Combining molecular intervention with *in vivo* imaging to untangle mechanisms of axon pathology and outgrowth following spinal cord injury.

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Abstract (136 words)

In vivo imaging of the spinal cord has allowed the observation of single axons over relatively long periods in the living mouse. After spinal cord injury, this methodology has helped to differentiate several pathological stages and tissue processes which impact axon morphology. In addition, the combination of *in vivo* imaging techniques with specific molecular intervention has shown that specific pathological axon changes can respond to distinct treatments. Combining *in vivo* imaging with molecular interventions is, hence, a powerful approach to extend our knowledge of the pathological processes leading to axonal loss. It also allows testing possible treatment options to, for example, increase axonal outgrowth. This review will provide a detailed description and critical examination of several studies that have combined the two methodologies in spinal cord injury research and pinpoints the specificities of the approach.

1. Introduction

Injuries to the spinal cord have catastrophic consequences for the patients affected. Spinal cord injuries trigger a cascade of pathological events affecting axons and surrounding cells that can be difficult to follow overtime in animal models, motivating the development of dynamic approaches such as *in vivo* imaging. In human pathology, in particular following accidents that compress the spinal cord, significant cavitation can be seen with gray and white matter damage. As the superficial white matter tracts of the spinal cord are easily accessible in animal models, most *in vivo* experimental optical imaging of the spinal cord has been performed in those superficial layers of the dorsal column. This has allowed obtaining high resolution imaging that has extended our knowledge on dynamic injury-induced processes.

Here we will expand on pre-existing reviews on (i) *in vivo* imaging of spinal circuits in invertebrates and vertebrates (Johannssen and Helmchen, 2013), and on (ii) methodological advances in *in vivo* imaging techniques (Laskowski and Bradke, 2013). In this review, we will specifically focus on advances obtained with *in vivo* imaging studies in the mouse spinal cord after injury, spotlighting those studies, which applied therapeutic intervention to better understand sequences of pathological and regenerative events.

The potential of combining an advanced imaging technique with molecular intervention to gain insight on pathological mechanisms has already been demonstrated over a decade ago (Kerschensteiner et al., 2005). Since this early publication, technical advances have been made, which open up a myriad of methodological possibilities related to the specificity of the injury, the microscopic setup, and the application of molecular interventions (Horton et al.,

2013; Ylera et al., 2009). All these methods can be applied to genetically modified mice, which offer great potential also in combination with viral vectors approaches to perform gene therapy. With methodological variety, new questions have become answerable, which will be discussed in this review.

2. Insights obtained from *in vivo* imaging on pathological processes induced by spinal cord injury

In vivo imaging allows tracking individual axons and the changes they undergo following injury in the spinal cord for periods up to 6 months post-injury (Lorenzana et al., 2015). The knowledge gained from *in vivo* imaging in spinal cord injury ranges from the axon's response to injury, i.e. axonal dieback and outgrowth, to the axon's interactions with other structures or cell types, such as glial and immune cells. An understanding of these pathological processes is necessary to enable interpretation of results on how molecular interventions affect spinal cord injury related mechanisms.

2.1. ***Axonal dieback***

In vivo imaging reveals that injury to the spinal cord is followed by a short initial phase, during which axons remain stable (Horiuchi et al., 2015; Kerschensteiner et al., 2005). The first observable morphological change after this stable phase is axonal dieback. Although axonal dieback following spinal cord lesions is a phenomenon already described with meticulous detail in early *ex vivo* studies of Ramon y Cajal (Ramon y Cajal, 1928), *in vivo* imaging studies have provided the scientific community with insights on specific phases of degeneration and its dynamic change over time. For example, by *in vivo* imaging, axonal dieback has been shown to strongly impact total axon length immediately after injury, but with a gradual decrease over time (Farrar et al., 2012). Further *in vivo* data indicates that this axonal dieback is a very heterogeneous process, where some axons dieback as a direct response to injury and others only weeks later (Dray et al., 2009; Farrar et al., 2012).

Axons die back through four different processes depending of the type of injury (i) pore-induced axon loss, (ii) acute axonal degeneration, (iii) slow axonal retraction and (iv) Wallerian degeneration (Dray et al., 2009; Kerschensteiner et al., 2005; Williams et al., 2014), illustrated in **Fig. 1**. These processes occur in isolation but can also affect one single axon consecutively or, when it comes to acute axonal degeneration and slow axonal retraction, in alternating phases (Lorenzana et al., 2015). This heterogeneity of axonal dieback processes makes it interesting to analyze the behavior of individual axons subjected to a single individual dieback process. The aforementioned observed gradual decrease in total dieback over time does not

necessarily imply that the degenerating processes also become slower with time; it might simply mean that fewer axons are affected by the process but degenerate at equal or faster rate. In fact, acute axonal degeneration and Wallerian degeneration at the beginning of and later in the injury, respectively, can occur at similar rates. Multiple *in vivo* observations and analyses of axons undergoing processes of dieback deliver sufficient detail to understand the rates and mechanisms (potentially also identify interplay) of individual dieback processes. In the following, we summarize the scientific advances made using *in vivo* imaging to understand the four identified processes of axonal dieback induced by different types of spinal cord injury models.

i. Acute Axonal Degeneration

In vivo imaging after transection or laser spinal cord injuries has shown that acute axonal degeneration lasts for less than 5 min and occurs anywhere in a time window of 2 min to 4 h following injury. This is a relatively quick process that leads to fragmentation of both the proximal and distal axon components (taken in relation to the lesion site) simultaneously. The proximal and distal segments fragment in directions opposing the injury, thus, retrogradely and anterogradely, respectively (Kerschensteiner et al., 2005; Lorenzana et al., 2015).

ii. Slow Axonal Retraction

The subsequent phase of slow axonal retraction also acts on proximal and distal axon components but, as the name implies, at slower rates and by retraction rather than fragmentation. Whereas fragmentation processes leave bits of axons behind for hours after the process is complete, slow axonal retraction is a “clean” process, retracting the entire axon end (Lorenzana et al., 2015). Hours after the injury, slow axonal retraction induces the formation of large, bulbous structures at the majority of severed axon stumps, termed retraction bulbs (Ertürk et al., 2007; He et al., 2016).

