

From the DEPARTMENT OF NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

**ON THE THERAPEUTIC POTENTIAL OF CANCER
DRUGS FOR SPINAL CORD INJURY**

Jacob Kjell



**Karolinska
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Cover: *Institutionen arbetar*, Mural by Peter Weiss (1916-1982), depicting the people at the Department of Pathology, headed by Folke Henschen, in 1944 at Karolinska Institutet. In the painting, the whole department is gathered in one room with experimental work and clinical practice in close connection to each other, which is the manner in which we have operated during the studies presented in this thesis. In the lower left corner, by the microscope, sits Gösta Hultquist, my granduncle.

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ABSTRACT

Spinal cord injury progresses in two stages. After the damage-causing physical event, comes an extended period when additional loss of cells and nerve fibers will occur and inflammatory and scar forming processes will come to prevail. The secondary events, however, also present a window of opportunity during which pharmacological intervention may decrease the extent of permanent neurological impairment. A few drugs have been tested clinically for such effects, but none is currently in use for spinal cord injured patients. Hence there is a need for additional therapeutic candidates.

This thesis addresses the lack of clinical candidates by investigating the possibility to reposition drugs in clinical use for other indications, by testing them in the acute stage of spinal cord injury. We evaluated the therapeutic potential of the three cancer drugs erlotinib, rapamycin, and imatinib. These drugs all inhibit receptor tyrosine kinase signaling and their respective molecular targets are likely to be involved in promoting the degenerative secondary events following the initial trauma. Hence these drugs offer a potentially fast translational process to serve as a first line treatment, protecting vulnerable tissue and allowing improved functional recovery.

In vitro, we characterized astrocytic cultures from adult rats and found that both growth conditions and choice of rat substrain will change astrocyte parameters and we further identified which of the tested substrains produce an astrocytic culture most similar to a human astrocytic culture (Paper I). We then characterized the spontaneous functional recovery of different rat substrains subjected to a mild contusion injury and found differences in recovery of hindlimb locomotion function, bladder function and sensory function with regard to mechanical stimuli (Paper III). The results should aid in optimizing the experimental and translational value of these in vitro and in vivo model systems.

To determine the therapeutic potential of erlotinib, rapamycin and imatinib, we administered the drugs per os with a 30 minute delay during the acute stage of a contusion injury in rats and monitored functional recovery. We found erlotinib treatment to accelerate bladder and locomotor recovery (Paper IV). We also characterized the spatiotemporal activation of the target of rapamycin, mTORC1, after the spinal cord injury. We found a biphasic activation of glial cells, primarily macrophages and microglia, revealing possible windows of opportunity for targeting mTORC1 with rapamycin in spinal cord injury (Paper V). However, acute treatment with rapamycin did not alter recovery of bladder function or locomotion (Paper IV).

We found that imatinib enhanced recovery of locomotion and bladder function by effectively reducing negative secondary events and rescuing spinal tissue, including axons (Paper II). To determine the possible clinical potential of imatinib we further delayed the initial administration of the drug, assessed motor and sensory recovery and searched for potential biomarkers in serum (Paper VI). We found imatinib to improve

hind limb locomotion when administered with a 4 hour delay and to improve bladder recovery even with a 24 hour delay. The 4 hour delay treatment had modest positive effects on recovery of mechanical and thermal sensory functions and we identified alterations of two cytokines/chemokines as candidate biomarkers.

In conclusion, further studies of erlotinib and rapamycin are needed in order to determine their therapeutic potential in spinal cord injury. Imatinib, however, stands out as a candidate drug for clinical trials in acute spinal cord injury.

LIST OF PUBLICATIONS AND MANUSCRIPTS

- I. Codeluppi S, Gregory EN, **Kjell J**, Wigerblad G, Olson L, and Svensson CI. Influence of rat substrain and growth conditions on the characteristics of primary cultures of adult rat spinal cord astrocytes. *Journal of Neuroscience Methods*, 197, 118–127, 2011
- II. Abrams MB, Nilsson I, Lewandowski SA, **Kjell J**, Codeluppi S, Olson L, and Eriksson U. Imatinib enhances functional outcome after spinal cord injury. *PloS One*, 7, issue 6, 1-12, e38760, 2012
- III. **Kjell J**, Sandor K, Svensson CI, Josephson A, and Abrams MB. Rat substrains differ in the magnitude of spontaneous locomotor recovery and in the development of mechanical hypersensitivity after experimental spinal cord injury. *Journal of Neurotrauma*, 30, 1805-1811, 2013
- IV. **Kjell J**, Pernold K, Olson L, Abrams MB. Oral erlotinib, but not rapamycin, causes modest acceleration of bladder and hindlimb recovery from spinal cord injury in rats. *Spinal Cord*, 52, 186-190, 2014
- V. **Kjell J**, Codeluppi S, Josephson A, Abrams MB. Spatial and cellular characterization of mTORC1 activation after spinal cord injury reveals biphasic increase mainly attributed to microglia/macrophages. *Journal of Brain Pathology*, Feb. 27, 2014 PMID:24576152 (Epub ahead of print)
- VI. **Kjell J**, Finn A, Hao J, Wellfelt K, Josephson A, Svensson CI, Wiesenfeld-Hallin Z, Eriksson U, Abrams MB and Olson L. Delayed imatinib treatment for spinal cord injury; functional recovery and biomarkers. (*Manuscript*)

PUBLICATIONS NOT INCLUDED IN THE THESIS

- Codeluppi S, Fernandez-Zafra T, Sandor K, **Kjell J**, Quingsong L, Abrams MB, Olson L, Gray NS, Svensson CI and Uhlén P. Interleukin-6 secretion by astrocytes is dynamically regulated by PI3K-mTOR-calcium signaling *Plos One*, 2014, 25;9(3) :e92649
- Abrams MB, Nilsson I, **Kjell J**, Lewandowski SA, Codeluppi S, Eriksson U and Olson L. Response to the report, "A re-assessment of treatment with a tyrosine kinase inhibitor (imatinib) on tissue sparing and functional recovery after spinal cord injury" by Sharp et al. *Experimental Neurology*, 2014, (in press)
- **Kjell J**, Codeluppi S, Olson L, Abrams MB. Chronic rat parvovirus serotype-1a (RPV-1a) infection enhances functional outcome in experimental spinal cord injury. (*Manuscript*)

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LIST OF ABBREVIATIONS

Aldh1L1	Aldehyde Dehydrogenase 1 Family, Member L1
AM	Astrocyte media
ANOVA	Analysis of variance
BBB	Blood-brain-barrier
BBB score	Basso, Beattie, Bresnahan-score
BCA	bicinchoninic acid
BSCB	Blood-spinal-cord-barrier
cDNA	Cyclic deoxyribonucleic acid
CDxx	Cluster of differentiation xx
Cnx-43	Connexion-43
CPP	Contact plantar placement
CSF	Cerebrospinal fluid
CSF-1	Colony stimulating factor-1
CSPG	Chondroitin sulfate proteoglycans
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
dpi	Days post injury
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GLAST	Glial high affinity glutamate transporter
GLT-1	Glutamate transporter-1
GS	Glutamine synthetase
H&E	Haematoxylin and Eosin
i.p.	Intraperitoneal
i.v.	Intravenous
Ig	Immunoglobulin

IGF-1	Insulin growth factor-1
IHC	Immunohistochemistry
IL-xx	Interleukine-xx
LFB	Luxol fast blue
MOPS	3-(N-morpholino)propanesulfonic acid
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
NeuN	Neural nuclei
NF	Neurofilament
NG2	neuron-glia antigen 2
NGF	Nerve growth factor
<i>p</i>	p-value
p	Phosphorylated
p.o.	Per os
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PLL	Poly-L-lysine
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time polymerase chain reaction
rcf	Relative centrifugal force
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
S100 β	S100 calcium-binding protein β
S6	Ribosomal protein S6
SCI	Spinal cord injury
SD	Standard deviation
SEM	Standard error of the mean
TBS	Tris-buffered saline
TNF- α	Tumor necrosis factor- α
Vi	Velocity at impact

INTRODUCTION

Current consensus in the field is that there will be no single miracle cure for spinal cord injury. Instead, stepwise improvement of different aspects of the pathology may eventually lead to a future “cure”. There is reason to believe that any successful future therapeutic intervention for spinal cord injury will include pharmacological components. Development of pharmacological interventions for spinal cord injury may benefit from the vast accumulated knowledge about drugs that are in clinical use for other indications. If any such drug is found experimentally effective, it could potentially reach patients faster.

To better predict which drugs may have positive effects on spinal cord injury, there is a need to understand the basic pathology of spinal cord injury. Much research has gone into understanding what happens and why. These findings have been the basis for many of the treatment interventions that have been tried experimentally and clinically, and contribute to our knowledge of what may and may not become a successful treatment.

THE SPINAL CORD

The spinal cord spans 2/3 of the spinal column and from the caudal part spinal nerves forming cauda equina connects the spinal cord to the lower lumbar and sacral segments. The spinal nerves exit the spinal column from in between the vertebrae to provide sensory and motor innervation of target areas. The spinal cord itself has a core of gray matter, where the nerve cell bodies reside and synaptic contacts are upheld, surrounded by white matter where different groups of axons project up to or from the brain in anatomically more or less well defined compartments. This compartmentalization of the spinal white matter tracts is important because lesions of the cord will cause distinctly different impairments depending on which tracts are lesioned and which tracts are spared. This also means that physical impact of a given magnitude may lead to quite different results depending on location. Moreover, the lipid-rich white matter differs from grey matter in terms of mechanical properties. If the spinal cord is subjected to impact injury, the force will be carried differently and typically cause greater destruction of neuronal cell bodies, while white matter is spared to a greater extent (Ichihara et al. 2001).

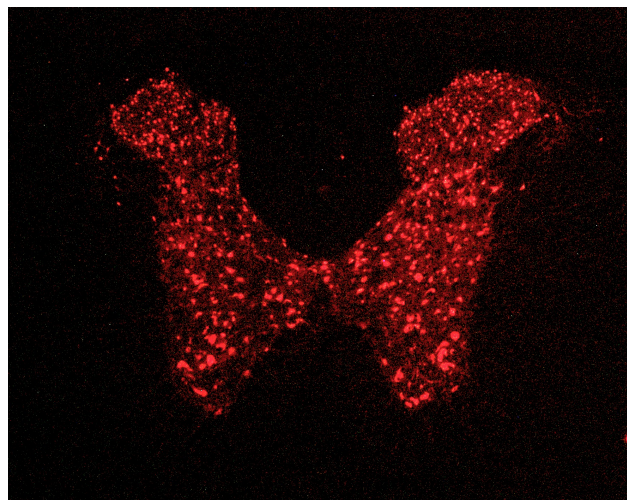


Fig 1. Neurons in the gray matter in a section from Th10, here visualized with NeuN.

Like the brain, the spinal cord is surrounded by protective meninges, rupture of which due to injury carries with it its own consequences and problems (Norenberg et al. 2004). Under the middle of the three meninges, in the subarachnoid space, resides the cerebrospinal fluid. Since one of its functions is to collect waste products secreted by the neural tissue, it can be used as a diagnostic tool after injury and possibly after therapeutic interventions (Ghoreschi et al. 2009; Hayakata et al. 2004; B. K. Kwon, Stammers, et al. 2010b; Guéz et al. 2003; Lubieniecka et al. 2011; Xie et al. 2013; Krishna et al. 2014). Spinal arteries and veins run along the cord and supply blood to different parts of the grey and white matter. Depending on the detailed course of blood vessels in a given individual and depending on how circulation becomes compromised after injury, an injury may hence render parts close to the injury more or less ischemic (Bingham et al. 1975; Martirosyan et al. 2011).

Blood vessels are surrounded by a basement membrane, mainly consisting of extracellular matrix (ECM) components such as collagen, laminin and proteoglycans, (Eriksdotter-Nilsson et al. 1986; Finlay et al. 1998). There is also ECM in the neural tissue where it plays an active part in the spinal cord, rather than just being a “glue” (Rutka et al. 1988; Busch and Silver 2007). The ECM, among other things, directs neural growth during development, creating inhibitory and growth promoting paths. After injury, the ECM is often inhibitory to nerve growth at and close to the injury.

Like the brain, the spinal cord also contains glial cells, including myelinating oligodendrocytes (Yamazaki et al. 2010), astrocytes, microglia, pericytes and ependymal cells. These cells are the major constituents of the spinal tissue and after injury these different cell types play different roles in the progression of the ensuing pathology and recovery.

SPINAL CORD INJURY

Spinal cord injury may not only be personally devastating, but may also be socially devastating and carries great socioeconomic costs. 12000 individuals suffer spinal cord injury each year in the US alone and those affected are often young male adults. However, the average age at injury has risen, and is now 42.6 years. Injuries mainly stem from vehicle accidents or falls, other common causes are violence and sports accidents (National Spinal Cord Injury Statistical Center 2010). Depending on the level and severity of a spinal cord injury, the result will be complete or partial paraplegia or tetraplegia. Tetraplegic patients need full-time care and support with all basic activities of living. For this group, therefore, also modest neurological improvements can significantly improve quality of life. Paraplegics can to a lesser or greater extent manage their daily undertakings and in this regard live a close to “normal” life. Most paraplegics have lost both normal bladder function and sexual function in addition to being unable to stand and walk. Although less known among the public at large, restoring bladder and sexual functions are at the top of the wish list for

paraplegics, while, until recently, these parameters have been given lesser priority in studies of rodent spinal cord injury models.

Many injured patients have problems with blood pressure regulation and there is a higher prevalence of cardiac disease (Myers et al. 2007; Montgomerie 1997; Byrne and Salzberg 1996). Decubitus ulcers are also common. In addition to loss of sensory, motor and autonomic nervous system functions, other neurological disturbances may develop, such as hypersensitivity, itching, pain or allodynia (Finnerup et al. 2003; Siddall et al. 2003; Defrin et al. 2001). The mechanisms behind several of these symptoms are not well understood, and it is of outmost importance that an intervention of any kind does not induce or aggravate any of these neurological problems (Hofstetter et al. 2005).

SECONDARY INJURY

The initial insult, causing rupture of spinal tissue, initiates secondary events that promote very limited, if any, regeneration and instead ultimately result in additional cell death, axon loss, chronic inflammation and scarring. Secondary injury progression begins with intracellular content being released into the extracellular microenvironment, initiating an inflammatory response and rupture of blood vessels, causing bleedings ischemia and edema. The blood-spinal cord barrier, similar in nature to the blood-brain barrier, is compromised after injury which further aggravates the situation and commonly affects a larger spinal cord territory than the primary insult (Figley et al. 2014; Hawkins and Davis 2005). Compromised circulation and other circumstances at the site of injury may lead to loss of oligodendroglia and thus myelin, which in turn impairs axon potential conductance, and indeed integrity of demyelinated axons.

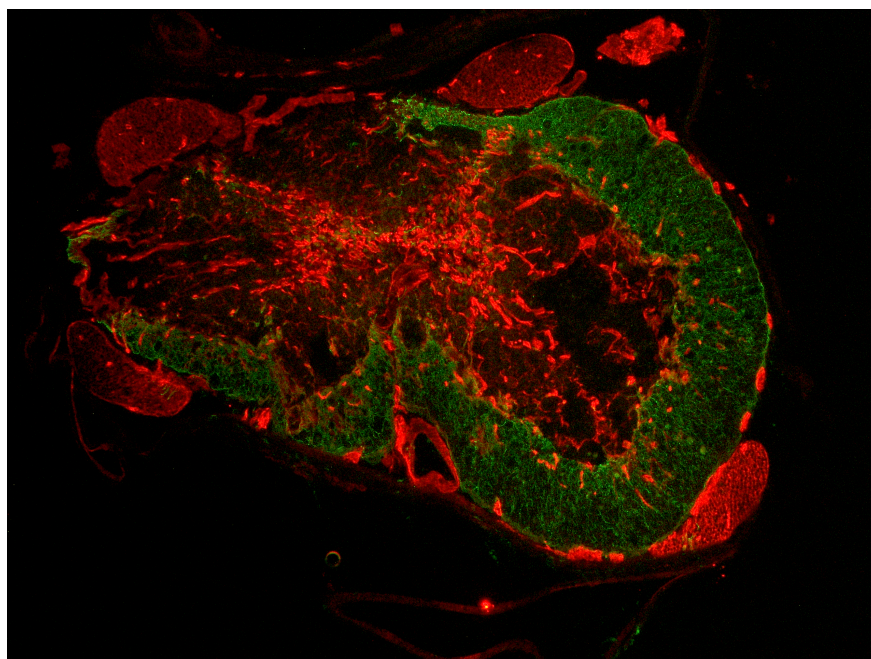


Fig 2. The injury site of the cord 5 weeks after a contusion injury in rat. Collagen 4 (red) can be seen in the epicentre of the injury, forming part of the scar. In the spared white matter there are reactive astrocytes (green), here visualized by their GFAP immunoreactivity.

Neuronal degeneration

Nerve cells are often destroyed during the primary insult, leading to loss of entire neurons, but the ischemic and compromised environment can cause further necrosis and apoptosis soon after injury. There was a debate concerning whether progression of cell death actually takes place, since it was known from early histological reports that many nerve cell bodies are lost within the first 24h after injury.

It was first believed that cell death was mostly due to necrosis, but then found to also be due to apoptosis, and perhaps mostly so, depending on type of injury (Crowe et al. 1997; Lou et al. 1998; Evelyne Emery et al. 1998; Zhang et al. 1997; Tator 1995; Beattie et al. 2000). To the extent that apoptosis is a major cause of neuron death at this early stage, the possibility remains that some of these neurons could be rescued. Neurons that die of necrosis are mostly located

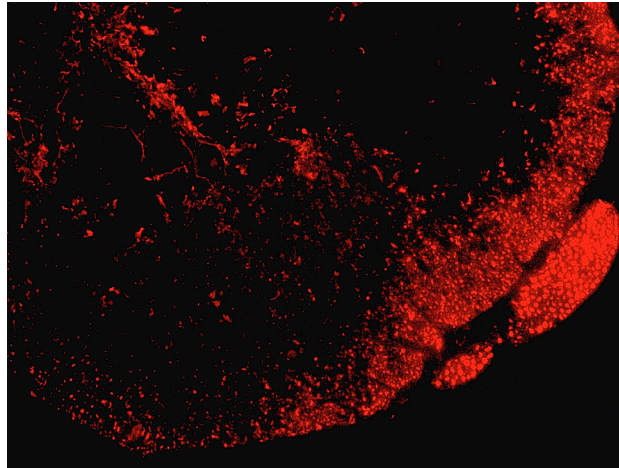


Fig 3. Spared axonal projections in a section of the injury site at 8 weeks after a spinal contusion injury in rat. A peripheral rim of axons, here visualized by NF-200, can be seen in the ventrolateral part of the injury site.

within the center of the injury, while neurons lost by apoptosis are located close to the injury epicenter. These necrotic and apoptotic events seem to be very close temporally in experimental setups. An increased presence of neurofilament fragments 4h after injury, suggests both necrosis and apoptosis, increased DNA fragmentation 8h after injury, suggests apoptosis (X. Z. Liu et al. 1997; Schumacher et al. 2000). Furthermore, caspase gene expression is present 8h post injury and neuronal loss is greatly exaggerated 6 compared to 3h after injury (Citron et al. 2000). At 24h after injury, neuronal loss is recognized as complete. To rescue any of these neurons therefore requires intervention taking this time frame into consideration.

As secondary events proceed, a prominent inflammatory response develops. While several aspects of the inflammatory response are needed, and perhaps even beneficial, the inflammatory response will also provide a chronically hostile environment for nerve fiber growth. Even though there may be some neuronal loss at later stages, most of the neurons cranial and caudal to the site of injury are viable and could potentially reconnect with neurons on the opposite side of injury, provided that a nerve growth permitting environment was provided (Evelyne Emery et al. 1998; P. Lu et al. 2012; R. P. Bunge et al. 1993; R. P. Bunge et al. 1997). Severed axons typically do send regenerate in the adult mammalian CNS, but are commonly inhibited and even repelled by the injury site. In fact, there seems to be no proper neuronal regeneration, as in

newly formed neurons in the spinal cord, as opposed to few areas in the brain (Bath and F. S. Lee 2010).

