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Running title: Delayed anti-Nogo-A antibody application

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Delayed anti-Nogo-A antibody application after spinal cord injury shows progressive loss of responsiveness

Abstract

Blocking the function of the myelin protein Nogo-A or its signalling pathway is a promising method to overcome an important neurite growth inhibitory factor of the adult CNS and to enhance axonal regeneration and plasticity after brain or spinal cord injuries. Several studies have shown increased axonal regeneration and enhanced compensatory sprouting along with substantially improved functional recovery after treatment with anti-Nogo-A antibodies, Nogo receptor antagonists, or inhibition of the downstream mediator RhoA/ROCK in adult rodents. Proof of concept studies in spinal cord injured macaque monkeys with anti-Nogo-A antibodies have replicated these findings; recently, clinical trials in spinal cord injured patients have been started. However, the optimal time window for successful Nogo-A function blocking treatments has not yet been determined. We studied the effect of acute as well as 1 or 2 weeks delayed intrathecal anti-Nogo-A antibody infusions on the regeneration of corticospinal tract (CST) axons and the recovery of motor function after large but anatomically incomplete thoracic spinal cord injuries in adult rats. We found that lesioned CST fibres regenerated over several millimetres after acute or 1 week delayed treatments, but not when the antibody treatment was started with a delay of 2 weeks. Swimming and narrow beam crossing recovered well in rats treated acutely or with a 1 week delay with anti-Nogo-A antibodies, but not in the 2 weeks delay group. These results show that the time frame for treatment of spinal cord lesions with anti-Nogo-A antibodies is restricted to less than 2 weeks in adult rodents.

Keywords: Nogo-A, spinal cord injury, plasticity, regeneration, sprouting, delayed treatment, recovery, motor function
Introduction

The failure of neurons to regenerate after axotomy in the central nervous system (CNS) is a major reason for the lack of substantial functional recovery after large brain or spinal cord lesions in adult mammals. Several factors contribute to this failure: Some CNS neurons are intrinsically reluctant to grow and to sufficiently upregulate regeneration-associated proteins following an injury (Plunet, et al., 2002). The formation of growth inhibiting scar tissue at the site of CNS injury and the presence of myelin associated growth inhibitors block the regeneration of injured axonal projections. Enzymes that degrade scar associated chondroitin sulphate proteoglycans (CSPGs) injected into the injured tissue led to enhanced fibre growth around injury sites (Yiu and He, 2006). The blockage of the myelin associated protein Nogo-A, a key growth inhibiting molecule in the oligodendrocyte cell membrane of adult higher vertebrates, by acute intrathecal infusion of neutralizing monoclonal antibodies or by peptides or fusion proteins blocking Nogo-A or its receptor NgR after spinal cord lesion led to enhanced sprouting and regeneration of injured axons accompanied by an improved functional recovery in adult rodents and macaque monkeys (Freund, et al., 2006, Gonzenbach and Schwab, 2008, Liebscher, et al., 2005, Schwab, 2004). Nogo knockout lines produced conflicting results and remain a subject of ongoing studies. While Nogo-A (Simonen, et al., 2003) and Nogo-A and -B (Cafferty, et al., 2010, Cafferty, et al., 2007, Cafferty and Strittmatter, 2006, Kim, et al., 2003) knockout lines showed an increased or partially increased regenerative and plastic phenotype in some labs, no effects were seen in Nogo knock-out lines generated in another laboratory (Lee, et al., 2009, Zheng, et al., 2003). Triple
knockouts for Nogo, MAG and OMgp showed major regrowth (Cafferty, et al., 2010), or only enhanced compensatory axon sprouting (or intraspinial plasticity) but no long-distance regeneration (Lee, et al., 2010). These mixed results could be explained by compensatory up-regulation of other Nogo splice variants (Simonen, et al., 2003) as well as other repulsive molecules (Montani and Schwab, unpublished observations), by the different genetic background of the mouse lines used by the different groups (Dimou, et al., 2006), and by different lesion paradigms used (Cafferty and Strittmatter, 2006). Constitutive, life-long genetic knockouts are known to often produce milder (or even no) phenotypes due to compensatory mechanisms which are less likely to occur after acute application of function blocking drugs, antibodies, peptides or fusion proteins. For a discussion of this issue, see (Schwab, 2010, Tuszynski, 2010).

As axons of axotomized upper motoneurons progressively retract from the lesion site (Pallini, et al., 1988, Seif, et al., 2007) and often atrophy (Wannier, et al., 2005), they may become less responsive to anti-Nogo-A treatment with increasing time after injury. In addition, scar formation and accumulation of CSPGs at the injury site may further impede successful regeneration (Busch and Silver, 2007) which could further reduce the efficacy of neurite growth enhancing treatments. Yet for use in human patients, determining the time frame for clinically successful interventions is pivotal, as victims of SCI can usually not be treated immediately after injury. Previous animal studies indicate that injured neurons may indeed retain the ability to regenerate for weeks or months after injury, if they are stimulated by adequate interventions (Houle, 1991, Kwon, et al., 2002, Ye and Houle, 1997, Ylera, et al., 2009). One week delayed treatment with the Nogo receptor antagonist NEP1-40 led to increased axonal regeneration and improved
locomotor function recovery which was comparable to acute treatment (Li and Strittmatter, 2003). However, the optimal time window for treatment with Nogo-A neutralizing agents is currently unknown.

