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Pathophysiology of Spinal Cord Injury Studied by *In Vivo* Optical Imaging

Patofyziológia poranenia miechy študovaná *in vivo* optickým zobrazovaním

Bachelor's thesis

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Poďakovanie

Týmito riadkami vyjadrujem poďakovanie vedúcej záverečnej práce, Mgr. Barbore Svobodovej, za odborné usmernenie, venovaný čas a ochotu. Ďakujem aj svojim priateľom, ktorí ma pri písaní práce podporovali a dali mi veľa cenných rád.

Vyhlásenie

Vyhlasujem, že som záverečnú prácu vypracovala samostatne a uviedla som všetky použité informačné zdroje a literatúru. Táto práca a ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

V Prahe, 9. 5. 2019 Sabína Vančíková

Abstract

Patients suffering from spinal cord injury experience physical, social, and vocational impairment. It is a condition often causing a permanent disability mainly due to axonal regeneration incapability in the central nervous system. The primary insult simultaneously damages cells in the lesion site and initiates a cascade of secondary cellular, vascular, and biochemical events extending the injury. These pathophysiological mechanisms are examined using multiple approaches. Novel imaging techniques complement classical histopathological methods and neuroanatomical tracing. Recent studies employ transgenic mice and two-photon microscopy to observe single cells in the injury site and the nearby vasculature *in vivo* longitudinally. *In vivo* optical imaging enables studying of axonal responses, such as degeneration, regeneration, and neurovascular interactions. It also gives an opportunity to assess the effects of applied drugs directly. New findings lead to a better understanding of the pathophysiology of spinal cord injury, resulting in the ability to develop other therapeutic strategies improving the outcome after injury.

Keywords: spinal cord injury, pathophysiological mechanisms, axonal regeneration, Wallerian degeneration, animal models, transgenic mice, *in vivo* imaging, two-photon excitation microscopy

Abstrakt

Pacienti trpiaci poranením miechy prežívajú fyzické, sociálne a pracovné ťažkosti. Je to ochorenie často spôsobujúce trvalé postihnutie hlavne ako následok zlyhania axonálnej regenerácie v centrálnom nervovom systéme. Primárne poškodenie súbežne ničí bunky v mieste zranenia a iniciuje kaskádu sekundárnych bunkových, cievnych a biochemických udalostí rozširujúcich poranenie. Tieto patofyziologické mechanizmy sú sledované niekoľkými prístupmi. Moderné zobrazovacie techniky dopĺňajú klasické histopatologické metódy a neuroanatomický tracing. Čerstvé štúdie využívajú transgénne myši a dvojfotónovú mikroskopiu k dlhodobému *in vivo* sledovaniu jednotlivých buniek v mieste poranenia a priľahlej cievnej sieti. *In vivo* optické zobrazovanie umožňuje štúdium axonálnych odpovedí vrátane degenerácie, regenerácie a neurovaskulárnych interakcií. Rovnako ponúka príležitosť priamo hodnotiť účinky aplikovaného liečiva. Nové poznatky vedú k lepšiemu porozumeniu patofyziológie poranenia miechy vyúsťujúceho do možnosti vývinu ďalších terapeutických stratégií zlepšujúcich dopad poranenia.

Kľúčové slová: poranenie miechy, patofyziologické mechanizmy, axonálna regenerácia, Wallerova degenerácia, animálne modely, transgénne myši, *in vivo* zobrazovanie, dvojfotónová excitačná mikroskopia

Abbreviations

AAD	acute axonal degeneration
BSB	blood-spinal cord barrier
CNS	central nervous system
DRG	dorsal root ganglion
GFP	green fluorescent protein
IL	interleukin
MP	methylprednisolone
NgR	Nogo-66 receptor
PKA	protein kinase A
PNS	peripheral nervous system
ROS	reactive oxygen species
SCI	spinal cord injury
THY1	Thy-1 cell surface antigen gene
TNF	tumor necrosis factor
WD	Wallerian degeneration
XFPs	fluorescent proteins
YFP	yellow fluorescent protein

Contents

Introduction

Spinal cord injury (SCI) is a debilitating injury with approximately estimated annual incidence being 40 to 80 cases per million population worldwide – according to etiology most of these cases are traumatic SCIs (World Health Organization, 2013). People suffering from this condition experience motor or sensory dysfunction of various severities, chronic pain and many other symptoms that are often permanent due to the regeneration failure of damaged axons. The cost of SCI is huge as it negatively affects patients' quality of life (not only their physical but also social and vocational well-being). Ongoing research into the treatment and functional recovery has led to the establishment of a large number of therapeutic strategies, however, there are no fully restorative therapies yet. Therefore, it is crucial to pursue an investigation of SCI, trying to understand the mechanisms of underlying events. A range of animal models and multiple techniques are employed to study SCI. Each method has several advantages and also some inherent limitations thus classical techniques are often complemented with novel ones. Recently, *in vivo* optical imaging approach revealed some fundamental properties of secondary responses to the initial SCI as it brought new and wider opportunities for examination of pathological events in the central nervous system (CNS). New findings help to understand this complex and dynamic process and may be useful for the development of potential treatments.

The aim of this thesis is to provide useful insights into the current studies of SCI, to briefly introduce common animal models and key research approaches, and to describe the latest knowledge about cell and vascular responses to SCI gained mainly due to *in vivo* optical imaging. The thesis is divided into four chapters. An overview of key events underlying traumatic SCI is given in the opening chapter. The two later chapters critically discuss animal models and SCI observation methods introducing its merits and drawbacks. The final chapter gathers and interprets certain findings on post-injurious pathophysiological responses.

1 Traumatic spinal cord injury

1.1 Epidemiology of spinal cord injury

The term 'SCI' refers to an event that damages the spinal cord (Figure 1.1) and results in changes in its function. These changes are either temporary or permanent and have rather devastating consequences. Traumatic SCI is triggered by an external physical impact and features injuries caused due to motor vehicle collisions, falls, sports or acts of violence. Motor vehicle collisions represent the leading cause, followed by falls and sports-related injury, whereas the minority of cases are caused by violence (Lenehan et al., 2012).

The consequences of human SCI depend on the indicators of spinal cord damage severity, which are very heterogeneous and include neurologic level (that is, cervical injuries are related to increased mortality by comparison with lower injury levels) and completeness of the lesion (Geisler, Jousse, Wynne-Jones, & Breithaupt, 1983). However, it often causes motor and sensory impairment. Hence, some patients may lose their independence, experience various physical and social difficulties and die prematurely in comparison with the age-matched control group (Krause, Sternberg, Lottes, & Maides, 1997). According to one study (Anderson, 2004) surveying what gain of function would dramatically improve lives of individuals living with a chronic SCI, patients with quadriplegia prioritized arm and hand function recovery, whereas paraplegics ranked sexual function restoration as the most important to their quality of life. These symptoms were preferred to the reduction of other commonly occurring symptoms (upper body strength and balance, bladder/bowel function, elimination of chronic pain, normal sensation and walking movement). Despite the progress in medicine, there is no cure for SCI. Partial function restoration is being designed in a step-by-step manner. Many therapies focusing on multiple targets have been tested on animal models or have reached clinical trials (Thuret, Moon, & Gage, 2006).

