently decrease neurite length. **C.** Neurite length was increased in primary neurons using the highest concentration of rADAM17 tested (3.7mM), (*p <0.05; **p <0.01; ***p< 0.001) (n= 137 cells per condition).

4.4.2 *In vivo* experiments

4.4.2.1 Inhibition of ADAM17 impairs locomotor recovery after spinal cord injury

To investigate the role of ADAM17 in functional recovery after SCI *in vivo*, we applied different concentrations of rADAM17 or vehicle solution, using different routes of application (Fig. 4.8). Firstly, after SCI, mice were randomly divided into three groups and immediately injected (i.p.) with one single concentration of rADAM17 (low concentration; 1.6µM), rADAM17 (high concentration; 16µM), or vehicle solution. Under these conditions, systemic application of rADAM17 did not influence the BMS score (Fig. 4.8A) or the rotarod performance (data not shown). In a second approach, we injected systemically (i.p.) rADAM17 during 7 days, however, none of the used concentrations affected the locomotor recovery of the mice assayed by the BMS (Fig. 4.8B) or the rotarod test (data not shown).

In a third experimental approach, immediately after injury we locally applied different concentrations of rADAM17, by placing a soaked gelfoam on top of the lesion. Under these conditions, low concentration rADAM17 (0.3mM) leads to a slight but significant increase in the BMS score during the first 3 days after injury compared to the vehicle group (Fig. 4.8C); later on, BMS scores became similar in both groups of mice. Conversely, high concentration rADAM17 (3.7mM) seems to reduce the BMS score compared to the vehicle condition (Fig. 4.8D), however, this difference is probably due to a low number of animals surviving during the experiment. No significant differences were seen among the three groups using the rotarod (data not shown).

To investigate the effect of the absence of functional ADAM17 after SCI *in vivo*, we applied daily injections (s.c.) of the ADAM17 inhibitor BMS-561392 (2.2mM/day during 15 days). This significantly reduces locomotion and coordination, as assayed by the BMS score (Fig. 4.9). No significant changes were seen using rotarod (data not shown).

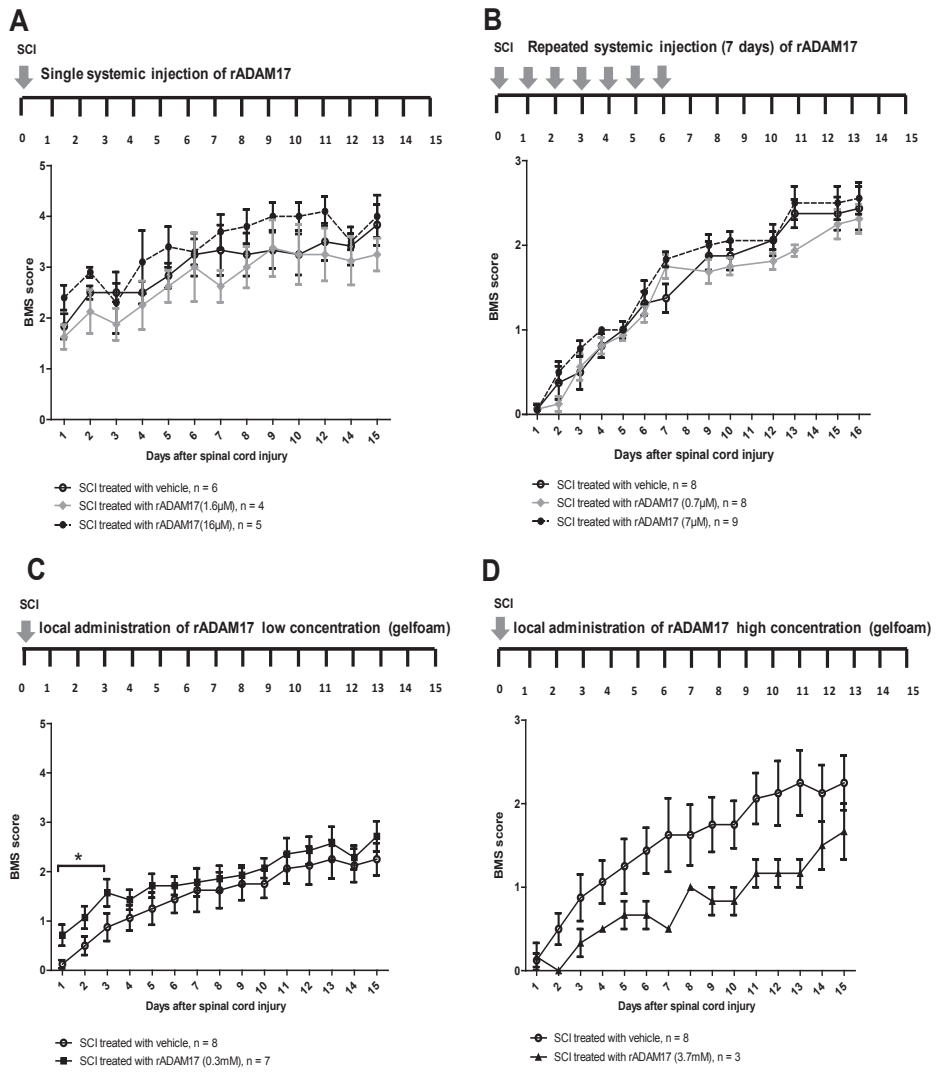

Fig. 4.8: Local application of rADAM17 transiently improves locomotion recovery after SCI. A. Mice received one single i.p. injection of rADAM17 (1.6µM or 16µM), or vehicle solution. No effects are seen. **B.** Mice were treated with daily i.p.

injections of rADAM17 (0.7µM or 7µM), or vehicle solution for 7 days, but this effect is not significant. **C**. Mice were locally treated with one single dose of rADAM17 (0.3mM), which significantly improves locomotor recovery in the acute phase after SCI (until 3 days after injury), (*p<0.05). **D**. Local application of rADAM17 (3.7mM) after SCI reduces the BMS score, but this does not affect locomotion.

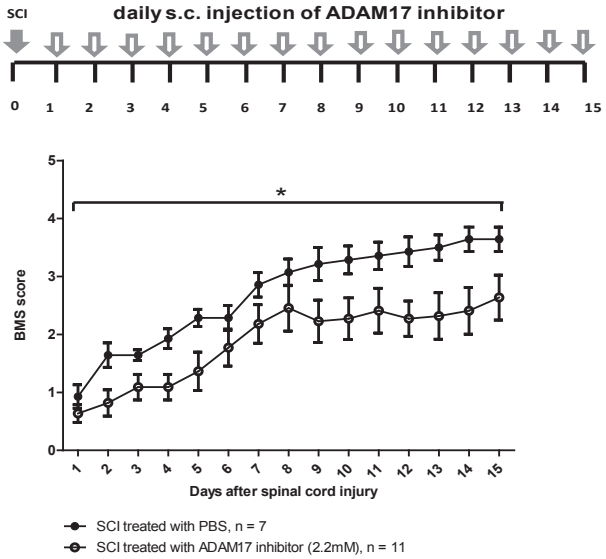

Fig. 4.9: Inhibition of endogenous ADAM17 reduces locomotor performance. Mice were daily injected (s.c.) with the specific ADAM17 inhibitor (2.2mM) for 15 days after SCI. This treatment significantly reduces spontaneous recovery after SCI as assayed by the BMS score (*p<0.05).

4.4.2.2 Inhibition of ADAM17 increases lesion size and astrogliosis after injury

Histological analysis of spinal cord cryosections 16 days after injury, from mice treated with the ADAM17 inhibitor BMS-561392, showed a significantly increased lesion size compared to PBS (control) mice (Fig. 4.10A-C). Astrogliosis, determined by GFAP intensity analysis, was significantly increased caudal to the lesion site in mice treated with ADAM17 inhibitor (Fig. 4.10D, E1-2). The area of demyelination (determined by MBP immunoreactivity) showed no differences between the groups (data not shown). T cell infiltration was also studied cranial and caudal to the lesion, and at the center of the lesion, however, no changes were seen in the

number of T cells between the two groups at any site analyzed (data not shown). In addition, we also assessed whether ADAM17 inhibition influences microglia/macrophages activation/infiltration. Mice treated with ADAM17 inhibitor displayed a significantly reduced expression of Iba1, especially close to the lesion center and caudally (Fig. 4.10F-H).

