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Delayed Anti-Nogo-A Antibody Application after Spinal Cord Injury Shows Progressive Loss of Responsiveness

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Abstract

Blocking the function of the myelin protein Nogo-A or its signaling pathway is a promising method to overcome an important neurite growth inhibitory factor of the adult central nervous system (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 begun. 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 fibers regenerated over several millimeters 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-week-delayed 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.

Key words: delayed treatment; motor function; Nogo-A; plasticity; recovery; spinal cord injury; sprouting

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 sulfate proteoglycans (CSPGs) injected into the injured tissue led to enhanced fiber 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 lesions 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 and Strittmatter, 2006; Cafferty et al., 2007,2010; 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-knockout 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 intraspinal plasticity), but no long-distance regeneration (Lee et al., 2010). These mixed results could be

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explained by compensatory upregulation of other Nogo splice variants (Simonen et al., 2003), as well as other repulsive molecules (Montani and Schwab, unpublished observations), produced by the different genetic backgrounds of the mouse lines used by the different groups (Dimou et al., 2006), and by the different lesion paradigms used (Cafferty and Strittmatter, 2006). Constitutive, lifelong genetic knockouts are known to often produce milder (or even no) phenotypes due to compensatory mechanisms that 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 and 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 spinal cord injury (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 that 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 (CST) fibers.

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 8–10 weeks) were kept in groups of 4–5 animals in standard cages on a 12-h 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) SCI.

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 1 or 2 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 assigned to either the IgG- or the anti-Nogo-A-treated groups). As different behavioral scores do not necessarily correlate, the readouts from the Basso-Beattie-Bresnahan (BBB) subscore and swim test were also used in cases for which 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 Figure 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 3 mg/mL. The anti-Nogo-A antibody 11C7 is monospecific for Nogo-A on Western blots, and does not cross-react 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 downregulation 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 mini-pumps (5 μL/h, ALZET® 2ML2; DURECT Corp., Cupertino, CA) connected to a subdurally-implanted catheter as previously described (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 (120 μL/200 g body weight; Janssen Pharmaceutica, Beerse, Belgium) and Dormicum (0.75 mg per 200 g body weight; Roche Pharmaceuticals, Basel, Switzerland). 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- to 10-week-old rats essentially as described previously (Liebscher et al., 2005), but with more extensive lesioning 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 behavioral assessment

The locomotor impairments varied substantially between rats, in spite of standardized surgical procedures. All animals