1.2 Mechanism of spinal cord injury

Primary injury

The initial mechanical trauma is generated by transection, compression, contusion, or laceration of the spinal cord. Primary injury begins within seconds as neurons and glial cells are instantly injured at the impact site, and the vasculature nearby is disrupted compromising

the blood-spinal cord barrier (BSB). Together, mentioned events lead to hemorrhages, ischemia, spinal cord swelling, and a cascade of other destructive events known as secondary injury.

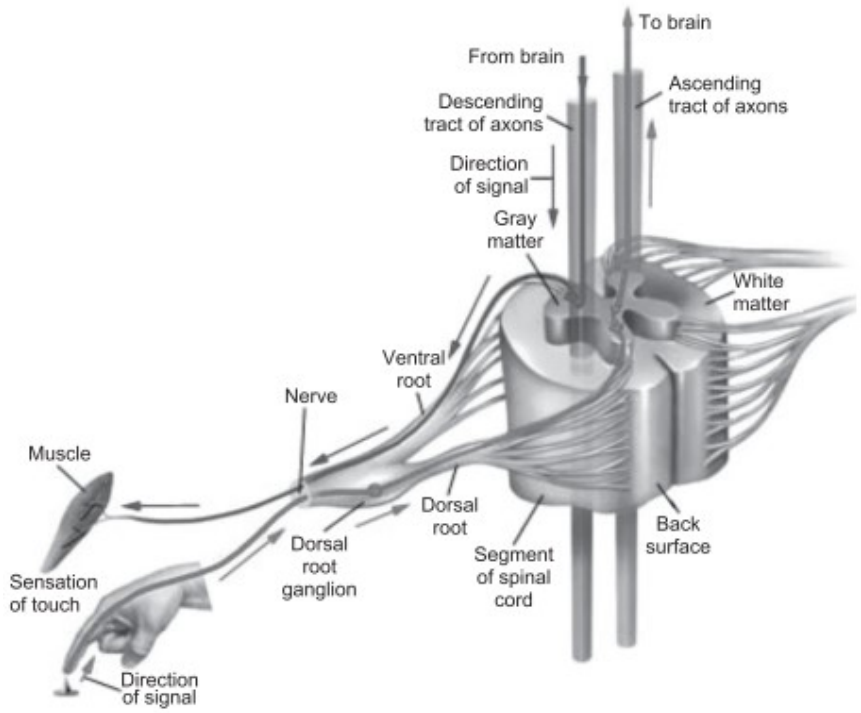

Figure 1.1 **Organization of the spinal cord.** The spinal cord is organized into the butterfly-shaped gray matter on the inside and a ring of white matter on the outside. The gray matter is mainly composed of neuronal cell bodies, while the white matter consists mostly of nerve axons and oligodendrocytes, which form the myelin sheath. The spinal cord is further divided into a large number of groups of nerve fibers called ascending and descending tracts. These tracts are responsible for transmitting specific information (motor or sensory stimuli) to and from the periphery respectively.
Modified from "Spinal cord injury," by Mcdonald, J. W., Becker, D., & Huettner, J., 2013, In A. Atala & R. Lanza (Eds.), *Handbook of Stem Cells* (Second Edi). Elsevier Inc.

Secondary injury

Secondary changes are responsible for further damage expanding the injury site and enlargement of the lesion area. This phenomenon, set in minutes after injury and lasting for months (Oyinbo, 2011), covers a continuation of events from the primary phase and also the emergence of new ones. These events happen due to vascular and biochemical effects and include immune system response (inflammation, edema, formation of free radicals), demyelination, axonal degeneration, and cell death (excitotoxic necrosis or apoptotic cell death). There is much interplay between secondary injury mechanisms; they are self-propagating and form a detrimental network (Oyinbo, 2011). Cell death by necrosis occurs

immediately during the acute phase, whereas cells in the surrounding tissue die during the secondary phase in the absence of an inflammatory response. The latter process, apoptosis, is a programmed pathway of cell death activated in neurons, oligodendrocytes, microglia, and astrocytes (Liu et al., 1997). The main trigger for apoptosis is calcium influx into cells activating enzymes that break down internal proteins (Ray, Hogan, & Banik, 2003). Increased number of apoptotic cells in the lesion site lasts the next few days (Paterniti et al., 2009) and apoptotic cells, in particular, oligodendrocytes, are spatially associated with degenerating axons (Casha, Yu, & Fehlings, 2001). Loss of oligodendrocytes leads to demyelination of axons surviving the initial trauma.

Inflammation is a critical event in the secondary phase as this response persists for several weeks and plays a vital role in the clearance of cellular debris (Fehlings & Nguyen, 2010). The previous breakdown of the BSB initiates rapid arrival of inflammatory cells (for example neutrophils and macrophages) to the injury site paralleled with activation of resident microglia. Activated microglia and macrophages produce many growth factors essential for tissue repair. Nonetheless, these cells secrete reactive oxygen species (ROS) and proinflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α, which worsen the injury. The significant increase in the concentration of those cytotoxic substances contributes to further destruction of healthy tissue (Donnelly & Popovich, 2008). For this reason, an uncontrolled immune system exacerbates spinal cord injury and inhibits axonal regeneration (Rossignol, Schwab, Schwartz, & Fehlings, 2007). Moreover, demyelinated axons are exposed to ROS and pro-inflammatory cytokines that lead to further cell death and conduction delay, which may cause inefficient neuronal communication (McTigue, 2008).

Another issue arises with glutamate, the most abundant excitatory neurotransmitter that is released in small quantities in the healthy spinal cord. However, injured axons and astrocytes release an enormous amount of glutamate that is accumulated in the extracellular space and causes overexcitation of neighboring cells through the activation of voltage-gated calcium channels. Cells suffering from such an excitotoxic shock admit waves of calcium ions and thus experience mitochondrial dysfunction, production of ROS and lipid peroxidation (Azbill, Mu, Bruce-Keller, Mattson, & Springer, 1997). Glutamate receptor-mediated excitotoxicity can adversely affect neurons as well as oligodendrocytes (Matute, Sánchez-Gómez, Martínez-Millán, & Miledi, 1997).

Chronic injury

The attempts to remyelinate axons, to rearrange vascularization, and to remodel neural circuits characterize the chronic injury phase. However, there are some limitations inhibiting regeneration after SCI. Cystic cavities, formed in the acute phase due to cell death and extracellular structure damage (Lagord, Berry, & Logan, 2002), fuse and put constraints on axonal regrowth and cell migration (Milhorat, Capocelli, Anzil, Kotzen, & Milhorat, 1995). Later, astrocytes proliferate in the surrounding of cystic cavities and their processes become interlaced. Astrocytes are the base of the glial scar, but it is also condensed with extracellular matrix proteins secreted by cells in the acute phase. These substances are growth inhibitory, and therefore glial scar negatively controls axonal regeneration (Ahuja, Martin, & Fehlings, 2016). Nonetheless, the glial scar has an advantageous function – it restrains the spread of cytotoxic molecules and inflammatory cells into adjacent tissue (Anderson et al., 2017).