We report that in adult rats the window of opportunity for treatment with anti-Nogo-A antibodies is clearly limited after spinal cord lesion and that delaying the application progressively reduces its effect on the functional recovery and the regeneration of corticospinal tract fibres.

Materials and methods

Animals and animal care

All procedures described herein were approved by the Veterinary Office of the Canton of Zürich, Switzerland. Adult female Lewis rats (180 - 200 g, aged 9 - 10 weeks) were kept in groups of 4 - 5 animals in standard cages on a 12 hours light/dark cycle with access to water and food ad libitum.

Experimental design

A total of 63 rats, divided into six groups were treated intrathecally for 2 weeks with anti-Nogo-A or control antibodies, starting immediately or with a delay of 1 or 2 weeks after an incomplete thoracic (T8) spinal cord injury.

The animals were handled and trained on the narrow beam and the swim test for 3 weeks prior to surgery. Preoperatively, a third of the rats were randomly assigned to the immediate treatment groups. The rats that received the treatment starting one or two weeks after spinal cord lesion were randomly assigned to the treatment groups in pairs according to their motor function deficits 6 days after injury (with the assigning
investigators blinded to the treatment group). The narrow beam performance was the principal measure used to randomize animals in pairs, i.e. animals with equal or similar scores were attributed to either the IgG or the anti-Nogo-A treated groups. As different behavioural scores do not necessarily correlate, the readouts from the BBB subscore and swim test were also used in cases were several animals had similar scores. This randomized assignment allowed the comparison of treatment groups with equal motor function deficits at the start of antibody application. All rats were number coded and kept in randomly mixed groups. All experimenters were blinded to the treatment throughout the experiment. The experimental design is shown in Fig. 1A.

**Antibodies, antibody administration, and CSF antibody concentration**

Mouse monoclonal antibody 11C7 directed against amino acids 623 - 640 of the rat Nogo-A sequence (Oertle, et al., 2003) and control monoclonal IgG antibodies directed against the plant protein wheat auxin were infused at a concentration of 3mg/ml. The anti-Nogo-A antibody 11C7 is monospecific for Nogo-A on Western blots and does not crossreact with other Nogo splice variants (Dodd, et al., 2005). The function blocking capacity of this anti-Nogo-A antibody is due to steric blockage of the interaction of Nogo-A with its receptor and the down-regulation of Nogo-A from the cell surface by internalization of the Nogo-A/antibody complex (Liebscher, et al., 2005, Weinmann, et al., 2006).

A total of 6 mg of antibody dissolved in 2 ml PBS was continuously delivered over 2 weeks into the intrathecal space using subcutaneously implanted osmotic minipumps (5 µl/h, Alzet 2ML2) connected to a subduraly implanted catheter as described before (Liebscher, et al., 2005).
CSF samples in the 1 week delay groups were collected by puncturing the cisterna magna immediately after pump removal. The CSF antibody concentration was determined with sandwich enzyme-linked immunosorbent assay.

**Spinal cord lesion surgery**

All surgical procedures were performed under anesthesia using Hypnorm (120μl/200g body weight, Janssen Pharmaceutics) and Dormicum (0.75mg per 200g body weight, Roche Pharmaceuticals). T-shaped lesions of the thoracic (T8) spinal cord that transected the dorsal, dorsolateral and ventromedial parts of the spinal cord were performed on 8 – 10 week old rats essentially as described previously (Liebscher, et al., 2005) but with more extensive lesion of the lateral funiculi. This lesion paradigm was chosen because it completely interrupts all parts of the CST, including the ventral fibers. This lesion paradigm produced well defined, moderate functional deficits with good recovery of motor and bladder function, thus keeping animal suffering to a minimum. Postoperatively, the bladder was manually expressed for 2 weeks. Antibiotics (Baytril, 5 mg/kg, Bayer AG, Leverkusen, Germany) were given subcutaneously for 7 days to prevent bladder infection.

**Exclusion criteria for behavioural assessment**

The locomotor impairments varied substantially between rats, in spite of standardized surgical procedures. All animals had complete lesions of the dorsal, dorsolateral and ventral funiculus containing the CST. To compare animals with similar functional deficits, rats with a performance of > 9 in the narrow beam test 6 days after experimental SCI were excluded post hoc (n=13). In addition, 2 rats with recurrent bladder infections
were excluded as well. The exclusions were done prior to statistical analysis on the number coded rats.

**Assessment of locomotor function recovery**

Locomotor function was scored directly (narrow beam test and BBB) or videotaped and analyzed on a computer (swim test). The number of animals was as follows: Animal numbers: acute Anti-Nogo-A: n = 9, acute IgG control: n = 7, 1 week delayed Anti-Nogo-A: n = 7, 1 week delayed IgG control: n = 9, 2 weeks delayed Anti-Nogo-A: n = 9, 2 weeks delayed IgG control: n = 7.

