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List of abbreviations

AR	adrenoceptor
Arg-1	arginase-1
BBB	blood-brain barrier
bFGF	basic fibroblast growth factor
BMCs	bone marrow cells
BMS	Basso Mouse Scale
BSCB	blood-spinal cord barrier
Ca	calcium
CAM	chorioallantoic membrane
CD	cluster of differentiation
CNS	central nervous system
CPA	carboxypeptidase A
CSPG	chondroitin sulphate proteoglycans
CST	corticospinal tract
Ctrl	control
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
Dpi	days post injury
E	epinephrine
EAE	experimental autoimmune encephalomyelitis
EC	endothelial cell
ECM	extracellular matrix
EPC	endothelial progenitor cell
EPSPs	excitatory postsynaptic potentials
Erk1/2	extracellular signal-regulated kinases 1 and 2
FCS	fetal calf serum
GFAP	glial fibrillary acidic protein
HBSS	Hank’s balanced salt solution
HPA	hypothalamic-pituitary-adrenal axis
Iba-1	ionized calcium binding adaptor molecule-1
IFN-y	interferon-y

IL	interleukin
IL13R	interleukin 13 receptor
iNOS	inducible nitric oxide synthase
ISO	isoproterenol
JAK	janus kinase
LCM	L929 conditioned medium
LPS	lipopolysaccharide
LVv	lentiviral vector
LY	LY294002 Akt inhibitor
Mϕ	macrophage
MAPK	mitogen-activated protein kinases
MBP	myelin basic protein
MC	mast cell
MCP	mast cell protease
MCP-1	monocyte chemoattractant protein-1
MHCII	major histocompatibility complex II
mMCP4	mouse mast cell protease4
mMCP6	mMCP6 mouse mast cell protease6
MMP	matrix metalloproteinase
MSC	mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGM	normal growth medium
NGS	normal goat serum
NE	norepinephrine
NF	neurofilament
NO	nitric oxide
PBS	phosphate buffered saline
PC	pericytes
PECAM-1	platelet endothelial cell adhesion molecule-1
PFA	paraformaldehyde
PICs	persistent inward currents
PI3Ks	phosphoinositide 3-kinases
P/S	penicillin/streptomycin
rIL-13	recombinant interleukin-13

ROI	region of interest
ROS	reactive oxygen species
SEM	standard error of the mean
SMC	smooth muscle cell
SNS	sympathic nervous system
STAT6	signal transducer and activator of transcription 6
TBI	traumatic brain injury
TGF-β	transforming growth factor-β
Th1	type 1 T helper cell
Th2	type 1 T helper cell
Tik2	tyrosine kinase 2
TNF-α	tumor necrosis factor-α
TLR	toll like receptor
U	U0126 ERK inhibitor
VEGF	vascular endothelial growth factor
VP	viral particles

Chapter 1

General introduction & aims

Chapter 1. Introduction and Aims

1.1 Spinal cord injury

Spinal cord injury (SCI) and the resulting paralysis are critical unsolved problems worldwide. It can result from contusion, compression, penetration, or maceration of the spinal cord. Most SCIs are caused by motor vehicle or workplace accidents (1). Being paralyzed by an SCI has many economic and social issues, as immobility significantly reduces the quality of life in all aspects (1-3). The neurological deficits following traumatic SCI are severe and often permanent due to the loss of ascending and descending axonal pathways, demyelination, and the lack of substantial axonal regeneration and plasticity. Following the primary mechanical insult, a cascade of cellular and molecular events occurs over the course of weeks causing destruction of initially spared spinal cord tissue (4).

The primary mechanical injury results already in extensive neuronal cell death and transsected axons. The secondary injury involves the initiation of an inflammatory response, leading to the infiltration of immune cells into the lesion area. Key players in this inflammatory reaction are microglia/macrophages, astrocytes and T cells. Other underlying mechanisms include the formation of free radicals, inflammation-mediated cell death, demyelination of surviving axons, glutamatergic excitotoxicity and ischemia (5, 6). These processes evoke additional tissue damage and limit the functional recovery after SCI (7). Later on, in the early chronic phase after SCI, a dense glial and fibrotic scar is formed by astrocytes that surround the central cavitation. Although scarring is regarded as advantageous in the acute phase, in a later stadium it will block axons to regenerate (8). This is because the scar tissue is rich in axonal growth-inhibitory molecules like chondroitin sulphate proteoglycans (CSPGs), which are released by hypertrophic astrocytes. Other inhibitors of axon regeneration are associated with myelin and extracellular matrix (ECM) compounds (9) (**figure 1.1**).

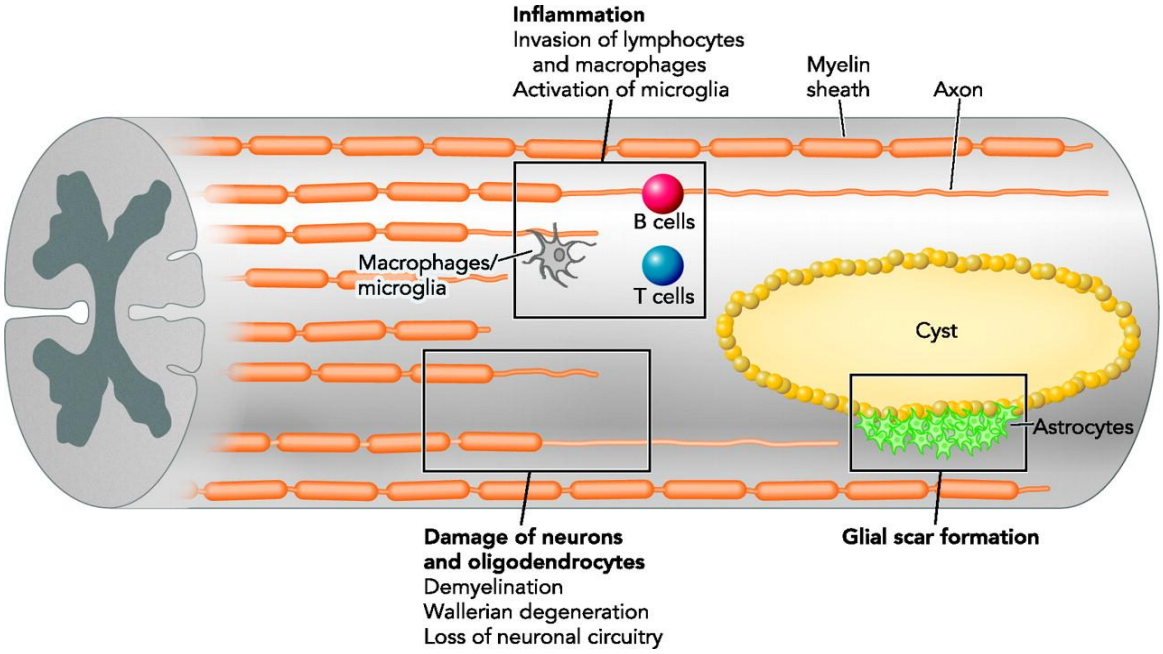

Figure 1.1: Schematic representation of the pathophysiology of spinal cord injury. The processes associated with SCI include a primary mechanical injury as well as a secondary injury. The primary mechanical insult results in neuronal and oligodendric cell death and damaged axons. The secondary injury cascade further aggravates the cellular damage. It is characterized by an inflammatory response, leading to the infiltration and activation of immune cells. Later on, a glial scar is formed with reactive astrocytes as major components. Figure adapted from (10)**.**

To complicate the pathology even further, the stress systems (e.g. hypothalamus-pituitary-adrenal axis and the sympathetic nervous system) will respond to SCI by hormonal and metabolic changes with direct and indirect effects on the inflammatory system. As described above, the inflammatory response in turn plays a major part in the increase of the secondary injury and the loss of function. Hence, the activation of the stress systems has a major influence on functional recovery after SCI.

Another important system that is affected, is the vasculature. After SCI, endogenous revascularization of the spinal cord is initiated. However, the new vessels are not beneficial, as they display morphological and functional anomalies. The newly formed capillaries have an unstable and leaky phenotype because they lack proper association with support cells like pericytes and astrocytes. Due to this instability, the neovessels will eventually degenerate two weeks after the initial trauma (11, 12). Nevertheless, adequate replacement of the damaged microvasculature should be promoted, since the nutrients and trophic factors required for neural regeneration can only be delivered to the site of injury when a functional vascular network is present.

Despite extensive research, there are currently no effective therapies available for SCI (13). Today, the treatment exists out of surgical interventions, that include spinal decompression and stabilization (14). Neuroprotective approaches are also applied and focus on preventing further progression of the secondary injury (e.g. anti-inflammatory drugs like methylprednisolone), whereas neuroregenerative treatments lay emphasis on repairing the broken neuronal circuitry of the spinal cord (15, 16), although with only minor effects. Rehabilitative training remains the standard method of therapeutic intervention in most countries to maximize functional recovery (17). Hence, further research is necessary to improve the effects of the current strategies and to invent new effective therapies.

Different experimental strategies can be followed to tackle the described secondary processes in order to stimulate recovery after SCI. A first strategy is to decrease the secondary damage caused by excitotoxicity which is due to glutamate release from damaged axons, free radicals, hemorrhage within the spinal column, inflammation, and apoptosis. This may be achieved using glutamate receptor antagonists to block excitotoxicity or high doses of the immunosuppressive drug methylprednisolone, although their efficacy is somewhat debatable due to massive side effects (17, 18). For example, short term high dose methylprednisolone has massive side effects like gastric bleeding and it can cause wound infection (19).

Secondly, stimulating axon regeneration is an important challenge in SCI research. Axon regrowth can be induced in several ways, including surgical reconnection of the lesioned spinal cord, targeting inhibitory factors that block axon formation (e.g. CSPGs and ECM components), or transplanting growth-promoting factors or stem cells that can stimulate regeneration (17, 18).

Thirdly, locomotion and autonomous functions may be improved by modulating neural network activity within the spinal cord. Following SCI, neural circuits for locomotion and autonomous functions are not lost below the lesion site, but lack descending signals from the brain. Pharmacological or electrical stimulation of selected pathways within the spinal cord may enhance such signals, thereby improving locomotion (18). Descending inputs to neural networks within the spinal cord include glutamatergic, noradrenergic, dopaminergic, and serotonergic pathways (20). These pathways could be pharmacologically manipulated in order to restore lacking descending signals from the brain. In addition, sensory inputs via dorsal root ganglions expressing receptors for ɣ-aminobuteryc acid (GABA) and glutamate could be modulated as they are responsible to convey peripheral signals to the spinal cord (21). However, there is little consensus on pharmacological strategies because individual drugs have both beneficial and deleterious effects in different studies (18).

The secondary injury is characterized by the influx of immune cells into the injured CNS. However, the effects of the immune cells are complex and even critical to repair. In the acute phase, macrophages, for instance, can exacerbate secondary injury by causing axonal dieback, but in the chronic phase, they can promote repair by clearing debris and by promoting remyelination, depending on the microenvironment and their activation state. Therefore, targeting immune-mediated secondary injury after the primary injury offers a therapeutic opportunity to treat traumatic SCI (22).

With these multifactorial processes in mind, it is worth exploring different mechanisms that can increase functional regeneration. In this thesis, we have focused on the neurotoxic, pro-inflammatory and regeneration-inhibitory spinal cord micro-environment to improve functional recovery after SCI. The neurotoxic environment has been targeted via adrenergic pathway modulation and stimulation of angiogenesis. The pro-inflammatory spinal cord micro-environment has been addressed via immunomodulation of macrophages. Lastly, we have focused on reversing the inhibition of regeneration via mouse mast cell protease 4.

1.1.1 SCI mouse models and assessment of functional recovery

1.1.1.1 The SCI hemisection mouse model

SCI animal models are indispensable to investigate the efficacy of therapeutic interventions and to reveal the underlying mechanisms. Mouse models of SCI offer the advantage of generating genetic mutations to identify genetic mechanisms which promote or prevent behavioral recovery.

Different SCI models exist and the choice of the model should depend on the study goals. An overview of the different SCI models based on the mechanism of injury, with their strengths and weaknesses, has been provided by the review of Cheriyan *et al* (23). Briefly, there exist contusion, compression, distraction, dislocation, transection or chemical SCI models. To create a contusion SCI model a transient force (e.g. weight-drop, electromagnetic force or air pressure) is applied to damage the spinal cord. Compression models are made by compression (e.g. with a forceps) of the spinal cord over an extended period of time. To obtain distraction models opposing traction forces are applied to stretch the spinal cord, whereas dislocation models are characterized by lateral displacement of vertebra. For chemical mediated SCI models, a chemical is applied to the spinal cord to induce injury. Finaly, transection involves partial (= hemisection) or complete severing of the spinal cord at a particular level.

In our research we use a hemisection SCI mouse model. This specific model is characterized by transection of the left and right dorsal funiculus, the dorsal horns and additionally the ventral funiculus (=T-cut) in order to completely transect the dorsomedial and ventral corticospinal tract (CST) in addition to several other descending and ascending tracts like the rubrospinal tract (24). Reticulospinal and vestibulospinal pathways in the ventral white matter and the ventral horn are spared, in addition to the spinothalamic tract in the lateral column, so rough touch, pain and temperature sensitivity are preserved (25). The model results into hindlimb paralysis and impaired bladder function, which makes it important to express the bladders daily for the first 2 weeks after which reflex bladder emptying gradually returns. Hindlimb locomotor function recovers over time to the point that animals can bear weight with their hindlimbs.

It is important to note that every animal model has its advantages and disadvantages. Given the complexity of human SCI, no animal model can cover all aspects of the injury. Therefore, all the results obtained during fundamental or pre-clinical research need verification in clinical studies.

One of the advantages of the hemisection model is that the injury creates a more-defined lesion and allows distinguishing between regenerated (newly formed) and spared fibers (26). Secondly, a hemisection or partial transection is more likely to be seen clinically compared to complete transection (23) and allows us to examine locomotor function. Given that hemisection results in a less severe injury than complete transection, postoperative animal care is also easier. In addition, the dorsal hemisection lesions typically fill in with a connective tissue matrix (25), which makes it a suitable model to study the scarring response after SCI as well.

One of the caveats of the hemisection model is its clinical relevance. The most common type of SCI in humans involves compressive impact (27). Therefore, for studies of neuroprotection and recovery, contusion injuries have become the golden standard. However, contusion models cannot by used for studies of axon regeneration.

Although contusions damage long ascending and descending tracts, there can be variable axon sparing, whereas surgical cuts allow tracts of interest to be targeted precisely (25).

Taken together, the T-cut hemisection mouse model has been chosen because the focus of our research group is axon regeneration, more specifically regeneration of the CST which is an import motor tract in humans (26).

1.1.1.2 The Basso Mouse Scale to assess functional recovery

Different outcome measures can be used to measure functional recovery after SCI. The grid walk test, beam walk test, Cat walk test or Digigait are all tests which measure coordination, paw placement and paw pressure. The Cat walk test or Digigait digitally record the parameters which has the advantage of less bias (28). However, during the first two weeks after SCI we cannot use these tests as they require that the mice can place their paws plantar. The Rotarod performance test is an additional test which can measure motor coordination. In this test the mice are placed on an accelerated rolling rod and the latency to jump/fall off the rod is automatically recorded by the action of the mouse dropping onto a trigger plate (29). This test is also digitalized. However, forepaw strength can bias the results.

Here, we have used the Basso Mouse Scale (BMS) to assess functional recovery. The BMS as a sensitive, valid and reliable measure of locomotor recovery specific for mice (30). It is 10-point scale going from 0 (= no ankle movement) towards a score of 9 (= normal locomotion), in which mice are scored by two investigators who are unaware of the experimental groups based on hind-limb movements made in an open field during a 4-minute interval. Locomotion in normal healthy mice, which have a BMS score of 9, is characterized by consistent weight supported stepping, a steady trunk and tail, and an easily recognizable pattern of forelimb and hindlimb movement (= coordination) (30). A step of the forelimb coincides with a step of the contralateral hindlimb in a reproducible pattern. Mice typically demonstrate up to three phases of locomotor recovery depending on the severity of the injury (30).

The early phase of recovery consists of the resolution of paralysis and/or paresis and goes from slight ankle movement to larger ankle movements before plantar placing occurres (BMS scores 1-2). Ankle movements are classified as slight or extensive based on whether the joint moved through less or more than half of the range of excursion.

The intermediate phase consists of recovery to plantar placing and the development of stepping (BMS scores 3-4). Plantar placement requires that the all digits of the paw are in contact with the ground. Weight support during plantar placement is scored when the hindquarters elevate. Stepping is defined as weight supported stance followed by forward limb advancement and then re-establishment of weight support. Dorsal stepping occurs when the dorsal rather than the plantar surface of the paw provides weight support. Stepping frequency as then catogarized as none, occasional (≤50%), frequent (>50%), or consistent (all of the time or fewer than five missed or dorsal steps).

In the late phase of recovery, the fine details of locomotion can be defined. Important parameters are coordination, paw position during stance, and the extent of trunk stability. These parameters can only be assessed in mice that demonstrate frequent or consistent stepping (BMS scores>4). Paw position is determined by assessing whether the middle digits of the hindpaw are parallel to the long axis of the body. Severe trunk instability is defined by postural deficits (e.g. extreme lean, waddlen collapse of the hindquarters) or by haunch hit, spasms or scoliosis. If less then 5 of these events occure the trunk instability is defined as mild. Normal trunk stability indicates that non of these events is present.

The output of the BMS is a semi-quantitative scale making statistical comparisons possible. We have based our statistical analyses on the argumentation proposed by Basso *et al* in the original paper on the BMS.

In the original development of the BMS, parametric statistics were used to analyze the BMS data, despite that they are ordinal and the magnitude of behavioral change between ranks may not be consistent. A generally exepted non-parametric test for repeated measures does not exist and the application of parametric statistics to ordinal data is valid if evidence indicates that each successive rank reflects a true increase in behavioral performance (30). Evidence of increasing behavioral performance across BMS ranks is apparent in the progression of high, intermediate and low performers through each category and the monotonic, ascending recovery curves for all strains and injury severities examined in this study (30). Therefore, parametric statistics are a reasonable option for analysis. Basso *et al* also make clear that it is important that the interpretation of BMS results is placed in a murine biological context rather than strictly according to the degree of numerical change. For instance, a treatment that changes the final BMS score from 3 to 4, although numerically small, would be biologically significant, reflecting an improvement from no stepping to stepping. Contrarely, a similar one-point increase from 1 to 2 may have less biological relevance.

Although the BMS has been developed for contusion injury, it can also be used with hemisection injury. The hemisection injury represents a more-severe injury in general meaning that the control groups without treatment or vehicle treatment can achieve a maximum score of 4 (31-34), whereas after contusion injury the control mice can obtain a score of 6 (30).

1.2 The role of stress pathways in functional regeneration after SCI

Stress is inevitable after SCI. The initial impact, the post-operative damage and the reduction in function are important stress factors amongst others. Stress in humans affects catecholamine (norepinephrine) and cortisol levels, major mediators of the two canonical pathways in a stress response, namely the systemic/adrenomedullary sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. In addition, there exists a complex brain-immune system interaction. The cortisol and (nor)epinephrine (NE and E) secreted by the HPA axis or the SNS are released into the peripheral blood by which they have excess to innate and adaptive immune cells. These will in turn modulate their secretion of cytokines and growth factors, that again may influence the stress pathways with a positive feedback loop.

The alpha (α)-adrenergic system (part of the SNS activated be NE and E) is one of the spinal systems that is activated after SCI. It can counteract pain-stimulating processes amongst others. However, after SCI, this system is damaged and therefore not functioning properly (35). Next to its involvement in locomotion and pain, the α-adrenergic pathway stabilizes reflex activation and spasms after SCI, both at the neural and the muscular level. More specifically, it regulates motor-neuron excitability, sensory synaptic transmission and muscle spasms (36-39). As described in **Chapter 2**, adrenoceptors (ARs; α-ARs and beta (β)-ARs) are valuable pharmacological targets for the treatment of CNS trauma.

Modulation of the α-ARs may improve locomotion directly by facilitating the transmission of descending motor and sensory inputs to neurons caudal to the lesion, or indirectly via immunomodulation to reduce the pro-inflammatory response after trauma in the CNS. It is important to note that this immunomodulation must be accompanied by other regenerative processes like neurite outgrowth, angiogenesis, synapse formation, degradation of the scar tissue, etc to improve functional recovery.

Immunomodulation and neuromodulation go hand in hand when attempting to activate and stimulate these processes.

Another branch of the adrenergic system, next to the α-AR pathway, is the β-AR pathway. For β2-AR agonists such as isoproterenol (ISO), salmeterol and clenbuterol, it has already been shown that they have neuroprotective effects and that they improve the neurological and functional outcome (40-44). The effects of β1-AR agonism in the context of SCI were not investigated before. Studies using rat and mouse transection SCI models show no effects on locomotion recovery by using a β2-AR agonist alone, although it was often a necessary co-factor to prevent muscle atrophy for example (45, 46). On the other hand, Zeman and colleagues thoroughly investigated the effects of β2-AR agonism after SCI in a rat contusion SCI model. They show that clenbuterol (=β2-AR agonist) improves locomotion and reduces tissue loss, compared to vehicle control. They suggest that activation of β2-ARs during the acute phase of injury stimulates glutathione-dependent antioxidative processes, that lead to reduced oxidative damage. However, further studies are needed to determine the affected cell types (47). All together, these data indicate the importance of β2-AR modulation for repair after CNS trauma.

Next to their neuroprotective effects, the relevance of β2-AR agonism to stimulate angiogenesis was recently confirmed as the expression of the receptors on endothelial progenitor cells (EPCs) and mature ECs coincides with increased proliferation and migration after stimulation with the non-specific β-AR agonist isoproterenol (48). The β2-ARs are most abundantly expressed on the vasculature and modulate the release of nitric oxide (NO), which in turn causes vasodilation (49). Damage to the vasculature and breakdown of the blood-brain barrier (BBB) are universal consequences after SCI (see **1.3 Angiogenesis after SCI**). Most importantly, the unfavorable regulation of pro-angiogenic and counterregulatory anti-angiogenic factors after CNS trauma is suspected to participate in the failure of revascularization and vessel stabilization (43). Vascular protection and revascularization are key to support survival of sprouting and regenerating axons (44, 50).

Summarized, these findings emphasize the important contribution of the sympathetic nervous system in CNS disorders and fuel the search for therapeutic strategies to modulate this system.

1.3 Angiogenesis after SCI

Within the human body, three mechanisms of blood vessel formation can be distinguished: vasculogenesis, angiogenesis and arteriogenesis. During embryonic development, a primitive vascular network is formed through vasculogenesis, i.e. de novo formation of blood vessels via the differentiation of hemangioblasts into ECs. This immature network is then expanded and remodeled into a more mature and functional vascular network via the process of angiogenesis. During angiogenesis, new blood vessels arise through the branching or elongation of pre-existing vessels. Finally, arteriogenesis comprises the increase of the luminal diameter of existing arteries by augmenting blood flow (51, 52).

Angiogenesis is not only a fundamental event during embryogenesis but also during adult life, as it is the main mode of blood vessel formation in processes like wound healing and ovulation (13). This multistep process is initiated by vasodilation of a pre-existing vessel. Next, mural cells such as pericytes (PCs) and smooth muscle cells (SMCs) detach, and the surrounding extracellular matrix (ECM) is degraded by proteases like matrix metalloproteinases (MMPs). As a consequence of this vessel destabilization, ECs begin to proliferate and migrate into the remodeled perivascular space. They assemble into a solid tube, which elongates until an adjacent vessel is encountered. The fusion or anastomosis of both vessels creates a continuous lumen, which is necessary to allow blood flow. Final vessel maturation is achieved through the deposition of a basement membrane and the recruitment of PCs and SMCs (51, 53). Within the healthy body, angiogenesis is controlled by a normal physiological balance between pro-angiogenic (e.g. vascular endothelial growth factor, VEGF; basic fibroblast growth factor, bFGF) and anti-angiogenic (e.g. angiostatin, endostatin) factors.

In general, the effect of the inhibitors is dominant over the stimulators and angiogenesis does not occur. However, during certain conditions such as wound healing and cancer, pro-angiogenic factors are produced in excess and the balance is shifted towards blood vessel growth (54).

After SCI , the vasculature and the blood-spinal cord barrier BSCB, the functional equivalent of the blood-brain barrier (BBB), are damaged. In the perilesional area, blood vessels surrounding the injury epicenter are typically damaged by shear stress. This causes ruptured and leaking blood capillaries, leading to hemorrhage and hyperpermeability of the BSCB, respectively. These processes induce further tissue loss by allowing the entrance of inflammatory cells and toxic molecules that exacerbate cell death (55, 56). Endogenous revascularization appears to be insufficient since newly formed vessels display morphological and functional abnormalities, further leading to vascular dysfunction, edema and death of neural cells (57, 58).

Preliminary data from our laboratory (generated in collaboration with the group of Prof. Dr. U. Himmelreich, KU Leuven) show that SCI results in a dramatic change in tissue perfusion and diffusion which is caused by the strong reduction in local blood supply and the resulting occurrence of cytotoxic edema both proximal and distal from the lesion site. Interventions that aim to promote regeneration of damaged axons or to rescue neural cells from apoptotic cell death might be negatively influenced by this lack of vascular supply, as all axons are in need of oxygen and nutrients, especially those that are in a regenerative state. In this context, it has been shown that stimulation of the pro-angiogenic AT2-pathway in a mouse SCI model promotes axonal plasticity and neuroprotection and thereby improved functional recovery (59). The unfavorable regulation of angiogenic and counterregulatory anti-angiogenic factors during the complicated course of vessel remodeling after SCI is suspected to participate in the failure of revascularization and vessel stabilization (43). Vascular protection and revascularization are, however, very important to support survival of sprouting and regenerating axons (60, 61). In addition, unsuccessful endogenous angiogenesis might limit the efficacy of other repair approaches.

For example, combinatorial treatment of stem cells and angiogenic factors such as VEGF are needed to enhance tissue sparing (and thus decreased cell death) after SCI (62).

β-ARs play a critical role in the modulation of CNS immunity (63) as well as in vascular tone regulation and neo-angiogenesis (64). So far, their role in the regeneration of a functional vascular system after CNS trauma has not been investigated. However, there is suggestive evidence that β-AR signaling may be a promising pharmacological target in the context of CNS trauma. Therefore, we have investigated the effects of the non-specific β-AR-blocker propranolol on functional recovery after SCI (**Chapter 3**). In addion, propranolol inhibits tubulogenesis of human brain endothelial cells (65). Furthermore, the expression of β2-ARs on different cell types in the CNS highlights that they may be interesting targets. Since massive microglia/macrophage infiltration is a hallmark of CNS trauma, it is of particular interest that the β2-AR agonists zilpaterol and clenbuterol induce the release of VEGF by macrophages, which can contribute to the stimulation of angiogenesis after SCI (66). β2-AR are also expressed on endothelial progenitor cells (EPCs), which are present in the systemic circulation. β2-AR stimulation results in EPC proliferation, migration, and differentiation, thereby enhancing neoangiogenesis and leading to an improved outcome in animal models of hindlimb ischemia (48).

These data indicate that β2-AR agonists are promising pharmacological tools to promote angiogenic sprouting after CNS injury. Therefore, we have investigated whether β2-AR agonists promote functional revascularization after SCI by stimulating angiogenesis (**Chapter 4**).

1.4 The macrophage phenotype after SCI

1.4.1 Macrophage response after SCI

SCI evokes an inflammatory response inside the lesion which aggravates the injury by causing additional tissue damage and neurodegeneration (67). Macrophages derived from both infiltrating monocytes and tissue resident microglia accumulate rapidly after SCI within the epicenter of the lesion. Hence, the role of these macrophages during SCI pathology has raised substantial interest among SCI researchers (68, 69).

Macrophages display a broad plasticity and can adapt their phenotype and functions depending on which type of stimuli they are exposed to. Consequently, the local microenvironment after SCI consists of different macrophage populations. Depending on the activation status and phenotype, macrophages may not only initiate the secondary injury cascades, but they also contribute to tissue repair. The two primary macrophage subsets are the classically activated M1 and alternatively activated M2 macrophages, which are considered as pro-inflammatory and anti-inflammatory, respectively (70, 71) (**figure 1.2**). Although this macrophage phenotype classification is broadly implemented, the dichotomy of M1/M2 is too simplistic. Many intermediate macrophage phenotypes exist. Depending on the stimulus, the consequent factors they secrete and the markers they express, they are classified as M2a, M2b and M2c macrophages amongst many others (69, 71) (**figure 1.2**). Despite the limitations of this linear classification, this concept still provides a useful framework to further define the role of macrophages during post-traumatic SCI. Lipopolysaccharide (LPS) and type 1 T helper cell (Th1) derived cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) promote macrophage differentiation towards an M1 phenotype. These cells secrete high levels of pro-inflammatory cytokines such as IFN-γ, TNF-α, interleukin (IL)-6, IL-23 and other toxic mediators such as reactive oxygen species (ROS) (e.g. nitric oxide (NO) and superoxide). These are crucial for host defense but also cause toxic damage to vital tissue.

Moreover, these cells contribute to axonal retraction and the formation of a growth-inhibitory glial scar. This creates a hostile environment at the lesion site, thereby suppressing the ability of axons to regenerate (69). Contrarily, type 2 T helper cell (Th2) derived cytokines like interleukin-13 (IL-13) polarize macrophages towards the alternative M2 phenotype. These M2s balance the pro-inflammatory microenvironment formed by M1 macrophages by producing anti-inflammatory cytokines such as transforming growth factor-β (TGF-β), IL-10 and IL-4 etc. Furthermore, they produce growth factors, promote angiogenesis, provide neuro/axonal trophic support and degrade the inhibitory glial scar components, resulting in the stimulation of long distance axon regeneration. Therefore, these M2 macrophages are able to modulate beneficially the harmful microenvironment associated with the secondary injury process of SCI (67, 69).

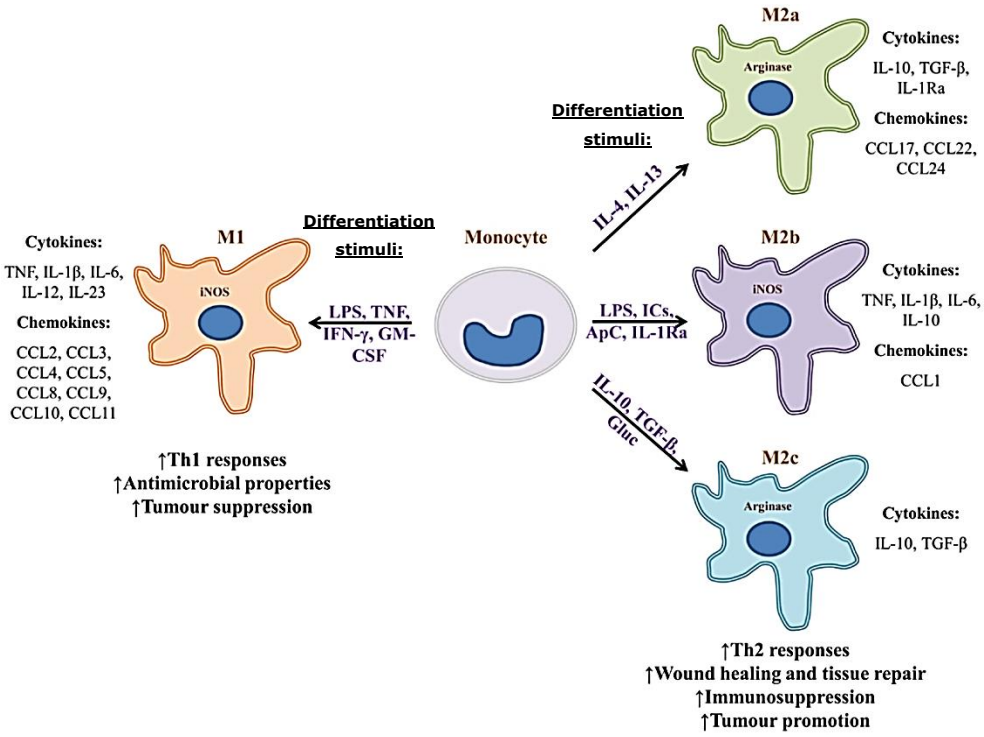

Figure 1.2: Schematic representation of the different macrophage phenotypes. In concert with other mediators, cytokines drive the fate of macrophages into a spectrum of inflammation-promoting "classically activated (M1)" to anti-inflammatory or "alternatively activated (M2)" macrophages. Stimuli, that act on monocytes to differentiate, can range from microbial substances to biochemical signals provided by the microenvironment of a given tissue. Macrophage subtypes release a different array of cytokines and chemokines that can either promote inflammation and sometimes tissue destruction, or wound healing and tissue repair. It is important to note that this macrophage scheme is too simplistic to represent the broad spectrum of macrophage phenotypes which are in addition reversible. Adapted from (71).

Although both M1 and M2 macrophages coexist during the first week after SCI in the lesion epicenter, only M1 macrophages persist at the lesion site until day 28. Recently, Kigerl *et al.* showed a rapid and prolonged M1 macrophage onset after SCI, which overwhelms the limited and transitory M2 macrophage response. Since M1 macrophages are neurotoxic and M2 macrophages rather induce axonal regeneration, this high M1:M2 ratio evokes major detrimental implications for the CNS repair after SCI (72). Therefore, reducing the M1 while increasing the M2 macrophage population during SCI might be an interesting new treatment approach to improve neuroregeneration (**Chapter 5**).

1.4.2 Immunomodulation by interleukin-13

Interleukin-13 (IL-13), a cytokine closely related to IL-4, is a canonical anti-inflammatory cytokine, which in some contexts can also act in a pro-inflammatory way (73). There are two types of IL-13 receptors, the first consists of a heterodimer containing IL-13Rα1 and IL-4Rα (which also binds IL-4) subunits (**figure 1.3**) (74). The second type is an IL-13 specific receptor and consists of an IL-13Rα2 chain (74, 75). Although IL-13 binds to the IL-13Rα2 chain with high affinity, it is considered to be primarily a decoy receptor given its short cytoplasmic tail and lacking signaling motif (74, 76). IL-13 first binds to IL-13Rα1 with a low affinity and then IL-4Rα is recruited to the complex which in turn generates a high affinity receptor (74, 77).

For this reason, signal transduction via the IL-4Rα is thought to be responsible for the majority of the functional characteristics of IL-4 and IL-13. However, IL-13 also possesses many unique effector functions which differentiates it from IL-4. For example, it has been shown that both IL-4 and IL-13 activate STAT3 and STAT6, while STAT1 is only activated by IL-13 (74, 78).

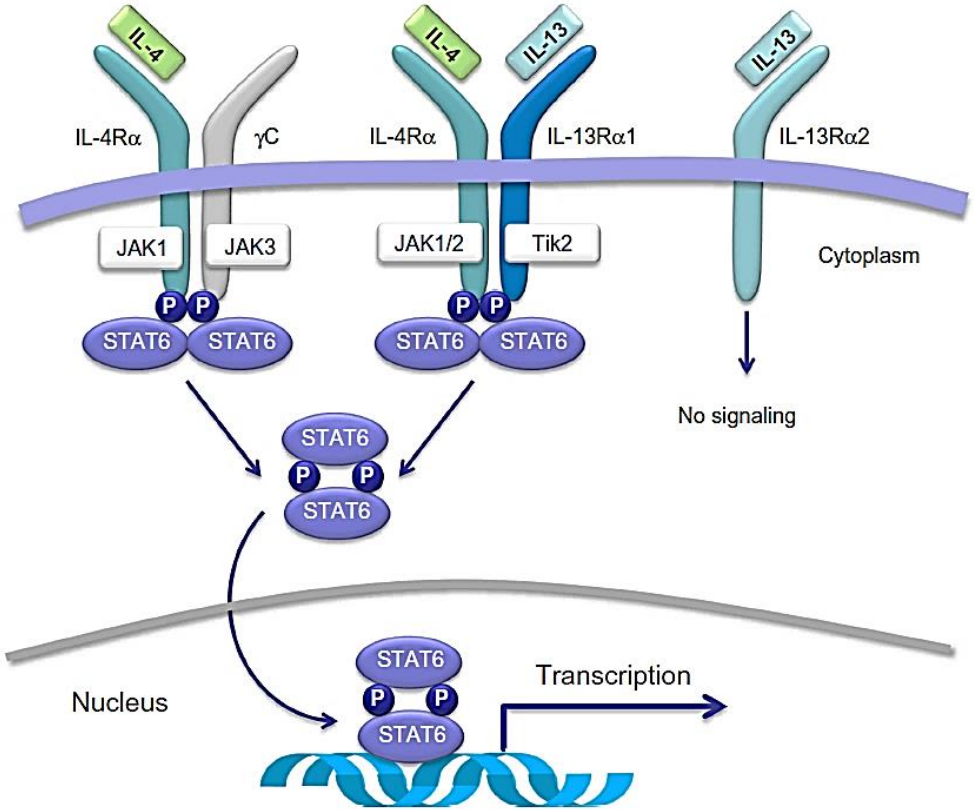

Figure 1.3: IL-13 membrane receptors and intracellular signaling pathways. Binding of IL-13 to IL-13Rα1 induces heterodimerization with IL-4Rα. This dimerization, which can also be triggered by IL-4, activates JAK1/2 and Tyk2 tyrosine kinases, that are responsible for phosphorylation of STAT-6. Phosphorylated STAT-6 dimerizes and migrates from the cytosol to the nucleus, where it binds to the promoter regions of IL-4/IL-13-responsive genes. IL-13 can also bind to its receptor α2-chain (IL-13Rα2), which is not coupled to any intracellular signaling pathway. The receptor complex constituted by IL-4Rα and γC chains, associated with JAK1/3 kinases, can be activated only by IL-4. Adapted from (74).

IL-13 is mainly secreted by Th2 cells, but also by other T cell subsets, mast cells, dendritic cells, microglia, and macrophages (75, 79). IL-13Rα1 is expressed on many different cell types, including B cells, mast cells, endothelial cells, fibroblasts, monocytes, and macrophages, but is absent on T cells (75). This indicates that IL-13 can influence a lot of cell types, and consequently various pathways, by binding to its receptors.

IL-13 plays an important role in the body's fight against parasites and cancer. On the other hand, it can also contribute to the pathophysiology of allergic diseases such as asthma by promoting immunoglobulin E production (reviewed in (80)). To illustrate its anti-inflammatory action, 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. nitric oxide (NO), reactive oxygen species (ROS)) both *in vitro* (81) and *in vivo*. For example, IL-13 has been shown to exert neuroprotective effects in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, by decreasing inflammatory cell infiltration and axonal loss as well as reducing clinical symptoms (82-84). Furthermore, preliminary data from our group have shown that IL-13 promotes neurite growth in primary neurons and organotypic brain slices *in vitro*. In the CNS, neurons and microglia work in synergy to reduce brain inflammation via induction of IL-13, which on its turn has also been shown to directly induce apoptosis in activated microglia (85). It therefore seems plausible that modulating the microglia/macrophage response after SCI by factors such as IL-13 may exert beneficial effects by down-regulating CNS regenerative-inhibitory factors (**Chapter 5**).

1.5 Mast cells and their proteases

As mentioned above (**figure 1.1**), a dense glial and fibrotic scar is formed around the lesion site after SCI which is non-permissive for axon regeneration. The scar tissue is rich in axonal growth-inhibitory molecules like CSPGs, inhibitors associated with myelin and ECM compounds. The importance of mast cells (MCs) and their proteases (MCPs) in the process of SCI has been studied extensively by our research group (31, 32, 86, 87). Results of these studies have already indicated a role for mouse mast cell protease 6 (mMCP6; mouse homologue of human tryptase) in scar tissue remodeling after SCI (32).