2 Experimental models of spinal cord injury

Since SCI is a severe condition associated with a permanent disability, there has been a massive amount of interest in designing therapies in order to improve neurologic function. A wide range of experimental models has been frequently used in the past years in an effort to better understand pathophysiological events accompanying SCI and to develop an effective treatment. Because of the ethical issues associated with the study of SCI in humans and the insufficient knowledge derived from clinical observations, most studies are based on animal models (Hassannejad et al., 2016).

2.1 Animal species

Selection of an appropriate animal model is demanding as many factors need to be considered. There is a large variety of animal species, which are used in research. Currently, rodents are used the most frequently (Sharif-Alhoseini et al., 2016). Advantages of rat models include low cost, easy handling and manipulation, well-understood anatomy, standardized analysis techniques (Kwon, Oxland, & Tetzlaff, 2002), and a good mimicking of human pathology as rats tend to form large cystic cavities at the lesion site (Lee & Lee, 2013). Mice and rats are alike in many ways, such as low cost and ease of care, and in addition to that mice have similar genomes to humans. However, mice do not develop cystic cavities. Although rats are larger and thus better models for carrying out surgeries and implanting devices (Talac et al., 2004), mice are popular SCI models nowadays thanks to recent advances in transgenic mouse models (Lee & Lee, 2013). Transgenic mice with SCI are the most useful models for *in vivo* imaging discussed later in this thesis.

Rodent models seem to be an appropriate means of concentrating on the pathophysiology of CNS, however, attempts to translate research results obtained from animal studies into clinical trials have revealed some discrepancies (Akhtar, Pippin, & Sandusky, 2008). Problems lie in inconsistency between laboratory-induced injury and real-life injury and problems with explaining functional outcomes in animals. There are also some anatomical and functional differences in axonal systems between rodents and primates. Rodents' spinal cord is significantly shorter, axonal regeneration for a few millimeters may contribute to functional recovery, but such distance may be insufficient in primates. Problematic interpretation of specific results also occurs (for example, a change of rat's forelimb-to-hindlimb coordination is irrelevant due to humans' bipedalism) and molecular signaling is dissimilar (Nout et al., 2012;

Kleitman, 2004). These constraints could be partially overcome by using a model closer to humans – larger animal models and non-human primate models (Kwon et al., 2015). Nonetheless, owing to moral and financial considerations (Kundi, Bicknell, & Ahmed, 2013), these larger mammals are used mainly as an intermediary model to confirm results from rodent models or to discover adverse effects of therapeutic treatment strategies before moving to clinical trials (Nout et al., 2012).

2.2 Spinal cord injury models

SCI models are selected specifically based on the design of particular research. Every model is appropriate for a concrete type of questions. According to the mechanism of SCI, injuries are divided into contusion, compression, transection, dislocation, distraction, photochemical, ischemic and excitatory models. As most human SCIs are caused by blunt trauma, the vast majority of studies are conducted on blunt trauma injury, for example, contusion (Sharif-Alhoseini et al., 2016). The most commonly used models are described below.

Contusion model

The contusion model is the oldest model (Anderson, 1982) and the preferred one for studying the pathophysiology of human SCI (Sharif-Alhoseini et al., 2016). The principle is to deliver a controlled mechanical force with an injury device to the spinal cord in order to damage and displace the spinal cord and to inflict transient injury in several severities. The difficulty to control whether particular axons are severed or spared is, however, the main drawback (Lee & Lee, 2013). There are many contusion models including injuries through weight-drop apparatuses, electromagnetic impactors, and pneumatic impactors.

Compression model

The compression model, in comparison, involves spinal cord compression over an extended period. That is the great merit due to the frequent occurrence of the prolonged compression in human SCI, and for this reason, the compression model is probably the most suitable. Clip compression, used in most cases, is simple, fast and provides a reliable simulation of clinical injuries (Jazayeri et al., 2013). Much less common is calibrated forceps compression as it does not possess the acute impact component happening in human SCI. Another model,

balloon compression, does not require a laminectomy, hence, causes no damage to the surrounding tissue (Chung et al., 2013).

Sometimes contusion-compression models are used to combine an acute initial insult and persisting compression of the spinal cord (Cheriyan et al., 2014).

Transection model

The transection model covers partial or complete interruption of the spinal cord pathways, therefore it is a good method to investigate axonal degeneration, regeneration or neuroplasticity rather than reflecting the pathophysiology of clinical SCI (Kundi, Bicknell, & Ahmed, 2013). To preserve tissue continuity and to reduce the physical harm to the spinal cord researchers often perform incomplete transection (Steward, Zheng, & Tessier-Lavigne, 2003). Most appropriately, the transection model is combined with neuroanatomical tracing and electrophysiological studies.

2.3 Region of spinal cord injury

Although human SCI mostly arises in the cervical region, just a fraction of injuries in animal models is located in that spinal level (Sharif-Alhoseini et al., 2016). Cervical injuries cause a high mortality rate as a result of respiratory impairment (Lane, Fuller, White, & Reier, 2008). Conversely, injury in the thoracic site appears to be safe, reliable, reproducible and highly studied (Rahimi-Movaghar, 2009). Nonetheless, the spinal cord lacks consistency across its levels in many characteristics such as diameter, degree of vascularization, size of sensory and motor neuron population or white/gray matter distribution (Pearse et al., 2005). Generalizing thoracic level injury findings to human cervical injury may thus be problematic, and further development of cervical injury models becomes highly recommended nowadays (Sharif-Alhoseini et al., 2016).

3 Approaches to spinal cord injury examination

SCI may be studied by several methods. As technology vastly improves over time, new methods are being developed. These technical refinements complement classical approaches and contribute to useful clarifications on pathophysiological events underlying SCI.

3.1 Classical methods

Investigation of SCI was long limited to static histopathological techniques or neuroanatomical tracing. Standard histological spinal cord analysis (for example, hematoxylin and eosin staining) was modified to specific histology relying on immunohistochemistry or *in situ* hybridization. Immunohistochemistry detects antigens (for instance, proteins unique to certain axonal populations) in tissue sections using specific antibodies allowing visualization (Evilsizor, Ray-jones, Lifshitz, & Ziebell, 2015). Contrarily, tracing techniques are based on endocytosis and axonal transport of tracers in either anterograde or retrograde direction. Anterograde axonal tracers are transported from the cell bodies to the axons. Examples of anterograde tracers are biotinylated dextran amine and cholera toxin B subunit (Alisky & Tolbert, 1994), while retrograde axonal tracers are fluorochromes, such as Fluoro-Gold or Fast-Blue (Richmond et al., 1994) and these are transported from axon terminals to the cell bodies. Eventually, tracers are visualized and distinguished on histologic sections. Classical approaches are certainly useful for neuroanatomical investigations, however, the examination of axonal regeneration exposed some limitations. One of the major weaknesses is these methods are end-point thus each animal provides only a partial information (Köbbert et al., 2000). This may cause misinterpretation of results due to comparing static data as SCI is not fully reproducible and synchronized. It is also very difficult to follow single axons using these methods. Together this necessitated the development of dynamic *in vivo* strategies, which combined with classical approaches offers new examination possibilities. To mention one, axonal tracers may be visualized by *in vivo* imaging today.