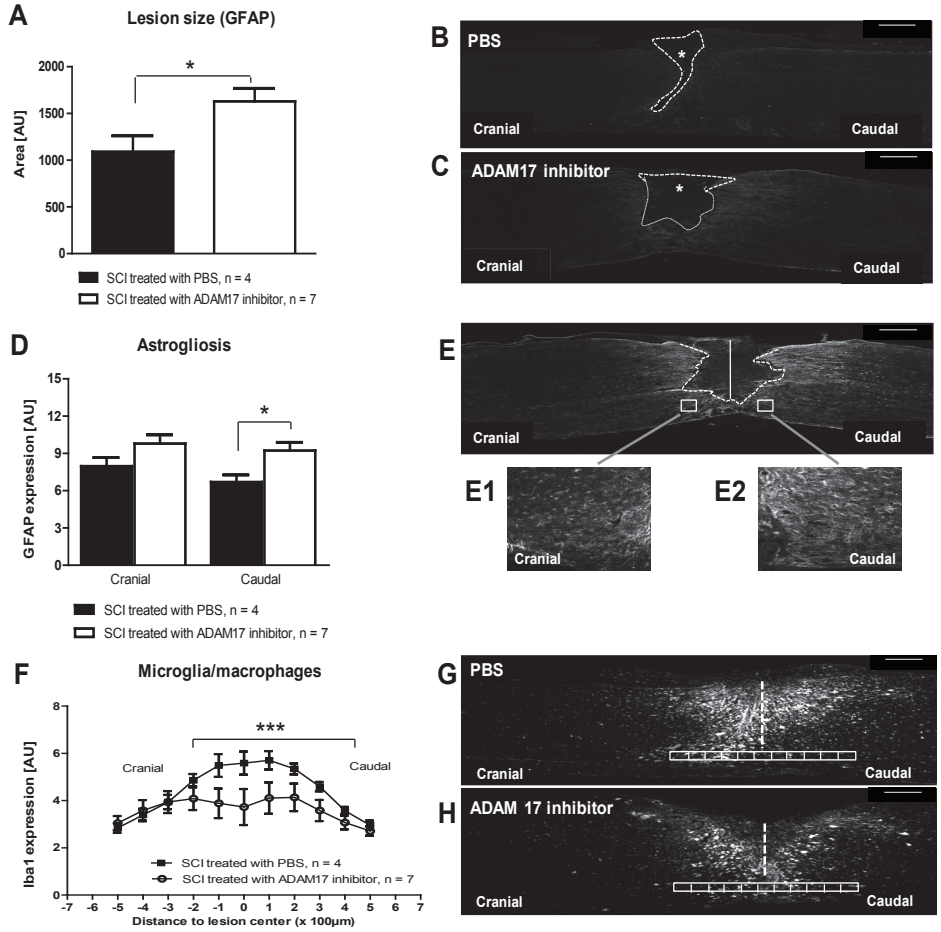

Fig. 4.10: Inhibition of ADAM17 after SCI, increases lesion size, astrogliosis and microglial/macrophage activation. A. Quantification of the lesion size after T-cut hemisection injury. ADAM17 inhibitor treated mice showed an increase in lesion size after 15 days of treatment (*p <0.05) **B-C.** Representative pictures of lesion area per group; the asterisk indicates the delineated area. **D**. Quantification of astrogliosis 100µm cranial and caudal to the lesion epicenter. There was a significant increase in gliosis caudal to the lesion site in mice treated with the ADAM17 inhibitor (*p <0.05). **E**. Representative picture showing the quantified squared region. **E1-E2**. Close up pictures of the quantified area showing GFAP immunoreactivity cranial and caudal to the lesion epicenter. **F**. Quantification of Iba1 immunoreactivity from 500µm cranial to

500µm caudal to the lesion epicenter. A significant decrease in Iba1 expression rostro-caudal to the lesion epicenter was seen after ADAM17 inhibition (***p <0.001). **G-H**. Representative pictures showing Iba1 expression around the lesion site. Scale bars = 100µm for B, C, E, G and H.

4.4.2.3 ADAM17 inhibition increases apoptosis of microglia/ macrophages in the spinal cord

ADAM17 is responsible for the cleavage of membrane-bound TNF-α in the soluble TNF-α form (Black et al., 1997). However, in our model ADAM17 inhibition did not significantly affect the expression of the two TNF-α forms (Fig. 4.11A-B). Inhibition of TNF-α after spinal cord injury leads to a decrease in apoptosis in the spinal cord (Genovese et al., 2006). Therefore, we investigated the expression of the pro-apoptotic marker Bax, and the anti-apoptotic marker Bcl-2. Inhibition of ADAM17 resulted in a slight but not significant decrease in Bcl-2 levels (Fig. 4.11C-D) and a significant increase in the expression of Bax (Fig. 4.11E-F)

Based on our previous *in vitro* results, we performed a double-labeling for active caspase-3 and CC-1 to identify apoptotic oligodendrocytes *in vivo* in SCI animals treated with the ADAM17 inhibitor BMS-561392. The analysis showed a slight, but not significant, increase in the number of apoptotic oligodendrocytes caudal to the lesion site (data not shown). In addition, we quantified apoptosis of oligodendrocytes from transgenic PLP-eCFP mice. Similarly, only a slight increase in the number of active caspase-3/PLP-double positive cells caudal to the lesion site was observed (Fig. 4.12A-B). Using double-labeling for active caspase-3 and CD11b to identify apoptotic microglia/macrophages, we found a significant increase in the number of apoptotic cells caudal to the lesion site (Fig. 4.12C-D). Finally, we measured viability using an MTT assay in primary oligodendrocyte and microglia cultures from mice deficient in ADAM17 (ADAM17ex/ex mice). As in our *in vivo* model, there was no difference in viability between wildtype and ADAM17-deficient oligodendrocytes (Fig. 4.12E). In contrast, survival of ADAM17-deficient primary microglia was significantly reduced by nearly 40% compared to cells from wildtype controls (Fig. 4.12F).

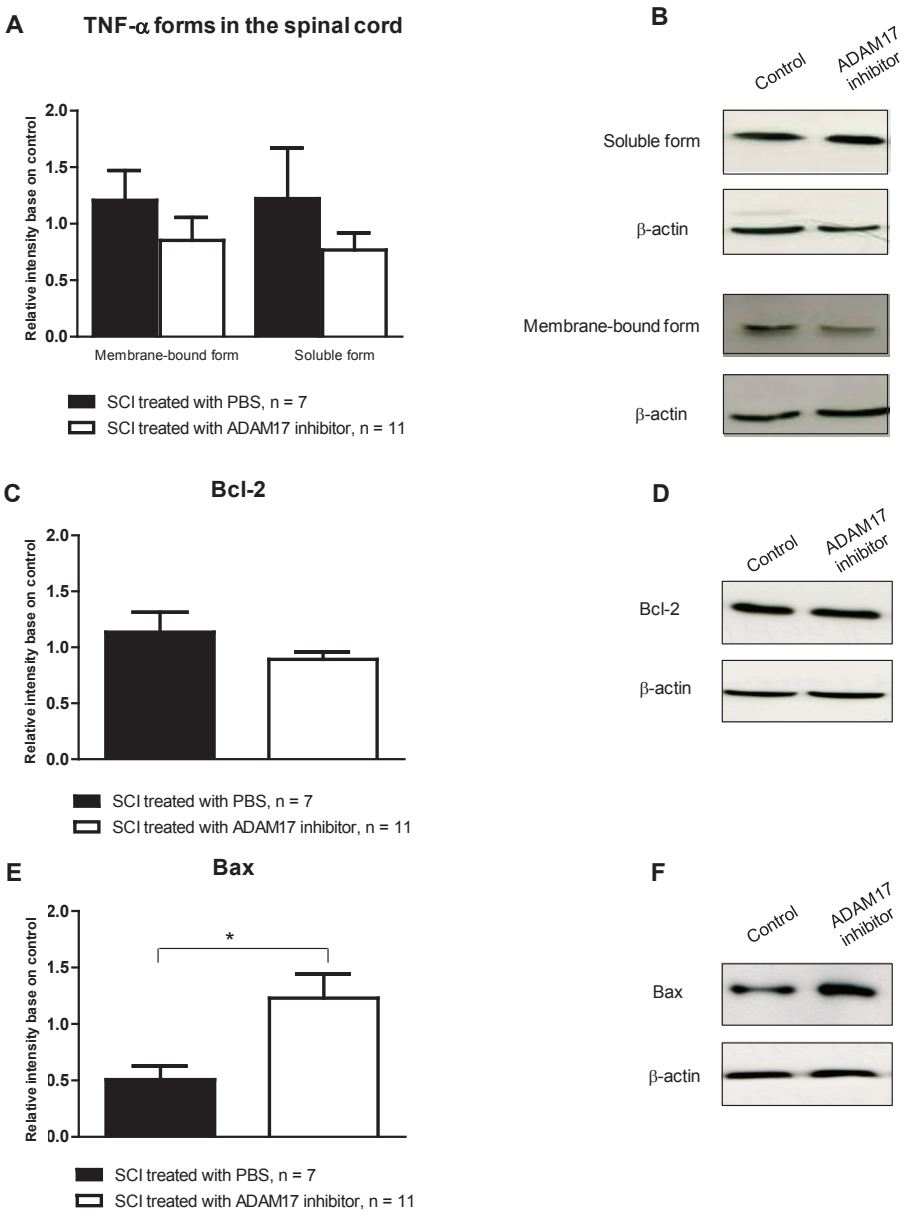

Fig. 4.11: ADAM17 inhibition increases apoptosis in the spinal cord independent of TNF-α expression. A. Densitometric analysis of homogenized spinal cord tissue around the lesion site for TNF-α membrane-bound and soluble forms after western blotting. No significant changes were seen in both TNF-α forms after inhibition of ADAM17, compared to the control condition. **B** Representative blots of both TNF-α forms and the loading control (β-actin) per treatment. **C**. Bcl-2 expression does not change after ADAM17 inhibition in the spinal cord. **D.** Representative blot of

Bcl-2 expression in both groups. **E**. Increased expression of the pro-apoptotic marker Bax in homogenized spinal cord tissue from mice treated with the ADAM17 inhibitor (* $p<0.05$). **F**. Representative blot of Bax expression in the control and ADAM17 inhibitor condition.