MCs from the periphery can infiltrate the CNS through a compromised BBB that is characteristic for many neuroinflammatory diseases. Considerable progress has been made in elucidating the crucial role of MCs in the pathology of several inflammatory CNS disorders (reviewed by Nelissen *et al*. (87)). Apart from their pro-inflammatory and mainly detrimental role in multiple contexts (88, 89), MCs also exert a number of important beneficial functions. We recently reported a favorable role of MCs after traumatic CNS injuries (86, 87). Experiments in knockout mice indicated that MCs support neuronal survival and functional recovery after CNS injury (86, 87). In particular, MCs appear to be protective in neuroregenerative processes following CNS trauma, due to their ability to degrade inflammation-associated cytokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6), and thereby temper 'detrimental' inflammatory processes (87). These immunomodulatory effects were at least partly mediated via mMCP4 (mouse homologue of human chymase), which we have investigated in this thesis more profoundly (**Chapter 6**). Apart from the immunomodulatory functions, mMCP4 also cleaves several physiological substrates that are important for tissue remodeling and it is involved in ECM degradation through direct cleavage effects or indirectly by activating other ECM-processing enzymes (i.e. matrix metalloproteinases [MMPs]) (90, 91). Therefore, we wanted to reveal its role in scar tissue remodeling after SCI (**Chapter 6**).

MCs are highly granulated immune cells that originate from multipotent hematopoietic precursors in the bone marrow (92). More than 25% of the total MC protein content consists of MCPs (e.g. MCP4) (91). These are stored in an enzymatically fully active form in the secretory granules and following MC degranulation, large amounts of active proteases are released into the extracellular space. Hence, MC proteases may be of significant importance in any condition in which MC degranulation occurs. The 3 major families of MC proteases are tryptases, chymases and mast cell carboxypeptidase A (CPA). Both tryptases and chymases belong to the family of serine proteases with endopeptidase activity (break peptide bonds of nonterminal amino acids [i.e. within the molecule]), whereas CPA is a metalloproteinase with exopeptidase activity (break peptide bonds of terminal amino acids) (93, 94). Each MC-specific protease is capable of degrading a multitude of different proteins (reviewed in (93)) and the availability of preferred substrates may vary at different stages of a given pathologic process. The MC specific proteases may thus exert dual roles, that on the one hand are proinflammatory, but on the other are protective.

MCs are well known for their role in allergic and anaphylactic reactions (95). In addition, MCs are also involved in processes of innate and adaptive immunity (96). MC progenitors circulate in the blood as differentiated MC precursors and then enter the tissue before maturing into tissue-specific phenotypes, according to the local microenvironment (97). Mature MCs can be found in a wide variety of tissues. They are typically located close to outer layers and barriers, such as the epithelia, mucosal membranes, and vascular walls where pathogens, allergens and other environmental agents are frequently encountered (95, 98, 99). In the mammalian brain, MCs are typically found in the meninges, choroid plexus, olfactory bulb, mesencephalon and the parenchyma of the thalamic-hypothalamic region. MCs generally reside alongside the blood vessels (reviewed in (100)). Following an appropriate stimulus, the content of the MC vesicles is released into the surrounding milieu. One of the most well-characterized stimuli to induce MC degranulation is the cross-linking of IgE molecules bound to the high-affinity IgE receptor, i.e. FcεRI [86].

MCs, however, can also be activated in response to several other types of stimuli, including anaphylatoxins C5a and C3a, neuropeptides (e.g. substance P), endothelin-1 and Toll-like receptor ligands (101). Another subset of mediators can be synthesized by MCs subsequent to their activation (102). These mediators include many cytokines, chemokines and other pro-inflammatory lipid-derived mediators like prostaglandins and leukotrienes (103). However, depending on the tissue in which they reside, MCs vary in their granule content (104), so that MCs found in different tissues contain different quantities and proportions of mediators (105).

1.6 Aims of this study

Spinal cord injury is a condition which involves all the important body systems. The primary injury develops into a secondary injury cascade for which currently no singular treatment can help. Hence, a multifactorial approach is needed. Therefore, it is worth exploring different mechanisms that can increase functional regeneration. In this thesis, we have focused on the neurotoxic (via adrenergic pathway modulation and stimulation of angiogenesis), pro-inflammatory (via immunomodulation of macrophages) and regeneration-inhibitory (via mouse mast cell protease 4) spinal cord micro-environment to improve functional recovery after SCI.

1.6.1 Beta-adrenoceptor modulation to analyze the effects of the neuroimmune stress pathway in SCI

As described in **chapter 2**, inevitably the sympathetic nervous system part of the stress systems will be activated after SCI. This will cause hormonal and metabolic changes with direct and indirect effects on the inflammatory system. Next, the inflammatory response will play a major part in the increase of the secondary injury and the loss of function. Preliminary data show that when we study the systemic effects of different stress pathway blockers *in vivo*, reduced functional recovery is achieved by blocking the β-ARs of the SNS. This indicates the importance of the β-adrenergic pathway for functional regeneration.

Therefore, we hypothesized that stimulating the β-adrenergic pathway provides neuroprotection and neuronal outgrowth in order to stimulate functional regeneration after SCI. The results are outlined in **chapter 3**.

1.6.2 Beta-adrenoceptor modulation to stimulate angiogenesis in order to improve functional regeneration after SCI

In CNS trauma, the importance of β2-AR modulation for neuroprotection and regeneration has been highlighted, although the effects on revascularization remain unclear. Vascular protection and revascularization are very important to support regeneration. Angiogenesis is associated with changes in EC proliferation and tube formation, controlled by ERK/MAPK and Akt signaling. Important regulators of these systems include hormones acting on G-protein-coupled receptors, such as β2-ARs. Therefore, the aim was to investigate the angiogenic capacity of the specific β2-AR agonist terbutaline *in vitro* as well as *in vivo* in our SCI mouse model. The pro-angiogenic effects of terbutaline on ECs derived from the CNS, namely bEnd.3-cells, were assessed by evaluating tube formation and proliferation. Underlying pathways were investigated by administration of specific inhibitors of ERK and Akt signaling. In order to investigate the general effects of terbutaline in an organotypic system, we have used the chick chorioallantoic membrane (CAM)-assay. These results are described in **chapter 4** of this thesis and were published in 2016 in the Journal of Cellular Physiology.

1.6.3 Adoptive transfer of IL-13 overexpressing macrophages to skew the M1 spinal cord micro-environment towards M2 to permit functional regeneration

After CNS injury, signaling pathways that polarize macrophages towards an neurotoxic M1 phenotype predominate. Next to their neurotoxic effects, they cause axonal dieback. Compared with M1 macrophages, M2 macrophages act pro-regenerative.

M2 macrophages promote more robust neurite outgrowth and they can enhance oligodendrocyte progenitor cell differentiation and hence remyelination. Neuroinflammatory responses may be beneficially influenced by M2a polarisation driven by IL-13.

Previous studies of our group, on MSCs as carriers of IL-13, already suggest that indirect effects via alternatively activated macrophages (M2) might decrease axon degeneration. Pro-inflammatory macrophages (M1) play a direct role in axonal retraction and destruction and an M2-conversion may have rendered these cells less destructive. In addition, it has been indicated that inhibition of macrophages improved recovery after neurotrauma. Moreover, it has been shown that macrophages can actively migrate to the SCI lesion site. Therefore, we have chosen for controlled immunomodulation, specifically targeted on macrophages, to improve functional regeneration after SCI and to reveal whether they are responsible for the IL-13 mediated neuroprotective effects. The results are described in **chapter 5**.

1.6.4 Mast cells protect from post-traumatic spinal cord inflammation in mice by degrading lesional scarring components via mouse mast cell protease 4

SCI is characterized by the formation of a glial and fibrotic scar at the lesion site. This scar creates a major barrier for regenerating axons and contributes significantly to the impaired functional outcome. Recent findings indicate that MCs protect the CNS after mechanical damage by suppressing detrimental inflammatory processes mediated via mMCP4, which also plays an important role in tissue remodeling and ECM degradation. Therefore, we have investigated in this study the effects of mMCP4 on scarring and recovery after SCI by using mMCP4 knockout mice. The findings are reported in **chapter 6**.

Chapter 2

The role of alpha-adrenoceptor modulation in central nervous system trauma

Based on:

Alpha-adrenoceptor Modulation in Central Nervous System Trauma: Pain, Spasms and Paralysis – An Unlucky Triad. Stefanie Lemmens, Bert Brône, Dearbhaile Dooley, Sven Hendrix and Nathalie Geurts. Medicinal Research Reviews, 35, No. 4, 653–677, 2015.

Chapter 2. The role of alpha-adrenoceptor modulation in central nervous system trauma

2.1 Abstract

Many researchers have attempted to pharmacologically modulate the adrenergic system to control locomotion, pain and spasms after central nervous system (CNS) trauma, although such efforts have led to conflicting results. Despite this, multiple studies highlight that α-adrenoceptors (α-ARs) are promising therapeutic targets because in the CNS, they are involved in reactivity to stressors and regulation of locomotion, pain and spasms. These functions can be activated by direct modulation of these receptors on neuronal networks in the brain and the spinal cord (neuromodulation). In addition, these multifunctional receptors are also broadly expressed on immune cells. This suggests that they might play a key role in modulating immunological responses, which may be crucial in treating spinal cord injury and traumatic brain injury as both diseases are characterized by a strong inflammatory component. Reducing the pro-inflammatory response will create a more permissive environment for axon regeneration and may support neuromodulation in combination therapies. However, pharmacological interventions are hindered by adrenergic system complexity and the even more complicated anatomical and physiological changes in the CNS after trauma. This review is the first concise overview of the pros and cons of α-AR modulation in the context of CNS trauma.

2.2 Introduction

2.2.1 Expression and multifunctional properties of the α-adrenoceptors in CNS trauma

2.2.1.1 Spinal cord injury (SCI) and traumatic brain injury (TBI)

SCI is a severe condition characterized by the loss of motor, sensory, and autonomic function below the lesion site. It can result from contusion, compression, penetration, or maceration of the spinal cord. Most SCIs are caused by motor vehicle or workplace accidents (1). Being paralyzed by an SCI has many economic and social issues, as immobility significantly reduces the quality of life (1-3).

Despite extensive research, there are currently no effective therapies available for SCI. Generally, three main experimental strategies are followed to stimulate recovery after SCI. The extent of the lesion is influenced by the primary insult and secondary damage from excitotoxicity due to glutamate release from damaged axons, free radicals, hemorrhage within the spinal column, inflammation, and apoptosis. Therefore, a first strategy is to tackle and reduce these secondary effects. This may be achieved using glutamate receptor antagonists to block excitotoxicity or high doses of the immunosuppressive drug methylprednisolone, although their efficacy is somewhat debatable (17, 18). For example, short term high dose methylprednisolone has massive side effects like gastric bleeding and it can cause wound infection (19).

Secondly, stimulating axon regeneration is an important challenge in SCI research. Axon regrowth can be induced in several ways, including surgical reconnection of the lesioned spinal cord, targeting inhibitory factors or transplanting growth-promoting factors or stem cells (17, 18).

Finally, locomotion and autonomous functions may improve by modulating neural network activity within the spinal cord. Following SCI, neural circuits for locomotion and autonomous functions are not lost below the lesion site, but lack descending signals from the brain.

Pharmacological or electrical stimulation of selected pathways within the spinal cord may enhance such signals, thereby improving locomotion (18). Sensory and descending inputs to neural networks within the spinal cord include glutamatergic, noradrenergic, dopaminergic, and serotonergic pathways (20). These pathways could be stimulated by pharmacological manipulation in order to restore lacking descending signals from the brain. However, there is little consensus on pharmacological strategies because individual drugs have both beneficial and deleterious effects in different studies (18). For instance, some studies showed that clonidine, an α-AR agonist, hinders walking ability, while others showed improved locomotor function and walking speed (106, 107).

However, despite extensive pre-clinical research, several clinical trials have failed to improve therapeutic outcome (13). Rehabilitative training remains the standard method of therapeutic intervention in most countries to maximize functional recovery (17). With this in mind, it is worth exploring the underlying mechanisms which increase functional recovery. Oh and colleagues have found that extracellular signal-regulated kinases 1 and 2 (Erk1/2) may be an important mediator for transmitting signals from the injury site to the neuronal cell body. They further suggest that activation of the Erk1/2 signaling pathway may be involved in enhanced outgrowth of CST axons after treadmill training (108). Erk1 and Erk2 are related protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade. This cascade participates in the regulation of a large variety of processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription. The same pathway is also activated by α-AR stimulation. Direct activation of Erk1/2 by α-ARs was reported in several studies (109-111). α-ARs may influence many processes involved in locomotion, pain and spasms via the Ras-Raf-MEK-ERK signal transduction cascade, and also by other mechanisms.

Another devastating disorder of the CNS is TBI, which resembles SCI in many aspects. In TBI, the primary mechanical insult to axons, neurons, glia, and blood vessels also results in a delayed secondary injury events due to neurochemical, metabolic, and cellular changes.

The secondary injury starts with an array of neurochemical events that produce toxic and pro-inflammatory molecules, such as oxidative metabolites and pro-inflammatory cytokines (IL-1) (112). These events cause lipid peroxidation, blood-brain barrier disruption, and cerebral edema, which can lead to local hypoxia and ischemia, secondary hemorrhage, herniation, and neuronal cell death (112). Taken together, events from this secondary injury account for neurological deficits observed after TBI, with substantial decrease in quality of life and high societal costs (112).

However, despite extensive efforts to develop neuroprotective therapies for this severe disorder, there have been no successful outcomes in human clinical trials (112). Examples of such therapies include a number of anti-inflammatory and multipotential drug treatment strategies that inhibit posttraumatic neuroinflammation and microglial activation (e.g. PPAR agonists), in addition to agents that reduce other secondary injury mechanisms such as edema formation and neuronal apoptosis (e.g. progesterone) (112). Severe TBI also leads to sympathetic hyperactivity leading to catecholamine excess, hypertension, abnormal heart rate, and agitation (113).

These findings emphasize the important contribution of the sympathetic nervous system in CNS disorders and fuel the search for therapeutic strategies to modulate this system. The activated sympathetic nervous system releases norepinephrine (NE) onto visceral targets, where it binds to either α- or β-ARs. Activation of these ARs by NE causes multiple downstream effects, such as modulation of movement and pain.

This review focusses on α-ARs. The expression of the α-ARs in the CNS and the immune system is summarized in **Table I**.

An increasing number of studies on α-AR modulation in functional recovery after CNS-trauma indicates the importance of these receptors in motor function, pain modulation and spasm regulation (**Table II**). In the CNS, receptor stimulation replaces missing descending signals, directly affecting locomotion, pain and spasms. Additionally, α-AR stimulation influences the inflammatory response which indirectly improves functional recovery.

This review summarizes this knowledge on α-AR modulation in recovery of motor function after CNS trauma, in particular in disorders such as SCI and TBI. In addition, a comprehensive scheme that helps to understand the pros and cons of α-AR modulation is represented in **Table III**.

2.2.1.2 The α-adrenoceptors

The α-ARs will be briefly introduced to explain the rationale for their modulation in SCI and TBI. For more comprehensive background information on this topic, we refer to chapter 10 in *Principles of Pharmacology* (114).

The α-ARs are G-protein coupled receptors which are subdivided into two major types: α1- and α2-ARs which are expressed in the peripheral nervous system as well as in the CNS.

The α1-ARs are classified into the subtypes α1A, α1B and α1D. The α2 ARs are subdivided into α2A, α2B and α2C (115). The α-ARs are located on both neural and non-neural tissue, where they respond to the catecholamines NE and epinephrine (E) (115). Dopamine may also act as a ligand for ARs, although only in supraphysiologic concentrations (114).

The sympathetic nervous system is the major source of NE production, responding to vigilance, stress, neuroendocrine stimuli and pain ("fight-or-flight" response). NE is secreted by sympathetic postganglionic neurons.

Analogous to these sympathetic postganglionic neurons are the chromaffin cells of the adrenal medulla. These produce and release E into the bloodstream when activated. Both E and NE act on all ARs, although with different affinities.

In the brain, NE also acts as a neurotransmitter. In response to stress stimuli, the locus coeruleus in the brainstem is activated. This nucleus projects noradrenergic axons to many different locations, including the cerebral cortex, limbic system and spinal cord, thereby creating a huge system of neuronal connections (114, 116). The locus coeruleus is involved in the regulation of attention, arousal, sleep-wake cycles, learning, memory, anxiety, pain, mood, and brain metabolism. It participates in a general arousal of the brain during "interesting" events in the outside world. The secreted NE can, for instance, make neurons of the cerebral cortex more responsive to salient sensory stimuli. Therefore, the locus coeruleus may function to increase brain responsiveness, speeding and optimizing information processing by sensory and motor systems (117, 118).

Activating the α-ARs leads to the modulation of cellular and synaptic properties resulting in spinal locomotor network adaptation (18, 118). The α-adrenergic pathway has been shown to initiate and facilitate the expression of spinal locomotor output, especially in a cat model of SCI. For example, intrathecal application of the α2-AR agonist clonidine can evoke hindlimb locomotion in acutely spinalised adult cats while walking on a treadmill. In general, α1-AR agonists are reported to have weaker effects than α2-AR agonists in acutely spinalised cats (18, 118). These data highlight the importance of the α-adrenergic pathway for modulation of the locomotion network after TBI or SCI. The α-adrenergic system is also one of the spinal systems which can counteract pain-stimulating processes. However, after SCI, this system is damaged and therefore not functioning properly (35). Next to their involvement in locomotion and pain, the α-ARs stabilize reflex activation and spasms after SCI, both at the neural and the muscular level. More specifically, they regulate motor-neuron excitability, sensory synaptic transmission and muscle spasms (36-39).

The expression of α-ARs in the CNS and on immune cells suggest that their ligands E and NE act as signaling molecules in both the nervous and immune systems (119). **Table I** shows an overview of the expression pattern and related functions of α-ARs in the CNS (neuromodulation) and on immune cells (immunomodulation).

Table I: Overview of the expression of α-adrenoceptors in the CNS and immune system and their related functions

α1-adrenoceptors in the CNS		
Expression	**Functions after receptor activation**	**Comments**
Postsynaptically in subcortical area of the brain and spinal motor neurons [120, 121]	- Involvement excitatory responses, locomotion control and changes in locomotion behavior, pain-control, spasms-activity and reflexes [36, 120-123]	Especially α1D-ARs are expressed in the cortex, hippocampus, olfactory bulb, dorsal geniculate and ventral posterolateral nuclei of the thalamus, with related functions in locomotor activity [121]
Astrocytes, microglia and neuronal cells [119, 124, 125]	- Regulating locomotion and neurotransmission [124] - Changes astrocyte function by changes in cytokine release, nerve growth factor production, glycogenolytic activity and amino acid uptake [119] - Changes microglia function by reducing expression of pro-inflammatory cytokines (IL-6 and TNF-α) [119]	Especially α1A-ARs are expressed on astrocytes and microglia [119] The effects of modulation of the expression of α1-ARs on neuronal cells are not clearly reported, although this expression could be related to locomotion changes and regulation of neurotransmission [118]
α1-adrenoceptors in the immune system		
Expression	**Related functions**	**Comments**
Human lymphoid tissues: spleen, bone marrow stromal cells, thymus, bone marrow, tonsils and synovial exudate [124]	- Immunomodulation [124]	
Lymphatic vessel-smooth-muscle cells [124]	- Regulating lymphoid cell trafficking [124]	
Lymphocytes, monocytes, macrophages [124, 126-129]	- Pro-inflammatory effects (increased IL-1β production) [124, 129]	
α2-adrenoceptors in the CNS		
Expression	**Related functions**	**Comments**
Pre- and postsynaptically [130, 131]	-Inhibiting neurotransmitter release[130, 131] -Regulating sympathetic tone, reactivity to environmental stressors and motor activities.[132, 133]	Functions were determined in studies on knock-out and overexpression of the α2C-AR subtype in mice [133]

	- Reduction of pain, spasms and reflexes [122, 123, 134, 135]	
Astrocytes [119, 125]	- Changes in astrocyte function by alterations in cytokine release, nerve growth factor production, glycogenolytic activity and amino acid uptake [119] - Increased expression on astrocytes in spastic animals; receptor stimulation suppresses glutamate release from these astrocytes and reduces spasms [135]	Especially α2A-ARs are expressed on astrocytes [119, 125]
Microglia, macrophages [119, 125]	- Modulating microglia function by reducing expression of pro-inflammatory cytokines (IL-6 and TNF-α) leading to pain relief [119, 136, 137]	Especially α2A-ARs are expressed on microglia [119, 125]
α2-adrenoceptors in the immune system		
Expression	**Related functions**	**Comments**
Lymphatic vessel smooth muscle cells [124]	- Regulating lymphoid cell trafficking [124]	
Lymphocytes [126]	- Decreasing T-cell responsiveness [126]	Especially α2A- and α2B-ARs are expressed on T-cells [126]

Abbreviations: AR, adrenoceptor; CNS, central nervous system; IL-1β, interleukin-1beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

α1-adrenoceptor expression in the CNS and the immune system

The α1-ARs are mostly found postsynaptically in the CNS, where they play an excitatory role. They generally mediate their responses through a G_q mechanism which activates phospholipase C, eventually leading to an increase of diacylglycerol and calcium influx. Multiple effects are possible due to the fact that the receptors are expressed at many different locations. For example, when α-ARs on smooth muscle cells are activated, smooth muscle contraction will follow. In general, they are found in subcortical areas of the brain such as the medial and lateral geniculate nuclei, reticular thalamic nucleus, dorsal raphe and on spinal motor neurons (**Table I**) (120-122). Here, they are involved in the control of locomotion behavior and pain (120-122).

Especially, the α1D-ARs are expressed in the cortex, hippocampus, olfactory bulb, dorsal geniculate and ventral posterolateral nuclei of the thalamus (121). Moreover, α1D-adrenoceptor signaling is required for stimulus-induced locomotor activity (121). Knock-out mice for these receptors display reduced wheel-running activity, exploratory rearing behavior and hyperlocomotion following acute amphetamine administration (121). In this study, amphetamines were used to block the reuptake of catecholamines (NE and E) and to increase their amount in the synapse (121). The α-ARs expressed in the ventral horns are involved in the reaction to pain stimuli. In the spinal ventral horn, noradrenaline, due to its action on the α1-adrenoceptor, facilitates pain behavior by increasing excitability of α-motoneurons (122). However, one has to take into account that pain assessment in animal studies is often based on the expression of motor behavior to potential painful stimuli. Therefore, it is important to exclude confounding effects induced by a change in motor behavior by noradrenergic compounds, instead of a change in pain sensation. In addition, stimulating α1-ARs in the spinal cord, with for example methoxamine, facilitates spasms activity and reflexes (36, 138). More specifically, α1-ARs are located on neuronal and glial cells, where they are involved in the regulation of locomotion and neurotransmission (**Table I**) (118, 124). They alter astrocyte function by targeting cytokine release, nerve growth factor production, glycogenolytic activity and amino acid uptake (119). In particular, α1A-ARs are expressed on astrocytes, where their stimulation affects astrocytic metabolism.

Stimulation of the α1A-ARs on the astrocytes leads to an increase in intracellular calcium which in turn increases glutamate uptake (125).The α1A-ARs are also expressed on microglia and stimulating these receptors with NE suppresses the expression of mRNAs encoding the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (119). This suggests that NE participates in the regulation of brain function by modulating microglia function (119).

In the immune system, α1-ARs are generally located in human lymphoid tissues, suggesting their involvement in immunomodulation (**Table I**) (124).

This hypothesis is supported due to their expression on lymphatic smooth-muscle cells, where they regulate lymphoid cell trafficking (124). The α1-ARs are also expressed on human lymphocytes, monocytes and macrophages (115, 121, 124, 126-129). Their stimulation on monocytes/macrophages induces a pro-inflammatory environment by increasing IL-1β production (129).

The expression of α1-ARs within both the CNS and the immune system suggests that these receptors may be suitable targets to treat conditions of TBI or SCI. Inflammation could be targeted using α1-AR agonists of receptors expressed on microglia and astrocytes. This would alter microglia or astrocyte function by reducing pro-inflammatory cytokine expression (119, 139). However, α1-AR stimulation on macrophages is associated with pro-inflammatory effects (124, 129). In addition, α1-AR stimulation on neurons of the spinal cord can aggravate pain behavior and facilitate spasms and reflexes.

Under physiological circumstances, spinal inhibitory systems, such as the α-adrenergic system, exist to counteract pain-stimulating processes (35). However, after SCI, this system is damaged. Excessive down-regulation of the α-adrenergic system or hyperactivation of compensatory pathways may contribute to the vicious circle of neuropathic pain. Therefore, stimulating or inhibiting these receptors should be considered cautiously depending on the context.

In the case of inflammation, a targeted approach on either astrocytes (stimulation leads to anti-inflammatory response) or macrophages (inhibition leads to an anti-inflammatory response), might be a promising alternative. To enhance functional recovery, however, immunomodulation alone is not sufficient. This immunomodulation must be accompanied by multiple processes in the CNS such as axon regeneration and synapse formation at the correct target. Stimulating these ARs in brain nuclei and in spinal motor neurons which are involved in locomotion control, may enhance or replace signals which are reduced or absent after CNS injury.

α2-adrenoceptor expression in the CNS and immune system

The α2-ARs are expressed pre- and postsynaptically and play an inhibitory role which is mediated through $G_{i/o}$ proteins, which in turn decrease cAMP and Ca^{2+} release. Furthermore, the presynaptic α2-ARs regulate neurotransmitter release by noradrenergic feedback inhibition (autoregulation) (115, 140).

In the CNS, α2-ARs regulate sympathetic tone and control vigilance, attention, reactivity to environmental stressors and motor activities, as shown in studies using α-AR knockout mice and mice with α-AR overexpression (132, 133). For example, lack of α2C-ARs was associated with increased amphetamine-induced locomotor activity, startle reactivity, aggression and forced-swimming test activity. Opposite changes were observed in the α2C-overexpressing mice.

These results suggest a role of this α2-AR subtype in processing sensory information and in the control of motor and emotional activities in the CNS (133). In the dorsal horns of the spinal cord, α2-ARs suppress pain signals by reducing the release of excitatory amino acids from afferent nerve fibers or by the induction of inhibitory potassium currents in postsynaptic spinal pain-relay neurons (122). In addition to their expression in the spinal cord, they are also present in the pons where they play a different role. Activating α2-ARs in the pons attenuates descending inhibition after nerve injury and increases pain (134).

The expression and the activation of the α2-ARs in the spinal cord is also correlated with a reduction in spasms and reflexes (135, 138, 141-143). The α2-ARs are found on astrocytes and are involved in regulating the function of these cells, such as stimulation of glycogenolysis (119, 125). More specifically, the α2A subtypes are expressed on both microglia and astrocytes (119, 125). They may regulate brain function by modulating microglia function, namely by reducing the expression of the pro-inflammatory cytokines IL-6 and TNF-α (119). Astrocytes show increased α2A-AR-expression in spastic animals. Stimulation of these receptors suppresses glutamate release from activated astrocytes and reduces spasms (135).

In the immune system, α2-ARs are expressed on lymphatic vessel smooth-muscle cells, where they regulate lymphoid cell trafficking (124). Furthermore, the expression of α2A-ARs and α2B-ARs on lymphocytes confirms their role in immunomodulation. Activating these receptors with NE decreases T cell responsiveness (126). These receptors are also expressed on activated macrophages, where their activation modulates the cytokine balance (↑ anti-inflammatory cytokine TGF-β1; ↓ pro-inflammatory cytokines IL-1β and TNF-α) and causes pain relief (136, 137).

The immunomodulatory role of α2-ARs via their expression on glial and immune cells, suggests that these receptors may be important targets for modulation after TBI or SCI. For instance, the inflammation-associated component after SCI or TBI could be indirectly reduced by altering microglia or astrocyte function with α2-AR agonists (119). In contrast to α1-ARs, which upon stimulation induce a pro-inflammatory profile in macrophages, α2-ARs are not expressed on macrophages. This suggests that modulating α2-ARs may have less adverse effects.

To conclude, the location of the ARs is of crucial importance. Their stimulation may decrease pain or spasms by acting on glial or immune cells, whereas pain is increased by acting on neuronal receptors in the pons. Stimulating the physiological inhibitory function of the ARs also would decrease locomotor activity.

2.3 The effects of α-adrenoceptor modulation in CNS damage

2.3.1 Effects of α-adrenoceptor modulation in CNS damage

The α-ARs are broadly expressed in the CNS and the immune system with related functions like control of locomotion behavior and immunomodulation, as discussed in part 1. Therefore, these receptors might be interesting targets after CNS damage. SCI and TBI lead to functional disability and have a strong inflammatory component, which both could be improved by α-AR modulation, considering the receptors' functions. Multiple studies were performed to unravel the effects of α-AR modulation after SCI and TBI and these are summarized in the next part. **Table II** gives a concise overview of these effects, which are also schematically represented in **Table III**.

Table II: Overview of the effects of α-adrenoceptor modulation on functional recovery in SCI and TBI

CNS injury model	**Receptor modulation**			
SCI	**α1-ARs**		**α2-ARs**	
	Agonism	**Anta-gonism**	**Agonism**	**Antagonism**
Effects on neuropathic pain	↑Neuropathic pain by postsynaptic hypersensitivity of the α1-ARs (144)	Unknown	-Analgesia -Neuropathic pain-relief and ↓allodynia by preganglionic neuron inhibition -↓Ca^{2+}, ↓cell excitation and ↓neurotransmitter release (145-152)	↑Neuropathic pain by presynaptic α2-AR dysfunction (144)
			-↓Mechanical hypersensitivity by altering cytokine balance (↓pro-infl; ↑anti-infl) (136, 153) -Inhibition of neuroimmune activation (137)	-↑Allodynia -↓analgesic effects on thermal and mechanical hypersensitivity -↑mechanical hypersen-sitivity -↑thermal hypersensiti-vity by antagonism of noradrenergic feedback inhibition (134, 153-155)
			-Noradrenergic feedback inhibition by spinal α2-ARs (↓neuropathic pain) -Neuropathic hypersensitivity by pontine α2-ARs	

			(↑neuropathic pain) [134]	
Effects on spasms/reflexes	↑Spasms by ↑Ca PICs [36, 156]	Inhibits spasms [36, 135]	↓Spasms [37-39, 135]	↑Spasms [36]
	↑Flexor reflex [138]	↓Flexor reflex [138, 157]	-↓Spinal reflexes -Muscle relaxant effect [138, 141-143]	↑Flexor reflex and stretch reflex [135, 157]
			Inhibits spasms by inhibiting EPSPs [36]	
			↓Spasms by suppression of glutamate release from activated astrocytes [135]	
Effects on locomotion	↑Motor neuron excitability [118]	↓Motor-neuron fire-ability [118, 158]	↑Motor-neuron excitability [118]	↓Motor-neuron excitability [118]
	Fast motor rhythm generation [159]		↑Recovery functions, such as hind limb locomotion [160-162]	Walking difficulties [160]
	Locomotor-like activity [163]		Preserved motor function and attenuation of spinal cord ischemia-reperfusion injury [164, 165]	Blockade locomotion [166]
	↑Regularity of hind limb stepping and interlimb coupling [167]		No induction of hind limb movements and ↓ non-locomotor and locomotor-like movements [106, 167]	

	↑ electromyographic activity by muscles involved in locomotion[168]		Improved locomotor function by administration of Clonidine [107]	
TBI	**α1-ARs**		**α2-ARs**	
	Agonism	**Anta-gonism**	**Agonism**	**Antagonism**
Effects on locomotion	Unknown	Motor deficit [169]	Motor deficit [169]	Facilitation of recovery [170]
		Delay in recovery [170-175]	Delay in recovery [176]	
		↓Beam walking performance [177]	↓Recovery beam-walking [170]	
		↑Hemiplegia [178]		
Other effects	Unknown	↓Cortical dysfunction [179]	-↑Alternating bursts of electromyographic activity -Alternating activation of the extensor and flexor musculature of the hind limbs (stepping) [180]	Neuroprotection [181]
			↓Spasms [182]	↑Cortico-motoneuronal excitability [183]
			Neuroprotection [184]	

Abbreviations: AR, adrenoceptor; Ca, calcium; CNS, central nervous system; EPSPs, excitatory postsynaptic potentials; PICs, persistent inward currents; SCI, spinal cord injury; TBI, traumatic brain injury.

Table III: Analyzing pain, spasms and paralysis after α-AR modulation

α1-adrenoceptor

	Agonism			Antagonism		
	+	+/-	-	+	+/-	-
SCI	↑ locomotion	↑ spasm activity	↑ pain reaction		↓ spasms	↓ motor neuron firing
		↑ flexor reflex			↓ reflexes	
TBI				prevention cortical dysfunction		↓ motor function

α2-adrenoceptor

	Agonism			Antagonism		
	+	+/-	-	+	+/-	-
SCI	↓ pain (spinal cord)	↓ spasms	↑ pain (pons)		↑ spasms	↑ pain
	neuroprotection	↓ reflexes			↑ reflexes	↓ locomotion
		↑↓ locomotion				
TBI			↓ locomotion	neuroprotection		
				↑ locomotion		

Color codes and arrows: white columns: improved functional outcome (+), gray columns: literature is complex (+/-), black columns: detrimental effects on functional outcome (-); ↑: increasing effects after AR modulation, ↓: decreasing effects after AR modulation. **Abbreviations**: TBI, traumatic brain injury; SCI, spinal cord injury.

2.3.1.1 Effects in SCI

Effects on neuropathic pain

Pain after SCI may be either neuropathic or nociceptive and these are caused by dysfunction of the nervous system or by damage to musculoskeletal or visceral systems respectively. A combination of both is also possible, depending on the extent and the level of the lesion. In addition, pain sensation is complicated by psychological and social factors (185). Therefore, pain has a strong very subjective component and is never the same amongst SCI patients, which complicates the design of effective drug therapies. Pain can arise from the spinal cord itself, the brain or peripheral structures. Neuronal and glial effects are involved and inflammation also plays a major role (122, 186).

The neuronal effects after SCI start off with excitotoxicity caused by overexposure to glutamate and overstimulation of the glutamate receptors. This overstimulation, in turn, can cause central sensitization in which dorsal-horn neurons become hypersensitive. After activation of the glutamate receptors, Ca^{2+} flows within the cell and activates downstream mediators (e.g. protein kinases). These factors phosphorylate mitogen-activated protein kinases (MAPKs), which activate proteins (transcription factors) involved in transcription and maintenance of the central sensitization (186). However, central sensitization is not only caused by overstimulation of the glutamate receptors. In general, SCI leads to increased expression or activity of ion channels, peptide receptors and neuro-immune factors, all contributing to dorsal horn neuron hyperexcitability resulting in central sensitization. Spinal inhibitory systems, such as the α-adrenergic system, exist to counteract these pain-stimulating processes (35). However, after SCI, this system is also damaged. Excessive down-regulation of the α-adrenergic system or hyperactivation of compensatory pathways may contribute to the vicious circle of neuropathic pain. The glial effects are associated with immune activation, which leads to the secretion of cytokines or other inflammatory mediators (e.g. IL-6 and substance P). These mediators activate astrocytes and microglia, which are normally in a resting state.

After activation, both astrocytes and microglia secrete substances which can bind to receptors on neurons in sensory circuits involved in pain sensation (reviewed in (186)).

The cause of pain after SCI is multifactorial and therefore difficult to manage. First-line treatments recommended for neuropathic pain include antidepressants, calcium-channel ligands, and topical lidocaine. Suggested second-line treatments are opioid analgesics, whereas third-line treatments include anti-epileptic medications amongst others (reviewed in (151, 187). As mentioned before, the α-ARs are involved in pain control. Their role in pain sensation associated with SCI has been comprehensively reviewed previously (122). The α-ARs located within the spinal dorsal horns are principally involved in the transmission through pain pathways. The dorsal horns relay information from ascending pain pathways and descending noradrenergic pathways. In the dorsal horns, α2-ARs suppress pain signals through the reduction of the release of excitatory acids from afferent nerve fibers or by the induction of inhibitory potassium currents in postsynaptic spinal pain-relay neurons. In the ventral horns, NE acts on the α1-ARs and aggravates pain behavior by increasing excitability of α-motoneurons.

Pharmacological studies and studies with genetic knock-out mice revealed a low tonic activity of the intrinsic noradrenergic pain regulatory system in the spinal cord. In contrast, after persistent injury, the role of the noradrenergic system acting on the spinal α2-AR is more significant. Therefore, it is suggested that the spinal α2-AR is involved in the noradrenergic feedback inhibition of sustained pain rather than the regulation of baseline pain sensitivity (122).

Normally, pain reduction after α2-AR stimulation is caused at the level of the spinal preganglionic neurons, where they are able to diminish sympathetic outflow to postsynaptic neurons by the noradrenergic feedback inhibition as described above (145, 153-155). Reduced intracellular Ca^{2+} in dorsal root ganglia neurons, which reduces cell excitation and neurotransmitter release, may provide another explanation (146).

Pain relief or reduced hypersensitivity after α2-AR stimulation may also be caused by a change in the balance between pro- and anti-inflammatory cytokines (such as decreasing the pro-inflammatory cytokine IL-1β or increasing the anti-inflammatory cytokine TGF-β1) (136, 137). For example, the α2-AR agonist clonidine modulates the cytokine balance (↑ anti-inflammatory cytokine TGF-β1; ↓ pro-inflammatory cytokines IL-1β and TNF-α) by acting upon activated macrophages that express these α2-ARs (136).

However, there are some conflicting results regarding increased pain after α2-AR agonism. These could be explained by the fact that spinal and pontine α2-ARs have opposite effects in pain-behavior after nerve-injury (134). In the spinal cord, nerve injury activates tonic noradrenergic feedback inhibition by stimulating α2-ARs and decreasing pain (134). In contrast, activating α2-ARs in the pons attenuates descending inhibition after nerve injury and increases pain (134). Finally, inhibition of α2-ARs with their antagonists inverts the pain-relieving effects after α2-AR agonism, by antagonism of the noradrenergic feedback inhibition in the spinal cord (153, 154).

In summary, hypersensitivity of α1-ARs is involved in neuropathic pain behavior after SCI, whereas α2-AR stimulation in the spinal cord reduces neuropathic pain. However, there are some conflicting results depending on the site of receptor modulation (pons vs. spinal cord) (**Table III**). Pain-reduction may be achieved by inhibiting α1-ARs or stimulating α2-ARs, although profound preclinical investigation is required.

Effects on spasms/reflexes

Similar to pain, spasticity after SCI could be caused by altered membrane channel and receptor properties of spinal neurons, increasing their excitability. In addition to neurons, there are muscular components which are also involved. On the neuronal level, many mechanisms could be responsible for exaggerated reflexes, including higher excitability of motoneurons and loss of pre-synaptic inhibition of Ia-afferent nerve fibers which normally excite the motoneurons via ionotropic actions (188, 189).

Muscular changes include an increase in the percentage of type IIx fibers (facilitate short-duration anaerobic activities) and changes in muscular tone (190). After SCI, descending overactivity causing exaggerated reflexes might be responsible for muscle hypertonia, which in turn leads to spastic movements (191). It is not fully clear whether these spastic movements are detrimental or beneficial for locomotion. Without the development of spastic muscle tone, some patients would be unable to walk because of the paresis (192). It has been debated whether hyperreflexia is correlated with the spastic movement disorder, because additional changes in muscle, ligament, and tendon properties occur in the latter (192). In conclusion, the cause of spasms after SCI is multifactorial and the outcome is further complicated by the site and the severity of the lesion. Therefore, therapies should attempt to focus on most, if not all, of these factors. For example, an incomplete SCI would not benefit from reflex inhibition, but rather from activation and training of residual motor function. On the other hand, immobilized patients with a complete lesion would benefit from reducing hyperreflexia and muscle tone (192).

