CHARLES UNIVERSITY

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Summary of the Dissertation

Enhancing axon regeneration and neuroplasticity after spinal cord injury:

Bridging the gap between development and disease

Zvýšení regenerace axonů a neuroplasticity po poranění míchy:

Využití poznatků z vývoje centrálního nervového systému k léčbě jeho poranění

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Enhancing axon regeneration and neuroplasticity after spinal cord injury:

Bridging the gap between development and disease

ABSTRACT

The precise wiring of the adult mammalian central nervous system (CNS) is determined during axon growth, guidance and neuroplasticity during and shortly after development. This intricate system is unable to regenerate when the adult spinal cord is injured. It's not well understood how to translate what is known about developmental processes into therapies for spinal cord injury (SCI). Therefore, effective therapies are difficult to find. This thesis aims to fill an important gap in our understanding of how developmental strategies for axonal growth and plasticity are exploited in SCI regeneration. In particular, we will contrast two approaches: (i) reducing the inhibitory environment that forms around the lesion, and (ii) exploiting the inhibitory environment for regeneration by forcing the overexpression of an appropriate integrin isoform in sensory neurons and allowing axons to grow on this environment. In this thesis, Aim 1, we used 4-methylumbelliferone (4-MU) to reduce the inhibitory environment formed around the lesion. The first step was to assess the potential adverse effects of long-term treatment. Using immunohistochemistry, proteomics, biomechanics, qPCR, behavioural tests and commercially available blood and urine tests, we found no irreversible adverse effects. Our next step was to test whether 4-MU could play a role in chronic SCI. 4-MU treatment reduced scarring after chronic SCI. However, the current dose was not sufficient to suppress SCI-induced chondroitin sulfate CS-GAG upregulation. Further dose adjustment will be required to improve functional recovery after SCI. In Aim 2 of this thesis, the integrin adhesion molecule together with its activator was expressed in sensory neurons using a viral vector. The sensory pathway was partially restored in the presence of this adhesion molecule. Many axons regenerated from the thoracic lesion to the brainstem. This is a distance of 4-5 cm. Taken together, these findings have implications for our understanding of the developmental mechanisms of spinal cord regeneration.

Key words:

4-methylumbeliferone, extracellular matrix, gene therapy, integrin, perineuronal nets, plasticity, regeneration, spinal cord injury

Zvýšení regenerace axonů a neuroplasticity po poranění míchy:
Využití poznatků z vývoje centrálního nervového systému k léčbě jeho poranění

ABSTRAKT

V průběhu vývoje centrálního nervového systému (CNS) a krátce po něm, dochází k růstu a vedení axonů, a zároveň k jeho plasticitě a tvorbě synaptických zapojení, které u dospělých savců pečlivě určují jeho přesné uspořádání a funkci. V dospělosti je tento proces ukončen a jistým způsobem zakonzervován. Po poranění míchy (spinal cord injury, SCI) dospělého jedince tento komplexní a složitý systém není schopen reagovat na poranění stejným způsobem, jako v období vývoje. Způsob, jak přenést poznatky o vývojových procesech do terapií po SCI, není dobře znám, což představuje obtížnost při hledání účinných léčebných postupů. V této práci se snažíme zaplnit důležitou mezeru v chápání toho, jak by bylo možné využít vývojové strategie růstu a plasticity axonů při regeneraci axonů po SCI, a to konkrétně dvěma přístupy: (i) Redukce inhibičního prostředí, které se tvoří okolo léze a (ii) naopak využitím inhibičního prostředí ve prospěch regenerace, a to vynucením nadměrné exprese příslušné izoformy integrínu v senzorických neuronech a umožnění růstu axonů po tomto prostředí. V této práci, pro cíl 1, jsme použili 4-methylumbeliferon (4-MU) ke snížení inhibičního prostředí vytvořeného kolem léze. Prvním krokem bylo posouzení možných nežádoucích účinků dlouhodobé léčby. Pomocí imunohistochemie, proteomiky, biomechaniky, qPCR, behaviorálních testů a komerčně dostupných testů krve a moči jsme nezjistili žádné nevratné nežádoucí účinky. Naším dalším krokem bylo otestovat, zda 4-MU může hrát roli při léčbě chronického SCI. Léčba 4-MU redukovala jizvu v chronické fázi SCI. Každopádně, námi prezentovaná dávka nebyla dostatečně účinná k potlačení SCI indukované upregulace CS-GAG. Ke zlepšení funkčního zotavení po SCI bude nutná další úprava dávky. V cíli 2 této práce byla adhezivní molekula integrín společně se svým aktivátorem exprimována v senzorických neuronech pomocí virového vektoru. V přítomnosti této adhezní molekuly došlo k částečné obnově

senzorické dráhy. Mnoho axonů se regenerovalo z hrudní léze do mozkového kmene. Jedná se o vzdálenost 4-5 cm. Dohromady nám tyto výsledky pomáhají pochopit, jak by se vývojové mechanismy daly využít v regeneraci míchy po SCI.

Klíčová slova

4-methylumbeliferon, extracelulární matrix, genová terapie, integrín, perineuronální sítě, plasticita, regenerace, poranění míchy

Table of Contents

1. BACKGROUND

1.1 Spinal cord injury

A spinal cord injury (SCI) is a serious condition, often leading to severe morbidity and permanent disability, that can affect anyone at any time, especially in daily life. The spinal cord has three main functions: It sends motor commands from the brain to the body, it sends sensory information from the body to the brain, and it coordinates reflexes. When an injury occurs, i.e., damage to the axons of the nerves that run through the spinal cord, it leads to the loss of motor and sensory functions and ultimately to paralysis, the severity of which depends on the degree of injury. However, SCI brings with it a high degree of health complications and limitations in daily life, such as breathing problems, impaired bladder and bowel functions, disturbances of autonomic functions and impairment of sexual functions.

The pathophysiology of SCI involves primary and secondary mechanisms of injury. Although both mechanisms are involved in the neurological dysfunction at SCI. After the initial irreversible mechanical injury leading to necrosis and destruction of neuronal connections, several secondary processes take place that may last for several months until a lesion cavity forms. The location and severity of the spinal cord lesion are used to classify the consequences of injury. Current research efforts are focused on these processes, which are thought to be reversible or modulable to some degree. Such modulation would be beneficial to the patient as it would prevent degenerative damage to the spared tissue, which is known to progress over several months after the actual injury. Secondary processes include oedema, blood-spinal cord barrier (BSCB) disruption, ischaemia, inflammation, oxidative stress, glutamate excitotoxicity and apoptosis, all of which occur immediately after the mechanical insult. The molecular cascades in these processes are interconnected and proceed via positive feedback loops or through the use of different molecular and cellular elements at different time points (Oyinbo 2011). In terms of time, SCI can then be divided into the acute phase (< 48 hours), the sub-acute phase (48 hours to

14 days), the intermediate phase (14 days to 6 months) and the chronic phase (> 6 months) (Ahuja et al. 2017).

The injury response following SCI resembles in many ways that of the developmental process. The first is axon guidance. During development, axons extend and navigate to form neural circuits, and after SCI, damaged neurons attempt to regrow axons and connect to new targets. It is also known that certain genes and pathways associated with neural development are reactivated following SCI. In the attempt to repair and regenerate damaged nerve tissue, this reactivation may play a role. Cell identity and function, both during development and in response to injury, may also change. There may be an attempt to recruit and activate different cell types, including stem cells, to participate in tissue repair after spinal cord injury. Finally, inflammation and activation of the immune system is part of the body's response to injury. There are also immune responses during development that affect how neural circuits form (Filous and Schwab 2018). However, the inflammatory response that follows a spinal cord injury can be either helpful or harmful. Despite these potential similarities, spinal cord regeneration in mammals is limited. Functional recovery after spinal cord injury is often only partial.

1.2 The struggle to make CNS axons regenerate

Where axons can regenerate, as in peripheral nerves, they can restore function spontaneously. In the CNS, however, axon regeneration fails. This is the main reason why paralysis and loss of sensation are permanent in conditions such as spinal cord injury. Regeneration of axons in patients with spinal cord injuries is one of the greatest hopes for restoring useful functions.

During development, neurons spread their axons throughout the nervous system and make connections to postsynaptic targets that are often quite distant from their origin. The ability of these young neurons to robustly extend their axons decreases dramatically in adulthood. This reduced intrinsic growth capacity is a key mechanism underlying the inability of adult CNS neurons to regenerate their axons

after injury (Blesch and Tuszynski 2009; Fawcett and Verhaagen 2018). The weak regenerative response after transection is not the only reason why CNS axons do not regenerate spontaneously; the environment surrounding CNS lesions also plays a crucial role in inhibiting axon growth.

Because CNS axons cannot regenerate spontaneously, sensory, motor and/or autonomic deficits are often permanent after CNS injury. Therefore, there is still a great unmet need for therapeutic strategies to improve the regeneration of injured CNS axons and thus improve function. Restoring regeneration and plasticity is therefore complex, and multiple inhibitory mechanisms must be considered. Regardless of the cause, we are now at a stage where the biology of regeneration failure is increasingly understood, and solutions are being found (Fawcett 2020). To enhance axonal growth and promote functional recovery, the various intrinsic and extrinsic factors that control axon regeneration and navigation in the inhibitory environment of the central nervous system must be identified (Afshari, Kappagantula, and Fawcett 2009).