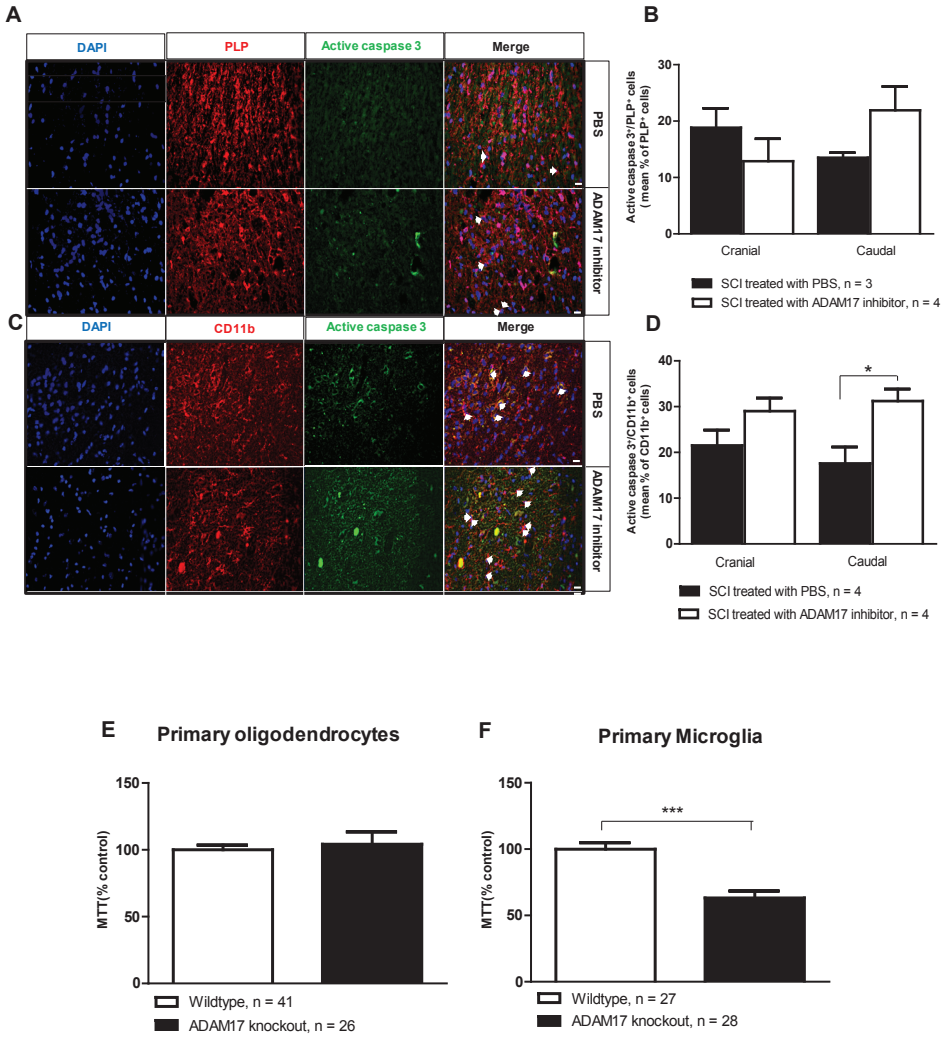

Fig. 4.12: ADAM17 inhibition increases microglial apoptosis *in vivo*. A. Representative pictures of PLP/active caspase-3 double staining. Scale bar = 20µm. **B.** Quantification of the number of apoptotic oligodendrocytes (PLP/active caspase-3 double positive) in PLP-eCFP transgenic mice in a squared area of 225 X 225 µm around the lesion site. There is a slight but not significant increase in the number or apoptotic oligodendrocytes caudal to the lesion site (225µm)**. C.** Representative pictures of $CD11b^+$/active caspase-3 caudal to the lesion site. The arrows show

apoptotic microglia in both groups. Scale bar = 20µm. **D.** Significantly increase in the number of apoptotic microglial (CD11b/ active caspase-3 double positive) cells caudal to the lesion site after inhibition of ADAM17 (*p<0.05). **E.** Survival of primary immature oligodendrocytes cultures from ADAM17 KO mice is not affected. **F.** Primary microglial cultures from ADAM17 deficient mice have a reduced survival compared to microglial cultures from wildtype animals (***p<0.001).

4.5 DISCUSSION

ADAM17 is involved in several cell signaling pathways which are crucial for survival and apoptosis, including signaling via TNFR, p75NTR, and EGFR. In the present study we have used the specific ADAM17 blocker BMS-561392 and ADAM17-deficient cell cultures to show that ADAM17 is a key survival factor for microglial cells *in vitro* and *in vivo*.

ADAM17 is best known for the shedding of TNF-α and its receptors (Black et al., 1997, Yang et al., 2006). After SCI, inhibition of both TNF-α forms with etanercept leads to a decrease in Bax and an increase in Bcl-2 expression and reduces apoptosis in the spinal cord (Genovese et al., 2006). The two forms of TNF-α show distinct binding affinities for TNFR-1 and TNFR-2. TNFR-1 has been primarily associated with apoptosis through the recruitment of TRADD, whereas TNFR-2 lacks a death domain and is associated with the anti-apoptotic effects of TNF-α (Wajant et al., 2003, McCoy and Tansey, 2008). There is increasing evidence that both TNF-α forms may play complementary roles. For example, membrane-bound TNF-α has a higher affinity for TNFR-2 and may therefore play a more important role in the regulation of cell survival (Wajant et al., 2003), remyelination after experimental autoimmune encephalomyelitis (Brambilla et al., 2011), and reduction of the inflammatory response in atherosclerosis (Canault et al., 2004), while soluble TNF-α has a higher affinity for TNFR-1 and may be more important for apoptosis and inflammation (Grell, 1995, Holtmann and Neurath, 2004). Therefore, the rationale of the present study was to modify the ratio of membrane-bound TNF-α and soluble TNF-α using the specific ADAM17 inhibitor BMS-561392, in order to improve functional recovery after SCI.

After SCI, inhibition of both TNF-α forms with etanercept decreases apoptosis, leading to an increase in Bcl-2 and a decrease in Bax expression in spinal cord homogenates (Genovese et al., 2006). We in contrast used a specific ADAM17 inhibitor. It has been suggested that the natural inhibitor of ADAM17, tissue inhibitor of metalloproteinases-3 (TIMP-3) may contribute to increased death of immature oligodendrocytes via ADAM17 in a cerebral ischemia model. The authors found indirect evidence for this by showing increased activity of ADAM17 and TNF-α in wildtype mice compared to TIMP-3-deficient mice, followed by an increased apoptosis of immature oligodendrocytes (Yang et al., 2011). However, in our SCI model, we did not find an increase in ADAM17 levels, at mRNA or protein levels in the spinal cord and motor cortex, respectively (data not shown).

Our *in vitro* results showed that ADAM17 inhibition promotes survival of astrocytes, while it decreases survival of microglia. In oligodendrocytes, we found a significant reduction of cell survival using two different cell lines; however, we did not find a clear effect in primary cultures of ADAM17 deficient mice, suggesting a selective effect of ADAM17 on different cell populations. In cortical neurons only rADAM17 had a dual effect on survival, depending on the concentration of the metalloproteinase. A different situation was observed for most of the cell types with the non-specific inhibitor of ADAM17, TAPI-1, which is also known to inhibit other metalloproteases (Antczak et al., 2008). Increased neurite length was measured in the presence of rADAM17. In addition, BMS-561392 concentration-dependently reduced neurite length in primary neuronal cultures. These results are in agreement with other reported studies, where ADAM17 induced neurite outgrowth and branching by the cleavage of different molecules involved in the control of neurite outgrowth (Ahmed et al., 2006, Kalus et al., 2006, Okamura et al., 2011).

As has been mentioned before, ADAM17 controls ectodomain shedding of various substrates; for this reason, we studied three selected signaling pathways associated to apoptosis, such as pro-NGF, p75NTR, TNF-α, and MAPK (Ware et al., 1996, Beattie et al., 2002a, Zhan et al., 2012). After SCI, increased pro-NGF production by activated microglia has been associated with apoptosis of oligodendrocytes via a p38 MAPK-dependent

mechanism (Yune et al., 2007) or via p75NTR (Beattie et al., 2002a). Since the effect of ADAM17 is cell-specific, we investigated *in vitro* each cell type separately. In our system, we did not find a change in the expression of pro-NGF or p75NTR between the control and the three concentrations of the specific ADAM17 inhibitor, neither in microglia or oligodendrocytes cultures. Interestingly, other signaling pathways associated with apoptosis, such as the well-known TNFR-1 and TNFR-2 signaling pathways, were not affected by ADAM17 inhibition in oligodendrocyte and macrophages/microglia cell line cultures.