Table II provides a summary of how α-ARs modulate reflex activation and spasms after SCI, at both the neural and muscular level. They regulate motor-neuron excitability, sensory synaptic transmission and muscle spasms (36-39). The brain stem provides most of the NE present in the spinal cord.

The NE stimulates spinal motor-neuron excitability by facilitating calcium-mediated persistent inward currents (Ca PICs) and inhibiting sensory afferent transmission to motor neurons via excitatory postsynaptic potentials (EPSPs). Thus, NE increases Ca PICs, which are crucial for sustained motor-neuron firing. Spinal cord transection eliminates most NE, thereby causing immediate loss of Ca PICs and an increase in sensory EPSPs to motoneurons. However, PICs recover with time. Together with the increased EPSPs, they trigger muscle spasms (36). Large, uncontrolled Na PICs also result in spasms (158). Stimulating α1-ARs with specific agonists (such as methoxamine) facilitates the Ca PICs and spasm activity (36). Furthermore, Sakitama *et al.* showed that α1-AR stimulation facilitates the flexor reflex (138).

On the other hand, antagonists have opposite effects (36, 138). Direct effects of α1-AR modulators on the regulation of muscle tonus and properties are not reported in these studies.

In contrast to α1-ARs, stimulating α2-ARs with their agonists (such as clonidine) mainly reduces spasms and reflexes (135, 138, 141-143). This can be caused by reducing the EPSPs that trigger spasms, as this will decrease sensory afferent transmission to motoneurons (36). Another possible reason for spasm reduction may be due to the suppression of glutamate release from activated astrocytes, which show increased expression of α2A-ARs in spastic animals (135).

Antagonism of α2-ARs, on the other hand, increases spasms and reflex activity, which may hinder coordinated locomotion (36, 135, 157). On the neuronal level, α2-AR agonists (e.g. tizanidine) reduce the release of the excitatory amino acids glutamate and aspartate from the presynaptic terminal of spinal interneurons. Here, they can also act on postsynaptic excitatory amino acid receptors (193). Aside from their neuronal actions, α2-AR agonists also display muscle relaxing activity (194).

In summary, α1-AR stimulation after SCI causes spasms and increases spinal reflexes, whereas inhibiting α1-AR has the opposite effect (**Table III**). However, direct effects on muscular properties are not reported.

In contrast, stimulating α2-ARs reduces spasms and spinal reflexes and relaxes muscles, indicating that these mediators act on multiple pathways. Inhibition of α2-ARs increases spasms and reflex activity (**Table III**). After injury, locomotion may be improved by reducing spasms and the underlying reflex and muscular property disturbances. This could be achieved by using antagonists for α1-ARs or agonists for α2-ARs. However, such approaches should be considered with caution, because as mentioned before the cause of spasms is multifactorial and influenced by the lesion type. For example, after an incomplete SCI, spastic muscle tone may be necessary for locomotion.

Effects on locomotion

The α-ARs were upregulated in the lumbar region of the spinal cord after complete spinal cord transection in a cat model (160). Locomotion recovery in the hind limbs was correlated to the time period for receptor upregulation (160). These results suggest the importance of these receptors for initiating and modulating locomotion after SCI. However, the literature is rather conflicting on the effects of stimulating or inhibiting α-ARs in locomotion (see **Table II**).

Reports on α2-AR stimulation show both locomotion recovery as well as impairment. *In vitro* studies show an increase in motor neuron excitability after α2-AR agonism (118). Furthermore, some α2-AR agonists were shown to stimulate locomotion in a limited number of SCI subjects (38, 39, 161). Improved locomotion may be a result of decreased spasticity or activation of the spinal circuitry (39). Bell *et al.* showed functional recovery with preserved cyto-architecture, decreased vacuolization and increased neuronal viability in the spinal cord when mice were treated with the α2-AR agonist dexmedetomidine after spinal cord ischemia-reperfusion injury (164). The exact mechanism responsible for this neuroprotective effect of dexmedetomidine is, however, unknown. Possible mechanisms include prevention of apoptotic processes or inhibition of lipid peroxidation (165). In contrast, α2-AR agonism with clonidine hinders walking ability (167). Furthermore, Lapointe *et al.* showed that clonidine does not induce hind limb movements in untrained mice with chronic SCI.

The authors conclude that clonidine is not a powerful central pattern-generator activator and may require additional factors and conditions to facilitate locomotor function recovery (106). On the other hand, multiple studies show that clonidine may improve locomotor function and walking speed in severely impaired people with incomplete SCI (107). Inconsistent effects may be caused by differences in SCI-models (complete vs. incomplete spinal cord section), application method of agonist (local vs. systemic), or receptor alterations (39, 160, 167). Furthermore, the treatment site also greatly influences the outcome, as agonists or antagonists have different effects when applied at spinal or supraspinal levels (39).

After complete spinal transection, there are only postsynaptic receptors present and their activation enhances locomotor activity. In contrast, after hemisection, also presynaptic receptors are detectable. These presynaptic receptors could for instance be inhibited by α2-AR agonists via noradrenergic feedback inhibition, which leads to reduced supraspinal input to the spinal circuitry and decreased locomotor activity (167). Blocking α2-ARs decreases motor-neuron excitability *in vitro,* causing locomotion blockage and walking difficulties in cats with SCI (118, 166, 195).

Current literature regarding α1-AR stimulation is more straightforward. Agonism of α1-ARs increases motor-neuron excitability and facilitates locomotor command-signal transmission in the lesioned spinal cord *in vitro* (118, 159, 163, 196). *In vivo* administration of the α1-AR agonist methoxamine improves the regularity of hind limb stepping and stabilizes interlimb coupling, thereby allowing locomotion for longer periods of time (167). Furthermore, stimulating α1-AR increases locomotor muscle electromyographic activity in paraplegic patients (168). However, administering antagonists decreases the motor-neuron fire ability (118, 158).

In conclusion, α1-AR agonists stimulate motor-neuron and muscle activity, whereas antagonism decreases motor neuron firing ability (**Table III**). The current literature on α2-AR modulation is not straightforward. Increased locomotor recovery and decreased locomotor ability are both reported after α2-AR stimulation **(Table III)**. On the other hand, α2-AR inhibition causes locomotion blockage (**Table III**).

2.3.1.2 Effects in TBI

The effects of α-AR modulation in TBI are summarized in **Table II**. The α1-AR expression decreases after TBI, more specifically in the cortex contralateral to the lesion and bilaterally in the dorsomedial hypothalamic and thalamic nuclei, as examined by receptor autoradiography for α1-AR binding with radioactive labeled [3H]prazosin (169). Furthermore, α1 AR-antagonists transiently worsen motor deficits after recovery (169).

Several studies show decreased motor recovery after α1-AR antagonism (170-172, 174-177, 197). For example, in animals which were recovered from beam walk deficits, a single administration of the α1-AR inhibitor prazosine transiently increased hemiplegic symptoms (172). Dunn-Meynell *et al.* suggested that a blockade of α1-ARs may lead to enhanced excitatory neurotransmission, which exacerbates behavioral deficits (177). In contrast, the study by Inoue *et al.* indicates that blocking α1-ARs prevents somatosensory deficits associated with brain trauma. In this study, the functional state of the traumatized brain was assessed with the deoxyglucose method, which is based on the finding that cortical depression of glucose utilization is correlated with somatosensory deficits (179).

There are no changes in α2-AR expression reported after TBI (169, 172, 174, 178). Stimulating α2-ARs negatively affects locomotion recovery after TBI. For example, α2-AR agonists transiently reinstate motor deficit following initial recovery (169, 172, 174, 178). Furthermore, Goldstein *et al.* showed a slower rate of recovery (via beam-walking measurement) after a single treatment with clonidine (α2-AR agonist) (170). However, effects of α2-AR agonists include reduced spasms, muscle stimulation and neuroprotection. For example, dexmedetomidine (α2-AR agonist) has a neuroprotective effect in an organotypic, hippocampal slice culture model for TBI (184).

Inhibition of α2-ARs facilitates recovery (170). α2-ARs antagonists also have neuroprotective effects. Models of cerebral ischemia, excitotoxicity and devascularization-induced neurodegeneration show that α2-AR antagonists provide neuroprotection (181). Furthermore, the noradrenergic system plays a role in promoting neuron survival in areas of the adult brain where neurogenesis persists (181). In addition, the excitability of motor neurons in the motor cortex is stimulated by α2-AR antagonists which have increased central NE. This process is closely linked to neuroplasticity (183).

In conclusion, α1-AR antagonism decreases functional recovery after TBI, however there are some conflicting data (**Table III**). The effects of α1-AR stimulation after TBI are currently unknown.

Literature on α2-AR stimulation mainly reports detrimental effects on motor recovery, whereas α2-AR antagonists are found to be neuroprotective (**Table III**). In other words, drugs that decrease NE release from presynaptic neurons (such as α2-AR agonists) or block α1-ARs on postsynaptic neurons impede functional recovery (such as beam-walking performance or behavioral deficits). On the other hand, drugs that increase NE release (such as α2-AR antagonists) facilitate recovery (176, 177, 198). Currently, extensive literature on the effects of α-AR modulation on pain and spasms after TBI is lacking. Therefore, more research is necessary to define a concise conclusion on the role of ARs in these pathologies.

2.3.1.3 Effects of confounders on alpha-adrenergic pathway modulation

Throughout this review, it has become clear that the effects of α-adrenergic pathway modulation on pain, spasms and paralysis are complex. Many reports demonstrate opposing effects of α-AR modulation on all three aspects, probably due to a different experimental set-up, interspecies differences and variations in lesion severity or level. In cats, the noradrenergic system seems to be the most important pathway for the initiation of locomotion (199). In a study by Barbeau and colleagues, the initiation and modulation of the locomotor pattern by noradrenergic, serotonergic and dopaminergic drugs was investigated in the cat model of chronic SCI.

Here, the noradrenergic pathway modified the timing, whereas the serotonergic and dopaminergic systems modified the output elements (e. g. increased tonic activity in all hindlimb muscles) (199). On the other hand, in rat and mouse SCI-models, serotonin and other agonists of 5-HT receptors have been shown to activate the locomotor circuitry (200, 201).

The response to different agonists of monoaminergic systems can also differ depending on the lesion type/severity. For example, when agonists of the different monoaminergic PWs were applied intrathecally in the chronic phase after a C4/C5 hemisection, particular parameters of hindlimb locomotion were mostly influenced in a negative, non-functional direction (e.g. increase in dragging) (202).

Contrarily, a complete thoracic SCI in rats lead to positive locomotor responses after treatment with monoaminergic agonists (200). This could be explained by a different number, sensitivity and distribution (pre- versus postsynaptic) of monoaminergic receptors in spinal cord injury models of various severity (203).

Filli *et al.* also highlighted differences between the fore- and hindlimb locomotor networks. They revealed a different responsiveness to monoaminergic agonists after unilateral cervical SCI (202). It was suggested that forelimb movements require more supraspinal commands controlling motor function, whereas hindlimb circuits function more autonomously by a central pattern generator (202).

2.4 Summary and future directions

The α-ARs are expressed on many cells within the CNS and the immune system. In the CNS, they are involved in mediating many diverse functions, ranging from reacting to stressors to the regulation of locomotor activity. Their expression on immune cells, on the other hand, is related to the regulation of inflammatory processes. All these aspects are crucial after varying types of CNS damage such as SCI and TBI. Both SCI and TBI are characterized by a strong inflammatory component. The primary injury is followed by a dramatic pro-inflammatory response which is the beginning of a complex process of secondary damage.

This is accompanied by substantial astrogliosis and the production of various inhibitory side-products (e. g. chondroitine sulphate proteoglycans as components of the glial scar), which impede axon regeneration and may negatively influence endogenous neurogenesis (204). Hence, it is hoped that limiting this pro-inflammatory response may create a more permissive environment for stimulating repair processes.

Table III gives a schematic overview of the effects of α-AR modulation in SCI and TBI. It is important to note that the table is an intentionally simplified representation, as α-AR pharmacotherapy is further complicated by the complexity of the numerous pathways within the noradrenergic system and by the inhibitory micro-environment after CNS trauma.

In addition, the table highlights the mutual effects of spasms and exaggerated reflex activity on functional outcome after SCI. Hypothetically, after α1-AR agonism, the negative effects (increased pain reaction and spasms/reflexes) outweigh the positive (improved locomotion), when spasms and exaggerated reflexes are considered to be detrimental for functional outcome, which is for example the case in completely paralyzed SCI-patients. However, it needs to be emphasized that spasms and reflexes can be essential for functional recovery in other situations (e. g. incomplete lesion). Contrarily, after α1-AR antagonism, the positive effects (decreased spasms/reflexes and prevention cortical dysfunctions) outweigh the negative (decreased locomotion), considering the same simplification.

After TBI, α1-AR antagonism decreases motor function, although it prevents cortical dysfunction. Agonism for the α2-AR has been reported to have both beneficial and detrimental effects on pain and locomotion, although neuroprotection adds up to the positive effects. After TBI, stimulation of these receptors decreases locomotion ability. Antagonism of α2-AR in SCI has been reported to decrease locomotion and to increase pain and spasms. For TBI, it improves locomotion and causes neuroprotection.

There is an ongoing discussion whether spasms are beneficial, detrimental or both, depending on context and the phase of the injury. It is a notorious problem that most studies do not distinguish between potentially beneficial or detrimental spasms.

The results of the studies summarized in this review indicate that ARs are valuable pharmacological targets for the treatment of CNS trauma. Modulation of the α-ARs may improve locomotion directly by facilitating the transmission of descending motor and sensory inputs to neurons caudal to the lesion, or indirectly via immunomodulation to reduce the pro-inflammatory response after trauma in the CNS. To enhance functional recovery, this immunomodulation must be accompanied by other regenerative processes like neurite outgrowth, angiogenesis, synapse formation, degradation of the scar tissue, etc.

Immunomodulation and neuromodulation go hand in hand when attempting to activate and stimulate these processes.

Although the complexity of the reported effects is high, a combination of α-AR modulators may be a promising therapeutic strategy for SCI and TBI. Importantly, additional parameters, such as type of injury (SCI or TBI), severity of lesion (e. g. partial or complete SCI), lesion level (e. g. cervical or thoracic SCI), time after injury, species differences and differences in receptor type and distribution before and after injury need to be considered when designing an effective treatment.

To conclude, pain, spasms, and paralysis appear to be closely connected within the adrenergic system. These parameters need to be carefully analyzed and balanced, given that all three units of the triad will be influenced by α-AR modulation. Hence, future research needs to focus on fine-tuning targeted approaches to improve functional recovery.

Chapter 3

Beta-adrenoceptor modulation to analyse the effects of the neuroimmune stress pathway in SCI

Chapter 3. Beta-adrenoceptor modulation to analyse the effects of the neuroimmune stress pathway in SCI

3.1 Abstract

Inevitably the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system of the stress systems will be activated after spinal cord injury (SCI). This will cause hormonal and metabolic changes with direct and indirect effects on the inflammatory system. Next, the inflammatory response will play a major part in the increase of the secondary injury and the loss of function.

Preliminary data show reduced functional recovery by blocking the beta-adrenoceptors (β-ARs) of the sympathetic nervous system (SNS). This indicates the importance of the β-adrenergic pathway for functional regeneration. We hypothesized that stimulating the β-adrenergic pathway provides neuroprotection and neuronal outgrowth in order to stimulate functional regeneration after SCI.

Neural viability and outgrowth experiments elucidate that propranolol (general β-AR antagonist) reduces primary neuron viability without effects on neurite outgrowth, whereas clenbuterol (β2-AR agonist) did not affect neuronal viability or outgrowth. In the SCI mouse model, propranolol decreased the functional outcome accompanied with a decrease in the number of $CD4^+$-T cells after SCI, whereas clenbuterol or xamoterol (β1-AR agonist) had no effects on functional recovery. Considering the detrimental effects of β-AR antagonism with propranolol on primary neuron viability *in vitro* and functional recovery and helper T cells *in vivo*, we suggest that the β-adrenergic pathway is indispensable for SCI recovery. Future studies are needed to provide insight in the pathways involved in SCI which are indirectly affected by the β-adrenergic system, in order to obtain a more targeted modulation.

3.2 Introduction

Stress is an inevitable part in SCI-patient's life. A generally accepted definition states that 'stress' is a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), which in turn activates a stress response. The direction of the stress response on the immune system is influenced by many factors, one being the duration of stress exposure. Acute stress exposure activates the fundamental defense mechanisms (fight-or-flight) and, hence, the immune system, whereas chronic stress exposure results in immune suppression (205).

Stress in humans affects cortisol and catecholamine ([nor]epinephrine) levels, which are the major mediators of two canonical pathways in a stress response, namely the hypothalamic-pituitary-adrenal (HPA) axis and the systemic/adrenomedullary sympathetic nervous system (SNS). These mediators will have a big influence on the immune system as there exists a complex brain-immune system interaction (206). Briefly, the cortisol and (nor)epinephrine secreted by the HPA axis or the SNS are secreted into the peripheral blood by which they have excess to innate and adaptive immune cells. These immune cells, in turn, will modulate their secretion of cytokines and growth factors, that again could influence the stress pathways via a positive feedback loop (207).

Taken together, the stress systems will react to SCI by hormonal and metabolic changes with direct and indirect effects on the inflammatory system. Next, the inflammatory response will play a major part in the increase of the secondary injury and the loss of function. By these means, stress has a big influence on functional recovery after SCI.

Moreover, preliminary data from our group have indicated that 30 min peri-operative hypothermic stress in mice leads to a dramatic increase in neurodegeneration and to a mortality up to 100% which can be prevented by perioperative warming.

In addition, we found that short-term hypothermic stress (4 min) leads to a significantly decreased functional recovery after SCI in mice, accompanied by increased corticosterone levels in serum samples of the hypothermic mice as compared to normothermic controls, indicating that the applied stress has a big impact on functional recovery.

These data urged us to study the systemic effects of different stress pathway blockers *in vivo*. Eventually, this led to reduced functional recovery when we blocked the beta-adrenoceptors (β-ARs) of the SNS, which indicated the importance of the β-adrenergic pathway for functional regeneration. We hypothesized that stimulating the beta-adrenergic pathway provides neuroprotection and neuronal outgrowth in order to stimulate functional regeneration in our T-cut hemisection mouse model.

The local effects of selected mediators (propranolol; a general β-AR antagonist, and clenbuterol; a β2-AR agonist) of β-ARs were studied using neurite outgrowth and neuronal survival assays *in vitro*. These experiments elucidated that propranolol reduces primary neuron viability, whereas clenbuterol did not affect neuronal viability or outgrowth. In our spinal cord injury mouse model, propranolol decreased functional outcome, accompanied with a decrease in the number of $CD4^+$-T cells, whereas clenbuterol, salbutamol (β2- AR agonist) or xamoterol (β1-AR agonist) had no effects on functional recovery. These results suggest that the β-adrenergic pathway is indispensable for neuron survival and functional recovery. However, further studies are needed to investigate whether targeted stimulation of the pathway can be a valid therapeutic strategy.

3.3 Materials and Methods

3.3.1 Primary neurons

3.3.1.1 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 (0.1 mg/ml) in Hank's balanced salt solution (HBSS), followed by mechanical dissociation 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% CO2 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).

3.3.1.2 MTT assay

To measure the effects of propranolol and clenbuterol on metabolic activity (cell viability/proliferation), we have used 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. Cells were incubated for 72h in neuron medium with selected concentrations of propranolol (0,01-100 µM; propranolol hydrochloride, Sigma-Aldrich, Overijse, Belgium) or clenbuterol (0,01-10 µM; clenbuterol hydrochloride, Sigma-Aldrich, Overijse, Belgium). After this incubation period, 1mg/ml MTT (Sigma, Diegem, 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 540nm.

3.3.1.3 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 either propranolol (0.1-10 μM) or clenbuterol (1-100 μM) was added to the neuron medium. After 72h, neurons were fixed with 4% paraformaldehyde (PFA) in 0.1M PBS, permeabilized using PBS containing 0.2% Triton X-100, and incubated with 10% normal goat serum (NGS) in PBS to block nonspecific binding. Cells were stained subsequently with primary monoclonal mouse anti-beta-III-tubulin antibody (0.8 μg/ml; Millipore, Brussels, Belgium) (overnight, 4°C) and goat anti-mouse Alexa 488 secondary antibody (1/250; Invitrogen, Belgium) (1h, room temperature). DAPI was used to visualize the cells nuclei, and coverslips were mounted onto glass slides using immu-mount (ThermoFisher Scientific, Belgium). Fluorescent pictures were taken of 6 randomly chosen fields per coverslip with the Nikon Eclips 80I and neurite outgrowth has been analyzed using Neurite Outgrowth Assay 6.1 (NEO 6.1; DCI labs, Keerbergen, Belgium).

3.3.2 Neurite outgrowth from collagen-embedded Organotypic entorhinal cortex slices

All slice culture reagents used in the two models described below were obtained from Gibco® (Invitrogen, Belgium) unless stated otherwise. Entorhinal cortex slices were prepared from P2 mice as described before (208). Briefly, brains were removed and placed in ice-cold dissection medium (MEM supplemented with 2 mM L-glutamine). Slices containing the entorhinal cortex region were prepared using a McIwain tissue chopper (350 μm thickness; H. Saur, Germany). Each entorhinal cortex slice was embedded in a single drop of collagen (type I, 2mg/mL; Sigma, Diegem, Belgium) and cultured for 48 hours in the presence or absence of propranolol (0.01-10 μM) or clenbuterol (0.01-10 μM). Neurite outgrowth was measured as described before. Picture processing was performed based on the Sobel algorithm after which the mean intensity was calculated in a standardized area of each slice (209).

3.3.3 Experimental spinal cord injury and β-AR modulator treatment

The *in vivo* experiment was performed using female C57BL/6j mice (9-10 weeks old; Janvier, France). They were housed in a conventional animal facility at Hasselt University under regular conditions, i.e. in a temperature-controlled room (20 ± 3 °C) on a 12 h light-dark schedule and with food and water ad libitum. 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.

A hemisection injury was performed as previously described (31). Briefly, mice were anesthetized after which a partial laminectomy was performed at thoracic level 8 to expose the spinal cord. A bilateral hemisection injury was induced to the spinal cord by using iridectomy scissors to transect left and right dorsal funiculus, the dorsal horns and additionally the ventral funiculus. This "T-cut" hemisection results in a complete transection of the dorsomedial and ventral corticospinal tract and impairs several other descending and ascending tracts. Afterwards, muscles were sutured and the back skin was closed with wound clips (Autoclip®, Clay-Adams Co., Inc.). Glucose solution (20%) was given after the operation to compensate for any blood loss during surgery. All mice were placed in a temperature-controlled chamber (33°C) until thermoregulation was established. Bladders were emptied manually until a spontaneous return of the micturition reflex. Mice were intraperitoneously injected twice a day (morning and evening) starting at 2 days before SCI until 3 days post injury with either propranolol (general β-AR antagonist; 20 mg/kg), xamoterol (β1-AR agonist; 2 mg/kg), clenbuterol (β2-AR agonist; 2 mg/kg) or the vehicle control saline (0.9% NaCl).

Locomotor recovery of the animals was determined by an investigator blinded to the experimental groups using the BMS (30). During the first week after injury, mice were scored daily and from the start of the second week until the end of the observation period, mice were examined every second day.

3.3.4 Immunohistochemistry and quantitative image analysis

At 21 dpi, mice were overdosed with Nembutal and transcardially perfused with Ringer solution containing heparin, followed by 4% paraformaldehyde in phosphate-buffered saline (PFA, pH 7.4). Spinal cords were dissected and dehydrated by incubation in 5% sucrose in 4% PFA, followed by a 30% sucrose solution in PBS. Next, the samples were embedded in Tissue-Tek O.C.T. Compound (Sakura, Belgium) and frozen in liquid-nitrogen cooled isopentane. Immunohistochemical stainings were performed on 10 µm thick saggital cryosections of these spinal cords. Spinal cord sections were blocked with 10% normal goat serum and permeabilized with 0.05% Triton X-100 in PBS for 30 min at RT. Then, the spinal cord sections were incubated with the primary antibodies mouse α-GFAP (Glial fibrillary acidic protein; 1/500, Sigma, Diegem, Belgium), rabbit α-Iba-I (ionized calcium binding adaptor molecule 1; 1/350, Wako, Neuss, Germany), rat α-CD4 (cluster of differentiation 4; 1/250, BD Biosciences, Erembodgem, Belgium) overnight at 4°C in a humidified chamber. Following repeated washing steps with PBS, spinal cord sections were incubated with goat anti-mouse Alexa 568, goat anti-rabbit Alexa 488 and goat anti-rat Alexa 568 secondary antibodies (1/250; Invitrogen) for 1 h at room temperature. Specificity of the secondary antibody was verified by including a control staining in which the primary antibody was omitted (data not shown). Autofluorescence was controlled for by omitting the primary and the secondary antibody. After removal of unbound antibodies, DAPI counterstaining was performed for 10 min and sections were mounted. Images were taken with a Nikon Eclipse 80i microscope and a Nikon digital sight camera DS-2MBWc.

Quantitative image analysis were performed on original unmodified photos using the ImageJ open source software. For standardization, analyses were performed on 6-9 spinal cord sections (per mouse) representing the lesion area, i.e. the lesion epicenter as well as consecutive sagittal sections, as previously described (31). Lesion size was evaluated using anti-GFAP immunofluorescence.

The T helper cell infiltration was evaluated by double staining against CD4 and Iba-1 in order to exclude $CD4^+$ microglial cells. Quantification of GFAP and Iba-1 expression was performed by intensity analysis using ImageJ open source software (NIH) within rectangular areas of 100 µm × 100 µm extending from 600 µm cranial to 600 µm caudal from the lesion epicenter. The infiltration of T helper cells was determined by quantifying all T helper cells in the entire spinal cord cryosection.

3.3.5 Statistics

Statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, Inc.). Data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. This test indicated that all data sets were not-normally distributed, except for the GFAP and Iba-1 expression data. Statistical differences between two groups were analyzed via the nonparametric Mann-Whitney U test and to compare multiple groups, a Kruskal Wallis test followed by a Dunn's Multiple comparison test was used. GFAP and Iba-1 expression were analyzed using regular two-way ANOVA. Data were presented as mean ± standard error of the mean (SEM). Functional recovery *in vivo* was statistically validated by day-per-day comparisons between groups by the Mann-Whitney U-test and were presented as box plots with whiskers indicating the minimum and maximum. Differences were considered statistically significant when $p<0.05$.

3.4 Results

3.4.1 Propranolol decreases primary neuron viability, whereas clenbuterol has no effects

Preliminary data of our research group indicated that the β-blocker propranolol worsened functional recovery after SCI. These data made us suggest that β-adrenoceptor agonism (e.g. with clenbuterol) improves functional recovery after SCI. First the local effects of propranolol and clenbuterol on primary neuron viability and neurite outgrowth in primary neuron culture and organotypic brain slices have been investigated.

To investigate the effects of the mediators on primary neuron viability, the metabolic activity was measured by means of an MTT-assay. Prior to the MTT-assay, the primary neurons were incubated with different concentrations of either propranolol (0.01-100 μM) or clenbuterol (0.01-10 μM). The control group did not receive any treatment; the cells were incubated with neuron medium. Propranolol decreased the metabolic activity of the primary neurons compared to control, in all concentrations used (**figure 3.1A**). In particular 100 μM propranolol acted neurotoxic with only 9% metabolic activity left compared to control (**figure 3.1A**). Therefore, it was decided not to use this concentration in follow up experiments. Clenbuterol, on the other hand, did not seem to be neurotoxic as it did not affect the metabolic activity of the cells (**figure 3.1B**).

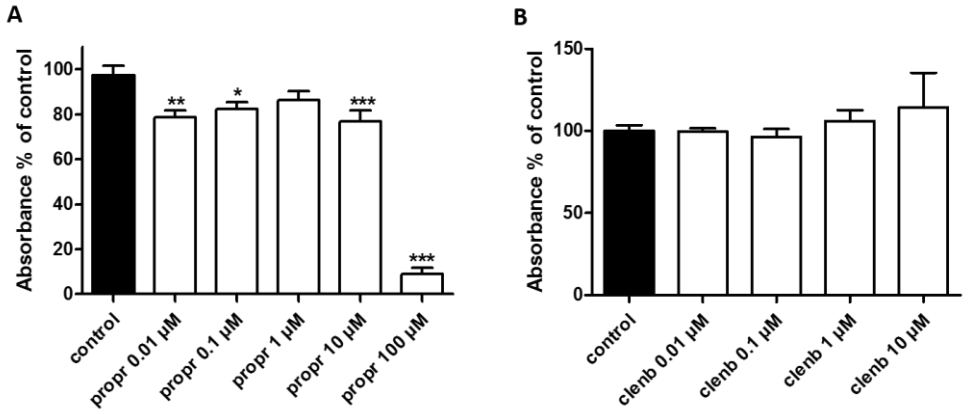

Figure 3.1: Propranolol decreases primary neuron viability, whereas clenbuterol has no effects. Primary neurons were treated for 72h with propranolol (0.01-100 µM) (A) or clenbuterol (0.01-10 µM) (B). The control contains neuron medium. **A)** Propranolol decreases primary neuron viability in all the concentrations used, with 100 µM being the most neurotoxic. **B)** Clenbuterol has no effects on primary neuron viability. Data were normalized to control and represented as mean ± SEM; n= 8-33 wells/condition: *p<0.05, **p<0.01, ***p<0.001.

3.4.2 Propranolol and clenbuterol do not affect neurite outgrowth in primary neuron culture.

To measure the local effects of the β-adrenergic mediators on neurite outgrowth, primary neurons seeded on cover slips in a 24-well plate were treated for 72h with either propranolol (0.1-10 µM) or clenbuterol (1-100 µM). The neurites were visualized by fluorescent beta-III-tubuline staining. Three parameters were quantified by the NEO-software, namely the mean number of neurites per neuron (mean # neurites/ neuron), the mean neurite length per neuron (mean neurite length (µm)/ neuron) and the mean number of branches per neuron (mean # branches/ neurons). We have selected these because we believe that the more neurites and branches and the longer they are, the better they can cross the lesion and synapse to the correct target. However, none of the three parameters was influenced by propranolol (**figure 3.2A-C**) or clenbuterol (**figure 3.2D-F**).

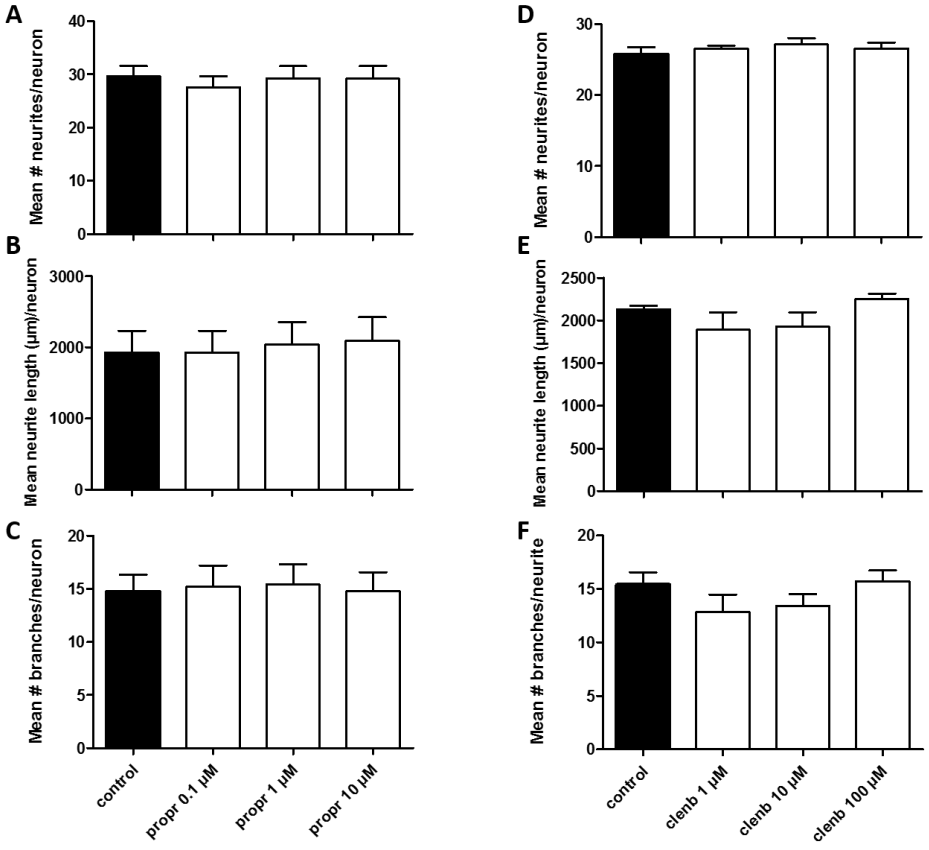

Figure 3.2: Propranolol and clenbuterol do not affect neurite outgrowth in primary neuron culture. Primary neurons were treated for 72h with propranolol (0.1-10 µM) or clenbuterol (1-10 µM). The control contains neuron medium. After beta-III tubuline staining, three parameters were quantified: the mean # neurites/neuron, the mean neurite length (µm)/neuron and the mean # branches/neuron. **A-C)** Propranolol did not affect the neurite outgrowth by the primary neurons, compared to control. **D-E)** Neither did clenbuterol. Data were presented as mean ± SEM; n= 4-12 wells/condition.

3.4.3 Propranolol and clenbuterol do not affect neurite outgrowth in entorhinal cortex slice culture

To investigate the effects of propranolol and clenbuterol on neurite outgrowth in a tissue context, organotypic entorhinal cortex slices have been used. Analyses of the neurites sprouting out of the slices after 48h incubation with the mediators did not reveal any significant differences between treatment or control (**figure 3.3A-B**).

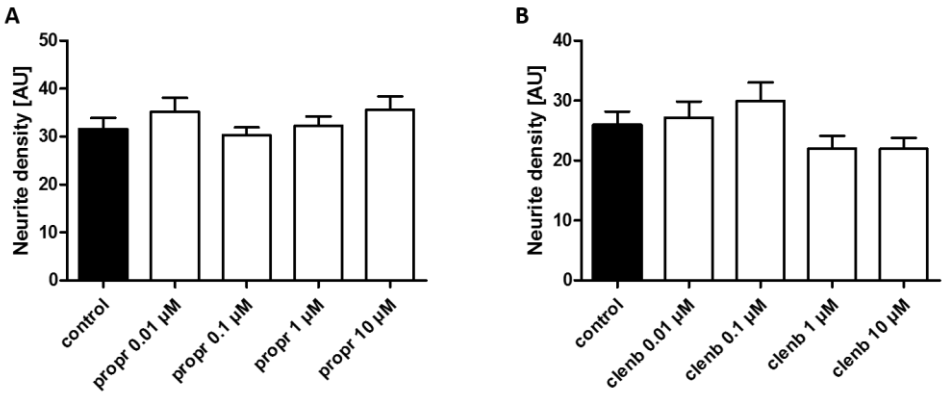

Figure 3.3: Propranolol and clenbuterol do not affect neurite outgrowth in entorhinal cortex slice culture. Entorhinal cortex slices were treated for 48h with propranolol (0.01-10 µM) (A) or clenbuterol (0.01-10 µM) (B). The control contains NGM. **A-B)** Neither propranolol or clenbuterol effect neurite outgrowth from the brain slices. Data were presented as mean ± SEM; n= 16-22 slices/condition.

3.4.4 β-adrenoceptor agonism with xamoterol or clenbuterol does not improve functional recovery after SCI, although antagonism with propranolol worsens the functional outcome

Preliminary data of our research group indicated that the β-blocker propranolol worsened functional recovery after SCI (**figure 3.4A**). This decrease in functional outcome was accompanied with a decrease in the number of T-helper cells (**figure 3.4E**), although the other important neuro-immune parameters after SCI lesion size, astrogliosis and macrophage/microglia infiltration) were not influenced (**figure 3.4B-D**).

Based on these data, we hypothesized that β-adrenoceptor agonism improves functional recovery after SCI. This hypothesis was addressed in our T-cut hemisection mouse model, treated with either xamoterol (**β1-AR agonist**) or clenbuterol (**β2-AR agonist**). Xamoterol and clenbuterol do not affect functional recovery after SCI, compared to the vehicle control, as no differences in the BMS were indicated (**figure 3.5A-B**).

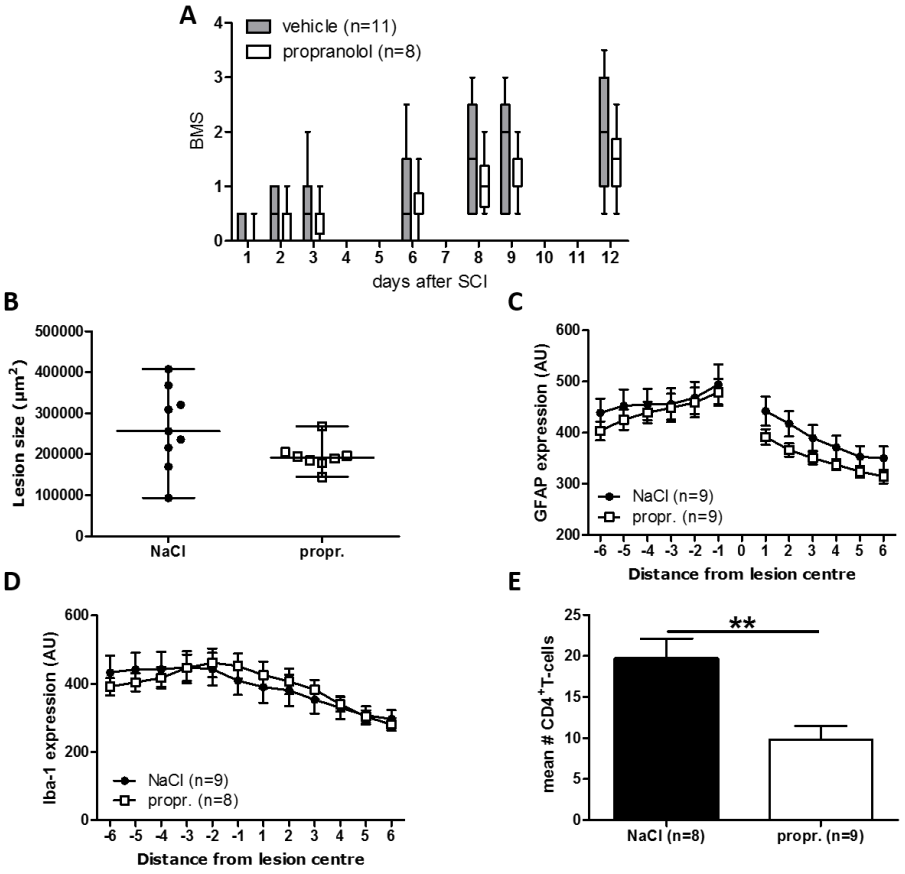

Figure 3.4: Propanolol decreases the number of CD4$^+$ T cells, whereas astrogliosis and phagocyte infiltration were not altered. A) Mice were injected (i.p.) twice a day, two days before until three days after SCI, with either propranolol (20 mg/kg) or the vehicle NaCl. Functional recovery was analyzed according to the BMS. A trend indicates reduces functional recovery after propranolol treatment, compared to vehicle control. The results are presented as box plots with the ends of the whiskers representing the minimum and maximum. **B-C)** Spinal cord cryosections were stained for GFAP after which the lesion size was measured by delineating the GFAP- lesion with ImageJ software. Astrogliosis was quantified by measuring the GFAP intensity by ImageJ. No differences were detected between the propranolol or the NaCl-treated group. **D)** Macrophage/microglia infiltration were quantified by measuring the intensity of the Iba-1 staining by ImageJ. No differences were detected between the different groups. **E)** Propranolol treatment significantly

decreased the number of $CD4^+$ T cells, compared to the control group. The data are presented as mean values ± SEM; n= as indicated in the figures; **p<0.01.