iii. Wallerian Degeneration

The final phase of degeneration, Wallerian degeneration, has been observed *in vivo* as early as 30 h (Kerschensteiner et al., 2005) and as late as 6 weeks post-injury (Farrar et al., 2012). Regardless of the heterogeneity of the lag phase, it leads to the degeneration of very long stretches of axons, while having a similar rate of degeneration as acute axonal degeneration (Dray et al., 2009). Additionally, Wallerian degeneration differs from acute axonal degeneration by merely fragmenting the distal and not proximal end of the axon. It starts at the injured end of the distal axon segment, leading to a thinning of the entire axon, progressing distally along the axon (Kerschensteiner et al., 2005). Interestingly, Wallerian degeneration is not restricted to clearly identified cut axons but can also occur in seemingly uninjured axons where it is initiated close to the lesion (Dray et al., 2009).

iv. Pore-Induced Axon Loss

In the contusion spinal cord injury model axons are not transected but damaged by blunt force. A recent *in vivo* imaging study has shown that this applied force creates mechanopores in affected axons, through which calcium can enter immediately after injury. The resulting increase in intra-axonal calcium coincides with axonal swelling, initiated mainly in the first 30 min post-injury, and is a predictor of axonal fragmentation. Although increased intra-axonal calcium level mostly precedes axon fragmentation, it does not invariably lead to it (Williams et al., 2014). More specifically, when elevated calcium levels become subsequently reduced and homeostasis restored by spontaneous sealing of mechanopores, pore-induced axon loss is prevented and the axon rescued long-term. In contrast, should mechanopores continue to disrupt an axonal membrane after contusion, it becomes increasingly likely for the axon to break (Williams et al., 2014). Whether pore-induced axon loss can be complemented by additional mechanisms of axon dieback such as those cited before remains to be explored.

2.2. Axonal outgrowth

The heterogeneity observed in axons' immediate dieback response can also be encountered in injured axonal outgrowth patterns, as illustrated in **Fig. 2**. First axonal sprouting has been observed as early as 6h following a pin lesion of the spinal cord (Kerschensteiner et al., 2005), and the majority of cut axons re-approach the pin lesion site one week post-injury (Fenrich et al., 2012). These sprouts can either be initiated at axonal endings, called terminal sprouts, or at nodes of Ranvier, called nodal sprouts. Both types of sprouts can grow quickly and produce axons with morphologies similar to unlesioned axons, with large caliber and a straight trajectory. Often when axons stop or fail to elongate this fast, they start a process of slow sprouting that generates rather thin new axons with more side branches. These thin, morphologically immature axons have been found to grow more in the center of a lesion, whereas regenerated unlesioned-like axons with few side branches grow at the rim of the lesion (Fenrich et al., 2012). This observation suggests that outer regenerating axons might get some directional information from neighboring intact fibers. In general, though, axons have been described to regenerate rather aimlessly (Dray et al., 2009; Kerschensteiner et al., 2005). Later elimination of regenerated axon branches has been observed (Dray et al., 2009). Where regeneration occurs in an aimless fashion, it is not surprising that some outgrowth might be functionally unnecessary and subsequently pruned. To our knowledge, *in vivo* data linking regenerated axonal morphology, such as small or large caliber fibers, with axon branch elimination is missing. Elimination of axon branches has been observed to involve fast fragmentation and slow retraction, similar to degeneration processes (Dray et al., 2009). The selective pruning rather than the total elimination of all regrown branches opposes the notion that a general inhibitory environment is the cause; it seems plausible that the origin lies in some (yet unidentified) intracellular signaling pathway.

2.3. Changes to the environment surrounding injured axons

Spinal cord injury does not solely cause morphological changes in axons; it also affects vasculature, causes immediate inflammatory responses and generates glial scars, which have been observed *in vivo*. Changes in vasculature can be visualized *in vivo* by dye injection into the tail vein. Neuroinflammatory responses have been studied using double transgenic mouse lines with neuronal and inflammatory fluorophore expression. The effect of glial scaring on axon morphology has been observed indirectly by varying the size of the scar to analyze how axons are differentially affected.

2.3.1. Vascular Changes

Spinal cord injuries do not solely disrupt neural transmission but also blood supply to the spinal cord by damaging blood vessels, leading to an immediate loss of vasculature which can be observed in the white matter *in vivo* (Dray et al., 2009). Hypoxia at the lesion site is reinforced by a progressive decrease in blood flow of the remaining vasculature, which has been studied *in vivo* for the first 2h following injury (Tang et al., 2015). As early as 3 days post-injury, attempts to compensate this disruption in blood supply occur by spontaneous angiogenesis within the first 400 μm surrounding the lesion epicenter, a process coincidental to axonal regeneration. There, many new vessel branches with small and medium diameters (from 6 to 30μm) appeared around the lesion peaking at 7 days. Neurovascular proximity peaks a week following injury, and axons proximal to vessels regenerate faster but not with increased directionality (Dray et al., 2009). Similar to regenerated axons, newly created vessels of varying sizes are not all maintained but undergo pruning, leaving only the stable ones intact 2 weeks post-injury (Dray et al., 2009). The visualization of axon–blood vessel interactions during *in vivo* imaging can support the evaluation of the efficacy of potential molecular interventions and treatments.

2.3.2. Neuroinflammation

Spinal cord injury induces blood-spinal cord barrier disruptions and release of inflammatory factors with, in particular, blood-derived macrophages attempting to infiltrate the injury site. Blood-derived macrophages, together with resident microglia, make up the two key players involved in neuroinflammation (Donnelly and Popovich, 2008). *In vivo* imaging studies have been helpful in elucidating the dynamic action of macrophages and microglia *in vivo* and in identifying their specific spatio-temporal distributions and role.

Microglial cells immediately respond to spinal cord injuries by starting to polarize and by extending their processes towards the lesion site, thereby creating a shielding sphere around the lesion. Within the next hour not only processes but also microglial and macrophage cell bodies start migrating towards the lesion. This migration is quicker in macrophages than in

microglia, a difference persisting at least for the first week post-injury (Evans et al., 2014). Albeit both macrophage and microglia increase in number, their populations peak at different times. Whereas the largest blood-derived macrophage population close to the lesion has been shown to occur at an early time point (approximately 1 week post-injury, depending on the type of lesion) (Evans et al., 2014; Fenrich et al., 2012), the microglia population peaks later (approximately at 3 weeks post-injury), where it forms a microglial scar (Dibaj et al., 2010). In addition, another *in vivo* imaging study revealed that migration is not the sole cause of an increase in microglial population; microglia also proliferates in proximity to the lesion (Fenrich et al., 2013). The contribution of these two processes, migration and proliferation, to increased cell population, however, is not yet known.

