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Genetic mutation of the semaphorin 3A receptor component neuropilin-1 in neurons does not enhance corticospinal tract regeneration

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Abstract

After a spinal cord lesion, a myriad of axon growth inhibitors present in myelin and in the neural scar contribute to the failure of injured axons to regenerate. Class 3 semaphorins are repulsive axon guidance cues that are induced in the meningeal fibroblasts that infiltrate the neural scar. Most if not all injured spinal cord neurons, including neurons that form the corticospinal tract (CST), continue to express the semaphorin receptor components neuropilin (Npn-1 and 2) and plexinAs. This had led to the hypothesis that semaphorin/neuropilin signalling limits axonal regeneration through the scar. To test this hypothesis we have investigated the regeneration of the CST in mice with an Npn-1-deletion specifically targeted to neurons and in littermate control mice. Mice deficient in Npn-1 in neurons do not exhibit enhanced regenerative growth of injured CST axons and do not display improved recovery of motor function as compared to control littermates. We therefore conclude that Npn-1/Sema3A signalling does not have a major impact on the failure of injured CST axons to regenerate. This illustrates that neuron-specific targeting of a single ligand-receptor pair (Sema3A/Npn-1) in the multi-component, divergent semaphorin/neuropilin/plexin signalling pathway is insufficient to enhance regeneration of the CST.

Introduction

Following a spinal cord lesion, axon growth inhibitors present in CNS myelin and in the neural scar are considered to contribute to the failure of injured CNS axons to regenerate. Nogo, Myelin-associated glycoprotein (MAG) and Oligodendrocyte myelin glycoprotein (OMgp) are the prototypical myelin-derived axon growth inhibitors. Chondroitin sulphate proteoglycans (CSPGs) and chemorepulsive axon guidance proteins of at least four gene families (ephrins, slits, RGM and semaphorins) are induced in cells of the neural scar and may act as major impediments to regenerative axon growth (reviewed by Giger 2010, Niclou et al. 2006, Fawcett 2006, Bolsover 2008).

The hypothesis that the failure of axon regeneration in the CNS is, at least to some extent, caused by molecular inhibitors, has been tested extensively. A majority of these studies have focused on the contribution of myelin-derived growth inhibitors. Two independent laboratories demonstrated that mice with a mutated Nogo gene have increased axon regeneration of lesioned spinal cord axons (Kim et al., 2003, Simonen et al., 2003, Dimou et al., 2006, Cafferty et al., 2010). A third laboratory, however, did not observe significant improvements in sprouting or axon regeneration (Zheng et al., 2003, Lee et al., 2009, Lee et al., 2010b). Genetic deletion of MAG or OMgp did not enhance CST regeneration, but had a modest effect on the sprouting of serotonergic fibers (Bartsch et al., 1995, Ji et al., 2008, Lee et al., 2010b). The myelin derived growth inhibitors bind to the Nogo-A receptor (NgR1) and paired Ig-like receptor B (PirB) (Fournier et
al., 2001, Atwal et al., 2008) and signal through co-receptors including p75\textsuperscript{NTR}, LINGO-1 and TROY (Wang et al., 2002a, Mi et al., 2004, Park et al., 2005, Shao et al., 2005). NgR1 knockout (KO) animals display a moderately improved regeneration capacity of certain spinal nerve tracts, although regeneration of the corticospinal tract (CST) was not affected (Kim et al., 2004, Zheng et al., 2005). In mice where p75\textsuperscript{NTR} or PirB was genetically deleted, regeneration of the CST was also unaffected (Zheng et al., 2005, Nakamura et al., 2011). The first efforts in dealing with the redundancy of these myelin inhibitors and their receptors have led to conflicting results (Cafferty et al., 2010, Lee et al., 2010b).

The inhibitory effect of scar-associated CSPGs on axon regeneration has been overcome by infusing the enzyme chondroitinase (ChABC) into the lesioned spinal cord. This enzyme removes the glycosaminoglycan (GAG) chains from the CSPGs and this produces a more permissive environment for injured axons that encounter the inhibitory neural scar. Following ChABC treatment a small number of transected axons was able to regrow back for several millimetres into and beyond the neural scar (Moon and Fawcett, 2001, Bradbury et al., 2002). These studies have been replicated in multiple injury paradigms and collectively demonstrate the potential of this approach (reviewed by Bradbury and Carter, 2011).