3.2 *In vivo* imaging methods

Dynamic *in vivo* imaging methods include several techniques, however, this thesis is focused on *in vivo* optical imaging (*in vivo* optical microscopy). Other techniques (for example, magnetic resonance imaging and its alternations) are not described here. *In vivo* microscopy allows longitudinal visualization of individual axons in the spinal tissue of living mice. This

approach has been made possible thanks to advances in transgenic technology and optical microscopy. As mentioned above, transgenic mice expressing high levels of at least one spectral variant of fluorescent proteins (together termed XFPs) in the nervous system are well-suited for *in vivo* imaging. Frequently, neurons are modified to express XFPs under the control of the gene promoter Thy1 (Laskowski & Bradke, 2013). The Thy-1 cell surface antigen gene (*THY1*) encodes a protein member of the immunoglobulin superfamily, expressed by cells in many parts of the nervous system, as well as by cells of the immune system, for example, thymocytes (Gordon et al., 1987; Morris, 1985). The Thy1 promoter can become a neuron-specific promoter by deleting a particular intron and thus selectively inhibiting expression in non-neuronal cells (Vidal, Morris, Grosveld, & Spanopoulou, 1990). While preparing transgenic mouse lines, it is essential to choose an ideal density of labeling depending on the specific experiment. Sparse labeling is recommended for tracing individual axons in the damaged spinal cord (Feng et al., 2000; Misgeld, Nikic, & Kerschensteiner, 2007). There are many other transgenic mouse lines in which glial cells, such as astrocytes, oligodendrocytes, and microglia, are labeled. Finally, some modifications of fluorescent labeling are available, one of them is multi-color imaging that allows observation of cellular (Crowe & Ellis-Davies, 2014) or neurovascular (Chen et al., 2017) interactions.

Recent progress in optical imaging enabled the observation of living cells to complement histological and immunohistochemical analysis. Although time-lapse microscopy was used for the first time a long time ago (Brayt, 1970), many adaptations have been made since then, including computerization of image analysis or improvement of microscope resolution. The pioneering *in vivo* imaging study employed conventional wide-field fluorescence microscopy (Kerschensteiner, Schwab, Lichtman, & Misgeld, 2005), followed by various studies using advanced technologies such as confocal and multiphoton (first published by Davalos et al., 2008) microscopy. The latter has some definite advantages as it allows deep tissue imaging with higher resolution and it is less phototoxic compared to wide-field microscopy (Crowe & Ellis-Davies, 2014).

The experimental procedure

Surgeries are usually carried out on adult transgenic mice while managing efficient anesthesia, analgesia and ventilation, and maintaining a constant animal's body temperature. Under these conditions, a laminectomy is performed at the proper spinal cord level to expose the dorsal column. The layer of dura matter in the laminectomy site is removed due to obtaining

an optimal fluorescence signal (Misgeld, Nikic, & Kerschensteiner, 2007). Then a group of axons is carefully transected applying selected method (a needle, iridectomy scissors or laser can be used). At this point, a potential pharmacological treatment may be applied (Ertürk, Hellal, Enes, & Bradke, 2007) if it is planned to study the effects of administered substance. Transected axons are imaged (Figure 3.1) using two-photon or multiphoton microscopy. After imaging, the animal can be either recovered for repetition of imaging or fixed tissue for further analysis can be obtained. In case of repeated imaging, muscle tissue and the skin are used to cover the laminectomy site post-surgery, and the skin is stapled. At first, there was a need for a repeated surgical opening of the injury site for each imaging session (Misgeld, Nikic, & Kerschensteiner, 2007). This approach, however, limits the number of potential time points accessible as it increases the possibility of infection and inflammation and damages surrounding tissue. Therefore, Farrar & Schaffer (2014) developed a method overcoming these pitfalls. Authors published a protocol for the implantation of a chronic spinal chamber (Figure 3.2) that allows chronic *in vivo* observations without requiring multiple surgeries. Two other research teams (Fenrich et al., 2012; Figley et al., 2013) independently reported the development of similar spinal cord window chambers. These three techniques slightly differ from each other in implantation materials, cost or surgical apparatus, though all of them enable longitudinal optical access to the spinal cord with the high frequency of imaging being limited only by animal's tolerance to multiple rounds of anesthesia. Later, Tang et al. (2015) developed a spinal stabilization device with an implanted window, which eliminates the artifacts caused by an animal's breathing and heartbeat. Post-operative, animals are kept warm in their cages and treated with analgesics or antibiotics (Laskowski & Bradke, 2013).

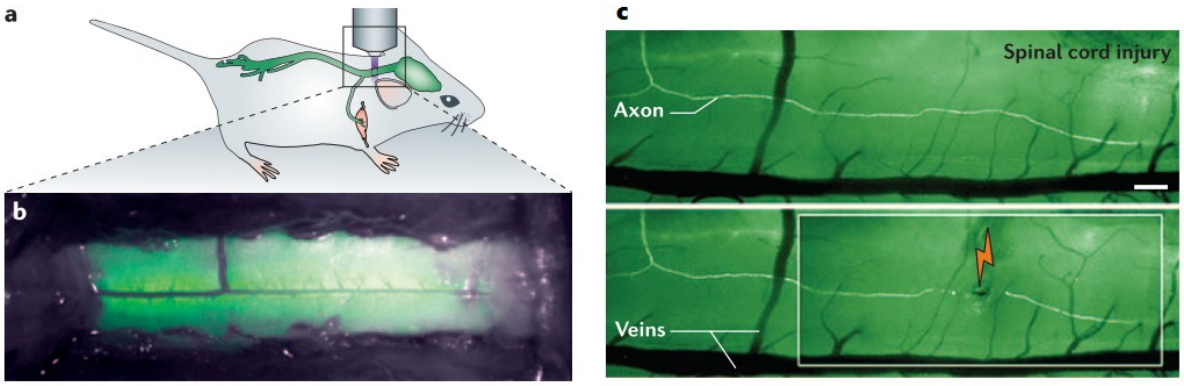

Figure 3.1 ***In vivo* imaging of mouse spinal cord.** (**a**) Schema showing the imaging procedure. (**b**) Viewing of the spinal cord in the laminectomy site. (**c**) *In vivo* visualization of a single axon in dorsal view. Flash, lesion. Top, before lesion; bottom, after lesion. Scale bar, 200 μm.

Modified from "Neuroimaging: *in vivo* imaging of the diseased nervous system," by Misgeld, T., & Kerschensteiner, M., 2006, *Nature Reviews Neuroscience*, *7*(6), 449.

Strengths of *in vivo* imaging

Strengths and advantages of *in vivo* imaging in contrast to classical methods are summarized in Table 1 below.

***In vivo* imaging approach**	**Classical approach**
longitudinal imaging	imaging at a single time-point
observation of cell dynamics	static view
direct real-time/repetitive observations of pathological events	only end-point analysis is available
time-lapse imaging of every animal model	comparing data obtained from multiple non-identical models
direct identification of axonal damage – transected, regenerated and spared axons may be distinguished	no clear distinction – a mixture of damaged and intact axons is seen
reliable assessment of treatment; before and after treatment visualization; identification of the drug's mechanism of action	only the outcome is observable; the drug's mechanism of action remains unknown

Table 1 **Comparison of two different approaches in the context of SCI examination: *in vivo* imaging versus classical methods**

Limitations of *in vivo* imaging

Although the benefits of *in vivo* imaging outweigh its disadvantages, the procedural limitations (reviewed in Laskowski & Bradke, 2013) need to be considered while interpreting acquired data. However, the drawbacks are being improved progressively.