Astrocyte reactivity

It has been debated how large a proportion of CNS tissue astrocytes represent compared to neurons, estimates ranging from 1:1 to 10:1 (Hilgetag and Barbas 2009). Nevertheless, astrocytes constitute a large population of CNS tissue and, in addition, human astrocytes are larger than astrocytes in other mammals (Sofroniew and Vinters 2010; Oberheim et al. 2006). Astrocytes maintain tissue homeostasis (Takano et al. 2006; Perea et al. 2009; Halassa et al. 2007; Seifert et al. 2006; Attwell et al. 2010; Sattler and Rothstein 2006; Iadecola and Nedergaard 2007; Simard and Nedergaard 2004; Nicoll and Weller 2003; Obara et al. 2008), including blood flow regulation, extracellular fluid dynamics and regulation of pH. These functions are also important to avoid excitotoxicity, both under normal and pathological conditions. Under pathological conditions astrocytes typically become reactive and may proliferate to cause astrogliosis, aggregates of astrocytes with increased amounts of the intracellular structural protein GFAP (Eng and Ghirnikar 1994). Astrogliosis is an invariable component of CNS injury and also found in many other CNS disorders and diseases (Hamby and Sofroniew 2010; Sofroniew 2009). It is present in e.g. Alzheimer's disease, to a limited extent in Parkinson's disease, and in relation to CNS tumors and stroke (Maragakis and Rothstein 2006).

After spinal cord injury, spared astrocytes in the vicinity of the injury site react to the resulting hypoxic environment and inflammatory signals (Brahmachari et al. 2006; Pekny and Nilsson 2005). The astrocytes become hypertrophic and there is a minor upregulation of GFAP, followed by greater GFAP upregulation and an increased number of astrocytes,

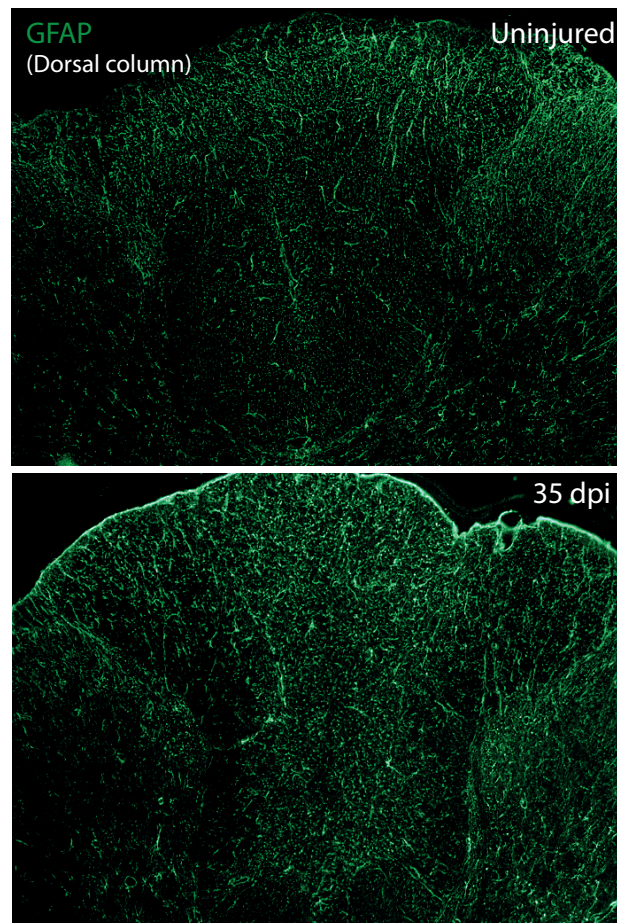


Fig 4. Astrocyte reactivity can be seen as far as 7 mm rostrally to the injury at 5 weeks after a spinal contusion injury in rat. Inflammation and demyelination are typically present in this area at this point in time.

around the perimeter of the injury site and areas with increased inflammatory activity, In rats this astrogliotic "scar" starts to manifest itself after 1-2 weeks and is fully developed by 3 weeks (Dusart and Schwab 1994; Sofroniew 2009). This has been confirmed to be the case in humans and astrogliosis has been found to remain chronically after the injury (Buss et al. 2007).

The dense rim of reactive astrocytes around the injury site is formed by local proliferation as well as migration from neighboring areas (Sofroniew and Vinters 2010; Sofroniew 2009). According to experiments in mice, ependymal cells also proliferate and migrate from the central canal and differentiate into astrocytes in the area of astrogliosis (Barnabé-Heider et al. 2010). The formation of the astrogliotic scar correlates with the deposition of a fibrotic scar, mainly consisting of basement membrane components such as collagen and laminin (Klapka and Müller 2006; Liesi and Kaupila 2002; Loy et al. 2002). However, the astrogliotic scar surrounds the fibrotic scar and astrocytes are rarely seen inside of the injury epicenter in contusion injuries with cavity formations (Göritz et al. 2011). Astrocyte reactivity can be triggered and maintained by inflammatory cytokines, such as TNF- α , IL-6, and INF- γ and growth factors such as TGF- β , PDGF, EGFR, and FGF (Z.-W. Li et al. 2011; Rabchevsky et al. 1998; Kahn et al. 1997; Merrill and Benveniste 1996). Moreover, astrocytes may produce most of these factors themselves and they can thus maintain a reactive phenotype through autocrine signaling (S. Lee et al. 2009).

The astrocytic scar counteracts nerve fiber growth (McKeon et al. 1991) to a large extent due to the deposition of chondroitin sulphate proteoglycans (CSPG) by the astrocytes. For instance, CSPGs of the ECM, are ligands of Nogo receptor 3 (NgR3), expressed by nerve fibers, thus inhibiting neurite growth after injury (Karlsson et al. 2013; Dickendesher et al. 2012). Indeed, enzymatic removal of CSPG allows growth cones to progress even through areas with reactive astrocytes (Bartus et al. 2011; Siebert n.d.; Rhodes and Fawcett 2004; Bradbury et al. 2002). On the other hand, astrocyte reactivity is important for containing the spinal cord wound (Faulkner et al. 2004; Sabelström et al. 2013). Inhibiting the formation of the astrocytic scar would further compromise the blood spinal cord barrier and allow widespread inflammation, resulting in uncontrolled excitotoxicity of the extracellular environment (Herrmann et al. 2008; Okada et al. 2006; Faulkner et al. 2004). Thus reactive astrocytes have a role in protecting and stabilizing the area around the scar that might otherwise constitute a risk for the surrounding intact spinal cord tissue.

Remyelination

Oligodendrocytes myelinate CNS axons (Peters 1964; Remahl and Hildebrand 1990) to provide saltatory conduction increasing signal velocity while reducing metabolic load (Poliak and Peles 2003). One oligodendrocyte can provide myelin segments to up to 60 axons, typically spatially separated from each other. Spinal cord injury will destroy many oligodendrocytes directly, and local ischemia and other pathological

circumstances may lead to death of additional oligodendrocytes, thus demyelinating segments of otherwise non-injured axons passing the site of damage (Mekhail et al. 2012). This severely impairs conduction properties and put the axons at risk for disruption. In contrast to neuronal apoptosis, oligodendrocytes show a biphasic apoptotic response, with the first phase occurring 4 - 24 hours after injury (Crowe et al. 1997; X. Z. Liu et al. 1997) and the second phase \approx 3 weeks after injury, presumably in response to prolonged periods of axon degeneration, and resulting in a second wave of demyelination (Griffiths and McCulloch 1983). Accordingly, apoptotic loss of oligodendrocytes is not restricted to the injury site, but stretches many millimeters caudally and rostrally, especially in the dorsal column in areas associated with Wallerian axon degeneration. Constituents of the oligodendrocytes such as Nogo, MBP and OmGP have been shown to restrict regeneration in CNS and blocking such inhibitory proteins has improved regeneration. Delaying myelin degeneration has been associated with poor tissue regeneration (Zhang and Guth 1997; Evelyne Emery et al. 1998; Zhang et al. 1996).

Spontaneous remyelination, first shown by Bunge et al, is present 1 week after injury and peaks 10 weeks after injury (M. B. Bunge et al. 1961; Totoiu and Keirstead 2005). Both demyelination and remyelination are thus long-lasting processes that have been shown to last past 60 weeks after injury in rats. The newly formed myelin sheaths have immature structural features with respect to internode length, and the ratio of axon diameter to myelin sheet thickness and may thus not provide full restoration of axon conduction properties (Franklin 2008; Gledhill and W. I. McDonald 1977; Ludwin and Maitland 1984). The remyelinating oligodendrocytes generally stem from a population of oligodendrocyte progenitor cells that migrate, proliferate and differentiate into mature oligodendrocytes (Ishii et al. 2001; McTigue et al. 2001). The oligodendrocyte progenitor cells are characterized by expression of the proteoglycan NG2 and are normally homogenously distributed in the spinal tissue,

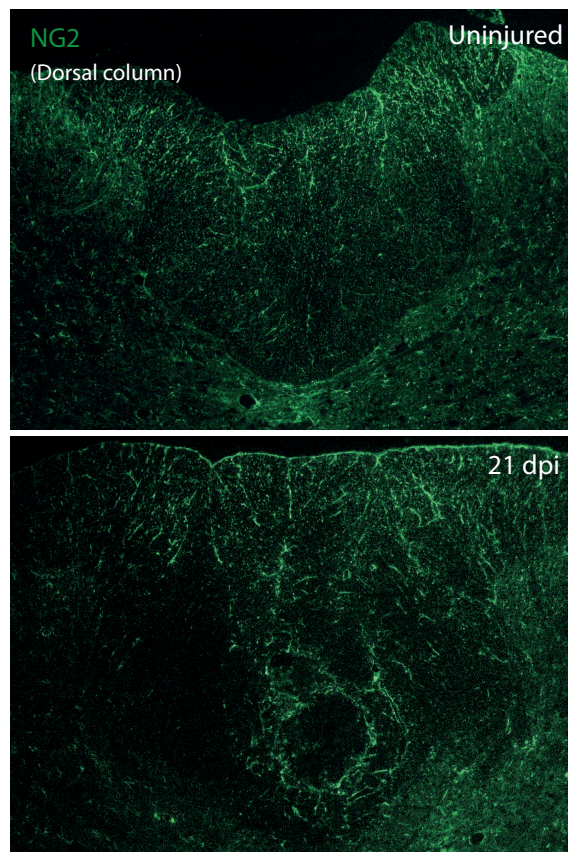


Fig 5. Oligodendrocyte progenitors cells are present in demyelinating areas of the dorsal column 7 mm caudal to the injury site, specifically around cavitations. OPCs are visualized with NG2.

except for a slightly higher abundance in white matter compared to gray matter (Dawson et al. 2003; Trotter et al. 2010; L. L. Jones et al. 2002; Kang et al. 2010). After injury, these cells can be found to cluster at sites of demyelination and it has been shown that neurotrophic factors and growth factors can promote oligodendrocyte progenitor cell recruitment and differentiation (McTigue et al. 1998; Sharma 2007). Areas of remyelination are associated with inflammation and revascularization, and it has thus been suggested that such processes promote the remyelination by oligodendrocyte progenitor cells. However studies also suggest that the inflammatory response could in part be responsible for the incomplete remyelination (Zhang and Guth 1997; Franklin 2008; B. Kwon et al. 2004; P. G. Popovich and T. B. Jones 2003).

Attempts to either use oligodendrocyte protective therapies such as minocycline with anti-apoptotic and anti-inflammatory effects or remyelination therapies such as treatment with neurotrophic factors have been experimentally successful in attenuating neurological deficits after spinal injury (McTigue et al. 1998; Stirling et al. 2004). Thus, enhancement of this process, as well as attempts to deliver for example Schwann cells to help remyelinate intact axons as well as to guide the regeneration of interrupted axons are important research areas (Guest et al. 2013; Guest et al. 2005; J. Sharp et al. 2010; J. W. McDonald et al. 1999; Mekhail et al. 2012).

Inflammatory response

The immune response after injury involves different types of inflammatory cells with different temporal activation and infiltration patterns (Donnelly and P. G. Popovich 2008). There are notable differences between the immune responses of rats and mice (Sroga et al. 2003). Rats appear more similar to humans than mice in terms of immune response and pathology. The inflammatory response in mice is also reportedly quite different to that of humans (Metz et al. 2000; Seok et al. 2013; Fleming et al. 2006; P. Popovich and Wei 1997; Kigerl et al. 2006). Since studies in this thesis are focused on rats, the inflammatory response to injury in rats will be discussed below.

Resident microglia are presumably the first immune cells to react to the primary injury (Watanabe et al. 1999; Hains and Waxman 2006). These ubiquitous cells are highly motile and able to proliferate and will accumulate in the damaged area as well as elsewhere in the spinal cord as a response to Wallerian degenerative events (Mothe and Tator 2005; Zai and Wrathall 2005; David and Kroner 2011; Watanabe et al. 1999). Microglia will produce inflammatory cytokines and chemokines. This cascade of proinflammatory cytokines and chemokines will affect the blood-spinal cord barrier, immune cell recruitment, and also the microglial cells themselves (Kreutzberg 1996; David and Kroner 2011). Microglia can change from a surveying state with ramified morphology, to a phagocytic stage with a roundish morphology, swollen by macrophagic inclusions of myelin fragments and other debris (see figure 6). At 24h post injury, microglia have small, but round morphology at the center of the injury site.

At later time points, they share a foamy and roundish morphology and can be difficult to distinguish from macrophages arriving from the circulation.

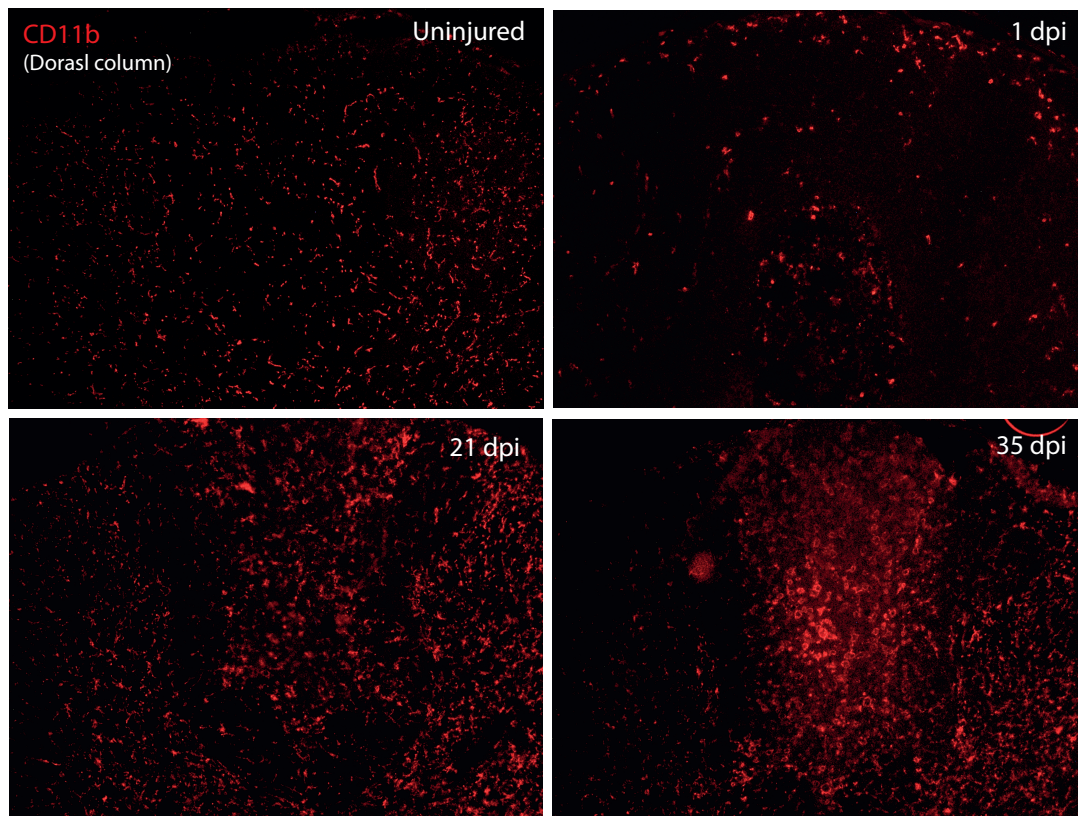


Fig 6. Microglia progressively change morphology after spinal contusion injury in rat and migrate to sites of demyelination and revascularization. Here, CD11b immunoreactivity visualizes microglia in the dorsal column 7 mm rostral to the injury site.

Neutrophils are the first inflammatory cells from the circulation to infiltrate the damaged spinal cord, with small numbers noted after 3h and large numbers 6h after injury (Taoka et al. 1997). This possibly suggests a role in the induction of neuronal apoptosis. However, lasting neural sparing and improved recovery by direct removal of neutrophils, has only been achieved in vivo when in unison with monocyte removal (S. M. Lee et al. 2011). Neutrophils are almost completely gone 3 days after injury, presumably removed through apoptosis due to their short life-time of ≈ 6 hours (Fleming et al. 2006).

Three days after injury blood monocyte-derived macrophages also start infiltrating the injury site and their numbers peak 7 days after injury (P. Popovich and Wei 1997; Blight 1992). These cells may phagocyte degenerating axons and have been ascribed proinflammatory properties that may further aggravate degenerative events (David and Kroner 2011). Furthermore, these cells, together with activated microglia, establish a chronic inflammatory state, suggesting that they do not promote resolution of inflammation. Notably, blood borne macrophages can with some certainty be distinguished from microglia with for example a marker for CD8 (P. G. Popovich et al. 2003).

Macrophages do not constitute a homogenous population. Certain populations, denoted M1 macrophages, promotes degeneration while another population, M2 macrophages, instead promotes regeneration (Kigerl et al. 2009). Thus, macrophages (as well as microglia) are polarized, and polarization can be shifted by for example pharmacological intervention (Guerrero et al. 2012; Hawthorne and P. G. Popovich 2011; Mercalli et al. 2013). Transplanting macrophages with an M2 phenotype in experimental spinal cord injury has been shown to promote regeneration (Rapalino et al. 1998). Furthermore, intervention producing an increased M2 response is generally associated with progressive amelioration of inflammation and improved functional recovery (Shechter et al. 2013; Miron et al. 2013; Nakajima et al. 2012).

T- and B-cells also participate in the inflammatory response to injury, though less is known about their roles and therapeutic potential. In rats, T-cells have been found to infiltrate progressively, primarily at the injury site, from 12h, peaking at 7 days after injury (P. Popovich and Wei 1997). T-cells remain at the injury site, but in lower numbers during the chronic inflammation. Indirectly, B-cells have been found to reside chronically in the injured spinal cord, as reflected by the presence of autoantibodies against CNS specific proteins (Hayes et al. 2002). Autoantibodies against for example myelin basic protein (MBP) as well as T-cells specific for MBP have been tested in experimental spinal cord injury models and found to improve locomotor function and reduce pathology (Huang et al. 1999; Hauben, Nevo, et al. 2000b; Hauben, Butovsky, et al. 2000a; T. B. Jones et al. 2004).

Revascularization/scarring

CNS vasculature consists of endothelial cells that are surrounded by a basement membrane, pericytes and astrocytic end-feet (Zlokovic 2008). This arrangement seals off the CNS from passive influx of small and large molecules and, together with microglia, forms the neurovascular unit and hence the blood brain barrier and the blood spinal cord barrier. The barrier maintains a milieu that allows neural networks to function properly while avoiding potentially damaging agents to enter the CNS. In neurodegenerative diseases, stroke, and, particularly, trauma, the barrier becomes permeable to macromolecules. In spinal cord injury, there is both rupture of the vasculature, causing bleedings, and breaches of the blood-spinal cord barrier that results in edema, and leakage of macromolecules (Griffiths and R. Miller 1974).