The studies on myelin inhibitors and CSPGs show that interfering with the signalling of neurite inhibitory molecules is a promising approach to promote axon regeneration in the CNS. But it is also clear that after the interventions described above, regeneration is still limited. It is likely that the induction of a number of repulsive axon guidance molecules in scar-associated cells could potentially prevent more profound regeneration (Luo et al., 1993, Fawcett and Asher, 1999, Pasterkamp et al., 1999). Evidence for the contribution of repulsive axon guidance molecules to regenerative failure has recently been demonstrated in mice with genetic deletion of EphA4 (Goldshmit et al., 2004). Additional evidence comes from experiments using neutralizing compounds or inhibitors that functionally interfere with RGMa or semaphorin3a (Hata et al., 2006, Kaneko et al., 2006).

In this article we have investigated the contribution of neuronal neuropilin-1 signaling to the failure of injured CST axons to regenerate. Semaphorins were originally identified as repulsive axon guidance molecules that act during CNS development (Kolodkin et al., 1993, Luo et al., 1993). In the adult nervous system, the secreted, class 3 semaphorins (Sema3s) are re-expressed by meningeal fibroblasts that form the core of the glial scar (Pasterkamp et al., 2001, De Winter et al., 2002b). Cultured meningeal fibroblasts do express high levels of Sema3A and meningeal fibroblasts of Sema3A KO animals are a more permissive substrate than WT cells for sensory axons (Chapter 2, Niclou et al., 2003). Moreover human peripheral nerve scar tissue contains Sema3A and neurons cultured on slices of neuroma tissue display increased neurite outgrowth when the Sema3A receptor expression in these neurons is reduced by short hairpin RNA-mediated knockdown (Tannemaat et al., 2007).
With the exception of Sema3E, all Sema3s act through binding to neuropilin, a protein that is part of a multimeric semaphorin receptor complex (He and Tessier-Lavigne, 1997, Kolodkin et al., 1997). Semaphorin receptors continue to be expressed by injured CST and DRG neurons (Gavazzi et al., 2000, De Winter et al., 2002b). Regenerating axons are therefore well-equipped to respond to these axon guidance cues. Adult sensory neurons retain their sensitivity to Sema3A in vitro and in vivo (Tanelian et al., 1997, Reza et al., 1999, Tang et al., 2004), however, the response of severed descending spinal cord axons to semaphorins is largely unknown. To determine to what extent the direct interaction between Sema3A and axonal Npn-1 contributes to impaired axonal regeneration, we generated knockout mice in which Npn-1 is specifically ablated in neurons just before birth and during early postnatal development. In contrast to constitutive Npn-1 KO mice, which die midway through gestation (Kitsukawa et al., 1997, Kawasaki et al., 1999), the genetic mutation of Npn-1 in late embryonal and early postnatal neurons results in viable mice that survive into adulthood. We show, that following CST lesion, disruption of Npn-1 in these neuron-specific Npn-1 mutants does not improve the outgrowth of CST axons. In addition, we do not observe enhanced recovery of motor function in lesioned conditional Npn-1 KO mice.

**Methods**

**Experimental animals**

The Npn-1 conditional knockout mice (Gu et al., 2003) were provided by Dr Alex L Kolodkin and Dr David D Ginty (The Johns Hopkins University School of Medicine, Baltimore, MD). Transgenic animals expressing Cre recombinase under control of the murine Neurofilament-H promoter (B6;129(FVB)-Tg(Nfh-cre)1Kul) were obtained from the Mutant Mouse Regional Resource Centres (MMRRC, Davis, CA). These mouse lines were crossbred to obtain Npn-1$^{+/−}$/NefhCre$^{+/−}$ mice, hereafter labeled as Npn-1 KO mice in a C56BL/6 background. Npn-1$^{+/−}$/NefhCre$^{+/−}$ littermates, hereafter labeled as wild type mice (WT) were used as control animals. Animals were housed in groups under standard conditions with food and water ad libitum and a 12h:12h light/dark cycle. Experimental procedures and behavioral tests were performed in accordance with the committee for laboratory animal welfare and experimentation of the Royal Netherlands Academy of Sciences.