Apart from mice's insufficient imitation of human pathology (absence of cystic cavities), employing mouse models in SCI observations requires experimenter's considerable skill in performing surgical procedures because mice are small in size and injuries should be as reproducible as possible. Each animal is slightly anatomically different thus it may be challenging to select the same injury location. Performed lesions must be minimal, ideally affecting only a few axons, to achieve satisfactory image quality. The imaging depth is limited as well – the two-photon laser scanning microscopy provides the deepest tissue imaging so far, and it is still possible just a couple of hundred μm below pia mater. Using two-photon imaging also partly overcame the issues with phototoxicity (Crowe & Ellis-Davies, 2014).

The surgery itself is hazardous due to anesthesia and increased risk of infection and inflammation. Tang et al. (2015) examined these potential effects of the implanted spinal window, and they found out it causes no significant damage to the axons, the microvessels, and the blood flow. Moreover, they showed the implantation of the spinal chamber leads to mild inflammatory response, but this response appears to be insignificant compared to inflammation caused by SCI. Lastly, Tang and coworkers (2015) did another important finding as they used a spinal stabilization device reducing movement alterations (e.g., breathing, heartbeat and muscle contraction) mentioned above.

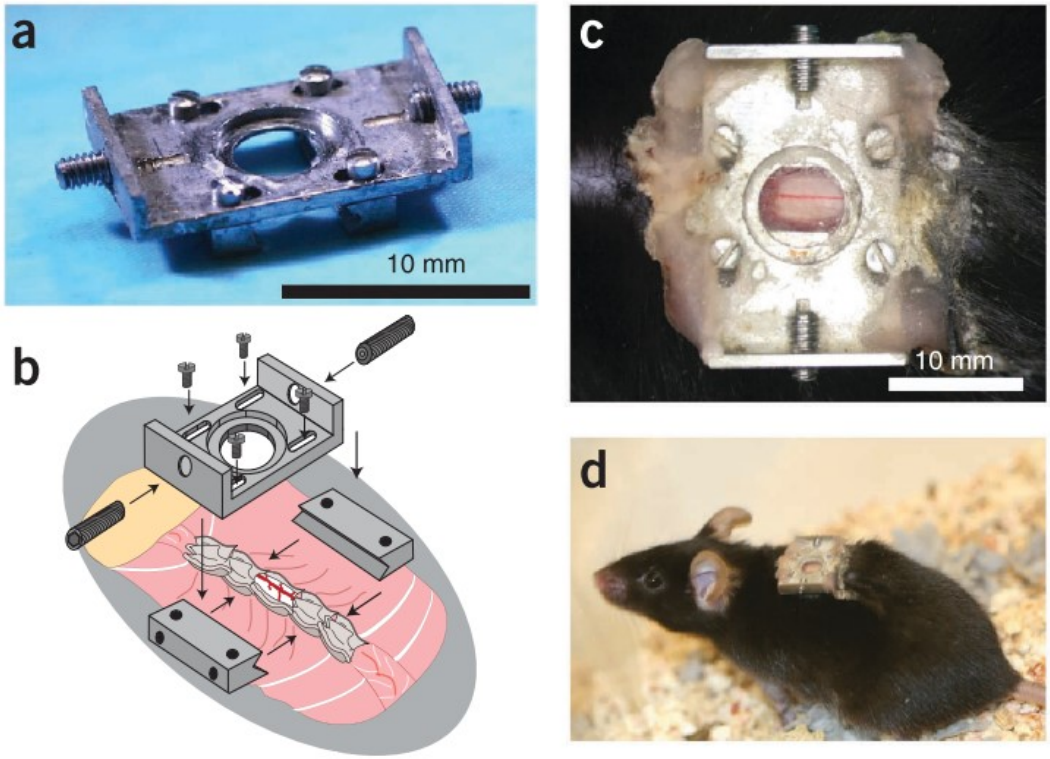

Figure 3.2 **Chronic spinal chamber for repetitive *in vivo* imaging of mouse spinal cord.** (**a**) Imaging spinal chamber consisting of several metal parts clamping the vertebrae, and a glass coverslip. Scale bar, 10 mm. (**b**) Schema showing the spinal chamber implantation. (**c**) Spinal cord imaged through the implanted chamber. Scale bar, 10 mm. (**d**) A mouse with an implanted chamber.

Modified from "Chronic *in vivo* imaging in the mouse spinal cord using an implanted chamber," by Farrar, M. J., Bernstein, I. M., Schlafer, D. H., Cleland, T. A., Fetcho, J. R., & Schaffer, C. B., 2012, *Nature Medicine*, *9*(3), 297–302.

4 Pathophysiological findings revealed by *in vivo* optical imaging

In vivo optical imaging technologies, introduced in the preceding section, has substantially contributed to our knowledge of physiological processes in the CNS as well as the behavior of various cell types under pathological conditions. Many protocols applying these techniques have been published in the past years on the injured or diseased brain and spinal cord. Several studies have revealed new findings on the pathophysiology of traumatic SCI.

4.1 Axonal degeneration, regeneration and regrowth

Unlike the peripheral nervous system (PNS), axon regeneration in the CNS is suppressed by the growth-inhibitory post-injurious environment combined with the limited intrinsic ability of neurons to regrowth axons (Chen & Zheng, 2014). A promising way to study this event is *in vivo* imaging of neuronal damage. Traumatic surgical axotomy in transgenic mice helps to understand numerous issues concerning axonal degeneration and regeneration. Fluorescently labeled axons in the spinal cord are transected and tracked afterward.

According to Augustus Waller's (1850) nerve transection experiments, axons degenerate distal to the injury site. This regulated cell-autonomous response (Coleman, 2005) is known as Wallerian degeneration (WD). Despite the fact the cellular mechanism is still unclear, distal axon ends are believed to undergo WD commonly, and proximal ends retract (axonal dieback) after axotomy. It has been quite difficult to perform direct observations on mammalian CNS axons, that is why the pathophysiology of these events remains unresolved. Kerschensteiner, Schwab, Lichtman, & Misgeld (2005) wanted to study this phenomenon through developing a method allowing to observe individual axons *in vivo* over time. They performed a laminectomy procedure and visualized transected GFP-labeled dorsal root ganglion (DRG) axons in transgenic mice over several spinal segments using wide-field microscopy. Authors reimaged individual transected axons at different time points and revealed symmetrical dieback of both axon ends in 24–30 hours after injury (Figure 4.1a). Reimaging required repetition of surgical procedure. Axon ends experienced a post-injurious stable phase lasting for 10–20 minutes and later went through sudden fragmentation, a process authors called 'acute axonal degeneration' (AAD; Figure 4.1b), followed by a slow axonal retraction resulting in the formation of retraction bulbs. After approximately 30 hours, the distal axon end degenerated due to WD, while the proximal axon end remained steady. This study published

by Kerschensteiner et al. (2005) showed more detailed insight on axonal dieback using *in vivo* optical imaging for the first time.