Loss of blood brain barrier integrity is characterized by upregulation of the basal membrane i.e. thickening of the membrane, an increased diameter of the vasculature and loss of endothelial tight junctions (Loy et al. 2002). Permeability peaks 30-60 min after injury and then decreases although it is still highly permeable 24 hours after injury, at which time many macromolecules have entered the parenchyma and become deposited in the extravascular compartment (Whetstone et al. 2003). Three days post injury, increased vascular permeability stretches many millimeters rostral and caudal to the injury site, and at the same time signs of revascularization can be seen (P. G.

Popovich et al. 1996; Figley et al. 2013). The permeability persists, but declines during the revascularization that takes place between 3 and 7 days after injury. It should also be noted that vascular permeability as well as revascularization is greater in the dorsal column rostrally and caudally to the injury site than in other white matter regions. Seven days after injury, deposition of basement membrane is evident at the injury site. However, most of the basement membrane is not associated with any blood vessel forming endothelial cells and hence does not support blood flow (Loy et al. 2002). While macrophages have been shown to direct angiogenesis, it does not seem as if the great infiltration of macrophages at the injury site supports stable revascularization. Instead, much of the basement membrane deposition will mature into a scar and persist chronically in the injured spinal cord (Silver and J. H. Miller 2004; Fantin et al. 2010) (see Fig 7). Nerve fibers can be found associated with laminin deposition at the site of injury after two weeks. Such neurites will later retract if the “sheet” of basal membrane does not become part of a capillary wall. To promote angiogenesis hence seems like a therapeutic option, but instead reducing revascularization has been shown to reduce edema, increase tissue sparing and improve functional recovery as seen with for example methylprednisolone (Xu et al. 1992).

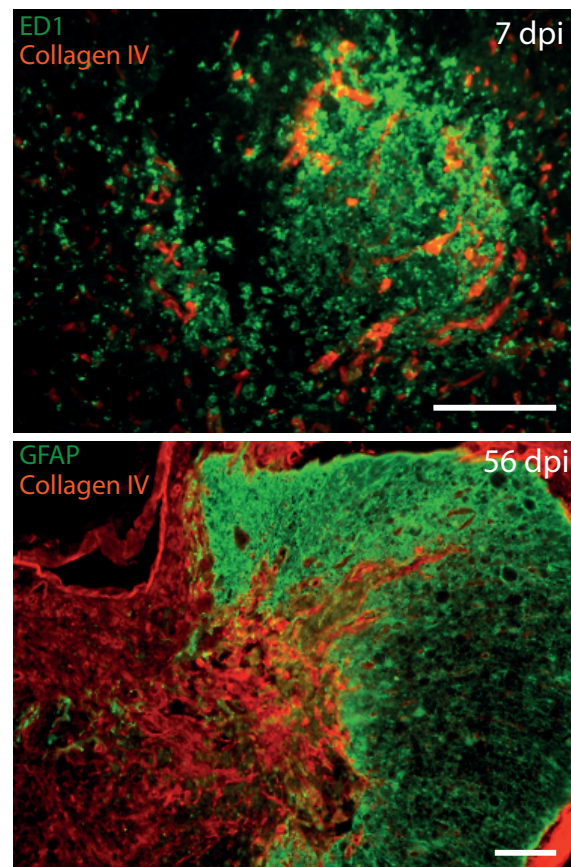


Fig 7. Signs of revascularization at the site of injury in the presence of macrophages that have infiltrated the spinal cord, 7 days after injury (upper image). However, 56 days after injury there is instead a dense fibrotic scar with surrounding astrogliosis at the injury site (lower image).

At two weeks after injury the blood spinal cord barrier has regained its integrity with respect to macromolecules. However, microvascular abnormalities as well as micromolecular permeability in the white matter around the injury site persists for longer periods of time (P. G. Popovich et al. 1996). The micromolecular permeability has been demonstrated to colocalize with clusters of microglia. Whether it is deleterious is unknown, although there has not been any observed impairments of behavior correlating to this event.

TREATMENT STRATEGIES

Treatment strategies for spinal cord injury are manifold and include to: (1) protect from secondary damage, (2) stimulate remyelination of axons, (3) stimulate and provide substrates/scaffolds for regeneration of injured axon pathways, (4) stimulate compensatory sprouting of remaining axonal systems, (5) establish neuronal relays across injury, as well as (6) replace lost nerve cells. Specific treatments often address several of these strategies. Neuroprotective treatment aims to reduce degenerative secondary events or increasing cell death resistance, while regenerative treatments aim to promote axon growth and proliferation of needed cell types. Therapy that promotes plasticity increases compensatory sprouting and even novel uses of remaining pathways. In our investigations of the therapeutic potential of cancer drugs, we have targeted the acute phase of the injury, primarily to promote neuroprotection. This in turn may indirectly promote axonal regeneration, as has been reported for EGFR inhibition (Yiu and He 2006; Koprivica et al. 2005; Ahmed et al. 2009).

Experimental strategies

Spinal cord injury models have been developed to mimic injuries found clinically such as contusion, compression and different degrees of spinal transection (Basso et al. 1996; Gruner et al. 1996; Schucht et al. 2002). We used a contusion model since it corresponds to the most common types of spinal cord injuries. Rodents are the most commonly used model animals even though for example zebra fish, salamander, cat, pig, and non-primate monkeys are also used. Rodents and humans do however differ with respect to some important neuroanatomical features (L. T. Brown 1974). For instance, the corticospinal tract that for the most part is located in the dorsal column in rats, is being located to a major extent in the dorsolateral white matter in humans (SAMLE and Schwab 1997; Martin 2005). Notably, the dorsal location in rats (and mice) renders the corticospinal tract particularly vulnerable to weight drop injury. There are also differences between rats and mice with respect to courses of pathology including the inflammatory response to injury (Sroga et al. 2003; Seok et al. 2013). This discrepancy is a central issue in translational research and we have chosen rats since they display a pathology which is more similar to that of humans (Metz et al. 2000).

In vitro models are used to study function of, and treatment options based on defined cell populations or tissues. For example, in paper I we aimed to characterize a spinal astrocyte culture system to have the possibility to determine molecular effects of cancer drugs on astrocytes, because these cells constitute in vivo targets for the cancer drugs of interest (Su et al. 2008; Codeluppi et al. 2009; Erschbamer et al. 2007). There are also slice culture preparations that can be used as an intermediate model system, and in which injury will progress without the involvement of the peripheral immune response (Ravikumar et al. 2012).

Current consensus is that a future treatment for spinal cord injury should include combinations of protective and regenerative therapies. However, different treatment strategies have mostly been investigated as separate interventions. This improves the prospect of finding a mechanism of action when there is an effect, but misses the possibility that certain effects may be seen, only when treatments are combined, and not by either component alone (Olson 2013). Pre-treatments can be used experimentally to obtain proof of concept for a protective effect of for example certain gene variants or drugs (Schumacher et al. 2000; Yip et al. 2010). Protective treatment generally targets one or several components of the secondary injury already in the acute stage of the injury, ultimately reducing excitotoxicity or increasing cell resistance towards excitotoxicity (Mattson 2003). Plasticity of neuronal connections can be induced through for example physical training, but also through inhibition of for example Nogo-A signaling (Behrman et al. 2006; Raineteau and Schwab 2001; Simonen et al. 2003). Regenerative therapies typically aim to induce long-distance axon growth, something that does not occur spontaneously after injury in adult mammals. Methods that have been employed to promote such growth include neutralization of axon growth inhibitors, peripheral nerve bridges, Schwann cell transplantation, neurotrophic factors, grafting cells that are genetically altered to express trophic factors and different sorts of artificial bridges (Olson 1997).

Since neural loss seems definite, there is today efforts to get the ependymal cells in the cord to differentiate to neurons and thus induce endogenous replacement of neurons (Moreno-Manzano et al. 2009). Also, stem cell therapies carry the promise of replacing the lost cells of the cord and have been successful experimentally (J. W. McDonald et al. 1999). Successful replacement of neurons, resulting in partial return of hind limb motility in the adult rat, was first demonstrated by Cheng et al using neuroanatomy-guided white-to-gray matter rerouting with autologous peripheral nerve bridges, secured by aFGF-containing fibrin glue, across a 5 mm gap in spinal cord (Cheng et al. 1996). Stem cells have been shown to induce axonal growth, not only through differentiation of the transplanted cells into neurons, but by secretion of different growth factors (P. Lu et al. 2003). Recently, combining neural stem cells and a large number of growth factors was shown to induce extensive neural sprouting, such that rats with complete spinal cord injury regained extensive movement of the hind limbs (P. Lu et al. 2012). This is promising indeed, although further preclinical optimization is needed before moving into clinical trials (Tuszynski et al. 2014).

Clinical trials

Clinical trials of systemic drug administration for spinal cord injury have demonstrated how difficult it can be to determine outcome. Drugs that have gone through sizeable clinical trials for spinal cord injury include Methylprednisolone, GM1 ganglioside, and Gacyclidine. Other drugs that have undergone clinical trials but where results have been inconclusive or where large clinical trials were never initiated include Fampridine

and thyrotropin-releasing hormone (Fehlings and Baptiste 2005; Steeves and Blight 2012).

There have been four clinical trials of Methylprednisolone for spinal cord injury, followed by controversy with respect to effects (Sayer et al. 2006). Methylprednisolone is a corticosteroid and thus has anti-inflammatory properties, although it was primarily its ability to reduce edema formation that was believed to confer the experimental improvements and that brought it to clinical trials (Ducker and ZEIDMAN 1994). During the four clinical trials, methylprednisolone was tested in different doses and using different administration regimens and it was concluded that the treatment was effective if administered within 8 hours after injury (Bracken et al. 1985; Bracken et al. 1992; Bracken et al. 1998; Bracken et al. 1990; Bracken et al. 1997). However, these results were based on post hoc analysis of a relatively small cohort and the treatment increased side effects such as pneumonia and sepsis. In the end, methylprednisolone was therefore not recommended as treatment for spinal cord injury.

GM1 ganglioside is a sphingolipid that had experimentally reduced edema and accelerated neural outgrowth (BOSE et al. 1986). The treatment was initiated within 72 h and did lead to promising results in a first clinical trial. However, a phase 2 trial did not reach endpoints and only minor indications of a beneficial effect were noted (Geisler et al. 1991; Geisler et al. 2001). Nevertheless, this clinical trial has provided extensive data on spontaneous recovery after spinal cord injury, which can help to provide directions for future clinical trials (Steeves and Blight 2012).

Gacyclidine is a competitive NMDA antagonist developed to counter glutamate toxicity directly after injury (Gaviria et al. 2000). The drug was administered as early as possible after injury, typically within 2 h, in an effort to harness the full extent of its potential neuroprotective effects. However, a phase 2 trial did not demonstrate any long-term improvements from Gacyclidine treatment (Tadie et al. 2003).

Thyrotropin-releasing hormone demonstrated positive effects in both preclinical and a small clinical trial, however, it has not had the opportunity to be tested in a large scale trial. The potassium channel blocker Fampridine did successfully decrease spasticity in a phase 2 trial, but effects were only noted in a subset of patients in a phase 3 trial and is not a standard treatment today.

Ongoing trials include neuroprotective therapy with minocycline, erythropoietin, and riluzole and regenerative therapy with anti-Nogo A antibodies and a C3 Rho inhibitor (Fehlings et al. 2012; Zoerner et al. 2010; Fehlings et al. 2011; Casha et al. 2012; Matis and Birbilis 2009). Cell transplantation has been tested on a large scale in China but did not meet international standards for either safety or efficacy (Dobkin et al. 2006). Instead, several small clinical trials with different types of cell transplants have started and may provide the spinal cord research community the useful data on safety and potential for efficacy (Pal et al. 2009; Saito et al. 2008; Tabakow et al. 2013; Guest et al. 2013; Guest et al. 2005; J. Sharp et al. 2010). There are also other

strategies to regain the ability to walk such as prosthetics and exoskeletons, including brain-device interfaces to control movements (Nicoletis 2012; Giszter 2008). To date, external devices have focused on motility, although sensing devices are also being developed. Other issues such as bladder control, erectile dysfunction, and pain symptoms may be more challenging to address.

Many trials have indicated beneficial effects in subgroups of the treated cohort. It is thus likely that not all patients will respond to any given treatment, emphasizing the need for pre-defined stratification variables that can be used in outcome analysis. Such variables may be genetic, related to the health status of the patient prior to injury, as well as to the injury itself and time to treatment. Biomarkers can be searched for in blood or CSF. MRI does not only aid in determining the extent of spinal tissue damage, but can also be used to monitor BBB permeability (Tatar et al. 2009; Flanders et al. 1990; Martínez-Pérez et al. 2013). Positron emission tomography may detect activation of certain glial cells and can detect glucose metabolism, and diffusion tensor imaging may image specific neural tracts (Floeth et al. 2013; Guo et al. 2012; Abourbeh et al. 2012; Kamble et al. 2011; Koskinen et al. 2013). Imaging methods are currently expensive and the techniques may not always have adequate resolution, but remains a promising future standard diagnostic tool.

Patients are classified according to the ASIA protocol based on motor and sensory assessment. A reliable prognosis of final outcome cannot be performed until ≈ 72 h after injury due to spinal shock masking motor and sensory capabilities (Anon 2008). It follows that neuroprotective treatments will most likely have to start before a reliable prognosis of final outcome can be done. The fact that a safe diagnosis cannot be made until long after initiation of drug treatment stresses the importance of methods to determine if effective concentrations of drug has reached key compartments such as blood or CSF and had cellular effects. Paper VI describes studies of how long a delay there can be until initiation of treatment with our candidate drug, Imatinib, and how to monitor that satisfactory doses of Imatinib have reached circulation and had cellular effects.

REPOSITIONING DRUGS FOR SPINAL CORD INJURY

Controversial results concerning efficacy and potential side effects has halted clinical use of methylprednisolone (Sayer et al. 2006). Hence there is no drug treatment in routine use that may improve recovery from spinal cord injury. The development of drugs for any disease, disorder or pathology is at least a decade-long process, very few candidates make it to clinical trials and fewer still have the desired effects (Ashburn and Thor 2004). In this respect, CNS disorders are especially challenging, the number of approved drugs have for a long time been in decline (Pangalos et al. 2007). Nine clinical trials in spinal cord injury have all concluded that drug effects were not robust enough, even though the tested drugs had been shown to have robust preclinical effects (Fehlings and Baptiste 2005). An example of how difficult it can be to predict outcome

came with a clinical stroke trial with the drug NXY-059, shown to have robust effects in a primate stroke model. Nevertheless, the drug failed to show any benefits in humans (Shuaib et al. 2007). Thus even the best imaginable model system can fail to predict outcome, highlighting the need for multiple drug candidates that can be tested in humans, also in spinal cord injury.

To reposition drugs that are already in clinical use for other indications for use in spinal cord injury has the advantage that the candidate drugs have already successfully passed all the checkpoints a drug must pass in order to become a drug for human use (Ashburn and Thor 2004). Recently, Riluzole, a treatment for amyotrophic lateral sclerosis, was swiftly repositioned to enter clinical trials for spinal cord injury (Fehlings et al. 2012).

Repositioning a drug that is well established for other indications, and that has been used for a long time, has many advantages. Acquired knowledge includes side effects, drug interactions, drug effects that can be used as biomarkers, as well as drug resistance issues and more. This information is particularly valuable in a clinical trial with acute drug treatment (Krishna et al. 2014). Here we have focused on repositioning imatinib, erlotinib and rapamycin, a group of drugs targeting RTK signaling, all used clinically as treatments against certain types of cancer (Tsao et al. 2005; Capdeville et al. 2002; Easton and Houghton 2006). These drugs have previously been repositioned for new indications and are extensively used clinically. Moreover, these drugs and their targets are still in focus of much experimental research, providing information about their effects and interactions, both in experimental models and humans.

RECEPTOR TYROSINE KINASE SIGNALING INTERFERENCE

There are 58 known receptor tyrosine kinases (RTK), divided into 20 subfamilies in humans (Lemmon and Schlessinger 2010). They are highly conserved throughout evolution, likely due to key roles in mitotic activity and energy homeostasis (Blume-Jensen and Hunter 2001). The RTKs are activated by ligands such as growth factors and pro-inflammatory cytokines, and also hormones.

A receptor tyrosine kinase consists of an extracellular receptor and an intracellular tyrosine kinase domain (TDK) (Ullrich and Schlessinger 1990; Schlessinger and Ullrich 1992). The extracellular receptors have different structural domains that with different affinity bind the respective ligands of a given subfamily. The intracellular tyrosine kinase domain is normally autoinhibited so that it cannot spontaneously bind ATP (Nolen et al. 2004; Huse and Kuriyan 2002). Receptor tyrosine kinases typically dimerize upon ligand binding, causing autophosphorylation of the tyrosine kinase domain, which initiates further signaling (Ullrich and Schlessinger 1990). While homodimers in their respective subfamilies are commonly formed, they also form heterodimers capable of similar intracellular signaling. Activation of a receptor tyrosine kinases induces 50 - 200 fold catalytic efficiency, which can be further increased 10

fold by autophosphorylation in the subsequent activation loop of the receptor (Cobb et al. 1989; Furdui et al. 2006).

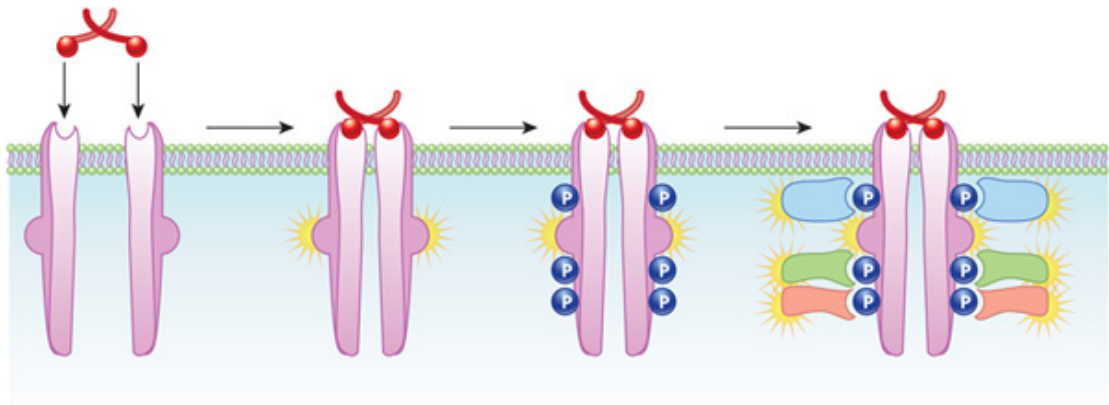


Fig 7. Receptor tyrosine kinase activation. (1) A Ligands bind to the two receptor tyrosine kinases. (2) RTK dimerization removes the autoinhibition of the phosphorylation sites of the tyrosine kinase domain. (3) Multiple tyrosines are phosphorylated. (4) Intracellular proteins bind the phosphorylated tyrosines, activating several intracellular signalling pathways. © 2010 Nature Education

There are both positive and negative feedback loops for these systems. Positive feedback loops include autocrine signaling by either production of a receptor tyrosine kinase ligand or cleavage of cell surface bound ligands that in turn may activate a RTK (Shilo 2005; Schulze et al. 2004). Receptor tyrosine kinase activation can also increase expression of the receptor tyrosine kinases or inhibit protein tyrosine phosphatases that normally could inhibit signaling (Ullrich and Schlessinger 1990; Ostman and Böhmer 2001). Negative feedback loops include internalization of the receptors or molecular interaction that negatively alters the affinity of the receptor for the ligand (Downward et al. 1985; Avraham and Yarden 2011). However, feedback loops are different depending on ligand, hence even if two ligands can activate the same cellular process they may cause different outcomes. For example, a ligand can induce a positive feedback loop that allows a sustained response, while a second ligand may induce a negative feedback loop resulting in a more transient response, as seen for NGF and EGF, respectively (Marshall 1995). Moreover, there are other situation-specific mechanisms that may alter the response of receptor tyrosine kinase activation. A mechanism that can be important in CNS trauma is the ability of ROS to transiently inhibit protein tyrosine phosphatases and thus reverse autophosphorylation (Tonks 2006; Reynolds et al. 2003). Malfunctioning receptor tyrosine kinase feedback causing constitutive receptor activation is common in cancer. These mechanisms, together with other causes for constitutive receptor tyrosine kinase activation such as chromosomal translocation or gain of function mutations, have prompted the development of pharmacological agents that interfere with receptor tyrosine kinase signaling (Blume-Jensen and Hunter 2001).