Microglial and macrophage interaction with axons *in vivo* has been analyzed under different settings. After a small laser induced lesion, microglia contacted and engulfed retraction bulbs in the first hours following injury (Dibaj et al., 2010). However, at later time points, during the first week post-injury, destructive interactions of microglia with axons have not been observed, and axons only die back when contacted by macrophages (Evans et al., 2014; Fenrich et al., 2013). This time dependent influence of microglia and macrophages on axonal fate can be best observed using *in vivo* imaging studies, and results could imply that microglia are more involved in the process of acute axonal degeneration, whereas macrophages play a larger role in Wallerian like degeneration. This hypothesis remains to be tested.

2.3.3. Glial Scar

The glial scar that forms in coincidence to the microglia activation also involves oligodendrocytes and astrocytes. Ylera and colleagues studied the effect of the glial scar on axonal outgrowth *in vivo* by varying the size of the lesion (2009). More precisely, they described how the glial scar can alter an axon's response to post-conditioned lesions. Conditioning lesions are an established lesion model which demonstrates the intrinsic capacity to regenerate of dorsal root ganglion (DRG) central axons (Neumann and Woolf, 1999). In a pre-conditioned lesion, the central branch of a DRG neuron is cut after a lesion to its peripheral branch. This triggers an increased regeneration of the DRG neuron's central branch (the anatomy of DRG neurons is illustrated in **Fig. 1**). When a peripheral branch, however, is lesioned after its central branch (post-conditioned lesion), the glial scar is the principal factor determining the regenerative fate of the lesioned axon. Therefore, when the spinal cord injury is a transection lesion, generating a large glial scar, the post-conditioned axons do not penetrate the lesion. If central axon branches are lesioned with a laser, creating a negligible glial scar, post- and pre-conditioned axons regenerate similarly (Ylera et al., 2009).

Another study showing the influence of the glial scar on regeneration involves lesions of DRG axons outside of the spinal cord. In this case, the injured central axon is imaged *in vivo* at the dorsal root entry zone, where the regenerating axons enter the spinal cord. The authors could

demonstrate *in vivo* that central axons regenerate until reaching this entry zone border (Di Maio et al., 2011). *Ex vivo* analysis revealed that axons fail to regenerate at the entry zone where the oligodendrocytic scar commences (Di Maio et al., 2011). The glial scar has also been observed to have beneficial functions following spinal cord injury (Rolls et al., 2009), such as tissue stabilization (Faulkner et al., 2004), regeneration induction (Anderson et al., 2016), or reparation of the blood brain barrier to inhibit inflammation (Bush et al., 1999). These effects have not yet been studied using *in vivo* imaging following spinal cord injury but astrocytes-specific transgenic mouse lines could be combined to imaging of the injured spinal cord *in vivo* to shed lights on the role of the glial scar over time as also done in the case of neuro-immune diseases (Bardehle et al., 2013; Herwerth et al., 2016).

3. Lessons learned from combining molecular intervention with *in vivo* imaging after spinal cord injury.

3.1. Using *in vivo* imaging to determine mechanisms of action of molecular interventions.

Several *in vivo* imaging studies demonstrate that treatment with molecular interventions (**Table 1**) leads to a shortening of the distance between the cut axon tip and the initial injury site. The selected drugs studied include calpain inhibitors and EGTA (Williams et al., 2014), the microtubule stabilizing epothilone B (Ruschel et al., 2015), the autophagy inducing agent Tat-Beclin 1 (He et al., 2016), the transcription factor STAT3 (Bareyre et al., 2011), the inhibitor of voltage-gated calcium channels pregabalin (Tedeschi et al., 2016), the corticosteroid medication methylprednisolone (Tang et al., 2015) and the neuroprotective steroid hormone progesterone (Yang et al., 2017). Which pathological processes are impacted by the treatments – prevention of axonal dieback or initiation of sprouting attempts – can only be determined accurately using *in vivo* imaging approaches.

Many *in vivo* imaging studies build on existing *ex vivo* and *in vitro* data to narrow down the question that will be addressed using *in vivo* imaging techniques. Calpain protease inhibitors and EGTA, for example, had been previously shown to be protective against degeneration *in vitro* (Wang et al., 2000; Zhai et al., 2003). However, only results from *in vivo* imaging experiments provided evidence that calpain protease inhibitors prevent acute axonal degeneration and pore-induced axonal loss in two separate injury models (Kerschensteiner et al., 2005; Williams et al., 2014). These therapeutic interventions therefore underlines that these two processes likely involve calpain proteolysis and, thus, elevated intra-axonal calcium concentration. Following contusion injury, it was shown that EGTA, which binds extracellular calcium, prevented pore-induced intra-axonal calcium elevations and axonal dieback (Williams

et al., 2014) when pools of axons were followed over time. This direct prevention of calcium influx illustrates a potential causal relationship between intra-axonal calcium elevation and axonal loss. Interestingly, axons started to degenerate more quickly after EGTA wash out than initially (Williams et al., 2014). These results indicate that EGTA does not lead to a recovery of the axons but only a temporary pause in axonal dieback. The study is an example of how combining molecular intervention with *in vivo* imaging helps to understand processes of the axon's response to injury.

Another example of a molecular intervention that was used in combination to *in vivo* imaging is the microtubule stabilizing drug epothilone B (Ruschel et al., 2015). In vitro assays had previously shown that microtubule fragmentation is one of the first pathological processes observable after axotomy, which can ultimately lead to degeneration (Tang-Schomer et al., 2010; Zhai et al., 2003). *In vivo* imaging revealed that stabilizing microtubules with epothilone B (Ruschel et al., 2015) and taxol (Ertürk et al., 2007), leads both to a reduction of axonal dieback and a decreased number of cut axons with retraction bulbs. Cytoskeletal components do not solely play a crucial role in degeneration but also impact axonal outgrowth, during which the cytoskeleton needs to supply the growth cones with the necessary machinery and membrane for expansion (Dent and Gertler, 2003). Stabilizing microtubules with epothilone B has been observed *in viv*o to also increase the number of regenerating axons following a spinal cord lesion (Ruschel et al., 2015).