2. AIMS AND HYPOTHESES

The main aim of this thesis was to investigate recapitulating developmental processes in the adult CNS to stimulate axon regeneration and functional recovery following SCI. Regeneration in the adult mammalian CNS is unsuccessful due to reduced intrinsic regenerative capacity of affected neurons, myelin-associated inhibitory factors and components of the glial scar (Silver and Miller 2004). This basically leads to two main therapeutic approaches. In simple terms, we can either remove the inhibitory environment in the CNS and thus increase plasticity, or we can use the inhibitory environment in favour of regeneration by over-expressing the right isoform of a molecule that can bind to the inhibitory environment around the lesion site and thus allow axons to pass through the lesion and thus promote regeneration.

2.1 Novel oral therapy for SCI: removing the inhibitory CSPGs to improve regeneration and plasticity

In the treatment of SCI, could oral treatment with 4-MU replace any CSPG removing therapies such as ChABC therapy?

- Since 4-MU is given orally, is long-term treatment safe in rats?
- Is the treatment with 4-MU at the current dose sufficient to re-open the window of plasticity in the chronic stage of spinal cord injury?

2.2 Using AAV-mediated overexpression of integrin α9 for sensory pathway reconstruction after SCI

After peripheral nerve injury, we have already seen that overexpressing integrin α9 with its activator kindlin-1 can allow axons to regenerate (Cheah et al. 2016).

Is it possible to use this approach to regenerate axons after spinal cord injury to help restore sensation?

3. METHODS

3.1 Experimental animals

For the Aim 1.1, ten-week-old healthy female Wistar rats (n = 24; 250 ± 30 g) were used for the pharmacological evaluation of potential 4-MU-mediated adverse effects. For the Aim 1.2, eight-week-old female Wistar rats (n=55, 250-300 g) were included in the study focused on the effect of 4-MU in the treatment of SCI in its chronic stage. For the Aim 2, 8-week-old female Lister-Hooded rats (n=36; 150-175 g) were used. Rats were housed in groups of two or three in cages with a 12-hour light/dark cycle and standard conditions of temperature (22 ± 2°C) and humidity (50% ± 5%). Rats had free access to tap water and food ad libitum.

3.2 SCI surgeries

All procedures were performed in accordance with relevant guidelines and regulations. All animal procedures were approved by the Ethics Committee of the Institute of Experimental Medicine of the Academy of Sciences of the Czech Republic (ASCR) and were performed in accordance with Act No. 77/2004 of the Czech Republic (ethics approval number: 13/2020). A power calculation based on previous studies was performed prior to the experiment to estimate the number of animals required. All work was performed in accordance with European Commission Directive 2010/63/EU and ARRIVE guidelines. Every effort was made to minimise pain and distress.

3.2.1 Contusion model of SCI

A commercially available Infinite Horizon SCI device (IH-0400 Spinal Cord Impactor device; Precision Systems and Instrumentation, Lexington, KY, USA) was used to induce moderate thoracic spinal cord contusion at the T8-9 level.

3.2.2 Dorsal column crush

Dorsal column crush was performed using fine Bonn forceps (Fine Science Tools) at the T10 level.

3.2.3 Tissue preparation

Animals were given an overdose of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to transcardial perfusion or to the collection of samples on dry ice.

3.3 Therapeutical interventions

3.3.1 4-MU treatment

2.5% (w/w) 4-MU was added to a chocolate-flavoured rat chow (Sniff GmbH, Germany) and prepared for use. This percentage would provide rats with 1.2 (±0.2) g/kg/day of 4-MU if the diet was consumed ad libitum.

3.3.2 AAV-mediated gene therapy

3.3.2.1 Preparation of AAV1 vectors

The plasmids AAV-SYN-α9-V5 and AAV-CMV-kindlin1-GFP were scaled and sequenced prior to AAV1 packaging as described previously (Hermens et al. 1999; Cheah et al. 2016). AAV1-SYN-GFP was purchased from Vigene (distributed by Charles River).

3.3.2.2 Direct injection into the DRGs

Injections into the DRGs were carried out prior to the SCI, however this was done on the same day and in quick succession. Two DRGs (L4 and L5) were exposed on the left side and 1 µL of viral vector was manually injected using a Hamilton syringe (Hamilton; specification: 33-gauge, 12 mm, PST3).

3.4 Behavioural tests

The following behavioural tests were used for assessing functional recovery and/or any adverse effects of treatment: Basso, Beattie and Bresnahan (BBB) test (Basso, Beattie, and Bresnahan 1995), Ladder Rung Walking test (Metz and Whishaw 2009), Maximum speed tests and treadmill rehabilitation, Rotarod, Grip tests, Thermal test (Plantar test), Mechanical pressure test (Von Frey test), and Tape Removal test

3.5 Immunohistochemistry

10-40 µm sections were permeabilized with 0.5% (v/v) Triton X-100 in 1X PBS for 20-80 min. After permeabilisation and/or avidin/biotin blocking, the tissue was

blocked in ChemiBLOCKER (1:10; Millipore cat. no. 2170), 0.3 M glycine, 0.2% (v/v) Triton X-100 in 1X PBS for 2 h. Fluorescence-conjugated secondary antibodies were used to detect primary antibodies after washes.

3.6 Tissue clearing for lightsheet microscopy

Four randomly selected DRGs from each group were cleared in ethyl cinnamate (Sigma-Aldrich, cat. no. 112372) as described previously by (Huang et al. 2019).

3.7 GAGs isolation

GAGs were isolated according to the protocol published by ('Perineuronal Nets: A Special Structure in the Central Nervous System Extracellular Matrix | SpringerLink', n.d.)

3.8 RT-PCR

RNA was isolated using the RNeasy Mini Kit (QIAGEN, #74804) according to the manufacturer's protocol. Subsequently, reverse transcription of RNA to complementary DNA (cDNA) was performed using TATAA GrandScript cDNA Synthesis Kit (TATAA Biocenter, #AS103c) according to the manufacturer's protocol. For qPCR, TaqMan® Gene Expression Assays (Life Technologies by Thermo Fisher Scientific, Waltham, MA, USA) were used for HAS1-3 and the GAPDH, all purchased from Applied Biosystems and used as recommended by the manufacturer. Amplification was performed on the qPCR cycler (QuantStudio® 6 Flex PCR System, Applied Biosystems® from Thermo Fischer Scientific, Waltham, MA, USA). The Ct values of each of the measured conditions were normalised to the GAPDH. The 2-ΔΔCt values were then expressed as described by Livak and Schmittgen (Livak and Schmittgen 2001).

3.9 Haematology and Biochemistry

Blood was collected from the retro-orbital sinus. Urine samples were collected manually. Samples were collected under anaesthesia. Samples were analysed by Synlab (Munich, Germany).

3.10 Proteomics

For assessment of renal toxicity, urinary proteins were assessed using the Kidney Toxicity 5-Plex Rat ProcartaPlex™ Panel 2 (Invitrogen cat. no. EPX050-30125-901). Samples were measured in accordance with the manufacturer's protocol.

The Th Complete 14-Plex Rat ProcartaPlex™ Panel (Invitrogen cat. no. EPX140-30120-901) was used to assess the T helper response. Samples were analysed on Bio-Plex 200 systems (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol.

3.11. Biomechanics

Biomechanical evaluation was performed in collaboration with the Czech Technical University, Prague, Faculty of Mechanical Engineering, Department of Mechanics, Biomechanics and Mechatronics, Radek Sedláček's group.

3.12 cFOS stimulation

Stimulation was performed as previously described by (Bojovic, Bramham, and Tjølsen 2016). Single subdermal needles (Rhythmlink) were used as electrodes and animals were perfused 2 h after electrical stimulation.

3.13 MRI

Ex vivo MRI imaging was performed to assess the completeness of the dorsal column lesion. The spinal cord was then imaged ex vivo using a 7T preclinical MRI scanner (MRS*DRYMAG 7.0T, MR Solutions, Guildford, UK). MRI was performed in collaboration with the Centre for Advanced Preclinical Imaging (CAPI) in Prague.

3.14 Microscopy and image analysis

Spinal cord sections were imaged on a LEICA CTR 6500 microscope. FAXS 4.2.6245.1020 software was used (TissueGnostics). Confocal images were obtained with the use of a Zeiss LSM 880 Airyscan microscope. Image analysis was performed using HistoQuest 4.0.4.0154 (TissueGnostics) and Fiji software (Schindelin et al. 2012). Light sheet data were processed using Huygens software

(Scientific Volume Imaging, The Netherlands, http://svi.nl) and Imaris microscopy image analysis software (Oxford Instruments).

3.15 Statistics

Data are expressed as mean ± SEM. Tukey's one- or two-way multiple comparison test was used to determine statistical differences between groups. A p-value of 0.05 was considered significant for all statistical analyses. GraphPad Prism (GraphPad 9 and after the update, 10 Software) was used for data processing and statistical analysis.

4. RESULTS

4.1 4-MU treatment at a dose of 1.2 g/kg/day is safe for long-term usage in rats

4.1.1 Body-wide downregulation of HA at 1.2 g/kg/day dose of 4-MU

Hyaluronan (HA) is not only one of the most important polysaccharide components of the extracellular matrix, it also plays a key role in the regulation of a large number of cellular processes and in the organisation of the tissue architecture (Kobayashi, Chanmee, and Itano 2020). A significant downregulation of staining intensity was observed in the tissues (brain, spinal cord, spleen, liver and kidney). This confirmed the effect of 4-MU in reducing HA and CS.

4.1.2 No adverse effects on haematological or biochemical parameters were observed with long-term administration of 4-MU at a dose of 1.2 g/kg/day

Our results suggest that no serious adverse changes in blood haematological or biochemical parameters were induced by long-term treatment with 4-MU.

4.1.3 1.2 g/kg/day dose of 4-MU does not affect animal behaviour

The grip strength test was used to assess neuromuscular function. It determined the maximum force that the animal could produce. Animals in the wash-out group had a significant reduction in the isometric contraction force of the forelimb compared to the placebo control group, but the reduction was mild. Motor function and forelimb-hindlimb coordination were assessed using the rotarod. Between the groups, no significant changes were observed.