**Swim test:** Intact rats use their hindlimbs and the tail for swimming, while their forelimbs are held immobile under the chin. Due to buoyancy, rats are able to swim even after a severe spinal cord injury. The basic swimming pattern with alternating hind limb strokes is usually not affected except for short periods during which rats swim in a ventroflexed position with often coupled hindlimb strokes (unpublished observation, manuscript under revision). This allows scoring the deviation from normal hindlimb usage and assessing its recovery over time as described by Liebscher (Liebscher, et al., 2005). Rats were videotaped while swimming in a Plexiglas basin (150 × 40 × 13cm, water temperature 28 – 30 °C). Swimming velocity was calculated by measuring the time required for swimming a distance of 60 cm. Hind limb usage was scored as described by Liebscher (Liebscher, et al., 2005): 4 = normal hind limb usage; 3 = hind limb strokes deviate laterally but hind limbs are underneath the body; 2 = hind paws are lateral to the body and the distance between the hind limbs is increased; 1 = large distance between hind limbs, i.e. the hind paws and legs are entirely lateral to the body.
Narrow beam test: To examine deficits in balance and fine motor control rats had to cross an elevated, tapered beam (1.4 m long) labeled with 24 equally spaced segments, from the wide (6 cm) to the narrow (1.5 cm) end. Intact rats have no difficulties crossing the beam in its entire length, whereas spinal cord lesioned rats step down onto a ledge fixed underneath as the beam is getting narrower, depending on their functional deficits. They were scored (0 – 24) according to the segment where they first stepped down. The average of 10 runs is reported.

BBB and BBB subscore: The hind limb locomotor recovery was assessed with the BBB Open Field Locomotor Scale (Basso, et al., 1995) by two blinded observers before and 1, 5 and 10 weeks after injury. The rats were individually placed in an open field for 4 minutes and joint movements, stepping capability, toe clearance, coordination, trunk stability and tail usage were scored. In addition, we determined the BBB subscore, which reflects toe clearance, hindlimb rotation and tail usage, regardless of coordination (Basso, 2004). The average score of the right and left hind limbs is reported for each animal.

Anterograde corticospinal tract tracing

Ten weeks after spinal cord injury, the corticospinal tract was anterogradely traced as described (Liebscher, et al., 2005). Briefly, a total volume of 2.0µl of 10% BDA (MW 10000; Molecular Probes, Eugene, OR) dissolved in 0.01M phosphate-buffered saline was injected at 4 sites of the hindlimb area of the sensory-motor cortex using a Hamilton syringe. Care was taken not to inject BDA into the lateral ventricles to avoid artefactual labelling of neurons via the CSF (Steward, et al., 2007). Three weeks later, the rats were
deeply anesthetized with pentobarbital and perfused with heparinized Ringer’s solution followed by 4 % paraformaldehyde. The spinal cords were dissected and processed as described (Liebscher, et al., 2005). Sagittal sections were cut at 50 µm on a cryostat and further processed by the avidin biotin method to reveal BDA labelled fibres using the semi-free floating technique (Herzog and Brosamle, 1997).

**Quantification of CST regeneration**

The number of BDA labelled corticospinal tract axons was counted at 0.5 mm, 2 mm, and 5 mm caudal to the lesion site on complete series of 50 µm thick sagittal sections at 400 x magnification for each spinal cord. If axons were arbourized, each segment was counted separately. A segment was defined as a continuous BDA-positive fibre that is not interrupted by branches. The vast majority of traced fibres were thin and followed an irregular course. Rarely, a small number of spared CST fibres were found in the ventral funiculus. They were clearly identified by their typical straight and regular trajectory and were not counted.

To correct for inter-individual tracing variability, the total number of BDA-labelled axons was quantified on 2 adjacent 50 µm cross-sections in the upper thoracic spinal cord several segments rostral to the injury site using a 63 x objective. The number of CST fibres counted caudal to the injury was then divided by the number of BDA-labelled fibres above the lesion to calculate the fraction of regenerated fibres. This calculated fraction of regenerated fibres was reported as percentage of regenerated fibres.

The animal numbers were as follows: acute anti-Nogo-A: n = 8, acute IgG control: n = 6, 1 week delayed anti-Nogo-A: n = 7, 1 week delayed IgG control: n = 6, 2 weeks delayed anti-Nogo-A: n = 11, 2 weeks delayed IgG control: n = 6.
Camera lucida reconstructions

The labelled corticospinal axons of three adjacent parasagittal spinal cord sections were projected onto a single plane and plotted together with the contour of the lesion and the spinal cord surface using a camera lucida tubus attached to the microscope.

Immunohistochemistry

To determine the up-regulation of the scar associated proteoglycan CS-56, rats were euthanized at 3, 7 and 14 days after SCI as described above. For each time point, 2 rats were used. The tissue was fixed and processed as described above and cut at 50 µm in the sagittal plane. Free floating sections were incubated with the primary monoclonal mouse IgM CS-56 (1:50, Sigma, Saint Louis, MO, USA) followed by a Biotin coupled donkey anti-mouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA, USA). The biotin coupled secondary antibody was detected using Cy3-conjugated streptavidin (1:300; Jackson ImmunoResearch, West Grove, PA, USA).