Molecular intervention using the autophagy-inducing peptide Tat-Beclin 1 has also been combined with *in vivo* imaging following spinal cord injury. Results indicate this drug's efficacy might also be rooted in microtubule stabilization (He et al., 2016). Under nonpathological conditions autophagy upholds cellular homeostasis by recycling unnecessary cytoplasmic components into basic building blocks (Yang and Klionsky, 2010), a process very likely to be of relevance during axon degeneration and regeneration. Previous studies investigating its effect on axonal morphology following central nervous system injury, however, have provided conflicting results (Gumy et al., 2010) of either impacting neurodegenerative (Knoferle et al., 2010; Koch et al., 2010) or regenerative (Rodríguez-Muela et al., 2012) processes. The *in vivo* imaging results following application of Tat-Beclin 1 directly to the injury site demonstrated a shortening of the distance between axon stumps and lesion site, as well as a reduction in the number of axons with retraction bulbs (He, et al., 2016). As these results are similar to those from direct application of microtubule stabilizing drugs, and because microtubule stabilization in vitro after treatment with Tat-Beclin 1 has been observed, a probable hypothesis is that Tat-Beclin 1 exerts its neuroprotective effect on axons by increasing autophagy, which in turn affects microtubular structure. Comparison of the differences in axon length and retraction bulb formation following axotomy reveals an epothilone-induced progressive increase (Ruschel et al., 2015), whereas the Tat-Beclin 1-induced increase evolves sharply at

1h post-injury and then remains approximately unaltered (He et al., 2016). One interpretation might be that epothilone largely impacts later stages of axon regeneration, whereas Tat-Beclin 1 prevents degeneration. A definitive conclusion, however, cannot be made and a more refined analysis observing the behavior of individual neurons and their change during different phases of axon degeneration and regeneration using *in vivo* imaging would be necessary.

A study examining the impact of the transcription factor STAT3 on single axon morphology over time following spinal cord injury using *in vivo* imaging and comparative analysis is reported in (Bareyre et al., 2011) and illustrated in **Fig. 3**. The authors were able to draw firm conclusions regarding the phasic regulation of axonal outgrowth by STAT3. By following individually labeled neurons after viral overexpression of STAT3 in DRG neurons subjected to pin lesions, Bareyre and colleagues demonstrated that the intervention specifically affected outgrowth rather than degeneration. Furthermore, the growth-promoting effects were shown to primarily target outgrowth initiation rather than extension. By comparing rates of regeneration of individual axons via observation at different time points following injury, they showed that STAT3 overexpression initiates earlier sprouting but does not affect outgrowth at later stages of the outgrowth process (Bareyre et al., 2011). This study pinpointed the mode of action of this specific transcription factor and highlighted the fact that *in vivo* imaging can help better understand how different stages of axonal dieback and outgrowth are targeted, which is important in finding any spinal cord injury treatments. The authors also investigated the role of STAT3 in regeneration of DRG neurons after a peripheral branch lesion. In line with the data following pin lesion in the spinal cord, deletion of STAT3 was shown to block peripheral regeneration. Again, they could demonstrate that this effect was temporary and that STAT3 deletion caused a delayed in outgrowth initiation rather than an impairment of axon elongation once the regenerative process had started (Bareyre et al., 2011). Addressing different pathological or regenerative stages in such investigations opens new options for combinatorial and time-dependent therapies. Previous *ex vivo* studies implicated STAT3 (Schwaiger et al., 2000); obtaining information of the speed of axonal outgrowth following STAT3 intervention with *ex vivo* studies would have been impossible. This observation stresses the advantages in the approach of following individual axons using *in vivo* imaging to infer axon dynamics. Dynamic analyses are already implemented after brain injury, e.g. measurement of spine turnover rate (Brown et al., 2007), and applied in the spinal cord following injury could yield in the future even more insights.

Another study used *in vivo* imaging in conjunction with pregabalin, a blocker of voltage gated calcium channels (Tedeschi et al., 2016), a medication already used for other diseases, such as neuropathic pain, epilepsy and anxiety (Shneker and McAuley, 2005). While administering pregabalin, which binds to the $\alpha 2\delta 2$ subunit of voltage gated calcium channels, the authors observed pools of axons and could see them closer to the lesion site when compared to vehicle

treated animals. Again, only refined analysis could prove that the shortening of the distance to the lesion was due to increased regeneration, as suggested by data on DRG neurons in culture treated with pregabalin.

The corticosteroid methylprednisolone is another example of a clinically-established medication, also in spinal cord injury. Although clinical trials claimed methylprednisolone to be effective following spinal cord injury if applied within 8 hours (Bracken et al., 1997, 1990), results from further studies in rodents challenged this view (Nash et al., 2002; Wells et al., 2003). These conflicting results have rendered necessary the unravelling of the precise effects of methylprednisolone and *in vivo* imaging studies have brought some advances. Such experiments have observed single axons over time and have revealed, for example, that axons of mice treated with methylprednisolone were closer to the injury site, compared to axons of vehicle treated mice. This difference was relatively constant from 8h until 48h post-injury (Tang et al., 2015), which might imply a role of the steroid hormone in preventing degeneration. This hypothesis is supported by the reduced intra-axonal calcium influx in methylprednisolone-treated axons compared to vehicle-treated axons *in vivo* (Tang et al., 2015). After injection of a tracer into the tail vein, the vasculature was mapped at the edge of the lesion. The authors could show that methylprednisolone treatment leads to an increased blood flow in vessels close to the lesion site (Tang et al., 2015). This effect of methylprednisolone on blood flow might compensate for the aforementioned general loss in vasculature following spinal cord injury. Similarly another study evaluated the effect of systemic treatment of mice with progesterone, another steroid hormone, on axon tip distance to injury evaluated on single labeled axons. They found that axons treated with progesterone were progressively closer to the lesion border than following vehicle treatment (Yang et al., 2017).

Most *in vivo* imaging studies applying molecular interventions focus on the injury-induced effects on axons, due to their clear functional relevance in recovery. These results can clearly benefit from complementary analysis. Dibaj and colleagues (2010) investigated the effect of molecular interventions also on microglia using *in vivo imaging*. Previous reports suggesting that ATP released from astrocytes could be responsible for microglial attraction following brain injury (Davalos et al., 2005) and that nitric oxide (NO) levels increase following spinal cord injury (Conti et al., 2007) prompted Dibaj and colleagues to investigate a possible interplay between purinergic and NO signaling, as well as microglial process attraction. By inhibiting the NO pathway and applying enzymes catalyzing the decompositions of ATP *in vivo*, they could completely prevent microglia from responding to spinal cord laser injuries (Dibaj et al., 2010). Conversely, the chemotaxis of microglial processes was increased when applying NO donors or ATP directly. Interestingly, this increased response was only observable when NO was

applied via an intraspinal local injection rather than global superfusion, illustrating that the mode of drug delivery can be crucial to the outcome.