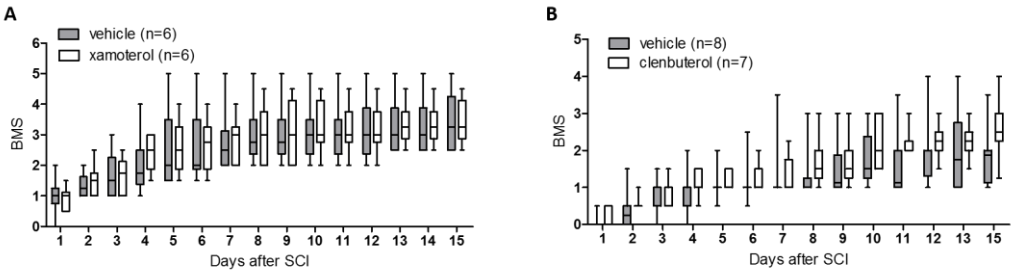

Figure 3.5: Xamoterol (beta-1 AR agonist) and clenbuterol (beta-2 AR agonist) do not affect functional recovery after SCI. Mice were injected (i.p.) twice a day, two days before until three days after SCI, with xamoterol (2 mg/kg) (A), clenbuterol (2 mg/kg) (B) or NaCl (vehicle control). Functional recovery was analyzed according to the BMS. **A)** β-1 AR agonism with xamoterol does not improve functional recovery after SCI, compared with control. **B)** Similarly, β-2 AR agonism with clenbuterol does not improve functional recovery after SCI. The data are presented as box plots with the whiskers indicating the minimum and the maximum; n= as indicated in the figure.

3.5 Discussion

In this study, we show that propranolol reduces primary neuron viability, whereas clenbuterol did not affect neuronal viability or outgrowth. Moreover, in our SCI mouse model, a trend indicates decreased functional outcome accompanied with a significant reduction in the number of $CD4^+$-T cells after SCI, whereas clenbuterol or xamoterol had no effects on functional recovery.

To investigate the underlying mechanisms more profoundly, we assessed the local effects of propranolol on primary neuron viability and neurite outgrowth. MTT-assay revealed that propranolol (β-AR blockage) reduced the metabolic activity of the primary neurons, whereas clenbuterol (β-AR stimulation) had no effects. Strikingly, neurotoxic effects of β-blockers have never been indicated before.

β-AR stimulation, on the other hand, provided neuroprotection in mixed neuron-astrocyte hippocampal cultures (210, 211). However, the direct local effects of β-AR modulators in primary neuron culture were never investigated before.

In our hands, neither of the mediators affected neurite outgrowth in primary neuron culture or entorhinal cortex slice culture, although others report positive effects of β-AR agonism on neuronal branching and growth cone formation (212). Similar to our results, Day *et al* did not found direct effects of β-AR agonism on primary neurons. Interestingly, they show that NE acting at glial β2-ARs induces neurite growth through the expression of soluble factors that elicit a neurotrophic action and increase neuronal complexity (213). These data indicate that β-AR stimulation may indirectly stimulate neurite outgrowth via astrocytes or other neuro-immune cells. However, we could not confirm these results in the entorhinal cortex slices, which is a complex organotypic *in vitro* model allowing the analysis of the interaction of neurons, astrocytes and microglia (214). The different results could be explained either by pharmacokinetic differences between the different compounds (NE vs. clenbuterol) or by important methodological differences (cell co-culture model vs. organotypic brain slice model) in the study of these factors.

In vivo, propranolol decreased the number of $CD4^+$ T cells after SCI. This reduction in helper T cells may dysregulate the action of $CD8^+$ T cells causing a Th1-response and axonal loss, as shown in a mouse model for multiple sclerosis (215, 216). Contrarily, others report promotion of differentiation towards Th2 cells after β-AR agonism (216, 217). In depth investigation is necessary to reveal whether β-AR antagonism leads to Th1 polarization. Anyway, β2-AR modulation on lymphocytes regulates the level of lymphocyte activity differentially, depending on the time of receptor engagement in relation to the activation and differentiation state of the cell, the molecular signaling pathway activated, and the cytokine microenvironment, which are all very variable in each stage of SCI (reviewed by (218)).

Previously, we have studied the systemic effects of different stress pathway blockers *in vivo* (unpublished data). Eventually, this led to reduced functional recovery when we blocked the β-ARs of the SNS with propranolol, which indicated the importance of the β-adrenergic pathway for functional regeneration. Therefore, we hypothesized that stimulating the beta-adrenergic pathway promotes functional regeneration in T-cut hemisection SCI mouse model. However, neither β1-AR agonism with xamoterol or β2-AR agonism with clenbuterol promoted functional recovery. The effects of β1-AR agonism in the context of SCI were not investigated before. Our β2-AR results confirm the results of other studies using rat and mouse transection SCI models. No effects on locomotion recovery were detected by using clenbuterol alone, although it was often a necessary co-factor to prevent muscle atrophy for example (45, 46). However, Zeman and colleagues thoroughly investigated the effects of clenbuterol after SCI in a rat contusion SCI model (47). They show that clenbuterol improves locomotion and reduces tissue loss, compared to vehicle control. They suggest that activation of β2-ARs during the acute phase of injury stimulates glutathione-dependent antioxidative processes, that lead to reduced oxidative damage. However, further studies are needed to determine the affected cell types (47).

Taken together, β-AR agonism with clenbuterol (β2- AR agonist) or xamoterol (β1-AR agonist) did not affect functional recovery or neuronal viability and outgrowth. However, the detrimental effects of β-AR antagonism with propranolol on primary neuron viability *in vitro* and functional recovery and helper T cells *in vivo* let us suggest the β-adrenergic pathway is indispensable for SCI recovery. Future studies are needed to provide insight in the pathways involved in SCI which are indirectly affected by the β-adrenergic system, in order to target relevant pathways more specifically (e.g. in time and place or cell-specific).

Chapter 4

Beta-adrenoceptor modulation to stimulate angiogenesis in order to improve functional regeneration after SCI

Based on:

The β2-Adrenoceptor Agonist Terbutaline Stimulates Angiogenesis via Akt and ERK Signaling

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Chapter 4. Beta-adrenoceptor modulation to stimulate angiogenesis in order to improve functional regeneration after SCI

4.1 Abstract

Angiogenesis is associated with changes in endothelial cell (EC) proliferation and tube formation, controlled by extracellular receptor-activated kinase (ERK)/mitogen activated protein kinase (MAPK) and Akt signaling. Important regulators of these systems include hormones acting on G-protein-coupled receptors, such as beta 2-adrenoceptors (β2-ARs). In central nervous system (CNS) trauma, the importance of β2-AR modulation has been highlighted, although the effects on revascularization remain unclear. Vascular protection and revascularization are, however, key to support regeneration.

We have investigated the angiogenic capacity of the specific β2-AR agonist terbutaline on ECs derived from the CNS, namely bEnd.3-cells. As angiogenesis is a multistep process involving increased proliferation and tube formation of ECs, we investigated the effects of terbutaline on these processes. We show that terbutaline significantly induced bEnd.3 tube formation in a matrigel in vitro assay. Moreover, administration of specific inhibitors of ERK and Akt signaling both inhibited terbutaline-induced tube formation. The proliferation rate of the ECs was not affected. In order to investigate the general effects of terbutaline in an organotypic system, we have used the chick chorioallantoic membrane (CAM)-assay. Most importantly, terbutaline increased the number of blood vessels in this in ovo setting. Although we observed a positive trend, the systemic administration of terbutaline did not significantly improve the functional outcome, nor did it affect revascularization in our spinal cord injury model.

In conclusion, these data indicate that terbutaline is promising to stimulate blood vessel formation, underscoring the importance of further research into the angiotherapeutic relevance of terbutaline and β2-AR signaling after CNS-trauma.

4.2 Introduction

The vascular system supports normal tissue function through the delivery of oxygen and nutrients, metabolic waste disposal and immune surveillance. Within the human body, three mechanisms of blood vessel formation can be distinguished: vasculogenesis, angiogenesis and arteriogenesis. Angiogenesis is not only a fundamental event during embryogenesis, but also during adult life, as it is a key process during wound healing (219). Under physiological conditions, angiogenesis does not take place because anti-angiogenic factors (e.g. angiostatin, endostatin) are in excess to pro-angiogenic factors (e.g. vascular endothelial growth factor, VEGF; basic fibroblast growth factor, bFGF). However, during wound healing and cancer development, pro-angiogenic factors are produced and the balance is skewed towards blood vessel growth (54). Adult angiogenesis is associated with endothelial cell (EC) proliferation and migration, controlled by extracellular receptor-activated kinase (ERK)/mitogen activated protein kinase (MAPK) and Akt signaling pathways (49, 220). In addition, EC apoptosis is strictly regulated during vascular growth by Akt signaling which protects ECs against apoptosis.

Damage to the vasculature and breakdown of the blood-brain barrier (BBB) are universal consequences after traumatic brain injury and spinal cord injury (SCI). Most importantly, the unfavorable regulation of pro-angiogenic and counterregulatory anti-angiogenic factors after CNS trauma is suspected to participate in the failure of revascularization and vessel stabilization (43). Vascular protection and revascularization are key to support survival of sprouting and regenerating axons (44, 50). Numerous angiogenic factors (e.g. vascular endothelial growth factor, VEGF) were already investigated for their effects after traumatic SCI, although with variable success (reviewed in (43)).

On the other hand, for beta 2-adrenoceptor (β2-AR) agonists such as isoproterenol (ISO), salmeterol and clenbuterol, it has already been shown that they have neuroprotective effects and they improve the neurological and functional outcome (40-44). These data indicate the importance of β2-AR modulation after CNS trauma, although previous studies did not focus on functional revascularization.

Beta-adrenoceptors are important G-protein-coupled receptors that belong to the adrenergic system and are involved in cardiac and vascular function. They are implicated in important signaling pathways during angiogenesis, such as the Akt- and ERK pathway mentioned above. The β2-ARs are most abundantly expressed on the vasculature and modulate the release of nitric oxide (NO), which in turn causes vasodilation (49). Their relevance to stimulate angiogenesis was recently confirmed as the expression of the receptors on endothelial progenitor cells (EPCs) and mature ECs coincides with increased proliferation and migration after stimulation with the non-specific β-AR agonist isoproterenol (48).

In this study, we have investigated the role of the short-acting and highly specific β2-AR agonist terbutaline (C12H19NO3) in the control of angiogenesis within the context of CNS trauma. Specifically β2-ARs were stimulated as this adrenoceptor subtype is abundantly expressed on the vasculature. It is expressed on ECs where it plays a role in vasodilation, EC proliferation, and EC migration. In contrast to clenbuterol and zilpaterol (specific β2-AR agonists), terbutaline is not reported to be anabolic, thereby avoiding adverse effects such as cardiac hypertrophy (221-223). Salbutamol is another specific β2-AR agonist correlated with angiogenesis. However, this agonist was reported to decrease the number of blood vessels in the chorioallantoic membrane (CAM)-assay (224). Therefore, we have selected terbutaline to investigate its angiogenic potential both in vitro and in vivo. Adult mouse brain ECs (bEnd.3) were chosen because they are derived from the brain and hence, they are more suitable to investigate angiogenesis in the context of CNS trauma and to relate our in vitro results with our in vivo results in a mouse model of SCI.

In a first set of in vitro experiments, we show that terbutaline stimulated tube formation of the bEnd.3 cells. Next, we evaluated whether terbutaline could enhance angiogenesis in the chorioallantoic membrane (CAM)-assay, which is an in ovo model for blood vessel development with a complexity that approximates the in vivo situation (225). Consistently, terbutaline treatment increased the number of blood vessels in the CAM assay. In conclusion, this ex vivo study suggests that terbutaline is a promising drug to improve angiogenesis via β2-AR stimulation.

Most importantly, enhanced tube formation at the cellular level as well as increased neo-vascularization in ovo suggest the importance of β2-AR stimulation to induce angiogenesis in order to improve functional regeneration, although in depth research is necessary to reinforce the effects.

4.3 Materials and Methods

4.3.1 Cell culture mouse brain endothelial cells

The bEnd.3 microvascular endothelial cell line was purchased from the American Type Culture Collection (Ampule passage No: 22; ATCC, USA). They were cultured at 37°C at 5% CO2 in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM; ATCC, USA) supplemented with 10% fetal calf serum (FCS; Life Technologies, Belgium), 100 IU.ml-1 penicillin and 100 µg.ml-1 streptomycin (P/S; Sigma-Aldrich, Belgium) in T-25 flasks (Greiner, Belgium). Medium change was performed every 3 days. When confluence was reached, the cells were sub-cultured after treatment with 0.05% trypsin/EDTA solution (Sigma-Aldrich, Belgium). All experiments were performed between passage No 22-30.

4.3.2 Tube formation assay

The effects of different concentrations of terbutaline (0.1; 1; 10 µM; Sigma-Aldrich, Belgium) on tube formation by the bEnd.3 cells were assessed using Ibidi angiogenesis µ-slides (Ibidi GmbH, Planegg/Martinsried, Germany). The inner wells of the µ-slide were coated with growth factor-reduced BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ). On top of the Matrigel 15,000 bEnd.3 cells were seeded, resolved in 50 µl of the desired experimental conditions. The cells were incubated with different concentrations of terbutaline (0.01; 1; 10 µM), terbutaline (1 µM) combined with the PI3K-inhibitor LY294002 (10 µM; Sigma-Aldrich, Belgium) or terbutaline (1 µM) combined with MEK-inhibitor U0126 (10 µM; Sigma-Aldrich). The inhibitors were added at the same time as terbutaline. DMEM without FCS was used as vehicle control. In addition, the pathway blockers have been applied to the controls to confirm their inhibitory capacity. The cultures were maintained for 6h at 37°C in a humidified atmosphere containing 5% CO2. Next, 3 representative pictures per well were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100, Japan) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG, Germany).

Image analysis was performed using the Angiogenesis Analyzer tool from ImageJ (National Institutes of Health, USA). Three parameters were quantified: the total branching length, the number of nodes and the number branches and segments. The tubes form a network. A segment is a tube delimited by two junctions (formed by one or multiple nodes), whereas a branch has an open ending. The total branching length is the sum of the lengths of the branches and segments in one picture. The number of nodes is the number of crossings between the segments. The number of branches and segments is the sum of the branches and segments in one picture. Per well, 3 representative pictures were analyzed and the mean values are presented.

4.3.3 MTT assay

The bEnd.3 cells were seeded in a 96-well plate (Greiner, Belgium) at a density of 7,500 cells per well in their normal culture medium. After attachment to the culture plate, cells were rinsed twice with phosphate buffered saline (PBS) and incubated with DMEM without FCS supplemented with different concentrations of terbutaline (0.01; 1; 10 µM), terbutaline (1 µM) combined with the PI3K-inhibitor LY294002 (10 µM; Sigma-Aldrich, Belgium) or terbutaline (1 µM) combined with MEK-inhibitor U0126 (10 µM; Sigma-Aldrich). DMEM without FCS was used as vehicle control. In addition, the pathway blockers have been applied to the controls to confirm their inhibitory capacity. After 6h or 72h of incubation, the different conditions were replaced by the conditions supplemented with 500 µg/ml MTT (MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Belgium). After 4h of incubation, the MTT solution was removed and a mixture of 0.01 M glycine in DMSO (Dimethyl Sulfoxide; Sigma-Aldrich, Belgium) was added to dissolve the formed formazan crystals. The absorbance was measured at a wavelength of 570 nm with a Benchmark microplate reader (Bio-Rad Laboratories, USA).

4.3.4 Chorioallantoic membrane assay

Fertilized chicken eggs (Gallus gallus; Wijverkens chicken farm, Halle, Belgium) were incubated for 3 days at 37°C in a humidified environment. On embryonic day 3 (E3), the developing CAM was detached from the egg shell by removing 3 ml of albumin. To evaluate whether the eggs were fertilized, a small window was created in the shell and immediately closed with cellophane tape. Six days later (E9), terbutaline (10; 50 and 250 nmol) was applied on plastic discs (8 mm diameter), which were allowed to dry under sterile conditions. Cortisone acetate (100 µg/disc; Dr. Ehrenstorfer GmbH, Germany) was applied to all discs in order to avoid an inflammatory response. The selected doses were based on earlier studies (226). After an incubation period of two days (E11), the CAM was carefully dissected to evaluate angiogenesis. Pictures of each CAM were taken with a stereomicroscope (Wild M3Z Stereomicroscope, Switzerland) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG). All vessels intersecting a concentric circle (radius 3.5 mm) positioned in the treated area were counted by 3 independent investigators who were blinded to the experimental conditions.

4.3.5 BrdU cell proliferation assay

The bEnd.3 cells were seeded in a 96-well plate (Greiner, Belgium) at a density of 20,000 cells per well in their normal culture medium. After attachment to the culture plate, cells were rinsed with PBS and incubated with DMEM without FCS supplemented with different concentrations of terbutaline (0.01; 1; 10 µM). BrdU incorporation was measured after 6 and 48, following the manufacturer's instructions (BrdU Cell Proliferation Kit, Merck Millipore, Belgium).

4.3.6 Experimental spinal cord injury and terbutaline treatment

The in vivo experiment was performed using female C57BL/6j mice (9-10 weeks old; Janvier, France). They were housed in a conventional animal facility at Hasselt University under regular conditions, i.e. in a temperature-controlled room (20 ± 3 °C) on a 12 h light-dark schedule and with food and water ad libitum.

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.

A hemisection injury was performed as previously described (31). Briefly, mice were anesthetized with 3% isoflurane (IsofFlo, Abbot Animal Health, Belgium) and received a subcutaneous injection of the analgesic buprenorphine Temgesic (0.1 mg/kg bodyweight; Val d'Hony Verdifarm, Belgium) before surgery. A partial laminectomy was performed at thoracic level 8 to expose the spinal cord. A bilateral hemisection injury was induced to the spinal cord by using iridectomy scissors to transect left and right dorsal funiculus, the dorsal horns and additionally the ventral funiculus. This "T-cut" hemisection results in a complete transection of the dorsomedial and ventral corticospinal tract and impairs several other descending and ascending tracts. Afterwards, muscles were sutured and the back skin was closed with wound clips (Autoclip®, Clay-Adams Co., Inc.). Glucose solution (20%) was given after the operation to compensate for any blood loss during surgery. All mice were placed in a temperature-controlled chamber (33 °C) until thermoregulation was established. Bladders were emptied manually until a spontaneous return of the micturition reflex.

After surgery, mice were distributed equally among the groups according to their Basso Mouse Scale (BMS) score. Mice were intraperitoneously injected twice a day, for 9 days with either terbutaline (5 mg/kg) or the vehicle control saline (0.9% NaCl). Locomotor recovery of the animals was determined by an investigator blinded to the experimental groups using BMS (30). During the first week after injury, mice were scored daily and from the start of the second week until the end of the observation period (21 days post injury [dpi]), mice were examined every second day.

4.3.7 Immunohistochemistry and quantitative image analysis

At 21 dpi, mice were overdosed with Nembutal and transcardially perfused with Ringer solution containing heparin, followed by 4% paraformaldehyde in phosphate-buffered saline (PFA, pH 7.4). Spinal cords were dissected and dehydrated by incubation in 5% sucrose in 4% PFA, followed by a 30% sucrose solution in PBS. Next, the samples were embedded in Tissue-Tek O.C.T. Compound (Sakura, Belgium) and frozen in liquid-nitrogen cooled isopentane. Immunohistochemical stainings were performed on 10 µm thick saggital cryosections of these spinal cords. Spinal cord sections were blocked with 10% normal goat serum and permeabilized with 0.05% Triton X-100 in PBS for 30 min at RT. Then, the spinal cord sections were incubated with the rat anti-CD31 primary antibody (1/1000; BD Pharmingen, BD Biosciences) overnight at 4°C in a humidified chamber. Following repeated washing steps with PBS, spinal cord sections were incubated with goat anti-rat Alexa 488 secondary antibody (1/250; Invitrogen) for 1 h at room temperature. Specificity of the secondary antibody was verified by including a control staining in which the primary antibody was omitted (data not shown). Autofluorescence was controlled for by omitting the primary and the secondary antibody. Images were taken with a Nikon Eclipse 80i microscope and a Nikon digital sight camera DS-2MBWc.

Quantitative image analysis were performed on original unmodified photos using the ImageJ open source software (NIH). For standardization, analyses were performed on 6-9 spinal cord sections (per mouse) representing the lesion area, i.e. the lesion epicenter as well as consecutive sagittal sections, as previously described (31). Spinal cord lesion areas are subdivided in different regions, i.e. the lesion epicenter, the rostral area and the caudal area. The CD31-positive area was determined at the three locations by selecting the stained area with ImageJ.

4.3.8 Statistics

For the tube formation assays, MTT assays and the BrdU-assays, Mixed model ANOVA was performed using JMP to exclude random effects caused by variability inside one group. Only when this requirement was met, additional statistical analyses were performed on the data set by using GraphPad. The data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. This test indicated that they were not-normally distributed. Therefore, statistical differences between two groups were analyzed via the nonparametric Mann-Whitney U test and to compare multiple groups, a Kruskal Wallis test with a Dunn's Multiple comparison test was used. Data were presented as mean ± standard error of the mean (SEM).

Functional recovery *in vivo* was statistically analyzed using two-way ANOVA for repeated measurements with a Bonferroni post hoc test for multiple comparisons. Histological analyses of blood vessel density (CD31) was statistically analyzed using the Mann-Whitney test. The data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. This test indicated that the BMS data set followed the Gaussian distribution. Data were presented as mean ± standard error of the mean (SEM). Additionally, in the attachments (figure S4.1), the BMS results are presented as box plots with the whiskers indicating the minimum and maximum. Day-to-day comparisons between the groups has been performed using the Mann-Whitney test.

Statistical analyses were performed using JMP Pro 11 and GraphPad Prism 5.01 software (GraphPad Software, Inc.). Differences were considered statistically significant when $p<0.05$.

4.4 Results

4.4.1 Terbutaline stimulates tube formation by mouse brain endothelial cells

First, the angiogenic capacity of terbutaline was investigated by the tube formation assay. Tube formation is an important step in the angiogenic process. It takes place after vessel destabilization, EC proliferation and migration. Studying this step in cell culture in vitro provides insight into which specific part of the blood vessel development is affected by the treatment The tube formation assay was performed on bEnd.3 cells, which express the beta-2 adrenoceptor (β2-AR) (data not shown). Terbutaline stimulation of these ECs resulted in increased tube formation as compared to the vehicle control (arrows; **figure 4.1A**). Three parameters were quantified: the total tube length (total branching length), the number of nodes (# nodes) and the number of tubes (# branches + # segments). In every condition with terbutaline, all these parameters were significantly increased (**figure 4.1B**). For instance, the total branching length increased with 68.3% when 1 μM terbutaline was applied (mean total branching length: 168.3% ± 11.65%; **figure 4.1B**), compared to the control condition (mean total branching length: 100% ± 5.12%; **figure 4.1B**). Summarized, terbutaline stimulates tube formation by adult mouse brain ECs.

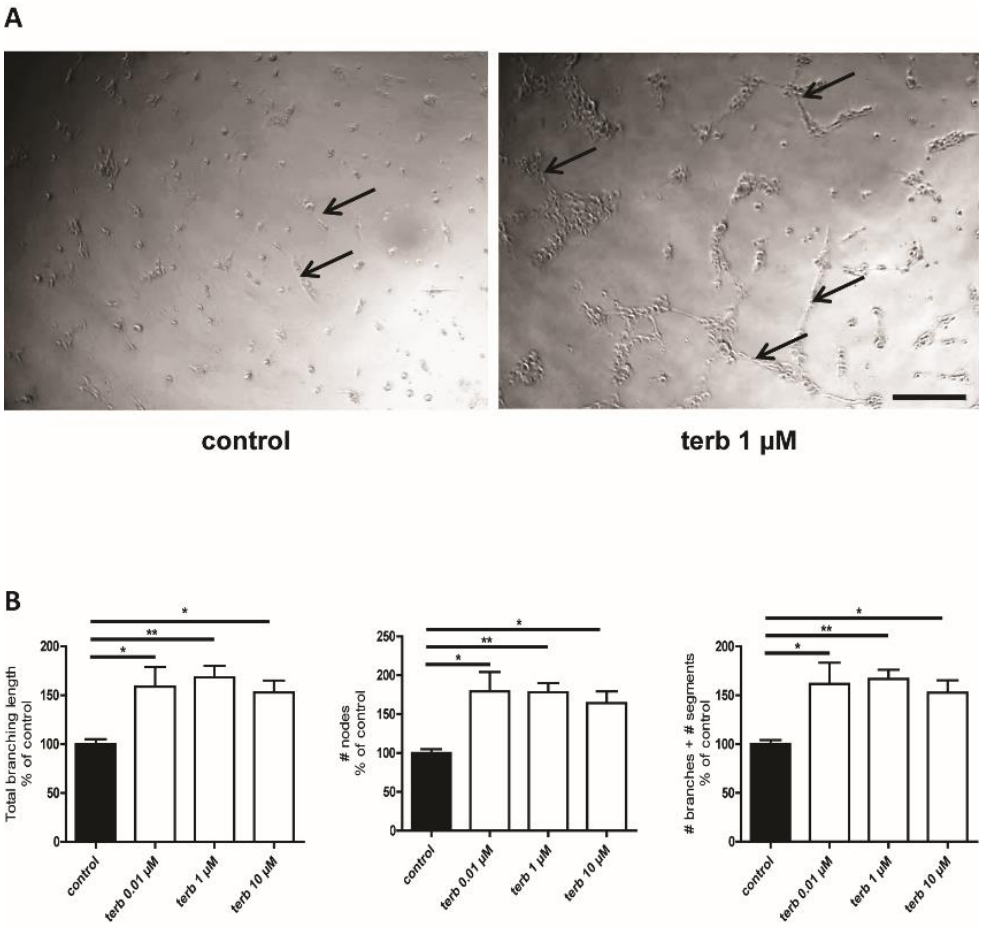


Figure 4.1: Terbutaline stimulates tube formation by bEnd.3 cells. A) The bEnd.3 cells, seeded on growth factor reduced (GFR)-matrigel in Ibidi-slides, were treated for 6h with different concentrations of terbutaline (0.01; 1; and 10 µM). Normal growth medium without FCS was used as vehicle to dilute terbutaline. Representative pictures are shown of the vehicle control-treated and the terbutaline 1 µM-treated ECs. Black arrows mark the formed tubes. **B)** Images were evaluated with the Angio-analyzer of ImageJ to quantify the total branching length, the number of nodes and the sum of the number of segments and branches. Scale bar= 500 µm. Data were normalized to control and represented as mean ± SEM; n= 8 wells/condition; *p<0.05 and **p<0.01.

4.4.2 Terbutaline stimulates tube formation via Akt and ERK signaling

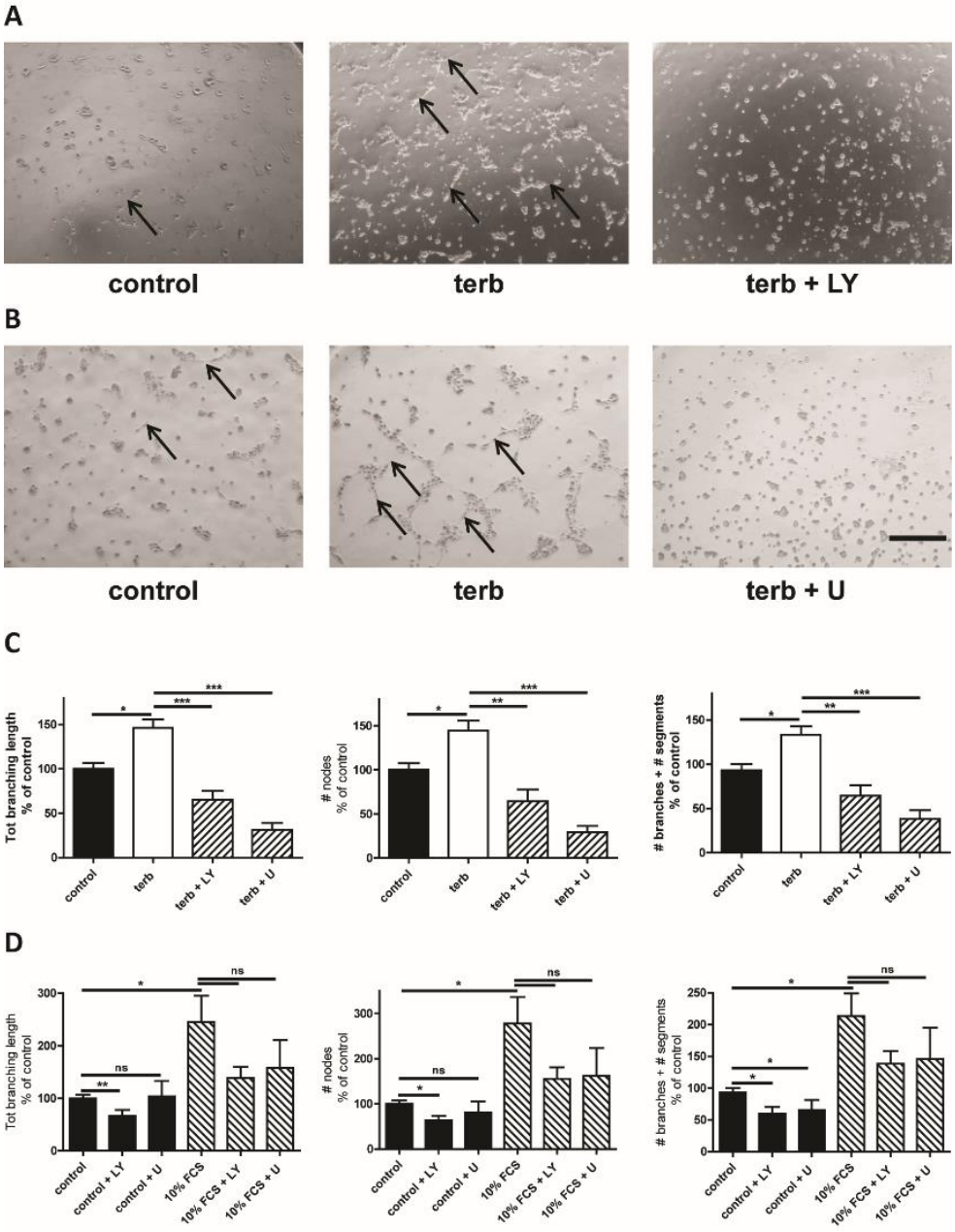
As terbutaline is a potent stimulator of tube formation in vitro, we aimed to unravel the pathways through which terbutaline exerts these effects. Therefore, we inhibited Akt or ERK, which are closely involved in the angiogenic process. LY294002 (LY) has been used because it is a potent inhibitor of phosphoinositide 3-kinases (PI3Ks), which are upstream of Akt in the Akt pathway. U0126 (U) is a highly selective inhibitor of MAPK/ERK kinase (MEK) 1 and MEK2, upstream of ERK1/2 in the respective pathway. Inhibition of both the Akt signaling or the ERK signaling significantly prevented tube formation induced by terbutaline (**figure 4.2A and 2B**, respectively). Quantification revealed that when LY was applied, the total branching length, the number of nodes and the number of tubes where reduced significantly by 80.99%; 80.1% and 68.73% respectively, compared to the condition where terbutaline alone was applied (terb 1 µM + LY 10 µM: 65.21% ± 10.31% vs. terb 1 µM: 146.2% ± 9.366% for the total branching length; terb 1 µM + LY 10 µM: 64.22% ± 13.4% vs. terb 1 µM: 144.4% ± 11.2% for the number of nodes; terb 1 µM + LY 10 µM: 64.47% ± 11.61% vs. terb 1 µM: 133.2% ± 9.481%; **figure 4.2C**).

Application of U reduced the tube formation by 114.64%; 115.25% and 95.16% for the total branching length, the number of nodes and the number of tubes respectively, compared to the condition where terbutaline alone was applied (terb 1 µM + U 100 µM: 31.56% ± 7.602% vs. terb 1 µM: 146.2% ± 9.366% for the total branching length; terb 1 µM + U 100 µM: 29.15% ± 7.172% vs. terb 1 µM: 144.4% ± 11.2% for the number of nodes; terb 1 µM + LY 10 µM: 38.04% ± 10.07% vs. terb 1 µM: 133.2% ± 9.481%; **figure 4.2C**). These results imply that the tube formation induced by terbutaline is dependent on Akt signaling and ERK signaling.

The inhibitors were also applied in the absence of terbutaline to evaluate their influence on tube formation as such.

The Akt blocker alone significantly reduced the total branching length, the number of nodes and the number of tubes compared to the vehicle control, whereas the ERK blocker only decreased the number of tubes (**figure 4.2D**).

However, the Akt blocker caused only a small inhibition of the standard tube formation, whereas it caused a large reduction of the terbutaline induced tube formation (e.g. 32,72% in the standard tube formation assay vs. 114,64% in the terbutaline induced tube formation for the total branching length). When applied to a condition (NGM + 10% FCS) known to induce tube formation, the blockers reduced the tube length, number of nodes and number of tubes, although not significantly, indicating their proper functioning (**figure 4.2D**).


A
control
terb
terb + LY
B
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terb + U
C
Tot branching length
% of control
# nodes
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# branches + # segments
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control
terb
terb + LY
terb + U
D
Tot branching length
% of control
# nodes
% of control
# branches + # segments
% of control
control
control + LY
control + U
10% FCS
10% FCS + LY
10% FCS + U
ns

Figure 4.2: Terbutaline stimulates tube formation via Akt and ERK pathway signaling. A-B) The bEnd.3 cells were seeded onto a GFR-matrigel and treated for 6h with terbutaline (1 µM), either or not in combination with the Akt blocker LY (10 µM) (A) or the ERK blocker U (100 µM) (B). The vehicle control contains NGM without FCS. Representative pictures are shown of the vehicle control, terb 1 µM, terb 1 µM combined with LY 10 µM and terb 1 µM combined with U 100 µM. Black arrows mark the formed tubes. **C)** Images were evaluated with the Angio-analyzer of ImageJ to quantify the total branching length, the number of nodes and the sum of the number of segments and branches. **D)** For the control conditions, the bEnd.3 cells were treated for 6h with the Akt blocker (10 µM) or the ERK blocker (100 µM) alone. The vehicle control contains NGM without FCS. The blockers were also combined with the normal growth medium of the cells (NGM) which contains 10% FCS. FCS is known to induce tube formation and can be considered as a positive control. Images were evaluated as described previously. The controls shown in these graphs are identical to the controls in figures A-C. For reasons of readability, they are shown in separate graphs. Scale bar= 500 µm. Data were normalized to control and represented as mean ± SEM; n= 24 wells/condition for the controls and terb 1 µM, n= 8-10 wells/condition for the combinations with LY and U; *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

4.4.3 Terbutaline slightly decreases the metabolic activity of the ECs via the ERK pathway

Measuring the metabolic activity gives a first impression of the proliferation capacity and cell viability. The ECs were incubated for 6h and 72h with terbutaline. The metabolic activity of the ECs after 6h incubation with terbutaline was only decreased when 10 µM had been applied, compared to the vehicle control. The other concentrations did not affect the metabolic activity of the cells (**Figure 4.3A**). After 72h incubation, the metabolic activity was decreased in all conditions (**Figure 4.3B**). Again, the involvement of the Akt and ERK pathway were assessed by blocking them with the respective inhibitors LY and U. Addition of the Akt blocker LY did not change the effects of terbutaline on the metabolic activity of the cells, although it decreased the metabolic activity of the cells on its own (**figure 4.3C and E**). In contrast, when the ERK blocker U was applied, the metabolic activity returned to levels similar to the vehicle control (**figure 4.3D**), whereas the blocker alone did not affect the metabolic activity of the cells (**figure 4.3F**).

To control for the proper function of the blockers, they were added to the normal growth conditions of the cells (NGM; with 10% FCS). The metabolic activity was reduced after application of the blockers, compared to the NGM without the blockers, indicating that the blockers did function properly (**Figure 4.3E-F**). Taken together, these results indicate that, on the short term (6h), terbutaline only slightly affects the metabolic activity when applied in higher concentrations (10 µM). The long term effects (72h) include reduction in the metabolic activity of the ECs, involving the ERK pathway.