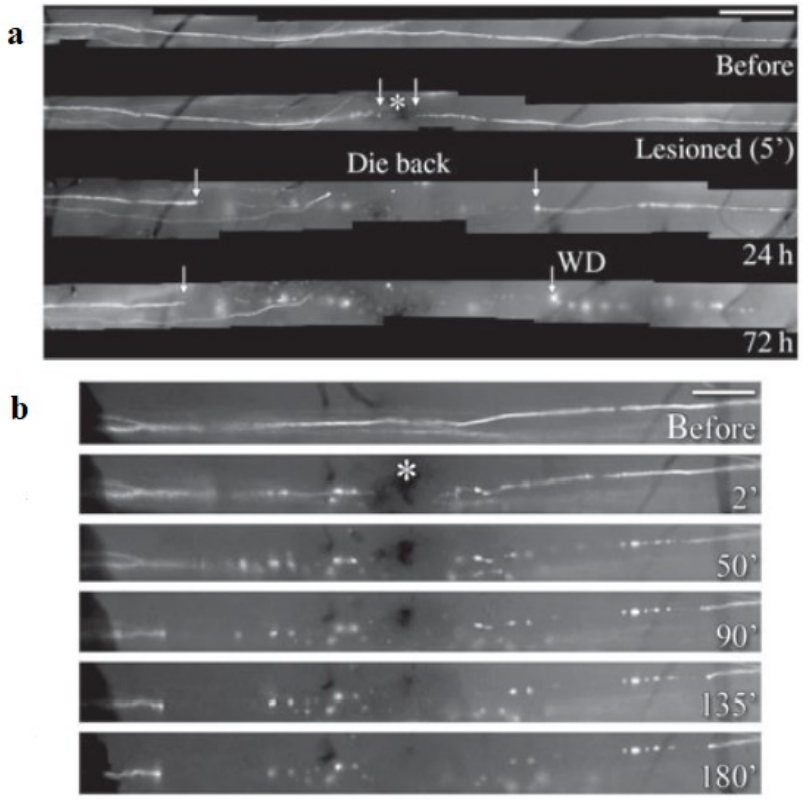

Figure 4.1 **Repetitive *in vivo* imaging of DRG axons degeneration.** (**a**) Changes in an injured axon branch imaged before, at 5 min, 24 h, and 72 h after SCI. Depicted events include dieback and WD. Asterisk, lesion; white arrows, axonal tips. Left, proximal axon end; right, distal axon end. Scale bar, 250 µm. (**b**) Imaging of an injured axon for 180 minutes. Depicted events include a steady phase (2 min), AAD (50 min) and later slow axonal retraction. Asterisk, lesion. Scale bar, 100 µm.
Modified from "*In vivo* imaging of axonal degeneration and regeneration in the injured spinal cord," by Kerschensteiner, M., Schwab, M. E., Lichtman, J. W., & Misgeld, T., 2005, *Nature Medicine*, *11*(5), 572–577.

Dray, Rougon & Debarbieux (2009) developed a similar *in vivo* imaging method that required surgery for each imaging session. However, there is a fundamental distinction between these two studies – Kerschensteiner et al. (2005) imagined a single sensory axon, whereas Dray and coworkers (2009) designed protocol allowing dynamic studies of degeneration and regeneration in populations of injured fluorescent DRG axons over several months based on two-photon microscopy. Research published by Dray et al. (2009) provided a time-lapse study of a population of approximately 40 GFP labeled axons after a minimal pin injury. Acquired data were analyzed quantitatively. Over half of the axons underwent AAD at the lesion site during the first 20 minutes, and this event was followed by WD supporting previous findings of Kerschensteiner et al. (2005). WD lead to a reduction on axon number gradually with rising

distance from the lesion site. A massive decrease at 400 μm eccentricity occurred by three days after injury, and a loss of 10% of the axons was visible 1,000 μm away from the lesion site on day 7. Results indicate that the area affected by WD is around fivefold compared to AAD.

Furthermore, both teams mentioned above and other authors examined posttraumatic axonal regeneration and regrowth. Kerschensteiner et al. (2005) found out one-third of proximal ends of stabilized transected axons initiated sprouting within the first two days after lesion. This growth is, however, considerably slower than post-injurious axonal growth rate measured in the PNS (Pan, Misgeld, Lichtman, & Sanes, 2003). Fenrich and coworkers (2012) studied axon growth dynamics following minimal SCI longitudinally using an implanted spinal glass window. Their time-lapse observations showed the lesion was refilled with 58% of neurons imaged on the uninjured spinal cord on day 46. According to morphology, these axons were divided into two types. The first group, occupying the outer edges of the lesion site, appeared to be similar to the uninjured axons, while axons staying in the center tended to follow curved trajectories.

Retraction bulb, a structure formed at the tips of lesioned axons in the paper published by Kerschensteiner et al. (2005), is a term relevant while discussing axonal regeneration and regrowth. While the counterpart of this structure, a growth cone, gives axon guidance during the development of the nervous system (Huber, Kolodkin, Ginty, & Cloutier, 2003) and its post-injurious formation at the tip of lesioned axon in the PNS is associated with axonal regeneration, a retraction bulb formed at the tip of a transected axon in the CNS is a nongrowing swelling (Hill, Beattie, & Bresnahan, 2001). Extension of axons associated with growth cones is possible thanks to an interaction of some cellular events, such as accumulation of mitochondria in the tip of actively growing axon (Morris & Hollenbeck, 1993) and the dynamics of microtubules (Dent & Gertler, 2003). On the contrary, the mechanism of retraction bulb formation and its effect on axonal growth incapability remained unclear for a long time as our knowledge was mostly based on *in vitro* models. Ertürk, Hellal, Enes, & Bradke (2007) wanted to find out more about the mechanism and carried out research contrasting responses of injured axons in the CNS and the PNS using *in vivo* imaging. Authors examined responses on sparsely GFP labeled DRG neurons as they form retraction bulbs at central axonal branches tips, and growth cones at peripheral axonal branches tips after axotomy. Results of this study showed that microtubule disorganization is a critical feature of retraction bulb formation. Application of the microtubule polymerization inhibitor, nocodazole, converted a growth cone into a retraction bulb-like

structure rendering axons unable to regenerate. Conversely, treatment with the microtubule polymer stabilizer, paclitaxel, prevented retraction bulb formation. These findings promoted numerous observations of cytoskeletal effects on axon regeneration and regrowth in the following years.

4.2 Vascular and axonal networks dynamics

Disruption of the vasculature in the spinal tissue, necrosis of blood vessel endothelial cells and hence deficiency in the blood supply are regular features of traumatic SCI. Restoration of high blood vessel density within a few days after injury was already shown in some immunohistochemical studies. These vessels often lacked connections with glial cells, and after one week, the vessel density lowered again (Imperato-Kalmar, Mckinney, Schnell, Rubin, & Schwab, 1997). Immunostaining works did not provide dynamic and spatial information on angiogenic response to SCI. Thus, *in vivo* imaging appears to be an effective way to study the behavior of the vasculature in the lesion site.