To determine the tissue penetration of the intrathecally infused antibodies, 6 rats treated with a delay of 2 weeks were euthanized 1 hour after pump removal. Three animals were used for each treatment group. The tissue was handled as described above and cut at 50 µm on a cryostat. Free floating sections were incubated with a rat-adsorbed anti-mouse antibody coupled to biotin (1:300, Jackson ImmunoResearch, West Grove, PA, USA). The biotin coupled secondary antibody was detected using the ABC-DAB system (Vector laboratories, Burlingame, CA, USA). The immunohistochemical staining procedure was done in the same batch for anti-Nogo-A and control antibody treated animals. The density
of antibody staining was quantified and color-coded with red indicating high antibody density, and dark blue indicating low antibody density.

**Assessment of lesion completeness**

All spinal cord lesions were reconstructed in the coronal plane to control for appropriate lesion size and shape. The lesions were reconstructed from the complete section series used for CST reconstruction at the site of the largest lesion extent and projected into a single coronal plane. The extent of the lesion was determined as percentage of the spinal cord cross-section using ImageJ software.

**Statistical analysis**

All statistical tests were carried out with SPSS 14.0. The locomotor tests were evaluated with a two-way repeated measures analysis of variance (ANOVA). The numbers of regenerated CST fibres was evaluated with the Man Whitney U test.

**Results**

**Lesion size and antibody distribution**

All the groups had similar lesion sizes, ranging between 40 % and 65 % injured tissue (Fig. 1B, C). In spite of standardized surgeries the lesion size varied between individual animals due to secondary effects like ischemic necrosis and bleeding. Control antibody and anti-Nogo-A antibody treated groups were not different in their lesion sizes, however. The 2 weeks delay groups, both anti-Nogo-A and control antibody treated, had slightly smaller lesions (P<0.05; 2-tailed T-test for independent samples) than the acute
and 1 week delay groups which might account for the slightly better performance in the narrow beam and the BBB score in the first weeks after injury.

Antibody concentrations in the CSF after 2 weeks of infusion in the 1 week delay groups ranged between 3 and 40 µg/ml and were similar in the anti-Nogo-A and the control antibody treated groups (Fig. 1D, 2-tailed T-test, 11c7: N = 11, control IgG: N = 12).

To rule out the possibility that scar tissue blocks the free distribution of antibodies within the CSF and the penetration of antibodies into the tissue 2 weeks after SCI we did immunohistochemical stainings of spinal cord and brain crosssections for mouse IgG in the 2 weeks delay group. The Immunohistochemical staining yielded dense signals in the spinal cord of anti-Nogo-A antibody treated animals and weak signals in control antibody treated animals, indicating that anti-Nogo-A antibodies, which bind to cell surface Nogo-A, are retained more efficiently in the tissue than the control antibody against wheat auxin (Fig. 1E). Moderate signals were also detected in the brains of anti-Nogo-A but not of control antibody treated rats. The strongest immuno-reactivity was observed at the spinal cord and brain surface (Fig. 1E). The lower antibody staining in the CNS parenchyma compared to the pial surface occurs because the intrathecally applied antibodies have to diffuse from the CSF into the parenchyma. These results are very similar to the ones obtained earlier (Weinmann, et al., 2006).

**Locomotor recovery**

The swim test showing the use and position of the hind limbs, the relatively difficult narrow beam test showing balance and precision of foot placement, and the BBB score for openfield locomotion were used to assess the effects of anti-Nogo-A antibody administration at 0, 1, and 2 weeks delays after spinal cord injury.
Narrow beam: The narrow beam paradigm (Fig. 2, top row) assesses different aspects of locomotor function; besides basic stepping function, successful crossing of the narrow-beam requires the capability to maintain balance. Six days after injury, the narrow beam scores were very low in all groups. Acutely treated rats with anti-Nogo-A antibody scored significantly higher than the control antibody group in the narrow beam test from 2 weeks on. The delayed treatment, however, did not improve the performance on the narrow beam: the slight recovery observed in the groups with delayed antibody application was equal for the anti-Nogo-A and the control groups. The lower score in the first week after injury in the acutely treated rats compared to the groups with delayed treatment is most probably due to subcutaneously implanted minipump in combination with the recent SCI. Early after injury, when balance and fine motor control have not recovered yet, the pump represents an irritation for the rats and reduces their ability to cross the beam.

Swim test: The groups with anti-Nogo-A antibody given acutely or with 1 week delay progressively improved their swimming velocity and their hind limb use over 3 - 4 weeks (Fig. 2, lower rows). In contrast, the animals with 2 weeks delayed anti-Nogo-A treatment, as well as the control IgG animals of all the groups did not improve swimming velocity and hind limb function. After injury, the swimming velocity was about 50 % of normal. It recovered to 61 % - 66 % in the acute and 1 week delayed anti-Nogo-A antibody treated groups. The hind limb score dropped to a very low level in all groups at 6 days after injury. The recovery of hindlimb function was significantly better in the acute and 1 week delayed anti-Nogo-A antibody treated groups compared to the
respective control groups. In contrast, the 2 weeks delayed anti-Nogo-A antibody treated group was not different from its IgG-control group.