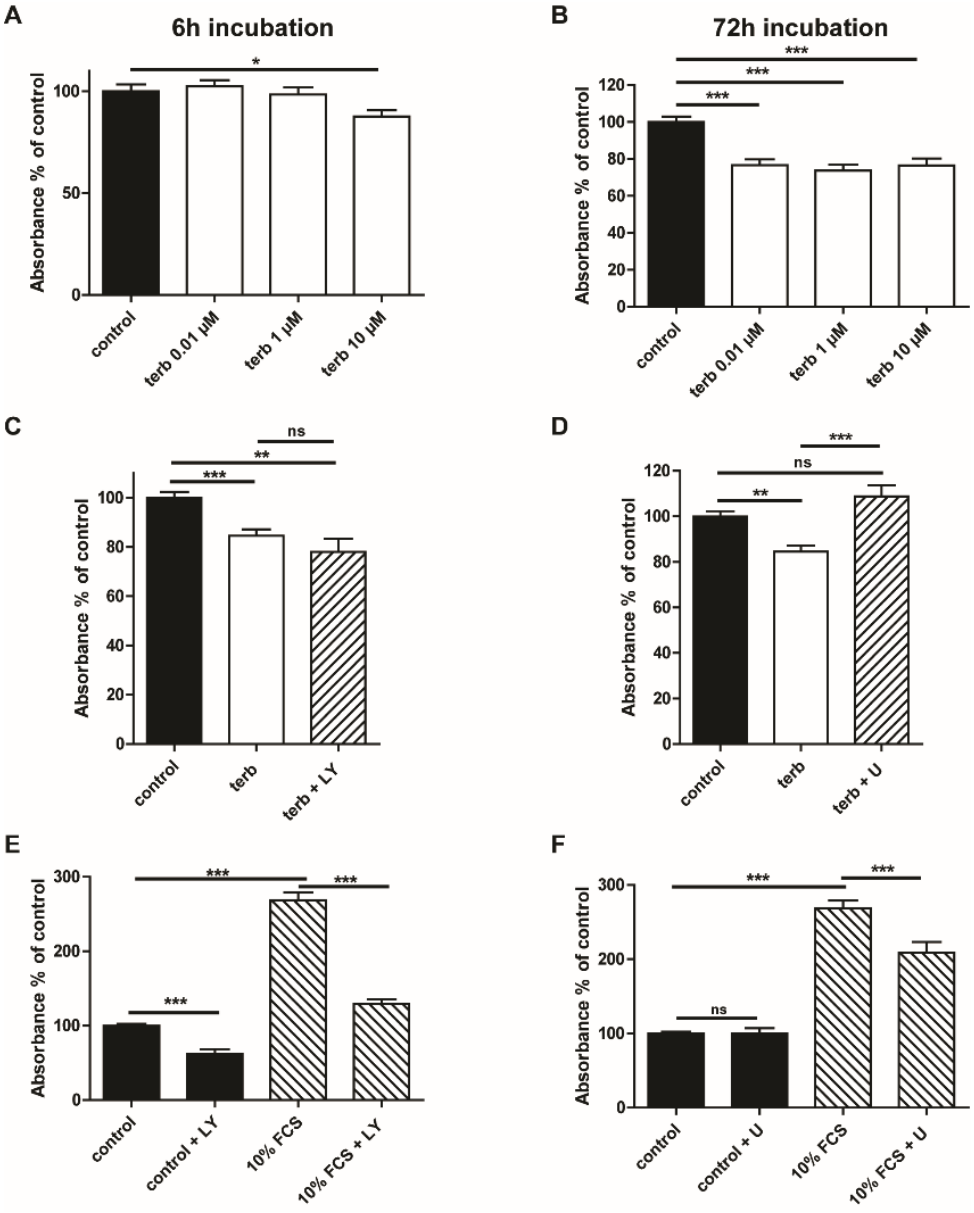

A
6h incubation
Absorbance % of control
control
terb 0.01 µM
terb 1 µM
terb 10 µM
B
72h incubation
Absorbance % of control
control
terb 0.01 µM
terb 1 µM
terb 10 µM
C
Absorbance % of control
control
terb
terb + LY
D
Absorbance % of control
control
terb
terb + U
E
Absorbance % of control
control
control + LY
10% FCS
10% FCS + LY
F
Absorbance % of control
control
control + U
10% FCS
10% FCS + U

Figure 4.3: Terbutaline slightly decreases the metabolic activity of the ECs via the ERK pathway. A-B) The bEnd.3 cells were treated for 6h or 72h with terbutaline (0.01; 1; 10 µM) after which their metabolic activity was measured by an MTT-assay. The vehicle control contains NGM without FCS. **C-D)** To investigate the effects of the Akt and the ERK pathway on metabolic activity (MTT-assay), terbutaline (1 µM) has been incubated in combination with the Akt blocker (10 µM; C) or the ERK blocker (10 µM; D) for 72h. **E-F)** The bEnd.3 cells were treated for 72h with the Akt blocker LY (10 µM; E) or the ERK blocker U (10 µM; F) alone. The vehicle control contains NGM without FCS. The blockers were also applied to the NGM containing 10% FCS. The controls shown in these graphs are identical to the controls in figure 4.3. For reasons of readability, they are shown in separate graphs. Data were normalized to control and represented as mean ± SEM; n= 22-30 wells/condition; *p<0.05; **p<0.01 and ***p<0.001.

4.4.4 Terbutaline does not affect the proliferation rate of the ECs

To determine whether terbutaline affects proliferation, a BrdU cell proliferation assay was performed. Therefore, the ECs were incubated with terbutaline for 6h and 48h. The data show that terbutaline does not affect the proliferation of the bEnd.3 cells after 6h and 48h (**figure 4.4A-B**). The time points were chosen because they correspond with the timing of tube formation and the timing of reaching a significant difference in proliferation between the vehicle control and the normal growth conditions with 10% FCS (**figure 4.4C-D**). In conclusion, terbutaline does not affect proliferation of bEnd.3 cells.

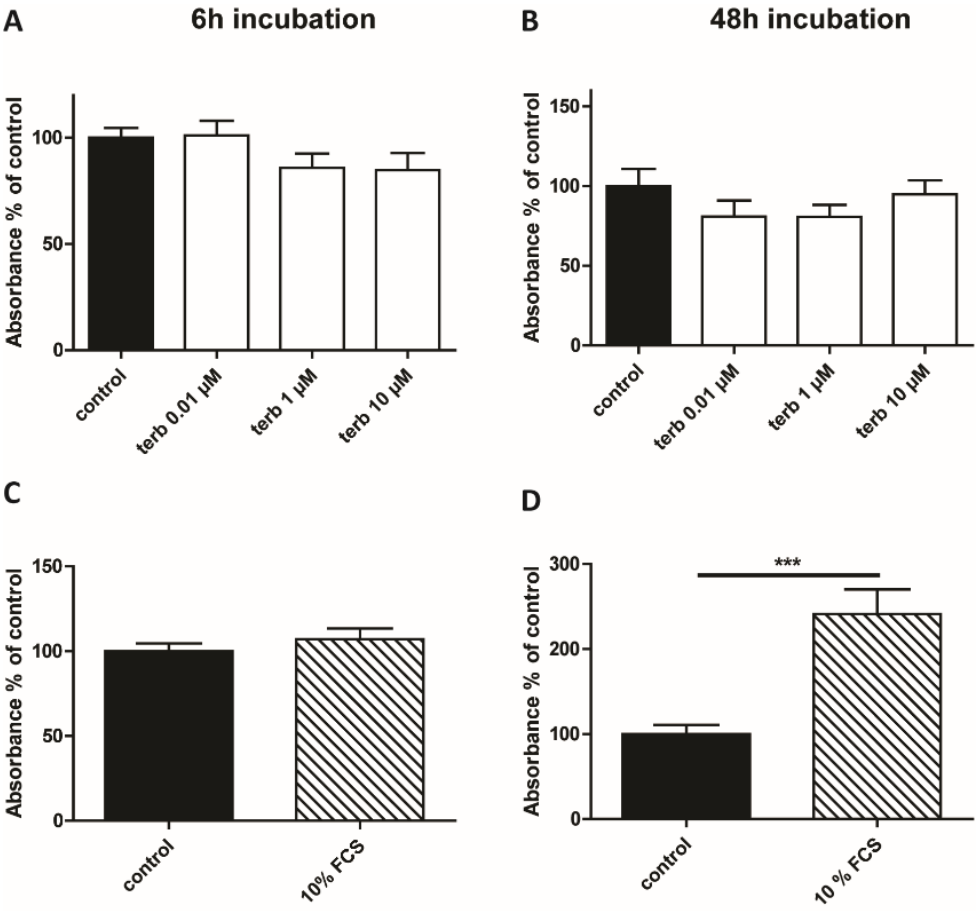
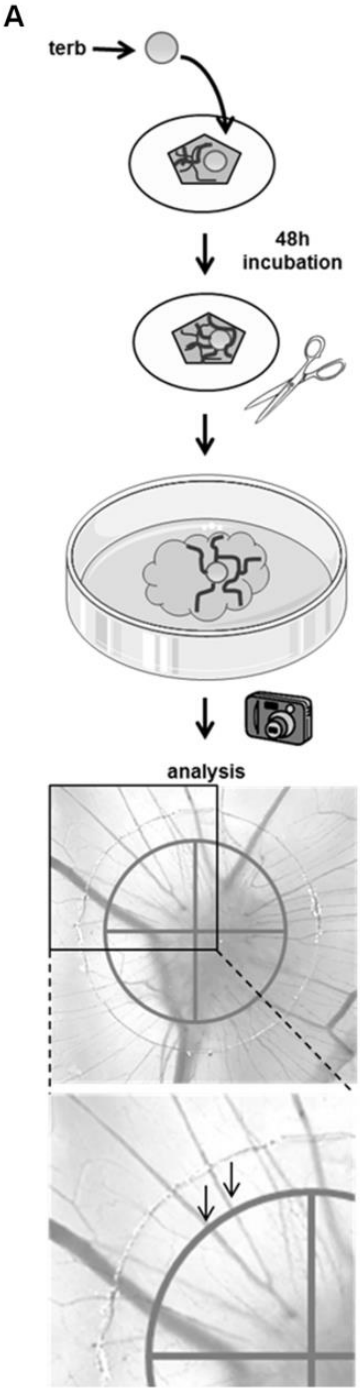
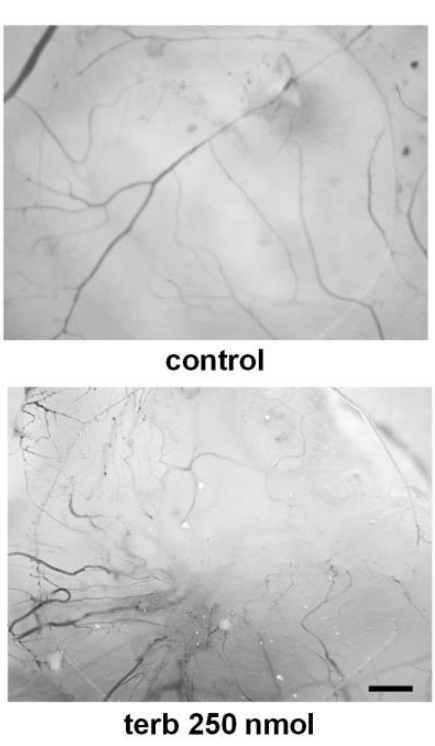


Figure 4.4: Terbutaline does not affect the proliferation of the bEnd.3 cells. A-B) The bEnd.3 cells were treated for 6h or 48h with terbutaline (0.01; 1; 10 µM). The vehicle control contains NGM without FCS. **C-D)** In addition, the bEnd.3 cells were grown in NGM with 10% FCS for 6h and 48h. The controls shown in these graphs are identical to the controls in figure A and B. To increase readability, they are shown in separate graphs. Data were normalized to control and represented as mean ± SEM; n= 10-15 wells/condition: ***p<0.001.

4.4.5 Terbutaline stimulates vessel formation *in ovo*

Next, we examined the more general effects of terbutaline on neo-vessel formation in a microcirculatory preparation, namely the chick chorioallantoic membrane (CAM). Briefly, on day 9 after fertilization, terbutaline has been applied on plastic discs (8-mm diameter), which were placed on the CAM. A solution of cortisone acetate (100 µg/disc) has been added to all discs to prevent an inflammatory response. A dried control disc loaded with dilution buffer (MQ) was used as vehicle control. After 48h incubation, the CAM was removed, and angiogenesis was evaluated. All vessels intersecting a concentric circle positioned in the treated area were counted (**figure 4.5A**). The results indicate that terbutaline increases the number of blood vessels in all the investigated concentrations, although only for 250 nmol the increase reached statistical significance compared to the vehicle control MQ (**figure 4.5B-C**). These data highlight that terbutaline not only stimulates tube formation in an isolated system of ECs in vitro, but it also significantly increases vessel formation in an organotypic and more general context.


A
terb
48h
incubation
analysis


B
control
terb 250 nmol

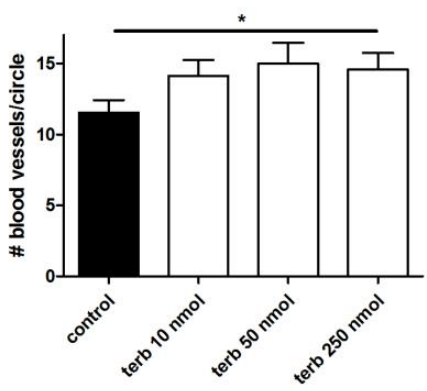

C
*
blood vessels/circle
15
10
5
0
control
terb 10 nmol
terb 50 nmol
terb 250 nmol

Figure 4.5: Terbutaline stimulates angiogenesis in the CAM-assay. A) On day 9 after fertilization, terbutaline has been applied onto the CAM on sterile plastic discs. A control disc loaded with dilution buffer (MQ) is placed on the CAM as vehicle control. After 48h incubation, the CAM is dissected and photographed. All vessels intersecting a concentric circle (radius 3.5 mm) positioned in the treated area are counted double blinded and independently by two investigators (arrows). **B)** Terbutaline stimulates blood vessel formation, compared to the vehicle control. **C)** Quantification reveals that terbutaline, when applied in an amount of 250 nmol, significantly increases the number of blood vessels, compared to the control. A trend towards increased angiogenesis is also visible for the other conditions, although statistical significance has not been reached. Scale bar: 500 µm. Data are represented as mean ± SEM; n= 12-23 CAMs/condition. *$p<0.05$.

4.4.6 Terbutaline alone does not affect functional or vascular regeneration after SCI

To unravel its potential as a therapeutic treatment strategy for CNS trauma, terbutaline has been applied in our SCI mouse model. Functional recovery has been assessed using the BMS scoring system. Systemic administration of terbutaline seems to increase the BMS score from day 3 post injury onwards, compared to the BMS of the control mice which only received NaCl, although the observed differences were not statistically significant (**figure 4.6A**). Additional statistical analyses comparing the groups day-to-day also did not reveal statistical significant differences (**Attachments, figure S4.1**).

To assess the effects of terbutaline on angiogenesis *in vivo*, we examined spinal cord sections for their CD31 expression (**figure 4.6B**). CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is a commonly used marker to detect ECs. Quantification of the CD31-positive area showed that both terbutaline- and NaCl-treated mice express CD31 at the same level perilesional and at the lesion site (**figure 4.6C**).

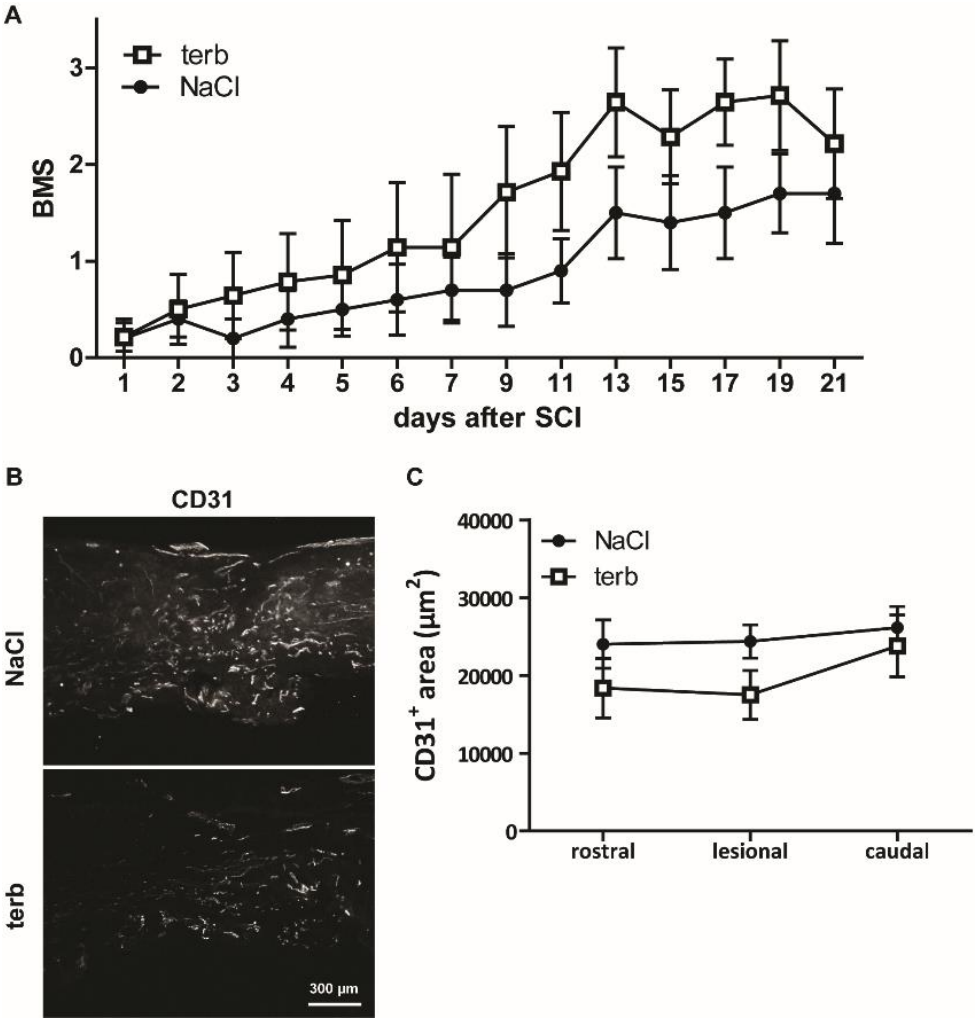


Figure 4.6: Terbutaline alone does not affect functional or vascular regeneration after SCI. A) The first 9 days after SCI, mice were injected twice a day with either terbutaline (5 mg/kg) or with the vehicle NaCl. Functional recovery was analyzed according to the BMS. The data are presented as mean values ± SEM; n= 5-7. **B)** Spinal cord cryosections were stained for CD31. Representative pictures at the lesion site are shown. Scale bar= 300 µm. **C)** A color threshold was set in order that the image analysis software could automatically detect the intensively stained CD31+ blood vessels. Quantification of the CD31+ area (µm²) is shown. The data are presented as mean values ± SEM; n = 6-9.

4.5 Discussion

In the present study, we show that terbutaline promotes angiogenesis by stimulating the β2-AR on bEnd.3 cells in vitro and stimulates blood vessel formation in the CAM assay, an in ovo model compromising of a complex vasculature (**figure 4.7**). We have specifically stimulated endothelial β2-ARs with terbutaline in order to stimulate tube formation. We have used the bEnd.3 cells as these are the most relevant cells to investigate in vitro the pro-angiogenic effects of terbutaline in the context of CNS trauma and to relate the data to our SCI mouse model. Although HUVEC cells are commonly used in in vitro angiogenesis assays, they are not ideal as a model for the vasculature of the adult mouse CNS.

Previous studies using the general non-specific β-AR agonist isoproterenol (ISO) either increased tube formation by mature ECs and EPCs or reduced tube formation in a different experimental set-up using different EC types and different outcome parameters (48, 49, 227, 228). Another complicating factor is the non-specific stimulation of both β1- and β2-ARs by ISO. Both signaling pathways exert distinct and often contradictory effects in multiple biological systems (64). In addition, the β2-AR is the most abundantly expressed AR subtype on blood vessels and ECs (64). It is therefore crucial to study selective β2-AR signaling in angiogenesis.

Three β2-AR agonists have been reported so far to play a role in angiogenesis. Clenbuterol and zilpaterol are known to stimulate the release of pro-angiogenic factors from macrophages (66). However, these are controversial agents because of their anabolic capacity (221, 222). In contrast to clenbuterol and zilpaterol, terbutaline is not reported to be anabolic, avoiding adverse effects such as cardiac hypertrophy (221-223). Salbutamol is another specific β2-AR agonist correlated with angiogenesis. However, it was reported to decrease the number of blood vessels in the CAM-assay (224). In contrast, specific β2-AR stimulation with terbutaline induced tube formation by bEnd.3 cells and blood vessel formation in the CAM.

Further analyses of the pathways involved in terbutaline-induced tube formation showed that the inhibition of both AKT and ERK significantly reduced the tube formation by bEnd.3 cells, implying that both pathways are important during EC tubulogenesis. This is in accordance with previous findings showing that AKT and ERK signaling are required for the tube formation of choroid-retinal ECs (229).

To explore the effects of terbutaline on other key steps of angiogenesis, we performed MTT-assays and BrdU-assays. The MTT-assay revealed that short term application (6h) of low doses of terbutaline (<10 µM) does not affect the metabolic activity. Long term application (48-72h; 0.01 µM-10 µM terbutaline) reduced the metabolic activity without effects on proliferation. These data suggest that terbutaline reduces the viability of the ECs. Indeed, it has been shown that stimulation of β2-ARs induces apoptosis of human aortic ECs via p38/MAPK signaling (227). Accordingly, we show that inhibition of MAPK/ERK signaling reversed the effects of terbutaline on metabolic activity, indicating the involvement of this pathway in terbutaline-mediated proliferation and cell viability. In addition, also other angiogenic factors, such as angiopoietin 2 (Ang2), transforming growth factor β (TGFβ) and angiotropin, play dualistic roles in angiogenesis. For Ang2 it depends on the co-stimulatory molecules. For instance, in the presence of VEGF, application of angiopoietin 2 mediates an increase in the capillary diameter, induces migration and proliferation of ECs. In the absence of VEGF, however, it causes apoptosis of ECs and regression of blood vessels (230). Like angiopoietin 2, TGF-β can act as an angiostatic or angiogenic molecule. Comparable to terbutaline, angiotropin is not mitogenic for ECs, but it promotes tube formation and stimulates angiogenesis in chorioallantoic membrane and cornea pocket assays (231).

To confirm the angiogenic potency of terbutaline in an organotypic model with fully functional blood capillaries, we applied the compound on the CAM. Here, the number of blood vessels was increased by 15% after terbutaline treatment compared to vehicle.

In contrast, the non-specific β-AR agonist ISO and the specific β2-AR agonist salbutamol reduced angiogenesis by 29-45% in the CAM-assay (224, 228). Moreover, Pullar *et al* showed an increase in angiogenesis after application of the β2-AR antagonist ICI 118,551 on the CAM (232). These studies, together with our data, highlight that terbutaline is the most promising specific β2-AR agonist to stimulate blood vessel formation.

The discrepancies could be explained either by pharmacokinetic differences between the different compounds or by important methodological differences in the study of these factors. As mentioned above, ISO non-specifically activates both β-adrenoceptor subtypes. For this reason, we have chosen to specifically stimulate the β2-ARs with terbutaline. In addition, the time points of application of the compounds, the incubation times as well as the method of analyses differ between the different studies. In previous studies the compounds were already applied on day 5 after fertilization (224, 228, 232). We applied terbutaline at day 9 after fertilization because only then is the capillary plexus of the CAM formed and associated with the overlying epithelial cells to mediate gas exchange. Before this time point, the blood vessels are immature and lack a complete basal lamina and smooth muscle cells. It is interesting to note that terbutaline stimulates angiogenesis in two models involving different species, namely tube formation by mouse ECs and blood vessel formation in the chick CAM. Since many angiogenic processes are evolutionary well-conserved, the results of this study, namely the pro-angiogenic capacity of terbutaline in two different species, give a first indication about the potential clinical relevance of the compound.

Finally, we have evaluated the therapeutic potency of terbutaline in vivo. Therefore, we applied terbutaline intraperitoneally in an SCI mouse model and monitored recovery of locomotor function in addition to revascularization. Although we observed a suggestive positive trend, the systemic administration of terbutaline did not significantly improve the functional outcome, nor did it affect revascularization. Interestingly, previous reports showed that β2-AR agonists like clenbuterol enhance functional recovery after SCI (45).

However, the effects on angiogenesis were not investigated. Although terbutaline and clenbuterol have similar chemical structures, they have different pharmacokinetic properties (233). These pharmacokinetic differences most likely have played a role in the observed discrepancies.

In addition, the use of clenbuterol has been associated with various adverse effects like tachycardia and muscle tremors (234). Therefore, it is currently not considered as a promising treatment strategy for SCI. The discrepancy between very promising in vitro findings and only a suggestive, non-significant improvement of the functional outcome in vivo may result from differences between the in vitro and in vivo situation as well as from the non-traumatic nature of the in vitro models (ECs and CAM) compared to the highly inflammatory post-traumatic nature of the in vivo injury model (SCI). After SCI, an overall non-permissive environment for the growth of axons and blood vessels is created due to the inflammation in combination with the lack of oxygen and nutrients, etc. We conclude that terbutaline as a monotherapy may not be potent enough to overcome these challenges and to induce proper revascularization of the spinal cord. A more complex approach is necessary, as adequate recovery of function can only be achieved when revascularization of the spinal cord is complemented with axonal regeneration and plasticity, elimination of glial scar tissue and remyelination.

An *in vivo* study by Thaker *et al*. suggested that terbutaline may indirectly influence angiogenesis by modulating VEGF secretion of non-endothelial cells. In this study mice bearing ovarian cancer cells were treated daily with 5 mg/kg terbutaline. The authors show that terbutaline treatment leads to higher VEGF production by the carcinoma cells, which resulted in enhanced tumor vascularization as revealed by CD31-staining (235). In addition, Iaccarino *et al*. have shown in two studies that β2-AR activation may either directly stimulate EC proliferation in vitro or indirectly by increasing their VEGF release (49, 227).

In our *in vivo* model it cannot be excluded that VEGF secretion was stimulated, however, we do not see any increase in vessel density (CD31) in the injured spinal cord after terbutaline treatment in vivo.

In conclusion, we provide novel evidence that the specific β2-AR agonist terbutaline can stimulate angiogenesis in vitro. The stimulation of tube formation is mediated via the ERK- and Akt-pathway. Moreover, terbutaline is able to stimulate neo-vascularization in ovo. Compared to other β-AR modulators, terbutaline appears to be the most promising compound as it acts specifically on the β2-AR, whereas ISO stimulates both β1- and β2-ARs. In addition, adverse effects like muscle hypertrophy can be avoided as unlike clenbuterol and zilpaterol, it is not reported to be an anabolic agent. Salbutamol should be avoided because it acts as an anti-angiogenic agent. Taken together, our findings make terbutaline the most promising β2-AR agonist to stimulate angiogenesis, whilst underlining the importance of further research into the angiotherapeutic relevance of terbutaline and β2-AR signaling in CNS trauma models. Most importantly, terbutaline increased the number of blood vessels in the *in ovo* setting, underscoring the general effects of terbutaline in an organotypic system. In conclusion, these data indicate that terbutaline is a promising β2-AR agonist to stimulate blood vessel formation.

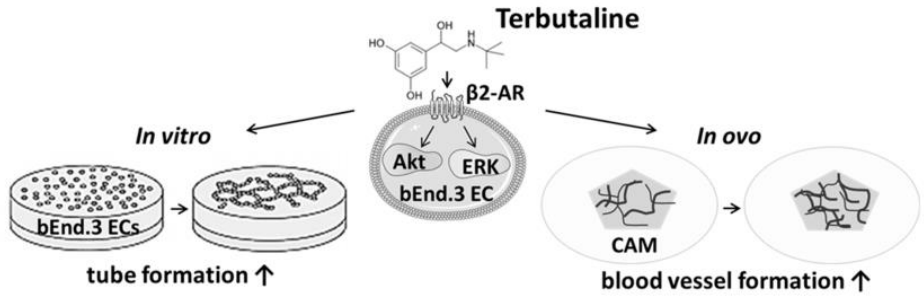


Figure 4.7: Terbutaline stimulates angiogenesis both in vitro and in ovo. Summarized, the short-acting, highly specific β2-AR agonist terbutaline significantly induced bEnd.3 tube formation in the matrigel in vitro assay. Administration of specific inhibitors of ERK and Akt signaling both inhibited terbutaline-induced tube formation.

Chapter 5

Adoptive transfer of IL-13 overexpressing macrophages to skew the M1 spinal cord micro-environment towards M2 to promote functional regeneration

Chapter 5. Adoptive transfer of IL-13 overexpressing macrophages to skew the M1 spinal cord micro-environment towards M2 to promote functional regeneration

5.1 Abstract

After SCI, classically activated M1 macrophages (Mϕ) dominate the spinal cord. The aim is to reduce these pro-inflammatory Mϕs because they secrete multiple pro-inflammatory and neurotoxic factors and attack dystrophic axons through direct interaction. We hypothesize that the therapeutic transfer of genetically modified Mϕs with a stable expression of high levels of IL-13 drives endogenous macrophages/microglia towards a beneficial phenotype in order to promote functional recovery after SCI. Therefore, we have transduced naive Mϕs in order to make them secrete IL-13 (IL-13-Mϕ). In addition, Mϕs were treated with recombinant IL-13 to induce an M2-Mϕ phenotype and with LPS to induce an M1-Mϕ phenotype. Transplantation of the IL-13-Mϕ immediately after SCI in the hemisection T-cut mouse model improved functional recovery accompanied with a reduction in lesion size and demyelinated area, compared to vehicle- and M2-Mϕ-treated mice. Further analyses revealed that the number of $CD4^+$ T-cells was reduced in both the IL-13-Mϕ and M2-Mϕ groups, compared to the vehicle group. Most importantly, after IL-13-Mϕ treatment, the number of arg-1^+ microglia/macrophages was significantly increased at the lesion site whereas the number of $MHCII^+$ cells was decreased. After M2-Mϕ treatment, the number of arg-1^+ microglia/macrophages was decreased at the lesion site. At the injection site, on the other hand, the number arg-1^+ and $MHCII^+$ microglia/macrophages was increased, which suggest that the transplanted M2-Mϕ do not migrate to the lesion site. A decrease in the number of macrophage-axon contacts in the IL-13-Mϕ mice may indicate a reduction in axonal dieback. In conclusion, our results show that transplantation of IL-13-Mϕ leads to improved functional and histological recovery in an SCI mouse model.

5.2 Introduction

The environment of an SCI lesion is extremely complex. The role of inhibitory effects of proteoglycans and myelin on axonal growth has been well established. However, the part of neuroinflammation in regeneration failure remains to be clarified, as the immune system in the CNS and its interplay with various cell types plays a main role in the multi-faceted pathology of SCI (236). After the primary injury, a secondary process sets in with injury-induced changes of oligodendrocytes and astrocytes and a robust and long-lasting inflammatory response dominated by macrophages (Mϕ), all inhibitory for regeneration. The Mϕs are mostly derived of two sources, namely CNS-resident microglia or blood monocytes (Mϕ precursors) migrating from the bone marrow or the spleen (237, 238). Microglia respond rapidly to injury by extending cellular processes or migrating towards the lesion site where they participate in scar formation (239). It has been suggested that this response serves a protective role, as pharmacologic or genetic inhibition of microglial activation exacerbates lesion pathology and impairs functional recovery (240). Two days after injury, monocytes bind to endothelial adhesion molecules and migrate to the lesion, guided by chemotactic gradients established by astrocytes (241). Shortly thereafter, these monocytes differentiate into tissue Mϕs. Data from several SCI animal models showed that the detrimental effects of intraspinal Mϕs predominate. Moreover, selective inhibition or depletion of Mϕ during the first two weeks postinjury significantly reduced the secondary injury, leading to improved recovery of sensory, motor, or autonomic functions (238).

However, in response to different combinations of factors in the extracellular milieu of the injured CNS (e. g. cytokines, cell fragments etc.) Mϕs can differentiate into functionally distinct cell subsets that differentially affect neuron survival and axon growth, namely "classically" (M1) or "alternatively" (M2) activated Mϕs (72). In addition, depending on the biological agents or conditions, different subtypes of alternatively activated macrophages may arise, with M2a, M2b, and M2c being the best characterized.

However, this classification is continuously under debate and needs fine-tuning on the single cell level *in vivo* (242).

After CNS injury, signaling pathways that polarize Mϕs towards an M1 phenotype predominate. The infiltration of axon-attacking M1-Mϕ greatly contributes to axonal retraction and the deleterious phenomenon known as axonal dieback (243). Compared with M1-Mϕs, M2-Mϕs promote more robust neurite outgrowth and they can enhance oligodendrocyte progenitor cell differentiation and hence remyelination (68, 72, 244), meaning that neuroinflammatory responses may be beneficially influenced by both M2a polarisation driven by interleukin (IL)-13/4 and M2c polarization driven by IL-10. Previous work from our research group showed potent pro-regenerative effects of IL-13 in the context of SCI (245). Therefore, in this study, we aimed to target the M1-Mϕs and drive them toward a pro-regenerative phenotype (M2) to limit axonal dieback and improve functional recovery.

We have focused on IL-13-mediated M2a polarisation of endogenous microglia and Mϕs. IL-13 is a canonical anti-inflammatory Th2 cytokine, closely related to IL-4 (246). Previously it has been shown to exert neuroprotective effects in EAE by decreasing inflammatory cell infiltration and axonal loss, accompanied with reduced clinical symptoms (84). After SCI in mice and human, IL-13 levels decrease significantly in the spinal cord and the blood circulation (247, 248). This drop in IL-13 levels after injury makes it plausible that application of IL-13 in the acute phase after SCI may have therapeutic potential. In addition, we have demonstrated that IL-13 stimulates neuronal survival and increases neurite outgrowth from primary neurons and organotypic brain slices *in vitro* (unpublished results). Moreover, we have shown that targeted delivery of IL-13 can produce *in vivo* induction of M2a-polarized macrophages, both in muscle tissue and non-traumatic brain. These results were achieved by delivery of IL-13 either by implantation of genetically engineered mesenchymal stem cells (MSCs) or by direct lentiviral vector (LVv) administration (249). Our group has also evaluated the effects of IL-13-expressing MSCs in an SCI mouse model.

The results of this study showed the first evidence that implantation of IL-13-expressing MSCs improved functional recovery by inducing M2a macrophages in the injured spinal cord (245). Despite this progress, the underlying mechanisms of the beneficial effects of IL-13 and M2a Mϕs following SCI are still unclear.

Similarly, Ma *et al.* have observed improved locomotor recovery after systemic injection of *in vitro* M2 polarized Mϕs in SCI rats (70). However, the findings of Kigerl *et al.* indicate that these M2-Mϕs do not maintain their phenotype after SCI (72). Therefore, although Ma *et al.* observed an improved functional recovery, we believe that a substantial number of the injected M2-Mϕs were repolarized towards an M1 phenotype because of the typical lesion microenvironment. For that reason, we have injected IL-13-overexpressing Mϕs (IL-13-Mϕ) because they can modulate the SCI inflammation continuously via the constitutive secretion of IL-13. We hypothesized that therapeutic transfer of genetically modified Mϕs with a stable expression of high levels of IL-13 drives endogenous Mϕs and microglia towards a beneficial phenotype in order to promote functional recovery after SCI. Therefore, we have transduced naive Mϕs (M0-Mϕs) in order to make them secrete IL-13.

Taken together, our results show that transplantation of IL-13-Mϕs leads to improved functional recovery, accompanied with a reduction in lesion size, demyelinated area and the number of $CD4^+$ T-cells in an SCI mouse model. Most importantly, after IL-13-Mϕ treatment, the number of arg-1^+ (M2) microglia/macrophages was significantly increased at the lesion site whereas the number of $MHCII^+$ (M1) cells was decreased. A decrease in the number of macrophage-axon contacts in the IL-13-Mϕ mice may indicate a reduction in axonal dieback. However, further research is necessary to track the transplanted Mϕ in order to investigate their interaction with endogenous microglia and Mϕs, in addition to neurons, to reveal the effects on neuron viability and axon regeneration.

5.3 Materials and Methods

5.3.1 Isolation and differentiation of bone marrow-derived macrophages

Bone marrow derived Mϕs were obtained by isolating murine primary bone marrow cells (BMCs) from both the tibia and femur of 9-16 weeks old C57BL/6J mice (Janvier, France) using a well established protocol (250). BMCs were collected by flushing the bone marrow cavities with sterile ice-cold 1xPBS. Cells were cultured for 10 days in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat inactivated fetal calf serum (iFCS) (FBS Superior, Biochrom AG, Germany), 1% penicillin/streptomycin (5000 units/ml penicillin, 5000 µg/ml streptomycin, Invitrogen, Belgium) and 15-30% L929 conditioned medium (LCM) at 37°C and 5% CO2. Culture medium was changed partially or completely every 3 days. Eventually, cells cultured in the above medium were defined as M0 macrophages (M0-Mϕ). For M1 polarization, M0-Mϕ underwent a 24 h incubation with 200 ng/µl LPS (Sigma, Belgium). Cells undergoing an incubation with 33,3 ng/µl recombinant IL-13 (rIL-13) (Sigma, Belgium) for 48h were defined as M2 macrophages (M2-Mϕ).

5.3.2 Lentiviral vector transduction

For the lentiviral vector (LVv) transduction of the Mϕ, cells were transduced with pIRES-mIL-13-puro viral particles (VP) provided by the Laboratory for Viral Vector Technology & Gene Therapy - Leuven Viral Vector Core of the KU Leuven, according to previously optimized procedures (245). Briefly, wells of an 24-well plate were seeded with 0.5 ml/well of the cell suspension (1,5 x 10^5 cells) in their standard culture medium and 10-20 µl/well of the LVv (correlated with a multiplicity of infection (MOI) of 36). Next, cells were incubated overnight at 37°C and 5% CO2 after which residual VPs were removed by performing a complete medium change. Antibiotic selection was performed with puromycin (2 µg/ml, InvivoGen, France) to select the transduced cells. The transduced Mϕ will be further named as IL-13-Mϕ.

Medium of the IL-13 Mϕ has been collected 72h after puromycine selection. This conditioned medium (CM) has been applied to M0 Mϕ to test whether it can polarize them.

5.3.4 Western blot analysis

Western blot has been performed as previously described (33). Briefly, total protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Belgium). Untreated Mϕ (M0-Mϕ) and rIL-13/LPS-stimulated Mϕ (M2-Mϕ/M1-Mϕ) were used as controls. Protein samples (4-8 µg) were separated on a 12% sodium dodecyl sulfate polyacrylamide gel for 40-50 min at 200 V. The proteins were transferred onto a 100% methanol-activated polyvinylidene fluoride membrane (Milipore, Belgium) for 90 minutes at 350 mA. After blocking the membrane for 60 min in a 5% non-fat dry milk in 1x Tris buffered saline supplemented with 0.05% Tween 20 (TBS-T), the membrane was incubated overnight with the primary antibodies for arginase-1 (Arg-1, 1:1000, 18354, Santa Cruz, USA), inducible nitric oxide synthase (iNOS, 1:500, N6657, Sigma, Belgium), beta-actin (1:5000, 47778, Santa Cruz, USA) at 4°C. After washing the membrane, it was incubated with the secondary antibodies rabbit anti-goat HRP (1:1000, P0260, Dako, USA) and Rabbit anti-mouse HRP (1:1000-1:5000, P0449, Dako, USA) at RT for 60 min. β-actin was used as an internal control. Signal detection was performed using the Pierce™ ECL Western Blotting Substrate (Thermo Scientific). Images were taken with the ImageQuant LAS 4000 Mini and analyzed with ImageQuant TL software (GE Healthcare, Belgium).

5.3.5 ELISA

Secretion of IL-13 in the medium by the IL-13-Mϕ was measured by using the mouse IL-13 ELISA Ready-SET-Go! kit (eBioscience, Austria), according to the manufacturer's instructions. Untreated Mϕ, M1-Mϕ and M2-Mϕ were used as controls.

5.3.6 Griess assay

Secretion of nitric oxide (NO) in the medium by the IL-13-Mϕ was measured by using the Griess Reagent System (Promega, USA), according to the manufacturer's instructions. Untreated Mϕ, M1-Mϕ and M2-Mϕ were used as controls.

5.3.7 Experimental spinal cord injury and locomotion tests

The *in vivo* experiment was performed using female C57BL/6j mice (9-10 weeks old; Janvier, France). They were housed in a conventional animal facility at Hasselt University under regular conditions, i.e. in a temperature-controlled room (20 ± 3 °C) on a 12 h light-dark schedule and with food and water ad libitum. 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. A hemisection injury was performed as previously described (31, 34, 245). Locomotor recovery of the animals was determined by an investigator blinded to the experimental groups using the BMS. During the first week after injury, mice were scored daily and from the start of the second week until the end of the observation period, mice were examined every two days.

5.3.8 Cell transplantation

For the transplantation experiment, the Mϕ were cultures in Nunc™ Dishes with UpCell™ Surface (Thermofisher Scientific, Belgium) to harvest them non-enzymatically. Cells were washed three times in vehicle (PBS or clear RPMI) and kept on ice until transplantation. The animals were divided into three groups: vehicle group, M2-Mϕ group and IL-13-Mϕ group. The intraspinal injections have been performed immediately after SCI. A motorized stereotaxic injector pump (Stoelting, Ireland) with a 34-gauge needle attached to a 10 µl Hamilton syringe was positioned 1 mm rostral to the lesion (245).

The needle was stereotactically inserted into the spinal cord at a depth of 1 mm and 10 000 cells in 2,5 µl vehicle were injected over a four minute time period (245). The vehicle group only received either PBS or clear RPMI. The needle was subsequently kept in place for an additional four minutes to allow pressure equilibration and to prevent backflow.