Among the MAPK signaling pathways, p44 MAPK or p38 signaling appears to be necessary for ADAM17 activation/trafficking (Diaz-Rodriguez et al., 2002, Soond et al., 2005) but at the same time ADAM17 controls EGFR and MAPK activation via the cleavage of EGFs substrates (Murthy et al., 2010). In the absence of MAPK signaling, ADAM17 interacts with its natural inhibitor TIMP3, leading to ADAM17 inhibition (Xu et al., 2012). Enhanced activation of p42/p44 MAPK and p38 MAPK in microglia/macrophages has been reported in the spinal cord, after SCI (Xu et al., 2006). In our study using a specific inhibitor to block ADAM17, we found a new communication mechanism between p44 MAPK signaling and this metalloprotease (Fig. 4.13). Although a possible link between p44 MAPK and ADAM17 was described before, to our knowledge we show for the first time that ADAM17 inhibition leads to decreased cell viability as well as decreased phosphorylation of p44 MAPK in microglia and oligodendrocyte cultures. Further studies will be required to clarify the relationship between ADAM17 activity and p44 MAPK signaling in microglia after SCI.

We also studied the effect of blocking ADAM17 *in vivo* after SCI. Surprisingly, in our SCI model, ADAM17 inhibition did not lead to differences in the levels of the two TNF-α forms in spinal cord homogenates. However, ADAM17 inhibition did significantly increase the number of apoptotic microglia/macrophage in the injured spinal cord by nearly 40% combined with a reduced histological and functional outcome after SCI. In line with these results we found a significant reduction of $Iba1^+$ microglia/macrophages levels after ADAM17 inhibition but no substantial change in MBP^+ and $CC\text{-}1^+$ oligodendrocyte cells. Microglial cells play a dual

role after CNS injury, participating as phagocytes to remove tissue debris and dead cells, as well as exacerbating tissue damage through the release of pro-inflammatory factors (David and Kroner, 2011). For example, after transient focal cerebral ischemia resident microglia cells play an important role in tissue clearance, compensating for the lack of entry of inflammatory cells (Schilling et al., 2005). After SCI, peripheral monocytes infiltrate the injury site and differentiate into macrophages. These infiltrating cells are necessary for recovery after SCI, an effect mediated though IL-10 secretion (Shechter et al., 2009). These data, together with the findings presented in this chapter, suggest that ADAM17 plays a key role in protecting beneficial microglia/macrophage and supports functional recovery after SCI possible via a p44 MAPK-dependent manner.

On the contrary, inhibition of ADAM17 promoted astrogliosis and increased lesion size after SCI. In addition, we detected a slight, but significant increase in functional recovery during the first three days after one single local administration of rADAM17. To our knowledge, this is the first time that endogenous ADAM17 has been reported to be used after SCI.

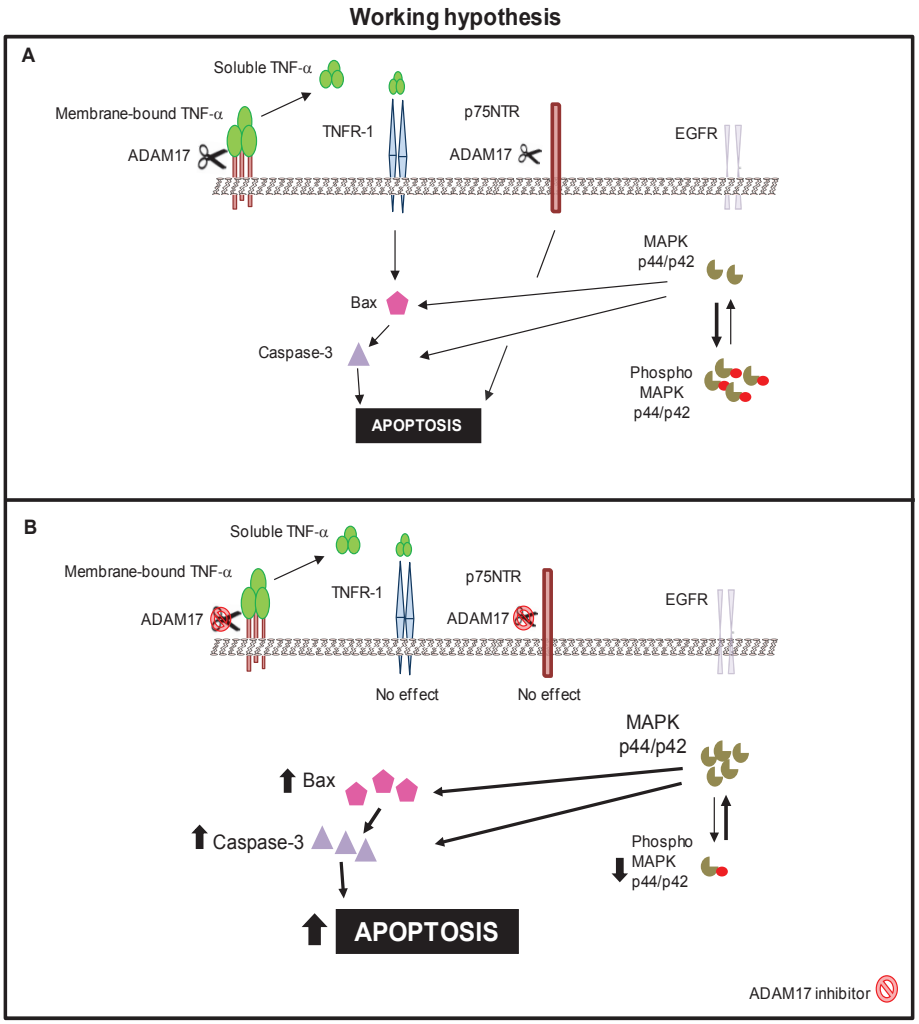

Fig 4.13: Blocking of ADAM17 induces inhibition/reduction of MAPKp44 phosphorylation in microglia and oligodendrocytes, increasing apoptosis. A. Model describing the ideal homeostatic situation when ADAM17 is active. ADAM17 cleaves membrane-bound TNF-α releasing the soluble TNF-α form; after which either the membrane-bound or soluble form of TNF-α can bind to TNFR-1 inducing activation of apoptosis. ADAM17 is also involved in the cleavage of EGFs substrates, leading to activation of EGFR and MAPKp44. These two pathways regulate the balance between apoptosis and survival. **B.** In the presence of BMS-561392, the levels of the soluble TNF-α form remain similar to the normal physiological conditions. However, there is an increased expression of apoptotic molecules such as Bax and caspase-3 (this is apparently independent of TNFR-1 and p75NTR activation). There is also a reduced phosphorylation of MAPKp44 after ADAM17 inhibition via a yet to be determined mechanism, leading to increased apoptosis.

CHAPTER 5

General discussion and conclusions

The mammalian CNS has a limited capacity to repair and to regenerate. SCI is a major pathological condition worldwide, associated with severe and permanent neurological damage and limited functional recovery. Mechanical damage of the spinal cord causes extensive neuronal loss, axonal damage, demyelination as well as increased production of "anti- and pro-inflammatory" factors. To date, there are still many open questions regarding the molecular mechanisms behind SCI-induced secondary injury processes, considering the large number of biochemical cascades that are involved in this pathology. In this thesis, the role of selected "anti- and pro-inflammatory" cytokines (i.e. IL-13 and TNF-α), as well as the metalloprotease ADAM17 were studied in the context of CNS regeneration and SCI. This final chapter provides a concise overview and general discussion of the main findings of this thesis.

5.1 IL-13: A POTENTIAL THERAPEUTIC TARGET FOR SCI

It has been reported that after CNS injury, either in the brain or the spinal cord, there is an extensive change in the expression of cytokines, that influences the immune response associated with secondary degeneration after injury (Donnelly and Popovich, 2008, Vidal et al., 2012). IL-13 belongs to the family of "anti-inflammatory" cytokines, whose levels are increased after brain (Dalgard et al., 2012) or spinal cord injury (Lee et al., 2010). Whereas a number of studies have reported beneficial effects of IL-13, such as reduction in the clinical severity and incidence of EAE (Offner et al., 2005), control of brain inflammation (Shin et al., 2004), reduction of Aβ levels in the brain (Kawahara et al., 2012) and promotion of recovery after SCI by modulation of the immune response (Nakajima et al., 2012), detrimental effects of IL-13 have also been documented, such as promotion of asthma (Yang et al., 2005), and neurotoxicity of hippocampal neurons (Park et al., 2009, Nam et al., 2012). In the same line, there is evidence that expression of IL-13Rα1 in dopaminergic neurons may contribute to their vulnerability and consequent loss in pathological settings, such as Parkinson's disease (Morrison et al., 2012).