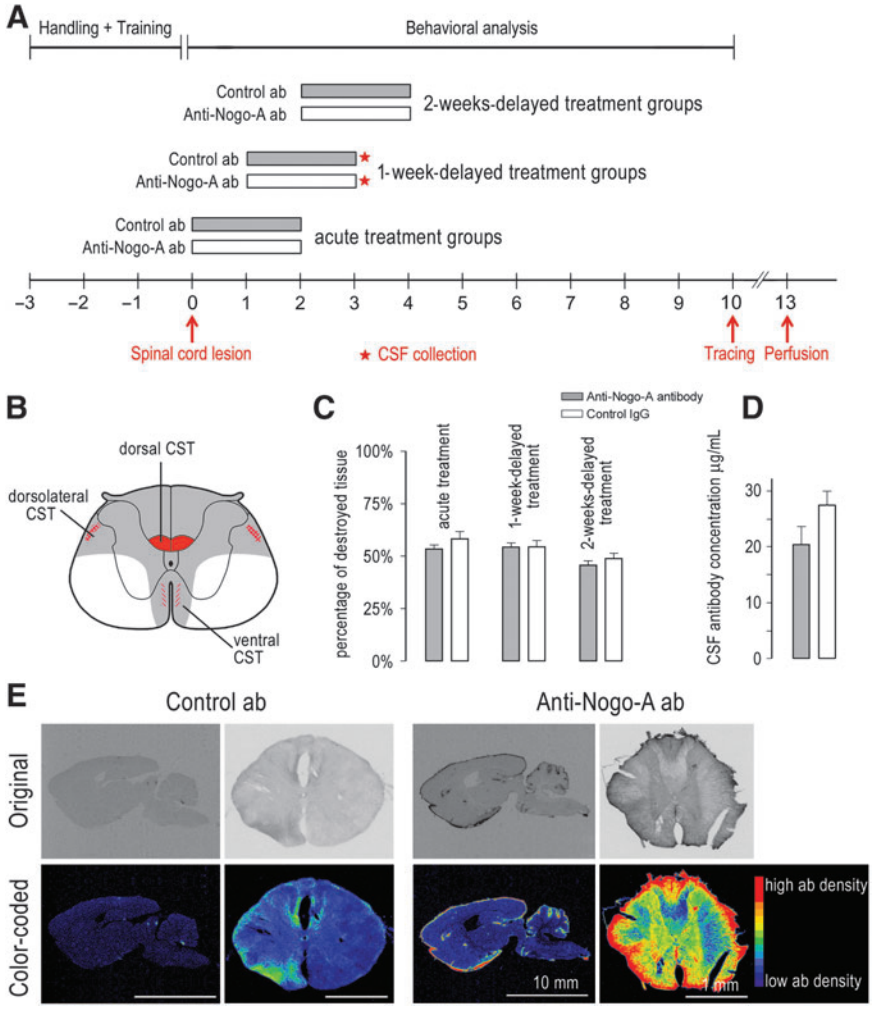

FIG. 1. Study design, lesion size, antibody tissue penetration, and cerebrospinal fluid (CSF) antibody concentrations. (**A**) Scheme summarizing the different treatment groups and the sequence of experimental steps. After a large, incomplete spinal cord injury at T8, adult rats were treated acutely 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 behavioral tests over a time period of 10 weeks after injury. Ten weeks after injury, the corticospinal tract (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 six 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 central nervous system (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 ± standard error of the mean. Antibody density in **E** is color coded: red indicates high antibody (ab) density, and dark blue indicates low antibody density.

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 on 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 with a computer (swim test). The number of animals was as follows: 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 their buoyancy, rats are able to swim even after a severe SCI. The basic swimming pattern with alternating hindlimb strokes is usually not affected except for short periods during which the rats swim in a ventroflexed position

with often coupled hindlimb strokes (Gonzenbach et al., 2010). This allows scoring the deviation from normal hindlimb usage and assessment of its recovery over time as described by Liebscher and associates (Liebscher et al., 2005). The rats were videotaped while swimming in an acrylic glass basin (150 × 40 × 13 cm, water temperature 28–30°C). Swimming velocity was calculated by measuring the time required for swimming a distance of 60 cm. Hindlimb usage was scored as described by Liebscher and colleagues (Liebscher et al., 2005): 4 = normal hindlimb usage; 3 = hindlimb strokes deviate laterally but the hindlimbs are underneath the body; 2 = hind paws are lateral to the body and the distance between the hindlimbs is increased; 1 = large distance between the hindlimbs (i.e., the hind paws and legs are entirely lateral to the body).

Narrow beam test. To assess deficits in balance and fine motor control, the 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 gets 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 hindlimb 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 min 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 hindlimbs is reported for each animal.

Anterograde corticospinal tract tracing

Ten weeks after SCI, the corticospinal tract was anterogradely traced as previously described (Liebscher et al., 2005). Briefly, a total volume of 2.0 μL of 10% BDA (MW 10000; Molecular Probes, Eugene, OR) dissolved in 0.01 M PBS was injected at four sites of the hindlimb area of the sensorimotor cortex using a Hamilton syringe. Care was taken not to inject BDA into the lateral ventricles to avoid artefactual labeling 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 previously 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-labeled fibers using the semi-free-floating technique (Herzog and Brosamle, 1997).