**Animal surgery**

*CST transection:* Animals were deeply anesthetized by an intraperitoneal injection of Hypnorm (0.1 mg/kg Fentanyl citrate/3.3 mg/kg Fluanisone HCl, Janssen Pharmaceuticals) and Dormicum (8.3 mg/kg Midazolam, Roche). During surgery, body temperature was maintained at 37°C using a heating pad. The spinal cord
was exposed by laminectomy of the C4 vertebra. The dura matter was opened using Vannas scissors followed by a bilateral lesion of the dorsal column using a micro knife transecting the dorsomedial CST, leaving the dorsolateral CST intact. Muscle layers were sutured and the skin was closed with Michell clips (Fine science tools). Postoperative analgesia was administered by one subcutaneous injection of Metacam (0.4 mg/kg, Boehringer Ingelheim). In animals of the sham procedure group a laminectomy was performed leaving the dura-mater and spinal cord intact.

**CST tracing:** CST fibers were anterogradely traced four weeks after surgery. To this end animals were anesthetized as described before and 0.8 µl of biotinylated dextran amine (BDA) solution (10% in PBS, MW 10.000, Invitrogen) was infused bilaterally into the primary motorcortex at a flow of 0.2 µl/min (coordinates 0.26, 1; 1.18, 1.25; 1.18, 1.8; 1.5, 1.8 millimeter anterior/posterior, lateral from bregma).

**Experimental groups:** All animals were 13 to 17 weeks of age on the day of surgery. A total of 14 KO and 12 WT mice received a bilateral CST lesion. The sham group consisted of 9 WT mice.

**Behavioural testing and evaluation**

All animals were tested 3 days before and 3, 7, 10, 14, 17, 21, 24 and 28 days after surgery.

**Narrow beam walk:** To evaluate regain of proper hind limb placements after CST lesion, a narrow beam test was performed. Animals were pre-trained for one week to cross a 8mm wide, 100cm long and 15 cm elevated beam. The narrow beam was flanked on both sides by a platform from which the animals initiated a run voluntarily. The total number slips and steps of both hind limbs were counted and averaged from 3 successful runs by two observers blinded to the experimental group.

**Forelimb grip strength:** Forelimb muscle strength was measured using the grip strength meter (TSE-systems). Mice were held by the base of the tail and positioned above the grip-bar. Upon lowering, the animal grabbed the bar after which the animal was gently pulled away from the bar. The maximum force at which the mouse released the bar was averaged for five trials. To correct for variations between animals, the muscle strength was normalised for the maximum strength observed at 3 days before the lesion.

**Rotarod:** To assess overall motor coordination, animals were paced on the Rotarod (Ugo Basile Biological Research Apparatus) rotating at a constant speed of 5 RPM. The rotation was accelerated to 40 RPM over a period of 5 minutes. The time the mice could remain on the rotating beam was recorded and normalised for the maximum performance at 3 days before surgery.

**Catwalk gait analysis:** Gait analysis was preformed as described before (Hamers et al., 2001, Hendriks et al., 2006). Briefly, mice were pre-trained for one week to cross a 100 cm long glass plate, confined by Plexiglas walls 35mm apart.
in a darkened room. Paw prints were recorded digitally and analysed using the Catwalk software. All analysed parameters were normalised for the performance at 3 days before surgery.

**Tissue preparation**

One week after CST tracing, animals were euthanized by injecting an overdose of Nembutal (sodium pentobarbital, Sanofi Sante) followed by transcardial perfusion of ice-cold saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer. The spinal cord and brain were dissected and post fixed overnight at 4°C followed by incubation in phosphate buffered saline (PBS) containing 0.25M EDTA and cryopreservation in PBS containing 25% sucrose. Tissue was embedded in OCT compound (Sakura), snap frozen in 2-methylbutane and stored at -80°C until sectioning. 20 µm Thick transversal cryosections at cervical level C1 were thaw mounted on Superfrost Plus slides (Fisher Scientific). The C2-C6 spinal cord segment was cryosectioned sagitally. All sections were dried and stored at -80°C until use.

**Histological analysis and quantification**

To determine the total number of BDA traced CST fibers, transversal sections were incubated in Tris-buffered saline (TBS), 0.2% Triton X-100, 5% bovine serum (block buffer) for 1 hour followed by incubation with streptavidin-Alexa488 (1:400, Invitrogen) in blocking buffer for 3 hours at room temperature. The sections were washed three times in TBS containing 0.1% Triton X-100. Tiled images of the dorsal column were captured using an Axioplan 2 microscope (Zeiss) with a 40x objective. The CST was outlined using Imagepro Plus (MediaCybernetics) and a grid was placed over the outlined area. Fibers were counted in approximately 25% of a systematic randomized selection of the outlined area.