In **chapter 2** we found a neurotoxic effect of high concentrations of IL-13 in hippocampal neurons under optimal growing conditions in brain slices. Meanwhile no effect of IL-13 was seen in primary cortical neuronal cultures. We recently found that IL-13 has no effect on survival of primary hippocampal neuron cultures; together, these data thus suggest an indirect effect of IL-13 on neuronal survival, mediated via other cells. In addition low concentrations of IL-13 seem to rescue hippocampal neurons from NMDA induced cell death *in vitro*. This is a very promising result and further research might either focus on the possible neuroprotective effects of low concentrations of IL-13 after traumatic CNS injury, or focus on other degenerative diseases in which the number and the integrity of hippocampal neurons is visibly affected (e.g. Alzheimer's disease, or temporal lobe epilepsy). Further research will also need to clarify the effects mediated by IL-13 on other cell populations that likely indirectly influence trophic support for the neuronal population. For example, after brain inflammation the activation of microglia cells exacerbates brain damage. The interaction between activated microglia and neurons is necessary for inducing IL-13 production in microglia cells in the damaged brain, leading to death of these microglia, thus supporting neuronal survival (Shin et al., 2004). Conversely, Park and collaborators reported that thrombin-induced neurotoxicity in hippocampal neurons was accompanied by an upregulation of IL-13 in activated microglia cells. IL-13-neutralizing antibodies reduced neuronal loss in the hippocampus by inhibiting NADPH oxidase activation and ROS production (Park et al., 2009). Similarly, IL-13-mediated oxidative stress has been implicated in death of hippocampal neurons (Nam et al., 2012). These results raise more questions about the specific signaling cascades involved in the beneficial or harmful responses induced by IL-13. It is tempting to speculate that IL-13 induced neurotoxic or protective signaling responses might be associated with: 1) the type of insult, which induces the activation of one or another specific signaling cascade over a certain cell population, and 2) the presence of certain factors (ROS, cytokines) around the affected area that might direct the final response.

In other CNS areas, such as the spinal cord, mesenchymal stem cells transplantation favored the development of alternatively activated

macrophages (M2 phenotype) by increasing the secretion of IL-13 and IL-14. This shift in the immune response reduced the inhibitory scar tissue formation, promoting axonal regeneration and functional recovery after SCI (Nakajima et al., 2012). Future research in our laboratory attempts to reveal the potential properties of stem cells secreting IL-13 as a therapy for SCI.

At the moment the direct effects of IL-13 on axonal regeneration have not been yet investigated. In **chapter 2** we present for the first time some evidence that IL-13 promotes neurite outgrowth *in vitro* via a JAK-dependent mechanism. Other Th2 cytokines, such as IL-4 and IL-10, are also known to promote neurite outgrowth in the PNS and CNS. For example, in a DRG outgrowth model, IL-4 modulates peripheral axon regeneration in a dose dependent manner (Golz et al., 2006), while IL-10 promotes axonal regeneration and functional recovery after SCI *in vivo* (Bethea et al., 1999). Our results using brain slices, demonstrated that the effect of IL-13 on promotion of neurite outgrowth was partially mediated by JAK1 signaling, which is associated with the IL-4Rα chain, that together with the IL-13Rα1 chains forms a functional IL-13 receptor (Hershey, 2003, Kelly-Welch et al., 2003). It is known that activation of JAKs by IL-13, especially JAK1 and Tyk2, results in phosphorylation of the cytoplasmic tyrosines in the IL-4Rα chain, followed by recruitment and phosphorylation of STAT6 and STAT3 to a lesser extent (Andrews et al., 2002, Hershey, 2003, Chiba et al., 2012). This is one of the most important transducing signaling pathways from the cell surface to the nucleus in response to cytokines.

The roles of IL-4, IL-13, IL-4Rα, or STAT6 in protective immunity against parasites have been widely studied using knockout animals (Spencer et al., 2001). However, little is known about the role of JAK1/STAT signaling after injury to the central nervous system. After SCI, there is an increase during the first 12 hours post injury, in JAK1 and STAT3 phosphorylation in neurons; later on, STAT3 activation increases in astrocytes and microglial cells in the spinal cord. IL-6 has been pointed out as one of the cytokines responsible for the activation of JAK1/STAT3, thus contributing to neuronal protection after injury (Yamauchi et al., 2006).

These results indicate that the activation of JAK/STAT signaling may play a beneficial role after injury to the CNS by promoting neuroprotection and

regeneration (**chapter 2**). Further research in this area will be needed to clarify if the role of JAK/STAT signaling through IL13 activation *in vivo* may also have a favorable outcome for regeneration after SCI.

Interestingly, local expression of IL-13 protein is significantly reduced 6 hours after SCI (our own unpublished observations). It is thus tempting to speculate that reconstitution of IL-13 may have a beneficial effect after SCI. In fact, we have recently demonstrated that systemic application of IL-13 for one week, starting one day before SCI, results in a better functional outcome in these animals; however, local application of IL-13 immediately after SCI decreases functional recovery as assayed by the BMS (unpublished observations from our laboratory). Nevertheless, local application of IL-13 at later time points may still be a promising therapy to improve functional outcome after SCI. It is important to consider the immunomodulatory effects of IL-13 on M1/M2 microglia/macrophage populations. At 7 days after SCI, the ratio between M1 and M2 microglia/macrophages increased markedly due to reduced M2 phenotype (Kigerl et al., 2009). IL-13 and IL-4 are among the main cytokines needed for M2 microglia/macrophages development. It is tempting to speculate that local application of IL-13 may be accompanied by IL-4 application at later time points to increase the development of the M2 phenotype and consequently reduce the destructive effects of microglia/macrophages, promoting a more permissive environment to achieve functional regeneration after injury. It is also necessary to clarify the effects of IL-13 on motor neuron survival, since on the one hand toxic effects have been observed in hippocampal neurons after IL-13 treatment (**chapter 2**) (Park et al., 2009, Nam et al., 2012), while it has been associated with preservation of neuronal integrity in the brain of multiple sclerosis patients on the other hand (Rossi et al., 2011).

5.2 TNF-α EFFECTS DURING THE ACUTE AND LATE PHASES AFTER SCI

The role of TNF-α after CNS injury is very controversial, since both beneficial and detrimental effects have been reported. For example, traumatic brain injury (TBI) in TNF-α-deficient mice, leads to a reduced neurological motor

function compared to wild-type mice (during the first week post injury). Wildtype mice started to recover from brain injury by 2-3 weeks post injury, whereas brain-injured TNF-α-deficient mice had persistent motor deficits for up to 4 weeks (Scherbel et al., 1999). Neuroprotective effects of TNF-α were also demonstrated in cerebral cortical neurons, where TNF-α protected against NMDA-induced neurotoxicity by increasing A-current density (outward potassium current) (Houzen et al., 1997). In hippocampal neurons, TNF-α improves neuronal survival and protects against neurodegeneration, since treatment with TNF-α inhibitors increased cell death (Shohami et al., 1997, Sriram et al., 2006). Moreover, double mutant mice for TNFR-1 and TNFR-2 have also shown to be protective against axotomy and toxic insults to dopaminergic neurons (Sriram et al., 2002, Wessig et al., 2005). Conversely, depletion of TNFR-1 in a transgenic Alzheimer's disease animal model (APP23 transgenic mice/TNFR-$1^{-/-}$) results in reduction of brain amyloid plaques and Aβ levels, preventing learning and memory deficits (He et al., 2007). In addition, treatment with TNF-α blockers increased neurite outgrowth and branching of hippocampal neurons *in vitro* (Neumann et al., 2002). In the same line, clinical evidence supports the concept that inhibiting the expression of TNF-a may result in cognitive improvement in Alzheimer's disease patients (Tobinick and Gross, 2008). Furthermore *in vivo* studies showed that inhibition of TNF-α improved spinal cord recovery (Genovese et al., 2006, Koopmans et al., 2009, Chen et al., 2011). For example, after administration of the TNF-α inhibitor etanercept, after SCI, there was a reduction of apoptosis of neurons and oligodendrocytes (Chen et al., 2011), as well as reduced tissue injury and neutrophil infiltration, leading to recovery of locomotion skills (Genovese et al., 2006, Chen et al., 2011). However, both TNFRs deficient mice showed worse functional outcomes after SCI (Kim et al., 2001).

In order to understand this "duality", we firstly studied the expression pattern of TNF-α mRNA after a T-cut hemisection injury. We found that TNF-α mRNA levels were increased immediately after injury, remaining relatively higher than untouched animals until day 7 (**chapter 3**). These results are in agreement with published reports using other SCI models, where TNF-α mRNA levels increased immediately after injury (Pineau and Lacroix,

2007). Strikingly, at the protein level there was a decrease in systemic and local TNF-α expression in injured animals, compared with untouched ones. A similar situation was recently reported, in a contusion injury model, where TNF-α protein levels increased 2 hours after injury, to begin to diminish 4 hours post-injury (Stammers et al., 2012). The mechanism behind this decrease is still unknown, however, it is known that TNF-α mRNA stability and translation may be affected by the expression of specific proteins involved in mediating the stability of conserved regions, such as AU-rich elements (ARE) (MacKenzie et al., 2002). These elements have been found in the 3'-untranslated region (3'-UTR) of transcripts encoding cytokines, oncoproteins, growth and transcription factors, as well as TNF-α (Caput et al., 1986, Kontoyiannis et al., 1999). For example, in synovial fibroblast-like cells carrying a high copy number of human TNF-α transgene, a high level of TNF-α mRNA, but not protein could be detected, indicating permanent suppression of translation mediated by ARE elements (Kontoyiannis et al., 1999). These results indicate that there are many check-points involved in the translation of TNF-α mRNA to TNF-α protein.

