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Histone H1 promotes regeneration after mouse spinal cord injury

Dissertation

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List of Abbreviations

APCs.....antigen presenting cells
ASIA..... American Spinal Injury Association
BCA..... Bicinchoninacid
Ca .....calcium
CHL1 ..... the close homolog of L1
CNS .....central nervous system
Conc .....concentration
DCs..... dendritic cells
DMEM .....Dulbecco's Modified Eagle's medium
DNA .....Deoxyribonucleic Acid
FBS..... fetal bovine serum
GFAP..... glial fibrillary acidic protein
HBSS..... Hank´s balanced salt solution
HRP .....diluted horseradish peroxidase
Iba1 …….. Ionized calcium-binding adaptor molecule 1
IFN-γ .....Interferon-gamma
IgCAM .....immunoglobulin cell adhesion molecule
IgG..... immunoglobulin G
IL-4 /-6 /-10 ……..... interleukin-4/-6/-10
K ..... potassium
LPS .....lipopolysaccharide
Mac2..... macroghage marker
mM .....Millimolar
Na .....sodium
NaOH .....natriumhydroxid
NCAM..... neural cell adhesion molecule
NG2 …… neuron-glial antigen 2
NO .....nitric oxide
NPCs.....neural precursor cells
NRP 2 …… neuropilin-2
NSAIDs .....non-steroidal anti-inflammatory drugs
O_2..... superoxide anion

PBS ..... phosphate-buffered saline
PBST ..... phosphate-buffered saline solution
PSA ..... polysialic acid
PVDF ..... polyvinylidene difluoride
RIPA ..... Radioimmunoprecipitation assay
RNA ..... ribonucleic acid
RT ..... room temperature
SCs ..... Schwann cells
SCI ..... spinal cord injury
SENAs ..... substrate adherent embryonic stem cell-derived neural aggregates
TGF-β ..... transforming growth factor-beta
TGF-β1 ..... transforming growth factor-beta1
TNF-α ..... tumor necrosis factor-alpha
ZNS ..... zentrales Nervensystem

Research hypothesis/question:

Based on the previous research we hypothesize that histone H1 will have a beneficial effect on regeneration if applied after spinal cord injury. In addition, we want to explore if this effect is PSA dependent.

1. Introduction

1.1 Epidemiology of spinal cord injury

The global incidence of spinal cord injury (SCI) is 8-246 cases / 1 million each year. The global prevalence is 236-1298 / 1 million, which is increasing all over the world in the past decades (Furlan et al., 2013). Although the total number of SCI increased with an increasing population in the past years, the incidence rate of acute traumatic SCI remained stable.

SCI is a traumatic event, which can result in temporary or permanent neurological dysfunction with tremendous impact on patient's physical and psychological state. It also can place an economic burden on health care systems (Rossignol et al., 2007; Singh et al., 2014; Thuret et al., 2006).

Regarding the causes of SCI there has been no uniformity between different countries (Ackery et al., 2004). A major cause of SCI is motor vehicle accidents in the developed countries with 58% of all SCI, followed by falls with 47% and violence (primarily gunshot wounds 14%). Less frequently seen are sport-related accidents, especially diving with 9% and iatrogenic SCI with 5% (Campos da Paz et al., 1992; Dincer et al., 1992; Hoque et al., 1999; Hart & Williams, 1994; Hulsebosch, 2002; Kang et al., 2017; Krassioukov et al., 2003; Martins et al., 1998).
Of course, there are also causes other than traumatic SCI resulting in spinal cord dysfunction, e.g. syphilis, multiple sclerosis, poliomyelitis, tuberculosis and neoplasm.

Until recently young people (20-40 years) represented the majority of SCI patients, nowadays an increase of SCI is more observed in older patients, especially due to falls and violence, more in males than females (2:1) and more in adults compared to children (Ackery et al., 2004; Bracken et al., 1981; Chen et al., 2015; Jain et al., 2015; Sekhon et al., 2001; Tator et al., 1993).

The most common injuries at cervical level affect C5 with 50% (Hachem et al., 2017). Thoracic and lumbar levels are affected with 35% and 11%, respectively. With advances of medical treatments in the past decades, life expectancy increased for patients with SCI. Life expectancy depends on the level and degree of injury, the heavier the initial damage of SCI, the worst is the outcome. Patients with the American Spinal Injury Association impairment scale (ASIA) grade D who are wheelchair

dependent have an estimated 75% of normal life expectancy, compared to the patients without wheelchair having 90% of normal life expectancy (Shavelle et al., 2015). There is an estimated number of more than 27 million people worldwide, who are living with long-term disabilities after spinal cord injuries (Spencer et al., 2019).

In general, the cost of treatment after SCI is patient-dependent, which is influenced by the severity of injury and the degree of disability (Jain et al., 2015). The economic burden on the health care system is huge, as SCI is associated with secondary complications as deep vein thrombosis, urinary and respiratory tract infections, chronic pain, osteoporosis, pressure ulcers and depression.
The average costs for patients with C1-C4 tetraplegia in the first year of injury were ranged in 2011 from $334,170 to $1,023,924 (Cao et al., 2011). In 2013, the total costs attributed to SCI were approximately $21.5 billion in the United States (Ma et al., 2014). The lifetime cost of a patient after SCI is about $2.35 million (Hachem et al., 2017).

1.2 Pathophysiology of spinal cord injury

SCI is a life changing acute trauma with temporarily or permanently damage of the spinal cord. The clinical outcome depends on the degree and the location of damage (Alizadeh et al., 2019). Higher cervical damage is associated with quadriplegia, whereas thoracic and lumbar lesions are more associated with paraplegia (Wilson et al., 2012).

As nearly all organ systems of the body are innervated by the spinal nerves, besides somatic (e.g. motor function) impairment there will also occur some unpleasant conditions like sensory loss, loss of bladder and bowel control, gastric ulcers, urinary tract infections, sexual dysfunction, respiratory problems, increased risk of deep vein thrombosis or chronic pain (Bunge et al., 1993; McDonald & Sadowsky, 2002; Hulsebosch, 2002; Kakulas, 1999; Thuret et al., 2006).

Following a SCI there are three phases of response: the primary, the secondary and the chronic injury phase (*Figure 1*).

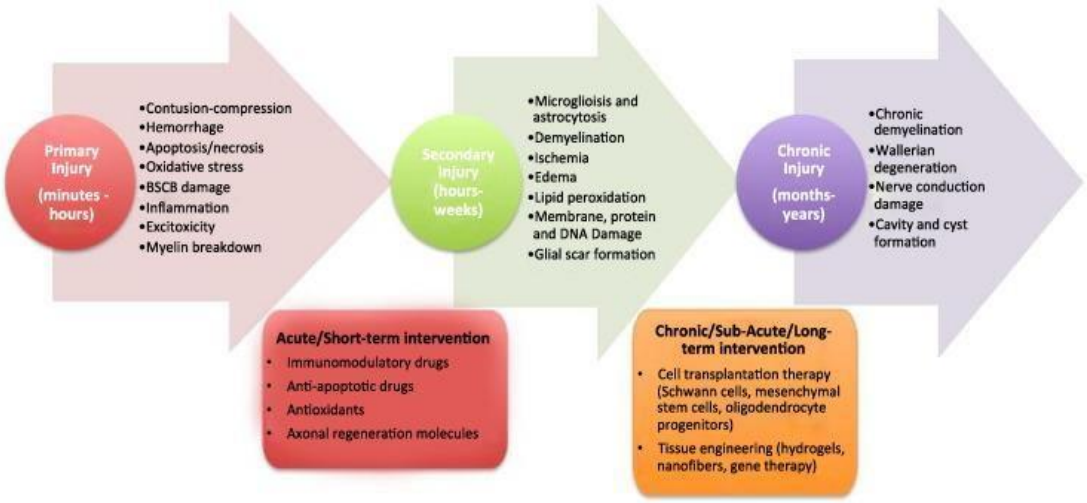

Figure 1.Three phases of response after spinal cord injury with time and different strategies of therapy (Kabu et al. 2015). Primary phase (contusion/compression, hemorrhage, inflammation, oxidative stress and myelin breakdown) starts immediately after injury occurs, which takes minutes to hours. It is followed by secondary phase (microgliosis/astrocytosis, demyelination, ischemia, edema, membrane/protein and DNA damage, glial scar formation), which takes hours to weeks. Finally, chronic phase (chronic demyelination, nerve conduction damage, cavity and cyst formation) occur, which takes months to years.

The primary phase of injury begins immediately by the mechanical force. This is the strongest predictor of overall prognosis (Oyinbo, 2011). Depending on the extend of the injury some irritation or even disruption of axons / blood vessels (hemorrhage) and breakdown of cell membranes (neurons and glial cells) will occur. These conditions result in pathophysiological changes with electrolyte disbalance, reduced or lack of normal blood flow and increased blood pressure into the lesion side, which causes a further mechanical force and damage of the tissue. The overall blood pressure diminishes, and a spinal shock will be the result, leading to vasospasm and formation of thrombi and ischemia, which can last for hours (Alizadeh et al., 2019; Hulsebosch, 2002; Thuret et al., 2006; Velardo et al., 2004).

The secondary phase starts a few hours later and can last over weeks or months (Ohab et al., 2006). The cascade of biochemical events leading to delayed cell death like electrolyte disturbance and edema from the acute phase can be persisting. As mentioned above, electrolyte disturbance with sodium (Na) influx into the cell cannot be balanced

by the sodium/potassium (Na/K) pump due to lack of energy.
Therefore, the sodium/calcium (Na/Ca) pump will be activated. This leads to cell death. One of the intracellular components that leak out of the cell is glutamate, which is a powerful excitatory neurotransmitter and responsible for activation of neurons. Uncontrolled release of glutamate will lead to toxic extracellular concentrations, which causes a cascade of activation and further cell death.
The inflammatory response is an important feature of this phase of injury. On the one hand it is a great system of defence against unknown and pathogenic agents. On the other hand, long duration of inflammation with its unspecific defence would have adverse effect on the nervous system. Inflammatory cells would attack the lesion side and phagocyte neurons, which cannot be rebuild. Instead, activation of astrocytes and microglia will cause scar formation, that can break down the previous connections and the result is dysfunction of nervous system (Bradbury & Burnside, 2019). With the invasion of inflammatory cells into the injured tissue local concentrations of cyto- and chemokines increase further. Also, inhibitory factors are expressed at the lesion sites, which expand the initial lesion and result in a loss of function (Hulsebosch, 2002; Sadowsky et al., 2002; Thuret et al., 2006).
In the chronic phase of injury, auto destructive mechanisms that cause cell death continue and the result is demyelination and glial scar formation, which hinders axonal growth and regeneration (Crowe et al., 1997). Scar formation is a complex process, wherein different cell types have their contribution. These are astrocytes, microglia and progenitor cells, macrophages as well as Schwann cells (Thuret et al., 2006). The aim of the scar formation is to isolate the damage and prevent further extension and neurodegeneration (Bush et al., 1999). Within the scar the ongoing processes fail to restore the normal architecture and composition of the damaged tissue. Axonal regeneration is inhibited by numerous growth-inhibiting factors, further demyelination and reduction of the functional capacity of the neurons will occur, which can result in spasticity and chronic pain (Hulsebosch, 2002; Silver & Miller, 2004).
Fortunately, a certain degree of repair has been seen in experiments of the past years. Specially, (peri)ependymal areas have a rich source of progenitors, that generate precursor cells to differentiate into neurons and glial cells (Azari et al., 2005; Yamamoto et al., 2001). There is almost no neurogenesis in the completely injured spinal cord, partial spinal cord injuries are followed by functional recovery resulting from remodelling which is based on axonal sprouting. A functional rearrangement in the

cortical sensorimotor occurs that makes the rubrospinal system compensate the loss of motor function (Raineteau & Schwab, 2001).