5.3.9 Immunohistochemistry and quantitative image analysis

Spinal cord cryosections (10µm; cut serially), were obtained from animals transcardially perfused 4 weeks post injury with ringer solution containing heparin, followed by 4% paraformaldehyde in 0.1M PBS, as previously described (32, 245). To determine lesion size, demyelination, gliosis, and inflammatory infiltrate, cryosections were pre-incubated with 10% serum in PBS containing 0.5% Triton X-100 for 30 minutes at RT. The following primary antibodies were then incubated overnight at 4°C: mouse GFAP (Sigma-Aldrich, Belgium; G3893), rabbit MBP (Merck Millipore, Belgium, AB980), rabbit Iba-1 (Wako, Germany; 016-20001), goat anti-Iba-1 (Abcam, UK; ab107159), rat anti-CD4 (BD Biosciences, Belgium; 553043), rabbit anti-neurofilament (NF) (ThermoFisher Scientific, Belgium; T.400.5) and rat anti-MHC-II (Santa Cruz; sc-59322). To identify alternatively activated microglia/macrophages, sections were permeabilized using 0.1% Triton X-100 for 30 minutes and treated with 20% serum in Tris-buffered saline (TBS, pH 7.5) for 2 hours. Incubation with primary goat anti-Arg-1 antibody (Santa Cruz, Germany; sc-18354), diluted 1:50 in TBS containing 10% milk powder (TBS-M), was performed overnight at 4°C. Following repeated washing steps, secondary antibodies were applied for 1 hour at RT. These consisted of: donkey anti-goat Alexa fluor 555 (Thermo Fisher Scientific, Belgium; A21432), donkey anti-goat DyLight 650 (Abcam, UK; ab96934), goat anti-rat Alexa fluor 568 (Thermo Fisher Scientific, Belgium; A11077), goat anti-rat DyLight 650 (Abcam, UK; ab6565), goat anti-mouse Alexa fluor 568 (Thermo Fisher Scientific, Belgium; A11004), goat anti-rabbit Alexa fluor 488 (Thermo Fisher Scientific, Belgium; A11008) and goat anti-rat Alexa fluor 488 (Thermo Fisher Scientific, Belgium; A11006).

DAPI (1:1000; Sigma-Aldrich, Belgium) counterstaining was performed for 10 minutes and sections were mounted using fluorescence mounting medium (DAKO, Belgium). Immunofluorescence analysis was performed using a Nikon Eclipse 80i fluorescence microscope and NIS-Elements Viewer 4.0 software was used for image processing.

For measurement of lesion size and demyelinated area, 5 to 7 serial sections per animal (WT BALB/c: 6-8 animals per group, WT C57BL/6: 9-10 animals per group) containing the lesion centre as well as consecutive rostral and caudal areas were analysed, as previously described (245). Briefly, lesion size was evaluated using anti-GFAP immunofluorescence, while the demyelinated area was evaluated using anti-MBP immunofluorescence, by delineating the area devoid of staining. Quantification of astrogliosis (GFAP expression) and microglial activation (Iba-1 expression) was performed using an intensity analysis with ImageJ open source software (NIH), within square areas measuring 100µm X 100µm extending 600µm rostral to 600µm caudal from the lesion epicentre. To quantify classically activated and alternatively activated microglia/macrophages at the lesion or injection site, sections were stained for MHC-II and Arg-1 respectively, and the number of positive cells has been counted caudally and rostrally as shown in the respective image in the results section. T-helper cells were identified by double staining against CD4 and Iba-1 in order to exclude $CD4^+$ microglial cells and quantified by counting the number of $CD4^+$ T cells throughout the entire spinal cord section as previously described (245). Quantification of microglia/macrophage and axon interactions was performed by counting the number of contacts between $neurofilament^+$ dystrophic axon bulbs and $Iba\text{-}1^+$ microglia/macrophages. Dystrophic axonal bulbs were identified based on their globular and bulbus morphology extending from an axon fibre as previosly described (8). A contact was determined when a cell-cell interaction was observed between a dystrophic axonal bulb and an $Iba\text{-}1^+$ cell which contained a $DAPI^+$ nucleus. Analysis was performed in two standardised areas rostral and caudal from the lesion epicentre and the mean number of contacts in these two areas was calculated per animal.

5.3.10 Statistics

Statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, Inc.) as previously described (32, 245). Data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. This test shows that all data sets are not-normally distributed, except for the BMS which follows the Gaussian distribution. Statistical differences between two groups were analyzed via the nonparametric Mann-Whitney U test and to compare multiple groups, a Kruskal Wallis test with a Dunn's Multiple comparison test was used.

Functional recovery *in vivo* was statistically analyzed using two-way ANOVA for repeated measurements with a Bonferroni post hoc test for multiple comparisons. GFAP, MBP, Iba-1 and Arg-1 expression were analyzed using regular two-way ANOVA. Data were presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant when $p<0.05$.

Additionally, in the attachments (figure S5.1), the BMS results are presented as box plots with the whiskers indicating the minimum and maximum. Day-to-day comparisons between the three groups has been performed by using the Kruskall-Wallis test followed by the Dunn's Multiple Comparison test when the data are not-normally ditirbuted and by using One-way Anova followed by Bonferroni's Multiple Comparison test when the data are normally distributed. Data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test.

5.4 Results

5.4.1 IL-13-Mϕs secrete IL-13 and polarize naive macrophages towards an alternatively activated macrophage phenotype (M2)

In a first part of this study, we verified the IL-13 secretion and the macrophage phenotype after lentiviral vector (LVv) transduction with the pIRES-mIL-13-puro vector. ELISA on the culture medium revealed that the IL-13 LVv transduced macrophages (IL-13-Mϕ) secrete significant more IL-13 (2 ng/ml) compared to the control groups: vehicle control (ctrl), recombinant IL-13 (rIL-13) stimulated macrophages (M2-Mϕ) and LPS stimulated macrophages (M1-Mϕ) (**figure 5.1A**). In fact, the ctrl- and M1-Mϕs do not secrete IL-13, whereas the M2-Mϕs contain low levels of IL-13 in their medium which is probably left over of the rIL-13, although we can not exclude they also secrete IL-13 because of their alternatively activated phenotype. Expression of Arg-1, an M2 marker, indicates that the IL-13-Mϕs have an alternatively activated macrophage phenotype (M2) (**figure 5.1B**). The low levels of NO_2-secretion and iNOS expression, which are both characteristics of classically activated macrophages (M1), confirm their M2-phenotype (**figure 5.1C-D)**. The M1-Mϕs secrete significantly more NO_2 and express significantly more iNOS compared to ctrl and IL-13-Mϕ, which indicates both tests work properly.

To investigate whether the IL-13-Mϕs can polarize naive M0-Mϕ towards M2, the M0s were incubated with the medium of the IL-13-Mϕ (CM). Western blot analyses revealed that after incubation with CM for 48h, the M0-Mϕ express arg-1 (**figure 5.1E**) and not iNOS (**figure 5.1F**), indicative of a M2-phenotype.

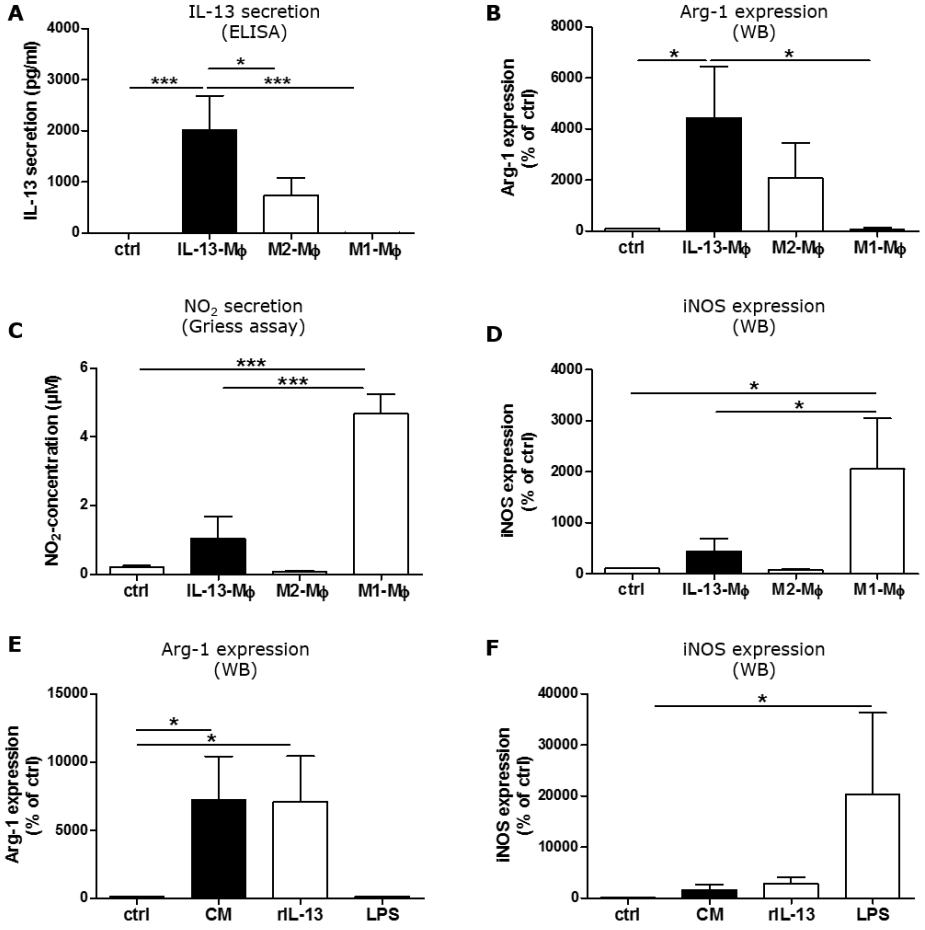


Figure 5.1: IL-13-Mϕs secrete IL-13 and polarize naive macrophages towards an alternatively activated macrophage phenotype (M2). A) IL-13 secretion in the culture medium has been measured by means of an IL-13-ELISA 72h after puromycine selection. IL-13-Mϕs secrete significantly more IL-13 in the medium compared to the different controls. Three different controls have been included in every experiment: not stimulated naive Mϕ (ctrl = M0), rIL-13 stimulated Mϕ (M2 positive control) and LPS stimulated Mϕ (M1 positive control). Data represent mean ± SEM; *p<0.05, ***p<0,001; n=22-23 wells/condition. **B)** Arg-1 expression has been determined by western blot analyses. IL-13-Mϕs express significantly more arg-1 compared to the ctrl and the M1-Mϕs. Data were normalized to control and represent mean ± SEM; *p<0.05, n=3-4 bands/condition analysed.

C) The Griess-assay has been used to measure the NO_2 secretion into the culture medium by the IL-13-Mϕ and the different controls. The IL-13-Mϕs do not secrete significantly more NO_2 compared to the ctrl, whereas the M1s secrete significantly more NO_2 compared to the IL-13-Mϕs and the ctrl which indicates the correct working of the Griess-assay. Data represent mean ± SEM; ***p<0.001, n=34-40 wells/condition. **D)** iNOS expression has been determined by western blot analyses. The IL-13-Mϕs do not express significantly more iNOS compared to the ctrl, whereas the M1-Mϕs do express significantly more iNOS compared to the IL-13-Mϕ and to control. Data were normalized to control and represent mean ± SEM; *p<0.05, n=3-4 bands/condition analysed. **E)** Naive M0-Mϕ have been incubated with CM from IL-13-Mϕs for 48h. Western blot analyses indicates they express arg-1 similarly to the M2-Mϕ and significantly higher compared to the ctrl. Data were normalized to control and represent mean ± SEM; *p<0.05, n=3 bands/condition analysed. **F)** Naive M0-Mϕs have been incubated with CM from IL-13-Mϕs for 48h. Western blot analyses indicates they express very low levels of iNOS, similarly to the ctrl. The M1-Mϕs express significantly more iNOS compared to the ctrl. Data were normalized to control and represent mean ± SEM; *p<0.05, n=3 bands/condition analysed.

5.4.2 Transplantation of IL-13-Mϕs improves functional recovery and reduces lesion size and demyelinated area after SCI

After verification of the IL-13-Mϕ phenotype, we investigated whether these Mϕs could improve functional recovery after SCI. In addition, we want to reveal whether the IL-13-Mϕs have a better therapeutic potential compared to M2-Mϕs as they have the tendency to become M1 *in vivo* in the pro-inflammatory lesion environment (72). Therefore, mice were injected perilesionaly with either vehicle, M2-Mϕs (=rIL-13 stimulated Mϕ) or IL-13-Mϕs (=Mϕs genetically engineered to continuously secrete IL-13). Functional recovery was measured during 4 weeks post inury using the BMS. The IL-13-Mϕ treated mice improved significantly better compared to the vehicle group and the M2-Mϕ treated mice (**figure 5.2A and figure S5.1 in the Attachments**). Lesion size quantification showed a significant decrease after IL-13-Mϕ treatment compared to vehicle treatment (**figure 5.2B**). Similarly, there is a trend towards a decreased demyelinated area after IL-13-Mϕ treatment compared to the vehicle control (**figure 5.2C**). M2-Mϕ treatment significantly reduced the demyelinated area compared to the vehicle.

Immunofluorescent stainings have been used to quantify the lesion size (**figure 5.2D-F**) and the demyelinated area (**figure 5.2G-I**). Summarised, these data show that on a functional level, IL-13-Mϕ treatment improves functional recovery, whereas M2-Mϕ treatment does not. On the histopathological level, the IL-13-Mϕs reduce lesion size and demyelinated area, whereas M2-Mϕs only reduce the demyelinated area.

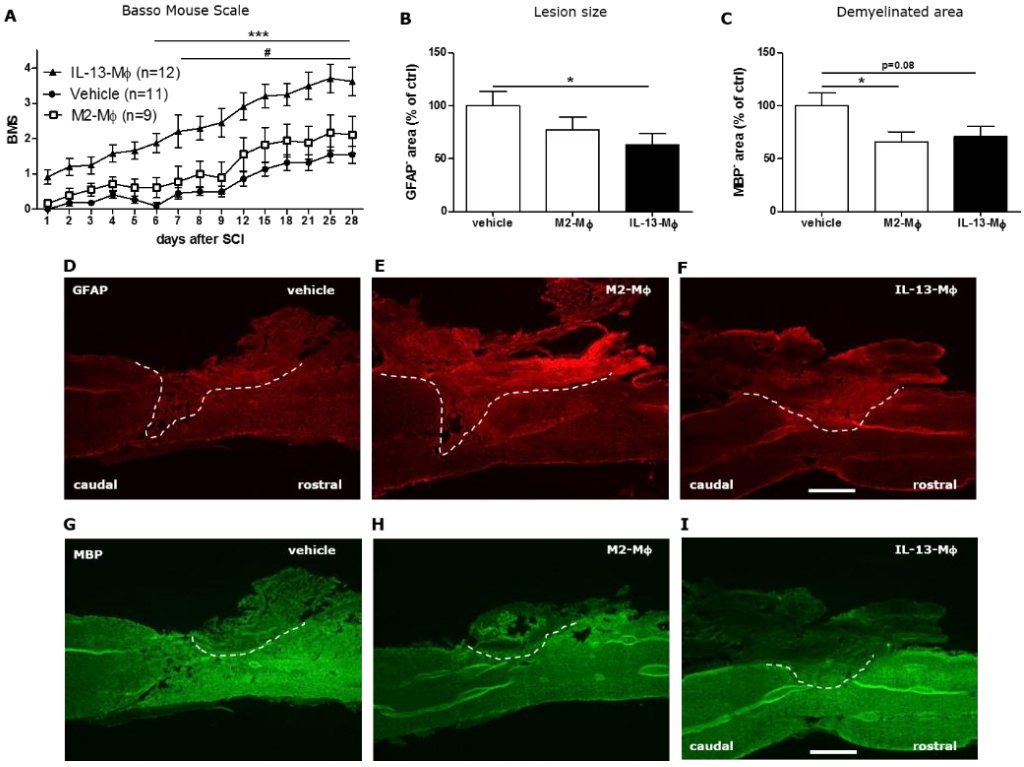


Figure 5.2: Transplantation of IL-13-Mϕs improves functional recovery and reduces lesion size and demyelinated area after SCI. A) Mice receiving transplantation of IL-13-Mϕs show a significantly increased BMS score following SCI, compared to M2-Mϕ and vehicle treated mice. Data represent mean ± SEM; *** $p < 0.001$ (IL-13-Mϕ vs. vehicle) and #$p < 0.05$ (IL-13-Mϕ vs. M2-Mϕ), n= 9-12 mice/group. **B-C)** Image analysis revealed a decrease in (B) lesion size and (C) demyelinated area in the IL-13-Mϕ treated animals, compared with the vehicle group. Data were normalized to control and represent mean ± SEM; *$p<0.05$, n= 11-13 mice/group. **D-I)** Representative images of the fluorescent staining for GFAP (D-F) and MBP (G-I) are shown of spinal cord sections including the injury epicentre of vehicle treated (D and G), M2-Mϕ treated (E and H) and IL-13-Mϕ treated mice (F and I). The lesion size and demyelinated area were determined as depicted by the dotted white line. Scale bar= 500µm.

5.4.3 Transplantation of IL-13-Mϕs has no effects on the presence of microglia/macrophages and astrogliosis at the lesion site

In a first attempt to understand the underlying mechanisms of the beneficial effects of IL-13-Mϕs on functional recovery and lesion size and demyelinated area reduction, we investigated the microglia/macrophage (**figure 5.3A**) and astroglial (**figure 5.3B**) responses 4 weeks after SCI. Therefore, Iba-1 (**figure 5.3C-E**) and GFAP (**figure 5.3F-H**) intensity was quantified 600 µm caudal and rostral from the lesion center (LC), in squares measuring 100x100 µm. There was no significant difference observed in the presence of microglia/macrophages between the different treatment groups (**figure 5.3A and figure 5.3C-E**). Additionally, there was no difference in astrogliosis between the different groups (**figure 5.3B and figure 5.3F-H**).

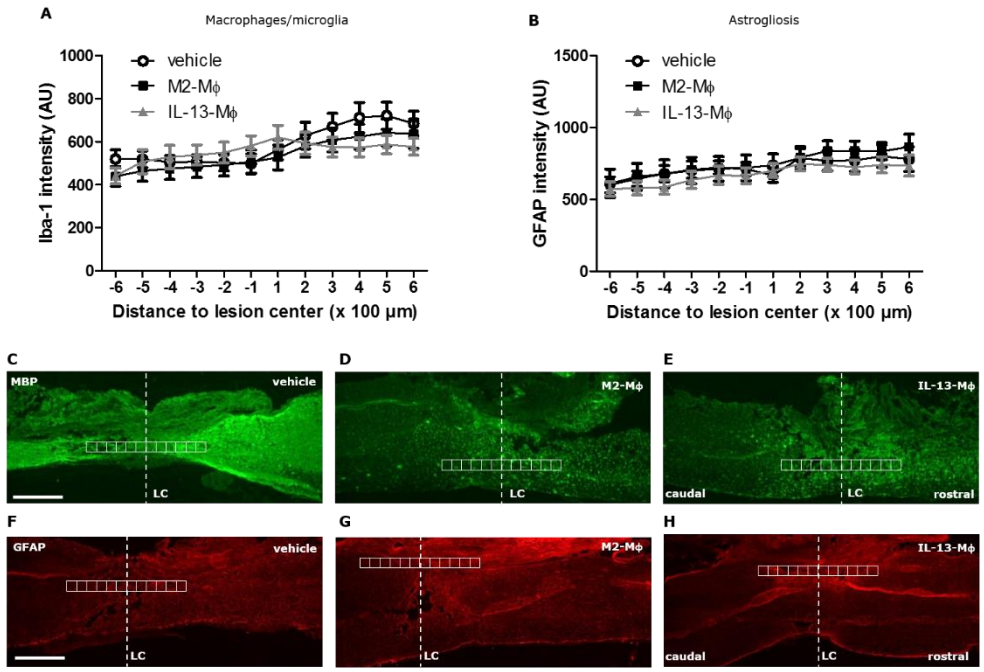


Figure 5.3: Transplantation of IL-13-Mϕs has no effects on the presence of microglia/macrophages and astrogliosis at the lesion site. A) There was no significant difference observed in the presence of microglia/macrophages between vehicle, M2-Mϕ or IL-13-Mϕ treated animals as measured by intensity analysis of Iba-1 (C-E). **B)** Similarly, there was no significant difference observed in astrogliosis between vehicle, M2-Mϕ or IL-13-Mϕ treated animals as measured by intensity analysis of GFAP (F-H). Data represent mean ± SEM; n= 11-14/group. **C-H)** All analyses were quantified within square areas of 100µm X 100µm perilesionally placed as indicated in the figure, extending 600µm rostral to 600µm caudal from the lesion center (LC). Scale bar= 500 µm.

5.4.4 Transplantion of IL-13-Mϕs increases the number of neuroprotective, alternatively activated macrophages and deceases the number of neurotoxic, clasically activated macrophages at the lesion site

To further investigate the effects of IL-13-Mϕ transplantation, the clasically activated microglia/macrophages (M1) and alternatively activated microglia/macrophages (M2) were quantified 4 weeks post injury at the level of the injection site and the lesion site by counting the $MHCII^+$ (**figure 5.4**) and $arg\text{-}1^+$ cells (**figure 5.6**) respectively. Counting the $MHCII^+$ microglia/macrophages (M1) at the injection site (indicated by I) (**figure 5.4A-C**) in the region of interest (ROI) (**figure 5.5A-B, E-F and I-J**) revealed that there is a trend towards an increased number after M2-Mϕ treatment, compared to vehicle and IL-13-Mϕ treatments (**figure 5.4D**)**.** On the other hand, quantifying the $MHCII^+$ microglia/macrophages at the lesion site (indicated by L) (**figure 5.4A-C**) in the region of interest (ROI)(**figure 5.5C-D, G-H and K-L**) revealed that their number is decreased after IL-13-Mϕ treatment, compared to the vehicle group (**figure 5.4D**)**.**

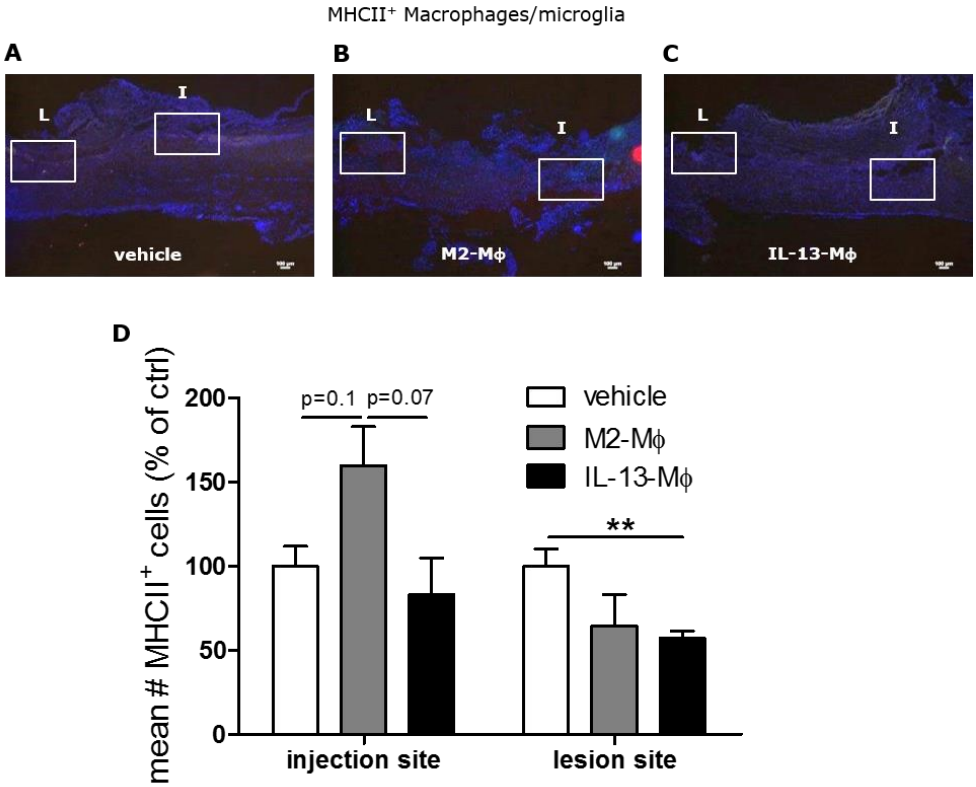


Figure 5.4: Transplantion of M2-Mϕ increases the number MHCII$^+$ microglia/macrophages at the injection site, whereas transplantion of IL-13-Mϕ deceases the number MHCII$^+$ microglia/macrophages at the lesion site. A-C) To identify the lesion (indicated by L) and injection site (indicated by I), we have included an overview of the section stained with DAPI (blue), MHCII (green) and arg-1 (red). Scale bars: A-C= 100 µm. **D)** Transplantion of M2-Mϕs increased the number MHCII$^+$ microglia/macrophages at the injection site, whereas transplantion of IL-13-Mϕs deceased the number MHCII$^+$ microglia/macrophages at the lesion site. Data were normalized to control and represent mean ± SEM; **p<0.01; n = 4-7/group.

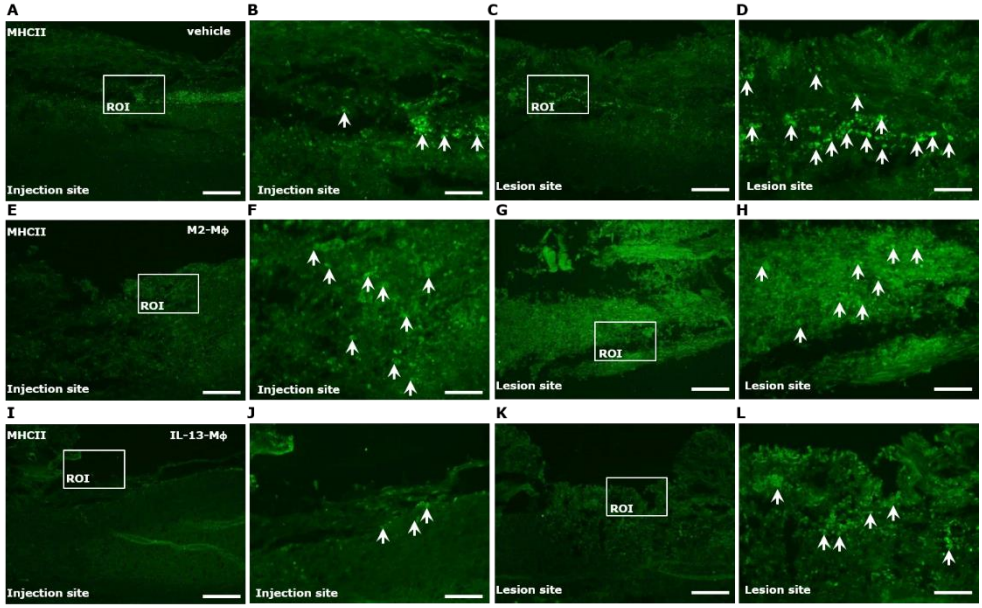


Figure 5.5: Transplantion of M2-Mϕ increases the number MHCII$^+$ microglia/macrophages at the injection site, whereas transplantion of IL-13-Mϕ deceases the number MHCII$^+$ microglia/macrophages at the lesion site. **A-L**) Representative images indicating the cell locations (ROI) at the injection site (A+E+I) and at the lesion site (C+G+K) are shown. B+F+J are higher magnifications of the ROIs in A+E+I; MHCII$^+$ cells are indicated by the arrows. D+H+L are higher magnifications of the ROIs in C+G+K; MHCII$^+$ cells are indicated by the arrows. Scale bars: A+E+I and C+G+K= 200 µm; B+F+J and D+H+L= 100 µm**.**

Quantifying the arg-1$^+$ microglia/macrophages (M2) at the injection site (indicated by I) (**figure 5.6A-C**) in the region of interest (ROI)(**figure 5.7A-B, E-F and I-J**) revealed that there is a trend towards an increased number after M2-Mϕ treatment, compared to IL-13-Mϕ treatment (**figure 5.6D**)**.** On the other hand, quantifying the arg-1$^+$ microglia/macrophages at the lesion site (indicated by L) (**figure 5.6A-C**) in the region of interest (ROI)(**figure 5.7C-D, G-H and K-L**) revealed that their number increased after IL-13-Mϕ treatment, indicated by a trend compared to the vehicle group and by a significant difference compared to M2-Mϕs (**figure 5.6D**)**.** On the other hand, the number of arg-1$^+$ cells was significantly decreased after M2-Mϕ treatment, compared to vehicle and IL-13-Mϕ treatment groups (**figure 5.6D**).

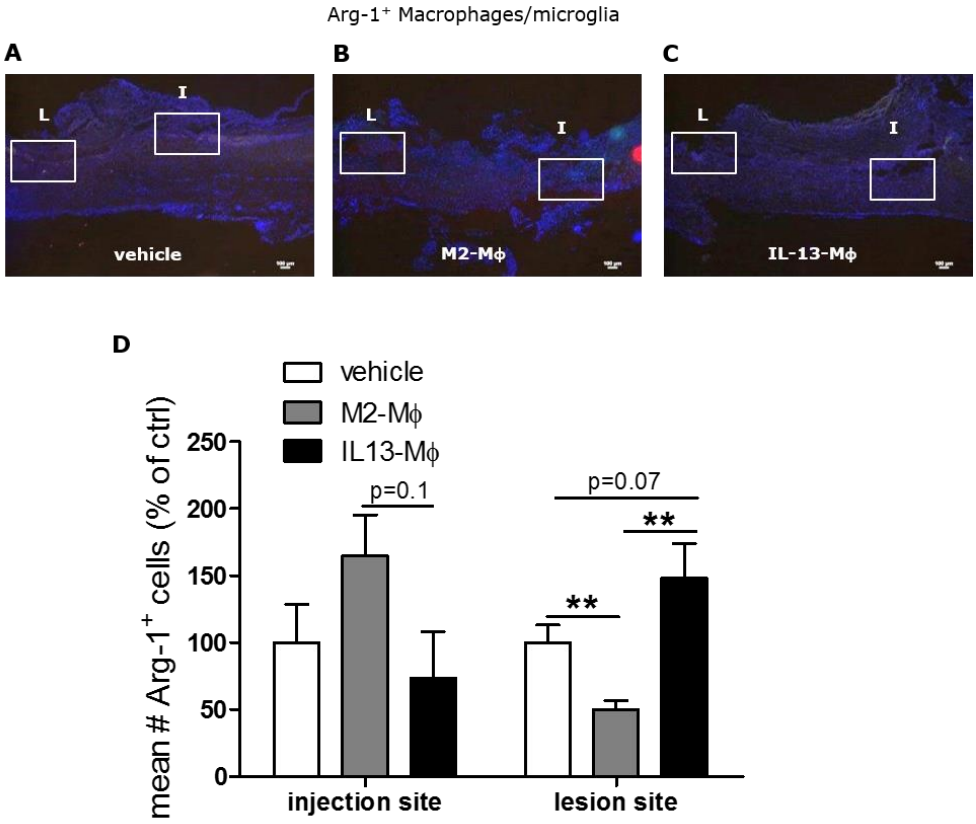


Figure 5.6: Transplantion of M2-Mϕs increases the number arg-1+ microglia/macrophages at the injection site, whereas transplantion of IL-13-Mϕs increases the number arg-1$^+$ microglia/macrophages at the lesion site. A-C) To identify the lesion (indicated by L) and injection site (indicated by I), we have included an overview of the section stained with DAPI (blue), MHCII (green) and arg-1 (red). Scale bare= 100 µm **D)** Transplantion of M2-Mϕs increases the number arg-1$^+$ microglia/macrophages at the injection site, whereas transplantion of IL-13-Mϕ increases the number arg-1$^+$ microglia/macrophages at the lesion site. Data were normalized to control and represent mean ± SEM; **p<0.01; n = 4-7/group.

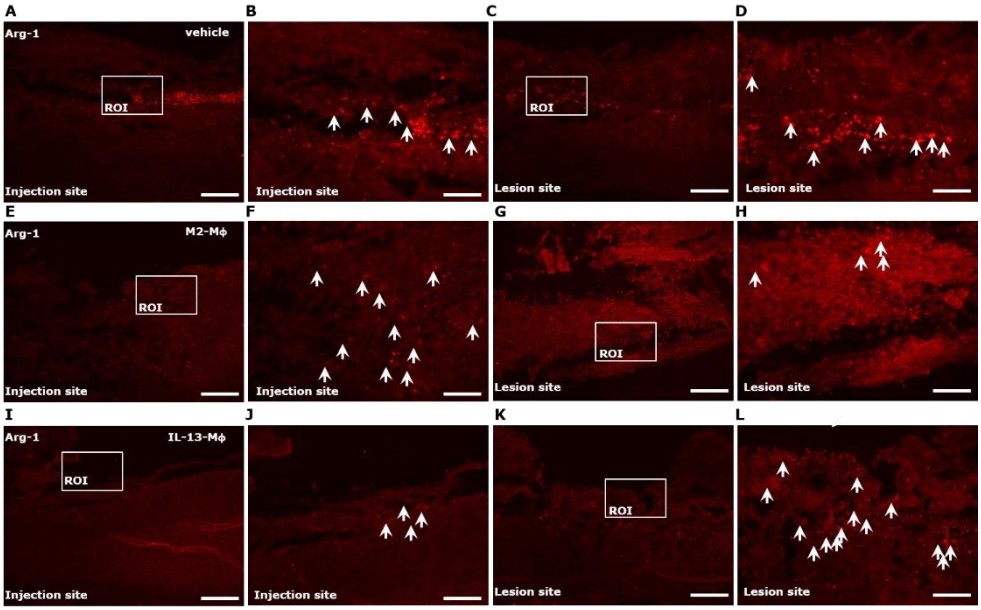


Figure 5.7: Transplantion of M2-Mϕs increases the number arg-1$^+$ microglia/macrophages at the injection site, whereas transplantion of IL-13-Mϕs increases the number arg-1$^+$ microglia/macrophages at the lesion site. A-L) Representative images indicating the cell locations (ROI) at the injection site (A+E+I) and at the lesion site (C+G+K) are shown. B+F+J are higher magnifications of the ROIs in A+E+I; Arg-1$^+$ cells are indicated by the arrows. D+H+L are higher magnifications of the ROIs in C+G+K; Arg-1$^+$ cells are indicated by the arrows. Scale bars: A+E+I and C+G+K= 200 µm; B+F+J and D+H+L= 100 µm**.**

5.4.5 Transplantion of IL-13-Mϕs decreases the number of CD4$^+$ T-cells in the spinal cord

Quantification of CD4$^+$ T-cells throughout the spinal cord revealed a significant decreas in the number of CD4$^+$ T-cells in the IL-13-Mϕ treated mice, compared to the vehicle treated controls (**figure 5.8**).

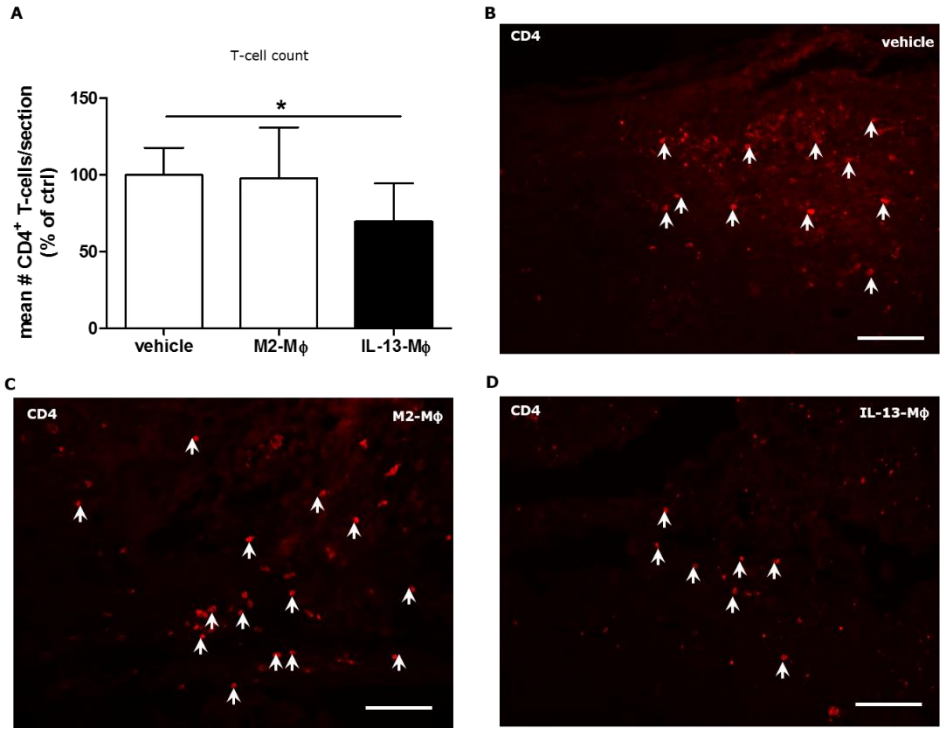


Figure 5.8: Transplantion of IL-13-Mϕ decreases the number of CD4$^+$ T-cells in the spinal cord. A) CD4 staining in spinal cord sections revealed a significant decrease in the number of CD4$^+$ T-cells in the IL-13-Mϕ treated group, compared with vehicle treated mice, 4 weeks after SCI. Data were normalized to control and represent mean± SEM; *p<0.05; n= 10-14 mice/group. **B-D)** Representative images of spinal cord sections of mice treated with vehicle (B), M2-Mϕ (C) or IL-13-Mϕ (D); arrows indicate the CD4$^+$ cells. Scale bar= 100 µm.

5.4.6 Transplantion of IL-13-Mϕs decreases the number of macrophage-axon interactions

Finally, we investigated how transplantation of IL-13-Mϕs might influence the microglia/macrophage-axon interactions using Iba-1 and neurofilament (NF) staining. We analysed two areas caudal and rostral from the lesion epicentre (indicated by the white boxes) (**Figure 5.9B-D**) and counted the number of microglia/macrophage-axon contacts (indicated by the white arrows in i-iii) (**Figure 5.9E-G**). We observed a significant decrease in the number of microglia/macrophage-axon contacts in the mice treated with M2-Mϕ and IL-13-Mϕ compared with vehicle control mice (**Figure 5.9A**). This reduction in the number of destructive macrophage-axon contacts suggests that both M2-Mϕ and IL-13-Mϕ may drive activated macrophages located at the lesion site to a more alternatively actived, neuroprotective phenotype.