*Open field locomotion:* The BBB score dropped from 21 to 7 - 10, the BBB subscore from 12 to 0 at 1 week after injury in all groups. Ten weeks after spinal cord lesion, most rats had consistently recovered stepping function, irrespective of treatment or treatment onset; they all ranked between 11 and 13 in the BBB score (Fig. 3, lower row), corresponding to no (11), occasional (12) or frequent (13) hindlimb – forelimb coordination, respectively. No significant difference with regard to fore limb – hind limb coordination, which depends largely on propriospinal connections running in the spared ventro-lateral tracts (Juvin, et al., 2005), was found between the treatment groups. As the BBB is not sensitive enough to detect relevant differences in the functionally important range between 10 – 14 points we determined the BBB subscore. In the BBB subscore, which reflects toe clearance, hindlimb rotation and tail usage (important for trunk stability and balance), the acutely and the 1 week delayed anti-Nogo-A antibody treated groups both reached significantly higher scores than the control antibody groups (Fig. 3, upper row). In contrast, in the 2 weeks delayed anti-Nogo-A antibody treatment group the BBB subscore was comparable to the control antibody group.

**Corticospinal tract regeneration**

The regeneration of the cut CST fibres under different treatment conditions was investigated 10 weeks after spinal cord lesion. Only animals showing a complete interruption of the dorsal, the dorsolateral and the ventral CST tracts were included in the analysis. A few animals were lost due to death during tracing surgery or due to tracing...
failure. In all analyzed spinal cords, the transected CST was slightly retracted from the lesion site and formed numerous retraction bulbs. Regenerating CST fibers grew in bridges of spared tissue around the lesion site and did not enter the lesion site, as described previously (Liebscher, et al., 2005). Labelled CST fibres observed below the lesion site were consistently of thin caliber and followed a tortuous course, mostly within the grey matter (Fig. 4A; Fig. 5). Most fibres arbourized extensively within the grey matter forming numerous collaterals. In control antibody treated rats, irrespective of the antibody infusion onset, very low numbers of labelled CST fibres were observed below the injury site, as indicated by a low percentage of 0.2 % - 1 % regenerating fibres (Fig. 4A, bottom row; Fig. B; Fig. 5, top row). In contrast, anti-Nogo-A antibody treated rats showed significantly higher numbers of labelled CST fibres at all analyzed levels below the injury site if the treatment was started acutely or with a delay of 1 week after injury (Fig. 4 and 5). The percentage of regenerated fibres was highest after acute treatment reaching means of 7.2 % – 10.9 % (p<0.01 at 0.5 mm and 2mm, p<0.05 at 5 mm). In the 1 week delay treatment group the regeneration was still significantly improved in the anti-Nogo-A compared to the control antibody group, reaching 3.9 % – 5.0 % (p<0.05 at 0.5 mm and p<0.01 at 2 mm and 5 mm). After 2 weeks delayed treatment, the percentage of regenerated fibres was significantly higher in the anti-Nogo-A than in the control antibody group 0.5 mm caudal to the injury (p<0.05), but not 2 and 5 mm (Fig. 4B).

CSPG immunoreactivity at the lesion site

To look for molecular mechanisms for the decreased anti-Nogo-A response at 2 weeks after injury we stained for the scar associated proteoglycan CS-56, which is known to be
upregulated after CNS injuries. Staining with the CS-56 antibody, which recognizes chondroitin-4- and chondroitin-6-sulphate proteoglycans (Avnur and Geiger, 1984), revealed very low levels of CSPG in the intact spinal cord (Fig. 6A). Slightly increased levels of CS-56 immunoreactivity were seen 3 days after injury; it was restricted to the lesion site and did not include neighbouring spared tissue (Fig. 6B). In contrast, 1 and 2 weeks after injury, the immunohistochemical signals for CS-56 had augmented substantially and spread to the neighbouring spared tissue (Fig. 6C, D). This time-course of CSPG expression is in agreement with earlier observations (Camand, et al., 2004).

**Discussion**

The results obtained with acute intrathecal anti-Nogo-A antibody infusion after spinal cord injury in adult rats confirmed earlier findings: regeneration of injured descending tract fibres e.g. of the CST, and enhanced recovery of locomotor functions. Delaying the start of intrathecal anti-Nogo-A antibody infusion after a lesion, however, led to a progressive loss of responsiveness to the treatment both on the cellular and the functional level. While regeneration and functional recovery were still strongly increased after a 1 week delayed anti-Nogo-A antibody treatment, the 2 weeks delayed treatment led to a minimal increase in fibre regeneration and no observably improved functional recovery compared to the control antibody infusion.

On the functional level, intrathecal anti-Nogo-A antibody administration started immediately after injury substantially improved several parameters of open field locomotion, in particular improved foot placement as assessed by the BBB subscore and increased ability to cross the narrow beam. Restoration of hindlimb motor control was
also reflected in the higher swimming velocity and improved hindlimb usage during swimming.

In contrast to Liebscher et al. we do not observe consistent forelimb/hindlimb coordination in the acutely treated anti-Nogo-A treated animals, resulting in similar overall BBB scores for both treatment groups. Forelimb/hindlimb coordination relies mainly on intact propriospinal connections which run in the lateral tracts. Our lesions were larger, i.e. 40% - 65% in the acute treatment groups compared to 40% - 50% in (Liebscher, et al., 2005) and destroyed the lateral tracts more substantially. This might explain why none of our animals recovered consistent forelimb/hindlimb coordination.