We can conclude that regeneration after SCI is a complex process, which is not only dependent on the primary, but also the secondary and the chronic phases of injury that can take months or in some cases years. It can have a tremendous impact on patient's physical and psychological state. Up to date there is unfortunately no wide range for treatment, so that more research is needed to explore novel therapeutic options (Hulsebosch, 2002).

1.3 The immune response

The immune system can be activated in two different pathways: the innate and the adaptive immune response (*Figure 2*). The innate immune response is a nonspecific initial fast response, which is triggered by neutrophils and macrophages/microglia as soon as pathogens enter the body. It is taking an important place in the first line of defense against an infection until the adaptive immune response is activated after 4-7 days (Janeway et al., 2001; Trivedi et al., 2006).

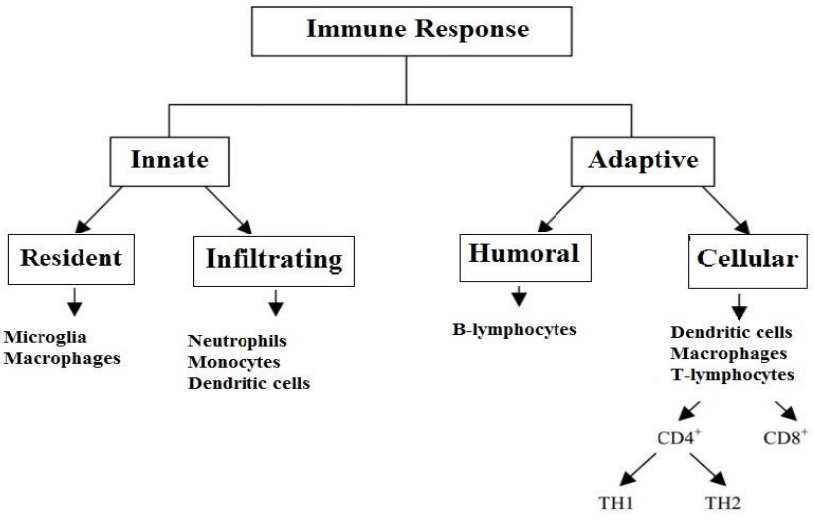

Figure 2. Adaptive and innate immune response (Trivedi et al., 2006). Innate immune system: The cells are either tissue specific or derived from a circulating pool of inflammatory cells. Adaptive immune system: The immune response consists of both humoral and cellular components. The humoral response consists of antibodies, which is mediated by B-

lymphocytes. The cellular response is mediated by T-lymphocytes, which are further classified into cytotoxic, $CD8^+$ and helper cells, $CD4^+$. $CD4^+$ develop into either TH1 or TH2 cells. TH1 cells produce pro-inflammatory cytokines (e.g. IFN-γ, TNF-α and IL-6). TH2 cells secrete anti-inflammatory cytokines (IL-4, IL-10 and TGF-β). Abbreviations: Interferon-gamma - IFN-γ; tumor necrosis factor-alpha - TNF-α; interleukin-6 - IL-6; interleukin-4 - IL-4; interleukin-10 - IL-10; and transforming growth factor-beta - TGF-β.

Neutrophils are heterogeneous cells with high functional plasticity, which also belong to the first defense line thanks to their high potency to sense, engulf and eliminate pathogens. They have surface receptors to recognize most pathogens and complement. They were initially seen as cells that can phagocyte pathogens, but recent studies support their regulatory contribution in the adaptive immune response. They release reactive radicals (superoxide anion (O_2^-) and nitric oxide (NO)) and cytokines as well as chemokines and other molecules. They can interact with T- and B-cells and possibly with antigen presenting cells (APCs) like dendritic cells (DCs) (Yang et al., 2019). Other cells of innate immune system (macrophages/microglia) recognize pathogens by their surface receptors, which bind the pathogen and enable them to engulf and digest them. Once activated, macrophages secrete cyto- and chemokines, which attract other cells like neutrophils, which in turn promote the process of inflammation further. This can also be done by complement proteins and their attractive properties. They bind to the surface of pathogens and make them recognizable for macrophages and therefore activate a cascade of reactions on the pathogens surface by their own (Janeway et al., 2001).

Microglia are acting by regulating brain development, maintenance of neuronal networks as well as elimination of pathogens and repair after injury. After injury, they proliferate and eliminate dead cells and all protein aggregates and soluble antigens as myelin debris that might cause damage in the central nervous system (CNS). They produce growth factors for neurite growth and anti-inflammatory and other cytokines like transforming growth factor-beta 1 (TGF-β1), which reduces the proliferation of astrocytes and impedes release of cytotoxic molecules that result in better neural survival (Meyers & Kessler, 2017; Trivedi et al., 2006). Recent studies showed a modulation of microglia-mediated neuroinflammation by TGF-β1 in rodents associated with a better functional outcome after intracerebral hemorrhage (Taylor et al., 2017).

In contrast, microglia also produce some inflammatory mediators like tumor necrosis factor-alpha (TNF-α), free radicals and interleukin-1, which cause some degree of damage after injury in the CNS. They promote neural cell death after injury, which cannot be abolished and increase thereby the loss of neurons, extend of initial injury and loss of function (Yang et al., 2004).

The role of macrophages in the secondary phase of injury is not easy to be noted as good or bad. Following an infection, they infiltrate after 1-2 days and reach a peak after 5-7 days (*Figure 3*).

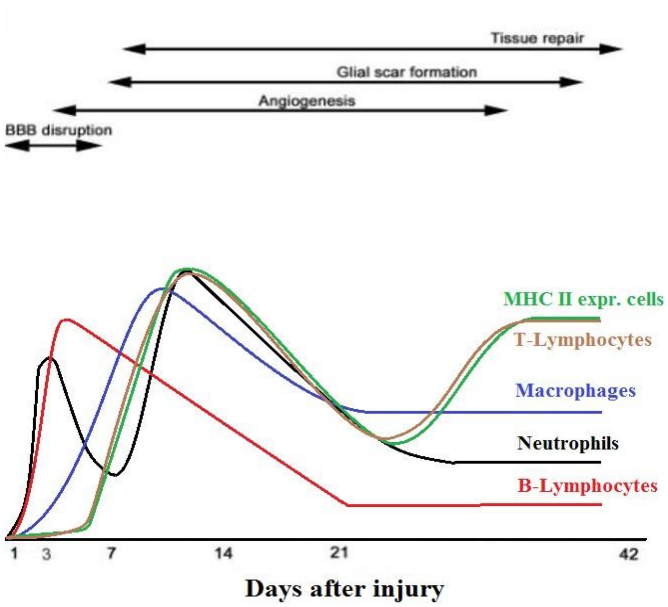

Figure 3. Schematic time-line of the infiltration of different immune cells after injury relative to secondary pathogenesis and wound healing (Trivedi et al., 2006). The summarized data from different studies addressed the timing of infiltration relative to early injury. Peak periods of infiltration occur at the same time with barrier disruption, angiogenesis and glial scar formation.

Macrophages fulfill many functions in inflammation and phagocytosis as well as regeneration by vascular remodelling and matrix rebuilding (Condeelis & Pollard, 2006; Hanahan & Weinberg, 2000).

The damage following an inflammation in this phase is specially caused by M1 type-macrophages by pro-inflammatory mediators as cytokines, neurotoxins and free

radicals, which are released during inflammation (Blight et al., 1992; Kigerl et al. 2009). DCs are cells that carry specific receptors on their surface which enables them to recognize numerous of pathogens. Once they bind pathogens on their surface, they can engulf and eliminate them intracellularly. Their main function is to present pathogens to the T-lymphocytes; they belong to the APCs and have the ability to eliminate pathogens which characterize them as components of first defense line. They not only initiate, but also support the ongoing inflammatory response, which may exacerbate secondary injury (Trivedi et al., 2006).

The innate immune response plays also an important role in initiating and directing the adaptive immune response (de Castro et al., 2004; Janeway et al., 2001; Sallusto & Lanzavecchia, 2002). The adaptive immune response is activated as soon as pathogens are presented to the lymphocytes by APCs.

1.4 Therapy of spinal cord injury

SCI can have a tremendous impact on patient's life, from partial temporary symptoms to even quadriplegia. Currently there is no restorative therapy. An individual therapy probably will not provide the cure, instead a combination of diverse strategies will result in a better outcome for patients after SCI. Experimental research aims at several approaches for potential therapies, from application of drugs to stem cell transplantation (Barnabe-Heider & Frisen, 2008; Thuret et al., 2006).
Several molecules are significantly identified in the therapy of neural damages, but only a few of them are investigated in *in vivo* models.

Most important molecules are neural cell adhesion molecule L1 (-L1), the close homolog of L1 (CHL1), the neural cell adhesion molecule (NCAM) and the glycan polysialic acid (PSA). They do not only play an important role in the development of nervous system, but also during neural repair and synaptic plasticity after injury and are therefore of big interest in therapeutic approaches in animal models of CNS (Irintchev & Schachner, 2011).
Cellular transplantation is also used in the *in vivo* models as a promising approach in the therapy of CNS after injuries, and different cell lines are applied yet. Transplantation of neural precursor cells (NPCs) in a mouse model after cortical brain injury showed a beneficial effect on regeneration by increased number of neurons and reduced glial scar formation (Lavdas et al., 2010). Another good candidate for cellular transplantation

would be Schwann cells (SCs). SCs are normally inhibited by astrocytes, but in *in vivo* models, there was a correlation between SCs with improved adhesive properties and an increased sprouting as well as better remyelination of nerve fibers and a significantly better functional outcome (Papastefanaki et al., 2016). Also transplantation of cultured adult olfactory ensheathing cells is being investigated as a promising procedure to re-establish glial pathways for the regeneration of severed axons after injury (Raisman & Li, 2007).

Early experiments with transplantation of stem cells were hindered by poor survival of the cells in the injured spinal cord after being transplanted. Embryonic stem cells as substrate adherent embryonic stem cell-derived neural aggregates (SENAs) seemed to bypass this problem. L1-overexpressing SENAs promote axonal growth, and showed increased neural survival and enhanced differentiation, and led to a better locomotor function, as well as a reduction of glial scar formation in mice (Chen et al., 2016; Cui et al., 2011).

Currently there are some drugs that are used after SCI. Specially corticosteroid drugs are used in the first response after SCI, which can be given in the first few hours after injury.

Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) and gabapentin, muscle relaxants, anti-depressants, and painkillers (e.g. narcotics) are used, dependent on patient's need and especially in the second phase of injury (Benzel, 2018; Thuret et al., 2006).

Finally physical therapy, which should begin soon after injury, plays an important role especially in the chronic phase and serves as a palliation (Gomara-Todra et al., 2014).

1.5 Polysialic acid

Structure and function of polysialic acid (PSA)

PSA is a well-characterised long polymer of the nine-carbon sugar neuraminic acid highly expressed on the surface of neurons and associated with the NCAM and other carriers like neuropilin-2 (NRP 2). The three major components that build PSA are: 5-N-acetylneuraminic acid (Neu5Ac), 5-N-glycolylneuraminic acid (Neu5Gc) and 5-deamino-3, S-dideoxyneuraminic acid (2-keto-3-deoxynonulosonic acid, Kdn) (*Figure 4*). The carbohydrate chains of PSA are strongly hydrated and negatively charged, and suggested to diminish cell adhesion and to increase their motility (Mishra et al., 2010).