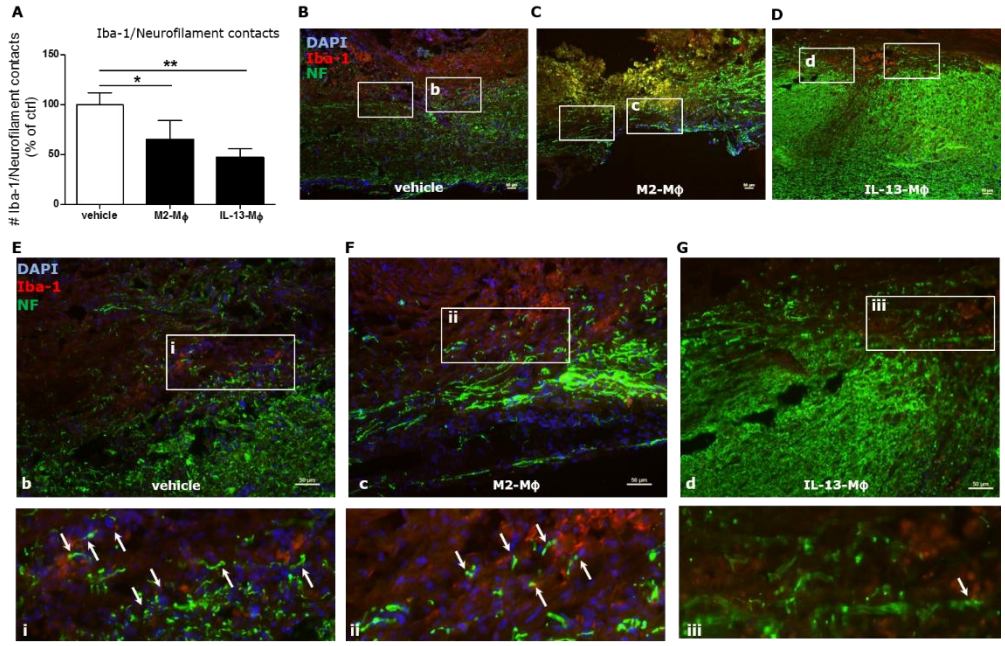


Figure 5.9: Transplantion of IL-13-Mϕ decreases the number of macrophage-axon interactions. A) Quantification of microglia/macrophage and axon contacts following staining for Iba-1 (red) and neurofilament (green) respectively, revealed a significant decrease in the number of contacts in both M2-Mϕ and IL-13-Mϕ treated mice compared with vehicle controls. Data were normalized to control and represent mean± SEM; **p< 0.01 and *p < 0.05; n = 10-13 mice/group. **B-G**) Overview images from vehicle (B), M2-Mϕ (C) and IL-13-Mϕ (D) treated mice indicate the areas (two white boxed regions) where microglia/macrophage and axon contacts were quantified rostral and caudal from the lesion epicentre. A larger magnification of the white boxes labeled (a, b, c) are shown in (E, F, G) respectively. The white boxed regions (i-iii) in images (E-G) are shown at a higher magnification underneath, to indicate examples of microglia/macrophage and axon contacts (arrows). Scale bar= 50μm.

5.5 Discussion

The aim of this study was to investigate whether lentiviral vector-mediated expression of the anti-inflammatory cytokine IL-13 by Mϕs improves functional regeneration via polarization of the lesioned spinal cord micro-environment towards an anti-inflammatory milieu. Previous studies of our group already indicated that IL-13, either via gene therapy or via MSCs as carriers, can improve functional outcome by induction of an alternatively activated Mϕ phenotype (M2) (245, 249, 251). However, it was not completely unraveled yet which phagocyte population was responsible for the neuroprotective effects as both a reduction in pro-inflammatory microglia as well as an increase in anti-inflammatory arginase-positive Mϕs became apparent after IL-13 treatment (245). A proposed mechanism of action was that indirect effects via alternatively activated Mϕs might decrease axon degeneration because pro-inflammatory Mϕs play a direct role in axonal retraction and destruction, therefore an M2-conversion may have rendered these cells less destructive (243). In addition, earlier studies have indicated that inhibition of Mϕs and not of microglia improved recovery after neurotrauma (243). Therefore, we have chosen for controled immunomodulation, specifically targeted at Mϕs, to improve functional regeneration after SCI and to reveal whether they are responsible for the IL-13 mediated neuroprotective effects. In addition, an increased potential for tumor formation in the CNS when using stem cells made us step aside from MSCs (252). Moreover, it has been shown that Mϕs can actively migrate to the SCI lesion site which is an additional advantage of using Mϕs instead of MSCs (253).

Here, we show that LVv mediated overexpression of IL-13 by Mϕs makes them secrete IL-13 levels comparable to the recombinant IL-13 levels necessary to polarize naive Mϕs towards M2 (254). In addition, they obtain an alternatively activated M2 phenotype and convert naive Mϕs to the M2 phenotype *in vitro* as well. After SCI, they improve functional recovery accompanied with a reduction in the lesion size and the demyelinated area.

In addition, IL-13-Mϕ treatment reduced lesional T helper cell infiltration and $MHCII^+$ macrophage/microglia (M1) infiltration, accompanied with an increase in $Arg\text{-}1^+$ macrophage/microglia (M2) at the lesion site. Last but not least, this treatment also reduced the number of axon-phagocyte contacts suggesting a reduction in axonal dieback (243).

Similarly, Ma *et al.* have observed improved locomotor recovery, decreased spinal cord lesion volume and increased preservation of axon myelination after adoptive transfer of *in vitro* M2 polarized Mϕs in SCI rats (70). However, the findings of Kigerl *et al.* indicate that these M2 Mϕs do not maintain their phenotype after SCI. They injected GFP^+ M2 Mϕs into SCI mice to investigate the effects of the lesion microenvironment on Mϕ polarization. Three days post-injection, confocal microscopy revealed that only 20-40% of the injected cells maintained their M2 phenotype (72). Therefore, although Ma *et al.* observed an improved functional recovery, we believe that a substantial number of the injected M2 Mϕs were repolarized towards an M1 phenotype because of the typical pro-inflammatory lesion microenvironment. For that reason, we have injected IL-13-overexpressing Mϕs because they can modulate the SCI inflammation continuously via the constitutive secretion of IL-13.

Several delivery methods can be used to get IL-13 at the lesion site, including direct protein injection, non-viral and viral gene therapy and implantation of genetically engineered cellular grafts, each of them having specific advantages and disadvantages. Whereas direct protein injection would be the most practical, it would require multiple injections as, in the case of IL-13, sustained therapeutic protein expression may be essential. In our hands, local application of recombinant IL-13 into the spinal cord had no effects, although systemic administration improved functional recovery. However, lesion size, demyelination and astrogliosis were not affected, ruling out any direct effects on lesion remodeling. Therefore, it was difficult to pin down the exact mechanism of recombinant IL-13 treatment (unpublished results).

The number of Arg-1$^+$ macrophages/microglia was increased at the lesion site (unpublished results), showing that IL-13 is a promising candidate for immunomodulatory therapy but the application method needed to be fine-tuned to obtain specific effects.

Alternatively, mechanical or chemical methods (e.g. electroporation, ultrasound or lipoplexes) may be applied to transfer plasmid DNA encoding the therapeutic protein of interest into inflammatory cells at the site of neuroinflammation. Nevertheless, these techniques are still poorly efficient and need further optimisation for *in vivo* application. On the other hand, gene transfer in the CNS by means of viral vectors is highly efficient in rodents but remains controversial in terms of clinical translation to humans, despite many efforts undertaken to control gene insertion, protein expression and/or unwanted immune reactions. In addition, Guglielmetti and colleagues found that LV injections resulted in a slight activation of microglia and macrophages and upregulation of MHCII expression in case of LV injections in control mice, most likely illustrating the response to the viral particles and the attempt to eliminate them (251). These results were in line with previous studies showing that LV transduction can elicit a transient pro-inflammatory response against the LV particles (255), mediated by the activation of toll-like receptor 7 and or toll-like receptor 9, which, respectively, recognize single stranded RNA and unmethylated CpG (256).

Transplantation of genetically engineered cell populations is an emerging methodological approach for in situ delivery of therapeutic proteins. We have already used MSCs as a cellular carrier to deliver IL-13 due to their relatively easy *ex vivo* culture and susceptibility for genetic modification. As described above this method also improved functional outcome by induction of an alternatively activated Mϕ phenotype (M2) (245). There was a strong indication that alternatively activated Mϕs might decrease axon degeneration and provide functional regeneration by these means. To confirm this hypothesis we directly applied alternatively activated Mϕs continuously secreting IL-13. Additionally, there might be less change for tumor formation and they can actively migrate to the lesion site.

T cell analysis showed a significantly lower number of $CD4^+$ T-cells in the animals treated with IL-13 overexpressing Mϕs and rIL-13 stimulated Mϕs. The specific subtype of T-cells is unclear due to the low number of T-cells present in the CNS (86). This impedes isolation of sufficient T-cells for flow cytometric analysis. Elevated arg-1 expression by Mϕs has previously been associated with decreased T-cell function. The expression of arg-1 caused a drop in extracellular L-arginine levels, accompanied with a loss of CD3ζ chain expression on activated T-cells resulting in impaired T-cell proliferation and decreased cytokine production (257, 258).

Surprisingly, we do not see a difference in the presence of Iba-1^+macrophages/microglia. A possible explanation could be an overall reduction in microglia as described previously by our group (245). The number of arg-1^+ phagocytes might be increased at the lesion site for the IL-13-Mϕ treated group, but with a reduction in the number of microglia this could level off the increase again. However, further research is necessary to address this question.

Finally, we suggest the improved functional recovery accompanied with histological recovery has been established by the IL-13-Mϕs polarizing the spinal cord micro-environment towards an anti-inflammatory milieu. We suggest that the injected anti-inflammatory IL-13 secreting macrophages migrate to the lesion site where they convert the local Mϕs towards an M2 phenotype, thereby creating a neuroprotective environment more prone to functional regeneration. To confirm their phenotype *in vivo*, we have to trace the Mϕs properly. Ongoing experiments with IL-13 secreting GFP^+ Mϕs will elucidate whether the injected Mϕs stay M2 and whether endogenous Mϕs are truly polarized by means of an arg-1 staining. By tracing the injected Mϕs it will also become clear if they migrate to the lesion site or exert their effects from a distance via a bystander effect. The shown reduction in axon-phagocyte contacts suggests that injecting polarized Mϕs makes the present phagocyte population less destructive, since following SCI, CNS axons undergo lengthy retraction from the lesion site and activated macrophages play a direct role in this retraction via destructive physical contact (243, 259).

Therefore, we speculate that a corresponding reduction in axonal dieback may have led to the improved histopathological and functional outcome observed following SCI. Axon regeneration needs to be investigated by tracing the cortical spinal tract. There are already clear indications from literature that alternatively activated Mϕs and microglia promote wound healing via the secretion of pro-regenerative factors such as IL-10, insulin growth factor-1 and vascular growth factor-A (260).

In conclusion, this study shows that perilesional transfer of IL-13 overexpressing Mϕs is a successful experimental approach to improve functional and histopathological recovery in the SCI mouse model.

Chapter 6

Mast cells protect from post-traumatic spinal cord inflammation in mice by degrading lesional scarring components via mouse mast cell protease 4

Based on:

Mouse mast cell protease4 suppresses scar formation after spinal cord injury

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[a]TV and SL contributed equally to this study

Chapter 6. Mast cells protect from post-traumatic spinal cord inflammation in mice by degrading lesional scarring components via mouse mast cell protease4

6.1 Abstract

Spinal cord injury (SCI) results in the formation of a glial and fibrotic scar which significantly impairs repair processes. Mast cells (MCs) protect the spinal cord after mechanical damage by reducing scar tissue formation via mouse mast cell protease6 (mMCP6) and by suppressing 'detrimental' inflammatory processes mediated via mMCP4. In addition to the immunomodulatory properties, mMCP4 also plays an important role in tissue remodeling and extracellular matrix degradation. Therefore, we have investigated the effects of mMCP4 on the scarring response after SCI. We demonstrate that the previously shown decrease in locomotor performance is associated with an increased lesion size. The expression of axon-growth inhibitory chondroitin sulphate proteoglycans was significantly increased in the perilesional area in mMCP4$^{-/-}$ mice compared to wildtype mice. In addition, the fibronectin-, laminin- and collagen IV-positive scar area was significantly enlarged in mMCP4$^{-/-}$ mice at the lesion center. Moreover, a degradation assay revealed that mMCP4 directly cleaves collagen IV *in vitro*. A suggestive trend also indicates direct cleavage of fibronectin. On the gene level, GFAP and neurocan were significantly higher expressed in the mMCP4$^{-/-}$ group compared to wildtype mice at day 28 and day 3 after injury respectively. In conclusion, our data highlight the scar-modulating properties of mMCP4 *in vivo*, thereby suggesting a new mechanism via which mMCP4 can stimulate recovery after SCI.

6.2 Introduction

Spinal cord injury (SCI) results in the formation of a glial and fibrotic scar which significantly impairs repair processes (8, 9, 261). The scarring response is an evolving process, with different cells participating (*e.g.* astrocytes, mast cells, endothelial cells), which results in the local secretion of extracellular matrix (ECM) components that form a dense scar (8, 262). This scar is characterized by the deposition of chondroitin sulphate proteoglycans (CSPGs) in the perilesional area (263-265), and the formation of a basement membrane-rich matrix in the lesion center (8, 266-268). Scar tissue formation is essential in the acute phase after injury to regain tissue integrity and to limit secondary tissue damage (266, 269), but it has a negative impact on repair processes at later time points. Scar remodeling therapies are therefore of great interest in current SCI research (19, 270-272).

Mast cells (MCs) are immune cells that are characterized by electron-dense granules in their cytoplasm in which preformed mediators are stored, including cytokines and MC-specific proteases (*i.e.* chymase and tryptase) (91, 273-275). They reside in virtually all organs, including the brain and spinal cord (276-279). As effector cells of the innate immune system, MCs from the periphery can also infiltrate the CNS through a compromised blood brain barrier that is characteristic for neuroinflammatory diseases and traumatic injuries. We recently reported a favorable role of MCs after traumatic brain- and spinal cord injury (32, 86, 247). Experiments in knockout mice indicated that MCs support neuronal survival and functional recovery (86, 247). In particular, MCs appeared to be protective due to their ability to degrade inflammation-associated cytokines such as interleukin 6 (IL-6), thereby tempering 'detrimental' inflammatory processes (247). These immunomodulatory effects were at least partly mediated via mouse mast cell protease4 (mMCP4), a MC-specific chymase (247). Moreover, we provided evidence that MCs reduce scar tissue formation after SCI via mMCP6, a MC-specific tryptase. Our findings indicate that mMCP6 reduces scarring by (in)direct cleavage of scar-related matrix components, and by suppressing the gene expression of several scar factors (32).

Apart from the immunomodulatory functions, mMCP4 is also involved in ECM degradation through direct cleavage effects or indirectly by activating other ECM-processing enzymes (e.g. matrix metalloproteinases) (90, 91). Considering the role mMCP6 played in scar remodeling and the ECM-degrading properties of mMCP4, we have investigated in this study whether mMCP4 improved recovery after SCI by targeting the inhibitory scar that is formed at the lesion site.

6.3 Materials and methods

6.3.1 Animals and experimental spinal cord injury

We used mMCP4 knockout (mMCP4$^{-/-}$; 10-12 weeks old; provided by Dr. Gunnar Pejler) mice, which were backcrossed for at least 10-generations on the C57BL/6 background (280). Wildtype (WT) C57BL/6j mice (10-12 weeks old; Janvier) were used as controls. A T-cut hemisection injury was performed as previously described (26, 247). Briefly, a bilateral hemisection injury of the spinal cord was induced by using iridectomy scissors to transect the corticospinal tract, which results in complete hind limb paralysis. All mice were housed in a conventional animal facility at Hasselt University under regular conditions, i.e. in a temperature-controlled room (20 ± 3°C) on a 12 h light-dark schedule and with food and water *ad libitum*; 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.

6.3.2 Immunohistochemistry

Spinal cord cryosections (14 µm), were obtained from animals transcardially perfused 28 days post injury (dpi) with ringer solution containg heparin, followed by 4% paraformaldehyde in 0.1M PBS. Immunofluorescent stainings were performed as described previously (32). The following primary antibodies were used: mouse anti-glial fibrillary acidic protein (GFAP) (1:500; Sigma-Aldrich), rabbit anti-laminin 1α (1:200; Abcam), rabbit anti-fibronectin (1:200; Abcam), rabbit anti-collagen type IV (1:200; Abcam) and mouse anti- CSPGs (1:200; Sigma-Aldrich). Next, the spinal cord sections were incubated with Alexa-labeled secondary antibodies, namely goat anti-mouse IgG Alexa 555, goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 568, goat anti-rat Alexa 488 and goat anti-mouse IgM Alexa 555 (1:250; all secondary antibodies were obtained from Invitrogen). Isotype control stainings were included and omission of the primary antibodies indicated specificity of the secondary antibodies (data not shown). Images were taken with a Nikon Eclipse 80i microscope and a Nikon digital sight camera DS-2MBWc (Nikon, Amstelveen, The Netherlands).

Quantitative image analyses were performed on original unmodified photos using the ImageJ open source software (National Institutes of Health, Bethesda, MD, USA). For standardization, analyses were performed on 5-8 spinal cord sections (per mouse) representing the lesion area, i.e. the lesion epicenter as well as consecutive sagittal sections, as previously described (32, 247). Representative photomicrographs are shown in Figure 6.1A. To maximise image readability, the contrast and brightness of the stainings was enhanced equally in the corresponding groups. Furthermore, the tissue section borders were highlighted using a white line for clarity.

6.3.3 Quantitative PCR analysis

The mice were anesthesized with 3% isoflurane (IsofFlo, Abbot Animal Health, Waver, Belgium) to perform SCI surgery to obtain spinal cord tissue for mRNA isolation at different time points post-injury. The mRNA expression levels of glial scar-associated components were investigated at different phases after SCI, namely the acute phase (2 dpi), the subacute phase (7 dpi), and finally the early stage of the chronic remodeling phase (28 dpi). At these selected time points, WT and mMCP4$^{-/-}$ mice were transcardially perfused with Ringer solution as described above. Healthy control mice (without SCI) were included in the analysis. Standardized areas of spinal cord tissue (5 mm cranial and 5 mm caudal to the lesion center) were collected and mRNA was extracted using the Paris Kit (Life Technologies), according to the manufacturer's instructions with minor modifications as described previously (32). Quantitative real time PCR was performed with the primers summed-up in Vangansewinkel *et al* (32).The gene expression levels are presented as fold change of the WT control condition.

6.3.4 Extracellular matrix degradation assays

Murine MCs were obtained as previously described (247, 281), by culturing primary bone marrow cells isolated from the femurs and tibia of WT or mMCP4$^{-/-}$ mice. Mature MCs were collected (5×10^4 cells), washed and stimulated for 30 min at 37 °C and 5% CO_2 with 1 µM ionomycine (Sigma-Aldrich) in 100 µl Tyrode buffer (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, 10 mM Hepes, 5.6 mM glucose and 0.01% BSA in MilliQ). MC degranulate was then collected and used for the ECM degradation assays. Murine recombinant fibronectin (1 µg; Abcam), laminin (0.5 µg; Millipore) or a CSPG-mix (CC117, 2 µg; Millipore) were incubated with 20 µl MilliQ or with 20 µl degranulate obtained from either WT or mMCP4$^{-/-}$ MCs for 48 h at 37 °C. The incubations for collagen IV were performed at 4°C to prevent polymerization. Next, the cleaved fragments were identified via Western blot analysis, performed as desribed previously (32). Western blotting for collagen IV was performed under native conditions (i.e. no denaturation step with β-mercaptoethanol). The following primary antibodies were used: anti-fibronectin (1:1000; Santa Cruz), anti-collagen type IV (1:2000; Abcam), anti-laminin (1:1000; Abcam) and anti-CSPGs (1:1000; CS56; Sigma-Aldrich). Next, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies: goat anti-mouse IgM and goat anti-rabbit (dilution 1:5000; all secondary antibodies were obtained from Dako). The protein bands were quantified via the ImageQuant TL software. To minimize bias due to differences in densitometric measurements between experiments, each control condition per experiment was set at 100% and is therefore lacking a standard error bar.

6.3.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). Data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. Histological differences between two groups were evaluated using the nonparametric Mann-Whitney U test. The *In vitro* degradation assays were analyzed with a 1-way ANOVA to compare multiple groups followed by a Tukey *post hoc* test. The Quantitative PCR data were analyzed using 2-way ANOVA with a Bonferroni *post hoc* test. Data were presented as mean ± SEM. Differences were considered statistically significant when $P < 0.05$.

6.4 Results

6.4.1 Increased lesion size and scarring response in mMCP4-/- mice after SCI

To investigate the involvement of mMCP4 in scarring more extensively, we subjected mMCP4$^{-/-}$ mice and their corresponding WT controls to a spinal cord hemisection injury. Here, we investigated whether the impaired functional outcome in mMCP4$^{-/-}$ mice after SCI was associated with a more severe lesion pathology. The lesion size (GFAP^{-} area) was significantly increased in mMCP4$^{-/-}$ mice compared to the controls at 28 dpi (**Figure 6.1Ai/ii, B**). Furthermore, we focussed on four key components of the scar, namely CSPGs in the perilesional area and fibronectin, laminin and collagen IV at the lesion center. The intensity of axon-growth inhibitory CSPGs was analyzed in a well-defined area around the lesion center (white encircled area in **Figure 6.1Aiii/iv**). Immunoreactivity for CSPGs was significantly higher in the perilesional area in mMCP4$^{-/-}$ mice compared to WT mice at 28 dpi (**Figure 6.1Aiii/iv, C**). In addition, also the area positive for fibronectin (Figure 1Av/vi, D), laminin (**Figure 1Avii/viii, F**) and collagen type IV (**Figure 6.1Aix/x, H**) was significantly enlarged in mMCP4$^{-/-}$ mice compared to control mice. The immunoreactivity of these scar components was comparable between the two experimental groups (**Figure 6.1A, E, G and I**).

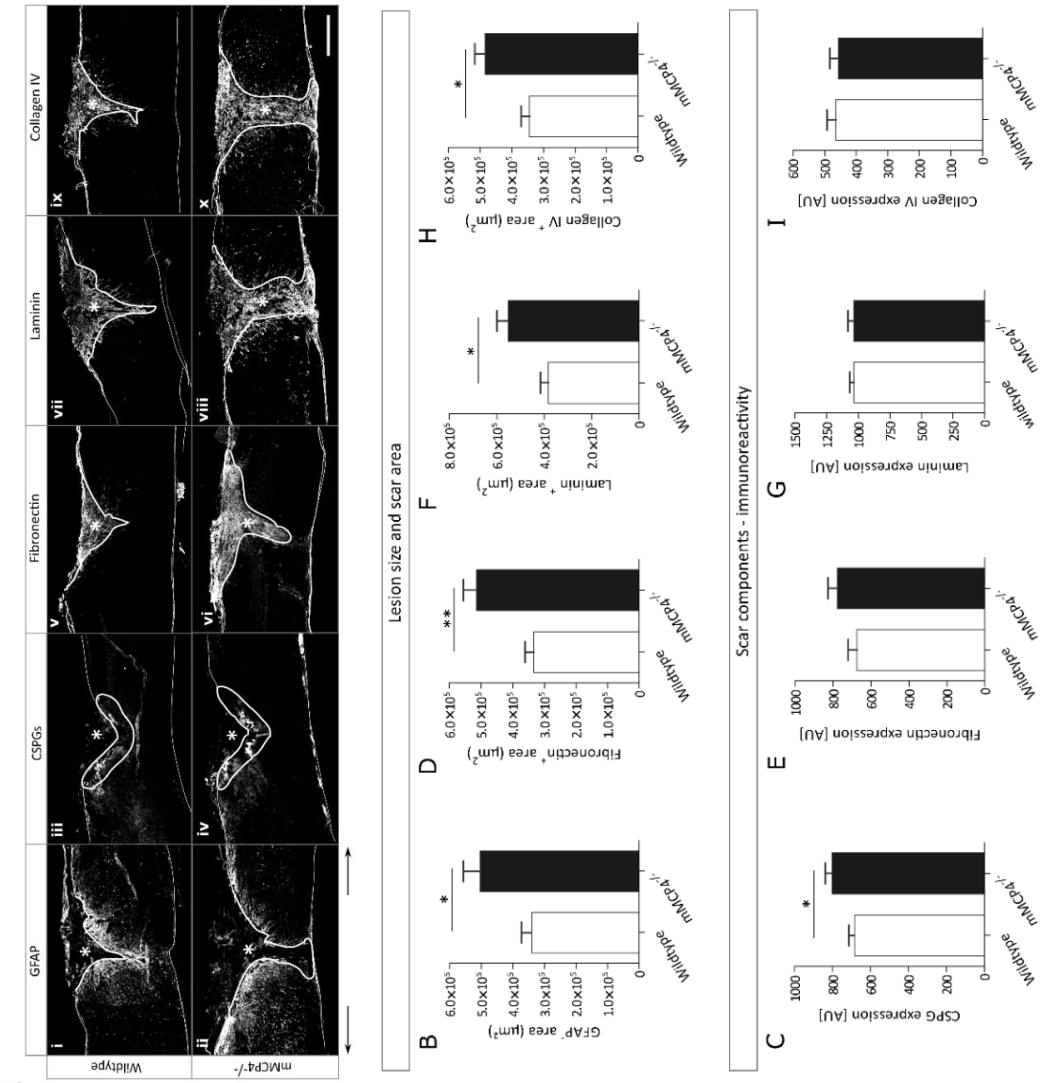
A
GFAP
CSPGs
Fibronectin
Laminin
Collagen IV
WildType
mMCP4-/-
i
ii
iii
iv
v
vi
vii
viii
ix
x
Lesion size and scar area
B
GFAP+ area (µm²)
D
Fibronectin+ area (µm²)
F
Laminin+ area (µm²)
H
Collagen IV+ area (µm²)
Wildtype
mMCP4-/-
Scar components - immunoreactivity
C
CSPG expression [AU]
E
Fibronectin expression [AU]
G
Laminin expression [AU]
I
Collagen IV expression [AU]

Figure 6.1: Increased lesion size and scar tissue formation in mMCP4$^{-/-}$ mice after SCI. A) Representative photomicrographs of the lesion size and deposition of scar components, marked by the white line, at the lesion site in mMCP4$^{-/-}$ and WT mice, respectively. Scale bars = 500 µm. **B)** The lesion size was significantly increased in mMCP4$^{-/-}$ (Aii) mice compared to WT mice (Ai). **C)** A significant increase in CSPG immunoreactivity was observed in the perilesional area in mMCP4$^{-/-}$ (Aiv), compared to WT mice (Aiii). **D)** An increased fibronectin-positive scar area was found in mMCP4$^{-/-}$ mice (Avi) compared to controls (Av). **E)** Fibronectin immunoreactivity was comparable between the two groups at the lesion center. **F-I)** Expression levels of basement membrane components were measured by performing immunofluorescence stainings for laminin and collagen IV. (F, H) The scar area for laminin and collagen IV was significantly increased in mMCP4$^{-/-}$ mice (Avii, ix) compared to WT mice (Aviii, x). (G, I) Immunoreactivity of these components at the lesion center was comparable between WT (Avii, ix) and mMCP4$^{-/-}$ mice (Aviii, x). Data were shown as mean ± SEM. WT control mice: n = 9; mMCP4$^{-/-}$ mice: n = 17. WT = wildtype. CSPGs = chondroitin sulphate proteoglycans. Asterisks indicate lesion center. * $p < 0.05$; ** $p < 0.01$.

6.4.2 Mouse MCP4 degrades scar-associated ECM components in vitro

An *in vitro* degradation assay was performed to determine which scar-associated ECM components are a direct substrate of mMCP4. Recombinant fibronectin, laminin, collagen IV or a mix of important CSPGs were incubated with degranulate collected from MCs that were obtained from either WT mice or mMCP4$^{-/-}$ mice. No cleavage of CSPGs was observed after incubation with MC_{WT} or $MC_{mMCP4}^{-/-}$ degranulate, indicating that mMCP4 does not directly cleave CSPGs in our *in vitro* model (**Figure 6.2A**). In contrast, fibronectin was cleaved after incubation with MC_{WT}. Fibronectin is visible via Western blot analysis as a 262 kDa protein band, which almost completely disappeared after incubation with MC_{WT} degranulate as compared to the control condition (**Figure 6.2B**). Furthermore, also cleavage fragments became visible at lower molecular weight levels after incubation with MC_{WT} degranulate (grey-boxed area, **Figure 6.2B**). When fibronectin was incubated with $MC_{mMCP4}^{-/-}$ degranulate a trend indicates that the cleavage is reduced compared to MC_{WT} degranulate. In addition, collagen type IV was cleaved after incubation with MC_{WT} degranulate, which resulted in a reduced expression of the 250 kDa protein band that corresponds with collagen IV (**Figure 6.2C**).

This effect was significantly reduced when collagen IV was incubated with $MC_{mMCP4}^{-/-}$ degranulate, indicating that mMCP4 directly cleaves collagen type IV. However, it is important to note that the intensity of the bands for collagen IV was low after a 48 h incubation period at 4°C. Laminin is a trimeric protein with a molecular weight of ~800 kDa that consists of an α-chain (400 kDa), a β-chain (200 kDa) and a γ-chain (200 kDa), which are visible as two protein bands on the blot. Laminin was cleaved after incubation by both MC_{WT} and $MC_{mMCP4}^{-/-}$ degranulate and no difference was observed between the groups, indicating that mMCP4 does not directly cleave laminin *in vitro* (**Figure 6.2D**).

6.4.3 Increased gene expression of scar-associated factors in mMCP4 knockout mice

Next, we addressed the question whether endogenous mMCP4 influences the gene expression of several scar-associated markers after SCI. Quantitative PCR analysis showed that GFAP mRNA levels increased slightly after injury in both WT and $mMCP4^{-/-}$ mice (**Figure 6.2E**). At 28 dpi, the expression level was higher for the $mMCP4^{-/-}$ mice compared to the WTs (fold change in expression vs. WT control condition: 3.590-fold vs. 1.786-fold) (**Figure 6.2E**). For the fibrous ECM component fibronectin the mRNA levels were decreased at 7 dpi in the $mMCP4^{-/-}$ mice (5.601-fold) compared with the WT mice (27.192-fold) (**Figure 6.2F**). Similarly, the expression levels of the basal lamina component collagen IV was decreased 7 dpi in the $mMCP4^{-/-}$ group compared to the WT group (**Figure 6.2G**). We also determined the gene expression profile of selected CSPGs, namely aggrecan, neurocan, and brevican (**Figure 6.2H-J**). The aggrecan mRNA levels decrease at 2 dpi, to increase at 7 dpi, after which they decrease again towards baseline levels. No differences were observed between WT and $mMCP4^{-/-}$ mice. Comparable to aggrecan, brevican mRNA levels were decreased 2 dpi and no differences were observed between the experimental groups. Neurocan mRNA levels are higher in the $mMCP4^{-/-}$ mice (2.069-fold) compared with WT mice (1.382-fold) at 2 dpi.

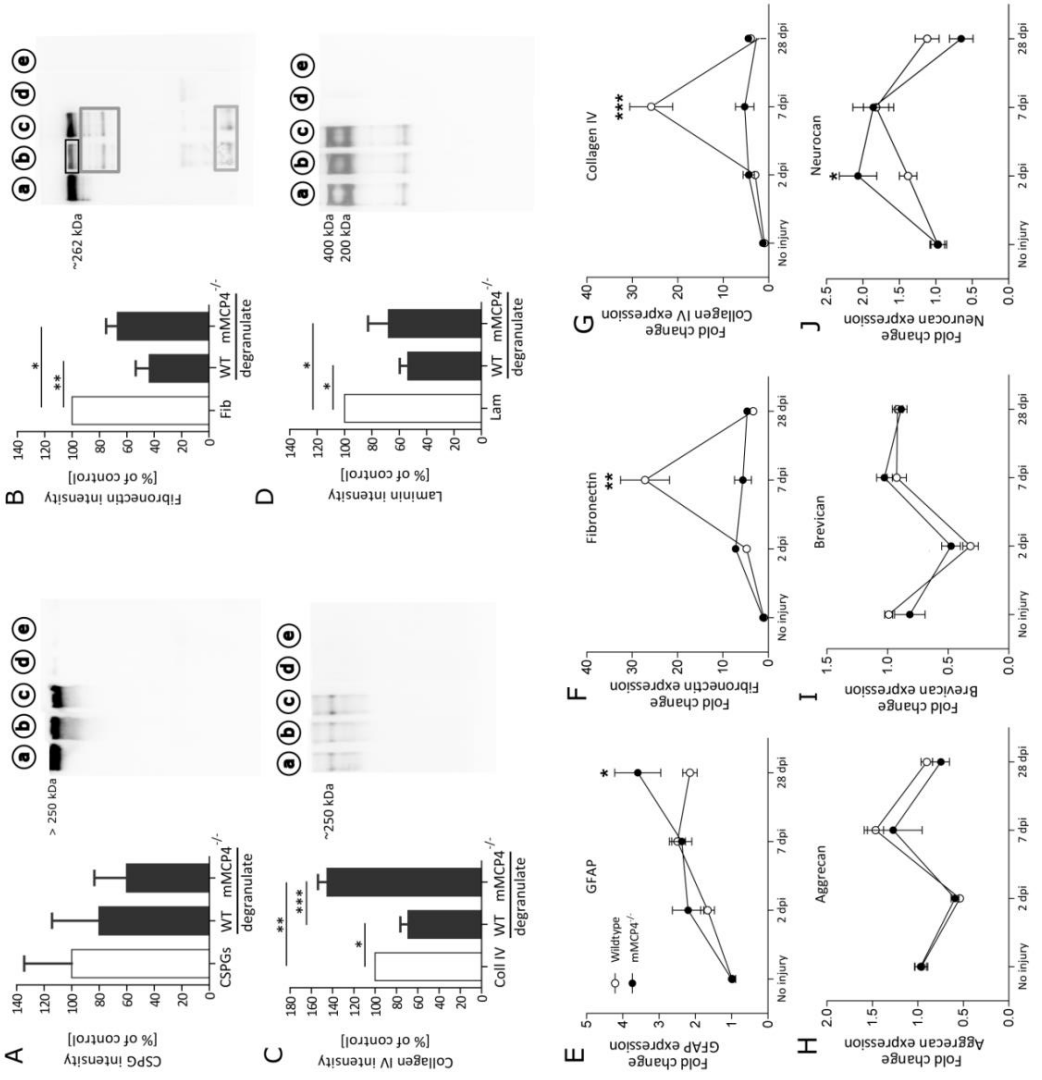
A
CSPG intensity [% of control]
CSPGs
WT
mMCP4-/-
degranulate
> 250 kDa
a b c d e
B
Fibronectin intensity [% of control]
Fib
~262 kDa
C
Collagen IV intensity [% of control]
Coll IV
~250 kDa
D
Laminin intensity [% of control]
Lam
400 kDa
200 kDa
E
GFAP
Wildtype
mMCP4-/-
Fold change GFAP expression
F
Fibronectin
Fold change Fibronectin expression
G
Collagen IV
Fold change Collagen IV expression
H
Aggrecan
Fold change Aggrecan expression
I
Brevican
Fold change Brevican expression
J
Neurocan
Fold change Neurocan expression
No injury
2 dpi
7 dpi
28 dpi

Figure 6.2: Mouse MCP4 degrades scar-associated ECM components *in vitro* and increases gene expression of scar-associated factors in mMCP4 knockout mice. A-D) Recombinant fibronectin, collagen IV, laminin or a CSPG-mix were incubated with MC_{WT} or $MC_{mMCP4^{-/-}}$ degranulate to measure protein degradation. (A) No cleavage of CSPGs was observed after incubation with MC_{WT} or $MC_{mMCP4^{-/-}}$ degranulate. (B) Fibronectin (Fib) was cleaved by both MC_{WT} and $MC_{mMCP4^{-/-}}$ degranulate (the grey boxed area indicates cleavage fragments), although with reduced (trend) cleavage by $MC_{mMCP4^{-/-}}$ degranulate compared to MC_{WT} degranulate. (C) Collagen IV (Coll IV) was cleaved by MC_{WT} degranulate, but not by $MC_{mMCP4^{-/-}}$ degranulate. (D) Laminin (Lam) was cleaved by both MC_{WT} and $MC_{mMCP4^{-/-}}$ degranulate. Data were normalized to control and are presented as mean ± SEM; n = 3-4/condition; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Legend figures: (a) = native protein only; (b) = native protein + MC_{WT} degranulate; (c) = native protein + $MC_{mMCP4^{-/-}}$ degranulate; (d) = MC_{WT} degranulate only; (e) $MC_{mMCP4^{-/-}}$ degranulate only. **E-J)** Expression levels of the glial scar-associated genes (E) GFAP, (F) fibronectin, (G) collagen IV, (H) aggrecan, (I) brevican, and (J) neurocan were measured by quantitative PCR analysis in spinal cord tissue from WT (white circles) and $mMCP4^{-/-}$ mice (black circles) at 2 dpi, 7 dpi, and 28 dpi. As a control, samples from mice that did not undergo surgery were included (no injury). (E) GFAP mRNA levels were significantly elevated in $mMCP4^{-/-}$ mice compared with WT mice at 28 dpi. (F) In contrast, fibronectin was increased in the WT group compared to the $mMCP4^{-/-}$ group at 7 dpi. (G) Similarly, collagen IV mRNA levels were elevated in WT mice. (H-I) From the CSPGs, aggrecan and brevican were not differentially expressed. (J) However, the expression of neurocan was increased in the $mMCP4^{-/-}$ mice compared to the WT mice at 2 dpi. Expression levels were normalized to the reference genes YHWAZ and CYCA and converted to fold change values vs. the WT control condition using the $2^{-\Delta\Delta CT}$ method. Data are presented as mean ± SEM; n = 5-9/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.5 Discussion

The importance of MCs in modulation of SCI pathology has already been indicated because they can support neuronal cell survival and they improve functional recovery after CNS injury via mMCP4. However, the underlying mechanisms are not completely unraveled yet. MMCP4 is a MC-specific chymase that degrades inflammatory mediators and thereby tempers detrimental inflammatory processes *in vivo* (86, 247). We recently provided evidence for excessive scar tissue formation at the lesion site in MC-deficient after SCI (32). In addition, we showed that endogenous mMCP6 has scar suppressing properties after SCI via indirect cleavage of axon growth-inhibitory scar components and alteration of the gene expression profile of these factors (32). Similarly, mMCP4 is also implicated in tissue remodeling and ECM degradation (90, 91). Therefore, this study was intended to unravel whether modulation of the inhibitory scar tissue at the lesion site could be an alternative pathway by which mMCP4 improves recovery after SCI.

Here, we show that the decline in hind limb motor function in mMCP4$^{-/-}$ mice is associated with a significantly increased lesion size and a stronger scarring response at the injury site. We investigated this scarring response more detailed by focussing on the following scar components: CSPGs in the perilesional area, and fibronectin, laminin and collagen IV at the lesion center. Expression levels of the CSPGs were significantly increased in the perilesional area in mMCP4$^{-/-}$ mice after injury. Moreover, enzymatic degradation of CSPGs can improve axon regeneration, synaptic plasticity and recovery after CNS injury, as demonstrated by others (271, 282, 283). This indicates the importance of CSPGs in SCI pathology. Hence, the impaired functional outcome in mMCP4 knockout mice may be related to the increased levels of CSPGs that are expressed in the perilesional area in these mice. In addition, we found a significantly increased area of several fibrous ECM and basal membrane components in the absence mMCP4 following SCI. This fibrotic scar is considered as an important barrier for CNS axons that have initiated a regenerative response after injury (267, 284).

Blocking the production of fibrotic scar components and stimulation of their degradation promotes regeneration of injured axons and improves functional recovery after CNS injury (267, 268, 285).