In order to understand the physiological relevance of decreased TNF-α protein levels after SCI, we reconstituted TNF-α to its "basal" levels, by injecting recombinant soluble TNF-α. Unfortunately, reconstitution of TNF-α did not influence locomotor recovery. It has been suggested that after SCI, TNF-α may play a harmful role during the acute phase, while in the chronic phase it may play a more beneficial role (Chi et al., 2008). In addition, under pathological conditions, such as chronic inflammation and autoimmunity (EAE), soluble TNF-α played a more detrimental role, while the membrane-bound form has a more protective one (Alexopoulou et al., 2006, Brambilla et al., 2011). However, in our SCI model administration of soluble TNF-α did not affect the expression of pro- and anti-apoptotic molecules, compared to the vehicle treated animals. Since these results (published and our own data) not only differ according to the expression of membrane-bound and soluble TNF-α forms, but also depend on the pathological settings, and on the expression of TNFRs, they raised even more questions about the dissimilar results obtained with TNF-α and anti-TNF-α treatments.

As mentioned above, blocking of TNF-α has beneficial effects during the acute phase (first minutes or hours) after SCI (Genovese et al., 2006, Chen et al., 2011). However, to our knowledge, there is a lack of information about the role of TNF-α at later time points which appears to us from a clinical point of view to be more relevant for spinal cord injured patients. In **chapter 3** we reported that TNF-α mRNA and protein levels have a tendency to increase again from 7 days onward after SCI, after first being significantly decreased. Similar results were reported for mRNA levels, by Pineau and Lacroix, who found increased TNF-α levels between 14-28 days after SCI (Pineau and Lacroix, 2007). Increased TNF-α production at the site of injury has been listed as one of the major agents to induce secondary injury after SCI (Oyinbo, 2011). Besides this, Chin and collaborators reported that TNF-α transgenic rats present an increase of apoptotic cells in the spinal cord during the acute phase (3 days onwards), while during the early chronic phase (around day 7) there was a decrease in tissue loss in TNF-α transgenic rats after SCI (Chi et al., 2008). Based on these results, we aimed to block peripheral TNF-α at 14 days after SCI, using three different doses as well as three different routes of administration. However, as reported in **chapter 3**, none of these approaches influenced functional recovery after SCI, as assayed by the BMS. These results indicate that TNF-α blocking during the early chronic phase after injury may not be effective, in contrast to what has been reported during the acute phase.

5.3 ADAM17 EFFECTS AFTER CNS INJURY

Proteolytic processing is extremely relevant for regulating the functional properties of cell surface proteins. Two families of zinc-dependent metalloproteases are highly involved in this process, namely the matrix metalloproteases and the ADAM proteases. We focused in this thesis on ADAM17, which cleaves a high number of different cell surface proteins involved in a variety of biological processes (**chapter 1** and **chapter 4**).
In the CNS ADAM17 is expressed by a variety of cells, such as neurons, oligodendrocytes, astrocytes, microglia/macrophages, and endothelial cells

((Skovronsky et al., 2001), and our own unpublished observations). ADAM17 has been implicated in nervous system development through activation of neural cell adhesion, and neurite outgrowth by cleavage of the cell-adhesion molecule L1 (Maretzky et al., 2005). ADAM17-deficient mice die close to birth (at around 17.5 days post coitum), and those born alive usually die within a few hours. The fetus presents with open eyelids, hair and skin abnormalities, and defects in epithelial maturation of multiple organs. The born mice are characterized by open eyelids, stunted vibrissae and wavy hair (Peschon et al., 1998). These findings highlight the importance of ADAM17 during development.

Several studies have shown differences in the expression levels of ADAM17 under pathological conditions such as osteoarthritis, rheumatoid arthritis, acute myocardial infarction, breast cancer, Alzheimer's and Prion diseases, mild cognitive impairment, and multiple sclerosis (Vincent et al., 2001, Arribas and Ruiz-Paz, 2005, Kim et al., 2008, Jiang et al., 2011). Inhibition of ADAM17 has been shown to protect neurons and brain tissue in an ischemic model of stroke, by reducing infarct size, soluble TNF-α expression and neurological deficits (Wang et al., 2004). Conversely, others have shown that ADAM17 can contribute to brain repair by inducing neuronal stem cell proliferation and migration (Rubio-Araiz et al., 2008). In another context, inhibition of ADAM17 decreased amyloid precursor protein levels without significant changes in Aβ levels (Kim et al., 2008), while pharmacological activation of ADAM17 reduced the production of Aβ42 (Meng et al., 2013). As for TNF-α, both neuroprotective and detrimental effects of ADAM17 have thus been reported. Further studies are needed to better characterize TNF-α and ADAM17 signaling mechanisms during brain injury and to establish under which circumstances ADAM17 may be beneficial or harmful.

In **chapter 4**, we show that ADAM17 is involved in the pathology of SCI. We provide evidence that ADAM17 inhibition reduces functional outcome after SCI, by increasing lesion size and reducing microglial/macrophages expression caudal to the lesion site. Moreover, local single concentration application of rADAM17 slightly improved BMS score during the acute phase (until day 3 after SCI). These results suggest that more than one local application of rADAM17 may be necessary to achieve a sustained locomotion

recovery after SCI. However, the concentration and timing of delivery must be carefully studied to avoid non-effective concentrations or toxic concentrations.

5.4 MOLECULAR MECHANISMS BEHIND THE PROMOTING EFFECT OF ADAM17 ON APOPTOTIC AND SURVIVAL PATHWAYS

ADAM17 controls ectodomain shedding of various proteins, among them TNF-α and its receptors, and pro-NGF and p75NTR, that play a fundamental role in apoptosis after SCI (Ware et al., 1996, Beattie et al., 2002a, Beattie et al., 2002b, Genovese et al., 2006, Chen et al., 2011); it is therefore not surprising that ADAM17 regulates apoptotic and survival pathways. In a cerebral ischemia model, ADAM17 was found to promote death of immature oligodendrocytes via TNF-α (Yang et al., 2011). In **chapter 4**, we reported that inhibition of ADAM17, using the specific inhibitor BMS-561392, increases both microglia and oligodendrocyte apoptosis *in vitro*, while *in vivo* (SCI model) only microglia/macrophages were significantly affected by ADAM17 inhibition. Microglia are cells with a dual role, in charge of cleaning cellular debris and toxic substances, thereby providing neuroprotection and facilitating regenerative processes after CNS injury (David and Kroner, 2011); on the other hand, microglia/macrophage activation also leads to secondary damage after SCI (Tian et al., 2007). These contradictory findings have led to a considerable debate regarding the beneficial (Schwartz et al., 1999, Schilling et al., 2005, Shechter et al., 2009)versus detrimental (Popovich and Hickey, 2001, Letellier et al., 2010, Iannotti et al., 2011) role of microglia/macrophages (mainly associated with inflammation) after CNS injury.

We found that apoptosis was independent of TNF-α signaling, since blocking of ADAM17 did not affect the expression levels of TNF-α (in both serum and spinal cord tissue). It has been shown before that after SCI, increased pro-NGF production by activated microglia is associated with apoptosis of oligodendrocytes via a p38 MAPK-dependent mechanism (Yune et al., 2007)

or via p75NTR (Beattie et al., 2002a). Therefore, we investigated these pathways *in vitro* using microglia and oligodendrocyte cell lines. Surprisingly, p75NTR, pro-NGF and TNFR-2 pathways were not affected by ADAM17 inhibition. Meanwhile, increased expression of TNFR-1 was found on the microglia membrane. Activation of TNFR-1 leads to the recruitment of TRADD with the consequent activation of caspases and ultimately cell death (Hsu et al., 1996, Locksley et al., 2001, Aggarwal, 2003). Both microglia and oligodendrocytes did not show a difference in TRADD expression. These results suggest that increased expression of TNFR-1 on microglia membranes was due to the non-shedding of TNFR-1 by ADAM17 inhibition, instead of increased activation of the receptor.

ADAM17 controls EGFR and MAPK p44/p42 activation via EGF substrate cleavage (Murthy et al., 2010). In the absence of MAPK signaling, ADAM17 interacts with its natural inhibitor TIMP3, leading to ADAM17 inhibition (Xu et al., 2012). Signaling via MAPK p44/p42 has been repeatedly associated with apoptosis (Ware et al., 1996, Beattie et al., 2002a, Zhan et al., 2012). In **chapter 4**, we found a decreased activation of MAPK p44 after ADAM17 blocking. It is tempting to propose a possible new communication mechanism between MAPK p44 signaling and ADAM17 in inducing apoptosis. Although a possible link between MAPK p44 and ADAM17 was described before in a different context, to our knowledge our results are the first to suggest the existence of a link between ADAM17 inhibition and decreased cell viability via MAPK p44.

It is remains an open question though, how ADAM17 inhibition is involved in decreased MAPK p44 activation leading to apoptosis in the end. However it is possible that blocking of ADAM17 leads to inhibition of EGFR signaling, reducing MAPK p44 activation. Recently, a relation between EGFR and microglia has been suggested. Using a contusion SCI model, inhibition of EGFR signaling reduced activation of microglia and astrocytes, by reducing MAPK activation (Qu et al., 2012). Secondly, a direct link between MAPK p44/p42 and caspase-3 activation has also been indicated. These studies suggested that activation of MAPK p44/p42 may lead to increased caspase-3 activation and consequent apoptosis (Allan et al., 2003, Zhuang and Schnellmann, 2006).