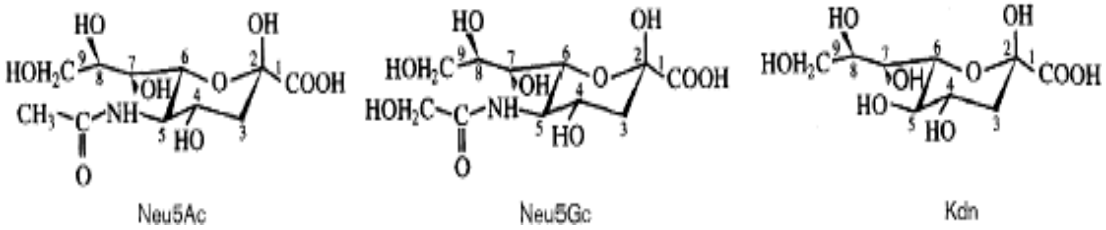

Figure 4. The structure of three components of PSA (Mühlenhoff et al. 1998). 5-N-acetylneuraminic acid (Neu5Ac), 5-N-glycolylneuraminic acid (Neu5Gc) and 5-deamino-3, 5-dideoxyneuraminic acid (Kdn).

PSA is a linear homopolymer of α2-8-linked N-acetylneuraminic acid with a helical conformation (Michon et al., 1987; Mühlenhoff et al., 1998; Rutishauser, 1989). The biosynthesis of PSA starts with a linkage between an α-2, 8-linked sialic acid and a terminal sialic acid component in an α-2, 3-linkage, which then continues up to 100 α-2, 8-linked sialic acid residues (Mishra et al., 2010).

This process is catalyzed by two sialyltransferases of the Golgi apparatus, namely ST8Sia-II and ST8Sia-IV (*Figure 5*). These enzymes are members of the sialyl transferase family and are the so-called type II trans-membrane glycoproteins that share the topological features common to other glycosyltransferases (Datta & Paulson, 1997). They consist of a cytoplasmic tail that goes through the membrane inside the Golgi - apparatus where the catalytic part is embedded. This catalytic part consists of three domains (*Figure 6*), the sialyl-motifs (***L***arge, ***S***mall and ***V***ery ***S***mall). The large one consists of 48-49 amino acids, is in the middle of the catalytic domain, which has eight invariant residues. The small one consists of 23 amino acids, is close to the C-terminal end of the enzyme and has two invariant residues. Their function is to bind the substrates (Datta & Paulson, 1997).

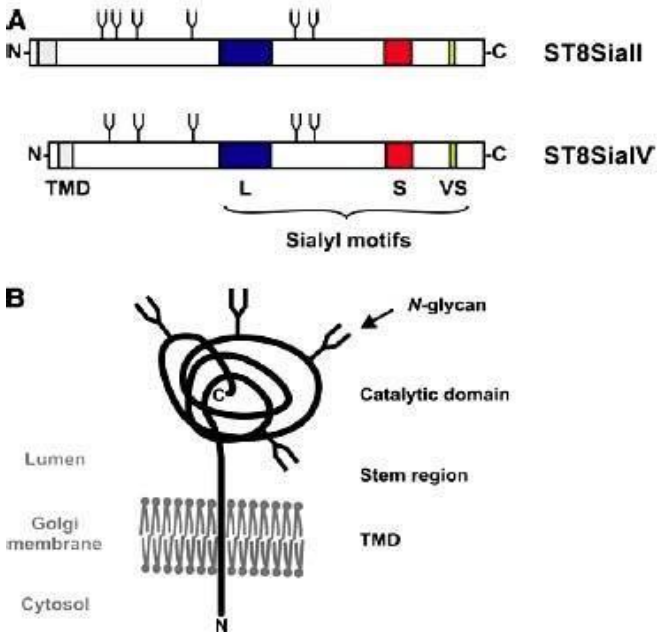

Figure 5. Polysialyl transferases; ST8Sia-II and ST8Sia-IV (Mishra et al., 2010). a) The trans-membrane domain (TMD) and the sialyl motifs large (L), small (S), and very small (VS) of the catalytic domain. The relative positions of the N-glycans are indicated by Y-shaped symbols. b) Type II transmembrane topology of polysialyltransferases.

ST8Sia-II and ST8Sia-IV are mostly selective to neural cell adhesion molecule, NCAM, the major carrier protein binds and of PSA. They catalyze the transfer of a-2,8-linked sialic acid components to α-2,3-or α-2,6-sialylated galactose, which are bound in α-1,4-linkage to N-acetyl glucosamine (Angata et al., 1998; Mishra et al., 2010). The attachment of PSA to N-glycans occurs at the 5^{th} Ig-like domain of NCAM (von der Ohe et al., 2002). Experimental work showed that the cooperation between these enzymes results in a higher number of PSA molecules and a higher degree of polymerization (Angata et al., 2002).

PSA emerges as a molecule with unique immunomodulatory potential. The carboxylate and acetamido groups are positioned in the enlarged helical structure, where different conformations of α-2, 8-linked PSA derivates are possible, which are due to the negative charge of the carbohydrate groups. The massiveness of the extended helical structure prevents disruption of these substituents (Mishra et al., 2010; Samuel & Bertozzi, 2004).

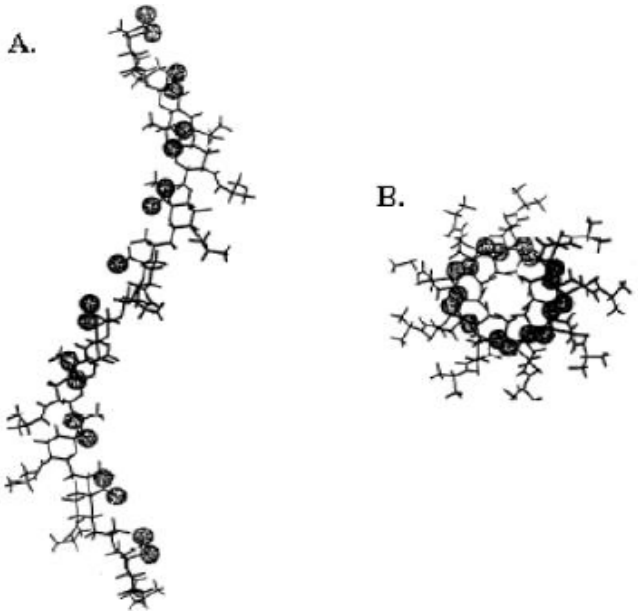

Figure 6. The helical structure of PSA (Mishra et al., 2010). The position of the carboxylate and acetamido groups of PSA and the massivness of the helix, which prevent disruption of the substituents. A: side view, B: top view.

Functions of polysialic acid

PSA has an inhibitory effect on cell adhesion when it is bound to NCAM. This inhibitory effect is independent of all other structural or functional properties of NCAM itself, but those mediated by IgCAM, a cadherin or an integrin (Fujimoto et al., 2001; Sadoul et al., 1983). Other negative regulatory interactions independent of NCAM could also be possible (Rutishauser et al., 1988). It has been observed that in absence of PSA the function of other cell adhesion molecules (L1/NgCAM) is positively changed (Acheson et al., 1991).

It has been proposed that the effect of PSA on cell adhesion can be found in its steric properties (Yang et al., 1994), where two mechanisms are assumed: PSA impedes trans-interaction of two opposing membrane-receptors and involves a cis-interaction of receptors on the same cell (*Figure 7*).

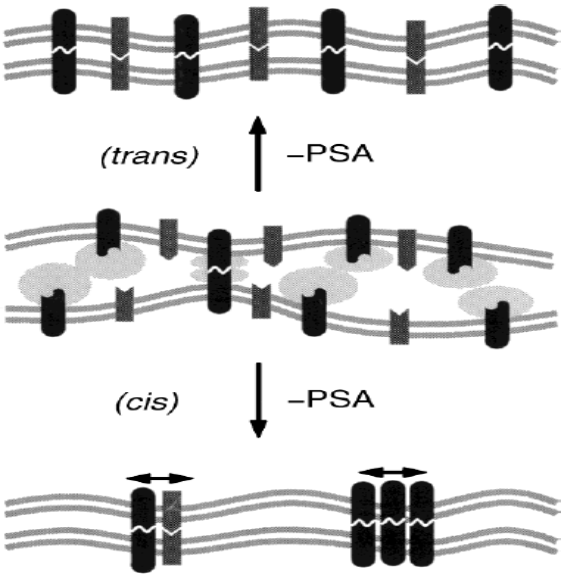

Figure 7. Two possible mechanisms of PSA in respect to cell-cell interaction (Mishra et al., 2010; Rutishauser, 1998). T*rans* mechanism: The highly hydrated polymer attached to NCAM serves as a barrier to membrane-membrane contact, which decreases the efficiency of interaction between complimentary receptors on opposing membranes. *Cis* mechanism: The steric action of PSA is local, where it affects cell-cell interactions by interference with a clustering of NCAMs (right) or by association of NCAM with other receptors on the same cells (left).

PSA plays an important role in several cellular mechanisms in the nervous system, i.e. behavior and growth of axons (Rutishauser, 2008).

During development of nervous system, there are three types of cell migration:

- radial cell migration (gliophilic)
- cell migration (neurons/glia) along axons (axophilic)
- co-operative streaming of neuronal precursors.

PSA seems to play a role in axophilic and co-operative cell migration, but not in gliophilic cell migration (Mishra et al., 2010).

Previous experiments showed that when PSA is removed by endoneuraminidase-N (Endo-N), axons seem to grow toward the slow muscle region. In general, the distribution of PSA is more prominent in the fast muscle regions compared to slow muscle regions (Landmesser et al., 1988). That means there should be other factors that contribute to attracting axon fibers in fast muscle regions, as PSA restricts interaction between neurons. PSA is the key factor in

some motoneurons when they are entering the period of axonal outgrowth. PSA is then removed by Endo-N and the result is the innervation of the appropriate muscle (Tang et al., 1992). A further important contribution of PSA is in the process of synapse plasticity.

1.6 Histone H1

Histones are basic proteins of the eukaryotic cell nuclei that pack and order the DNA into structural units called nucleosomes, which together form chromatin (*Figure 8*). Without histones, the unwound DNA in chromosomes would be very long, which would not be very functional.