Already discussed work by Dray et al. (2009) also observed the dynamics of neurovascular interactions and its impact on axon regrowth for the first time using dual-color imaging. Firstly, the work supported previous knowledge demonstrating huge posttraumatic angiogenesis, largest by day 7 (Figure 4.2a). In week two, newly formed vessel branches rapidly remodeled and only a small number of them remained stable. It proves the significant plasticity of the vascular network. Secondly, this study revealed DRG axon sprouts growing in close proximity to the new blood vessels (Figure 4.2b). This affinity for blood vessels influenced elongation of axon sprouts. Axons following blood vessels elongated almost eight times as fast as those non-following blood vessels. Nonetheless, blood vessels do not guide axons toward the lesion site.

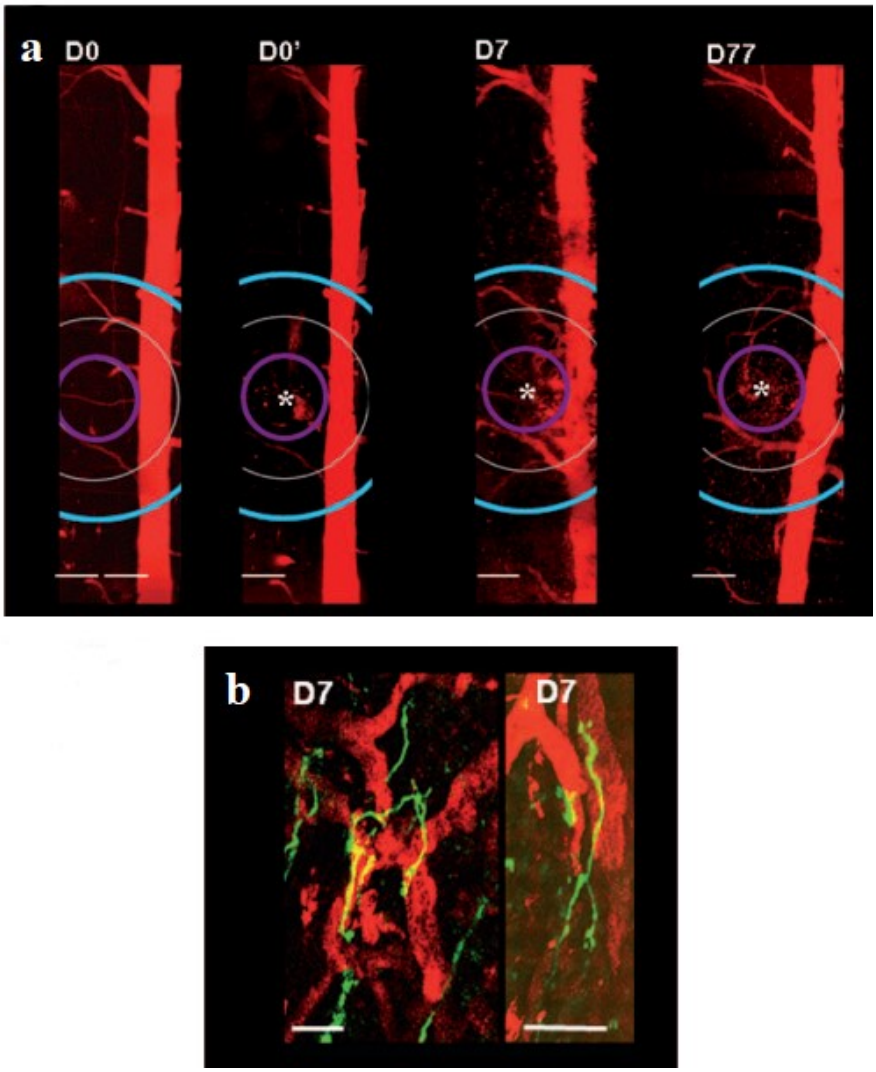

Figure 4.2 **Repetitive *in vivo* imaging of vascular and axonal networks.** (**a**) Vascularization of the spinal cord imaged before (D0), immediately after (D0'), on day 7 (D7) and day 77 (D77) after SCI. Asterisk, lesion. Scale bar, 200 µm. (**b**) Axon sprouts growing along newly formed blood vessels on day 7 (D7). Scale bar, 50 µm. Modified from "Quantitative analysis by *in vivo* imaging of the dynamics of vascular and axonal networks in injured mouse spinal cord," by Dray, C., Rougon, G., & Debarbieux, F., 2009, *Proceedings of the National Academy of Sciences*. *106*(23), 9459–9464.

4.3 Therapeutic strategies after spinal cord injury

Methylprednisolone treatment

SCI remains a devastating problem for our society as novel pharmacological treatments and surgical procedures are insufficient to completely restore function, in spite of significant medical progress. Understanding of pathophysiological mechanisms underlying future

complications, however, helps to develop promising therapeutic strategies alleviating secondary injury. For instance, there was an attempt to clinically apply a method based on inhibition of the Nogo-66 receptor (NgR) by phosphorylating its ectodomain. Infusion of protein kinase A (PKA) and ATP in the injured spinal cord area aided locomotor recovery (Suehiro et al., 2013). From all the potential strategies, the most successful is methylprednisolone (MP) treatment.

MP, a synthetic corticosteroid, relieves inflammation and suppresses the immune system. It is used to treat a wide range of medical condition such as arthritis; skin, eye, kidney, and intestinal disorder; severe allergies and asthma (AHFS Patient Medical Information, 2017). Since the first study demonstrated the effects of MP on acute SCI (Hall & Braughler, 1981), many authors have focused their attention on this drug. Both animal studies and human trials proved the efficacy of MP in animal models or patients with SCI (Bracken et al., 1990), therefore, it has been commonly used in clinical treatment as recommended neuroprotective agent (Bracken, 2012). However, high-dose of MP can cause side effects, such as infection, bleeding, trouble sleeping, and vomiting (Failli et al., 2012).

While developing new therapeutic strategies, researchers have to optimize some fundamental problems including the suggestion of a therapeutic window or determining a safe dosage to avoid adverse side effects. It is crucial to establish an optimal therapeutic window that in the context of spinal cord damage means the time range between SCI and the application of a treatment, during which the treatment is still effective (Danton & Dietrich, 2005). Farrar et al. (2012) imagined fluorescently labeled mice DRG axons *in vivo* for five weeks after SCI using an implanted spinal chamber. They observed heterogeneity in axon dieback at longer timescales. Authors believe their findings and other upcoming studies could be beneficial in proposing the most suitable therapeutic window by connecting this heterogeneity with regenerative responses to therapy.

Another essential issue in designing therapeutic treatment is to explore the effectivity of the particular agent, in this case, MP. In 2012, Felleiter and colleagues published a retrospective cohort study showing no clear statistical distinction between SCI patients with and without MP therapy thus the use of high-dose MP is relatively controversial.

The study of MP impact on SCI was at first confined to tracing techniques, which required extraction of the injured spinal tissue after death. These *in vitro* methods did not provide data on dynamic changes of SCI after application of MP treatment. Recently, two

studies independently dealt with the matter by *in vivo* observations. In both these studies, implanted spinal chambers were used, and spinal cords of transgenic mice were observed over multiple hours using two-photon microscopy. As they compared sham group with saline-treated and methylprednisolone-treated mice groups, Zhang et al. (2014) used a traumatic compression SCI model, whereas Tang et al. (2015) used a traumatic hemisection SCI model.