3.2. Advantages of combining *in vivo* imaging with molecular interventions after spinal cord injury.

As seen in the previous paragraph, *In vivo* experiments can greatly differ in the way data are analyzed either following single axons or bulk of axons over time. Most of the other differences between studies lie in their imaging setup. Older studies, for example, used wide-field microscopy, whereas more recent studies use two-photon microscopy, which enables working with denser labels due to better optical sectioning. The way of administration of the therapeutic intervention can differ and *in vivo* imaging might even allow determining the most advantageous drug delivery (Dibaj et al., 2010). Other types of drug administration include systemic administrations, through intraperitoneal and subcutaneous injections, and overexpression with adeno-associated viruses (Bareyre et al., 2011). Different types of injury used in the experiment can yield different results. Differences in observations as to when, for example, a pathological process starts might be explainable by differences in lesion models or even be specific to one model as the pore-induced axon loss for contusion injuries. Small lesions such as laser-induced lesion have the advantage of making the tracking of individual objects easier than larger lesions. These are, however, less comparable with clinical spinal cord injury, which is better modeled by contusion models. Hence, one needs to weigh benefits against disadvantages of each lesion model before designing the experiment.

All *in vivo* studies presented in this review make use of transgenic mouse lines expressing fluorescent proteins under the Thy1 promoter (Thy1-XFP) and exhibiting distinct patterns of labeled neurons (Feng et al., 2000). Some *in vivo* studies have used Thy1 mouse lines with sparse DRG labeling, such as the Thy1-GFP-M mouse line, which makes tracking of individual axons across long imaging periods easier. In order to visualize microglia (Dibaj et al., 2010), CX3CR1-GFP (Jung et al., 2000) were crossed with a Thy1-YFP mouse line. The two studies analyzing changes in calcium levels both used mouse lines with genetically encoded calcium indicators under the Thy1 promoter, Thy1-TNXXL (Williams et al., 2014) and Thy1-GCaMP (Tang et al., 2015). In both calcium indicators fluorescent proteins are bound to a calcium/binding domain; in TNXXL mouse lines this is troponin C (Mank et al., 2008) and in GCaMP mouse lines calmodulin (Zariwala et al., 2012). In Thy1-TNXXL mice, results are obtained as ratiometric measurements of FRET signals while in GCaMP mouse lines, outcomes are visualized using single wavelengths. New mouse lines with genetically encoded calcium indicators are constantly generated to yield best imaging results with increased

baseline fluorescence, dynamic range and affinity for calcium (Chen et al., 2013). This new state-of-the-art technology could bring new insights into the long-term effect of distinct molecular interventions on uninjured or injured axons with the potential to remodel.

Some *in vivo* studies combined the *in vivo* observations with *ex vivo* analysis. This is a powerful way of benefitting from both methodologies. For example, in the *in vivo* study identifying STAT3 as a possible initiator of axonal outgrowth following spinal cord injury, the authors were successful at re-identifying axons that were scanned *in vivo,* so that they could complete their analysis at a later time point, lying outside of the time realm of *in vivo* imaging (Bareyre et al., 2011). The STAT3 also study demonstrated possibilities for therapeutic intervention and showed that regeneration initiation and maintenance are two different processes that can be targeted individually. Other studies have proven successful to find new treatment options, such as the epothilone study (Ruschel et al., 2015), or to reveal new principles of spontaneous remodeling, such as application of the calcium chelating agent EGTA (Williams et al., 2014).

In general, combining molecular intervention with *in vivo* imaging following spinal cord injury has several advantages:

(1) It is optimal to reveal the best drug application paradigm to yield maximal efficacy (Dibaj et al., 2010).

(2) It is useful for identifying the mode of action of therapeutic intervention on specific phases of axonal growth (Bareyre et al., 2011) allowing a better combination of multiple treatments following spinal cord injury (Dibaj et al., 2010). Many researchers increasingly recognize the importance of sequentially tackling different stages of the events after trauma (Alto et al., 2009; Anderson et al., 2018; Fouad et al., 2005; Lu et al., 2004). *In vivo* imaging (either performed continuously or at different time-resolutions) also offers a powerful tool to understand how cell or tissue response to a single molecular intervention is altered by an additional subsequent intervention.

(3) It allows collecting data at many time points following the injury, thereby allowing for the precise determination of an effective window for therapeutic intervention without the need to increase the animal sample size.

(4) It permits discovering new therapeutic interventions targeting a specific pathological event. For example, an analysis on calcium recovered axons, as in the study by Williams and colleagues, would not have been possible without knowing the axon's history, in this specific case that the axon previously exhibited high intra-axonal calcium levels (Williams et al., 2014). Also, measuring the individual axon's rate of regeneration, such as in the STAT3 study, relies on identifying the same structure over time using *in vivo* imaging (Bareyre et al., 2011). Due to the heterogeneity in the pathological development following spinal cord injury, one could also think of molecular

interventions only affecting neurons that have, for example, previously undergone acute axonal degeneration but not slow axonal retraction.

4. Conclusion

Important advances have been made since the first publication combining *in vivo* imaging of the spinal cord with molecular manipulation after injury. These combinatorial studies necessarily have to be based on the understanding gained from *in vivo* studies of spontaneous pathological processes affecting axons, glial and immune cells, as well as vasculature. Manipulation of these processes yields insight on their differences and expanded information on their related chemical and physiological basis. Knowledge necessary to provide new therapeutic possibilities could also be expanded, and the validity of already established therapeutic interventions, such as methylprednisolone were probed (Tang et al., 2015).
It must be noted that *in vivo* microscopy does not offer any improvement in spatial resolution compared to confocal microscopy and can also in thin samples produce some non-negligible level of photodamage. Therefore, it is always necessary to determine when the use of in vivo imaging is appropriate and can lead to new insights. Hence, when designing *in vivo* spinal cord experiments with molecular manipulation after spinal cord injury one must carefully choose the adequate experimental setup when it comes to the type of molecular intervention, the timing of intervention, the administration of intervention, the type of injury model, the mouse line used and the type(s) of analyses. The development of additional reporter mouse lines and genetic labeling with viruses will allow the scientific community to gain new insight on pathological events and cell-cell interactions following spinal cord injury *in vivo*. New labeling techniques, together with super resolution imaging such as super-resolution shadow imaging, in which extracellular matrix is labeled and, hence, all cell bodies and processes visible (Tønnesen et al., 2018), might soon be applicable in an *in vivo* setting. This would allow direct visualization of general tissue response to molecular intervention in spinal cord injury. An interesting spinal cord injury-induced mechanism, which has not yet been analyzed using *in vivo* imaging, is remodeling, a process where uninjured axons start sprouting to form detour circuits bridging the lesion (Bareyre et al., 2004). So far, data from remodeling after injury originate from regions deep in the spinal cord, which are not accessible by conventional *in vivo* methods. Using lenses to reach deeper tissues as in the brain (Barretto et al., 2009) is not feasible because removing tissue for lens placement will induce severe spinal cord injury. New developments in reaching ventral areas of the spinal cord involve window implantation above the lateral spinal cord (Cartarozzi et al., 2018). Combining this with a three-photon microscopy