In summary, our results indicate that ADAM17 is a key factor in maintaining survival of beneficial microglia/macrophages after SCI. Future studies will be needed to clarify dissect the role of glial cells and the relation between activation of ADAM17 and MAPKp44 after CNS injury, to facilitate the development of new therapies or drugs that can favorably modulate the immune response.

5.5 THE ROLE OF IL-13 AND ADAM17 IN PROCESSES OF NEUROREGENERATION

In **chapter 2** and **chapter 4,** we have used different *in vitro* screening techniques to understand the role of IL-13 and ADAM17 in the CNS. *In vitro* models are relatively simple and efficient for the analysis of neuroprotection in the CNS. Although they cannot replace *in vivo* models, they are useful to complement them and allow exploration of molecular mechanisms (Kumaria and Tolias, 2008). To investigate the regenerative properties of IL-13 in neuronal outgrowth we used in **chapter 2** two well-characterized models: primary cultures of dissociated cortical neuronal cells and organotypic brain slices (Hechler et al., 2006). The first one has the advantage of allowing to study neuronal and glial cells independently, with the disruption of intercellular connections as the main disadvantage (Kumaria and Tolias, 2008). In the second model, neurite connections and local circuitry are preserved, as well as the morphological architecture of the brain tissue, resembling more closely the *in vivo* situation (Kumaria and Tolias, 2008, Su et al., 2011). Therefore, organotypic hippocampal slice cultures have been used before as a model for experimentally induced neurodegeneration and neuroprotection (Cho et al., 2007). Another important factor to keep in mind is the age of the samples; while primary cultures are mostly isolated from embryos, organotypic brain slices are more often prepared from early postnatal animals, since slices from adults have a high cell mortality (Su et al., 2011).

From both models it is tempting to speculate that the effect of IL-13 on neurite outgrowth seems to be a direct effect on neurons and not an indirect

effect via glial cells, since the percentage of contaminating cells in our primary cultures is negligible compared to the number of neurons and neuronal progenitor cells present. *In vitro*, EC axons preserve the capacity to regenerate, allowing measurement of growth and reinnervation (Hechler et al., 2006). Using both models we saw an increase in neurite growth and reinnervation in the presence of IL-13. However, future *in vivo* experiments using axonal tracers, such as biotinylated dextran amine for example, will allow us to investigate further the effect of IL-13 on neurite growth after SCI and the possibility of other cell types involved. To study neuronal death we also used both primary cultures and organotypic brain slices. In **chapter 2** we showed that IL-13 does not influence viability of immature primary neurons, while there is a suggestive protective effect of IL-13 on cell toxicity induced by NMDA. Future experiments will need to determine whether this effect of IL-13 is a direct effect on neuronal populations or mediated by glial cells; our preliminary results using primary hippocampal cultures (data not shown) suggest that the protective effect of IL-13 observed in hippocampal slices is likely mediated by other cell types present in the hippocampal tissue.

In **chapter 4** we studied the effect of the selective ADAM17 inhibitor, BMS-561392, on growth of primary neuronal cultures. We observed a significant reduction of neurite growth when ADAM17 was blocked, while there was an increase in growth in the presence of rADAM17; future studies will need to address the *in vivo* effect of ADAM17 on regeneration of axons after SCI. As for IL-13, we investigated the role of ADAM17 in cellular death using primary cultures, but also in cell lines. Both populations showed consistent results regarding the strong effect of ADAM17 on microglial survival. There was a good correlation between *in vitro* and *in vivo* data in microglial cells; *in vitro*, using immortalized BV-2 cells and primary cultures of microglial cells, as well as *in vivo* after SCI, ADAM17 promoted survival of microglial cells.

5.6 MODULATION OF PHAGOCYTES BY CYTOKINES AND GROWTH FACTORS

The role of microglia/macrophages after CNS injury is still controversial, since both beneficial (Schilling et al., 2005, Shechter et al., 2009) and detrimental effects (David and Kroner, 2011) have been described. Macrophages/microglia are divided into two groups according to their activation phenotype, i.e. M1 (classically activated) or M2 (alternatively activated) (Gordon, 2003). After SCI, the microenvironment of the injured cord favors polarization towards an M1 macrophage/microglia phenotype rather than M2. Cells with an M1 phenotype are known to exert only a moderate axon growth promoting-effect, while M2 macrophage/microglia are able to significantly promote axon growth (Kigerl et al., 2009). There is evidence that microglia can produce growth factors such as insulin-like growth factor (IGF) 1 and 2, brain-derived growth factor (BDNF) and TGF-α, and glial cell line-derived neurotrophic factor (GDNF) in the CNS (Batchelor et al., 2002, Butovsky et al., 2006a, Suh et al., 2013), as well as cytokines (Hanisch, 2002). In a striatum lesion model, it was proposed that macrophages/microglia generate a gradient of BDNF and GDNF towards the wound edges, controlling dopaminergic sprouting after injury (Batchelor et al., 2002).

Under pathological conditions, the relationship between cytokines and growth factors can influence the secretion of other molecules able to modulate the immune environment. In addition, cytokines can regulate the production of growth factors; for, example IL-4 and IL-13 are known to upregulate the production of IGF1 in macrophages/microglia (Wynes and Riches, 2003). Moreover, IL-13 is able to induce proliferation of human bronchial epithelial cells *in vitro,* via redistribution of TGF-α (Booth et al., 2001) in an ADAM17-dependent mechanism (Booth et al., 2007). Intrathecal administration of TGF-α has been shown to improve functional outcome after SCI, by increasing proliferation and infiltration of astrocytes, as well as axonal growth (White et al., 2011). Thus, it is tempting to speculate that *in vivo* application of IL-13 might induce an increase in functional outcome after SCI by stimulating TGF-α production via microglia/macrophages, thus

influencing the proliferation of astrocytes. Future studies will need to clarify the veracity of this hypothesis. On the other hand, a more "pro-inflammatory" environment, induced by LPS injection, suppressed BDNF production in a model of Parkinson's disease *in vivo,* resulting in a prolonged activation of microglial cells and eventually in progressive damage to dopaminergic neurons (Zhou et al., 2012); in contrast, for example, *in vitro* TNF-α treatment had a neuroprotective effect in cultures of primary astrocytes, by increasing GDNF and BDNF production (Saha et al., 2006). Thus, these findings suggest that M2 microglia/macrophage phenotype expressing growth factors, such as BDNF, play an important role in axonal regeneration after CNS injury.

5.7 GENERAL CONCLUSION

The results presented in this thesis offer new insights in the role of IL-13 and ADAM17 after SCI. Nowadays, combining different therapies is thought to be a more promising strategy to promote regeneration after injury, than the delivery of one single molecule. Here we present evidence that IL-13 is a potent inducer of neuronal outgrowth while ADAM17 is a strong survival factor for microglial cells. To our knowledge, no overlapping function exists between IL-13 and ADAM17, which could increase the changes of both molecules to promote functional recovery after SCI independently of each other. There is however one publication which shows that IL-13-induced proliferation and TGF-α shedding of airway epithelial cells is mediated by ADAM17. This phenomenon seems to be mediated by a redistribution of TGF-α to the apical surface (where ADAM17 is constitutively expressed) (Booth et al., 2007), which suggest a possible synergistic effect. It is therefore tempting to speculate that a combined therapy, for example local application of ADAM17 immediately after injury can increase the rate of cell survival after SCI, followed by a later application of IL-13 alone or in combination with ADAM17 at 1 week after injury (Donnelly and Popovich, 2008) to promote recovery of damaged axons after SCI. Another interesting approach might be local application of ADAM17 at different time points. The results

presented in this thesis showed that ADAM17 is a key survival factor for macrophages/microglial cells as well as a promoter of axonal growth for neurons *in vitro*. Both characteristics make ADAM17 a promising candidate to achieve axonal regeneration and functional recovery after SCI.

Summary

SCI is devastating pathology associated with a significant impact on life expectancy and quality, as well as a considerable economic cost. To date there is no cure available; hence there is an urgent need to identify efficient therapies. In order to achieve this, it is important to clearly identify the temporal and spatial role of specific target molecules; it may well be that some molecules play a regenerative role during the acute phase, while a more harmful effect may be observed at later stages, or vice versa.

In this thesis, we firstly examined the potential effects of the "anti-inflammatory" cytokine IL-13 on neuroregenerative processes. We show that IL-13 is a potent promoter of neurite growth and neuronal survival *in vitro* **(chapter 2)**. The data presented in this thesis, together with own *in vivo* data indicating beneficial effects of IL-13 after SCI, make IL-13 a promising candidate to promote regeneration after CNS injuries. However, more studies are needed to determine the optimal time window and dosage for IL-13 application, as well as the possibility of co-application of IL-13 with other molecules.