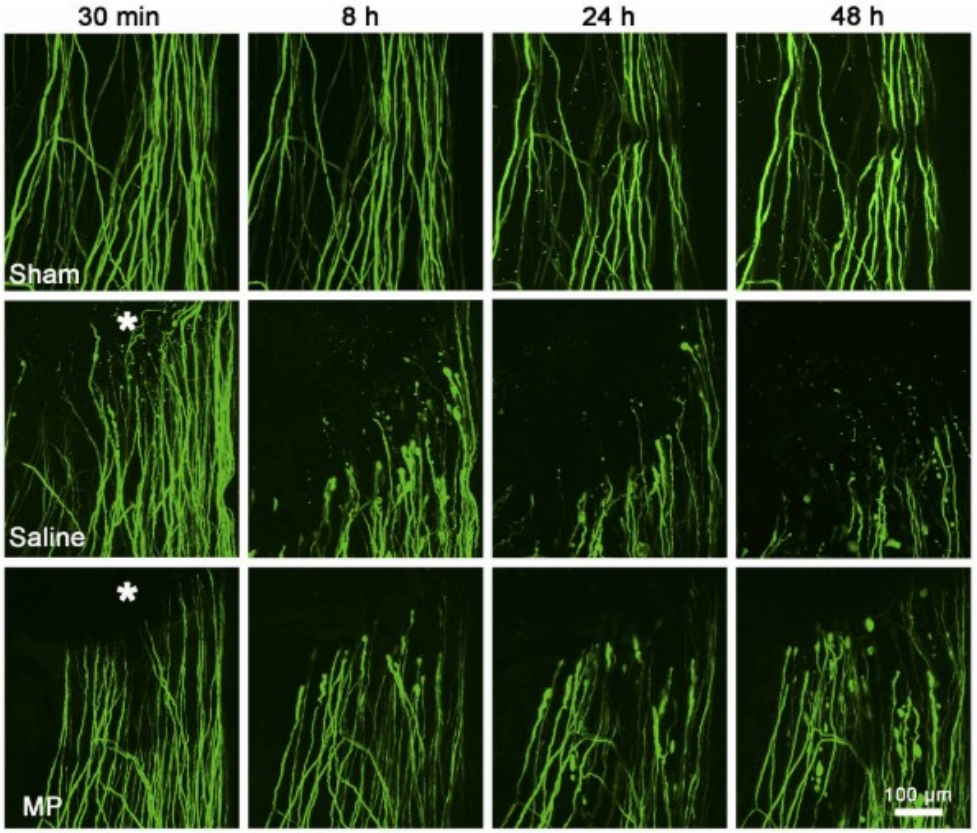

Figure 4.3 **Repetitive *in vivo* imaging of axonal dieback after hemisection SCI.** Images of the sham group, saline-treated group and MP-treated group at 30 min, 8 h, 24 h, and 48 h post-injury. Asterisk, lesion site. Scale bar, 100 µm.

Modified from "*In vivo* two-photon imaging of axonal dieback, blood flow, and calcium influx with methylprednisolone therapy after spinal cord injury," by Tang, P., Zhang, Y., Chen, C., Ji, X., Ju, F., Liu, X., … & Zhang, L., 2015, *Scientific Reports*. *5*, 9691.

Mentioned studies showed consistency while demonstrating that early application of MP lowers the accumulation of microglia and macrophages at the injury site, and consequently moderates axonal damage. In addition, Tang and coworkers (2015) focused on other pathological events belonging to secondary injury cascade and noticed further beneficial effects of MP. Most importantly, the results within the MP-treated group indicated a reduced posttraumatic axonal dieback (Figure 4.3), a higher neuronal and microvessels number, an increased microvascular blood flow, and a significant reduction of calcium influx after SCI in

comparison to the saline-treated group. In summary, MP-treatment attenuates secondary injury and therefore might be an effective treatment for acute traumatic SCI.

Neuropreservative strategy

Although the majority of possible therapies for SCI is concentrated on developing strategies regenerating interrupted axons or reducing effects of secondary injury events, an alternative way would be to prevent axons being disconnected after injury. Williams et al. (2014) tracked YFP-labeled spinal axons *in vivo* within the first hours after spinal cord contusion injury to find out whether it is possible to rescue compromised axons. They discovered a variety of post-injurious axon morphologies – unaffected, swollen and broken/degenerated axons. All broken axons were earlier swollen, however, some of the swollen axons finally maintained their integrity. It implies there is a critical time window, observed to last for several hours, during which swollen axons either disintegrate or spontaneously recover. This period is open to a potential neuropreservative intervention as a part of a multistep therapeutic strategy.

Moreover, Williams and colleagues (2014) examined what influences the fate of axons and they revealed that increases in intra-axonal calcium levels relate to axonal fragmentation. This finding is consistent with another study showing calcium rise is the noteworthy cause of axon degeneration in SCI (Stirling & Stys, 2010). In contrast, axons that were able to restore the standard level of intracellular calcium recovered and were protected from degeneration. The study consequently identifies manipulation of intra-axonal calcium level as an efficacious method to preserve connectivity of axons after traumatic injury.

Conclusion

Unresolved pathophysiology of SCI and the fact this condition is still without a cure requires further study. This thesis introduced up-to-date knowledge about mechanisms of SCI and present possibilities of examination. Investigation of SCI had been long time limited to end-point analyses and post-mortem histology. The great progress has been made in genetic engineering spreading the idea of using transgenic animal models, especially transgenic mouse lines, and advances in optical microscopy has led to the development of dynamic optical imaging strategy. This novel method enabled studying of cellular and vascular responses due to *in vivo* visualization of individual cells and other structures over time by employing advanced techniques like two-photon microscopy or procedures such as spinal cord window implantation. It also opened up an opportunity to directly observe mechanisms of drugs administered to the lesion site. In spite of several limitations, *in vivo* optical imaging approach is a promising way to reveal the behavior of affected cells and to better understand events occurring after the primary insult. New findings considerably broaden our previous knowledge and therefore may help establish effective treatments.

This bachelor's thesis also aimed to summarize and interpret results of recent studies focused mainly on *in vivo* observations of SCI pathophysiology. That review was subdivided into a few topics. Findings associated with axonal degeneration, regeneration and regrowth clarified the mechanism of post-injurious transected axons degeneration in the CNS. At first, axons experienced a sudden symmetrical fragmentation, AAD, followed by retraction bulbs formation, and WD of distal axon ends. Studies on vascular and neuronal network dynamics demonstrated posttraumatic angiogenesis and enormous plasticity of vessels. The proximity of blood vessels promoted axonal regeneration. Finally, the part about therapeutic strategies showed that MP-treatment attenuates effects of secondary injury events and thus confirmed its therapeutic potential. It also gave an optimistic view of developing neuropreservative therapies in the future.

In summary, this thesis is written as a comprehensive review of current SCI studies presenting the latest knowledge about the pathophysiology of this devastating condition. It gives an insight into multiple events that are being studied using *in vivo* optical imaging approach. Hence, it might be useful for inspiring researchers to conduct further investigations of this phenomenon as many questions remain unclear.

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