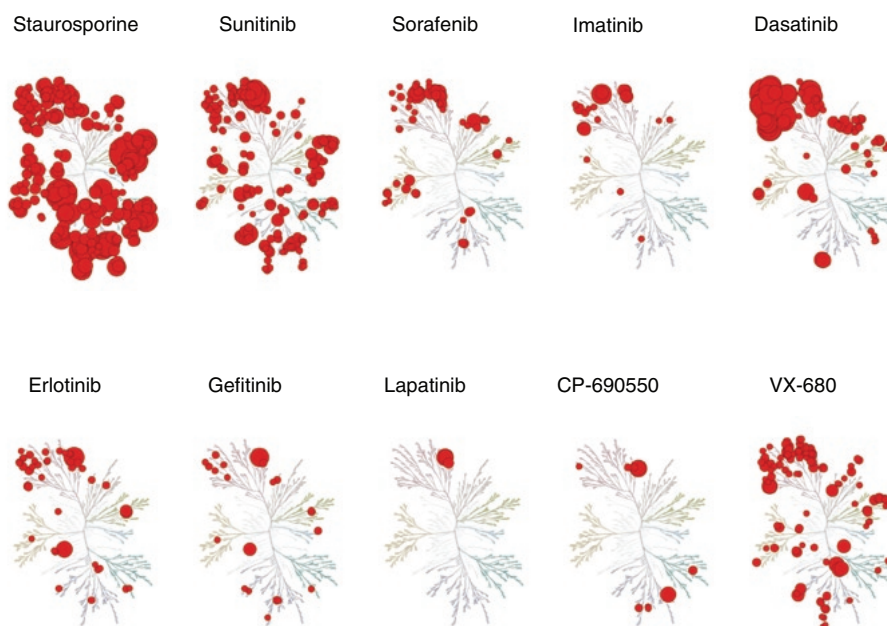


Fig 8. Receptor tyrosine kinase binding profile of RTK inhibitors. Staurosporine is a pan-inhibitor of RTKs, CP-690550 and VX-680 are experimental protein kinase inhibitors, while the other compounds are FDA-approved kinase inhibitors. The red circles represent the binding profile of these kinase inhibitors for different members of the RTK family and the size of the circles defines the affinity of the inhibitors to these kinases. From Dr. Ghoreschi with permission (Ghoreschi et al 2009).

There are mainly two categories of drugs; small molecular inhibitors that target the ATP binding site, and monoclonal antibodies that interfere with RTK signaling (Imai and Takaoka 2006). The monoclonal drugs have been developed due to their selectivity, since the small molecule inhibitors have been shown not to be selective due to tyrosine kinase domain similarities (Reichert and Valge-Archer 2007; Fabian et al. 2005). However, that small molecular inhibitors lack exclusive selectivity need not be a disadvantage, but could be advantageous. Firstly, it may be advantageous for a drug treatment to have multiple or additive effects. Secondly, in cancer therapy patients commonly develop drug resistance, sometimes due to selective mutations, but sometimes due to compensatory mechanisms (Lemos et al. 2008; Thomas et al. 2004; Marchetti et al. 2008). Notably, even though small molecular inhibitors typically have a wide spectrum of receptor tyrosine kinase interference, they are generally tolerable at effective doses (Ghoreschi et al. 2009). By using a drug that acts on several receptor tyrosine kinase targets, one is already using a combination treatment of sort. Thus such compensatory mechanisms may be fewer and has perhaps lead to the success of applying these drugs to different cancers, as well as other diseases. This reasoning is further strengthened by the fact that monoclonal antibody therapy for cancer has been found to need complementary chemotherapy in order to be effective, due to compensatory cell signaling (Reichert and Valge-Archer 2007).

In this thesis we aimed to determine the therapeutic potential of the small molecular inhibitors imatinib, erlotinib and rapamycin, studied in a rat weight drop spinal cord

injury model. Imatinib and erlotinib target TDKs of certain RTKs and rapamycin targets a kinase further downstream of most RTKs. Thus these drugs share most of the above-mentioned properties, while each drug also has its own individual characteristics. Imatinib, erlotinib, and rapamycin, are commercially available as Glivec, Tarceva, and Sirolimus, respectively.

Imatinib

Imatinib was first developed to combat chronic myeloid leukemia by inhibiting the constitutive activation of the BCR-Abl tyrosine kinase enzyme (Capdeville et al. 2002). This was the first small molecule inhibitor aimed towards a receptor tyrosine kinase to be FDA-approved. The drug is commercially available as Glivec, or Gleevec, and is also FDA approved for other diseases than CML, such as c-Kit positive gastrointestinal stromal tumors (Siehl and Thiel 2007). Imatinib was supposed to selectively inhibit the BCR-Abl tyrosine kinase formation, but has later proven to inhibit many other receptor tyrosine kinases. Accordingly, imatinib has a wide array of effects with therapeutic potential in several diseases and disorders (Pardanani and Tefferi 2004). Imatinib not only inhibits c-Kit, but also c-Fms, TGFR, and PDGFR, and it ameliorates symptoms of asthma, lung fibrosis, dermal fibrosis, hypereosinophilia, and autoimmune arthritis (Berlin and Lukacs 2005; Daniels et al. 2004; Cools et al. 2003; Zoja et al. 2006; Pardanani et al. 2003; Distler et al. 2007; Paniagua et al. 2006; Balachandran et al. 2011; Seggewiss et al. 2005; Dewar et al. 2005). Many of these effects have been attributed to direct effects on the immune system, including mast cells, macrophages, and T-cells, and imatinib has been shown to change the cytokine secretion profile of these immune cells.

Our interest in imatinib as a potential therapeutic treatment for spinal cord injury primarily came from evidence of the ability of the drug to normalize the BBB in a stroke model, through inhibition of PDGFR- α signaling (Su et al. 2008). Moreover, imatinib has recently been found to ameliorate disease progression in a rat model of multiple sclerosis (Z Adzemovic et al. 2013).

Erlotinib

Erlotinib is a reversible EGFR/HER1 inhibitor and its primary clinical application is non-small cell lung cancer (Shepherd and Pereira 2005). Interestingly, erlotinib has been found effective in lung cancers without an EGFR mutation, even though more effective when EGFR is highly expressed. Erlotinib has been repositioned for pancreatic cancer which is its second clinical application (Moore et al. 2005). Furthermore, erlotinib has been tested as a combination treatment in several other cancers such as prostate, hepatocellular, biliary and breast cancer, but to what extent it is truly effective as a mono or combination treatment in these diseases needs to be determined (Philip et al. 2006; Gravis et al. 2008; Philip 2005; Dickler et al. 2009). Erlotinib has been shown to also inhibit JAK2 (V617F), present in polycythemia vera, which is a myeloproliferative disorder (Z. Li et al. 2007).

Oral erlotinib bioavailability increases with food intake and the drug is metabolized in the liver by CYPs that can for example be induced by smoking (Frohna et al. 2006; Hamilton et al. 2006). Smoking hence increases clearance of the drug and as a result a smoker needs a larger amount of drug to have the desired effect.

EGFR inhibition has also reportedly shown beneficial effects in the CNS. EGFR inhibition can reduce the CSPG deposition by astrocytes and further promote neural growth by reduction of inhibitory signals from myelin or CSPG, presumably due to additional indirect effects generated by the astrocytes (Koprivica et al. 2005; B. Liu et al. 2006; Ahmed et al. 2009; Erschbamer et al. 2007). We sought to determine the therapeutic potential of systemic administration of erlotinib since a non-reversible EGFR inhibitor, PD168393, improved functional outcome after local administration in a spinal cord injury in rat (Erschbamer et al. 2007).

Rapamycin

Originally an antifungal agent, Rapamycin is now used in the clinic to avoid transplant rejection. There has been promising results treating different cancers, even though rapamycin is generally regarded as an immunosuppressant (Law 2005). Rapamycin binds FK506-binding protein of 12 kDa (FKBP12), one of the components of mTOR complex 1 and thus inhibits the complex formation. mTOR is part of the phosphoinositide 3-kinase (PI3-K)-related kinase family and forms two active complexes, mTORC1 and 2. mTORC1 is formed with raptor (regulatory-associated protein of mTOR) and four other components (Laplane and Sabatini 2009). The complex can be activated by growth factor induced AKT activation (Potter et al. 2002; Bhaskar and Hay 2007) that signals through tuberous sclerosis complex (TSC) 2 and Rheb activation (Long et al. 2005; Codeluppi et al. 2009).

mTORC1 orchestrates many basic functions related to energy homeostasis and transcription and thus mTOR dysfunction is associated with a variety of disorders. Evidence for its involvement has been found in for example glioblastoma (Akhavan et al. 2010), breast cancer (Zhou et al. 2007), and lung cancer (Carretero et al. 2007). The mTOR pathway has also received increased attention in CNS disorders such as Alzheimer's disease (Caccamo et al. 2010), Parkinson's disease (Santini et al. 2009) and ischemic stroke (Pastor et al. 2009). Experimentally, rapamycin or derivatives thereof have relieved or reduced the magnitude of symptoms associated with the above mentioned diseases, as well as expanding life expectancy in mice (Harrison et al. 2009). Our studies of the therapeutic potential of rapamycin were primarily prompted by its ability to reduce astrocyte reactivity in ischemic spinal cord injury and to inhibit a downstream target of both imatinib and erlotinib (Buck et al. 2006; Burchert et al. 2005; Codeluppi et al. 2009).

AIMS

The primary aim of this thesis was to determine the therapeutic potential of cancer drugs that inhibit receptor tyrosine kinase signaling, in a rat spinal cord contusion injury model. A secondary aim was to increase knowledge about basic pathology in experimental models, in order to facilitate translational approaches in spinal cord injury.

Specifically we aimed to:

- Determine how different rat substrains influence properties of spinal cord astrocyte cultures and recovery from spinal cord contusion injury.
- Monitor mTORC1 activation after spinal cord contusion injury
- Determine if imatinib, erlotinib and rapamycin, three cancer drugs interfering with receptor tyrosine kinase signaling, might improve functional outcome after spinal cord contusion injury in rats.

MATERIALS AND METHODS

All animal work has been approved by the Northern Stockholm Animal Ethical Committee and performed in accordance with the Helsinki declaration.

IN VIVO TECHNIQUES

Animals

Rats

The studies presented here have primarily used rats, as opposed to mice, due to the higher translational value of rats in studies of spinal cord injury. We used exclusively Sprague-Dawley rats, but tested different substrains obtained from the vendors Scanbur (Germany), Harlan (Netherlands) and Charles River (Germany). Females were chosen to reduce severity of urinary infections and to ease manual bladder emptying.

Transgenic mice

PDGFR α ^{+/GFP} mice on C57BL/6J genetic background were used in study II.

Surgery

Rats were subjected to spinal cord contusion injury using a dedicated instrument (NYU Impactor, Keck Center for Neuroscience) as previously described (GRUNER 1992; Basso et al. 1996). Rat weights at the time of experimental contusion injury was 180-300g. Prior to surgery, animals received analgesics (bruprenorphin 0.015 mg/kg, Temgesic, 0.03 mg/kg, i.p.). Under anesthesia (isoflurane) we exposed the dorsal surface of the spinal cord through laminectomy of the spinal column at T10 and caudal half of T9. By dropping a 10 g weight onto the exposed spinal cord we induced a mild or moderate contusion injury, depending if the weight was dropped from 12.5 or 25 mm. The computerized system of the NYU Impactor ensured that the severity of the injury was standardized. The system allowed determination of impact force based on measurements of height, time (Ct), and impact velocity (Vi). Rats with errors exceeding 5% of expected values were excluded. In the final stage of the surgery, muscle and skin was sutured, and animals allowed to recover in cages heated by a heating pad.

Post operative care

Analgesic treatment (bruprenorphin, Temgesic, 0.015 mg/kg, i.p.) was administered once daily for 3 days and prophylactic antibiotics (0.6 mg/kg trimethoprim, Borgal, Hoechst, AG) for 7 days. The bladder was emptied manually twice daily until the animal regained bladder function. Animals had access to food and water ad libitum and were housed three per cage. The temperature was maintained at 24–26°C with 12 h light.

Drugs and delivery

Glivec (Novartis), Sirolimus (Wyeth) and Tarceva (Roche) were administered per os using gavage. Glivec was prepared as a suspension and administered daily at a dose of 250 mg/kg. We grinded the Glivec pills and mixed the powder with PBS. The suspension was then heated to 37 °C for 5 min and then spun at 13000 ref. The supernatant was transferred to a new tube and later administered as an initial full dose. For the following consecutive days of treatment, the daily dose was divided into 1/3 in the morning and 2/3 in the evening. Tarceva was similarly prepared, with water instead of PBS, and administered at a dose of 5 mg/kg per day. Rapamycin was purchased as a suspension that we diluted with PBS and administered at a dose of 1.5 mg/kg per day.

Blood sampling

The rats were put in a heated cage for 15 min prior to blood sampling. Animals were then transferred to a plastic constrainer and 250 µl blood was extracted from the tail vein. The blood samples were incubated in room temperature for 20-30 min and then spun at 4800 rpm for 10 min at 4 °C. The serum (the supernatant) was collected and transferred to – 80 °C.

CSF sampling

Under isofluorane anesthesia we exposed the dura mater caudal to the skull bone. CSF was extracted from cisterna magna using a syringe, transferred to a tube, snap frozen on dry ice, and stored at – 80 °C. Animals were sacrificed after the procedure.

Bladder emptying and recovery assessment

After spinal injury, rats loose ability to empty their bladder and hence the bladder has to be manually emptied. This is carried out by applying gentle upward pressure to the bladder region at the lower most part of the belly using the index and middle fingers. The amount of expelled residual urine was taken as a measure of bladder dysfunction.

Behaviour tests

Scoring of open field locomotion

We observed the rats in an open field to determine ability to use hind limbs after injury according to the BBB (Basso, Beattie, Bresnahan) scoring system (Basso et al. 1995). The BBB score rates hind limb function from 0 (no function) to 21 (normal gait). We also assessed the animals using the BBB subscore that combines scores for paw position, toe clearance, trunk stability and tail position (Lankhorst et al. 1999). Animals were scored during 4 min sessions (commonly weekly) by two experienced experimenters blinded to the group identity of the rats. BBB scoring levels are strictly

defined and a training video is provided to train personnel. In fact, meta analysis by statisticians suggest the scores behave as linear data (Scheff et al. 2002).

Automated gait analysis

To acquire additional stepping parameters for mildly injured animals, we used an automated gait analysis walkway (Catwalk, Noldus) (Hamers et al. 2001). The system records limb coordination, called regularity index, taking different stepping patterns into consideration. The walkway also records several individual stepping parameters such as base of support (width between paws), stride length (length between placements of a paw), swing time (time that the paw is not touching the walkway), and stance time (time that the paw touches the walkway). The rats are required to perform three complete runs, defined as continuous locomotion along the measurement area of the walkway. The scores are then averaged and typically normalized to pre-surgical measurements. The automated measurements of limb coordination can also be used to validate observational assessment of coordination from the open field test (Koopmans and Deumens 2005). This was done in Papers III and VI.



Fig 9. Automated assessment of gait was performed using the Noldus® Catwalk XT. Courtesy Noldus Information Technology BV, www.noldus.com

Mechanical sensitivity

To determine if injured rats in Papers II and VI develop hypo- or hypersensitivity to mechanical stimuli, we applied pressure to the hind paws using von Frey filaments (Stoelting, Wood Dale, IL). Prior to surgery, rats are habituated to the testing environment, consisting of a Plexiglass enclosure on top of a wire mesh floor. At the

time of testing animals were allowed to habituate for 30 min and then tested with calibrated von Frey filaments, with approximately logarithmical incremental force (0.4, 1, 2, 4, 6, 8, 10 and 15g), according to the “up-down” method. A 50% probability of paw withdrawal was calculated using measures averaged from both hind paws into one score per animal (Dixon 1980; Chaplan et al. 1994). The tests were typically performed weekly after spinal contusion injured animals had regained weight support and the values were later normalized to pre-surgery measurements.

Thermal sensitivity

In Paper VI we assessed hypo- or hypersensitivity to cold stimuli by applying a cold spray to the hind paws or a shaved area at the level of injury. The animal was assessed using a scale with four steps (0-3), corresponding to the reaction to the cold spray. A normal response (1), corresponds to moving the affected hindlimb or giving a localized response i.e. transient skin twitch. Score 0 is no response at all, while 2 is moving away from the cold spray and 3 is moving away and vocalizing. This score can thus determine different levels of hypersensitivity, but only one level of hyposensitivity.

Histochemical techniques

Spinal cord preparation

Animals were deeply anesthetized and transcardially perfused via the ascending aorta with 50-75 ml of calcium free Tyrode solution, containing 0.1 ml of heparin to avoid blood clotting, followed by perfusion with 4% paraformaldehyde in 0.1 M PBS and post-fixation in the same solution for 1h. Alternatively, Tyrode/heparin perfusion was followed by hydroextrusion of the spinal cord from the vertebral canal using a syringe with distilled water or Tyrode solution, and in vitro fixation (4% paraformaldehyde in 0.1 M PBS) for 2h. All post-fixed tissue was transferred to a 0.1 M PBS solution containing 10% sucrose, which was exchanged once for 4 days.

The cords were divided into segments that were embedded (OCT compound, Tissue-Tek, or NEG 50, Richard Allan Scientific) and frozen on dry ice. The frozen segments were sectioned on a cryostat to produce 14 µm (in situ hybridization) or 20 µm (immunohistochemistry, histochemistry) sections.

Histology

To determine amount of tissue and myelin that remains after injury to the cord, both Haematoxylin and Eosin (H&E) staining, and Luxol fast blue (LFB) staining was used. For H&E slides were first immersed in a 0.5% haematoxylin solution for 2.5 min at room temperature. Slides were then differentiated in a solution of 1% HCl in 70% alcohol for 10-15 seconds. After rinsing in cold water for 10 min, slides were immersed in 1% eosin solution for 2 min. A second short rinsing of the slides in cold water preceded subsequent dehydration in 70%, 95% and 99.5% alcohol, and lastly xylene. For LFB staining slides were immersed in an LFB solution, containing solvent blue 17, 95% ethyl alcohol and glacial acetic acid (5%), at 56°C for 16h. Sections were then rinsed first in 95% ethyl alcohol and then in distilled water. Sections were subsequently

differentiated first in a 0.05% lithium carbonate solution for 30 seconds and then in 70% ethyl alcohol for 30 seconds. After washing slides in distilled water, the differentiation of the slides was continued in 95% ethyl alcohol for 5 min, and in 99.5% alcohol for 2x5min followed by xylene for 2x5min. Slides from both procedures were mounted with Entellan (VWR International, West Chester, Pennsylvania, PA, USA) at the end of the staining procedure.