Sagittal sections were blocked as described above and incubated overnight at 4°C with Rabbit-anti-GFAP (1:1000, DAKO) in block buffer. The following day, sections were washed three times in TBS containing 0.1% Triton X-100 and incubated with goat anti-Rabbit-Cy3 (1:400, Jackson Immunoresearch) and streptavidin-Alexa488 (1:400, Invitrogen) for 3 hours at room temperature. The sections were washed 3 times in TBS containing 0.1% Triton X-100 and coverslipped. Tiled images were captured from every second section using an Axioplan 2 microscope (Zeiss) with a 20x objective.

Using the GFAP IHC signal, the ventral, caudal and rostral borders of the lesion site were identified. Using these three reference points, the center of the lesion was determined. In all images containing BDA positive CST fibers and lines were placed in dorsal-ventral orientation in the core of the lesion and 0.25, 0.50, 0.75 and 1.0 mm rostral and caudal from the lesion center using Imagepro Plus.
(MediaCybernetics). The number of CST fibers running through the white matter that crossed these lines was counted.

**Statistics**

All results are expressed as mean ± SEM. For the CST fiber index and narrow beam test, statistical significance was tested with a Kruskal-Wallis test followed by a Mann-Whitney U post hoc test. The rotarod, grip strength and catwalk experimental data were tested with an ANOVA analysis with a Bonferroni post hoc test. A value of p < 0.05 was considered significant.

**Results**

**Characterisation of Npn-1<sup>f/f</sup> / NefhCre<sup>+/−</sup> mice**

Mice that are homozygous for the loxP flanked exon 2 of Npn-1 and carry one allele of NfhCre (Npn-1<sup>f/f</sup> / NefhCre<sup>+/−</sup>) are born at Mendelian distribution and survive into adulthood.

**Histological analysis of injured CST fibers**

To analyse the location of CST fibers, animals were traced by injecting BDA in the motor cortex. One week after tracing, animals were perfused and spinal cord tissue was processed for immunohistochemical analysis. One knock out and one wild type animal died during CST lesion surgery. One knockout animal died during the BDA tracing procedure. Due to tissue processing error, histological analysis was not possible on 2 KO, 2 WT and 3 sham group animals. Therefore complete analysis was carried out on 10 KO, 10 WT and 5 sham animals. In all lesioned animals the dorsal CST lesion was complete. The total number of traced CST fibers was counted in transversal sections on cervical level C1 (Fig. 1b). We observe no significant differences in the C1 fiber counts in lesioned WT and KO animals (WT:1035 ± 150, KO:1276 ± 313). The number of CST fibers at 1, 0.75, 0.5, 0.25 and 0 mm caudal and rostral to the center of the lesion was determined in sagittal sections (Fig. 1a,c). To correct for variation in tracing efficiency, the CST fiber index was calculated by dividing the fiber counts proximal and distal to the lesion by the total number of CST fibers at the C1 cervical level (Fig. 1d). In all animals the CST fiber index observed 1 mm caudal to the lesion was similar to non-injured animals at the corresponding cervical level. In lesioned animals the number of fibers decreased as they advanced into the GFAP positive area around the injury site. In a few animals, a minute fraction of the fibers reached to or beyond the core of the lesion. Overall, we did not observe significant changes in fiber indexes at fixed distanced from the lesion core when comparing WT and KO animals (Fig. 1d).
Figure 1. CST fibers regenerate equally in Npn-1 knockout and wild type animals. Immunohistochemical double staining was used to visualize BDA traced CST fibers (green) and GFAP (red) in sagittal sections in the lesioned area (a, c). The total amount of CST fibers at cervical level C1 (b) was used to correct for tracing variability. Quantification of CST fibers at set intervals of the injury site (d) revealed no differences between wild type and knockout animals. Scale bar: 250 µm (a) and 50 µm (b,c)
Behavioural analysis