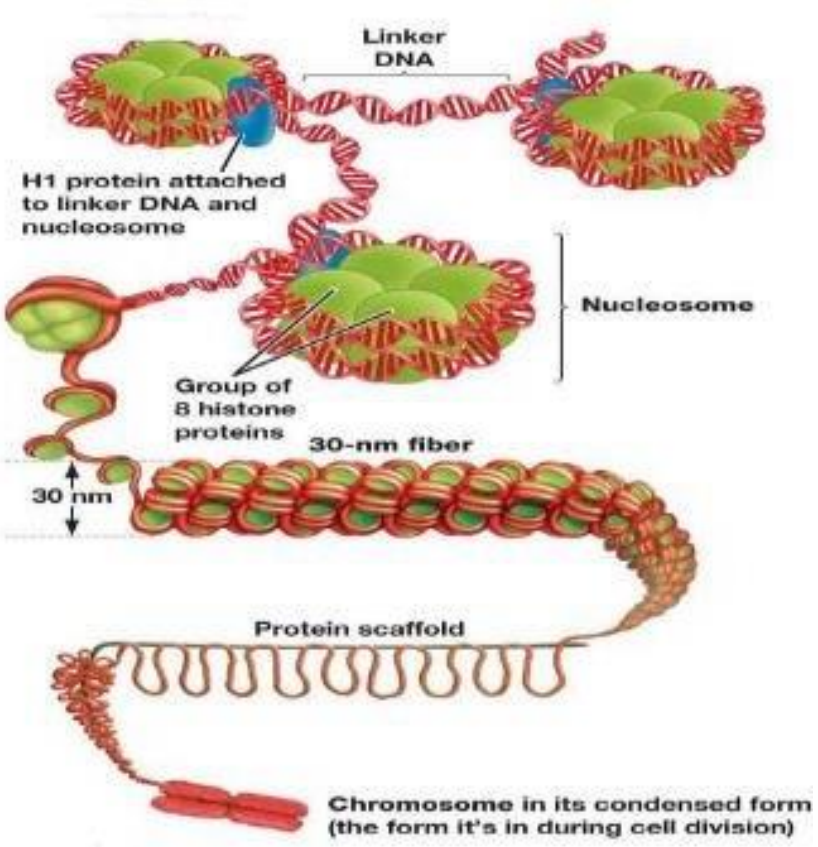

Figure 8. Schematic representation of histone, nucleosome and chromatin (Eukaryotic Chromosome Structure). The length of DNA must be compacted in order to fit it inside nucleus. The compacting of DNA is accomplished by binding to different cellular proteins. This result in the formation of chromatin, which can be defined as highly condensed chromosomes.

Histones do not only keep DNA strands together, but also play an important role in gene regulation. There are six major histone families identified so far: H1, H2A, H2B, H3, H4 and H5. Histone H1 is also known as the linker histone, which is related to histone H5 as they have a similar structure and function. Histone H5 is only found in avian

erythrocytes (Bhasin et al., 2006; Cox et al., 2005; Hartl et al., 1988; NCFBI 2017). Compared to other histones, histone H1 is the most prominent protein in the nucleus. Histone H1 binds to the linker DNA, which is required for further packing and stabilizing of chromatin (Alberts et al., 2002; Mishra et al., 2010). There are different subtypes of histone H1 (H1 a-e and H1), that have been identified, where based on functional differences H1 a/c can be seen as a different subset to H1 b/d and e (Lennox & Cohen, 1983). Extranucleary histone H1 has been localized in the cytoplasm (Reichhart et al., 1985) and at the cell surface of neurons by anti-histone H1 antibodies ANA108 (Bolton & Perry, 1997).
It has been suggested that histone H1 also acts as an acute phase protein like lipopolysaccharide (LPS)-binding protein with antibacterial properties, when infections occur (Hiemstra et al., 1993).

Histone H1 had been shown to be an interaction partner of PSA using an unconventional anti-idiotype approach and direct binding of histone H1 to PSA could be observed (Mishra et al., 2010). It has been shown that histone H1 is present at the cell surface of cerebellar neurons and SCs and that this extracellular histone H1 has a beneficial effect on neural precursor cell migration, neurite outgrowth, as well as SC proliferation and process elongation. Increased functional recovery and motoneuron reinnervation by histone H1 after femoral nerve injury indicates its importance in regeneration *in vivo* (Mishra et al., 2010).

2. Rationale and aims of the study

It has been shown that extracellular histone H1 has beneficial effects on neural precursor cell migration, neurite outgrowth and Schwann cell proliferation and process elongation. Increased functional recovery and preferential motoneuron reinnervation after peripheral nerve injury elicited by histone H1 indicated its importance in regeneration after peripheral nerve injury *in vivo* (Mishra et al., 2010). Therefore, I wanted to confirm and extend these observations to regeneration after spinal cord injury.
The aim of this study was to evaluate if there is a beneficial effect of histone H1 on regeneration after spinal cord injury. We also were interested if histone H1 in the context of regeneration after spinal cord injury acts dependent or independent of its

interactions with PSA.

The specific aims of the study were:

1. To assess astrocyte activation upon histone H1 treatment after spinal cord injury;
2. To assess activation of microglia/macrophages upon histone H1 treatment after spinal cord injury;
3. To assess neural precursor cell proliferation upon histone H1 treatment after spinal cord injury;
4. To assess migration of astrocytes within a scratch-wound assay upon histone H1 treatment;
5. To assess if enzymatic removal of PSA by EndoN has an effect on migration of astrocytes within a scratch-wound assay upon histone H1 treatment.

3. Materials and Method

3.1 Antibodies and reagents

Histone H1 was purchased from Merck Chemicals GmbH (Darmstadt, Germany; Cat# 14-155) and Hölzel Diagnostika Handels GmbH (Cologne, Germany; Cat# CS-CS426).
Antibodies, which were used for immunoblot analysis and/or immunohistochemistry were:
- Rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Sigma-Aldrich, Taufkirchen, Germany; Cat# G3893)
- Rabbit anti-neuron/glia antigen 2 (NG2) antibody (AN2 1E6) (Niehaus, Stegmuller, Diers-Fenger,& Trotter, 1999)
- Rabbit anti-Ionized calcium binding adaptor molecule 1 (Iba1 antibody; Wako Chemicals, Neuss, Germany; Cat# 019-19741)
- Rabbit anti-Mac2/galectin3 antibody (Thermo Fisher Scientific, Darmstadt, Germany; Cat# 14-5301-81)
- Rabbit anti-Ki67 antibody (Synaptic Systems, Göttingen, Germany; Cat# 398 103)
- Anti-PSA antibody 735 (Frosch, Gorgen, Boulnois)
- Cy3-conjug. goat anti- rabbit sec. antibody and a horse radish peroxidase-conjug. secondary antibody; (Dianova - Hamburg, Germany).

3.2 Animals

C57BL/6J wild-type female mice were used in all experiments. For the biochemical experiments 2-7-day-old female mice and for *in vivo* experiments 3-month-old female mice were obtained from the central animal breeding facility of the Universitätsklinikum Hamburg-Eppendorf. The mice had no sign of any disease prior to the injury and treatment. All mice were kept in the specific pathogen-free laboratories under standard laboratory conditions on an artificial 12 h light/dark cycle with food and

water supply *ad libitum*. The experiments were conducted in accordance with the German and European Community laws on protection of experimental animals (permission number Org-679 and animal project number 098/09). All procedures we used were approved by the responsible committee of The State of Hamburg. The animal treatments, acquisition of data as well as all analyses were performed in a blinded fashion.

3.3 Surgical procedures

The mice were anesthetized by intraperitoneal injection of ketamine / xylazine (Parke-Davis/Pfizer, Karlsruhe, Germany). An eye ointment was applied to protect the eyes of mice from drying during anesthesia and irritation after that. The back of the mice were shaved and disinfected with an antiseptic solution. A dorsal incision was made through the skin, the underlying tissues were removed so that vertebral bone plate was visible. Laminectomy was performed with forceps (Fine Science Tools, Heidelberg, Germany) at thoracic level of T7-9. After exposing the spinal cord, a compression device was used to elicit compression injury (Curtis et al., 1993; Steward et al., 2003). The device consisted of one pair of forceps mounted in a stereotaxic frame, which compressed the spinal cord (100%, according to the operational definition of Curtis et al. (1993) for 1 second. This was time-controlled by an electromagnetic device. After that 1 µl of histone H1 (concentration 100 µg/ml, which was diluted with PBS) were applied at both rostral and caudal side of the lesion. The control mice were treated with PBS. The skin of the mice was subsequently surgically closed using 6-0 nylon stitches (Ethicon, Norderstedt, Germany). Postoperatively, the mice were kept warm for at least 24 hours to prevent hypothermia. The animals were provided with water and standard food. Manual bladder voiding was performed during the whole postoperative period at least once each day.

3.4 Preparation of spinal cord tissue

Six weeks after SCI, mice were anesthetized with sodium pentobarbital (Narcoren®, Merial, Hallbermoos, Germany, 5 µl/g body weight, i.p.), followed by an incision of the chest and transcardial perfusion initially with physiologic saline for 60 seconds, then followed by formaldehyde (4% formaldehyde and 0.1% $CaCl_2$ in 0.1 M

sodium cacodylate buffer with pH 7.3). After 15 minutes of perfusion (about 100 ml per mouse), the mice were left for about 2 hours at room temperature (RT). After that the spinal cords were dissected, post-fixed overnight (18-22 hours) in the same fixative at 4°C. For cryoprotection 15% sucrose solution was added to the fixative, put for 2 days at 4°C. The samples were put to freeze for 2 minutes in 2-methyl-butane (isopentane) pre-cooled -25°C to -30°C in the cryostat. Sections (25 µm thick) were cut from the spinal cord on a cryostat (Leica CM3050, Nussloch, Germany), after that they were collected on SuperFrost Plus glass slides (Roth, Karlsruhe, Germany).

3.5 Histology and immunohistochemistry

The immunohistochemical stainings were performed (as described by Irintchev et al., 2005) on sections with a thickness of 25 µm. The sections were stored at -20°C and air-dried for 30 minutes at 37°C before use. A solution of 10 mM sodium citrate was prepared (pH 9.0, adjusted with 0.1 M NaOH) and preheated in a jar up to 80°C in a water bath. For antigen-demasking the sections were incubated at 80°C for 30 minutes in the sodium citrate solution, were then taken out of the jar and left to cool down at RT. Blocking of unspecific binding sites for the secondary antibody and permeabilization was then performed. The sections were incubated in PBS containing 0.2% Triton X-100 (Fluka, Buchs, Germany), 0.02% sodium azide (Merck, Darmstadt, Germany) and 5% normal goat or donkey serum (Jackson ImmunoResearch Laboratories, purchased via Dianova, Hamburg, Germany) at RT for one hour. After one hour the blocking solution was aspired and the slides were incubated with following primary antibodies: Rabbit anti-GFAP (1:5000); rabbit anti-NG2 (1:2000); rabbit anti-Iba1 (1:500); rabbit anti-Mac2 (1:10000); rabbit anti-Ki67 (1:500). They were diluted in PBS containing 0.5% lambda-carrageenan and 0.02% w/v sodium azide in PBS. The slides were incubated for 3 days at 4°C in a screw-cap staining jar (30 ml capacity, Carl Roth). After that, they were washed 3 times in PBS (15 minutes each time) before an appropriate secondary antibody was applied. The sections were then incubated with the second antibody goat anti-rabbit (1:200) in PBS-carrageenan at RT for 2 hours. As a secondary antibody we used goat anti-rabbit IgG conjugated with Cy3 (Jackson ImmunoResearch Laboratories, purchased via Dianova, Hamburg, Germany). After a subsequent wash in PBS, cell nuclei were stained for 10 minutes at RT with bis-benzimide solution (Hoechst 33258 dye, 5 µg/ml in PBS, Sigma-Aldrich).

Finally, the sections were washed again 2 times in PBS (each time for 5 minutes) and then mounted with Fluoromount G (Southern Biotechnology Associates, Biozol, Eching, Germany) for one hour at RT and then stored in the dark at 4°C.