Immunohistochemistry

Immunohistochemistry was performed by first incubating slides in blocking solution with serum from the same species as the secondary antibody. The slides were then incubated with a primary antibody (table 1) and the primary antibodies were then identified by appropriate, fluorescence-labeled secondary antibodies (Cy3,Cy2, DyLight™ 488, DyLight™ 555, Jackson Laboratories). An anti-fade agent was included in the mounting medium (Prolonged gold® with or without DAPI, Invitrogen).

Table 1. Antibodies used for immunohistochemistry

Antibody	Specificity	Host	Source
Albumin	Albumin protein	chicken	Affinity Bioreagents
Aldh1L1	Acetaldehyde dehydrogenase 1	mouse	neuroMab
CD206	Mannose receptor	rabbit	Abcam
CD25	Alpha chain of the IL-2 receptor	mouse	Serotec
CD3	Cluster of differentiation 3	mouse	Serotec
CD45	Protein tyrosine phosphatase, receptor type, C	mouse	Serotec
CD45Rα	Protein tyrosine phosphatase receptor alpha	mouse	Millipore
CD5	Cluster of differentiation 5	mouse	Serotec
CD8	Cluster of differentiation 8	mouse	Abcam
CS-56	Chondroitin sulfate	mouse	Sigma
ED1 (CD68)	Cluster of differentiation 68	mouse	Serotec
Fibronectin	Fibronectin	rabbit	Chemicon
GFAP	Glial fibrillary acid protein	mouse	DAKO
GFAP	Glial fibrillary acid protein	rabbit	Sigma
GLT-1	Glutamate transporter-1	rabbit	Cell signaling
Iba-1	ionized calcium-binding adapter molecule 1	goat	Abcam
IgG	Immunoglobulin G	rat	Santa Cruz
Ki-67	Ki-67 protein	rabbit	Abcam
Lectin	Lectin protein	biotinylated (goat)	Vector laboratories
MPO	Myeloperoxidase	rabbit	Abcam
MPO	Myeloperoxidase	goat	Bio site
Nestin	Nestin protein	mouse	Millipore

NeuN	neuronal nuclei	mouse	Chemicon
NF-200	Neurofilament-200	chicken	Chemicon
NG2	neuron-glial antigen 2	mouse	Millipore
NG2	neuron-glial antigen 2	rabbit	Millipore
OX42 (CD11b)	Integrin alpha M	mouse	Serotec
OX6 (CD74)	class II histocompatibility antigen gamma chain	mouse	Serotec
PDGFR- α	Platelet-derived growth factor receptor alpha	rabbit	Cell signaling
PDGFR- β	Platelet-derived growth factor receptor beta	rabbit	Cell signaling
pS6 (S235/236)	phosphorylated ribosomal protein S6 (Serine 235/236)	rabbit	Cell signaling
S100 β	S100 calcium binding protein beta	rabbit	Abcam
S6	Ribosomal protein S6	rabbit	Cell signaling
SMI-312	Pan-axonal marker	mouse	Covance

Stereology

Stereology was performed on spinal sections with H&E staining to determine volumes of spared tissue and volume of cystic cavities caudal to the injury, and also with LFB staining to determine the volume of spared myelin. The experimenter was blinded to section identity and stereological analysis was based on the Cavalier principle (Stereologer, SPA Inc, MD).

Biochemical biomarker analysis

Levels of cytokines and chemokines were analyzed in serum and cerebrospinal fluid (CSF) from rats using immunoassay kits (MesoScale Discovery, Gaithersburg, MD, USA). The cytokine/chemokine kits were tested and validated for both matrices following manufacturer's instructions. Rat MCP-1 and MIP-3 α levels were measured using custom made kits and concentrations of INF- γ , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-13, KC/GRO and TNF were measured using a commercially available multiplex (10-plex) immunoassay kits (Cat No N05044-1).

All samples were randomized before assay procedures. Briefly, plates pre-coated with antibodies towards the analytes of interest, were blocked with the provided diluent for 60 min, washed and thereafter serum or CSF samples, diluted 1:4 respectively in assay diluents, were added to the wells and the plates were incubated for 2 h. Following washing of the plates, MSD Sulfo TAG secondary antibody mixtures were added and the plates were incubated for an additional 1.5 hrs. All incubations were performed at room-temperature. After a final washing step, read buffer (2X) was added and the plates analyzed (SECTOR Imager, SI6000, MesoScale Discovery). Lower limit of quantification was 52 pg/mL for IL-1 β and IL-2, 42 pg/mL for IL-5 and IL-6, 1 pg/mL for IL-4, 11 pg/ml for KC/GRO and between 4.3-6.1 pg/mL for TNF, INF- γ , IL-10 and IL-13. LOQ was 39 pg/mL for MIP-3 α and 624 pg/mL for MCP-1 in the custom made plate.

IN VITRO TECHNIQUES

Cell culture preparation

Primary rat astrocyte cultures

To determine the effect of media on the astrocyte phenotype after culture, we tested media from four different vendors (Gibco, Sigma, Hyclone, AM) in paper I. Furthermore, we used two Sprague-Dawley substrains from Harlan and Charles River to determine if there were phenotypic differences. Except for these two changing parameters, the cell culture preparation remained the same as described in the flow chart (figure 1). Our cell culture preparation is a modified

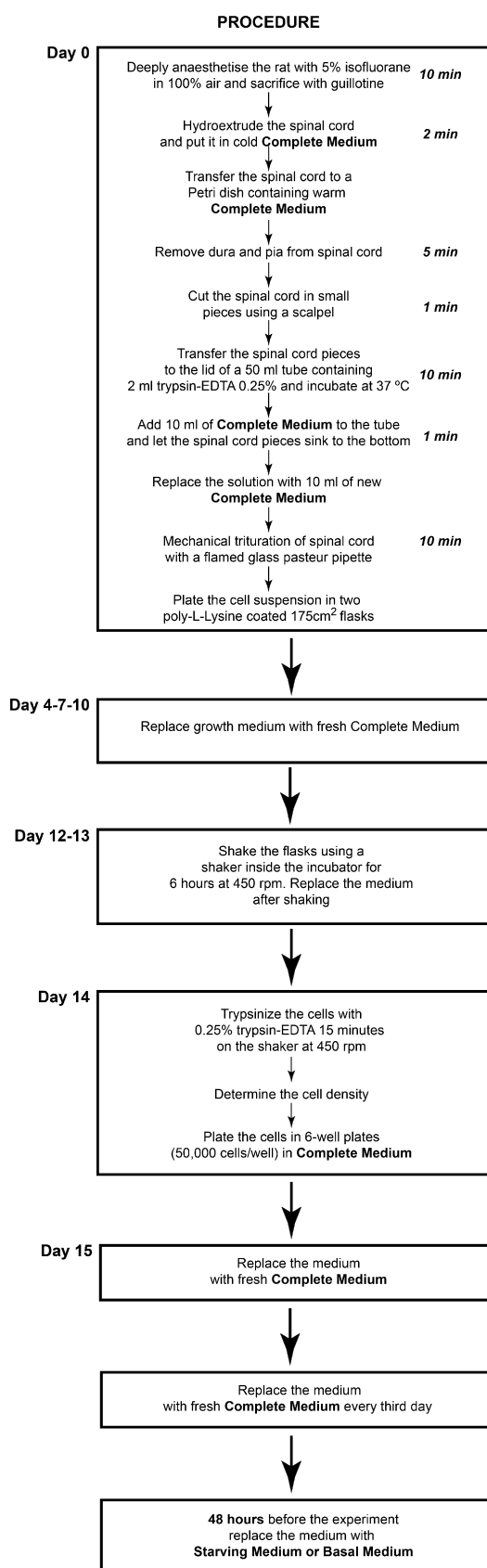


Figure 10. Flow chart describing the procedure for preparation of primary adult astrocytes.

version of a protocol previously described by Tawfik et al and was also used in paper IV (Tawfik et al. 2006). The solutions and compounds used during the procedure were trypsin-EDTA (Sigma), PLL (Sigma), Laminin (Sigma), and Trypan blue solution (Sigma).

Primary adult human astrocyte culture

Human astrocytes were used in paper I and were purchased from 3H Bioscience (1820). The astrocytes were cultured by thawing the vial with the cell suspension in a 37°C bath and then transferring the cells into 37°C AM media in a 75 cm² tissue culture flask. The following day the media was exchanged to remove residual DMSO and then continuously every third day. At 90% confluency, we trypsinized and plated the cells as described for the primary rat astrocyte cultures.

Western blot

Western blot was performed in paper IV from primary rat astrocyte cultures. After the experimental procedure, the confluent cell layer was homogenized in RIPA lysis buffer with added protease and a cocktail of phosphatase inhibitors (Roche, Mannheim, Germany) and collected in Eppendorf tubes. The cell suspension was sonicated and centrifuged, after which the supernatant was transferred to new tubes. We determined gel-loading dilution by measuring protein concentration using a BCA-assay. After diluting the samples to the same concentration, using LB x1 and adding 10 % DTT, we boiled the samples for 3 min before loading them into a 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). MOPs buffer (NuPage, Invitrogen) was used as running buffer and samples ran until reaching end of gel (Novex western blot system, Invitrogen). We transferred our sample tracer onto a PVDF membrane (Millipore, Temecula, CA, USA) using a transfer buffer mix (NuPage). To fluorescently label the transfer membrane, we first incubated it in 50% TBS and 50% blocking buffer (Licor, Lincoln, NE, USA) for 30 min and then incubated the membrane in primary antibodies in 50% TBS-0.1% tween and 50% blocking buffer. After washing the membrane in TBS-0.1% tween, we incubated it with secondary antibodies (Odessey IRDye 680 and 800, Licor) for 1 h. Lastly, we washed the membrane with TBS-0.1% tween and once with TBS before being scanned (IR scanner, LI-COR).

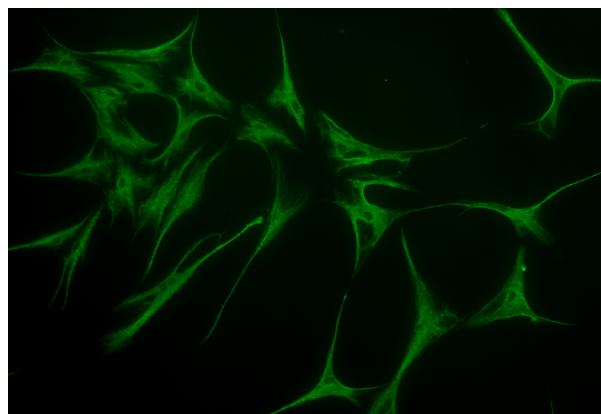


Fig 11. Cultures of primary rat astrocytes visualized with immunoreactivity to the structural protein vimentin.

qPCR

qPCR was performed in paper I on primary rat astrocyte cultures. The astrocytes were lysed (Trizol®, Invitrogen) and RNA was extracted using a phenol-chloroform extraction protocol provided by the vendor (Invitrogen). cDNA was created from the RNA using reverse transcription with random hexanucleotide primers (Applied Biosystems). PCR amplification was performed using a TaqMan master mix (Invitrogen). The standard curve method was used to quantify GLT-1, GS, CNX-43, GFAP and S100 β mRNA. GAPDH had been found to be a stable housekeeping gene for our primary astrocyte cultures and was used for normalization in case of possible RNA concentration differences.

DATA ANALYSIS

Image processing

Image processing included quantifying different properties of objects in digital images of microscopic fields of view or in other cases to adjust color properties for enhanced visibility of the object. An image processing program (FIJI, Fiji.sc) was used to quantify color intensity, object area or object length. There were no alterations of image properties if color intensity was quantified. An image editor (Illustrator®) was used to alter brightness and contrast.

Statistics

For comparison of parametric data or data that were normally distributed we used Student's t-test, ANOVA or two-way ANOVA. The analyses of variance were followed by Bonferroni post-hoc tests. For comparison of non-parametric data we used the Mann-Whitney U-test or the Kruskal-Wallis test. Data were typically presented as mean \pm SD or SEM. p value <0.05 was considered statistically significant and values were annotated as *, ** and *** for p values <0.05 , <0.01 , and <0.001 , respectively.

SUMMARY OF RESULTS

Paper I: Choice of media and host substrain alters astrocyte culture parameters

- The use of AM medium during culture of primary astrocytes results in minimal microglia contamination.
- Culture media and substrain affects mRNA expression of astrocyte-associated genes in primary astrocyte cultures.
- A rat substrain was identified to express the same astrocyte-associated markers, as well as similar levels of mRNA expression of astrocyte-associated genes, as human astrocytes.

Paper II: Immediate oral treatment with imatinib enhances recovery from SCI

- Imatinib improves recovery of bladder function and locomotor function.
- Imatinib rescues spinal tissue and axons, while attenuating astrogliosis, macrophage infiltration, and chondroitin sulphate proteoglycan deposition.
- Imatinib normalizes the blood-spinal cord barrier and reduces number of PDGFR- α and β positive cells.

Paper III: Rat substrains differ in recovery from SCI

- Substrains can have different (or similar) recovery of locomotor function, sensory function and bladder function after SCI.
- Substrains can differ with respect to loss of axons, white matter, and degree of morphological deformation, even if the inflammatory status is similar.

Paper IV: Oral Erlotinib, but not Rapamycin, causes modest acceleration of recovery from SCI

- Erlotinib accelerates bladder function recovery and locomotor recovery.
- Rapamycin does not alter recovery of bladder function or locomotion.

Paper V: SCI causes biphasic alterations of S6 phosphorylation

- There is biphasic increase of S6 phosphorylation in glial cells and a biphasic decrease in neurons.
- Microglia/Macrophages are the predominant cell population responsible for the glial increase in S6 phosphorylation.
- Proliferating cells are typically positive for phospho-S6 24h after injury.

Paper VI: Delayed oral imatinib treatment enhances functional outcome and induces changes in cytokine response after SCI

- Delaying initiation of treatment by 4h still improves recovery of hind limb function. Delaying initiation of treatment by 4, 8, or 24h also accelerates recovery of bladder function.
- Treatment has no negative impact on mechanical or thermal sensitivity or body weight after injury.
- Treatment induces an increase of macrophage-associated cytokines after 7 days in uninjured and injured animals.
- Treatment induces activation of macrophages in spleen, thymus and bone marrow at 7 days in injured and uninjured rats. Treatment decreases macrophage load at the site of injury in the spinal cord.

RESULTS AND DISCUSSION

With no currently available treatment for spinal cord injury, there is an urgent need for an intervention that could reduce the final functional deficits caused by spinal cord injury (Sayer et al. 2006). If one could reposition drugs already in use, this would have the potential advantage of easier progression into clinical trials than purely experimental drugs (Ashburn and Thor 2004). In an effort to reposition drugs for spinal cord injury, we set out to determine the therapeutic potential of three drugs in clinical use for cancer. The three drugs imatinib, erlotinib, and rapamycin, interfere with growth factor induced receptor tyrosine kinase signaling. Targeting the receptors antagonized by these three drugs with other compounds have recently been shown to reduce degenerative secondary events in, or as seen in, spinal cord injury (Erschbamer et al. 2007; Codeluppi et al. 2009; Su et al. 2008). These drugs thus seem to have clinical potential in acute spinal cord injury. Treatment would be expected to protect spinal tissue from secondary degeneration, rather than inducing regeneration of injured axons or even collateral sprouting from remaining axons. As a result of the efforts presented in this thesis, we propose a new candidate treatment for spinal cord injury that should be of considerable interest for clinical trials.

To determine the therapeutic potential of acute treatment with imatinib, erlotinib or rapamycin after spinal cord injury, we revealed additional aspects of importance to both research and clinical trials in spinal cord injury. We specify translational value and parameters of importance for inter-experimental comparison of in vivo and in vitro techniques. In connection to the molecular targets of the three cancer drugs, we also add information about the pathological process during secondary events after spinal cord injury,. Finally, we specify parameters important to reproducibility of experimental research in spinal cord injury and treatment related variables for group stratification in a potential clinical trial.

MODEL CHARACTERIZATION

We set out to characterize an in vitro model and certain aspects of our in vivo model for experimental spinal cord injury. In doing so, we acquired fundamental knowledge about basic biology and pathology of experimental spinal cord injury and were able to determine suitable pre-conditions for studies on the therapeutic potential of cancer drugs.

In vitro

We wanted to characterize an in vitro system with astrocytes and determine the most appropriate pre-conditions and translational value, since receptor tyrosine kinase signaling from our targets of interest was present in astrocytes (Erschbamer et al. 2007; Codeluppi et al. 2009; Su et al. 2008). A modified version of an astrocyte culture

preparation previously described by Tawfik et al was used to characterize several aspects that may be of importance to the outcome of continued experiments with these cultures (Tawfik et al. 2006).

A primary concern with respect to cell culture systems is to avoid contamination or make it minimal. Microglia can contaminate an astrocyte culture and give rise to cytokine expression patterns that would not occur in pure astrocyte cultures (S. Hu et al. 1999). To identify the pre-condition with the least amount of microglia contamination, we tested four culture media from different vendors (Hyclone, Sigma, Gibco, or AM). We also determined if different media composition, defined as different percentages of vendor growth factor mix in media (100%=complete, 10%=starving and 0%=basal), and different substrains would affect microglia numbers. We found that the AM medium results in the least amount of microglia contamination compared to media from the other vendors, independent of the percentage of growth factor mix in the media. The minimal microglia contamination in the AM medium is probably due to the hydrocortisone that it contains, which is known to inhibit microglia proliferation (Ganter et al. 1992). Surprisingly, we found that the cultures obtained from the different substrains caused different magnitudes of microglia contamination. Spinal tissue from the Harlan rat substrain gave rise to less contamination compared to the Charles River rat substrain. However, this was of no concern if the astrocytes were cultured in the AM media, since the contamination then became neglectable for both substrains. This was confirmed by analyzing our cell cultures for expression of cytokine IL-1 β after incubation in either AM medium or medium from Sigma. In accordance with the microglia profile for culture growth in the respective media, we found expression of IL-1 β from astrocytes cultured in medium from Sigma, but not from astrocytes cultured in AM medium. It should be noted that our results cannot determine if the IL-1 β expression was directly due to microglia or indirectly through microglia interactions with the astrocytes.

We went on to determine if our primary cultures of rat astrocytes expressed common astrocytic markers and whether they did so in the different media compositions (complete, starving or basal media). We analyzed levels of GS, GFAP, GLT-1, CNX-43 and S100 β . We found expression of these genes in all media. However, we found the levels of expression to be media dependent for GS and CNX-43. Thus we conclude that media can alter the phenotype of primary rat astrocytes.

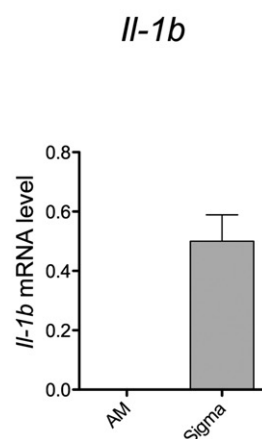


Fig 12. IL-1 β expression in astrocytic cultures.

Culturing primary astrocytes in AM medium that results in cultures devoid of microglia contamination did not result in any IL-1 β expression.

Astrocytes cultured in Sigma medium, containing microglia, express IL-1 β .

Figure from Paper I.

So far, the astrocyte cultures in the AM medium had the least microglia contamination and proportionally highest expression of astrocyte genes. We next determined which rat substrain corresponded best to corresponding human astrocyte phenotypic traits in different compositions of AM media. We arrived at two conclusions. First, astrocytes from the Harlan rat substrain seem to be more similar to human astrocytes than astrocytes from the Charles River substrain. Second, astrocytes from the Harlan substrain (and human astrocytes) react to starvation. Four of our genes, GS, GFAP, CNX-43 and S100 β are considered pro-inflammatory, because they have been found to be up-regulated under inflammatory conditions in vitro and in vivo (do Carmo Cunha et al. 2007; Benton et al. 2000; Cronin et al. 2008; Eng and Ghirnikar 1994; I.-H. Lee et al. 2005). Conversely, GLT-1 is considered anti-inflammatory (Tawfik et al. 2006). We found no change in GLT-1, but since levels of GFAP and GS were reduced in starving or basal media, these astrocytes can be considered to go from a reactive state in complete media to a more quiescent state in starving or basal media. Since pre-incubation in starving or basal media is done prior to any stimulation with for example growth factors, it is of interest that growth factors may induce a more reactive state in cultured astrocytes.