approach (Horton et al., 2013) might open the door to observe and understand pathological mechanisms of spinal cord injury and neuronal remodeling approaches in the gray matter. *In vivo* imaging in spinal cord injury research is a quickly developing field that has provided new insight and discoveries. In combination with molecular intervention this field will target fundamental knowledge on pathological processes following injury and provide opportunities for development of promising therapies.

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Figure and table descriptions

Figure 1: Axonal dieback following spinal cord transection and contusion injuries as observed using *in vivo* imaging studies. *In vivo* imaging studies in the spinal cord are performed on central branches of DRG neurons (top). Contusion injuries (left) have been observed using *in vivo* imaging to cause calcium influx through mechanopores. These mechanopores either seal, and the axon is rescued, or they are maintained and the axon

breaks. Injury by laser or transection can cause acute axonal degeneration and slow axonal retraction with retraction bulb formation consecutively or in alternating phases. Wallerian degeneration occurs later and only affects the distal part of the axon. Times indicated are earliest and latest observations of the respective pathological processes taken from different *in vivo* imaging studies*. In vivo* imaging is commonly performed on large superficial myelinated axons originating from sensory neurons in the dorsal root. Those neurons can be visualized in transgenic mouse line, in which a neuron-specific fragment of the Thy1 promoter drives cytoplasmic expression of specific fluorophores (Thy1-XFP) in subsets of neurons, including the dorsal root (DRG). Axons in this figure (also in the following figures) are represented without their myelin sheath.

Figure 2: Axonal outgrowth and pruning following spinal cord injury as observed using *in vivo* imaging studies. Limited axonal regeneration can occur at nodes (left) or at distal ends of axons (right). Sprouting has been observed to occur aimlessly, generating axon branches with high caliber and straight trajectory and axon branches with small caliber and more side branches. Both types of axon branches are pruned at a later time point while the exact mechanism of axon branch elimination remains unknown.

Figure 3: *In vivo* imaging reveals that STAT3 initiates axonal outgrowth but does not cause increased axonal extension rates at later time points after spinal cord injury. *In vivo* imaging of a transected peripheral branch of the DRG shows maintained axonal outgrowth with growth cone formation (blue). Deleting STAT3 in those neurons inhibits outgrowth initiation following injury but axons still extend similarly as unaltered axons at later time points (dark green). In central branches of DRG neurons, transection is only followed by very limited axonal outgrowth and subsequent extension. STAT3 overexpression in DRG neurons, though, initiates outgrowth but does not maintain increased rates of outgrowth later (bright green). Hence, during the axon extension phase, normal axons and STAT3 overexpressing axons regenerate similarly.

Table 1: **Summary of methodological setups, analysis and results for experiments combining *in vivo* imaging with molecular intervention.** Experiments are arranged by publishing date. Observational analysis (o) refers to descriptive changes without quantifications in the original publication. Same samples analysis (ss) refers to studies in which the same single structures are followed and analyzed across time for control and treatment group. Variable sample analysis (vs) refers to samples in control or treatment group that were analyzed as bulk and therefore might be more heterogeneous over time. The time interval of

analysis presented in the table only refers to timepoints used for analysis. Additional scanning might have been performed and examples shown in between time points.