3.6 Analysis of GFAP and NG2 expression, cell activation and proliferation

The expression of glial fibrillary acidic protein (GFAP) and neuron-glial antigen 2 (NG2) was measured by selecting 100 µm of each side (rostral and caudal) from the lesion (using images obtained with a Zeiss Axiophot microscope 40× objective) and estimation of the area with immunopositive structures normalized to the total image area (set as 100%) using Image-J as described (Wu et al., 2012). Six longitudinal sections per animal were analysed. To calculate the group mean values, mean values from individual animals were used. GFAP is the best marker for activated astrocytes following injuries of the CNS. (Eng & Ghirnikar, 1994; *Jacque et* al., 1978). It is involved in many processes in the CNS, which include astrocyte-neuron interactions by providing energy nutrition for survival, development, differentiation and regeneration (Brenner, 2014*;* Hampton et al., 2004*;* Parpura et al., 2012; Rost et al., 2016; *T*ardy et al., 1990; Wang et al., 2004*;* Zhang et al., 2017*).*

NG2 is expressed on the surface of NG2-positive glial and oligodendrocyte precursor cells. In the past years different studies indicated the inhibitory role of NG2 on axonal regeneration and growth after injury of CNS. Once NG2 glial cells are activated, they contribute to the glial scar formation, which is considered a barrier to regenaration and axonal growth (Romulo de Castro et al., 2005; Zhi Jiang Chen et al., 2002).

To quantify the proliferating Ki67-immunoreactive cells on longitudinal, parasagittal sections were counted. Rostral and caudal to the lesion, all Ki67-positive cells were counted approximately 500-750 µm from the lesion site on each side of the lesion and calculated per area unit (mm^2). Ki67-antigen is a cell cycle related nuclear protein, that is expressed only by proliferating cells in all phases of an active cell cycle (G1, S, G2 and M phase), but absent in resting cells (G0). Ki67 is normally used as a neuronal marker of cell cycling and proliferation.

To quantify the activation of microglia/macrophages, anti-Iba1 and anti-Mac2 antibodies were used. Iba1 is a macrophage/microglia-specific calcium-binding protein, which is involved in the activation of quiescent microglial cells. Although it is expressed in both activated and quiescent cells, the degree upon activation is higher

(Imai & Kohsaka, 2002). Following an injury of the CNS axons will be damaged. Following activation of immune reaction, as myelin-debris impedes regeneration it has to be removed by microglia. Mac2-antigen is an approximately 30 kDa carbohydrate-binding protein expressed on the surface of inflammatory macrophages and other macrophage cell lines, including microglia (Dong & Hughes, 1997) and thus can be used to stain activated microglia and macrophages.

3.7 Astrocyte cell-culture

Astrocytes were cultured from the cerebral cortices of wild-type female mice (C57BL/6J), which were 1-2-day-old as described (Jakovcevski et al., 2007). Brains were dissected and freed of the meninges, then mechanically dissociated by pipetting up and down through a fire-polished Pasteur pipette (successive through200,150,and30 µm nylon mesh filters; VWR,Darmstadt,Germany) and then plated on the tissue culture dishes or plates (Greiner BioOne,Frickenhausen,Germany) coated with poly-L-lysine (PLL, 0.01 mg/ml in H_2O). The cells grew in Dulbecco's Modified Eagle's Medium (DMEM with high glucose, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 200 nM glutamine, 1% Penicillin/Streptomycin at 37°C with 5% CO_2. The medium was changed three times every week. After 7 days the cultures were ready for following experiments.

3.8 Scratch-wound assay

The capacity for migration of astrocytes was examined by scratching confluent astrocyte monolayers to induce natural cell migration, as described (Cory, 2011; Yuan et al., 2015). The astrocyte monolayers were scratched by a sterile 200-μl pipette tip across the surface. The detached cells were removed immediately by washing 3 times with Hank´s balanced salt solution (HBSS) and fresh medium was added. After that, the cells were kept in culture for additional 72 h in the medium with 1% FBS and 10 µl of histone-H1 (conc. 2,0 µg/µl), 11,11 µl of EndoN (conc.1,8 µg/µl), 13,33 µl of EndoN+histone- H1 (conc. 1,5 µg/µl) or 11,11 µl of HBSS (conc.1,8 µg/µl) as the control group. The protein concentrations had been previously determined by BCA test.

Images of the closing gapes were acquired directly after the injury (0 h) and 8, 24, 48 and 72 h under microscope (Zeiss, AxioVision).

3.9 Collecting astrocyte tissue

After scratch-wound assay (72 hours later), we have removed the medium of each well and collected the tissue (astrocytes) as followed. We used 2 ml of a Radioimmunoprecipitation assay (RIPA)-buffer with 80 µl of a protease-inhibitor. Subsequently we put 200 µl of this solution into each well, scrubbed the tissues from the plate away and collected them into 1,5 ml reaction vessels. They were kept in ice, then centrifuged for one hour and subsequently freezed for western blot later.

3.10 SDS-gel electrophoresis and western blot analysis

A Mini Trans-Blot (BioRad) was used for western blot analysis to transfer proteins from the 8% acrylamide gel onto a 0.45 µm nitrocellulose membrane (Schleicher and Schuell) to detect separated proteins with corresponding antibodies. An electro blotting "sandwich" was put together according to the manufacturer´s instructions. Each lane was loaded with 20 µl (15 µl of a sample + 5 µl of sample buffer). After 5 minutes in thermomixer at 95°C, it was put to run at 75V. We prepared a transfer buffer (200 ml blot buffer + 1400 ml H2O + 400 MeOH) and poured it in the jar with PVDF membrane in methanol. Electrophoretic transfer of proteins was done within 90 minutes at constant 100 V on ice. The nitrocellulose membrane was put for one hour in blocking solution(4% skim milk powder in phosphate-buffered saline solution pH 7.4 with 0.01% Triton X-100 (PBST)) at RT. The membrane was incubated overnight at 4°C under agitation in appropriate dilution in PBST with primary antibody (anti-PSA 735 (1:1000). Then the membrane was washed 3 times (for about15 minutes) with PBST at RT. After that, a diluted horseradish peroxidase (HRP)-conjugated secondary antibody (40 ml of 4% milk in PBST + 4 µl of a secondary antibody) was applied in PBST. The secondary antibody solution was aspired and the membrane washed 3 times with PBST. The immunoreactivity was visualized by using a chemiluminescence detection system (ECL) and detected with ImageQuantTM LAS 4000 mini (GE Healthcare).

3.11 Microscopy and photographic documentation

All images used in my experiments were obtained with a confocal laser-scanning microscope (Zeiss LSM510 crypton-argon) equipped with an objective lens (60 x oil-immersion). Images were scanned with a resolution of 512x512. The photos were edited by using Adobe Photoshop 8.0 software (Adobe Systems Inc., San Jose, CA).

3.12 Statistical analysis

The statistical analysis of the histological and immunohistological studies were restricted to compare mean values of same genotypes by using two-tailed *t* test for independent groups, after the normalcy of distribution was confirmed by Shapiro-Wilk test, and the equal variance was tested. The mean value was calculated by using approximately 6 measurements per animal, which was a representative value. So, for all comparisons the degree of freedom was determined by the number of animals, the accepted level of difference between groups was 5%. The statistical analyses were performed by using the SPSS Statistics 11.5 software (IBM Corporation, Somer, NY).

4. Results

4.1 Stereological analysis of the spinal cord

To determine the reaction of astroglia, microglia/macrophages, and cell proliferation, immunostainings with antibodies to NG2, GFAP, Iba1, Mac2 and Ki67 were performed. To quantify the effect of histone H1, different areas rostral and caudal to the injury were measured with and without (control) treatment or histone H1 treatment.

After spinal cord injury, astroglia cells are activated and migrate to the lesion site to keep the process of inflammation going. Astrocytes express high levels of GFAP and NG2 (Alonso, 2005; Hampton et al., 2004; Rost et al., 2016; Wang et al., 2004).

4.1.1 GFAP expression

GFAP is an intermediate filament protein, which is primarily expressed in the CNS in astrocytes. I measured the density of GFAP-positive structures in mice with and without treatment of histone H1 or vehicle, 6 weeks after SCI *(Figure 9)*. The results showed that GFAP expression was down-regulated in mice that were treated with histone H1 compared to vehicle-treated mice both rostral and caudal to the injury site.

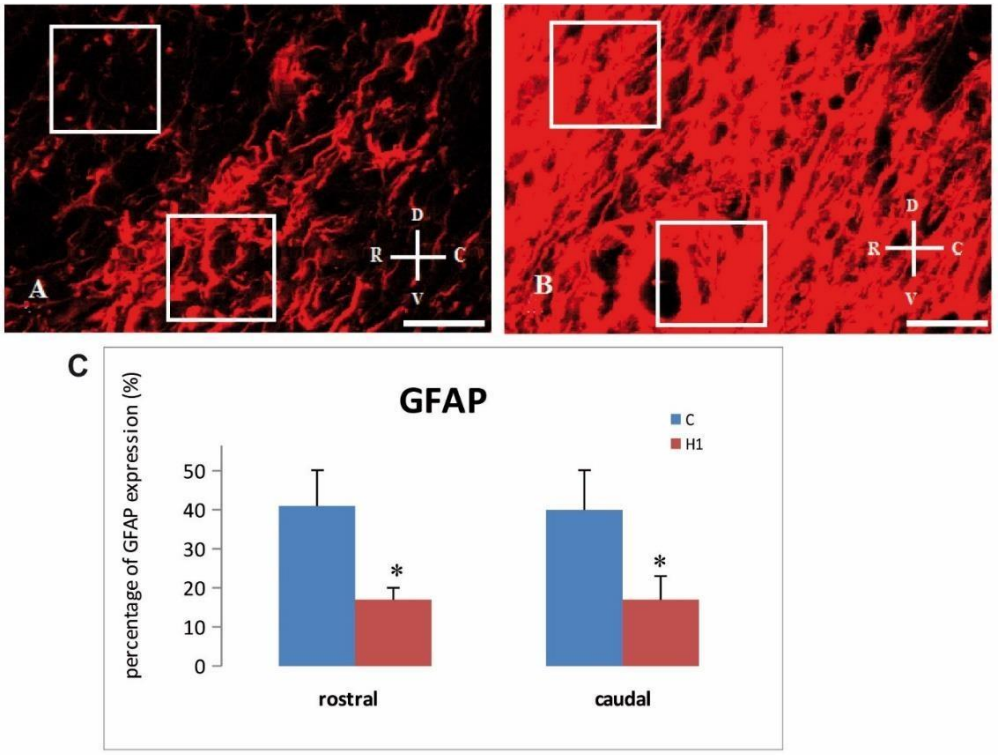

Figure 9. Analysis of GFAP expression in the spinal cord 6 weeks after injury. (A, B) Representative confocal images of GFAP-stained sections from histone H1 treated mice (A) and vehicle treated mice (B). R: rostral; C: caudal; V: ventral; D: dorsal. Rectangles indicate representative areas used for quantification of the immunostained area relative to the total rectangle area.
Scale bars: 100 µm. (C) Bar graph shows percentage of the GFAP stained area at the lesion site of histone H1 (H1)- and vehicle (C)-treated mice 6 weeks after SCI. N=6 in each group, the number of slices in each group was 36. Mean numbers + SEM from both groups, asterisks indicate significant differences from the control group. *$p < 0.05$, two-tailed t-test.