Finally, we compared astrocyte associated protein expression in astrocytes from the Harlan substrain with human astrocytes. We found a similar expression of the five markers tested, GFAP, Aldh1L1, Nestin, GLT-1 and S100 β . There was no apparent discrepancy in morphology; however, we found human astrocytes to be larger than Harlan rat astrocytes, which is in line with previous findings (Oberheim et al. 2009).

In conclusion, we determined that AM medium is appropriate to use for astrocyte cultures. In our case, the use of AM medium results in a culture able to produce results specifically reflecting the astrocytes, due to minimal microglia contamination and robust expression of astrocyte associated genes and proteins. Furthermore, for these conditions, astrocytes from the Harlan substrain seem to have the highest translational value. Thus we have found an in vitro procedure for producing primary rat astrocytes that may be used to determine effects of receptor tyrosine kinase signaling.

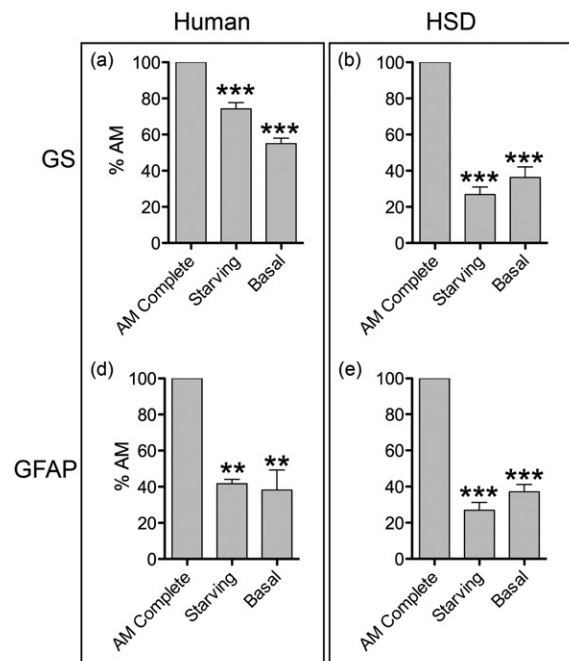


Fig 13. Harlan astrocytes share phenotype characteristics with human astrocytes.

Harlan astrocytes reacted similar to starvation as human astrocytes for the investigated gene expression. Above figure, depicting GS and GFAP gene expression comparison, is from Paper I.

In vivo

In Paper II we determined the effect of imatinib treatment on hindlimb locomotion by scoring of hind limb function after a moderate injury. However, in Papers IV and VI we used mild injury in order to be able to confirm scores by using automated gait analysis. Moreover, we aimed to determine effects on certain sensory functions after imatinib treatment in Paper VI, which is only possible with a mild injury that permits hindlimb weight support. Bladder recovery was the third functional parameter of importance, since improvement of this parameter was one of our main findings in Paper II.

Interestingly, it has previously been found that there exists differences in functional recovery amongst different strains of rat after spinal contusion injury (Mills et al. 2001). Even substrain differences have been found in other rodent models of CNS pathologies (Yoon et al. 1999; Swerdlow et al. 2000; Nicholson et al. 1994; deLuca et al. 2010). Hence, to determine if there were any substrain differences and to find the most appropriate substrain for locomotion, bladder and sensory assessment after mild contusion injury to the spinal cord, we set out in Paper III to determine the spontaneous functional recovery of these parameters for three Sprague-Dawley rat substrains.

We investigated the Sprague-Dawley substrain used in Paper II, from the vendor Scanbur, and two common Sprague-Dawley substrains, also studied in Paper I, from the vendors Harlan and Charles River. Assessing recovery of bladder, locomotion and sensory function until 8 weeks after injury, we found there to be similarities, but also robust differences among these substrains.

In assessing locomotion in an open field using BBB scoring, we found that the Scanbur and Charles River rat substrains had similar recovery of both BBB scores and subscores, while the Harlan rat substrain regained hindlimb function to a greater extent than the other two substrains. Limb coordination as determined by BBB scoring was confirmed by automated assessment of coordination (regularity index). However, these automated coordination measurements were slightly higher for the Charles River rats compared to Scanbur rats. The automated assessment device (Noldus Catwalk) can determine many different stepping parameters and we found the final measurements for

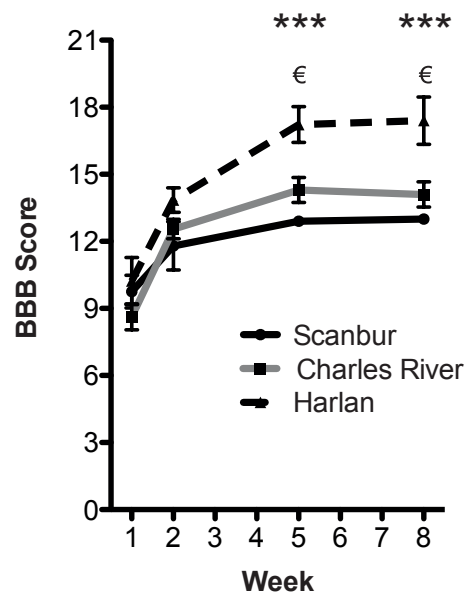


Fig 14. Substrains differ in locomotor recovery. The Harlan, Scanbur and Charles River rat substrain locomotion was assessed by BBB scoring for 8 weeks after injury and with automated measurements of coordination (regularity index) during week 6,7 and 8. Here the observed coordination is exchanged for the regularity index in order to produce new BBB scores. Figure from Paper II.

both base of support and stride length to be more normalized for Harlan rats compared to Scanbur and Charles River rats.

When investigating possible differences in sensory recovery in Paper III, we tested mechanical sensitivity of the hind paws using von Frey filaments. Both Scanbur and Charles River rats became hypersensitive after injury, and this hypersensitivity normalized by week 6 and 7 for Charles River and Scanbur rats, respectively. However, Harlan rats did not become hypersensitive and were instead slightly hyposensitive at week 8. Furthermore, bladder recovery was faster in the Harlan rat substrain, compared to the other two substrains, with seemingly similar recovery rates. These results show that the Harlan substrain is not as suitable as the Scanbur and Charles River rats for testing potential treatments under these conditions, since the magnitudes of the functional deficits caused by injury were too small. Even though Scanbur rats and Charles river rats recovered in a similar fashion, we conclude that Scanbur would be most appropriate to use, since it had lower Regularity index levels and a longer time window of hypersensitivity, compared to Charles River rats.

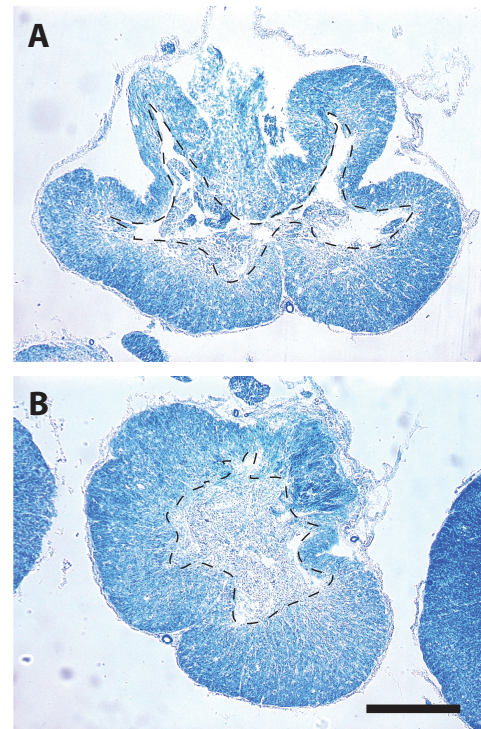


Fig 15. Spinal cord injury affects morphology differently in different substrains. Representative picture of tissue sections from the injury site of two Sprague-Dawley substrains from the vendor (A) Scanbur and (B) Harlan. Figure from Paper II.

We also found histological correlation with these functional discrepancies, including white matter sparing, neurofilament loss and distinct morphological differences. The morphological differences are interesting, since the tissue sparing pattern seen in Harlan rats may signify sparing of specific motor axons and constitute a better indicator of degree of locomotor recovery than the volume of spared white matter (Raineteau and Schwab 2001; L. T. Brown 1974; Metz et al. 1998; Schucht et al. 2002).

The differences between substrains found in Paper III may have several causes, such as genetic factors, geometry of the spinal canal, differences in dura properties etc. However, we did not observe any differences in the inflammatory response. We do confirm substrain differences for spinal contusion injuries; however, we can only speculate about the possibility of substrain differences for other types of experimental spinal cord injuries. The results were not only important for our choice of substrain for future experiments with a mild injury, but shows the importance of determining basic parameters when using new injury types, assessment methods, different strains and even substrains. Indeed, it is not uncommon to find discrepancies between laboratories

in the literature (X. Z. Liu et al. 1997; Ozawa et al. 2002; Yu et al. 2000). Our results suggest that such differences may not only be due to site specific differences, such as for example surgical skill or differences in assessment of functional recovery, but possibly also to the use of different substrains.

In conclusion, we have determined pre-conditions for an in vitro and an in vivo model that carries considerable translational value. This allowed further investigations of the therapeutic potential of imatinib, erlotinib, and rapamycin, but also highlights some important aspects of these model systems to the research field as a whole, especially concerning the use of different substrains.

THE THERAPEUTIC POTENTIAL OF RECEPTOR TYROSINE KINASE SIGNALING INTERFERENCE

Receptor tyrosine kinases are primarily activated by different growth factors and pro-inflammatory cytokines and are thus the target of many cancer drugs (Levitzki and Gazit 1995; Zwick et al. 2001; Gschwind et al. 2004). Much is known about the receptor tyrosine kinases signaling pathways and about drugs that interfere with signaling from these ligands.

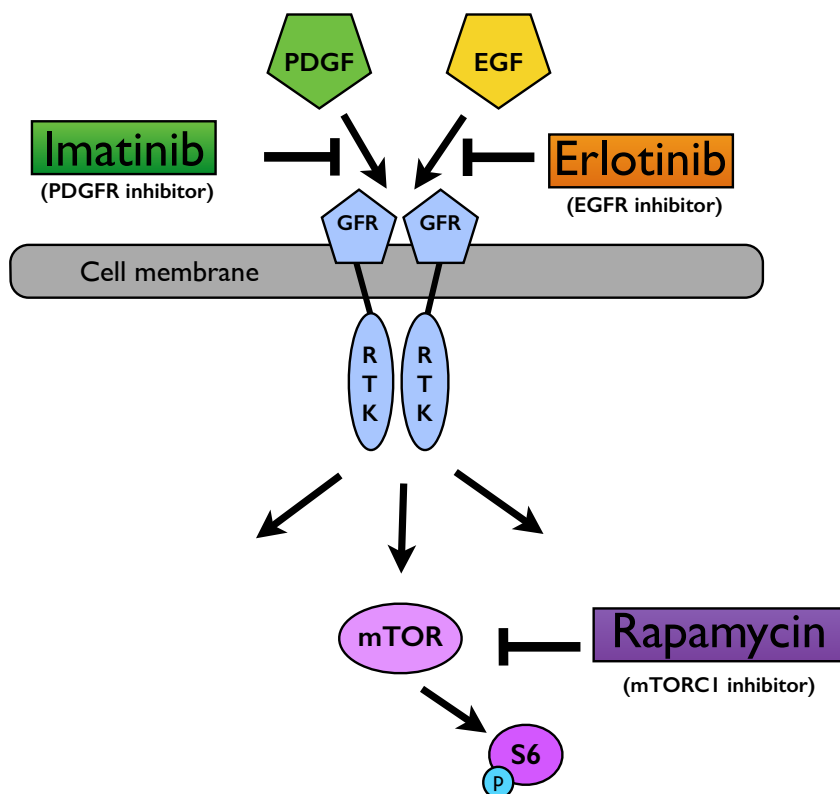


Fig 15. The three cancer drugs imatinib, erlotinib and rapamycin and their respective molecular targets. Figure modified from Paper IV.

Research into the role of growth factors in the intact and compromised CNS has revealed distinct, but also overlapping, roles which might be exploited for therapeutic interventions (Reuss et al. 2003; Andrae et al. 2008; Rosenstein et al. 2008; Korsching 1986; Werner and Leroith 2014; Unsicker and Strelau 2000; Greenberg et al. 2009). However, repositioning an antagonist, as opposed to an agonist of RTK signaling for spinal cord injury may seem counterintuitive. Many experimental interventions aimed at improving outcome after spinal cord injury have focused on promoting nerve growth with nerve growth factors such as NGF, NT3 and BDNF (Lindsay 1988; Tuszynski et al. 1996; Kim et al. 1996; Oudega and Hagg 1996; Hefti 1986). While the NGF family of growth factors stimulates neurite growth, we focused on growth factors with other effects, and targeting other cells of the CNS. This approach focuses on rescue, rather than repair, and aims to reduce the harmful secondary events that take place acutely after injury (Tsai and Tator 2005).

We decided to investigate the therapeutic potential in spinal cord injury of the three RTK signaling antagonists imatinib, erlotinib and rapamycin. on the basis of evidence for the ability of this class of drugs to reduce certain harmful secondary events in, or as seen in, SCI (Codeluppi et al. 2009; Su et al. 2008; Erschbamer et al. 2007). Imatinib acts upon PDGFR, and Erlotinib on EGFR, while Rapamycin inhibits a downstream kinase complex of both growth factor receptors, called mTORC1. Imatinib, erlotinib and rapamycin are all used clinically against different forms of cancer (Tsao et al. 2005; Wood et al. 2002; Bjornsti and Houghton 2004). An additional advantage of these drugs is that they can be administered systemically as opposed to locally.

We set out in Papers II, IV, V, and VI to determine the therapeutic potential of imatinib, erlotinib and rapamycin in a well defined spinal contusion injury model. We found that imatinib appears suitable for clinical application and provide a treatment time window as well as possible biomarkers for effect of this drug.

Below we present the results for each treatment separately, but there were certain experimental conditions that applied to all the studies of the three treatments. Specifying these conditions are important, since they do provide a rationale for the experimental set up and will be a central part of the discussion of our results. We have used similar drug administration protocols, and, we have used doses that should be clinically translational. Cancer drugs can give severe side effects if given in too high doses, especially since they are given systemically, and thus there is a maximum tolerated dose for each drug (Gurney 1996). This restricts the possible doses worth testing experimentally in planning for clinical trials (Hoshino-Yoshino et al. 2011). None of the tested drugs penetrate well into the intact CNS. However, a treatment window presents itself because injury will compromise the blood-spinal-cord-barrier and make it permeable to small molecules at least during the first week after injury (Habgood et al. 2007; Figley et al. 2014). It should be noted that because we treated the animals systemically rather than locally, improved recovery after spinal injury is not necessarily due to effects exerted directly at, or near the injury site. However, the marked local improvement of spinal cord morphology seen in the imatinib studies,

strongly suggests that local effects had contributed. It should also be noted that we did not test for additive or synergistic effects of any of the four possible combinations of erlotinib, imatinib and rapamycin.

Erlotinib

A study from our group had previously demonstrated the potential of improving recovery after a spinal contusion injury by local inhibition of EGFR using PD168393 (Erschbamer et al. 2007). EGFRs was found to be expressed in activated microglia or macrophages and astrocytes after spinal cord injury, thus leading to the interpretation that local treatment with the EGFR inhibitor improved recovery by altering the secondary response to injury for these cell types. These were very intriguing results; however, to move an experimental treatment into clinical trials takes many years and local administration can be difficult to implement clinically.

Erlotinib is the active compound of the commercially available cancer treatment Tarceva and its molecular target is also the EGFR. If systemic treatment with erlotinib of experimental spinal cord injury would have effects similar to those found with the experimental EGFR inhibitor, this would be of significant translational value. With this rationale we tested if oral erlotinib treatment, starting 30 min after injury and given once daily for 5 days, would improve functional recovery after spinal cord contusion injury. We assessed hindlimb locomotor function and bladder recovery and found an accelerated recovery of both these functional parameters. However, at the end of the 10 week observation period control animals had recovered BBB scores and

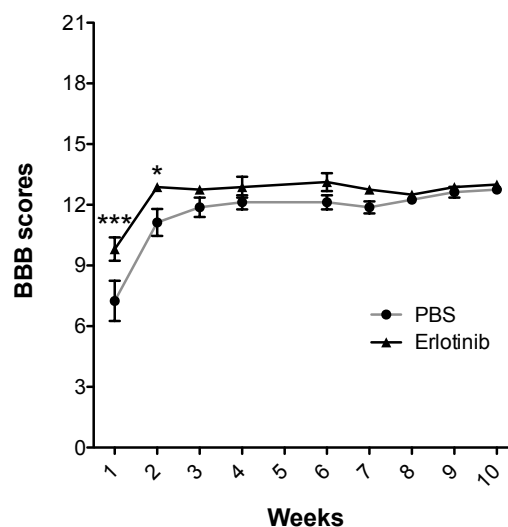


Fig16. Erlotinib accelerates locomotor recovery. Hindlimb locomotor recovery was assessed using the BBB score for 10 weeks after injury. Figure modified from Paper IV.

bladder function to the same extent as erlotinib-treated animals. Since we subjected the rats to a mild injury we were also able to perform automated assessment, but only when a majority of the animals had regained hind limb weight support, which our rats typically did after a mild injury at week 2,.

The treatment did not alter weight loss of the animals and no obvious side effects of the drug were detected. Hence, we have no reason to believe that the drug dose induced severe side effects that could have compromised possible functional improvements as the weeks progressed. It therefore seems the drug had early positive effects that were not strong enough to raise function of treated animals above the level that was finally

reached also by non-treated animals, whereas Erschbamer et al. found that a limited period of local PD168393 treatment lead to permanently improved functional recovery.

Erlotinib is a reversible inhibitor in comparison to the irreversible and very potent inhibitor used by Erschbamer et al, which may explain the lesser effects of erlotinib (Levitzki and Mishani 2006). Furthermore, it is possible that the dose was lower at the injury site with our systemic treatment, although we used the highest dose reportedly tolerable without side effects (EMA Tarceva report). If this dose was too low, a large increase of dose would not be an option for systemic administration of the current galenic formulation. Notably, there are ways of increasing the dose without increasing toxicity, such as nanoencapsulation (Marslin et al. 2009). The treatment period was 5 days, similar to what had been used when administering imatinib in Paper II, but shorter than the two weeks used by Erschbamer et al. An increased treatment period could perhaps have improved the outcome; however, such a schedule could also result in increased toxicity and would need careful investigation. Nevertheless, there seems to be room for further optimizing erlotinib treatment for SCI. It cannot be excluded that this drug may be useful in combination with other drugs to produce lasting functional improvements. Our results do not exclude clinical usefulness of this drug, with the current treatment schedule or in combination with other drugs, in spinal cord injury.

Rapamycin

The mTOR pathway is one pathway downstream of receptor tyrosine kinases such as EGFR and PDGFR. Rapamycin inhibits mTORC1, and had been found to reduce astrocyte reactivity in a model of ischemic spinal cord injury (Codeluppi et al. 2009). To be able to reduce astrocyte reactivity might be beneficial also in a spinal cord contusion injury. The effects we found in Paper II and later in Papers IV and VI might have been due to reduced activity of mTORC1. This motivated us to determine if rapamycin would have similar beneficial effects as had been found for treatment with imatinib. Furthermore, if rapamycin would produce beneficial effects, they might be additive or synergistic in combination with erlotinib or imatinib. Prior to any behavioral experiments to determine therapeutic potential of rapamycin, we characterized mTORC1 activation after spinal cord contusion injury. In doing so, we hoped to determine a potential treatment window, as well as learn more about mTORC1 activation after injury.