When the start of the antibody application was delayed by 1 week after injury, an improved functional recovery was still observed in the swim test and the BBB subscore where the extent of recovery was comparable to that obtained with acute treatment. In contrast, however, the performance on the narrow beam did not improve in the 1 week delayed treatment group. Balancing on and crossing the narrow beam likely requires substantial supraspinal control such as tactile input from the whiskers, visual information, vestibular and cerebellar input for balance, and correct foot placement and grip control. In contrast, the control of swimming may require less supraspinal commands and depends to a larger extent on the spinal central pattern generator. The lower percentage of regenerating CST fibres in the 1 week delayed compared to the acute treatment with anti-Nogo-A antibodies (5% versus 10%) may explain the lack of improved recovery of narrow beam crossing in the 1 week delayed anti-Nogo-A antibody treated animals. This lower, but in comparison to control animals increased number of regenerated CST fibres,
may still be sufficient to control swimming, but not for the more demanding beam crossing task.

In the 2 weeks delay groups the lesion size tends to be slightly smaller than in the other groups explaining the slightly better motor performance of these groups. One could argue that this slightly smaller lesion size in the 2 weeks delay groups might have led to sufficient spontaneous motor function recovery and thereby disguised the treatment effect of anti-Nogo-A antibody treatment. Data from Liebscher (Liebscher, et al., 2005) suggest that this is not the case: rats with the same lesion paradigm but smaller lesion size (40% - 50% in Liebscher compared to 40% - 60% in the 2 weeks delay group in our study) showed a clear response to acute anti-Nogo-A antibody treatment. Therefore, the lack of functional recovery is most likely due to the 2 weeks delayed treatment.

In parallel to the functional readouts, a progressive loss of responsiveness to anti-Nogo-A antibodies with time after the lesion was observed anatomically with regard to the regeneration enhancing effect of the antibody treatment. Acute and 1 week delayed treatment robustly increased regeneration below the injury site: higher numbers of BDA-labelled CST fibres with the irregular morphology typical of regenerating fibres were found 0.5 mm, 2.0 mm and 5.0 mm caudal to the lesion site in the anti-Nogo-A antibody treated groups. These regenerated fibres arbourized extensively in the grey matter and formed numerous varicosities, suggesting that they formed synaptic connections and integrated into the spinal circuitries. The 2 weeks delayed treatment led to a minimal increase in the number of labelled CST fibres in the area immediately caudal to the injury site, and no increase further below, indicating that the regeneration enhancing effect on CST fibres was substantially decreased.
Several factors may explain why a delayed treatment with anti-Nogo-A antibodies is less effective. First, axotomized CNS neurons show a transient lesion induced growth response after injury which subsides after 1-2 weeks and turns into cellular atrophy (Plunet, et al., 2002). Neutralizing Nogo-A in the early post-injury phase may allow these sprouting fibres to elongate and find targets that supply retrograde trophic signals to the growing neurons, thus sustaining growth and stabilizing the new connections. However, once the lesion-induced intrinsic growth effort has stalled, delayed blockade of Nogo-A may be insufficient to promote long-distance regeneration. Some types of CNS neurons, such as rubrospinal neurons, can be stimulated to grow by neurotrophins even in an atrophic, shrunken state up to 1 year after axotomy, suggesting that combined neurotrophic factors plus anti-Nogo-A antibody treatments may be one way to extend the regeneration permissive time window (Kwon, et al., 2002, Ylera, et al., 2009). For example, the combination of the neurotrophin NT3 and anti-Nogo-A antibodies showed improved CST regeneration even 2 months after SCI (von Meyenburg, et al., 1998). A second reason for the lack of pronounced regeneration after a 2 week delayed treatment may be the formation of scar tissue with its scar-associated growth inhibitory molecules. Different species of CSPGs are secreted and accumulate within 1-4 weeks after injury around CNS lesion sites (Jones, et al., 2002, Tang, et al., 2003). Some persist at the injury site for several months contributing to the barrier for regenerating axons (Jones, et al., 2002, McKeon, et al., 1999, Tang, et al., 2003). Early blockage of myelin inhibition, before scar associated inhibitory proteins are deposited in large amounts, may therefore be crucial.
A possible interference of scar tissue with anti-Nogo-A antibody distribution by the CSF circulation could be ruled out by measuring antibody concentrations in the CSF taken from the cisterna magna and by immunohistochemical staining for mouse IgG in the spinal cord and brain tissue. The high anti-Nogo-A and control antibody concentrations, between 20 - 30 µg/ml in the CSF in the 1 week delayed treatment animals, as well as the good antibody penetration into the brain and spinal cord tissue in the 2 weeks delayed treatment groups indicate that the antibodies were well distributed throughout the CNS.