4.1.1 NG2 expression

I measured the density of NG2-positive structures in mice with histone H1 or vehicle treatment 6 weeks after SCI *(Figure 10).*

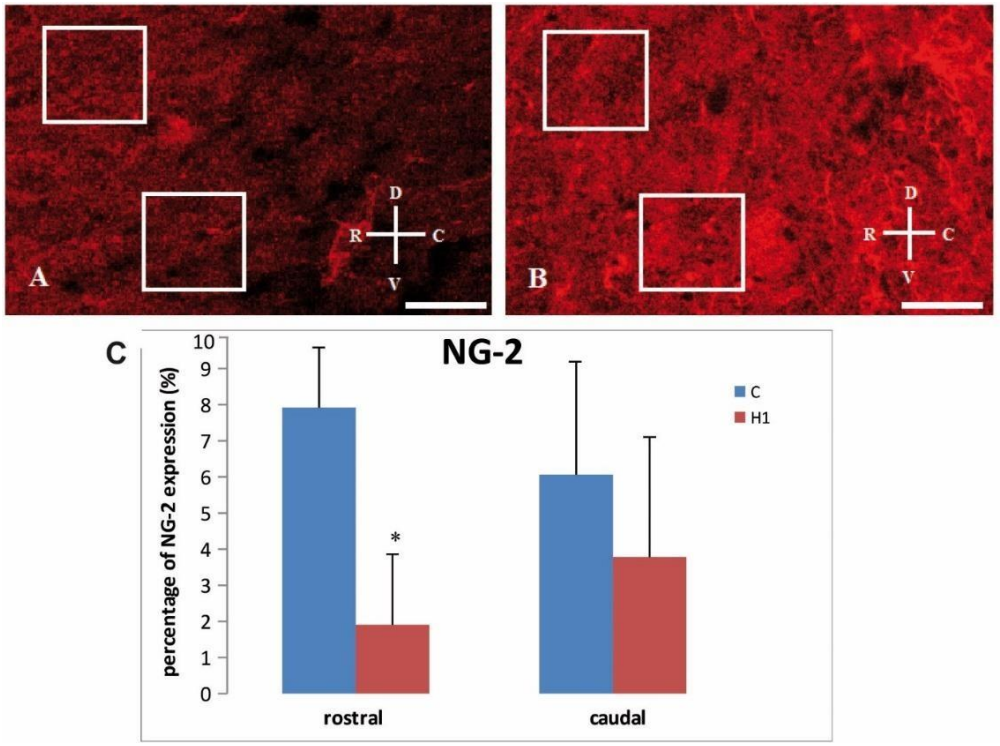

Figure 10. Analysis of NG2 expression in the spinal cord 6 weeks after injury.
(A, B) Representative confocal images of NG2 stained sections from the rostral part of the lesion site of histone H1 (A) and with vehicle treated (B) mice (40 x objective). R, rostral; C, caudal; V, ventral; D, dorsal. Rectangles indicate representative areas used for quantification of the immunostained area relative to the total rectangle area. Scale bars: 50 µm. (C) Bar graph shows percentage of NG2 immunostained area in histone H1 (H1)- and vehicle (C)-treated mice 6 weeks after SCI. Shown are mean values + SEM from both groups. N=6 in each group, the number of slices in each group was 36. The asterisk indicates a significant difference from the control group. *$p < 0.05$, two-tailed t-test.

Results show that NG2 expression was down-regulated in histone-H1-treated mice compared to vehicle-treated mice. It should be noted that the low expression of NG2 was statistically significant (*$p < 0.05$, two-tailed t-test) only in the rostral part of the lesion site.

4.1.2 Iba1-positive cells

Activated microglia and macrophages express Iba1 and Mac2. They are mainly responsible for activation and maintenance of the inflammation and therefore glial scar formation (Okada et al., 2018; Zhou et al., 2014).

Iba1 is a macrophage/microglia-specific calcium-binding protein involved in the activation of quiescent microglial cells (Imai & Kohsaka, 2002; Okada et al., 2018). As a result of this activation, the protein expression is enhanced and is observed at high levels in activated microglia. This allows reliable evaluation of both resting and activated microglial cells (Imai & Kohsaka, 2002).
The density of Iba1 immunoreactive cells were evaluated in histone H1-treated and vehicle-treated mice 6 weeks after SCI (*Figure 11*). Quantification showed a lower density of Iba1-positive cells rostral but not caudal to the lesion site after histone H1 treatment when compared to vehicle treated mice.

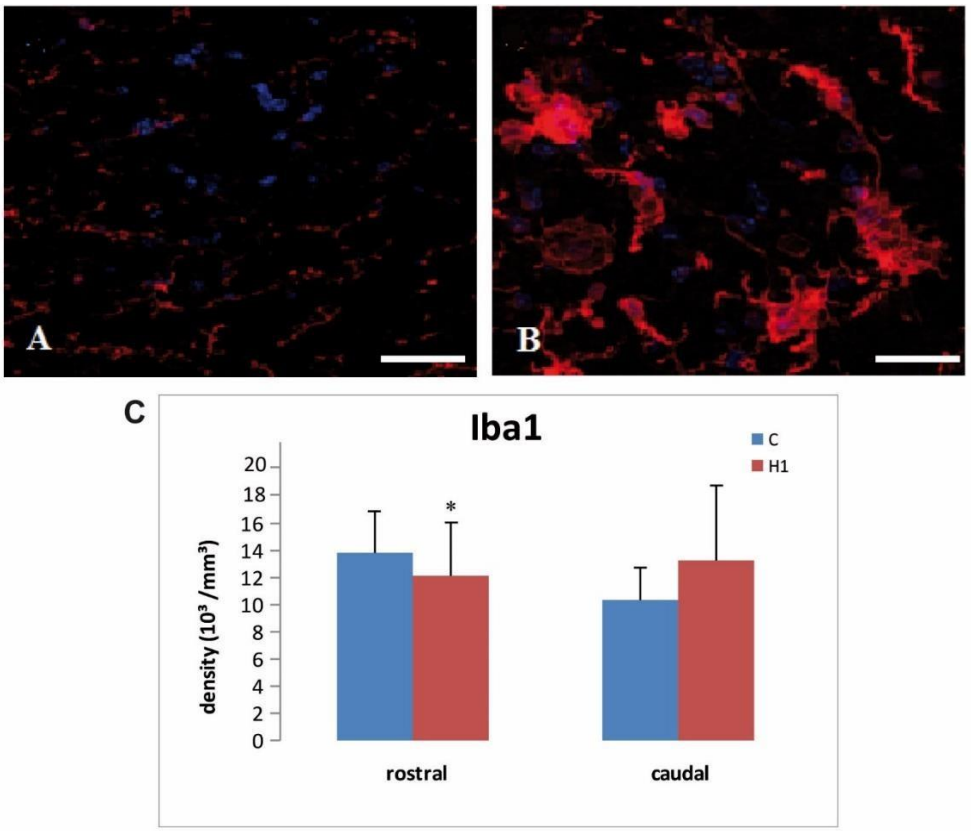

Figure 11. Analysis of Iba1 expression in the spinal cord 6 weeks after injury. (A, B) Representative confocal images of rostral parts of the lesion site from histone H1-treated mice; 10 x objective (A) and 40 x objective (B). Scale bars: 100 µm (A) 20 µm (B). Counting frame size 60x60 µm. *(C)* Bar graph shows quantification of density of Iba1-positive microglia in histone H1- (H1) and vehicle (C)-treated mice 6 weeks after SCI. N=6 in each group, the number of slices in each group was 36. Shown are mean values + SEM from both groups. The asterisk indicates a significant difference from the control group. *p < 0.05, two-tailed t-test.

4.1.3 Galectin3/Mac2-positive cells

Next, I measured the density of Mac2-positive microglial cells in mice with histone H1 or vehicle treatment 6 weeks after SCI *(Figure 12).*

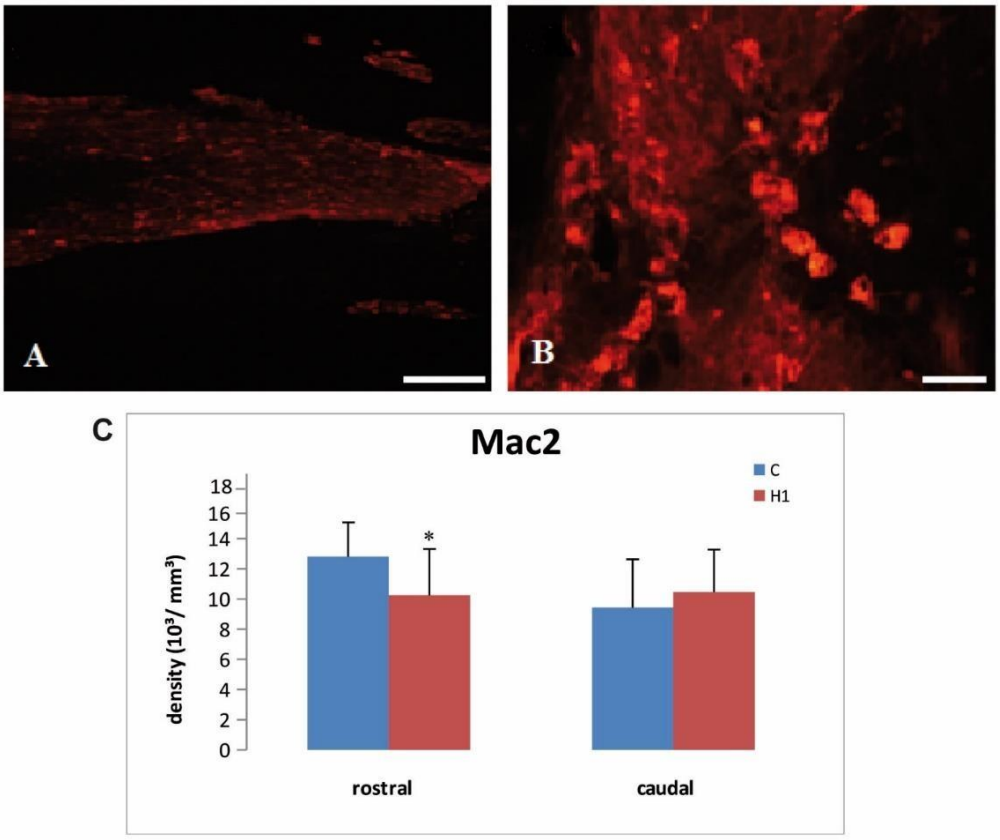

Figure 12. Analysis of Mac2 expression in the spinal cord 6 weeks after injury. Representative confocal images of the rostral part of the lesion site from histone H1-treated mice; (A) 10x objective and (B) 40x objective. Scale bars:100 µm (A) 20 µm (B).(C) Bar graph shows quantification of density of Mac2-positive microglia in histone H1- (H1) and vehicle (C)-treated mice 6 weeks after SCI. N=6 in each group, the number of slices in each group was 36. Shown

are mean values + SEM from both groups. The asterisk indicates a significant difference from the control group. *p < 0.05, two-tailed t-test.

The analysis showed that the number of Mac2-positive cells was lower rostrally to the lesion site in histone H1-treated mice than in vehicle-treated mice whereas levels of Mac2 were not changed caudal to the lesion site (*p < 0.05, two-tailed *t*-test).

4.1.4 Ki67-positive cells

Cell proliferation was quantified by measuring the expression of Ki67 by counting Ki67-positive cells, rostral and caudal to the lesion site. The number of Ki67-positive cells was increased 6 weeks after treatment with histone H1 both rostral and caudal to the lesion site *(Figure 13)*. Both were statistically significant (*p < 0.05, two-tailed *t*-test). The results indicate a stimulation of cell proliferation in the injured spinal cord by treatment with histone H1.