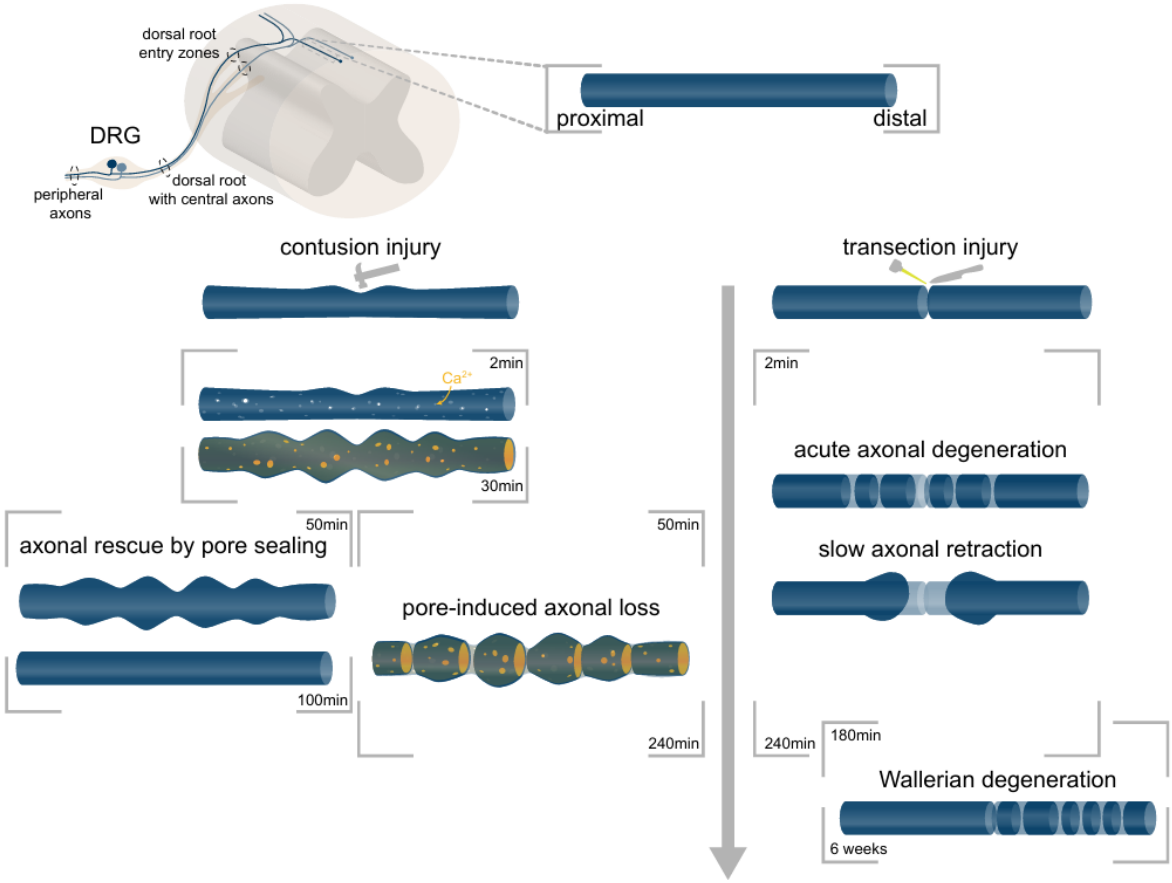

Figure 1

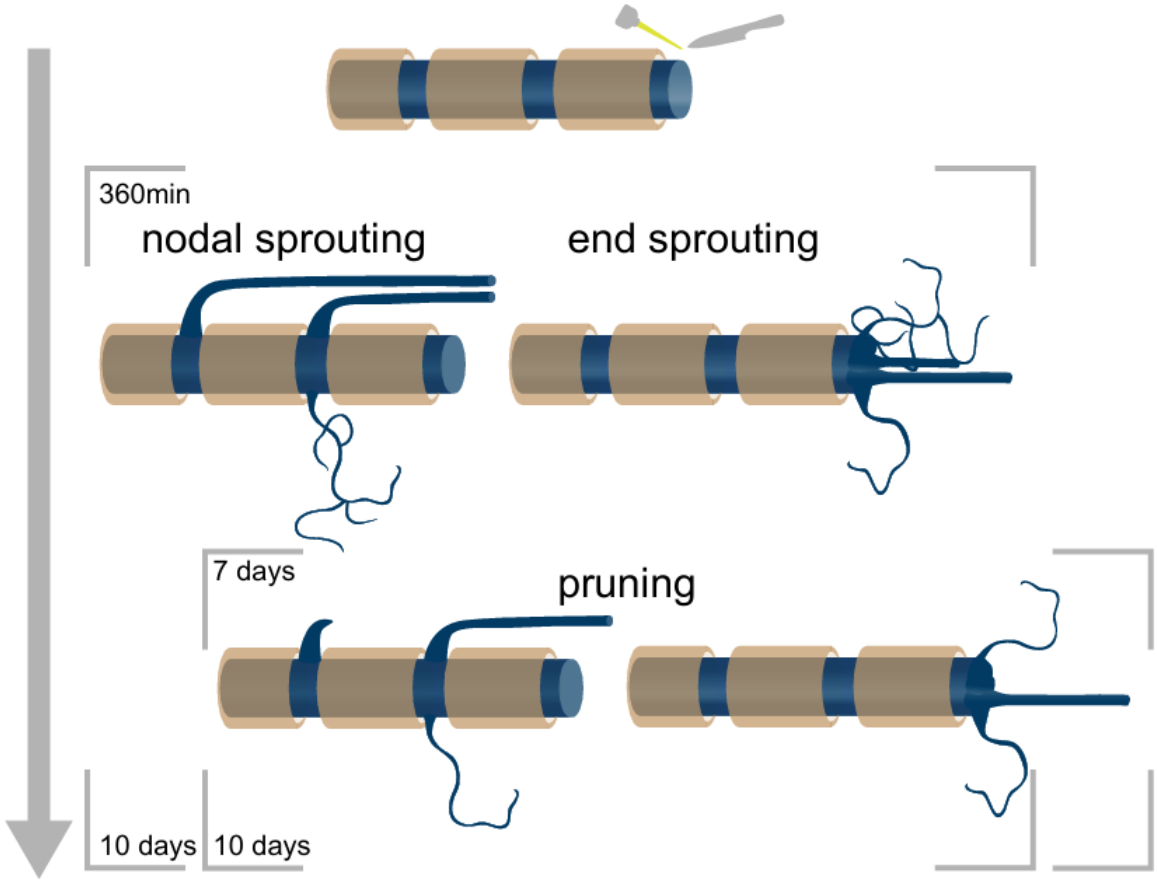

Figure 2

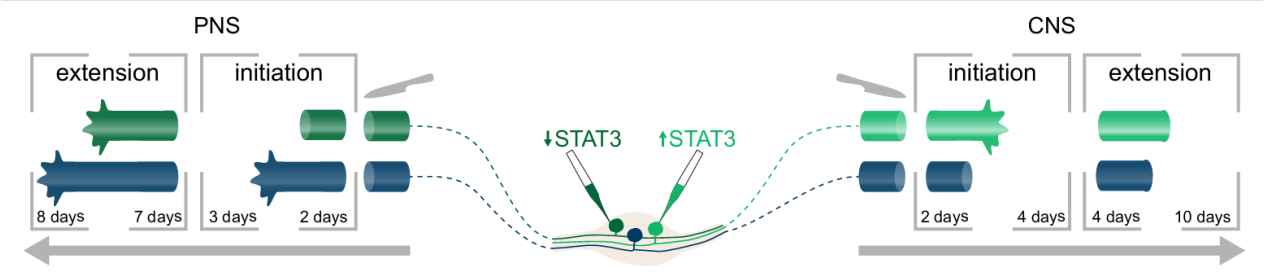

Figure 3

Injury paradigm	Imaging			Molecular intervention			Analysis			Effect of intervention	Citation
	Labeling	Microscope	Location	Drug	Administration	Time interval	o/ss/vs	Time interval	Analyzed matter		
Dorsal column pin lesion	Thy1-GFP S mouse line	Widefield microscopy	C3 to C6	Calpain inhibitors (EST and calpain inhibitor III)	Superfusion	20min preincubation until end of imaging	ss	5min and 30min post injury	Lesioned axon tip to injury distance	Block of acute axonal degeneration	(Kerschensteiner et al., 2005)
Dorsal column transection	Thy1-GFP S mouse line	Widefield microscopy	T11 to T12	Microtubuli stabilizing drug (taxol)	Topical application	0h to 6h post injury (once per h)	vs	0h to 6h post injury (each h)	% of lesioned axons with retraction bulb	Progressively decreasing % of axons with retraction bulb when compared to control	(Erturk et al., 2007)
Dorsal column laser-induced lesion	Crossed Thy1-YFP with CX3CR1-EGFP mouse line	2 P microscopy	C4 to C5	NO synthase inhibitors (L-NAME, MB); NOS scavenger (PTIO); sGC inhibitors (ODQ, MB), ATP hydrolase (apyrase)	Superfusion	15min to 45min preincubation until end of imaging	vs	3min and 60min post injury	Change in number of pixels with microglia positive signal in an area around the lesion border	Decreased microglia infiltration at 60min when compared to control, effect is reversible after washout with CSF	(Dibaj et al., 2010)
				NO donors (SPNO, SNAP); cGMP analogue (8-Br-cGMP); ATP	Superfusion						
Injection-induced lesion				NO donor (SPNO); cGMP analogue (8-Br-cGMP); ATP	Intraspinal injection	At start of imaging		0/5min and 45min post injection		Increased microglia infiltration at 60min when compared to spinal cords with control injections	
Injection-induced lesion				ATP (intraspinal injection) and sGC inhibitor (ODQ) or NO donor (SPNO) (spinal cord superfusion)		15min to 45min preincubation before injection	vs	5min and 45min post injection		Same microglia infiltration as in injection alone	
				NO donor (SPNO) (intraspinal injection) and ATP hydrolase (apyrase) (spinal cord superfusion)						Decreased microglia infiltration in comparison to injection alone	
				NO donor (SPNO) (intraspinal injection) and ATP (spinal cord superfusion)						Increased microglia infiltration in comparison to injection alone	
Dorsal column pin lesion	Thy1-GFP S mouse line	2 P microscopy	cervical spinal cord	Transcription factor STAT3, STAT3c	Gene therapy with AAV injection into DRG	AAV injection at 12 days pre injury, strong viral expression at 2 days post injury	ss	2 and 4 days post injury	Speed of axon regeneration	Increased axon regeneration speed in comparison to animals injected with a control virus	(Bareyre et al., 2011)
		2 P (in vivo) and confocal microscopy (ex vivo)						4 (2 P) and 10 (confocal) days post injury		Same axon regeneration speed as in animals injected with a control virus	
Mild contusion injury on dorsal column	Thy1-TNXXL mouse line	2 P microscopy	L4 to L5	Chelating agent with high affinity for Calcium (EGTA)	Superfusion	0min to 60min post injury	vs	Pre injury time point and continuously from 2/8min until 240min post injury	% of axons with unchanged, elevated and recovered calcium levels	Axons are prevented from reaching elevated calcium states and a majority of those with already elevated calcium levels recover; after washout populational distribution based on calcium levels is similar to untreated immediately after injury	(Williams et al., 2014)
									% of axons with unchanged, swollen, broken and recovered morphology	Axons are prevented from breaking; % of axons breaking increases twice as quickly as non-treated immediately after injury	