4.1.4 Long-term 4-MU treatment at the current dose does not affect the biomechanical properties of tendons and skin, but does affect the biomechanical properties of bone

To further characterise the systemic effect mediated by 4-MU, we decided to investigate the biomechanical properties of bones, skin and tendons after long-term 4-MU treatment. Our results suggest no significant changes in the measured parameters, except for the external stiffness of the bones, where we observed a significant reduction in the stiffness of the measured femurs. External stiffness represents the resistance to deformation under the applied load. These results suggest

that the reduced level of HA and GAGs in the bones leads to a reduction in the water content, making the bones less resistant to deformation.

4.2 4-MU at a dose of 1.2 g/kg/day reduces glial scar but is insufficient to induce functional recovery after chronic SCI

4.2.1 Although 4-MU reduces GAG synthesis and perineuronal nets (PNNs) in the intact spinal cord, 4-MU (1.2 g/kg/day) is not sufficient to downregulate the increased production of CS after SCI

GAGs were extracted from the dissected spinal cords (Fig. 1A). The results showed that 4-MU treatment alone and 4-MU plus daily treadmill training (significantly reduced GAG levels compared to placebo. In the treated animals, rehabilitation did not affect the efficacy of 4-MU in downregulating GAG synthesis. Interestingly, daily treadmill exercise alone also showed a modest but non-significant reduction in GAG levels compared to placebo, suggesting that rehabilitation (or exercise) may independently reduce GAG levels. In the wash-out group, the total amount of GAGs recovered to a level similar to that of the rehabilitation group, suggesting a partial return of GAGs (Fig. 1A). In comparison to the placebo group, the 4-MU and/or rehabilitative effect on GAGs levels was tested.

We also quantified the degree of HA downregulation using HABP staining (Fig. 1B, C). Sections at Th6 and Th10 and around the level of Th8, the focus of subsequent experiments, were subjected to histochemical staining. In the 4-MU-treated group, the intensity of HABP was significantly decreased at Th8 and Th6 sections compared to the placebo group. At the Th10 level, there was no significant difference between the 4-MU and placebo groups. After 8 weeks of wash-out, HA remained at low levels at Th8 and Th6 compared to the placebo group, with some tendency for HA production to return, but not to reach the level of the placebo group (Fig. 1B, C).

Quantification of HAS mRNA expression in spinal cord samples by qPCR (Fig. 1D) did not show a significant down-regulation of HAS gene expression in the 4-MU

group. However, clear trends of downregulation of HAS gene expression were observed in the 4-MU group compared to all other groups. In the wash-out group, HAS gene expression reached placebo levels in combination with the rehabilitation group, suggesting a recovery of normal GAGs expression after the wash-out period.

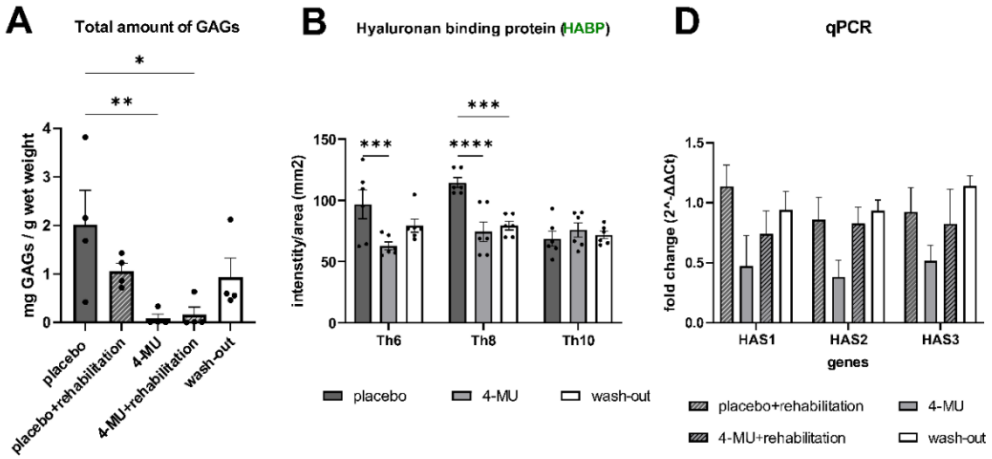

Fig. 1. 4-MU reduces HA and CSPG synthesis in non-SCI animals even after 8 weeks of feeding. (**A**) Bar graph showing the total amount of GAGs extracted from spinal cords. Values are presented as mean ± SEM; * $p < 0.05$, by one-way ANOVA, Dunnett's post-hoc test. (n = 4 animals per group). (**B**) Quantification of fluorescence images. Sections were stained with HABP and the signal intensity in the grey matter was analysed. (**C**) Bar graph showing the fold change in the expression of HAS 1-3 genes (as 2^-ΔΔCt). Values are presented as mean ± SEM; two-way ANOVA, Tukey post-hoc test in all 4 groups (n = 4 animals per group).

We next investigated the effect of 4-MU treatment on perineuronal nets (PNNs) in the ventral horns. This was done by co-staining for WFA and ACAN. Spinal cord sections from all groups were stained for WFA and ACAN (Fig. 2) and the number of positive cells was counted in the ventral horns up to the central canal. Similar to the biochemical assays, 4-MU treatment and treadmill exercise independently reduced the total number of WFA+ive cells in the spinal ventral horns (Fig. 2). The combination of both induced a greater down-regulation. We also observed that the number of WFA+ive and ACAN+ive neurons returned to control levels after the wash-out period (Fig. 2). This suggests that the current dose of 4-MU at 1.2 g/kg/day or rehabilitation can effectively reduce PNNs in the uninjured spinal cord.

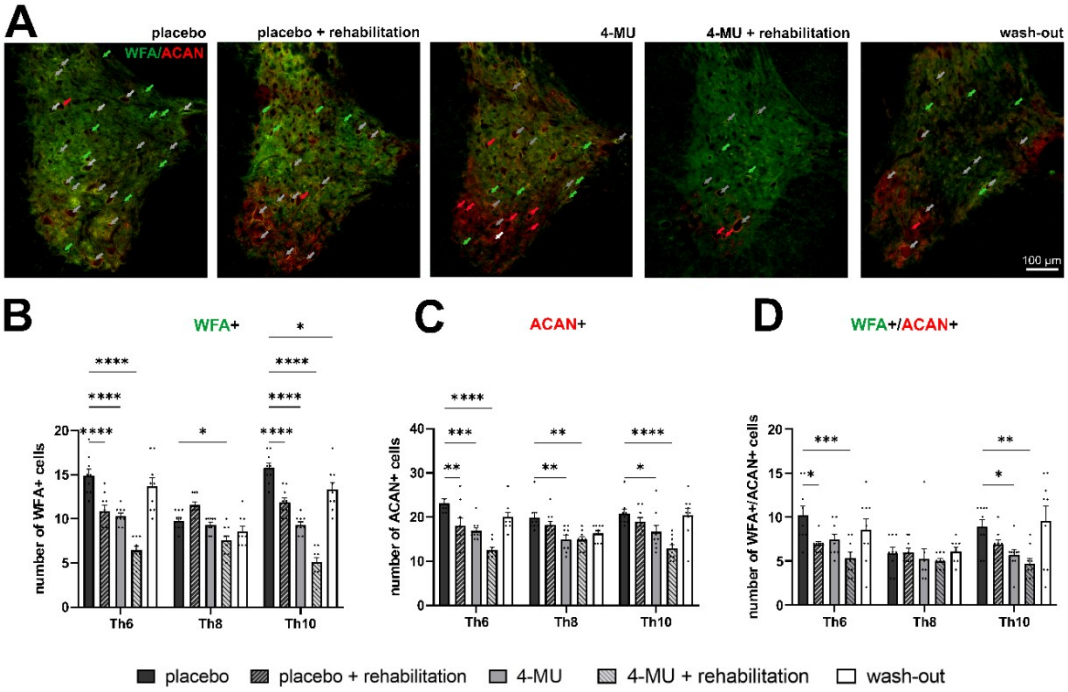

Fig. 2. Down-regulation of PNNs after 8 weeks of 4-MU feeding in uninjured animals. (**A**) Representative fluorescent images showing WFA and ACAN+ive PNNs around cells in the ventral horns and their colocalization in thoracic spinal cord (Th8) in uninjured animals in all groups after 8 weeks of feeding and after 8 weeks of wash-out period. Green arrows indicated the WFA+ive PNNs enwrapped cells; red arrows indicated ACAN+ive PNNs enwrapped cells and grey arrows indicated cells where WFA/ACAN positive signal colocalizes. Scale bar 100 µm. (B–D) Quantitative analysis of (A). Data showed mean ± SEM (n = 3 animals per groups). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA, Dunnett's multiple comparison.

Having found that a dose of 4-MU of 1.2 g/kg/day in combination with rehabilitation was effective in reducing PNNs in the spinal cord, we next investigated whether this dose was sufficient to reduce the increased expression of inhibitory ECM in the injured spinal cord. Rats were subjected to a moderate SCI induced by a 200 kdyn impact at the Th8 level of the spine. Six weeks after injury, the animals were randomly divided into two groups. The groups received a daily diet containing 4-MU or placebo for 8 weeks. In addition, both groups received task-specific rehabilitation for the 16 weeks concurrent with the oral 4-MU treatment (i.e., 8 weeks during 4-MU treatment and 8 weeks after) in order to prime appropriate re-connection from the potentially enhanced neuroplasticity. First, we evaluated the

amount of HA within the spinal cord using HABP. After 4-MU treatment, we observed a trend towards lower HA levels throughout the spinal cord. Even after an 8-week wash-out period, the intensity remained reduced.