Quantification of corticospinal tract regeneration

The number of BDA-labeled CST 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× magnification for each spinal cord. If axons were arborized, each segment was counted separately. A segment was defined as a continuous BDA-positive fiber that is not interrupted by branches. The vast majority of traced fibers were thin and followed an irregular course. Rarely, a small number of spared CST fibers 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-labeled axons was quantified on two adjacent 50-μm cross-sections in the upper thoracic spinal cord several segments rostral to the injury site using a 63× objective. The number of CST fibers counted caudal to the injury was then divided by the number of BDA-labeled fibers above the lesion to calculate the fraction of regenerated fibers. This calculated fraction of regenerated fibers was reported as a percentage of regenerated fibers.

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 labeled 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 upregulation 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, two 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-Aldrich, St. Louis, MO), followed by a biotin-coupled donkey anti-mouse secondary antibody (1:200; Jackson ImmuoResearch, West Grove, PA). The biotin-coupled secondary antibody was detected using Cy3-conjugated streptavidin (1:300; Jackson ImmuoResearch).

To determine the tissue penetration of the intrathecally-infused antibodies, six rats treated with a delay of 2 weeks were euthanized 1 h 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). The biotin-coupled secondary antibody was detected using the ABC-DAB system (Vector Laboratories, Burlingame, CA). 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 a 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 fibers was evaluated with the Mann-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 and C). In spite of standardized surgeries, the lesion size varied between individual animals due to secondary effects like ischemic necrosis and bleeding. However, control antibody- and anti-Nogo-A-antibody treated groups were not different in their lesion sizes. The 2-weeks-delayed groups, both anti-Nogo-A- and control antibody-treated, had slightly smaller lesions ($p < 0.05$ by two-tailed *t*-test for independent samples) than the acute and 1-week-delayed groups, which might account for the slightly better performance on 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-delayed groups ranged between 3 and 40 μg/mL, and were similar in the anti-Nogo-A- and the control antibody-treated groups (two-tailed *t*-test, anti-Nogo-A antibody 11c7: $n = 11$, control IgG: $n = 12$; Fig. 1D).

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 cross-sections for mouse IgG in the 2-week-delayed 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 immunoreactivity 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 hindlimbs, the relatively difficult narrow beam test showing balance and precision of foot placement, and the BBB score for open field locomotion were used to assess the effects of anti-Nogo-A antibody administration at 0-, 1-, and 2-week delays after SCI.

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

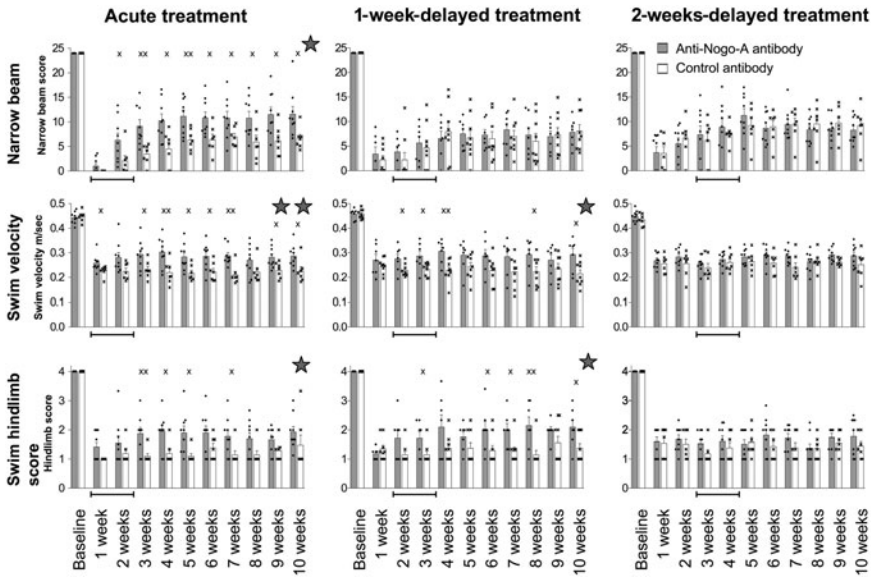

FIG. 2. Locomotor performance on the swim test and the narrow beam test before (baseline) and after injury. Upper row: Narrow beam test. The rats crossed a tapered narrow beam from the broad to the narrow end. Antibody treatment time is indicated by black horizontal bars. Middle row: Swim velocity in m/sec. 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 was large and the animals used their forelimbs to compensate for the weak or failing hindlimbs. Means ± standard error of the mean 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 analysis of variance are indicated with large stars above the bar graphs. Treatment effects at single time points are indicated with small crosses (*$p \leq 0.05$, **$p \leq 0.01$).