To reveal direct actions of mMCP4 on the scar components, *in vitro* degradation assays were performed. Its ability to activate metalloproteinases (286, 287), process pro-collagen (288), and to degrade matrix components (90, 287) suggests that mMCP4 has the potential to influence the structure of connective tissues. Several studies in other experimental models, such as obstructive nephropathy and atherosclerosis, have shown that MC chymase has anti-fibrotic actions by degrading fibronectin (280, 287, 289, 290). Chymase can also directly degrade components of the basement membrane, including collagen types IV and V, laminin, and elastin (291). Accordingly, we show that collagen IV is a substrate of mMCP4 *in vitro*. A trend also suggests direct cleavage of fibronectin, whereas the data also indicated that laminin or CSPGs (i.e. aggrecan, neurocan, versican) are no direct substrates of mMCP4. These findings suggest that indirect effects might be involved via which mMCP4 promotes scar remodeling after SCI. MC chymase has the ability to activate other ECM-processing enzymes, in particular matrix metalloproteinases (MMPs). It can activate pro-MMP1 and pro-MMP3 (292, 293) which in turn activate other MMPs, resulting in ECM degradation. In addition, chymase is able to convert pro-MMP9 and pro-MMP2 to their active forms (287), which both have an important role in matrix turnover and tissue remodeling (294).

Besides the classic functions of a protease, mMCP4 may act as a potent regulator of gene expression similar to mMCP6 (32). Therefore, we analyzed the gene expression profiles of the glial scar associated genes GFAP, fibronectin and collagen IV in spinal cord tissue from WT and mMCP4$^{-/-}$ mice at several time points after injury. Furthermore, we determined the gene expression profiles of aggrecan, neurocan, and brevican. We found an elevated GFAP expression in the mMCP4$^{-/-}$ group. In addition, neurocan gene expression was increased at 2 dpi in the mMCP4$^{-/-}$group compared to the WT group, which corresponds with the increased immunoreactivity against CSPGs on the protein level.

Neurocan is involved in the formation of specialized ECM structures called perineuronal nets (PNNs) (295). These PNNs are a condensed layer of pericellular matrix which aggregates and wraps around the soma and proximal dendrites of some neurons in the CNS, where it preserves synapses and prevents unwanted neuronal plasticity (296). Surprisingly, in the absence of mMCP4, the gene expression of fibronectin and collagen IV stayed at baseline level compared to the gene expression by WT mice which is increased at 7 dpi. At 28 dpi, the expression of fibronectin and collagen IV by WT mice also decreased back to baseline. However, at the protein level the fibronectin positive and collagen IV positive area were increased in the mMCP4$^{-/-}$ group. The discrepancy seen between mRNA and protein levels is a well-known phenomenon, as protein expression is regulated transcriptionally as well as post-transcriptionally. Moreover, a growing class of non-coding RNAs called microRNAs (miRNAs), is involved in post-transcriptional regulation of genes (297). From these results we suggest that endogenous mMCP4 may directly or indirectly influence the gene expression of several scar-associated ECM components after SCI. However, additional studies are needed to reveal which gene regulatory mechanisms of mMCP4 are involved in CNS injury and repair.

To conclude, we demonstrate here that mMCP4-deficiency is associated with exacerbated scarring levels and impaired recovery after SCI. Our data suggest that MCs promote scar remodeling after SCI via mMCP4, which implies a new potential mechanism via which mMCP4 can support recovery after CNS injury. However, it remains speculative whether mMCP4 cleaves ECM components directly or indirectly after injury. An example of an indirect scenario could be that the immunomodulatory effects of mMCP4 may suppress the scarring response. Previously, we found that mMCP4 has immunomodulatory functions by cleaving pro-inflammatory mediators (e.g. IL-6) and mMCP4-deficiency resulted in increased levels of IL-6 *in vivo* (247, 298, 299). Proinflammatory cytokines (e.g. IL-6), in turn, are the initial triggers of astrogliosis. Reactive astrocytes (astrogliosis) are the main source of CSPGs. Hence, the lack of mMCP4 in our mouse model and the consequent increase in IL-6 may explain the increased deposition of CSPGs.

Chapter 7

Conclusions & general discussion

Chapter 7. Conclusions & general discussion

SCI is a devastating condition leading to disability of multiple body systems. Hence, to improve functional recovery, immunomodulation must be accompanied by regenerative processes like neurite outgrowth, angiogenesis, synapse formation, degradation of the scar tissue, etc. Additional parameters such as type of injury (e.g. contusion or transection), severity of lesion (e. g. partial or complete SCI), lesion level (e. g. cervical or thoracic SCI) and time after injury all need to be considered when designing an effective treatment. Paralysis is also accompanied with pain, spasms, and other visceral dysfunctions like the absence of bladder control. Stimulating one (e.g. locomotion) could exaggerate the other (e.g. pain) as they are closely correlated, meaning that the experimental therapies need to be carefully analyzed and balanced (**chapter 2**). Hence, future research needs to focus on fine-tuning targeted approaches to enhance functional recovery. It is clear that a multifactorial approach is needed, but first in depth investigation of different mechanisms that can increase functional regeneration is essential.

7.1 Beta-adrenoceptor modulation is not an effective therapeutic strategy to improve functional outcome after SCI

As described in **chapter 2**, after SCI the stress systems will cause hormonal and metabolic changes with direct and indirect effects on the inflammatory system. Next, the inflammatory response will play a major part in the increase of the secondary injury and the loss of function. Preliminary data show that when we study the systemic effects of different stress pathway blockers *in vivo*, reduced functional recovery is achieved by blocking the β-ARs of the SNS. This indicated the importance of the β-adrenergic pathway for functional regeneration. Therefore, we hypothesized that stimulating the β-adrenergic pathway provides neuroprotection and neuronal outgrowth in order to stimulate functional regeneration after SCI. In this study, we showed that propranolol reduced primary neuron viability, whereas clenbuterol did not affect neuronal viability or outgrowth.

Moreover, in our SCI mouse model, a reduction in functional recovery was accompanied with a decrease in the number of $CD4^+$-T cells, whereas clenbuterol or xamoterol had no effects on functional recovery (**chapter 3**).

The reduction in T-helper cells after β-AR blockage with propranolol might dysregulate the activity of $CD8^+$ T cells and polarize the lesional environment towards pro-inflammatory (Th1 mediated) which might be an explanation for the decrease in functional recovery. However, we could not thoroughly investigate the T cells as their number is very low in the spinal cord which makes isolation and analysis difficult (31).

Next to its role in neuroprotection and regeneration, the β2-adrenergic pathway is closely involved in angiogenesis. Therefore, in the next chapter, we focused on β2-adrenergic pathway modulation to improve functional revascularization because vascular protection and revascularization are key to support recovery after SCI.

Angiogenesis is associated with changes in EC proliferation and tube formation, controlled by ERK/MAPK and Akt signaling. Important regulators of these pathways are the β2-ARs. Therefore, the aim was to investigate the angiogenic capacity of the specific β2-AR agonist terbutaline *in vitro* as well as *in vivo* in our SCI mouse model (**chapter 4**). We show that terbutaline promotes angiogenesis by stimulating the β2-AR on bEnd.3 cells *in vitro* and stimulates blood vessel formation in the CAM assay, an *in ovo* model consisting of a complex vasculature (**figure 4.7, chapter 4**). Further analyses of the pathways involved in terbutaline-induced tube formation showed that the inhibition of both AKT and ERK significantly reduced the tube formation by bEnd.3 cells, implying that both pathways are important during EC tubulogenesis. This is in accordance with previous findings showing that AKT and ERK signaling are required for the tube formation of choroid-retinal ECs (229). Long term application of terbutaline reduced the metabolic activity of bEnd.3 cells without any effects on proliferation. These data suggest that terbutaline reduces the viability of the ECs.

Indeed, it has been shown that stimulation of β2-ARs induces apoptosis of human aortic ECs via p38/MAPK signaling (227). Accordingly, we show that inhibition of MAPK/ERK signaling reversed the effects of terbutaline on metabolic activity, indicating the involvement of this pathway in terbutaline-mediated proliferation and cell viability. At the CAM the number of blood vessels was increased by 15% after terbutaline treatment compared to vehicle. These data highlight that terbutaline is effective to stimulate blood vessel formation, althought it did not improve functional outcome or revascularization after SCI.

In contrast, previous reports showed that β2-AR agonists like clenbuterol enhance functional recovery after SCI (45). However, the effects on angiogenesis were not investigated. Although terbutaline and clenbuterol have similar chemical structures, they have different pharmacokinetic properties (233). These pharmacokinetic differences most likely have played a role in the observed discrepancies. In addition, the use of clenbuterol has been associated with various adverse effects like tachycardia and muscle tremors (234). Therefore, it is currently not considered as a promising treatment strategy for SCI. After SCI, an overall non-permissive environment for the growth of axons and blood vessels is created due to the inflammation in combination with the lack of oxygen and nutrients, etc. We conclude that terbutaline as a monotherapy may not be potent enough to overcome these challenges and to induce proper revascularization of the spinal cord.

To summarize this part, β-AR stimulation did not improve functional recovery after SCI, although it stimulated blood vessel formation *ex vivo* and inhibition showed detrimental effects on neuron viability, indicating that modulation of the pathway has important implications for SCI pathology. However, β-adrenoceptor modulation is not an effective strategy to stimulate functional outcome after SCI, at least not as a monotherapy.

7.2 IL-13 overexpressing macrophages to skew the spinal cord micro-environment towards anti-inflammatory is a successful therapeutic strategy to permit functional regeneration

Several studies report beneficial effects of Mϕs after SCI (300), although others suggest detrimental effects of Mϕs to CNS regeneration (301). Moreover, depletion or inactivation of Mϕs has led to neuroprotection, increased regeneration, and improvements in motor, sensory, and autonomic functions, which highlights the therapeutic potential of immunomodulation of these cells (238, 302). After CNS injury, signaling pathways that polarize Mϕs towards an M1 phenotype predominate. In addition to their neurotoxic effects, they cause axonal dieback. In contrast to M1-Mϕs, M2-Mϕs act pro-regenerative. They promote neurite outgrowth and they can enhance oligodendrocyte progenitor cell differentiation (68, 72, 244). Hence, neuroinflammatory responses may be beneficially influenced by both M2a polarisation driven by IL-13 and M2c polarization driven by IL-10. Previous work from our research group showed potent pro-regenerative effects of IL-13 in the context of SCI (245). Therefore, we have focused on IL-13-mediated M2a polarisation of endogenous microglia and Mϕs.

Previous studies of our group with MSCs as carriers of IL-13 already suggest that indirect effects via alternatively activated Mϕs might decrease axon degeneration (245). However, the underlying mechanisms of the beneficial effects of IL-13 and the induced M2a Mϕs following SCI remained unclear. Moreover, it has been shown that Mϕs can actively migrate to the SCI lesion site which is an advantage of using Mϕs instead of MSCs (253). Therefore, we have chosen for controlled immunomodulation, specifically targeted on Mϕs, to improve functional regeneration after SCI and to reveal whether they are responsible for the IL-13 mediated neuroprotective effects. Others also observed improved locomotor recovery, decreased spinal cord lesion volume and increased preservation of axon myelination after systemic treatment with *in vitro* M2 polarized Mϕs in SCI rats (70).

However, we believe that a substantial number of the injected M2 Mϕs was repolarized towards an M1 phenotype by the pro-inflammatory lesion microenvironment (72). For that reason, we have injected IL-13-overexpressing Mϕs based on the idea that they can modulate the SCI inflammation continuously via the constitutive secretion of IL-13.

We hypothesized that therapeutic transfer of genetically modified Mϕs with a stable expression of high levels of IL-13 drives endogenous Mϕs and microglia towards a beneficial phenotype in order to promote functional recovery after SCI. In **chapter 5**, we show that IL-13-Mϕ treatment indeed improves functional recovery accompanied with a reduction in the lesion size and demyelinated area, reduced T-helper cell infiltration and $MHCII^+$ microglia/macrophage infiltration accompanied with an increase in the number of $Arg\text{-}1^+$ macrophages/microglia and a reduced number of axon-phagocyte contacts.

We suggest that the injected IL-13-Mϕs migrate towards the lesion site where they convert locally the destructive M1 Mϕs into M2, continuously, via the secretion of IL-13, thereby creating a neuroprotective environment more prone to functional regeneration. Contrarily, *in vitro* induced M2-Mϕs remain at the injection site where they polarize again into M1. To confirm the phenotypes *in vivo*, we have to trace the Mϕs properly. Ongoing experiments with IL-13 secreting GFP^+ Mϕs will elucidate whether the injected Mϕs stay M2 and whether endogenous Mϕs are truly polarized towards M2. By tracing the injected Mϕs, it will also become clear if they migrate to the lesion site or exert their effects from a distance. The shown reduction in axon-phagocyte contacts may be related to decreased axonal dieback (259), which already suggests that the transplanted IL-13-Mϕs make the local phagocyte population less destructive. Therefore, we speculate that a reduction in axonal dieback may have led to the improved histopathological and functional outcome observed following SCI. Tracing the cortical spinal tract will reveal the effects of IL-13-Mϕ treatment on axon regeneration. In conclusion, this study shows that perilesional transfer of IL-13 secreting macrophages is a successful experimental approach to improve functional and histopathological recovery in the SCI mouse model.

7.3 Modulating mouse mast cell protease4 and the lesional scar is a promising therapeutic strategy to improve functional recovery after SCI

SCI is characterized by the formation of a glial and fibrotic scar at the lesion site. This scar creates a major barrier for regenerating axons and contributes significantly to the impaired functional outcome. Recent findings indicate that MCs protect the CNS after mechanical damage by suppressing detrimental inflammatory processes mediated via mMCP4, which also plays an important role in tissue remodeling and ECM degradation. Therefore, we have investigated the effects of mMCP4 on scarring and recovery after SCI by using mMCP4 knockout ($mMCP4^{-/-}$) mice.

Previously, we have shown that the absence of mMCP4 results in impaired functional recovery after a spinal cord hemisection injury (87). We show here that the decline in hind limb motor function in $mMCP4^{-/-}$ mice was associated with a stronger scarring response at the injury site (**chapter 6**). We investigated this scarring response in more detail by focussing on the following scar components: CSPGs, fibronectin, laminin and collagen IV. Expression levels of axon-growth inhibitory CSPGs were significantly increased in the perilesional area in mMCP4 knockout mice after injury. Hence, the impaired functional outcome in mMCP4 knockout mice may be related to the increased levels of CSPGs. Moreover, the enzymatic degradation of CSPGs can improve axon regeneration, synaptic plasticity and recovery after CNS injury, as demonstrated by others (271, 282, 283). This indicates the importance of CSPGs in SCI pathology.

To investigate whether these CSPGs or other scar related components are direct substrates of mMCP4, we performed *in vitro* degradation assays. The obtained results indicated that mMCP4 did not directly cleave CSPGs, fibronectin or laminine, although immunohistological analyses of these components *in vivo* indicated they were increased at the lesion site after injury in the $mMCP4^{-/-}$ mice. This suggest that mMCP4 has indirect effects on the scarring response after SCI.

Profiling of scar related gene expression by qPCR showed that GFAP expression was elevated in the mMCP4 knockout group. In addition, neurocan gene expression was increased at 2 dpi in the mMCP4 knockout group compared to the WT group. Surprisingly, in the absence of mMCP4, the gene expression of fibronectin and collagen IV stayed at baseline level compared to WT mice at 7 dpi. From these results we conclude that endogenous mMCP4 may directly or indirectly influence the gene expression of several scar-associated ECM components after SCI. However, additional studies are needed to reveal which gene regulatory mechanisms of mMCP4 are involved in CNS injury and repair.

To conclude, our study suggests that MCs promote scar remodeling after SCI via mMCP4. However, it remains speculative whether mMCP4 cleaves ECM components directly after injury. Another scenario is that the immunomodulatory effects of mMCP4 may indirectly suppress the scarring response after CNS injury. For example, it is well known that proinflammatory cytokines (e.g. TNFa, IL-6) are the initial triggers of astrogliosis after SCI. Reactive astrocytes (astrogliosis), in turn, are the main source of CSPGs that are produced at the lesion site after injury. In our previous study, we showed that mMCP4 cleaves IL-6 *in vitro* and mMCP4-deficiency resulted in increased levels of IL-6 *in vivo*. In this way, the lack of mMCP4 in our mouse model may explain the increased deposition of CSPGs via increased IL-6 or other pro-inflammatory cytokines. Hence, our data imply a new potential scar-remodeling mechanism via which mMCP4 can support recovery after CNS injury, in addition to the immunomodulary effects that we have reported before (86, 87). Future research should aim at defining the therapeutic validity of MCPs (e. g. application of recombinant MCP4) to improve recovery after SCI.

7.4 Future perspectives

7.4.1 What are the next steps to translate the local delivery of IL-13 to the clinic?

In this thesis, we have shown that perilesional injection of IL-13 overexpressing Mϕs is a promising method to change the detrimental pro-inflammatory environment towards a pro-regenerative milieu accompanied with improved functional recovery. However, with translation to a clinical setting kept in mind, this approach might be too fundamental.

Several cell types can be used as carriers of IL-13. In previous studies we have already used MSCs giving promising results. However, there are still several safety concerns, such as tumorogenicity, associated with stem cell transplantation which limits their applicability in the clinic. Therefore, Mϕs might be a better option.

The use of allogeneic, instead of autologous IL-13-secreting cells, would increase the accessibility of cell therapy because it would reduce the period of time necessary to generate the cells (off-the shelf principle). Our colleagues have already shown that IL-13-secretion from allogeneic MSC grafts can prolong the immunological survival of the cells compared with the survival of control allogeneic MSCs (249). However, their therapeutic efficacy following SCI still needs to be investigated. The same approach might be interesting with Mϕs, as their use has some benefits compared to stem cells. Besides the absence of tumorogenicity and the migration capacity of Mϕs, an advantage is that they can be generated in a relatively short period of time (303). Additionally, they can be safely intravenously delivered, whereas intravenous administration of MSCs has some contra-indications because they invade the lungs. It has been shown that macrophage/dendritic cell populations can safely pass the lung circulation (304). Therefore, validation of intravenous administration of IL-13 secreting macrophages is a valuable next step to make the first translation towards the clinic.

7.4.2 How are IL-13 secreting Mϕs exerting their improving effects after SCI?

In chapter 5 we show that mice treated with IL-13 overexpressing Mϕs have a significant improvement in both functional and histopathological outcome following SCI. However, the question regarding how IL-13 secreting Mϕs are exerting these beneficial effects, still remains unanswered.

The approach with Mϕs was chosen to answer the question whether the mechanism of IL-13 is via Mϕs or microglia (245). Considering the promising effects, we assume that Mϕs play a big part. However, thorough tracing of the injected IL-13-Mϕs is necessary to investigate their migration capacity and to determine whether they stay polarized and polarize other phagocytes *in vivo*. We also can not rule out a direct effect of IL-13 on microglia apoptosis, as previous results of our research group have indicated a reduction in microglia after treatment with IL-13 secreting MSCs (245). To investigate the mechanism of action of IL-13 directly, future experiments will include transplantation of IL-13-producing cells in IL-4 receptor type II knockout ($IL4RII^{-/-}$) mice. The IL-4 receptor type II binds both IL-4 and IL-13 (305). Hence, its knockout will rule out IL-13 effects.

7.4.3 How could we therapeutically target mMCP4/chymase?

Studies have shown that chondroitinase ABC (ChABC), an enzyme of bacterial origin, supports axonal growth after SCI by degrading ECM components of the glial scar (271, 306). However, administering ChABC to the injured CNS is not without significant risks concerning safety, biodistribution and thermal stability (307, 308). Therefore, the search for more suitable treatment strategies is ongoing. Our results from specific MCP-knockout mice suggest a beneficial role for endogenous MCPs after CNS injury. The therapeutic potential of exogenously administered MCPs remains to be unraveled (31, 86). Preliminary data indicate that administration of mMCP6 in the SCI mouse model slightly improves SCI (Tim Vangansewinkel *et al*, manuscript in progress).

Therefore, future studies should test the administration of MCPs, in particular mMCP4 and mMCP6 (or combined), to stimulate axonal regeneration after SCI by degrading inflammation-associated cytokines and remodeling ECM scar tissue, ultimately leading to improved functional recovery.

However, there are some pitfalls. MCPs are not commercially available. Therefore, recombinant mMCP4 and mMCP6 have to be produced in house. Although other mMCPs exist, we specifically selected mMCP4 and mMCP6 because they are considered as the closets functional homologues to human α-chymase and β-tryptase, respectively (273). The production of recombinant MCP6 is well-established in our lab via protocols of our collaborators (309). Unfortunately, production of MCP4 was not feasible yet.

Summarized, after optimization of the production of recombinant MCPs, in depth investigation of the effects of their administration is necessary before we can conclude this is a relevant treatment strategy.

7.4.4 Could we combine these promising strategies?

It is clear that a multifactorial approach is needed to tackle spinal cord injury. However, the majority of studies investigate the effect of a single treatment after experimental SCI. Several different drugs show effects of a similar magnitude, although still none of these experimental drugs were effective enough to reach the clinic. To obtain additive or synergistic effects, one needs to combine treatments targeting different mechanisms. However, as soon as treatments are combined, matters get complicated.

When a combinatorial treatment is planned, the useful effects of a given drug need to be fully understood from a single mono-treatment study, although one cannot predict the effects of a combination from a mono-treatment study. When two drugs are combined, they may act independently of each other or they can target the same mechanisms. Unfortunately, they can also interact negatively. In addition, there may be situations in which neither of two drugs is effective alone, while the combination has an effect.

It follows that combining three or more treatments is associated with a correspondingly larger number of possible outcomes, in addition to the adverse effects that one should bare in mind. To start and to simplify the challenge of combining treatments one may focus on combining treatments that, when used as single approach, had positive effects and are unlikely to interact with each other (310, 311).

In this thesis, we have focused on the neurotoxic (via adrenergic pathway modulation and stimulation of angiogenesis), pro-inflammatory (via immunomodulation of macrophages) and regeneration-inhibitory (via mouse mast cell protease 4) spinal cord micro-environment to improve functional recovery after SCI, although we did not combine the different strategies yet. Against all experimental throwbacks, future research should focus on combination therapy considering the principle of highly active antiretroviral therapy (HAART) for HIV infection as a set example that combination therapy is effective. HIV infection has of course a completely different pathology compared to SCI. However, also for HIV no monotherapy was effective enough. Now, with HAART, HIV-1 infection is manageable as a chronic disease in patients who have access to the medication and who achieve durable virologic suppression (312). Similarly, SCI would benefit from a combination therapy to tackle the different affected mechanisms.

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Summary

Summary

Spinal cord injury and the resulting paralysis are critical unsolved problems worldwide. The primary injury develops into a secondary injury cascade for which currently no treatment can help. The secondary injury is characterized by an inflammatory response, leading to the infiltration and activation of immune cells. Later on, the formation of a glial scar is a major bottleneck for regenerative processes. To enhance functional recovery, immunomodulation must be accompanied by regenerative processes like neurite outgrowth, angiogenesis and degradation of the scar tissue, amongst others. It is clear that a multifactorial approach is needed, but first in depth investigation of different mechanisms that can increase functional regeneration is necessary.

As a first approach, we modulated the stress systems after SCI. As described in **chapter 2**, inevitably the sympathetic nervous system (SNS) of the stress systems will be activated after SCI. This will cause hormonal and metabolic changes with direct and indirect effects on the inflammatory system. Next, the inflammatory response will play a major part in the increase of the secondary injury and the loss of function. In **chapter 3** we hypothesized that stimulating the β-adrenergic pathway (part of the SNS) provides neuroprotection and neuronal outgrowth in order to stimulate functional regeneration after SCI. However, β-AR agonism with clenbuterol (β2- AR agonist) or xamoterol (β1-AR agonist) did not affect functional recovery or neuronal viability and outgrowth. Summarized, the detrimental effects of β-AR antagonism with propranolol on primary neuron viability *in vitro* and functional recovery and helper T cells *in vivo* let us suggest the β-adrenergic pathway is indispensable for SCI recovery.

Secondly, we aimed to improve functional revascularization after SCI via modulation of the β-adrenergic pathway. We show that terbutaline promotes angiogenesis by stimulating the β2-AR on bEnd.3 cells *in vitro* and stimulates blood vessel formation in the CAM assay *in ovo,* with only a suggestive, non-significant improvement of the functional outcome *in vivo* (**chapter 4**).

Taken together, β-AR stimulation did not improve functional recovery after SCI, although it stimulated blood vessel formation *ex vivo* and inhibition showed detrimental effects on neuron viability. Therefore, we conclude that β-adrenoceptor modulation is not an effective therapeutic strategy to improve functional outcome after SCI.

In a third approach, we modulated the inflammatory response directly. In **chapter 5,** we show that perilesional transfer of IL-13 secreting macrophages (IL-13-Mϕ) is a successful experimental treatment to improve functional and histopathological recovery in the SCI mouse model. We suggest that the injected anti-inflammatory IL-13-Mϕs migrate to the lesion site and convert the local destructive M1-Mϕs to M2, creating a neuroprotective environment more prone to functional regeneration. We showed an increase in the number of neuroprotective arg-1 positive microglia/macrophages and a decrease in the number of axon-attacking MHCII positive microglia/macrophages by IL-13-Mϕ treatment at the lesion site. In addition, there was a decrease in the number of macrophage-axon contacts in the IL-13-Mϕ treated mice, suggesting a reduction in axonal dieback. Hence, we speculate that a reduction in axonal dieback may have led to the improved histopathological and functional outcome.

Lastly, SCI is characterized by the formation of a glial and fibrotic scar at the lesion site. This scar creates a major barrier for regenerating axons and contributes significantly to the impaired functional outcome. Therefore, we have investigated the effects of mMCP4 on scarring and recovery after SCI by using mMCP4 knockout mice. The results described in **chapter 6** suggest that mast cells promote scar remodeling after SCI via mMCP4. However, it remains speculative whether mMCP4 cleaves ECM components directly or indirectly after injury. It might be that the immunomodulatory effects of mMCP4 may indirectly suppress the scarring response after CNS injury. For example, the absence of mMCP4 increased the expression of pro-inflammatory cytokines (e.g. IL-6) which, in turn, can increase the deposition of CSPGs. In summary, our data imply a new potential scar-remodeling mechanism via which mMCP4 can support recovery after CNS injury, in addition to the immunomodulary effects that we have reported before.

Future research should aim at defining the therapeutic validity of MCPs (e.g. application of recombinant MCP4) to improve recovery after SCI.

In conclusion, we have demonstrated in this thesis that immunomodulatory therapy using IL-13 secreting macrophages provides a great therapeutic potential for the treatment of SCI. However, further research is still required to identify the mechanisms behind these effects.

SCI needs a multifactorial approach, therefore we have focused on different strategies to tackle the damage to different mechanisms caused by the secondary injury. The focus of future research should be combination treatments, starting with the ones that have singular positive effects and are unlikely to interact with each other.

Nederlandse samenvatting

Nederlandse samenvatting

Ruggenmergschade en de resulterende verlamming kunnen op de dag van vandaag nog steeds niet genezen worden. Na een ruggenmergletsel ontwikkelt de primaire schade zich snel in een al maar groter wordende secundaire schade waarvoor er nog steeds geen behandeling bestaat. Deze secundaire schade wordt gekenmerkt door een ontstekingsreactie die leidt tot de instroom en activatie van allerlei ontstekingscellen. Later wordt er een actief litteken gevormd, gekenmerkt door reactieve astrocyten, dat regeneratieve processen belemmert. Om functioneel herstel te kunnen bekomen moet immuun-modulatie samengaan met regeneratieve processen zoals onder meer neurieten-uitgroei, bloedvatvorming en afbraak van het groei-inhiberende littekenweefsel. Het is duidelijk dat een multifactoriële aanpak nodig is, maar eerst moeten verschillende enkelvoudige mechanismen grondig onderzocht worden.

Ten eerste hebben we het stress systeem willen beïnvloeden na ruggenmergschade. Het sympathisch zenuwstelsel, deel van het stress systeem, wordt onvermijdelijk geactiveerd na een ruggenmergletsel (**hoofdstuk 2**). Dit veroorzaakt allerlei hormonale en metabole veranderingen met directe en indirecte effecten op het immuunsysteem en dus ontstekingsreacties. Deze ontstekingsreacties, op hun beurt, dragen bij aan de beschreven secundaire schade en functieverlies. In **hoofdstuk 3** stellen we de hypothese dat het stimuleren van het β-adrenerge systeem (deel van het sympathisch zenuwstelsel) zorgt voor neuroprotectie en neurieten-uitgroei om zo functionele regeneratie te bekomen. De resultaten tonen echter dat β-adrenoceptor (AR) stimulatie met clenbuterol (β2-AR agonist) of xamoterol (β1-AR agonist) geen effecten heeft op functioneel herstel, neuronale viabiliteit of neurieten-uitgroei. Propranolol (β-AR antagonist), daarentegen, verminderde neuronale viabiliteit *in vitro. In vivo* verminderde propranolol functioneel herstel en reduceerde het het aantal $CD4^+$ T-cellen, wat ons doet besluiten dat het β-adrenerge systeem toch een rol speelt na ruggenmergschade.

Ten tweede hebben we via modulatie van het β-adrenerge systeem, functionele bloedvatvorming na ruggenmergschade willen verbeteren. In **hoofdstuk 4** tonen we dat terbutaline bloedvatvorming stimuleerde *in vitro* via stimulatie van de β2-AR op bEnd.3 cellen. Daarenboven stimuleerde het ook bloedvatvorming op de chorio-allantois membraan van het kippenei. Echter, bij het ruggenmergschade muismodel, leidde het niet tot een verbetering van de bloedvatvorming of functioneel herstel.

Vanuit de resultaten van hoofdstuk 3 en 4 besluiten we dat β-adrenoceptor modulatie geen veelbelovende therapeutische strategie is om functioneel herstel na ruggenmergschade te verbeteren.

In een derde benadering hebben we de ontstekingsreactie direct willen beïnvloeden. In **hoofdstuk 5** tonen we aan dat perilesionale injectie van IL-13 secreterende macrofagen (IL-13-Mϕ) een succesvolle experimentele behandeling is om functioneel en histopathologisch herstel te kunnen bekomen in een ruggenmergschade muismodel. Als mechanisme stellen we voor dat de geïnjecteerde IL-13-Mϕ migreren naar de lesie en daar destructieve macrofagen (M1-Mϕ) veranderen naar M2, om zo een neuroprotectieve omgeving te creeëren die functioneel herstel bewerkstelligt. Dit wordt bekrachtigd omdat we een stijging zagen in het aantal neuroprotectieve arg-1 positieve micoglia/macrofagen en een daling in het aantal destructieve MHCII positieve microglia/macrofagen bij de lesie. Daarnaast was het aantal macrofaag-axon contacten afgenomen, wat doet vermoeden dat er een daling is in axonale retractie. We suggeren dan ook dat een daling in axonale retractie aan de basis ligt van het histopathologische en functionele herstel.

Ten slotte hebben we het gliale en fibrotisch litteken, dat gevormd wordt na ruggenmergschade, willen moduleren. Dit litteken zorgt namelijk voor een barrière tegen axonregeneratie en draagt bij tot het verslechteren van functioneel herstel.

Aangezien muis mestcel protease 4 (mMCP4) een rol speelt in dit proces in andere ziektebeelden, hebben we met behulp van mMCP4 knockout muizen de effecten onderzocht van mMCP4 op littekenvorming en functioneel herstel na ruggenmergschade. De resultaten beschreven in **hoodstuk 6** tonen aan dat mestcellen (MCs) litteken-remodellering bevorderen na ruggenmergschade via mMCP4. Het is alleen nog niet duidelijk of mMCP4 de extracellulaire matrix componenten direct of indirect afbreekt. Het zou ook kunnen dat de immuun-modulerende effecten van mMCP4 een rol spelen in het onderdrukken van het litteken. In het knockout muismodel zorgt de afwezigheid van mMCP4 immers voor verhoogde expressie van inflammatoire cytokines zoals IL-6, wat op zijn beurt de afzetting van chrondoitine-sulfaat proteoglycanen (CSPGs) doet toenemen. Deze CSPGs zijn verantwoordelijk voor het littekenweefsel. Samengevat impliceren onze data een nieuw litteken-remodellerend mechanisme via mMCP4 dat functioneel herstel na ruggenmergschade bevordert. Dit mechanisme staat naast en hangt samen met de immuun-modulerende effecten van mMCP4 die eerder door ons gerapporteerd werden. Toekomstig onderzoek zal moeten uitwijzen of mestcel proteasen, bijvoorbeeld via de toediening van recombinant MCP4, waardevol zijn om functioneel herstel te bekomen.

In conclusie, hebben we in deze thesis aangetoond dat immuun-modulerende therapie met IL-13-Mϕ een goed therapeutisch potentieel biedt voor de behandeling van een ruggenmergletsel. Voortgezet onderzoek is wel nog nodig om de onderliggende mechanismen aan het licht te brengen. Het is duidelijk dat ruggenmergschade een multifactoriële benadering vereist. Wij hebben ons alvast geconcentreerd op verschillende strategieën. De focus van toekomstig onderzoek moeten liggen op het combineren van behandelingen, te beginnen met diegenen die enkelvoudig positieve effecten hebben en waarvan geweten is dat ze niet met elkaar reageren.

Curriculum Vitae

Curriculum Vitae

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Publications

Publications from this work

Lemmens S, Kusters L, Bronckaers A, Geurts N, Hendrix S
The beta2-Adrenoceptor Agonist Terbutaline Stimulates Angiogenesis via Akt and ERK Signaling
Journal of cellular physiology, 2017, Epub 2016/07/13. **IF: 4.155**

Lemmens S, Brone B, Dooley D, Hendrix S, Geurts N
Alpha-adrenoceptor modulation in central nervous system trauma: pain, spasms, and paralysis--an unlucky triad
Medicinal research reviews, 2015;35(4):653-77, Epub 2014/12/30. **IF: 8.431**

Co-authored publications

Vangansewinkel T, Geurts N, Quanten K, Nelissen S, **Lemmens S**, Geboes L, Dooley D, Vidal PM, Pejler G, Hendrix S
Mast cells promote scar remodeling and functional recovery after spinal cord injury via mouse mast cell protease6.
FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2016;30(5):2040-57, Epub 2016/02/27. **IF: 5.299**

Geurts N, Vangansewinkel T, **Lemmens S**, Nelissen S, Geboes L, Schwartz C, Voehringer D, Hendrix S
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Journal of leukocyte biology, 2016;99(4):579-82, Epub 2015/11/19. **IF: 4.289**

Nelissen S, Vangansewinkel T, Geurts N, Geboes L, Lemmens E, Vidal PM, **Lemmens S**, Willems L, Boato F, Dooley D, Pehl D, Pejler G, Maurer M, Metz M, Hendrix S.
Mast cells protect from post-traumatic spinal cord damage in mice by degrading inflammation-associated cytokines via mouse mast cell protease4.
Neurobiology of disease, 2014;62:260-72, Epub 2013/10/01. **IF: 5.82**

Poster Presentations

2015: 3rd International Spinal Cord Repair – ISCORE 2015
Barcelona, Spain
Stefanie Lemmens, Lauren Kusters, Annelies Bronckaers, Nathalie Geurts and Sven Hendrix
The β2-adrenoceptor agonist terbutaline stimulates angiogenesis

2015: The immune-brain axis: from molecules to behavior
Diepenbeek, Belgium
Stefanie Lemmens, Nathalie Geurts and Sven Hendrix
Beta-2 adrenoceptor stimulation to promote angiogenesis for spinal cord regeneration

2014: EMBO Development and regeneration of the spinal cord
Sitges, Spain
β2-adrenoceptor agonists do not improve functional recovery after spinal cord injury
Stefanie Lemmens, Sven Hendrix, Nathalie Geurts, Tim Vangansewinkel, Dearbhaile Dooley, Myriam Gou Fabregas

Grants

2013: PhD Grants for Strategic Basic Research Flanders Innovation and Entrepreneurship

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Attachments

Attachments

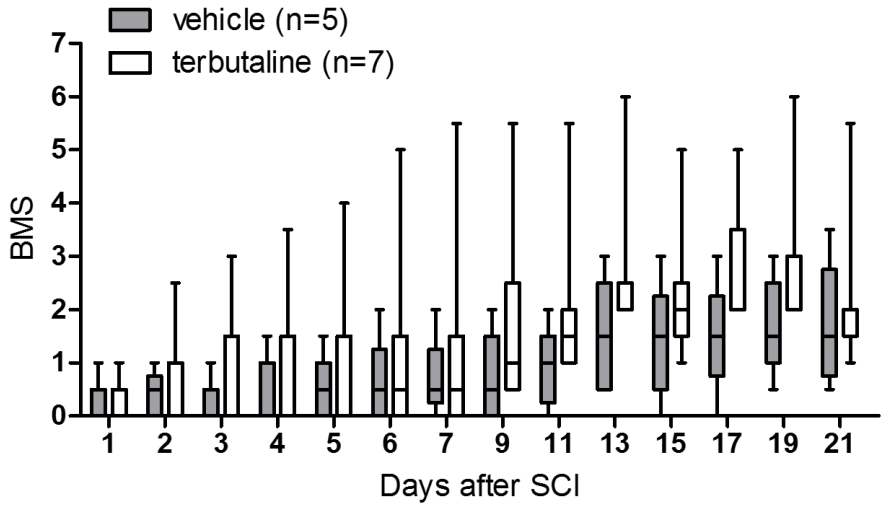

Figure S4.1: Terbutaline does not affect functional after SCI. The first 9 days after SCI, mice were injected twice a day with either terbutaline (5 mg/kg) or with the vehicle NaCl. Functional recovery was analyzed according to the BMS. The data are presented as box plots with whiskers indicating the minimum and maximum and day-to-day differences between groups were evaluated with the Mann-Whitney test considering them as significant with $p<0.05$; n= 5-7.

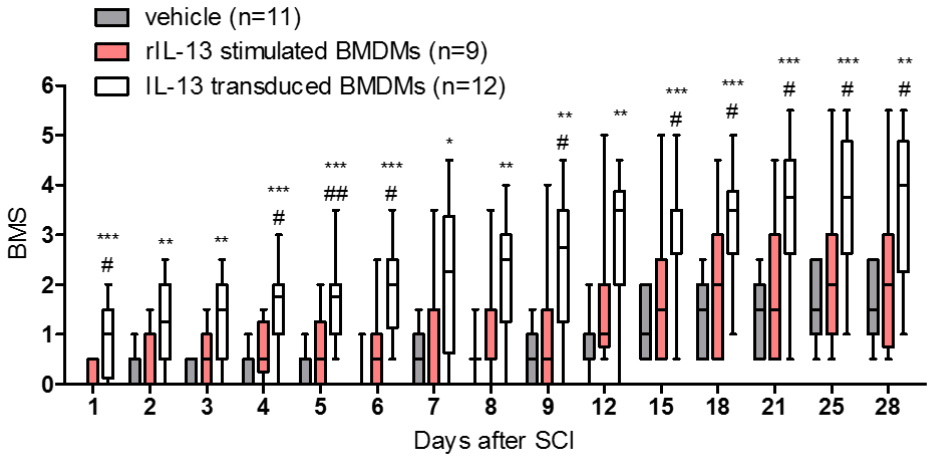

Figure S5.1: Transplantation of IL-13-Mϕs improves functional recovery after SCI. Mice receiving transplantation of IL-13-Mϕs show a significantly increased BMS score following SCI, compared to M2-Mϕ (differences are indicated by #) and vehicle treated mice (differences are indicated by *). Data are represented as box plots with whiskers indicating the minimum and maximum. Day-to-day differences between groups were analysed using the Kruskall Wallis test followed by Dunn's Multiple Comparison test until day 12, when the data were not normally-ditributed. From day 15 onwards the data follow the normal distribution and day-to-day differences between groups were analysed using One-way Anova followed by Bonferroni's Multiple Comparison test; *p<0.05, **p<0.01, ***p < 0.001 (IL-13-Mϕ vs. vehicle) and #p<0.05, ##p<0.01 (IL-13-Mϕ vs. M2-Mϕ), n= 9-12 mice/group.

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