Regenerating fibres of interrupted tracts and compensatory growth of unlesioned axons have to form meaningful connections with correct and often distant target cells in the spinal cord. During development, growing axons are guided by a complex set of molecular cues that allow the correct formation of neuronal circuits (Canty and Murphy, 2008). Subsequent activity-dependent mechanisms then fine tune and stabilize the circuitry in the late phases of development (Hua and Smith, 2004, Martin, 2005). Little is known about the mechanisms that could guide regenerating axons to functionally meaningful targets or stimulate intact neurons to form new compensatory connections after an adult CNS injury. These mechanisms may be absent or failing in the denervated, non-functional spinal cord, with increasing time after injury. It is noteworthy, however, that enhancement of compensatory fibre growth with formation of functionally meaningful connections by anti-Nogo-A antibodies can occur after stroke even if the antibody application was delayed by 1 or 4 weeks (Markus, et al., 2005, Seymour, et al., 2005, Tsai, et al., 2007, Tsai, et al., 2010). In contrast to the recovery from spinal cord
lesions, stroke recovery may depend less on axon tract regeneration and on fibres that regenerate through a CSPG-rich lesion area.

Muscle spasms are a common consequence of spinal cord injury. Their occurrence in a rat model for muscle spasms was greatly reduced after anti-Nogo-A antibody treatment (Gonzenbach, et al., 2010). Importantly, 1 or 2-weeks delayed treatment with anti-Nogo-A antibodies was equally effective as acute treatment. This Anti-Nogo-A-antibody mediated reduction of muscle spasms in delayed treatment might be due to increased plasticity of intraspinal neuronal circuits and not due to long-distance regeneration of transsected axons.

An increase of intraspinal plasticity, e.g. increased numbers of midline crossing fibres after unilateral CST lesions has been observed as early as 1 week after SCI after anti-Nogo-A antibody treatment (Bareyre, et al., 2002). This intraspinal plasticity could also contribute to the early treatment response seen in the acutely treated group, perhaps combined with early regenerating fibers (regeneration velocity can be up to 1 mm/day (Schnell and Schwab, 1990), although the initial delay until fibers start to grow is unknown).

In spite of standardized surgeries the variation of performance level and lesion size is substantial in spinal cord lesion experiments due to secondary injury effects like ischemic necrosis and bleeding, which may limit the conclusions of this study. However, all treatment groups had similar lesion sizes, ranging around 50% injured tissue. In addition, the random assignment to the delayed treatment groups in pairs according to the initial motor function deficits ensured valid control groups.
The present results show that the time frame for successful treatment of spinal cord injured adult rats with Nogo-A blocking agents is restricted to less than 2 weeks after injury. Our findings are of clinical importance, since a limited time frame for successful Nogo-A blocking treatment is to be expected in human spinal cord injured patients as well. The duration of this time window may differ substantially between humans and rodents; the degenerative and regenerative changes after injury follow a markedly different time course in man and rat. For example, the spinal shock phase lasts for a few hours to about 2 days in rats, but from days to 4 weeks in humans (Ditunno, et al., 2004, Hiersemensel, et al., 2000). The time course of motor function recovery after incomplete lesions is more protracted in humans who show motor improvements over several months, whereas rats usually reach a plateau 4 - 6 weeks after injury. Although the time frame for successful treatment may thus be wider in humans than in rodents, our results indicate that the treatment’s success is larger if it is started early after injury. This present study also implies that anti-Nogo-A antibodies alone may not be very effective in the chronic stage of spinal cord injury. Strategies combining different treatments which tackle the problem on different levels e.g. neuronal growth stimulation as well as scar suppression may provide the key to overcoming the blockage of regeneration in chronic para- and tetraplegic patients.

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Author disclosure Statement

No competing financial interests exist. The anti-Nogo-A antibody was provided by
Novartis Pharma AG.

References


Figure legends

Figure 1: Study design, lesion size, antibody tissue penetration and CSF antibody concentrations. (A) Scheme summarizing the different treatment groups and the sequence of experimental steps: after a large, incomplete spinal cord injury at T8, adult rats were treated acutely or with a delay of 1 or 2 weeks with either a monoclonal antibody against Nogo-A or with a control antibody. Locomotion was assessed with different behavioural tests over a time period of 10 weeks after injury. Ten weeks after injury, the CST was anterogradely traced to assess the regeneration of lesioned axons. (B) Scheme of the T-shaped spinal cord lesion at T8. This lesion (grey) interrupts the major CST in the dorsal funiculus as well as the minor projections in the dorsolateral and ventral funiculus (red). The lateral and ventral funiculi are partially spared and act as bridges for regenerating axons. Examples of reconstructed lesions are shown in figure 5. (C) Lesion extent in the 6 experimental groups. (D) Mean antibody concentration (µg/ml) in the CSF collected from the cisterna magna after 14 days of intrathecal infusion, started 1 week after spinal cord injury. (E) Antibody tissue penetration into brain and thoracic spinal cord of injured rats after 14 days of continuous infusion started 2 weeks after injury. The anti-Nogo-A antibodies penetrated well into the CNS tissue and were retained at high and intermediate levels in the spinal cord and brain parenchyma, respectively. Control antibody levels were low in the spinal cord and almost undetectable in the brain suggesting that they were washed out quickly as they did not bind to the CNS tissue. Bars indicate mean ± SEM. Antibody density in (E) is color-coded: red indicates high antibody (ab) density, dark blue indicates low antibody density.