Narrow beam walk
To analyse the regain of coordinated paw placement after CST injury, we subjected the animals to a narrow beam test. After one week of pre-training, all animals were able to cross the 8 mm wide beam with less than 5% misplacements or slips of the hind limbs (Fig. 2). Three days after the lesion, wild type animals were not able to properly place their hind limbs on the beam in 65.6 ± 8.3% of combined hind limb steps, where knockout animals made 84.2 ± 3.5% slips. At day 10 post injury, wild type animals had recovered to making 16.2 ± 1.8% slips from which point on they slightly recovered to 11.2 ± 1.3% at 28 days post injury. In contrast, knockout animals recovered to 32.8 ± 7.8% slips at 10 days post injury and gradually improved to making 20.2 ± 5.5% slips at 28 days post injury. Overall, upon CST injury, we observed an increase in hind paw slips in knock out as compared to wild type animals. This difference reached significance at 7, 14, 21 and 24 days post injury.

Figure 2. Narrow beam walk analysis shows decreased coordinated hind paw placement in Npn-1 knock out mice. After CST lesion, Npn-1 KO mice showed 84.2±3.5% of combined hind limb steps, where wild type animals made 65.6± 8.3% slips. For the remainder of the experiment the animals displayed a similar recovery profile, maintaining the difference in hind limb function. (* p<0.05 significance between KO and WT)
Coordinated motor performance was measured using the Rotarod system. Three days after CST lesion, wild type and knockout animals performed at 59.6±5.3% and 46.9±5.9% respectively of their performance recorded before surgery (Fig. 3). Similar to the performance on the narrow beam test, knockout animals showed an increased loss of motor coordination upon CST lesion as compared to wild type animals. This trend was persistent throughout the duration of the experiment, but did not reach significance.

Forelimb grip strength
To measure forelimb grip strength, animals were pre-trained for one week before CST lesion. Three days after lesion wild type and knockout mice had maintained 63.9±5.0% and 59.7±4.7 respectively of their maximum grip strength where sham animals performed at 90.6±11.5% of their initial strength (Fig. 4). The loss of grip strength of lesioned animals did not significantly change throughout the duration of the experiment. Unexpectedly, sham group grip strength started decline from 10 to 17 days post operation to 62.9±4.8%
Catwalk

Automated gait analysis was performed using the catwalk system. We quantitatively analysed several specific gait parameters. The regularity index, the fraction of normally placed step sequences, was unchanged. The base of support of the front limbs decreased significantly to 75.5 ± 3.7% and 81.3 ± 3.4% in lesioned wild type and knockout animals on the third day after CST lesion and recovered completely on day seven for the remainder of the experiment (data not shown). The lesion had no effect on the base of support of the hind limbs. The stride length of the front and hind paws decreased as a response to the lesion (Fig. 5a, b). This decrease was significant for the front paws of knockout animals from 7 until 21 days after injury, and in hind paws of knock out animals at 10 and 21 days after injury. The wild type animals showed a trend towards complete recovery at 24 days after injury. The knockout group stride length recovery is delayed and is significantly different from the wild type injured animals at 20 and 24 days after injury for the front paws and at 24 days after injury for the hind paw measurements. Again, similar to the rotarod and narrow beam test, knockout animals have an increased response to the lesion that seems to persist throughout the duration of the experiment and reaches significance at the later time points due to the lack of recovery.

Figure 4. Front paw grip strength does not recover after CST lesion. Three days after CST lesion, front paw grip strength was reduced to 63.9±5.0% and 59.7±4.7 in WT and Npn-1 KO animals respectively. This loss in grip strength did not change during the remainder of the experiment. Unexpectedly, the sham group showed a decline in grip strength starting at day 10 after surgery.
In this study we have examined regeneration of the injured CST in conditional Npn-1 deficient mice by analysing the regrowth of transected CST fibers and functional recovery. We have done so by using an animal model in which the Npn-1 gene is ablated specifically in neurons around the time of birth. Our studies show that knocking out Npn-1 does not promote the regeneration of CST fibers. Conditional Npn-1 knockout animals appear to have an increased loss of function.

Discussion

Figure 5. Impaired stride length recovery in CST lesioned Npn-1 KO animals. Upon CST lesion WT and Npn-1 KO mice show a decrease in fore (a) and hind limb (b) stride length. This motor impairment recovers completely in WT animals where in KO animals this trend of recovery is delayed. (* p<0.05 significance between KO and WT)
upon CST injury. The recovery of motor function in KO and WT mice is similar, but a subtle difference in gait behaviour suggests a delayed recovery of KO animals.