However, when we assessed the efficacy of 4-MU treatment in reducing the CSPG-rich inhibitory environment formed around the lesion. Our results showed no significant difference between the 4-MU and placebo groups after a further 8 weeks of no treatment but daily rehabilitation (Fig. 3). These results suggest that the strong upregulation of CSPGs after SCI made the dose of 1.2 g/kg/day of 4-MU insufficient to suppress their production in the injured spinal cord.

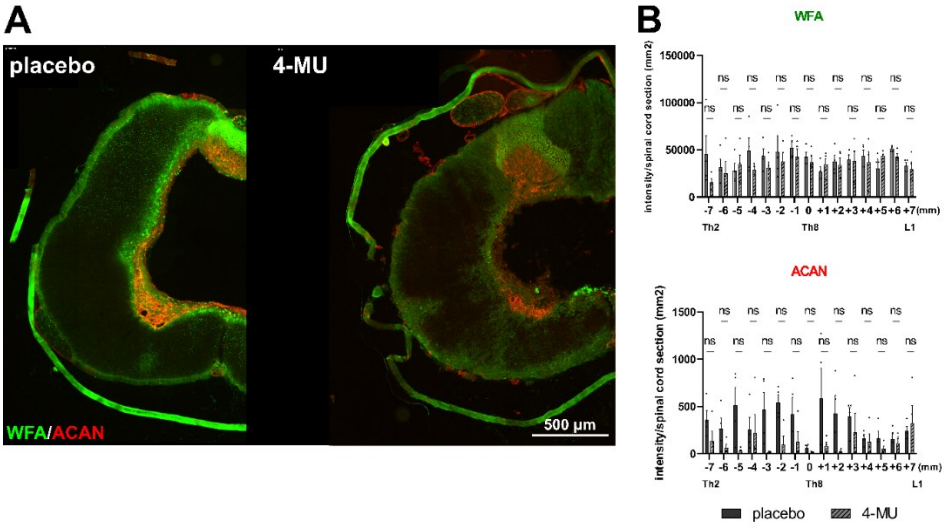

Fig. 3. Immunofluorescent double staining of WFA and ACAN suggested that 4-MU at the current dose of 1.2 g/kg/day was not sufficient to downregulate the increased production of CS after SCI. (**A**) Representative fluorescence images showing the area of WFA+ive (in green) and ACAN+ive (in red) around the centre of the lesion. (**B**) Quantitative analysis (A). Individual data points and their mean ± SEM are presented (n=4 animals per group). ns by two-way ANOVA, Sidak's multiple comparison test.

4.2.2 4-MU at 1.2 g/kg/day reduces glial scar in chronic SCI

HA is upregulated by astrocytes in neuroinflammation (Back et al. 2005) and is produced by both neurons and glia in the CNS. Given our observation of HA downregulation around the lesion cavity, we next focused on the effect of 4-MU on the glial scar after SCI. A quantitative analysis of the GFAP+ive area was carried out to assess the glial scar surrounding the lesion cavity on cross sections (Fig. 4). A

significant decrease in astrogliosis was observed in the 4-MU treatment group at a dose of 1.2 g/kg/day in comparison to the placebo group.

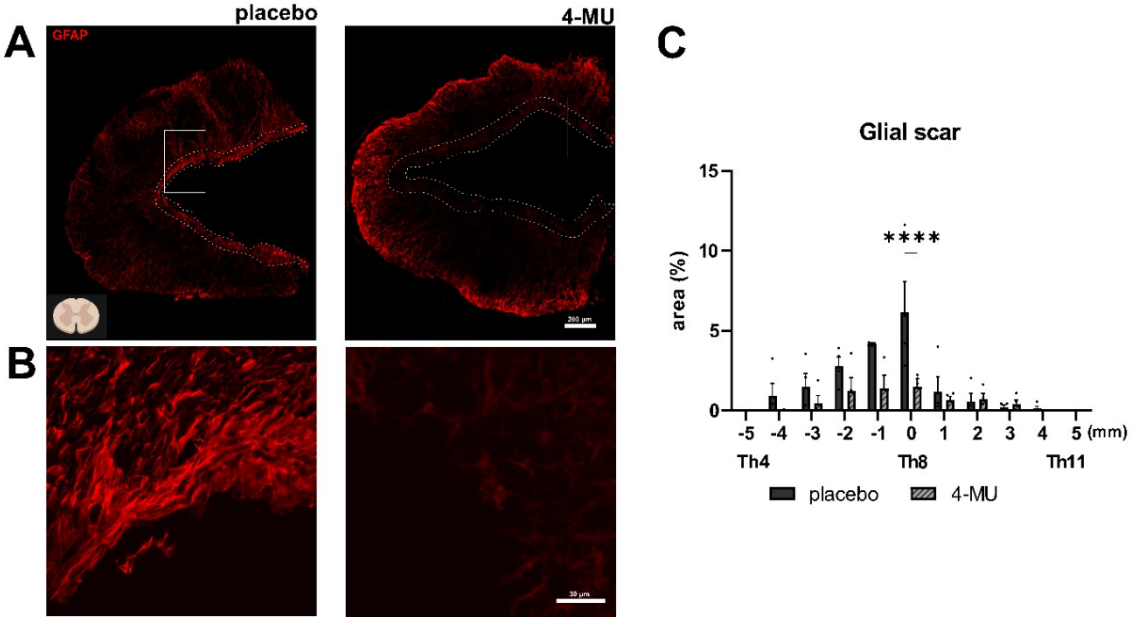

Fig. 4. The area of glial scar around the lesion site was reduced by 4-MU treatment. (**A**) Representative fluorescence images of the lesion epicentre staining for GFAP in chronic SCI treated with 4-MU and placebo. (B) Magnified images showing structural changes in GFAP+ive scar tissue after 4-MU treatment compared to placebo-treated animals. Scale bar: 30µm; (C) Quantification of (A). Values are presented as mean ±SEM; **** $p<0.0001$ by two-way ANOVA, Sidak post hoc test. (n= 4 animals per group).

4.2.3 4-MU at a dose of 1.2 g/kg/day promotes the sprouting of serotonergic fibres at a distance from the injury, but has no effect on the density of synapses around the site of the lesion

Serotonin (5-HT) plays an essential role in the control of sensorimotor functions. We therefore sought to determine whether 4-MU treatment alters serotonergic innervation. We observed a significant increase in 5-HT+ive puncta in the ventral horns above the lesion (4-MU 259.25 ± 12.3 vs. placebo 208.58 ± 4.06), and below the lesion (4-MU 270.67 ± 15.17 vs. placebo 203.96 ± 6.01). The results suggest that 4-MU treatment leads to downregulation of HA and promotes long-term synaptic plasticity after chronic SCI in combination with rehabilitation during the 8-week

wash-out period. However, there was no significant difference in the synaptic density between the groups.

4.2.4 4-MU at a dose of 1.2 g/kg/day is not sufficient to improve functional recovery in the chronic phase of SCI

We tested whether axonal sprouting induced by 4-MU and daily rehabilitation would lead to functional recovery in the chronic stage of SCI, based on biochemical results showing that 4-MU abolishes plasticity-limiting perineuronal networks. There were no significant differences or indications of a trend towards improvement at any time point. This suggests a lack of 4-MU mediated recovery.

4.3 Expression of α9 integrin allows for the reconstruction of the sensory pathway of the spinal cord after injury

4.3.1 AAV transduces integrin and kindlin in DRG neurons

The ratio of GFP- and/or V5+ive cell bodies to βIII-tubulin+ive cell bodies was counted to determine transduction efficiency. The transduction efficiency was similar for the three vectors and ranged from 28 to 38% for the single vectors and from 20 to 25% for the co-transduction with both the α9 and the kindlin-1 vectors.

4.3.2 α9-K1 axons regenerate across lesions in the bridges of connective tissue

Axons from the α9-K1 group were observed to cross the lesion, re-enter CNS tissue and continue growing rostrally up the spinal cord. Within the lesions, many axons were seen in GFAP-negative connective tissue strands and bridges, and in the meningeal/connective tissue roof covering most lesions. A few regenerating axons grew around the base of the lesion. At the interface of the connective tissue with the rostral lesion edge, axons often showed axonal tangles with changes in trajectory (Fig. 6). However, once established in the CNS tissue, axons followed a fairly straight trajectory. Some axons did not have a re-entry into the CNS tissue. Instead, there was growth along the spinal cord in the meninges (Fig. 6). We measured 849 ± 64 axons 1 mm rostral to the lesion edge in the α9-K1 group.