Figure 2: Locomotor performance in the swim test and the Narrow beam test before (baseline) and after injury. Upper row: Narrow beam test. Rats crossed a tapered narrow beam from the broad to the narrow end. Antibody treatment time is indicated with black
horizontal bars. Middle row: Swim velocity in m/s. Three runs over 60 cm were averaged. Bottom row: Score for hindlimb function during swimming. Intact rats swim with their hindlimbs underneath the body. After the incomplete thoracic spinal cord injury the distance between the hindlimbs is large and the animals use their forelimbs to compensate for the weak or failing hindlimbs. Means ± SEM are shown; black dots and crosses represent single animal values of anti-Nogo-A antibody and control IgG treated groups respectively. Significance levels of the treatment effect (anti-Nogo-A antibody versus control IgG) as determined by repeated measures ANOVA are indicated with large red stars above the bar graphs. Treatment effect at single time points are indicated with small stars. *p ≤ 0.05, ** p ≤ 0.01.

**Figure 3: Open field locomotion** Upper row: BBB subscore measuring toe clearance, hindlimb rotation and tail usage independent of coordination. Lower row: BBB score. Bars indicate means ± SEM; black dots and crosses represent the single animal values of anti-Nogo-A antibody and control antibody treated animals, respectively. Significance levels of the treatment effect (anti-Nogo-A antibody versus control) are indicated with stars above the bar graphs and were calculated with repeated measures ANOVA. *p ≤ 0.05. ab = antibody.

**Figure 4: CST axons caudal to the lesion after acute and 1 or 2 weeks delayed anti-Nogo-A or control antibody treatment.** (A) Photographs of BDA-traced CST fibres in the lower thoracic spinal cord taken at increasing distances caudal to the lesion site. Regenerating fibres are typically fine, follow an irregular course and often arbourize extensively in the grey matter. (B) Quantification of CST axons 0.5 mm, 2.0 mm, and 5.0 mm caudal to the lesion site. The average numbers of BDA-labelled axons caudal to the lesion site were divided by the total number of labelled CST fibres for each animal in the dorsal corticospinal tract and reported as % regenerating fibres. Black dots and crosses represent the single animal values of
anti-Nogo-A antibody and control antibody treated animals, respectively. Bars indicate means ± SEM; *p<0.05, **p<0.01; Man Whitney U tests.

Figure 5: Camera lucida reconstructions of paramedian sagittal sections through the spinal cord lesion site of rats that received acute, 1 or 2 weeks delayed anti-Nogo-A antibody treatment, or acute control IgG treatment. Three adjacent sections were superimposed. Left: reconstructed coronal spinal cord sections showing the lesion extent (grey) of the respective animals. Substantial numbers of BDA labelled CST fibres are found caudal to the injury site after acute and 1 week delayed anti-Nogo-A infusion. After 2 weeks delayed infusion, some fibres were seen close to the injury site, but not at more caudal levels.

Figure 6: Photomicrographs of CS-56 immunoreactivity on paramedian sagittal spinal cord sections at different time points after injury. (A) In intact animals the CSPG levels were low. (B) 3 days after injury the CSPG levels were slightly increased at the lesion site. Bridges of intact tissue with low levels of CS-56 are indicated by black arrows. (C, D) 1 and 2 weeks after injury, CS-56 levels were substantially increased and spread from the lesion edge into the surrounding tissue including the ventral tissue bridges. Inset in (B): the dotted line on the coronal spinal cord section shows the position of the paramedian sagittal sections in B - D.
Figure 1

A

Handling + Training  |  Behavioural analysis

Control ab | Anti-Nogo-A ab
2 weeks delayed treatment groups

Control ab | Anti-Nogo-A ab
1 week delayed treatment groups

Control ab | Anti-Nogo-A ab
acute treatment groups

Spinal cord lesion
CSF collection
Tracing Perfusion

B

C

D

E

Control ab | Anti-Nogo-A ab

Original

Colour-coded

264x385mm (300 x 300 DPI)
Delayed anti-Nogo-A antibody application after spinal cord injury shows progressive loss of responsiveness (doi: 10.1089/neu.2011.1752)

This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.
Figure 3

The figure shows the effects of different treatment delays on the blood-brain barrier (BBB) score. Three treatment groups are compared: acute treatment, 1 week delayed treatment, and 2 weeks delayed treatment. The graphs display the BBB subscore and BBB score over time (baseline, 1 week, 5 weeks, 10 weeks).

Key points:
- Acute treatment shows a significant reduction in BBB subscore compared to control, with maintenance of effects over the tested time points.
- 1 week delayed treatment also shows a reduction in BBB subscore, but the effect is less pronounced compared to acute treatment.
- 2 weeks delayed treatment shows no significant change in BBB subscore, indicating a progressive loss of responsiveness.

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### Table

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<th>0.5 mm</th>
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<tr>
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<tr>
<td>1 week delayed</td>
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#### Figure 4

A. Spinal cord level caudal to injury site

B. % fibers regenerating

- 0% 10% 20% 30% 40%
- acute control
- acute Anti-Nogo
- 2 weeks delayed
- 1 week delayed
- acute treatment
Figure 5

<table>
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<tr>
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<tr>
<td></td>
<td>acute application</td>
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