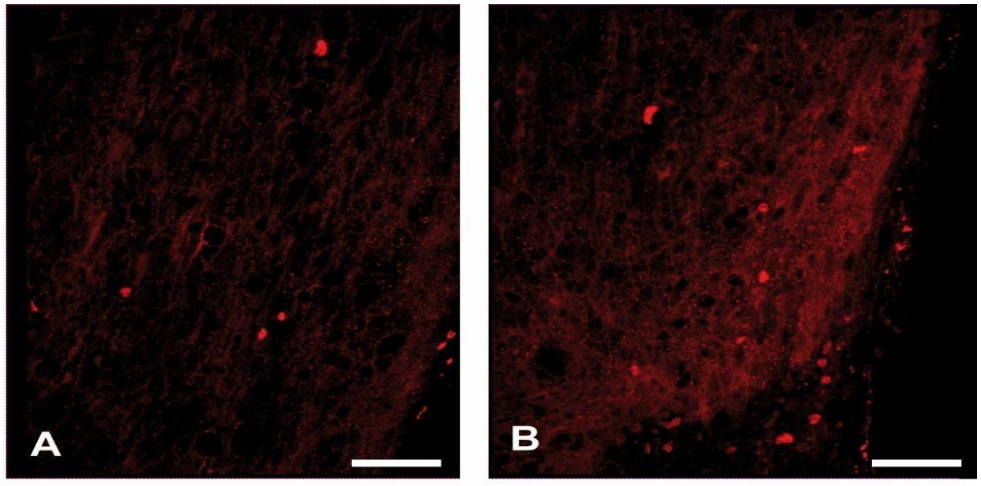

Figure 13. Analysis of Ki67 expression in the spinal cord 6 weeks after injury. Representative confocal images of Ki67-positive cells in histone H1 (A) and vehicle (B) treated mice. Scale bars: 100 µm. (C) Bar graph shows quantification of density of Ki67-positive cells in histone H1- (H1) and vehicle (C)-treated mice. N=6 in each group, the number of slices in each group was 36. Shown are mean values + SEM from both groups. Asterisks indicate significant differences from the control group. *$p < 0.05$, two-tailed *t*-test.

In summary, from the results it can be concluded that histone H1 has a stronger impact on the activation of astrocytes and cell-proliferation, but only partially on activation of microglia and macrophages (rostral part) after spinal cord injury.

4.2 Astrocyte migration assay

Following spinal cord injury, the formation of the scar, the so called 'glial scar' is an important factor that diminishes regeneration after injury by limiting re-growth of axons through the lesion. Astrocytes have not only some beneficial effects after injury by limiting the extent of the damage *(*Adams & Gallo*,* 2018*;* Gaudet & Fonken,2018; Lietal., 2012; Okadaetal., 2018; Quraishe et al., 2018; Renault- Mihara et al., 2008; Saadoun et al., 2005; Sun et al., 2013), but they also contribute to limiting axonal re-growth by the scar formation. As spinal cord injury can be simulated in vitro by a scratch wound of astrocyte cultures (Holtje et al., 2005; Liang et al., 2007; Yoo et al., 2016), we cultured astrocytes and performed a migration assay after scratch wounding and treatment of astrocytes with histone H1 and EndoN, to analyze the dependence of potential histone H1 effect on PSA .

The analysis showed that migration of astrocytes after a scratch assay during the 72 h follow-up period was slower when cells had been treated with histone H1 and histone H1+EndoN. The strongest effect seems to be in the last group (*Figure 14*). The differences were statistically significant (*$p < 0.05$, two-tailed ^{t}-test). My results indicate that histone H1 plays an inhibitory role in migration of astrocytes and that PSA has its *partial* contribution to this effect. This effect might contribute to an attenuated formation of the glial scar *in vivo*.

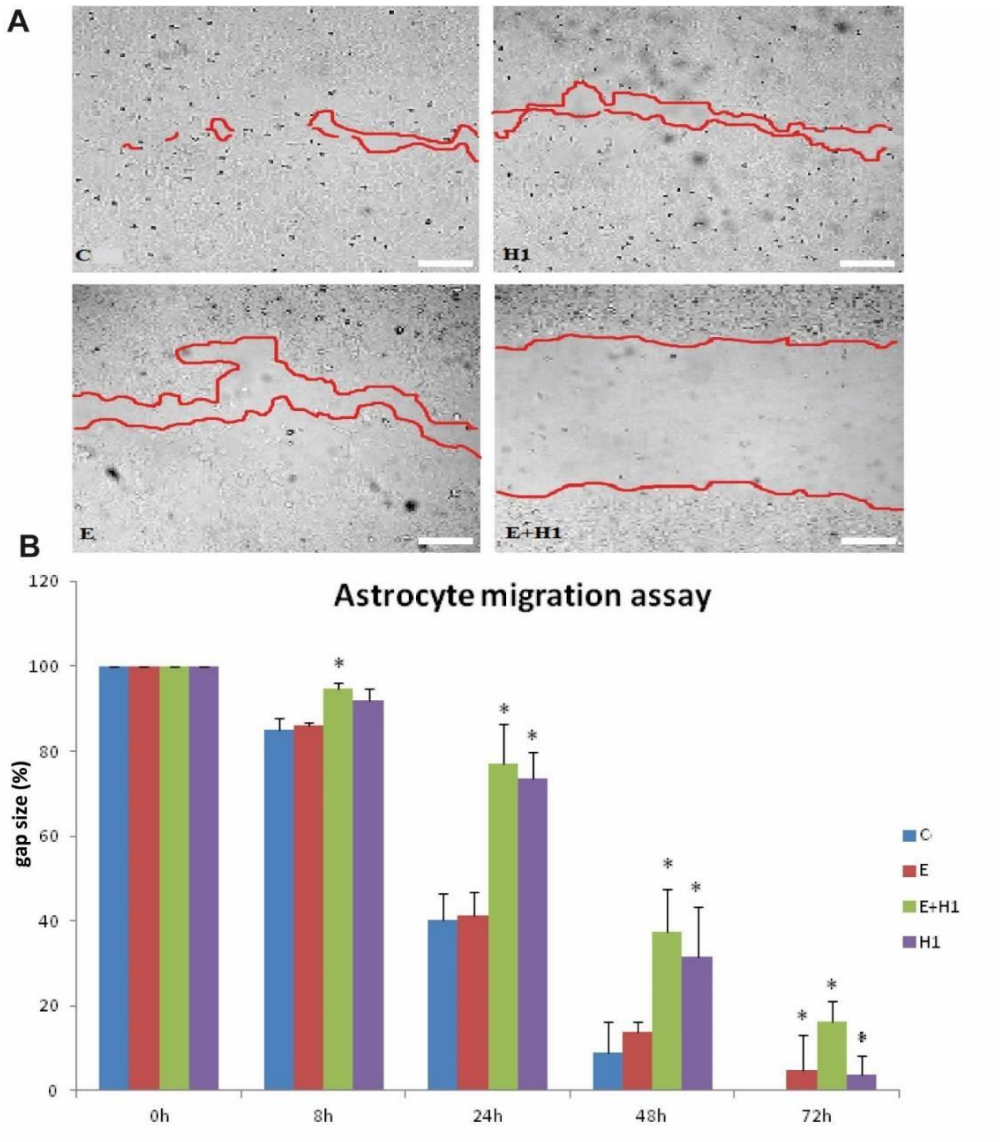

Figure 14. Scratch-induced migration of astrocytes *in vitro*. Four groups of astrocytes were treated with vehicle (Con), histone H1 (H1), EndoN (E) and EndoN+histone H1 (E+H1). The migration of cells from both sides of the 'scratch-gap' towards the other side was followed up for 72 hours. The concentrations we added were: 10 µl of histone-H1 (conc. 2,0 µg/µl), 9 µl of EndoN (conc.1,8 µg/µl), 7,5 µl of EndoN+histone-H1 (conc. 1,5 µg/µl) and 9 µl of HBSS (conc.1,8 µg/µl).

(A) Representative images after 72 hours for each group. *Scale bars: 100 µm.*(B) A bar graph with mean values + SEM from four independent experiments for quantification of the average distance between the two borders of the gap after scratch and treatment with vehicle (Con), histone H1 (H1), EndoN (E) and EndoN + histone H1 (E+H1). Asterisks indicate significant differences from the control group (n = 6 wells per group; 5 pictures of each well were taken). * $p < 0.05$, two-tailed *t*-test.

4.3 Western blot

Subsequently I performed a western blot to determine the levels of PSA in the cells and could prove that PSA could not be detected in both EndoN and EndoN+H1 treated groups. The complete enzymatic removal of PSA by EndoN could be confirmed (*Figure 15*).

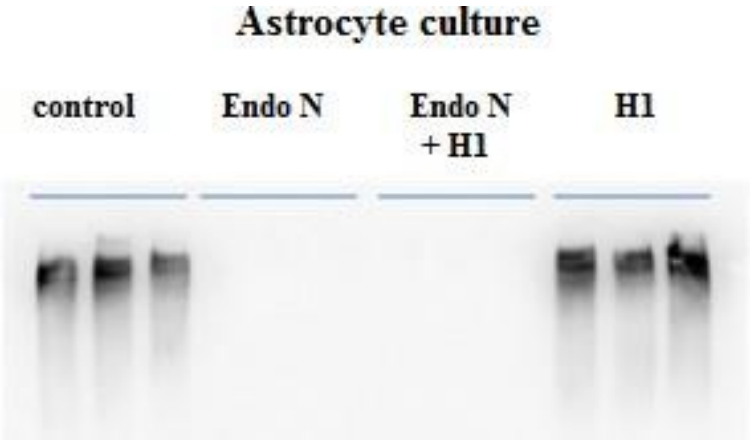

Figure 15. Immunoblot of homogenates of four groups of astrocytes, which were treated with vehicle (control), EndoN, EndoN + histone H1 (EndoN+H1) and histone H1 (H1). The membrane was loaded with 20 µl of each sample. Subsequently the membrane was incubated with an antibody that recognizes PSA. PSA was not detected when the treatment included EndoN.

5. Discussion

Our experiments mainly focused on the potential beneficial effect of histone H1 on glial scarring after spinal cord injury and wether this effect is PSA-dependent. Here, I report that histone H1 has a beneficial effect on regeneration after spinal cord injury, especially by decreasing astrogliosis and inflammatory response, as well as by increasing cell proliferation. Furthermore, I saw an increase of the beneficial effect of histone H1, especially when PSA was removed by EndoN. Therefore, it can be suggested that the interaction of histone H1 with PSA on the cell surface is at least partially responsible for regeneration after SCI and that there should be other pathways, which PSA competes with.