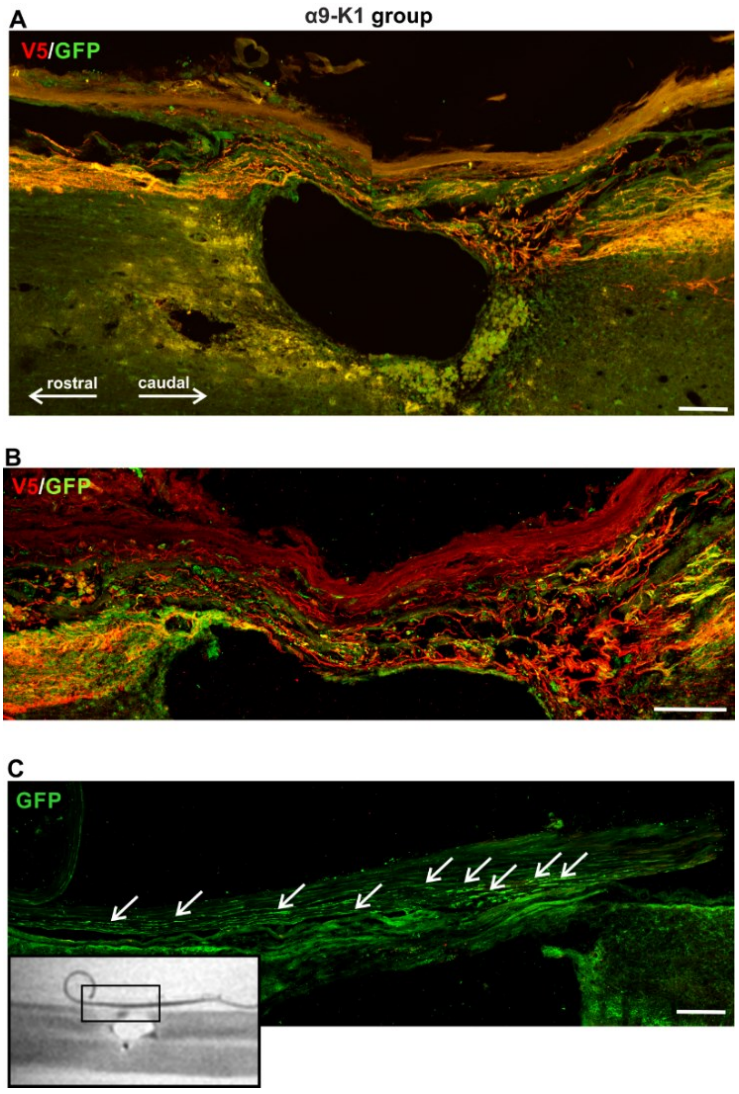

Fig. 6. Co-expressing α9-integrin and kindlin-1 promotes regeneration of sensory axons (**A**) An example of spinal lesions in T10 from the α9-K1 group. Approaching the lesion from the caudal side are many red (α9-V5 stained) axons. Some random growth occurs when the axons enter the bridge over the top of the lesion, which consists mainly of meninges, and then the axons enter the CNS tissue again for rostral growth on the left; 200 µm. (**B**) An example of an axon that passes through a fine strand of connective tissue. There is a region of wandering growth as the axons re-enter CNS tissue at the rostral end; 100 µm. (**C**) Some axons continue to grow in the meninges next to the CNS tissue. Where the detail comes from is shown in the lower left MRI image; 50 µm.

4.3.3 α9-K1 axons regenerate to the brain stem

Regenerating axons were seen rostral to the T10 lesion in animals injected with both integrin α9 and kindlin-1. Many of these axons were seen to extend all the way up to the spinal cord. These regenerating axons were therefore following a different path to that of the sensory axons that had not been injured. Along this regenerating pathway, there were frequent branches that extended into the grey matter of the dorsal horn. The distance of axon regeneration from the thoracic lesions was up to 5 cm. Almost all regenerating axons rostral to the lesion stained for both α9-V5 and kindlin-1-GFP (Fig. 7). This contrasts with axons caudal to the lesion, where there was a significant proportion of single-stained axons (Fig. 7). The implication is that

only axons with both alpha9 and kindlin-1 were capable of regeneration through the lesion. Regeneration index 5mm above lesion was approximately 0.5.

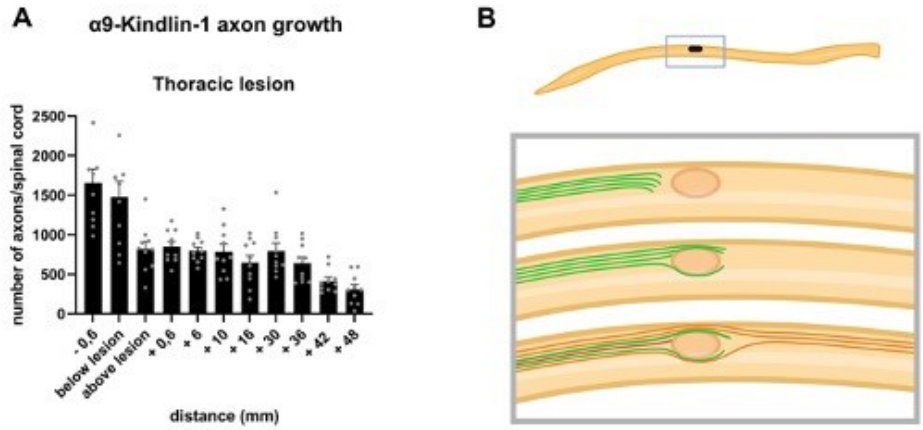

Fig. 7. Distant regeneration in the spinal cord co-expressing alpha9 integrin and kindlin-1. (**A**) Bar graphs show the number of axons after thoracic injury with L4, L5 DRG injections. Bar graphs show data with mean ± SEM (n= 7-12 animals per group). (B) Schematic representation of key results of Aim 2. Only the α9-K1 group showed substantial regeneration beyond the lesion into the rostral cord. Axons in the laminin-containing connective tissue at the core of the lesion regenerated in the kindlin group. Created with BioRender.com.

4.3.4 α9-K1 axons regenerate through tenascin-C containing tissue

Immunolabelling for GFAP, laminin and tenascin-C was performed to examine the substrate on which α9-kindlin-1 V5- positive axons were growing. The connective tissue strands and the roof in lesions through which axons regenerated were totally or partially GFAP negative. There was usually a clear border with GFAP positive CNS tissue. Laminin and tenascin-C staining was seen in the connective tissue through which axons grew across the lesions. The boundary between the connective tissue and the CNS was less clear with tenascin staining. This is because the perilesional CNS tissue also expresses tenascin (Fig. 8). In the kindlin-1 group, GFP+ive regenerated axons were observed in association with the laminin+ve connective tissue substrate. However, they were not able to grow back into the CNS tissue (Fig. 8). In summary, within the lesion, axons expressing a9-V5 and kindlin-1 appeared to regenerate preferentially through laminin and tenascin-C positive connective tissue structures and were then able to re-enter and grow within tenascin-

C expressing CNS tissue. Axons expressing kindlin-1 alone contained activated forms of the integrins expressed endogenously by sensory neurons, which are laminin and fibronectin receptors. These axons grew where laminin was present but did not re-enter the laminin negative CNS tissue.

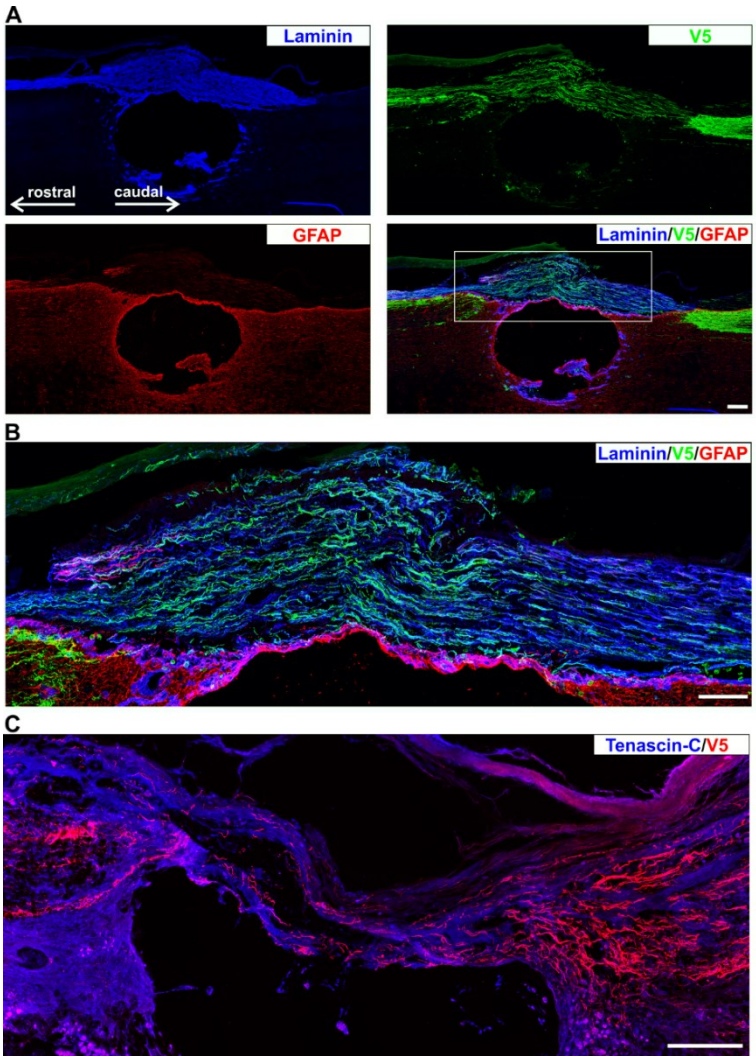

Fig. 8. α9-integrin kindlin-1 axon regeneration by laminin-111 isoform and tenascin-C positive tissues. (**A**) The GFAP-ve and tenascin+ve laminin+ve bridge that develops over the lesions is mostly derived from connective and/or meningeal tissue. Within the connective tissue bridge, tangled axons and axons crossing the lesion from the α9-K1 group can be seen; 200 µm. (**B**) The detailed image shows the bridge region with growing axons with morphology typical of regenerating axons, and partially with the retracting bulb; 50 µm. (**C**) Growth of axons in the α9-K1 group through strands of connective tissue that are stained for tenascin-C.; 200 µm.