				Calpain inhibitor (calpain inhibitor III)		60min to120min post injury		Pre injury time point and continuously from 2/8min until 480min post injury	% of axons with unchanged, elevated and recovered calcium levels	Calcium levels are unaffected by the drug; Reduced number of broken axons; Reduced risk of axons with elevated calcium levels at 100min post injury to fragment	
									% of axons with unchanged, swollen, broken and recovered morphology		
				Calcium channel blocker (bepridil hydrochloride, nimodipine, ω-conotoxin GVIA), glutamate receptor antagonist (CNQX disodium salt, MK801 maleate)		30min pre injury to 60min post injury		At first post injury time point	% of axons with unchanged, elevated and recovered calcium levels	Unchanged	
									Cumulative axon breakdown rate	Unchanged	

Table 1

Dorsal column transection	Thy1-GFP M mouse line	Widefield microscopy	T12	Microtubule stabilizing drug (epothilone B)	i.p.	Injected 1 day pre injury; remained at stable levels for ~6 days	VS	0 to 4 days post injury (once a day)	% of axons with retraction bulb	Progressively decreasing percentage of axons with retraction bulb when compared to control	(Ruschel et al., 2015)
									Axon tip to injury distance	Progressively decreasing distance to injury site when compared to control	
							O	0h and 6 h, 1 and 4 days post injury	Axon dynamics (dying back or regenerating)	Less axons die back, more axons regenerate when compared to control	
Unilateral transection	Thy1-YFP H mouse line	2 P microscopy	T12	Therapeutic agent for treating spinal cord injury (methylprednisolone)	Topical application	30min, 6h and 24h post injury	SS	30min, 8h, 1 day, 2 days post injury	Axon tip to injury distance	Constantly decreased distance to injury from 8h onwards when compared to control	(Tsai et al., 2015)
	Thy1-YFP H mouse line with i.v. Texas red dextran injection						VS	Successive line scanning at 30min to 120min post injury (each 30min)	Rodon method to measure red blood cell velocity	Progressively increasing red blood cell velocity whereas the control group exhibits decreasing red blood cell velocity with time	
									Vascular lumen diameter	No difference between treatment and control group	
	Thy1-GCaMP mouse line						SS	30min to 120min post injury (each 30min)	Calcium levels (intracellular change in fluorescence) of lesioned axons	Progressive decrease in elevated calcium response after injury in comparison to control mice	
Dorsal unilateral, superficial transection	Thy1-YFP M mouse line	2 P microscopy	C4 to C5	Autophagy inducing peptide (Tat-Beclin 1)	Superfusion	0min to 60min post injury	VS	0h to 5h post injury (each h)	% of axons with retraction bulb	Constant decrease in axons with retraction bulbs when compared to spinal cords treated with scrambled peptides	(He et al., 2016)
									Axon tip to injury distance	Constantly decreased distance to injury from 8h onwards when compared to spinal cords treated with scrambled peptides	
Dorsal column crush	Thy1-GFP M mouse line	2 P microscopy	T12	Alpha2delta2 subunit of voltage gated calcium channels blocker (pregabalin)	i.p.	Injected 7 days pre injury three times per day	VS	Immediately until 3 days post injury (each day)	Axon tip to injury distance	Progressively decreased distance to injury from 1 day onwards when compared to animals treated with saline	(Tedeschi et al., 2016)
Dorsal hemisection	Thy1-YFP H mouse line	2 P microscopy	T11	Neuroprotective steroid hormone (progesterone)	i.p. (1h post injury), s.c. (3h, 24h, 48h post injury)		SS	30min, 1 day, 2 days, 3 days post injury	Axon tip to injury distance	Progressively decreased distance to injury from 1 day onwards when compared to spinal cords treated with vehicle	(Wang et al., 2017)