The presence of the chemorepulsive protein Sema3A in the neural scar that forms after injury to the CNS is well established but the relative contribution of Sema3A to the plethora of growth inhibitory molecules encountered by injured CNS axons has not been thoroughly studied. Studying spinal cord regeneration in mice lacking the Sema3A or Npn-1 gene would be a bona fide approach to determine the contribution of Sema3A-Npn-1 signalling to the inhibition of axonal regeneration. However, mice that lack the Sema3A receptor Npn-1 do not survive beyond developmental stage E12.5 as a result of extensive neural and vascular defects (Kitsukawa et al., 1997, Kawasaki et al., 1999). Similar nervous system defects have been reported for mice that lack the Sema3A gene (Behar et al., 1996, Taniguchi et al., 1997). Although most Sema3A KO mice die a few days after birth (Behar et al., 1996), the few mice that survive could be used for regeneration studies although these mice may have developmental abnormalities of the nervous system. However, besides the possible behavioural changes due to the developmental abnormalities of the nervous system, the constitutive knockout of Sema3A could have an effect on regeneration by directly affecting migration of cells that form the neural scar, by influencing neovascularization (Joyal et al., 2011) or oligodendrocyte precursor cell migration (Spassky et al., 2002, Williams et al., 2007, Syed et al., 2011). To study the repulsive effects of Sema3A on injured neurons only, we have generated a mouse model in which Npn-1 is mutated in neurons starting at embryonal stage E18.5 by using neurofilament-H promoter driven Cre recombination in a conditional Npn-1 KO mouse. The Nefh-cre expression is initiated around birth (Hirasawa et al., 2001), at which time the majority of the nervous system has fully developed. Although the CST continues to develop during the first two weeks after birth, the majority of CST fibers have grown past the thoracic level T1 at postnatal day P1 (Gribnau et al., 1986). While the anatomy of the CST at the cervical level of the spinal cord and overall motor performance of non-lesioned KO animals were not different from WT animals, we cannot rule out that other parts of the nervous system are affected by knocking out Npn-1 perinatally.

Four weeks after transection of the CST, we did not observe differences in CST fiber regeneration in WT and KO mice. Furthermore, Npn-1 KO animals do not show an improved recovery using various motor skill tasks. We did however observe an increased deficit in KO animals directly after CST injury. The acute nature of this phenotype indicates an underlying developmental difference between KO and WT animals that is only uncovered after injury. From these experiments it is clear that eliminating Npn-1 signalling alone does not influence the functional and histological outcome of a CST lesion. A number of factors may account for the absence of an effect on regeneration in Npn-1 KO animals. First, besides Npn-1, corticospinal neurons also express neuropilin-2 (Npn-2), the receptor for Sema3B, 3C and 3F, rendering the regenerating CST axons sensitive to the other members of the class 3 Semas that are expressed in the scar (De
Winter et al., 2002b). Secondly, the presence of other growth inhibitory molecules may mask the contribution of, or be dominant over the effects of Sema3A. This idea finds some support in the observations showing that it is possible to improve neurite outgrowth by interfering with Sema signalling in relatively simple tissue culture based models. Primary neurons on a monolayer of Sema3A KO meningeal cells display improved neurite outgrowth, and inhibiting Npn-2 signalling using blocking antibodies allowed neurites to cross the astrocyte-meningeal cell boundary in vitro (Shearer et al., 2003, Niclou et al., 2006). In contrast to the situation in vivo, myelin-derived inhibitors do not play a role in these simplified in vitro regeneration assays, while semaphorins are more prominent constituents of in these in vitro models.

The redundancy of inhibitory molecules that play a role in the failure of spinal cord regeneration is currently an important point of discussion in the field. Eliminating multiple inhibitory pathways could provide a way to move forward and opening possibilities for improving spinal cord repair. Two studies have now reported the effects of deleting multiple myelin inhibitors with varying success. Studies by the Strittmatter laboratory have reported that double knockout of myelin and OMgp does not improve CST fiber regeneration and functional behaviour, but does have a synergistic effect on both parameters in the absence of nogo (Cafferty et al., 2010). Studies by the Zheng laboratory, however, do not confirm these results in similar experiments (Lee et al., 2010b). The same group recently reported a second study in which two different classes of inhibitory molecules are targeted to promote axonal regeneration by knocking out NgR and plexinA3/A4, the two co-receptors for class 3 semaphorins (Lee et al., 2010a). They showed that interfering with one class of molecules, or both at the same time, did not enhance regeneration of injured axons. Currently there is extensive debate about how mouse strain, approach of genetic mutation, location and method of injury application and technical details in the procedure of the histological analysis play a role in the conflicting results that are reported (Dimou et al., 2006, Cafferty et al., 2007, Steward et al., 2007, Lee et al., 2009, Lee et al., 2010a, Lee et al., 2010b, Schwab, 2010).