4.3.5 Regenerating axons form a functional synapse across the lesion

To assess functional connectivity across the lesion, spinal cFOS expression was visualised after electrical stimulation of the median and sciatic nerves. cFOS is an immediate early gene and a well-established marker of neuronal activity induced transcription. Results are presented as percentage ratio of number of cFOS+ive cells above lesion to number of cFOS+ive cells below lesion. Compared to the GFP (7.754 ± 1.334% after thoracic SCI) and kindlin-1 (9.650 ± 1.313% after thoracic SCI) groups, a higher percentage of cFOS+ive cells was observed in the α9 - kindlin-1 group (44.380 ± 2.684% after thoracic SCI).

4.3.6 α9-K1 restores sensory function

To investigate the recovery of sensory behaviour, forelimb and hindlimb function tests were performed in animals with T10 lesions/DRG lumbar injections. Soft mechanical pressure (Von Frey), heat (plantar/Hargreaves) and tape removal tests were used (Fig. 9).

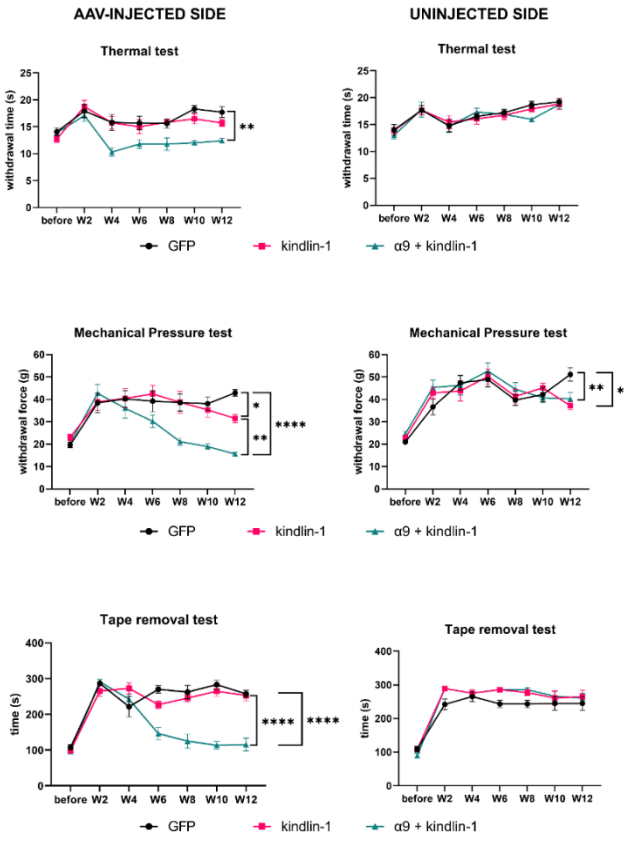

Fig. 9. α9-K1 led to recovery of sensory functions. After thoracic lesions, only in the α9-Kindlin group and only on the treated side, there was a recovery of heat sensation, pressure sensation and tape removal. Data show mean ± SEM (n=10-12 animals per group). ns $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, **** $p < 0.0001$, two-way ANOVA, Tukey's multiple comparison test.

5. DISCUSSION

5.1 4-MU treatment at a dose of 1.2 g/kg/day is safe for long-term usage in rats

The first objective was to assess systemic effects after long-term treatment with 4-MU followed by a 9-week wash-out period. The results showed that there was a reduction in the levels of HA and CSPGs. The results suggest that long-term treatment with 4-MU is well tolerated and does not appear to interfere with normal physiology. 4-MU has previously been identified as an inhibitor of the synthesis of HA. HA is an un-sulphated glycosaminoglycan. The result is a decrease in HAS mRNA levels (Vigetti et al. 2009). Here, we report that 4-MU is also an inhibitor of CS synthesis. The key building block of CS is the same monosaccharide, GlcA. As CS is a key inhibitory molecule for neural regeneration and plasticity, our results suggest that 4-MU may have potential as a novel non-invasive treatment for nervous system disorders. In our experiments, we had a systemic route of administration. It was assumed that the whole body of the animal would be affected. We had in mind: HA is expressed at high levels throughout the body and is conserved throughout evolution. HA is a simple linear polysaccharide. It has a variety of biological functions. By interacting with various molecules, HA maintains tissue homeostasis and organises the structure of the ECM. The exceptional biophysical and biomechanical properties of HA contribute to tissue hydration. It mediates the diffusion of solutes across the extracellular space and maintains tissue lubrication. As reviewed in (Dicker et al. 2014), the binding of HA to cell surface receptors activates a large number of signalling pathways that regulate cell function, tissue development, the progression of inflammation, wound healing responses and tumour biology. Looking more closely at the potential pathophysiological changes mediated by 4-MU, we investigated the systemic effect of 4-MU treatment at a dose of 1.2 kg/g/day, which showed no irreversible adverse effects from long-term administration of 4-MU.

5.2 4-MU as novel therapy for chronic SCI

Then, we investigated whether a dose (1.2 g/kg/day) of 4-MU would be sufficient to reduce PNNs in the ventral horns and promote sprouting and functional recovery in chronic SCI. We found that the oral dose of 1.2 g/kg/day (4-MU) was sufficient to reduce PNNs and HA in uninjured animals, but that it was not sufficient to suppress the strong upregulation of CSPGs after SCI, so that no functional recovery was observed.

Many studies have focused on regeneration strategies after SCI in recent years. These strategies are often based on the targeting of PNNs and the manipulation of the glial scar to attenuate the inhibitory properties of its environment. Current strategies range from proteolytic manipulation of the ECM to targeting specific ECM components by synthesising inhibitory ECM molecules after SCI (Burnside and Bradbury 2014). One of the most studied approaches is enzymatic ECM modification using ChABC. By degrading CS chains to disaccharides, removing CSPG inhibition in the glial scar and removing PNNs as a brake on plasticity, ChABC benefits both acute and chronic SCI conditions (Wang et al. 2011). Spinal cord injured animals show improved recovery, both anatomically and functionally, following ChABC treatment (Bradbury et al. 2002; Wang et al. 2011). Functional recovery after SCI is further enhanced when restoration of plasticity is combined with rehabilitation (Wang et al. 2011). However, there are several drawbacks to using ChABC. The main disadvantages of using this bacterial enzyme are its thermal instability and short half-life, which requires multiple or continuous intrathecal administrations (Nori et al. 2018), the potential for the body to develop an immune response, and the difficulty of dosing. In addition to ChABC, Keough and colleagues (Keough et al. 2016) tested a subset of 245 drugs that were known to penetrate the CNS and exhibit oral bioavailability. None of these 245 compounds was shown to have sufficient ability to overcome oligodendrocyte precursor cell inhibition of CSPG.

We therefore investigated whether the administration of 4-MU would reduce the synthesis of both HA and CS, thereby facilitating neuroplasticity. Indeed, both anatomically by histochemistry and biochemically by GAG quantification, we observed a down-regulation of HA and CS. Our data suggest that 4-MU, in combination with daily training, is a suppressor of GAG synthesis. Administration of 4-MU resulted in removal of PNNs in the ventral horns using PNN markers including WFA and ACAN. After a wash-out period of 8 weeks, PNNs reappeared. It is likely that CS synthesis is less sensitive to UDP-GlcA deficiency, which explains why PNNs reappeared after 8 weeks of wash-out while HA levels remained low. CS is synthesised in the Golgi apparatus where UDP-GlcA sugars are transported into the Golgi lumen with high affinity. HA is synthesised directly at the cytoplasmic membrane (Nagy et al. 2015).

We used a thoracic contusion injury that mimics the most common closed SCI in humans, sparing some axons around a central cavity and ablating dorsal corticospinal tracts (CSTs) critical for human motor control (Basso 2000). In the chronic phase, starting 6 weeks after injury, 4-MU was administered (Kjell and Olson 2016). At this stage, the glial scar is well established. CSPGs are upregulated and the acute immune response has subsided (Hu et al. 2010). 4-MU treatment was accompanied by daily treadmill rehabilitation for consolidation of appropriate synaptic connections and pruning of others (Oudega, Bradbury, and Ramer 2012). After the 8 weeks of treatment and rehabilitation, rehabilitation was continued for a further 8 weeks as a wash-out period. This wash-out period allows the PNNs to reform and stabilise de novo synapses and consolidate anatomical plasticity (Al'joboori, Edgerton, and Ichiyama 2020; Wang et al. 2011), while the continued rehabilitation prunes random connections, supports appropriate connections and removes inappropriate connections (Fawcett and Curt 2009). There was a robust reduction in the glial scar surrounding the cavity following oral administration of 1.2 g/kg/day 4-MU. Throughout the wash-out period, this reduction was sustained.

We analysed the intensity of the 5-HT signal to assess the potential of 4-MU to remove the plasticity brake formed by PNNs. Away from the lesion, we observed increased 5-HT sprouting. However, this sprouting did not result in a significant difference in synapsin immunoreactivity within the ventral horns above and below the lesion between 4-MU treated and placebo animals. The effect of 4-MU on the mediation of changes in other cellular composition was also investigated. Assessment of functional recovery showed no significant differences between the 4-MU and placebo groups, even with continued rehabilitation over the next 8 weeks. As 4-MU-mediated PNNs ablation has been demonstrated in the mouse hippocampus to improve memory in ageing mice (Dubisova et al. 2022), we hypothesised that the lack of functional recovery after SCI in this study was due to the lower dose of 4-MU administered (1.2g/kg/day versus 2.4g/kg/day) and the strong CS-GAG upregulation after injury. A higher dose of 4-MU combined with rehabilitation should be tested to see if this improves recovery after SCI.