We induced a moderate spinal cord injury and determined mTORC1 activation by immunohistochemistry of downstream phosphorylation of ribosomal protein S6 1 day and 1, 3, 4, 6, 8, and 17 weeks after injury. The investigated cell types included astrocytes, neurons, oligodendrocyte progenitor cells, microglia, activated microglia/macrophages, as well as proliferating cells.

In agreement with previous work, we found certain neuronal populations with constitutive mTORC1 activation, especially the motor neurons of the ventral horns (see

Fig 17) (Cota et al. 2006; Norsted Gregory et al. 2010). In contrast, few glial cells displayed any mTORC1 activation in uninjured animals. However, it was this group of cells that displayed increased activation of mTORC1 in response to injury. One day after injury we observed mTORC1 activation in many glial cells, while there was a reduction of the number of neurons that displayed mTORC1 activation in the medial grey matter. At this time point, mTORC1 activation is quite homogenous in white matter and we found that all examined glial cell types expressed pS6. Interestingly, we found microglia, rather than astrocytes to constitute the largest proportion of glial cells expressing pS6 and thus presumably mTORC1 activation (see Fig 17). We identified no or very few neutrophils displaying mTORC1 activation, while most of the cells defined as proliferating by being Ki-67-positive also expressed pS6. Proliferating cells at this early time after spinal injury have previously been identified as different forms of glia (Zai and Wrathall 2005; Lytle and Wrathall 2007).

For the later time points, we found a reduction of mTORC1 activation at 1 week after injury, followed at 3 weeks by a second longer period of elevated mTORC1 activation. At this time, pS6-positive glial cells were found not only in and around the injury site, but also, to a lesser degree, rostral and caudal to the injury site. The majority of the mTORC1 activation was now localized to areas of inflammatory activity and while astrocytes, oligodendrocyte progenitor cells, and ramified microglia were represented, the population of activated microglia and/or macrophages was now the major contributor to the observed mTORC1 activation. This scenario, present at 3 weeks after injury,

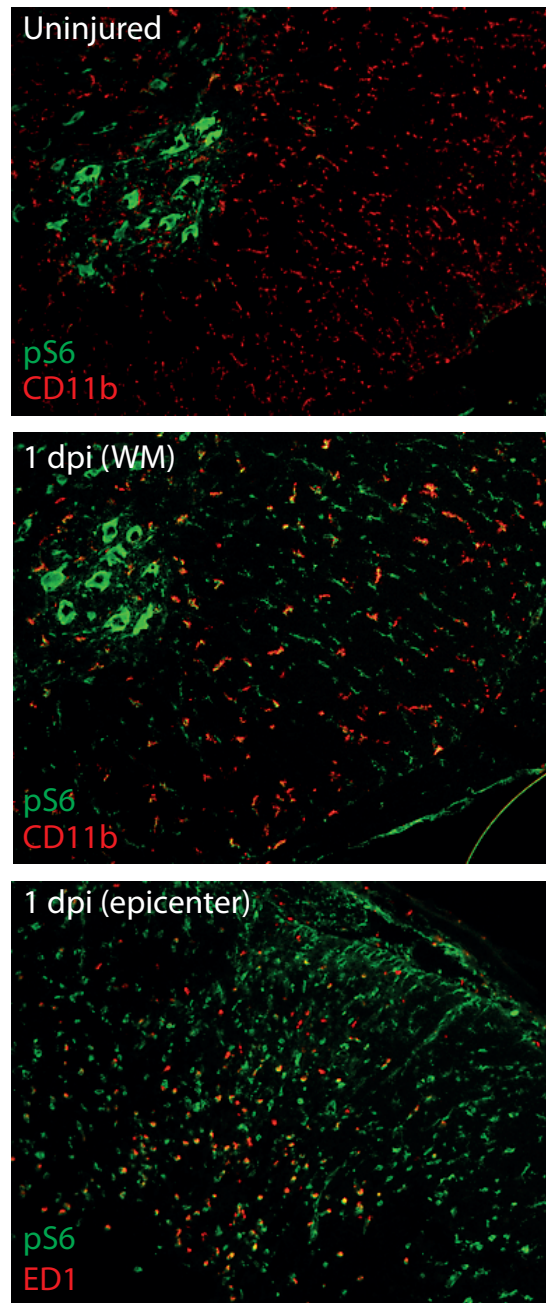


Fig 17. mTORC1 activation mainly in inflammatory cells. Representative pictures of pS6 immunoreactivity and markers for microglia (Cd11b) and macrophages (Ed1) at 1 day after injury compared to tissue from an uninjured animal.

reached a peak of mTORC1 activation at 6 weeks after injury, and was no longer present at 17 weeks after injury. In addition to this reduction in glial mTORC1 activation at 117 days after injury, we found fewer neurons displaying mTORC1 activation caudal to the injury site.

In conclusion, there seems to be two phases of mTORC1 activation, the first phase dominated by microglia and the second phase dominated by foamy activated microglia or macrophages. Hence we have identified two treatment windows for inhibition of mTORC1 activation that could be targeted by rapamycin. It is interesting to find immune cells to be the dominant population of cells that display mTORC1 activity during its biphasic increase since previous studies have mostly focused on neurons and astrocytes. Nevertheless, our characterization spurs more questions concerning the role of mTORC1 activation in inflammatory cells after injury. mTORC1 activation can promote cytokine expression, cause pro-inflammatory activation and even polarize activated microglia or macrophages towards a phenotype that can promote either degeneration or regeneration (Chen et al. 2012; D.-Y. Lu et al. 2006; Mercalli et al. 2013; Russo et al. 2009). Whether this is true for the microglia/macrophage populations that display mTORC1 activation after spinal cord injury remains to be examined. Some aspects of the inflammatory response may be negative, other positive for recovery after spinal cord injury. Therefore, it may be just as important that we have determined time points when mTORC1 activation is low in these immune cells (David and Kroner 2011; Kigerl et al. 2009; Rapalino et al. 1998).

Under certain circumstances, neurite growth has been found to be stimulated by mTORC1 activation, although current observations from genetic models are not sufficient to consider this approach a candidate for clinical application (K. Liu et al. 2010; Meikle et al. 2008). Nevertheless, the functional improvements seen in experimental spinal cord injury with treatments that increase ATP have been attributed to mTORC1 activation, and it is not far fetched to speculate that some of the benefits of growth factor treatment might be mediated by activation of this pathway (L. Y. Hu et al. 2010). We have contributed by providing a temporal map that may be used to improve regeneration of neuronal circuits in SCI by targeting mTORC1. It is possible that cell specific modulation of mTORC1 could be a beneficial treatment option. It is interesting to note that there can be convergent neuro-immune signaling that promotes beneficial synergistic effects from one ligand activating the mTORC1 pathway both in neurons and inflammatory cells (Gensel et al. 2012). In conclusion, we find mTORC1 activation is spatiotemporally located to several injury induced secondary events, such as for example proliferation, inflammation, and astrogliosis, which may serve as a foundation for further research into the therapeutic potential of this pathway in SCI.

In our pre-clinical attempts to determine the therapeutic potential of rapamycin in SCI, we targeted the first phase of mTORC1 activation, as we had done for the imatinib and erlotinib treatments. We induced a mild injury and with a 30 min delay, we administered the first dose, followed by daily doses for two weeks. Hind limb locomotion was scored and gait parameters were also registered. We also assessed bladder recovery by measuring residual urine. We did not find any effects of rapamycin on any of these functional parameters. Likewise, there seemed not to be any effects on weight and no indications of drug toxicity. Shortly after finalizing this experiment, Sekiguchi et al reported improved locomotor outcome in response to acute rapamycin treatment in mice with spinal cord injury (Sekiguchi et al. 2011). Hence we decided to also study a cohort of rats with a more severe injury. However, acute rapamycin treatment after a moderate spinal cord contusion injury also failed to cause any overt beneficial effects on either recovery of hindlimb locomotion or bladder function.

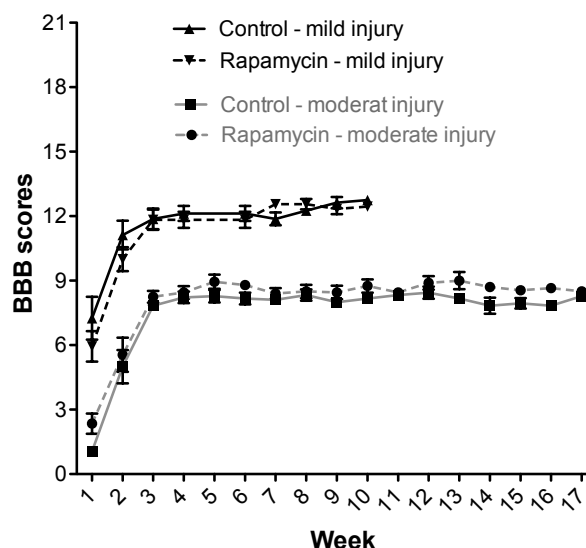


Fig 18. **BBB scores from rapamycin experiments.** Hindlimb BBB scores for mild (12.5 mm) and moderate (25 mm) spinal contusion injury. Figure modified from Paper IV.

We conclude that a clinically translational dose of rapamycin during 14 days does not improve recovery of gait or bladder function in our experimental models. We did not, however, determine if there were any effects on sensory function, which is a possibility since mTORC1 has been shown to have a role in generating pain symptoms in models of nerve injury (Géranton et al. 2009). The discrepancy between our results and those of Sekiguchi et al could perhaps be ascribed to species differences, since mice and rats mount different inflammatory responses to spinal cord injury. The rapamycin dose was quite similar, 1.5 mg/kg in our study and 1 mg/kg in the study by Sekiguchi et al, but Sekiguchi et al gave only one initial dose after injury, while we administered the drug daily for two weeks. In light of these results, we suggest that further work is needed in order to determine if rapamycin may have any therapeutic potential and if mTORC1 may be responsible for the beneficial effects of erlotinib or imatinib.

Imatinib

After spinal cord injury the blood-spinal cord barrier becomes permeable and leaky to large molecules, which disturbs the extracellular milieu and contributes to edema. In

the CNS, tPA seems to be necessary and sufficient to cause BBB permeability (Keck et al. 1989; L. F. Brown et al. 1995; Yepes et al. 2003). Interestingly, tPA has been found to induce production of PDGF-CC that was shown to trigger PDGFR- α signaling leading in turn to increased BBB permeability in a model of stroke (LI et al. 2000; Fredriksson et al. 2004; Su et al. 2008). Interestingly, Su et al also found that Imatinib, a cancer drug in clinical use, counteracted the BBB permeability by its ability to block PDGFR- α . The blood-spinal cord barrier breach is a rapid secondary event in spinal cord injury that is thought to aggravate other secondary events. Hence, we hypothesized that improving the integrity of the barrier using imatinib would protect tissue after spinal cord injury.

In Paper II we subjected rats to a moderate injury and administered imatinib in doses corresponding to high but tolerable doses in humans. An initial dose was delivered 30 min after injury and was followed by daily doses for 5 days after injury. We assessed hindlimb locomotor function, sensorimotor function and recovery of bladder function and found the imatinib improved all these functional parameters. Hindlimb locomotor function was monitored using BBB scoring. We found that most imatinib treated animals regained the ability to support weight, while most of the control animals did not. Sensorimotor capability was tested using the contact plantar placement (CPP) score. Imatinib treatment increased the ability to position the hind paw in response to a light touch to the dorsal face of the foot. We used amount of residual urine as a measure of bladder recovery, and found that imatinib caused a dramatic acceleration of bladder recovery. Imatinib treatment allowed full recovery of the ability to empty the bladder, while this was not the case for all control animals.

Since we believed that the rapid-onset 5-day treatment would be protective, we expected to find more tissue sparing, including more spared axons. We found this to be the case and the proportion of axonal sparing was even greater than the tissue sparing caused by imatinib, suggesting that even a small increase in tissue sparing may significantly impact

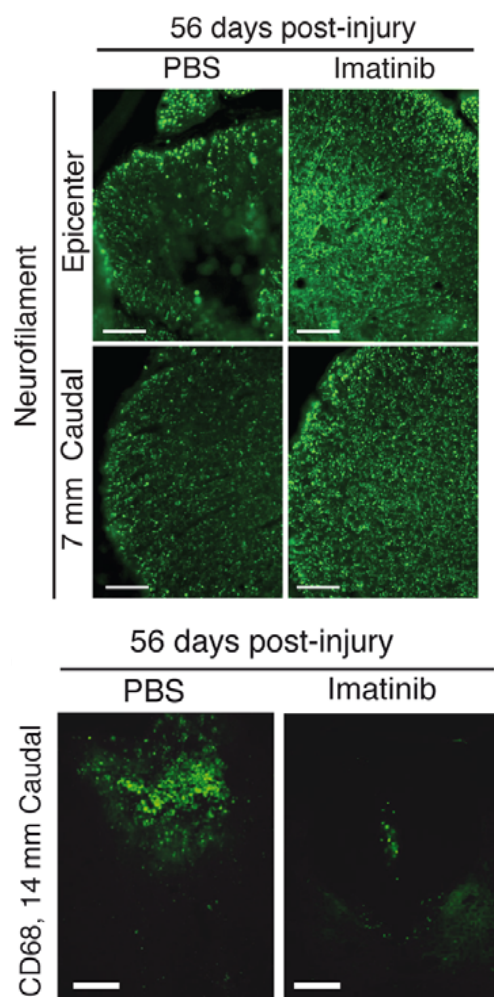


Fig 19. Imatinib spares axons and reduces inflammation. 56 days after injury we determined greater immunoreactivity of NF-200, while we found less ED1 immunoreactivity, in the imatinib treated group compared to the control group. Figure from paper II.

survival of axonal projections. We also observed a reduction of the activated microglia and macrophages in treated animals at the final time point of the experiment, 56 days after injury. This was also seen 5 days after injury, the last day of treatment. However, the number of neutrophils was not affected by treatment at 24h after injury.

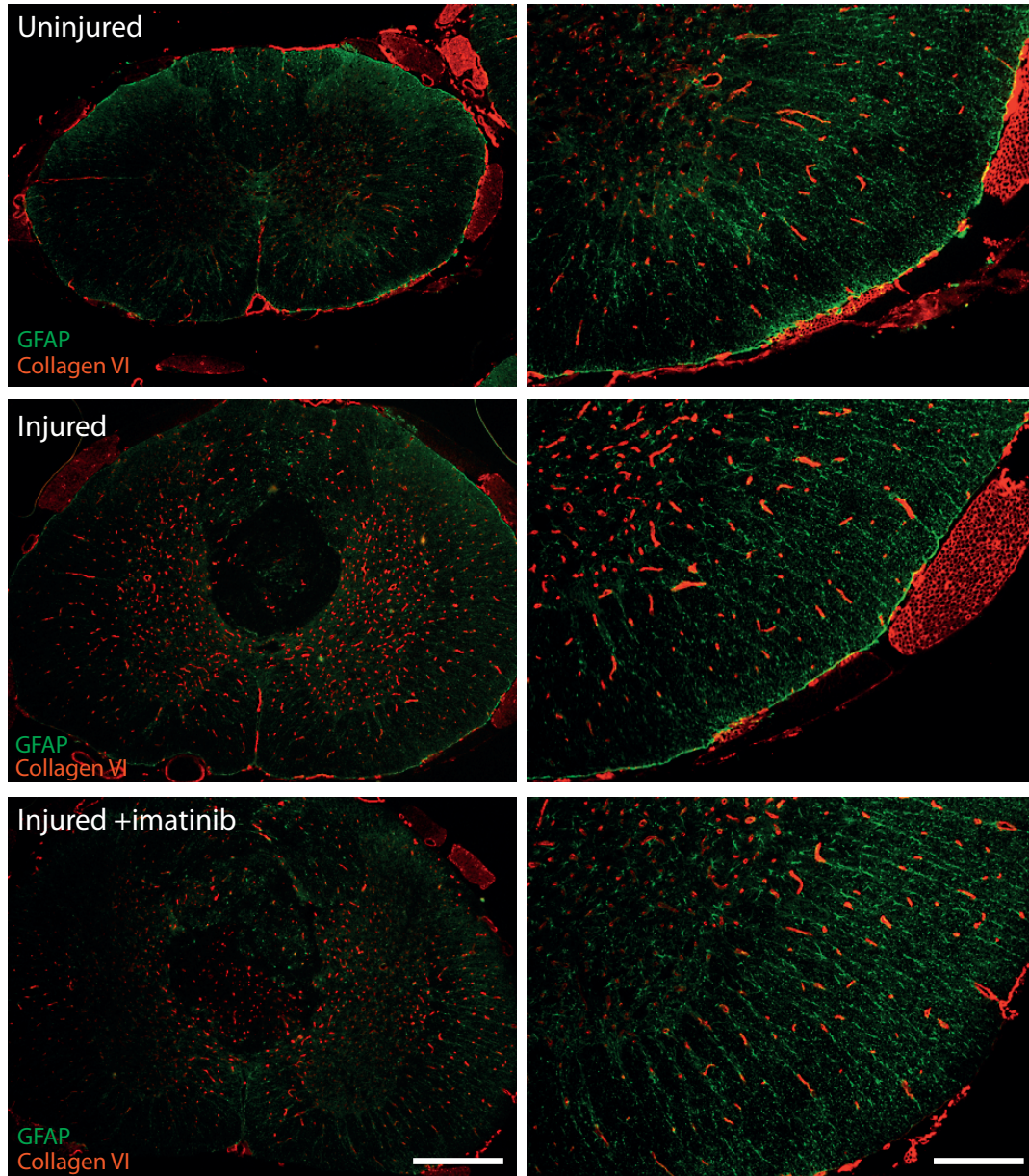


Fig 20. Imatinib attenuates vascular collagen VI production after injury. 1 day after injury we find that the increase of collagen is reduced by imatinib treatment both caudal and rostral to the injury site. Representative pictures 7 mm caudal to injury.

Since we assumed one effect of imatinib would be to reduce blood-spinal cord permeability, we determined leakage of albumin and IgG into the spinal cord parenchyma at the site of injury, hallmarks of a disrupted barrier (Seitz et al. 1985; Poduslo et al. 1994; Jiang et al. 1992). We confirmed that imatinib improved vascular integrity and also found that imatinib reduced edema formation. In line with these

observations, we observed that endothelial tight junctions were normalized and that PDGFR- α and PDGFR- β were less expressed in spinal tissue from animals treated with imatinib. PDGFR expression was clearly affected even at 24h after injury and imatinib also normalized collagen expression around spinal cord blood vessels (see Fig 20.) (Loy et al. 2002). Our results point to a robust effect on the neurovascular unit (Dore-Duffy et al. 2000; Stanimirovic and Friedman 2012). Together, this suggests that imatinib improved blood-spinal cord barrier integrity and that this may have caused or contributed to the reduction of tissue destruction.

From the literature, it seems probable that imatinib can counteract pericyte migration away from the blood vessels towards the injury site, an effect that may be part of the blood-spinal cord barrier normalization effect (Dore-Duffy et al. 2000; Göritz et al. 2011; Song et al. 2009; Su et al. 2008). This in turn, may lead to less injury-derived cytokines, chemokines and other molecules entering the blood stream, thus reducing arrival of infiltrating inflammatory cells to the site of injury. Clearly, imatinib may have additional effects, possibly acting directly on the immune cells (Z Adzemovic et al. 2013; Pardanani and Tefferi 2004). For example, in Paper II, we find that activated microglia express PDGFR- α . Importantly, imatinib was administered systemically, which means the macrophages that infiltrate the spinal cord at around day 3 after injury could potentially have been affected already when residing in lymphoid organs such as bone marrow and spleen.