Improved regeneration after spinal cord injury with decreased astrogliosis and increased cell proliferation was observed after applying small organic compounds mimicking PSA or PSA-overexpressing Schwann cells to the injured spinal cord in rodents (Marino et al., 2009; Mehanna et al., 2010; Papastefanaki et al., 2007). In other experiments, an inefficient regeneration in NCAM-deficient mice after spinal cord injury has been observed (Saini et al., 2016). These and other studies suggested that PSA has a beneficial effect upon spinal cord injury. Histone H1 was demonstrated as a binding partner of PSA, interacting directly with PSA (Mishra et al., 2010). Histone H1 has been demonstrated in the nucleus (Doenecke et al., 1997), in the cytoplasm (Parseghian & Luhrs, 2006) and also extracellularly at the surface of several cell types, especially in the context of injury and regeneration (Brix et al,1998; Henriquez et al., 2002; Watson et al., 1995). It has been shown that histone H1 is present at the cell surface of cultured cerebellar neurons and Schwann cells. The application of histone H1 promotes neural precursor cell migration, neurite outgrowth and Schwann cell proliferation (Mishra et al., 2010). These favourable effects depend on the interaction of histone H1 with PSA (Mishra et al., 2010). Application of histone H1 increased functional recovery after femoral nerve injury, indicating its beneficial effect *in vivo* (Mishra et al., 2010). Additionally, histone H1 improves locomotor recovery after spinal cord injury when pumped through an osmotic pump into the spinal cord. Beneficial effects of histone H1 in spinal cord injury extend from the enhanced locomotor recovery after injury, to better cholinergic and monoaminergic reinnervation, to decreased astrocyte activation after injury (Kleene et al., 2019). Notably, in depth the effects of histone H1 on astrocytes both *in vitro* and *in vivo* were studied. The results from RNA expression profiling

indicated that histone H1 treatment of cultured astrocytes up-regulates the expression of mRNAs encoding proteins which regulate gliogenesis, migration, apoptosis/cell death and cell proliferation as well as, more generally, transcription, translation, and metabolism. This analysis also showed down- regulation of expression of mRNAs encoding proteins which control gliogenesis and impair functions during nervous system development, such as neurogenesis (Kleene et al., 2019). Therefore, histone H1 may affect scar formation in the injured adult spinal cord by reducing gliogenesis and activation of astrocytes. Moreover, histone H1 alters the expression profile of mRNAs of proteins that regulate neurogenesis, neuronal differentiation, axonal pathfinding and neuronal cell death as well as angiogenesis, wound healing and immune system functions (Kleene et al., 2019). It is possible that histone H1 triggers astrocytes to generate an environment that promotes neuronal survival, axonal regrowth, sprouting and/or sparing, which improve regeneration after spinal cord injury. Regarding spinal motoneuronal functions, it is likely that histone H1 with its support on survival *in vitro* allows survival *in vivo* under pathological stress condition in spinal cord injury (Grossman et al., 2001; Lee et al., 2014; Xu et al., 2005). Interestingly, the levels of several mRNAs which encode proteins associated with increased astrocyte activation and reactive gliosis, including GFAP, NG2 and CHL1 (Alonso, 2005; Cregg et al., 2014; Middeldorp & Hol, 2011; Moeendarbary et al., 2017; Rost et al., 2016; Sofroniew & Vinters, 2010; Sun & Jakobs,2012; Wu et al., 2010; Yang & Wang, 2015) are decreased by histone H1 in cultured astrocytes. In *vivo*, histone H1 decreases the levels of the regeneration-hostile molecules GFAP, NG2 and CHL1 (Dou & Levine, 1994; Fidler et al., 1999; Filous et al., 2014; Hackett & Lee, 2016; Levine, 2016; Tan et al., 2006; Ughrin et al., 2003; Yang & Wang, 2015) rostrally and caudally to the lesion site. These findings support the assumption that histone H1 reduces scar formation *in vivo* by astrocytes by regulating the expression of axon growth inhibiting factors and gliosis-enhancing proteins.

In previous studies it was shown that PSA had beneficial effects when applied after spinal cord injury as a glycomimetic peptide (Marino et al., 2009; Mehanna et al., 2010), PSA mimicking compound (Saini et al., 2016), and in the form of PSA-overexpressing Schwann cells (Papastefanaki et al., 2007). Most prominent effects of PSA application were an enhanced motor performance after injury (Mehanna et al., 2010; Papastefanaki et al., 2007; Saini et al., 2016), enhanced monoaminergic reinnervation (Marino et al., 2009; Mehanna et al., 2010; Papastefanaki et al., 2007),

enhanced cholinergic reinnervation (Mehanna et al, 2010) and decreased astrocyte scarring upon injury (Marino et al., 2009; Papastefanaki et al., 2007). In the current study we found that treatment with histone H1 affected all those parameters in a similar manner. However, when we applied histone H1 *in vitro* upon enzymatic removal of PSA from astrocytes, it enhanced the outcome, suggesting that histone H1 effect on astrocyte migration is not PSA-dependent. I here addressed the impact of histone H1 treatment on glial scar formation using an astrocyte scratch assay paradigm. This *in vitro* assay is commonly used to simulate *in vivo* scar formation, since two-dimensional cell cultures are much easier to quantify and have been shown to correlate well with the *in vivo* experiments (Chen et al., 2007; Jakovcevski et al., 2007). The decrease in astrocyte scar formation after histone H1 application is comparable with the similar results reported for effects of PSA on scar formation *in vitro* (Marino et al., 2009) and *in vivo* (Papastefanaki et al., 2007). The relationship of the upregulation of histone H1 and downregulation of polysialic acid and their contribution to the reactive astrocyte injury response need further investigation.

However, the decreased PSA expression on the NCAM of the glial scar is associated with the increased histone H1 protein expression, and therefore likely represents a functionally relevant response of histone H1 to spinal cord injury. Interestingly, when PSA was enzymatically removed using EndoN, it enhanced the ability of histone H1 to alleviate astrocyte scarring. Thus, we can conclude that the effects of histone H1 on astrocytes, at least considering their migration, are not dependent on its binding to PSA. It may be important to further study the mechanisms behind this effect, i.e. if it is intrinsic property of histone H1 molecule, or due to interaction with other binding partners (e.g. proteoglycans).

Next, I examined the effects of histone H1 on astrocyte scar formation *in vivo*, using immunostainings for GFAP and NG2, proteins highly upregulated by astrocyte activation (Alonso, 2005; Rost et al., 2016). Histone H1 treatment lowered the expression of GFAP both rostrally and caudally to the lesion site, while the effect on NG2 was significant only in the rostral region. Combined with the observation that histone H1 increases cell proliferation in the vicinity of the lesion site *in vivo*, this suggests that astrocytes do not proliferate less, but that the activation of astrocytes is ameliorated upon treatment with histone H1.

Our previous study offered several lines of evidence that histone H1 improves neurite outgrowth and axonal regeneration via PSA-dependent mechanisms (Mishra et al.,

2010). For instance, application of the PSA antibody or enzymatic degradation of PSA abolished the enhanced neurite outgrowth from cerebellar neurons (Mishra et al., 2010). Similarly, *in vivo* experiments showed that histone H1 improves regeneration of injured femoral nerve of mice (Mishra et al., 2010). Similar positive effects on recovery after femoral nerve lesion in mice using PSA-mimicking peptides were observed (Mehanna et al., 2009). However, extracellular histone H1 has been shown to bind to other molecules, potentially important in regeneration. For example, cell surface heparan sulfate proteoglycans (Watson et al., 1999), lipopolysaccharide (Bolton and Perry, 1997; Hampton et al., 1988), amyloid precursor protein (Potempska et al., 1993) and β-amyloid in amyloid plaques (Duceet al., 2006) bind to histone H1. It is worthwhile to mention that similar to histone H1, PSA also binds to heparan sulfate proteoglycans (Storms and Rutishauser, 1998) and that the heparan sulfate-dependent interaction between heparan sulfate proteoglycans, in particular perlecan, and extracellular histone H1 plays an important role in skeletal muscle development and regeneration (Henriquez et al., 2002). Importantly, my findings that the presence of PSA is not essential for the effect of histone H1 on migration of the cultured astrocytes suggest that there is an additional, PSA-independent mechanism by which histone H1 exerts its beneficial effects in spinal cord injury.

Another notable effect of histone H1 treatment in spinal cord injured mice reported here is an enhancement of the progenitor cell proliferation in the lumbar spinal cord. After injury several cell types proliferate in the spinal cord, including oligodendrocytes, astrocytes, microglia/macrophages, and neuronal progenitors present around the central canal (Marichal et al., 2009; McTigue & Stokes, 2001). Although I could not distinguish between the proliferating cell types, two particularly interesting cell types that could contribute to the difference in proliferation rate observed between the histone H1 treated mice and the controls are oligodendrocyte progenitors and neuronal progenitors. It was previously shown that Schwann cells treated with histone H1 proliferate more than non-treated cells (Mishra et al., 2010), and PSA was shown to increase proliferation of Schwann cells and oligodendrocytes *in vitro* and improves remyelination after femoral nerve injury *in vivo* (Mehanna et al., 2009). It is possible that histone H1 would have a similar effect on oligodendrocytes as it has on Schwann cells, considering their similar function. On the other hand, ependymal cells around the central canal strongly express PSA and were shown to give rise to mature neurons (Marichal et al., 2009). It is tempting to speculate that these are the cells that get

stimulated to proliferate upon histone H1 treatment.

6. Summary

6.1 English

Taken together my data show that histone H1 has beneficial effects in a mouse spinal cord injury model and indicate that it has a promising therapeutic potential for treatment of CNS injuries. To which extent the effects of histone H1 in spinal cord injury are PSA-dependent remains an open question. Considering the specific aims of this study, the following conclusion can be drawn:

1. The activation of astrocytes, estimated by GFAP and NG2 expression at the lesion site is reduced upon histone H1 treatment after spinal cord injury;
2. The activation of microglia/macrophages, estimated by Iba1 and Mac2 expression upon histone H1 treatment after spinal cord injury is decreased as well, particularly rostrally to the lesion site;
3. There is an increase in neural precursor cell proliferation upon histone H1 treatment after spinal cord injury, based on Ki67-proliferating cell antigen expression;
4. Scratch-wound assay shows a significant decrease in astrocyte motility to close the gap upon histone H1 treatment;
5. The enzymatic removal of PSA by EndoN has an enhanced effect on migration of astrocytes in the scratch-wound assay upon histone H1 treatment, thus astrocytes show a decrease in migration upon treatment with histone H1 after removal of PSA: Thus, this effect is deemed PSA-independent.

6.2 Deutsch

Zusammenfassend zeigen meine Ergebnisse die positiven Auswirkungen von Histon H1 in einem ‘mouse spinal cord injury model’ und somit das mögilche therapeutische Potential für die Behandlung der Verletzungen des zentralen Nervensystems (ZNS). Inwiefern diese Wirkung PSA-abhängig ist, bleibt vorerst offen. Aufgrund unseren Daten können wir folgende Schlussfolgerungen ziehen:

1. Die Aktivierung von Astrozyten, geschätzt durch GFAP- und NG2-Expression an der Läsionsstelle, ist nach Histon H1 Behandlung nach einer Rückenmarksverletzung verringert;
2. Die Aktivierung von Mikroglia/Makrophagen, geschätzt durch Iba1- und Mac2-Expression nach einer Histon H1 Behandlung nach einer Rückenmarksverletzung, ergibt sich ebenfalls verringert, insbesondere rostral zur Läsionsstelle;
3. Es zeigt sich weiter eine Zunahme der Proliferation von neuralen Vorläuferzellen nach einer Behandlung mit Histon H1 nach einer Rückenmarksverletzung, dies aufgrund der erhöhten Expression des Ki67-Zellantigens;
4. Der ‘scratch-wound assay’ zeigt eine signifikante Abnahme der Astrozytenmotilität nach einer Behandlung mit Histon H1;
5. Die enzymatische Entfernung von PSA durch EndoN hat einen verstärkten Einfluss auf die Migration von Astrozyten im ‚scratch-woud assay‘ nach einer Behandlung mit Histon H1, wobei die Astrozyten nach Behandlung von Histon H1 und Entfernung von PSA eine deutlich verringerte Migration zeigen: Somit wird dieser Effekt als PSA-unabhängig angesehen.

7. References

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9. Curriculum Vitae

‘’Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt’’

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