5.3 Using AAV-mediated overexpression of integrin α9 for sensory pathway reconstruction after SCI

In the last part of this thesis, we have shown that α9-K1 transduced neurons regenerated their neurons vigorously through the largely fibroblastic environment of the lesion core of the injured rat spinal cord. The axons then extended back into the CNS tissue of the spinal cord, where they regenerated down to the level of the spinal cord, a distance of more than 4 cm from the thoracic lesions. In the grey matter of the dorsal horn, where synapses were evident, many axonal branches grew. On stimulation, neurons in the dorsal part of the spinal cord upregulated cFOS, indicating that functional connections were being formed. The light touch, the heat and the removal of the tape - the full battery of behavioural tests - indicate the full recovery of the behaviour.

The sensory neurons of the DRG are capable of regeneration, as evidenced by their ability to regenerate axons within the PNS. The extensive regeneration of axons in the current study suggests that α9-K1 transduction activates mechanisms that

regulate regeneration in the CNS environment. Up-regulation of the RAGs gene expression programme (Chandran et al. 2016) is associated with sensory regeneration in the PNS. Regeneration without expression of the RAGs programme is unlikely, as several of the molecules expressed in the RAGs programme are required for successful regeneration. Transduction of sensory neurons with α9-K1, even in the absence of axotomy, results in the expression of the RAGs programme, thus priming the neurons for successful regeneration (Cheah et al. 2023). However, for long-distance spinal sensory regeneration to occur, expression of the RAGs programme alone is not sufficient. Crushing peripheral nerves prior to lesioning the central sensory branch upregulates the RAGs programme and leads to increased local sprouting of severed axons, but not long-distance growth (Neumann and Woolf 1999). There is therefore a need for an additional element. The events of cell migration are dependent on growth-promoting receptors on the cell surface that bind to ligands in the environment (Caswell and Norman 2006). This receptor-ligand interaction leads to signalling, increased cytoskeletal dynamics associated with focal adhesions, which in turn lead to mechanical traction and migration (Ridley 2011). Integrins are the major cell surface adhesion molecules which are inducers of migration. Adult DRG neurons express several fibronectin- and laminin-binding integrins (α3β1, α4β1, α5β1, α6β1 and α7β1), with the laminin receptor α7β1 playing a major role in axonal regeneration in peripheral nerve (Werner et al. 2000). However, in the acute SCI, laminin and fibronectin are found in fibroblasts in the core of the lesion, surrounding blood vessels and in the meninges, but not in the central nervous system (Orr and Gensel 2018). Tenascin-C and osteopontin, which are not partners for the integrins expressed in adult DRGs, are the integrin ligands in reactive CNS tissue. The major migration-inducing integrin for tenascin-C and osteopontin is α9β1, and it is therefore not surprising that α9-K1 transduction allows axon regeneration to occur (Høye et al. 2012). Expression of kindlin-1 alone activates endogenous integrins expressed by DRG neurons. These integrins bind to laminin and fibronectin. Therefore, kindlin-1 alone allowed regeneration of sensory

axons into the core region of the lesion, but not into CNS tissue containing tenascin-C, for which sensory axons have an integrin receptor but very little laminin. Only when neurons were transduced with α9-K1 to provide an activated form of this tenascin-C-osteopontin receptor, did regeneration into the lesion and on to CNS tissue occur. The uninjured axons below the lesion in the α9-K1 transduced animals expressed both α9 and K1 in most cases, but some were positive for only one of these. Only axons expressing both α9 and K1 were seen above the lesion, indicating that successful CNS regeneration requires this combination. However, in animals with lesions in the thoracic spinal cord, we observed a decrease in V5 signal in the spinal cord, which may indicate that integrin trafficking becomes weaker with distance.

The axons that regenerated through the spine lesion were associated with strands and bridges of GFAP-negative tissue that was shown to contain cells of the meninges and blood vessels, perivascular and fibroblastic origin. These cells expressed laminin and tenascin-C, providing a suitable. growth substrate for endogenously expressed kindlin-1-activated laminin-binding integrins and α9-expressing neurons. At the rostral interface of the lesion core and CNS tissue, axon growth tended to be chaotic, indicating exploratory behaviour within the disturbed tissue. Within the cord rostral to the lesion, regenerating axons in the α9-K1 group were found particularly at the dorsal column/dorsal horn border, with some axons growing wanderingly through white matter. This is different from the normal path of sensory axons and is also different from the path through the white matter that was taken by axons that regenerated after α9-K1 and a dorsal root crush (Cheah et al. 2016). Although it is not clear why the white matter/grey matter boundary should provide this, we speculate that the path disruption associated with growth through the lesion causes axons to seek a permissive path.

Approximately 1000 axons regenerated 2-5 cm from the lesions into the hindbrain in our study and in the previous study using dorsal root crush rather than spinal lesions (Cheah et al. 2016). Several data show that these are regenerated rather than

unlesioned axons: i) Axons in the lesion pass through GFAP negative fibroblastic tissue. Uninjured axons would be surrounded by CNS glia; ii) Axons rostral to the lesion follow a different route than unlesioned axons; iii) Regeneration progresses over time, which is well illustrated by the behavioural tests, where the very slow improvement is seen before week 8, where the sudden improvement appeared in the α9-K1 group but not in the K1-only and GFP groups, and this improvement remained until the end of the experiments, suggesting that the transduced axons only reached the particular targets after 8 weeks.

After stimulation of the sciatic nerve, the peripheral branch of the DRG injected with α9-K1, we tested the ability of these synapses to stimulate neurons in the spinal cord by observing the upregulation of c-FOS in propriospinal neurons. In the α9-K1-treated animals, the number of c-FOS neurons after stimulation was much higher than in the control animals, indicating connections between the regenerated axons and spinal cord neurons. This was demonstrated by sensory recovery testing. In particular, we saw eventual full recovery in fine touch, heat and tape removal tasks in the α9-K1 lumbar injected group. The time course of recovery in this and our previous experiment (Cheah et al. 2016; 2023) was similar to the time course of axon regeneration. As the animals do not appear to see the tape on their hind paws, recovery in the tape removal task is particularly informative. Instead, it appears that sensory detection triggers the removal of the tape from the hind paw. The sensation must reach the brain, which then carries out the removal process, in order to perceive that there is tape on the hind paw.

Almost complete reconstruction of the spinal sensory pathway has been achieved employing the strategy of using an activated integrin to induce sensory regeneration. It is important to note that in the present study, axons were able to regenerate over a length that would allow growth across a human injury, as the lesion length in human SCI varies from 1 to 7 cm (Dalkilic et al. 2018). The regeneration index, which compares the number of axons below and above the lesion, shows that 50% of the axons regenerated through the lesion area. Once through the lesion, the number of

axons did not decrease significantly up to the high cervical cord. However, due to the lack of innervation of the medullary sensory nuclei, complete reconstruction was not achieved. Methods to re-innervate these nuclei have been identified using chondroitinase digestion and neurotrophin-3 expression (Massey et al. 2006) and could be used to achieve complete tract reconstruction. However, functional recovery, including tape removal, which requires sensory information to reach the brain, was almost complete despite the lack of innervation of the sensory nuclei. If α9-K1 could be delivered to descending spine axons, it is likely that regeneration would be possible. However, in these highly polarised neurons, integrins are restricted to the somatodendritic domain and excluded from the axons, so this repair strategy cannot currently be directly applied to descending motor pathways (Andrews et al. 2016). Strategies have been identified to allow integrins to transport to motor axons, which may make it possible to reconstruct motor pathways (Petrova et al. 2020; Nieuwenhuis et al. 2020).

6. CONCLUSION

In conclusion, the results of this thesis show that the regeneration of axons in the CNS is inhibited by a large number of intrinsic and extrinsic factors. It has been shown that no single intervention is sufficient to fully regenerate damaged axons in the adult mammalian CNS because these factors act in parallel. Our aim has been to show that, in principle, there are two main therapeutic approaches to the treatment of SCI. Put simply, you could remove the inhibitory environment around the lesion site, thereby increasing plasticity, or you could use the inhibitory environment to promote regeneration by overexpressing the correct isoforms of molecules that are capable of binding to the inhibitory environment around the lesion site, thereby allowing axons to pass through the lesion. Both approaches have something in common, namely that axons in the adult CNS can be regenerated by reactivating the processes that trigger axon growth during development.

We have shown that 4-MU is safe for long-term use on rats. Furthermore, 4-MU can effectively downregulate HA throughout the body, with no detectable. side effects. We have also observed that 4-MU at a dose of 1.2 g/kg/day is effective in the downregulation of PNN (as shown by anti-aggrecan staining). The further question was whether 4-MU at the current dose would be sufficient for the promotion of anatomical plasticity and recovery after SCI.

In the next part, we focused on the chronic stage of SCI to see if 4-MU could be used to treat it. The main reason for the use of the chronic phase is that by then the scar is fully established and it is more clinically relevant for the potential future translation to the treatment of the chronic phase than is the acute phase of SCI. We hypothesised that 4-MU would not only reduce the glial scar surrounding the lesion, but also remove the PNNs around certain types of spinal neurons, thus re-opening a window of plasticity. We also expected that task-specific rehabilitation, in our case treadmill training, could remodel, adapt and reorganise axonal sprouting after training, leading to recovery of a motor skill after SCI. We investigated that 4-MU at the current dose is able to reduce the scar around the lesion, but after injury there

is an upregulation in the production of CSPGs around the lesion. Despite the fact that 4-MU at the current dose reduced scar even in the chronic stage, it was not sufficient to reduce upregulated CSPGs after SCI. Thus, we did not observe functional recovery, but the 5-HT staining showed some increased sprouting in the 4-MU treated animals. These results suggest that perhaps the higher dose would lead to functional recovery. However, a further study would be needed to test this hypothesis.