Interfering with growth inhibitory molecules using pharmacological inhibitors, blocking antibodies and peptides appears to have been more successful than genetic perturbation studies. In particular the class of myelin inhibitors and their receptors have been rewarding targets. Using the neutralizing antibody IN-1, the soluble NgR-ecto receptor domain, and neutralizing peptide NEP1-20, has proven to be successful in a variety of regeneration paradigms (Schnell and Schwab, 1990, GrandPre et al., 2002, Fischer et al., 2004, Li et al., 2004). The reason why neutralising agents are more effective than genetic mutation of the myelin inhibitors or its receptors is continuously under debate. One of the possible explanations is that the effects of the blocking reagents may extend beyond target neutralization. Also there may be roles of Nogo and NgR beyond growth inhibition. The unimpressive regenerative capacities of the various mutant mice as compared to acute intervention with blocking agents, could also be explained
by compensatory mechanisms that can occur in mutant animals (reviewed by Teng and Tang, 2005, Schwab, 2010).

Inhibition of non-myelin inhibitors also led to enhanced regeneration. The use of EphA4 blocking peptide or a RGMA blocking antibody has shown to have a significant effect on CST axon regeneration and functional improvement (Hata et al., 2006, Fabes et al., 2007). Antibodies blocking Sema3A function have been described but have not been used in spinal cord injury research (Shirvan et al., 2002). The Sema3A inhibitor SM-216289 or xanthofulvin has been found to be beneficial to olfactory nerve and spinal cord injury (Kikuchi et al., 2003, Kaneko et al., 2006). Administration of SM-216289 after a spinal cord transection improves raphespinal axon but not CST regeneration. The inhibitor also increases Schwann cell migration into the lesion area, which leads to myelination of regenerating axons, contributing to the positive effects of SM-216289 administration. Finally, the inhibitor has beneficial effects on angiogenesis and motoneurons survival. This inhibitor is not entirely selective for Sema3A as it does bind to matrix metalloprotease-2 and the epidermal growth factor receptor, and thereby possibly modulates the extracellular matrix (ECM) and cell adhesion, migration and proliferation. Therefore, it is evident that the inhibitor acts on a multitude of regeneration related processes.

The precise mechanism behind the inhibitory effects of proteoglycans is not well understood, but enzymatic degradation of the glycosaminoglycan side chains of CSPGs that are part of the ECM of the neural scar improves the outgrowth of injured axons, and facilitates functional recovery (Bradbury et al., 2002). Besides that proteoglycans themselves are inhibitory, the ECM could be a scaffold that binds Sema3A (De Wit et al., 2005) or other inhibitory proteins (reviewed in De Wit and Verhaagen 2007). Consequently, degradation of the ECM should also relieve the hypothesized contribution of the Sema3A to the inhibitory environment of the neural scar.

Interfering with down stream signalling of inhibitors such as Rho and PKC or stimulating the intrinsic growth capacity by elevating cAMP level is a promising method to deal with the redundancy in inhibitory mechanisms by neutralizing a broad spectrum of inhibitors (Dergham et al., 2002, Neumann et al., 2002, Qiu et al., 2002, Sivasankaran et al., 2004).

It is important to realize that regeneration of the central nervous system fails for multiple reasons. The idea that inhibitory molecules in myelin and in the neural scar are major factors is well established and progress has been made in finding ways to neutralize some of these molecules. However, in addition to inhibitory control at least two other components of the failure of repair mechanisms deserve attention: the lack of positive influences at a lesion site and the inadequate intrinsic response of CNS neurons to axon injury. A logical combination therapy would include a neurotrophic treatment (e.g. a neurotrophic and/or protective factor), a treatment that promotes axon growth from within an injured neuron (e.g. cAMP) and an anti-inhibitory treatment that overcomes inhibition by myelin and scar-derived inhibitors.