Imatinib is not specific for the receptor tyrosine kinase of the PDGFR, and has been shown to also interfere with the c-kit receptor and TGF- β receptor. Through these receptors, imatinib has been shown to improve outcome in diseases related to a hyperactive immune system, other than leukemia (Balachandran et al. 2011; Helbig and Kyrz-Krzemień 2011; Berlin and Lukacs 2005). Hence imatinib could potentially have several direct effects via receptor tyrosine kinase inhibition other than improving vascular integrity through PDGFR. The multitude of targets for imatinib may indeed be the reason for its effectiveness in our spinal cord injury model. Moreover, it is not necessarily the direct effects, but perhaps indirect effects of imatinib, or a combination thereof, which has caused the observed tissue preservation. Such effects can be of primary importance for the therapeutic potential of imatinib. Changes in secondary events, or non-direct effects, may also have predictive value in clinical settings. As we continued with preclinical experiments in Paper VI, we kept this in mind for our experimental setup.

In Paper VI we had two main aims; to determine if imatinib treatment seemed reasonable to use clinically and if so, could we provide variables for group stratification specific for this treatment that would further improve its translational value. Immediate administration of imatinib will not be possible clinically and thus we aimed to determine if a delayed treatment would still carry the beneficial effects we had observed with the 30 min delay. We settled for a 4 hour delay, since this would allow many spinal cord injured persons to reach the clinic. We also wanted to make sure that our treatments had no negative effects on sensory function or promoted development of

pain symptoms (Hofstetter et al. 2005). We chose mild injury, to allow tests of sensitivity to mechanical stimuli and temperature. We extended the duration of the treatment period from 5 to 14 days, since we performed a pilot study that determined a 14-day treatment period to have no negative effects, and might possibly confer further benefits in terms of functional recovery.

In assessing locomotion with a 4h delay of treatment after a mild injury, we could use both observational and automated assessment. We did find the treatment to improve limb coordination and hindlimb function, with the BBB score and subscore, respectively. Automated measurements confirmed the improvements found for limb coordination with the BBB score and revealed improvements of other measurements of hindlimb function. Bladder recovery was also improved, similarly to what we observed with a 30 min delay. Hindlimb sensory function was not affected negatively and the group treated with imatinib had consistently more normal scores for both sensitivity to mechanical and thermal stimuli compared to the control group, suggesting a possible beneficial effect of treatment. Weight curves also suggested beneficial effects of imatinib. In conclusion, imatinib seems suitable for application as an acute treatment for spinal cord injury.

Following up on the delayed treatment, we next tested if imatinib still had beneficial effects if initiation of treatment was delayed 8 or 24 hours. Defining treatment windows can also be used to stratify a clinical trial cohort (B. K. Kwon, Okon, et al. 2010a; Krishna et al. 2013). We found that an 8 and even a 24h delay of initiation of imatinib treatment still improved bladder recovery in a seemingly similar way as when treatment start was delayed 4h. However, with the two longer delays, we no longer observed improvements of hindlimb function. On the one hand, these results emphasize the importance of initiating treatment as early as possible. On the

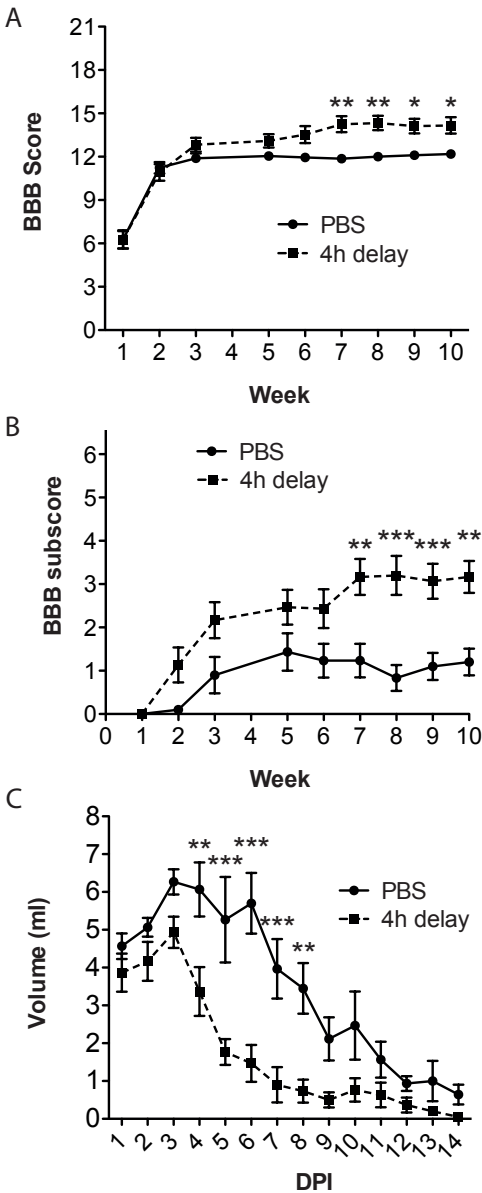


Fig 21. Improved functional outcome with a 4h treatment delay. We assessed hind limb locomotion during 10 weeks after injury and bladder recovery during two weeks after injury. (A) BBB score (B) BBB subscore (C) Residual urine. Figure reorganized from Paper VI.

other hand, we do find robust functional improvements of bladder function even with a 24 hour delay, suggesting that patients could be included also with such a long delay of treatment start. In case of a clinical trial, it will be important to stratify patients during post-hoc analysis with respect to time until the first dose of imatinib.

The reason why imatinib did not lead to beneficial effects on locomotion when treatment was delayed by 8 hours is not known. It is tempting to conclude that only the 4 hour delay was able to rescue sufficient neural tissue, since this is the time after injury when neurons and glia start to undergo apoptosis (Lou et al. 1998; Crowe et al. 1997; Shuman et al. 1997; Evelynne Emery et al. 1998). In particular, the loss of oligodendroglia in the injured area may aggravate loss of descending and ascending axons that run across the site of injury or impair impulse flow in such axons. It follows that any treatment for spinal cord injury that aims to protect and rescue spinal tissue, should be administered during the time window between primary injury and programmed neuronal cell death. It should be noted that we did not test a 6 h delay, and also that acceptable delay times might be longer in larger animals than in rodents. Furthermore, imatinib requires 1-2 hours to become bioavailable when administered orally (Peng 2004), hence an i.v. solution could theoretically give similar results, as found here with a 4h delay, with a 5-6h delay.

Experimentally, we have defined parameters, such as injury severity and animal strain or substrain; however, such defined and predictable parameters are not present clinically. Patients will all have different injuries with different recovery patterns; they can be fast or slow metabolizers for certain drugs, there is considerable genetic variability between individuals (possibly differences in CNS vulnerability, plasticity and ability to recover from injury), they may need other drugs that interfere with a drug such as imatinib, and they can, depending on the injury, have impairments of the gastrointestinal system and thus impaired drug absorption (Haouala et al. 2011; Gondim et al. 2010). Hence it would be advantageous, especially in the case of a clinical trial of acute drug treatment for SCI, to have an easily measurable biomarker for a known effect of the drug (B. K. Kwon, Stammers, et al. 2010b). Imatinib has previously been shown to affect levels of many inflammatory cytokines (Z Adzemovic et al. 2013; Daniels et al. 2004; Mokhtari et al. 2011; Hayashi et al. 2012). The expression of cytokines in CSF and neural tissue after injury has been investigated extensively, but circumstances may not always allow CSF sampling and any change of e.g. cytokine values in CSF may still not necessarily related to levels in the injured area of the spinal cord (Bromander et al. 2012; Stammers et al. 2012; B. K. Kwon, Stammers, et al. 2010b; Csuka et al. 1999; Shechter et al. 2013). Blood is the most easily acquired analyte and is used clinically for analysis of S100 β patients with spinal cord injury (Marquardt et al. 2006). Hence we aimed to determine if there were changes in inflammatory biomarkers in serum during the time of treatment that could be used as a proxy biomarker for imatinib, indicating that effective doses have reached the circulation.

We selected a 4h delay for the imatinib treatment. To be able to determine if a possible biomarker could also be a “disease” biomarker, we compared lesioned animals with and without imatinib treatment, a group that received sham surgery, as well as an uninjured control group to define basal cytokine values. We took serum samples 1, 3 and 7 days after injury in injured groups and analysed for MCP-1 and MIP3- α .

Both MCP-1 and MIP3- α were found to be elevated at day 1 for all groups that had undergone surgery, compared to basal levels. However, both MCP-1 and MIP3- α was further increased 7 days after injury in the group that received imatinib in comparison to the other groups that either had similar or lower values compared to day 1 after injury. Furthermore, inspecting cytokine levels in individual animals in the group that received imatinib treatment, we found a significant difference between day 1 and 7 after injury for both MCP-1 and MIP3- α . This suggests that an individual can be its own control using these biomarkers, which would be an advantage in a clinical setting. We also found MIP3- α to be a more reliable biomarker than MCP-1, which is in line with previous observations for MCP-1. MCP-1 has previously been shown to increase in leukemic patients receiving imatinib for long periods of time (Hayashi et al. 2012). Here we have shown that this is also the case in spinal cord injury, even though SCI itself increases the levels of this cytokine, and we have identified a time when to monitor this increase. Furthermore, the fact that MCP-1 has been found to act similarly in humans, renders it more likely that our findings will translate to humans. It is reassuring that we discovered MIP3- α to react similarly to MCP-1, and its robust response suggests MIP3- α could be a useful biomarker.

We harvested lymphoid organs and spinal cords 7 days after injury for immunohistochemical analysis of immune cells. Interestingly, we found an increased activation of macrophages in the lymphoid organs; spleen, bone marrow, and thymus, while at the same time confirming the suppression of inflammation and amount of inflammatory cells at the site of injury in the spinal cord. We found that the activation of macrophages was due to imatinib, by administering the drug to uninjured animals,

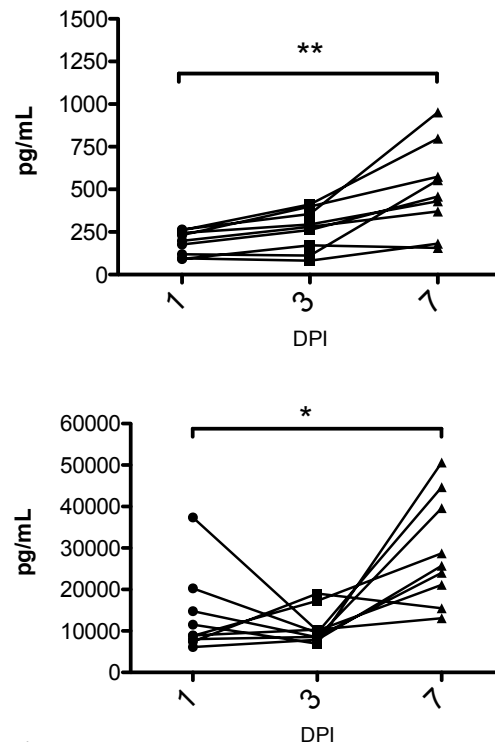


Fig 22. **Imatinib increases macrophage-associated factors with time.** We measured levels of MIP3a (top) and MCP1 (bottom) during treatment at 1, 3 and 7 days post injury (DPI). Each line represents values of an individual animal. Figure from Paper VI.

and seeing similar effects in the lymphoid organs, and by noting that that no such activation was present 1 day after injury for the imatinib group. The correlation between elevated levels of both MCP-1 and MIP3- α and the activation of macrophages in the lymphoid organs strongly suggest a direct association, especially since both MCP-1 and MIP3- α are macrophage associated cytokines that are expressed by the macrophages in for example the spleen (Deshmane et al. 2009; Ruth et al. 2003). It is beyond the scope of this study, but perhaps this event could have a role in causing the beneficial effects of imatinib for spinal cord injury, as well as for the drug effects in other applications.

In conclusion, we have found biomarkers that could be used as stratification variables in a clinical trial. The activation of macrophages in spleen, thymus and bone marrow may be of interest for clinical applications of this drug, but if this has anything to do with the decrease of inflammatory activation at the site of injury in the spinal cord remains to be investigated.

As part of a NIH effort to replicate studies with potential to advance into clinical trials, Paper II was recently re-assessed by Sharp et al (K. G. Sharp, Yee and Steward 2014b). They confirmed the improved recovery of bladder function and there were also tendencies of greater tissue sparing and a reduction of the inflammatory response in the imatinib treated group at 56 days after injury. They did not replicate the robust effect on locomotion found by us in Paper II. However, Sharp et al were not able to reproduce the degree of injury and corresponding locomotor recovery of non-treated animals as in Paper II, even though the same substrain, impactor and injury severity was used. Their control group had similar or higher locomotor scores then the imatinib treated animals had in our study. Hence, the locomotor tests were to some extent not suitable for their condition. We have recently published a detailed commentary to the re-assessment study by Steward et al of our Paper II (Abrams et al 2014). Notably, the group of Sharp et al. have

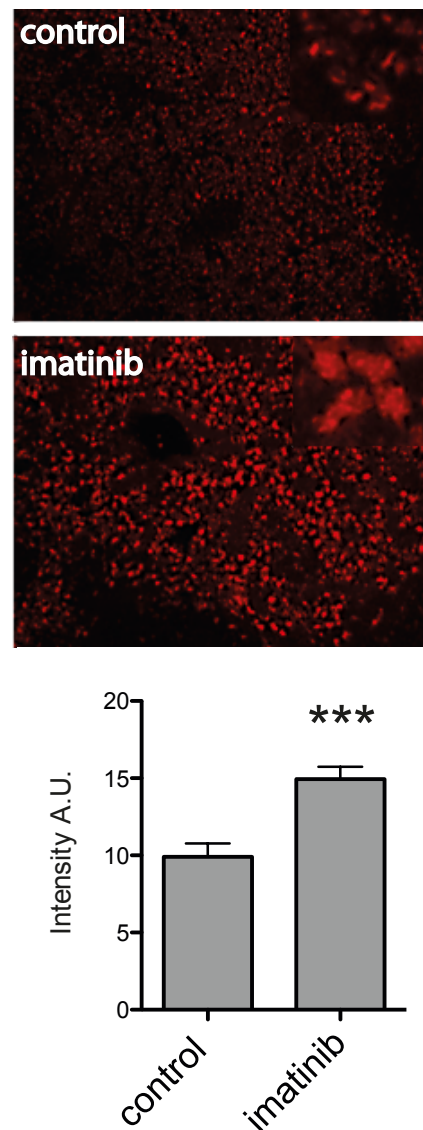


Fig 23. Imatinib treatment activates macrophages in lymphoid organs. 7 days after injury we found macrophages in spleen, bone marrow and thymus to display more ED1 immunoreactivity. Representative pictures of spleen from animals given (a) PBS and (b) imatinib. (c) Quantification of ED1 immunoreactivity. Figure modified from Paper VI.

not been able to fully reproduce any of eight studies so far (K. G. Sharp et al. 2012; K. G. Sharp, Yee and Steward 2014b; K. G. Sharp et al. 2013; Erschbamer et al. 2012; Steward et al. 2006; Steward et al. 2008; Steward et al. 2012; K. G. Sharp, Yee and Steward 2014a). Thus we conclude as in Paper III that it is important to properly characterize experimental outcome and make sure that the lesioned control group has a degree of impairment suitable for the detection of drug-induced improvements.

STUDY CONCLUSIONS

Paper I: Growth conditions and substrain origin can improve translational value of astrocyte cultures

- Astrocyte growth conditions are important determinants of astrocyte properties in vitro.
- Astrocytes obtained from different rat substrains can have different properties and be more or less comparable to human astrocytes.

Paper II: Imatinib is a potential candidate for clinical trials in SCI

- Immediate initiation of oral imatinib treatment rescues spinal tissue and improves functional outcome.
- Immediate initiation of oral imatinib treatment improves blood-spinal cord barrier integrity, reduces subacute inflammation, and rescues axon pathways.

Paper III: Rat substrains can have different experimental and translational value in SCI research

- Choice of rat substrain may influence functional and histological outcome in experimental SCI.
- Substrain differences is one factor that may explain inter-experimental differences.

Paper IV: Receptor tyrosine kinase inhibition may counteract secondary negative effects of SCI

- Erlotinib accelerates recovery, but Rapamycin does not. Further work is needed to determine whether these compounds may be of interest in the development of treatment strategies for SCI.

Paper V: Possible windows of opportunity for targeting mTORC1 in spinal cord injury.

- There is a biphasic alteration of mTORC1 activation in the injured spinal cord.
- mTORC1 activation in microglia /macrophages may underlie contradictory effects of mTORC1 modulation. Further research is warranted to determine the role of mTORC1 activation in microglia/ macrophages after SCI.

Paper VI: Imatinib is a candidate for clinical trials in SCI; candidate proxy biomarkers have been identified

- Clinical application of imatinib in SCI seems feasible because initiation of treatment can be delayed from 4 (locomotion) up to 24 (bladder function) hours and because no major side effects were found.
- Time of administration seems important for outcome; treatment should be initiated as early as possible.
- Cytokine levels in blood, on an individual patient basis, may serve as a proxy marker of effective levels of imatinib and might also be used as a variable for group stratification.

CONCLUDING REMARKS

We set out to determine the therapeutic potential of repositioning three cancer drugs for spinal cord injury. The drugs inhibit certain receptor tyrosine kinases or part of their common downstream signaling. Parts of this work were focused on characterizing in vitro and in vivo models and particularly aspects of their experimental and translational value. As a result, we were able to obtain more information about the basic pathology and define possible treatment windows with respect to our chosen drug treatments. Both erlotinib and rapamycin will need further work to determine their possible therapeutic potential. For imatinib, we conclude that the drug has therapeutic potential and is suitable for clinical application. Our results suggest that early inhibition of receptor tyrosine kinases in spinal cord injury counteracts some negative secondary events and may lead to tissue sparing and improved functional recovery. Perhaps more can be done to improve the therapeutic potential, such as controlling certain secondary events that may expand the treatment window or counteract any possible negative effects for these or other therapeutic pharmacological agents for spinal cord injury. Another interesting aspect for future studies is combining treatments, in the hope of additive or synergistic effects.

We are entering a somewhat hopeful era of spinal cord injury research where, similar to imatinib, several other therapeutic interventions are advancing towards clinical trials or are already being tested in patients. However, to determine outcome of acute drug treatment in patients with spinal cord injury is a demanding task. After finding a cancer drug with therapeutic potential for spinal cord injury, we attempted to address some of the anticipated clinical issues. In Paper VI, we determined stratification variables that may be of clinical value for our therapeutic candidate drug imatinib. We also uncovered effects of imatinib on cellular phenotypes and functions that may prove important clinically, for example when planning to combine Imatinib with other pharmacotherapies.

Very recent work suggests that patients with chronic spinal cord injury with no remaining motor function, may have small numbers of remaining descending, (and possibly ascending), but silent axon pathways (Angeli et al. 2014). It was shown that epidural stimulation of the lumbar spinal cord, presumably by increasing sensitivity of spinal circuits, allowed such patients to regain a degree of control of leg movements in response to sound or visual cues, proving that the commands that led to leg movements more than 2 years after injury came from the brain. It was also shown that training strengthened these pathways to the extent that one patient was finally able to move his legs without support of epidural electrical stimulation of the lumbar spinal cord. These very interesting results suggest that it may become very important to rescue even very limited numbers of seemingly useless axons across the site of injury. Indeed, Imatinib rescued axons across the site of injury that would otherwise be lost.

The main aim for the studies in this thesis was to find a potential candidate for clinical application. In imatinib we have found such a candidate. To reposition drugs is one of the primary missions of NIH for different diseases and disorders in their quest of “supporting scientific studies that turns discoveries into health” (Collins 2011). The possibility to reposition drugs is increasingly explored for different diseases and disorders. Our data suggest it may be a useful approach for spinal cord injury.

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