As mentioned above, one way to recapitulate the developmental stage is not to remove the inhibitory (CSPGs-rich) environment. However, in attempts to achieve axon regeneration, this is not the only option available to us. During axonal pathfinding, developing axons navigate the extracellular environment, extending to postsynaptic targets to form a functional synapse. The mechanism by which they navigate is a type of classical cell migration and follows a specific set of rules. When James Fawcett's laboratory at the University of Cambridge tried to regenerate axons, they came up with a simple idea. The idea was to try to recapitulate this developmental stage in cell cultures and after dorsal root crush in the rat model by overexpressing the particular integrin isoforms that are key to axon pathfinding during development. We did a follow-up study in a rat model of SCI. The first step was to overexpress the α9 integrin with its activator kindlin-1, which was injected directly into the DRGs to try to promote sensory axon regeneration. The reason why sensory pathways were chosen for this study is simple. SCI patients have more health problems than just the inability to walk, and at the same time the neurons are in the DRGs, not at the lesion site, which gave us the idea that the sensory pathways might be a better target. In this part of the thesis, we showed that AAV-mediated overexpression of integrin α9 together with kindlin-1 can lead to partial reconstruction of sensory pathways after SCI. In addition, we observed that axons passing through the lesion were able to form functional synapses above the lesion. We also observed behavioural improvements in the treated animals. However, as the sensory axons did not reconnect in the sensory nuclei in the spinal cord, we did not

achieve full pathway reconstruction with this treatment. This opens up room for follow-up studies - to use ChABC (or any other approach to locally dissolve the PNNs and CSPGs rich environment in the sensory nuclei) and then use this approach to reconstruct the sensory pathways connected to the bladder and perineum innervation (as this would be an amazing step forward for the patients if the bladder sensation would work again) and last but not least to try to reconstruct not only the sensory pathways but also the corticospinal tract.

In conclusion, this thesis has provided some insights that may change the field of axon regeneration somewhat and allow us to move forward in developing new experimental treatments that hopefully can move from the bench to patients and improve their daily lives

7. SUMMARY

Following SCI, several developmental principles come into play to either promote or inhibit spontaneous regeneration, and manipulation of these has the potential to contribute to functional recovery. In this work, by considering not only altering the inhibitory environment of the injured spinal cord, but also forcing the overexpression of the appropriate integrin isoform that allows regenerating axons to grow onto the inhibitory scar, thereby promoting sensory tract regeneration, we have explicitly provided a novel developmental input to the field of CNS repair.

Finding an effective way to reduce the inhibitory environment is one of the key approaches in the treatment of SCI. We focused on investigating a drug that can be administered orally with a simple dosage, which has been a goal for some time - 4-MU. 4-MU is already approved for biliary therapy in humans, but only for the short term, whereas in SCI it is a long-term treatment. Long-term 4-MU treatment at a dose of 1.2g/kg/day appears to be associated with no apparent adverse effects in a rat model. However, the current dose was insufficient for effective reduction of CSPGs in the scar around the lesion.

We know that there is a remarkable communication between the growth machinery that exists during development and that which exists in the adult. When we used AAV vectors to overexpress the integrin α9 together with its activator Kindlin-1, which is crucial for developmental axon pathfinding, we were able to use the inhibitory environment around the lesion to promote axon growth to the molecules present there. We were able to partially reconstruct the sensory pathways after the dorsal column crush.

8. SHRNUTÍ

Mícha po poranění prochází procesy, které mohou určitým způsobem připomínat vývoj CNS. Tyto procesy, pak mohou samotnou spontánní regeneraci buď podporovat nebo brzdit. Určitá manipulace s těmito mechanismy může výrazně přispět k funkčnímu zotavení. Tato disertační práce se zaměřuje na zkoumání toho, jak snížení inhibičního prostředí poraněné míchy a „znovuotevření" kritické periody ovlivňují funkční zotavení. Zároveň se zabývá otázkou, zda lze toto inhibiční prostředí, v některých případech, využít ve prospěch regenerace. V práci ukazujeme, že po vyvolání nadměrné exprese příslušné izoformy integrínu, která hraje roli při navigaci axonů během vývoje, umožňuje regenerujícím axonům růst po jinak inhibičních proteinech jizvy, kterou využívají jako lešení. Tato práce přináší nový pohled na regeneraci axonů po poranění míchy, který využívá rekapitulaci vývojového stadia.

Hledání účinného způsobu, jak snížit inhibiční prostředí, patří mezi efektivním přístupům při léčbě poranění míchy. Zaměřili jsme se na studium léku, který lze podávat perorálně s jednoduchým dávkováním - 4-MU. Tento přípravek je již schválen jako choleretikum a antispasmodikum pro krátkodobou léčbu u lidských pacientů, ale zároveň je využíváno experimentálně pro léčbu celé řady nemocí a poruch. Nicméně, v případě poranění míchy, je potřeba léčba dlouhodobější. Naše experimenty naznačují, že dlouhodobá léčba4-MU v dávce 1,2 g/kg/den nevykazuje zjevné nežádoucí účinky u potkanů. Je však nutné poznamenat, že současná dávka není dostatečná pro efektivní snížení CSPG v jizvě kolem poranění na docílení funkčního zlepšení po míšním poranění.

Víme, že existuje pozoruhodná komunikace mezi růstovým mechanismem, který funguje během vývoje, a mechanismem, který je aktivní v dospělosti. Při využití vektorů AAV k nadměrné expresi integrínu $\alpha 9$ spolu s jeho aktivátorem Kindlinem-1, klíčovým pro navigaci axonů během vývoje, jsme úspěšně využili inhibiční prostředí v okolí poranění k podpoře růstu axonů. Dosáhli jsme částečné rekonstrukce senzorických drah po dorsální hemisekce.

9. LITERATURE REFERENCES

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10.OVERVIEW OF PUBLICATIONS AND ATTENDED CONFERENCES

10.1 Dissertation-relevant publications

1. **Štěpánková K**, Mareková D, Kubášová K, Sedláček R, Turnovcová K, Vacková I, Kubinová Š, Makovický P, Petrovičová M, Kwok JCF, Jendelová P, Machová Urdzíková L. 4Methylumbeliferone Treatment at a Dose of 1.2 g/kg/Day Is Safe for Long-Term Usage in Rats. Int J Mol Sci. 2023 Feb 14;24(4):3799. doi: 10.3390/ijms24043799. PMID: 36835210; PMCID: PMC9959083. **IF: 6.208**
2. **Štepánková K*#**, Chudíčková M*, Šimková Z, Martinez-Varea N, Kubinová Š, Machová Urdzíková L#, Jendelová P#, Kwok JCF#. Low oral dose of 4-methylumbelliferone reduces glial scar but is insufficient to induce functional recovery after spinal cord injury. Sci Rep. 2023 Nov 6;13(1):19183. doi: 10.1038/s41598-023-46539-5. Erratum in: Sci Rep. 2024 Jan 8;14(1):785. PMID: 37932336; PMCID: PMC10628150. **IF: 4.6**

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10.2 Dissertation-relevant publications but not included

1. Smith NJ, Doody NE, **Štěpánková K**, Fuller M, Ichiyama RM, Kwok JCF, Egginton S. Spatiotemporal microvascular changes following contusive spinal cord injury. Front Neuroanat. 2023 Mar 21;17:1152131. doi: 10.3389/fnana.2023.1152131. PMID: 37025098; PMCID: PMC10070689. **IF: 2.9**
2. Machova Urdzikova L, Cimermanova V, Karova K, Dominguez J, **Stepankova K**, Petrovicova M, Havelikova K, D Gandhi C, Jhanwar-Uniyal M, Jendelova P. The Role of Green Tea Catechin Epigallocatechin Gallate (EGCG) and Mammalian Target of Rapamycin (mTOR) Inhibitor PP242 (Torkinib) in the Treatment of Spinal Cord Injury. Antioxidants (Basel). 2023 Feb 3;12(2):363. doi: 10.3390/antiox12020363. PMID: 36829922; PMCID: PMC9952296. **IF: 7.767**

3. **Stepankova K**, Jendelova P, Machova Urdzikova L. Planet of the AAVs: The Spinal Cord Injury Episode. Biomedicines. 2021 May 28;9(6):613. doi: 10.3390/biomedicines9060613. PMID: 34071245; PMCID: PMC8228984. **IF: 4.717**
4. Krupa P, **Stepankova K**, Kwok JC, Fawcett JW, Cimermanova V, Jendelova P, Machova Urdzikova L. New Model of Ventral Spinal Cord Lesion Induced by Balloon Compression in Rats. Biomedicines. 2020 Nov 5;8(11):477. doi: 10.3390/biomedicines8110477. PMID: 33167447; PMCID: PMC7694490. **IF: 4.717**

10.3 Attended conferences

1. **The 13th Conference of The Czech Neuroscience Society 24-25 November 2021 in Prague:**

   Poster: Manipulating perineuronal nets and the glial scar: 4MU as a therapy for chronic spinal cord injury
2. **FENS Forum 2022 | International Neuroscience Conference 9-13 July 2022 in Paris:**

   Poster: Oral administration of 4-methylumbelliferone combined with rehabilitation promotes anatomical plasticity and functional recovery in chronic stage of spinal cord injury.
3. **Czech-BioImaging Annual Scientific Conference 4-5 October 2022 in Hustopeče:**

   Oral presentation: Label-free pSHG/THG microscopy for the visualization of axon regeneration after spinal cord injury
4. **Scientific conference | Second faculty of Medicine 12-13 November 2022 in Prague:**

   Oral presentation: The reopened window of plasticity promotes axonal regeneration after spinal cord injury

5. **Neuroscience 2022 | Society for Neuroscience 12-16 November 2022 in San Diego:**

   Poster: Orally administered 4-methylumbelliferone promotes anatomical plasticity and functional recovery in the chronic stage of spinal cord injury