Secondly, we present evidence that anti-TNF-α therapies have a specific time window of effectiveness. TNF-α mRNA levels do not correlate with TNF-α protein levels, since an increase of TNF-α mRNA expression was observed during the acute phase after SCI., while protein levels were decreased. Interestingly, functional recovery of locomotion skills have been observed using anti-TNF-α drugs during the early acute phase. However, during the early chronic phase, blocking of TNF-α did not influence functional outcome as assayed by the BMS **(chapter 3)**.

Finally, we investigated the role of the metalloproteinase ADAM17 in the pathology of SCI. We are the first to show that ADAM17 displays a cell-dependent effect on survival, with ADAM17 inhibition mainly reducing the viability of microglial cells (*in vitro* and *in vivo*). In addition, ADAM17 was found to be involved in the promotion of neurite growth in cortical neuronal cultures, and importantly, ADAM17 inhibition decreased functional outcome after SCI **(chapter 4)**. These promising results strongly suggest a therapeutic potential for ADAM17 as a modulator of microglial survival and activation to promote regeneration after CNS injury.

Together, our findings justify further studies on the therapeutic potential of both IL-13 and ADAM17 in the treatment of CNS injuries.

Nederlandse samenvatting

Ruggenmergschade is een ernstige aandoening, die wordt geassocieerd met een belangrijke impact op de levensverwachting en de kwaliteit van leven, en die vanuit economisch oogpunt nadelig is. Tot op heden is er geen therapie beschikbaar; er is dus dringend nood aan de ontwikkeling van efficiënte behandelingen. Hiertoe is het belangrijk om de exacte rol van specifieke targetmoleculen te bepalen in de tijd en ruimte, aangezien de mogelijkheid bestaat dat bepaalde moleculen een meer regeneratieve rol spelen in de acute fase, terwijl deze in een latere fase schadelijk zijn, of omgekeerd.

In deze doctoraatsthesis werd als eerste het effect van het anti-inflammatoire cytokine IL-13 onderzocht op processen betrokken bij neuroregeneratie. We tonen voor het eerst dat IL-13 een sterke stimulator is van neurietgroei en neuronale overleving *in vitro* (**hoofdstuk 2**). De data voorgesteld in deze thesis, tezamen met data van onze onderzoeksgroep die aantonen dat IL-13 bevorderlijk is voor herstel na SCI *in vivo*, suggereren dat IL-13 een veelbelovend kandidaat-molecule is ter bevordering van regeneratie na schade aan het centraal zenuwstelsel (CZS). Echter, uitgebreid onderzoek is essentieel om het optimale tijdsvenster en de juiste dosering voor het toedienen van IL-13 te bepalen, alsook de mogelijkheid voor het toedienen van IL-13 in combinatie met andere factoren.

In een tweede luik hebben we aangetoond dat anti-TNF-α therapieën een specifiek tijdsafhankelijk effect vertonen in de context van ruggenmergschade. Bovendien correleren TNF-α mRNA niveaus niet met TNF-α eiwit niveaus, aangezien een toename in TNF-α mRNA niveaus werd waargenomen in de acute fase na SCI, maar TNF-α eiwit niveaus daarentegen daalden. Eerder werd wel functioneel herstel van motorische vaardigheden gezien bij gebruik van anti-TNF-α middelen tijdens de acute fase. Echter, onze data tonen aan dat blokkeren van TNF-α in de vroege chronische fase geen effect heeft op functioneel herstel zoals bepaald werd met de BMS score (**hoofdstuk 3**).

Tenslotte werd onderzocht of het metalloproteinase ADAM17 een rol speelt in de pathologie na ruggenmergschade. We zijn de eerste om aan te tonen dat ADAM17 een cel-specifiek effect op overleving vertoont, waarbij inhibitie van ADAM17 voornamelijk de viabiliteit van microglia verlaagt (*in vitro* en *in*

vivo). Daarnaast blijkt ADAM17 ook belangrijk voor het bevorderen van neurietgroei in corticale neuronale culturen, en belemmerde de inhibitie van ADAM17 het *in vivo* functioneel herstel na ruggenmergschade (**hoofdstuk 4**). Deze veelbelovende resultaten duiden sterk op het therapeutisch potentieel van ADAM17 als modulator van de viabiliteit en activatie van microglia ter bevordering van regeneratie na schade aan het CZS.

Samengevat rechtvaardigen onze bevindingen verder onderzoek naar het therapeutisch potentieel van zowel IL-13 als ADAM17 voor de behandeling van CZS schade.

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Curriculum vitae

Curriculum vitae

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Overview of scientific activities

Publications:

Vidal P.M., Lemmens E., Avila A., Vangansewinkel T., Chalaris A., Rose-John S., Hendrix S. ADAM17 is a survival factor of microglial cells after spinal cord injury in mice. Submitted.

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Vidal P.M., Lemmens E., Dooley D., Hendrix S. The role of "anti-inflammatory" cytokines in axon regeneration. Cytokines Growth Factor Rev, 2013; 24(1):1-12. Epub 2012.

Vidal P.M., Lemmens E., Geboes L., Vangansewinkel T., Hendrix S. Late blocking of peripheral TNF-α is ineffective after spinal cord injury in mice. Immunobiology, 2013 Feb; 218(2):281-4. Epub 2012.

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Hendrix S., Nelissen S., Lemmens E., Vangansewinkel T., **Vidal P.M.**, Willems L., Boato F., Dooley D., Pejler G., Maurer M., Metz M. Mast cell protect from post-traumatic spinal cord inflammation in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4. Immunology, 2012; 137:548-549.

Lemmens E., **Vidal P.M.**, Nelissen S., Vangansewinkel T., Hendrix S. Interleukin-13 stimulates neurite outgrowth *in vitro* in primary neurons and organotypic brain slices while it worsens clinical outcome after spinal cord injury *in vivo*. Glia, 2011; 59:S91.

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Oral presentation:

Vidal P.M., Lemmens E., Boato F., Nelissen S., Vangansewinkel T., Hendrix S. IL-13 is a promoter of neurite outgrowth in primary neurons and organotypic brain slices. 14th Euron PhD days, October 7–8, 2010, Hasselt, Belgium.

Selected scientific meetings:

Vidal P.M., Lemmens E., Avila A., Vangansewinkel T., Hendrix S. ADAM17 inhibition increases microglia death after spinal cord injury in a MAPK dependent manner. PhD symposium: "Cell-based therapies in cental nervous system pathology", April 12th, 2013, Hasselt. Belgium.

Vidal P.M., Lemmens E., Avila A., Vangansewinkel T., Hendrix S. Inhibition of ADAM17 increases microglial death after spinal cord injury. 5th Annual meeting of the Society for Neuroscience, October 13-17, 2012, New Orleans, US.

Avila A., **Vidal P.M.**, Nguyen L., Rigo J. Glycine receptor activation influences interneuron cell migration during early development of the cerebral cortex. 5th Annual meeting of the Society for Neuroscience, October 13-17, 2012, New Orleans, US.

Vidal P.M., Lemmens E., Vangansewinkel T., Hendrix S. ADAM17 inhibition increases oligodendrocytes death after spinal cord injury. 8th FENS forum of Neuroscience, July 14-18, 2012, Barcelona, Spain.

Nelissen S., Geboes L., Lemmens E., Vangansewinkel T., **Vidal P.M.**, Willems L., Boato F., Dooley D., Pejler G., Maurer M., Metz M., Hendrix S. Mast cells protect from post-traumatic spinal cord inflammation in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4. 8th FENS forum of Neuroscience, July 14-18, 2012, Barcelona, Spain.

Nelissen S., Lemmens E., Vangansewinkel T., **Vidal P.M.**, Willems L., Boato F., Dooley D., Pejler G., Maurer M., Metz M., Hendrix S. Mast cells protect from post-traumatic spinal cord inflammation in mice by degrading inflammation-associated cytokines via mouse mast cell protease-4. PhD symposium "Cytokines and cell trafficking in immunological disorders", February 9th, 2012, Hasselt, Belgium.

Avila A., **Vidal P.M.**, Nguyen L., Rigo JM. The Glycine receptor is expressed and functionally active in migratory interneurons and influences early cortical development. 4th Annual meeting of the Society for Neuroscience, November 12-16, 2011, Washington DC, US.

Lemmens E., **Vidal P.M.**, Nelissen S., Vangansewinkel T., Hendrix S. Interleukin-13 stimulates neurite outgrowth in vitro in primary neurons and organotypic brain slices while it worsens clinical outcome after spinal cord injury in vivo. 10th European meeting on Glial cells in Health and disease, September 13-17, 2011, Prague, Czech Repulic.

Avila A., **Vidal P.M.**, Nguyen L., Rigo JM. Glycine receptor activation influences early cortical development. 8th IBRO world congress of Neuroscience, July 14-18, 2011, Florence, Italy.

Vidal P.M., Nelissen S., Geboes L., Lemmens E., Hendrix S. Late phase TNF-α treatment does not affect functional outcome after spinal cord injury. 8th IBRO world congress of Neuroscience, July 14-18, 2011, Florence, Italy.

Vidal P.M., Lemmens E., Boato F., Nelissen S., Vangansewinkel T., Hendrix S. IL-13 stimulates neurite outgrowth in primary neurons and organotypic brain slices. 10th international congress of Neuroimmunology, October 26–30, 2010, Sitges, Spain.

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