beam requires the capability to maintain balance. Six days after injury, the narrow beam scores were very low in all groups. Rats acutely-treated with anti-Nogo-A antibody scored significantly higher than the control antibody group on the narrow beam test from 2 weeks on. The delayed treatment, however, did not improve 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 the subcutaneously implanted mini-pump in combination with the recent SCI. Early after injury, when balance and fine motor control have not yet recovered, 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 a 1-week delay progressively improved their swimming velocity and their hindlimb 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 hindlimb 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 hindlimb 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 their respective control groups. In contrast, the 2-weeks-delayed anti-Nogo-A antibody-treated group was no different from its IgG-control group.

Open field locomotion. The BBB score dropped from 21 to 7–10, and 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, regardless of treatment or treatment onset; most 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 forelimb–hindlimb coordination, which depends largely on propriospinal connections running in the spared ventrolateral 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-week-delayed anti-Nogo-A antibody treatment group, the BBB subscore was comparable to that of the control antibody group.

Corticospinal tract regeneration

The regeneration of the cut CST fibers 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

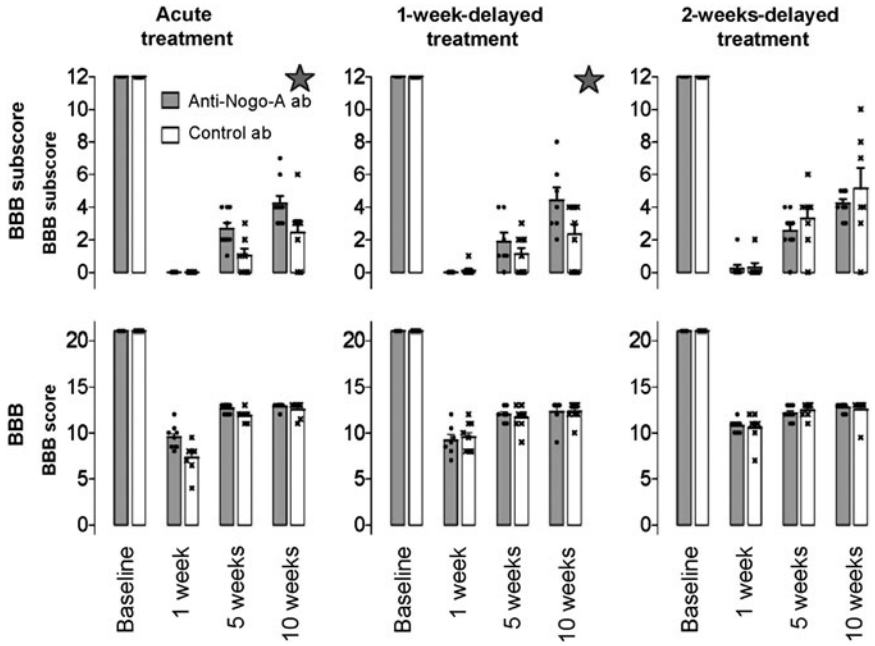

FIG. 3. Open field locomotion. Upper row: Basso-Beattie-Bresnahan (BBB) subscore measuring toe clearance, hindlimb rotation, and tail usage, independent of coordination. Lower row: BBB score. Bars indicate means ± standard error of the mean; black dots and crosses represent the single animal values of 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 analysis of variance (*$p \leq 0.05$; ab = antibody).

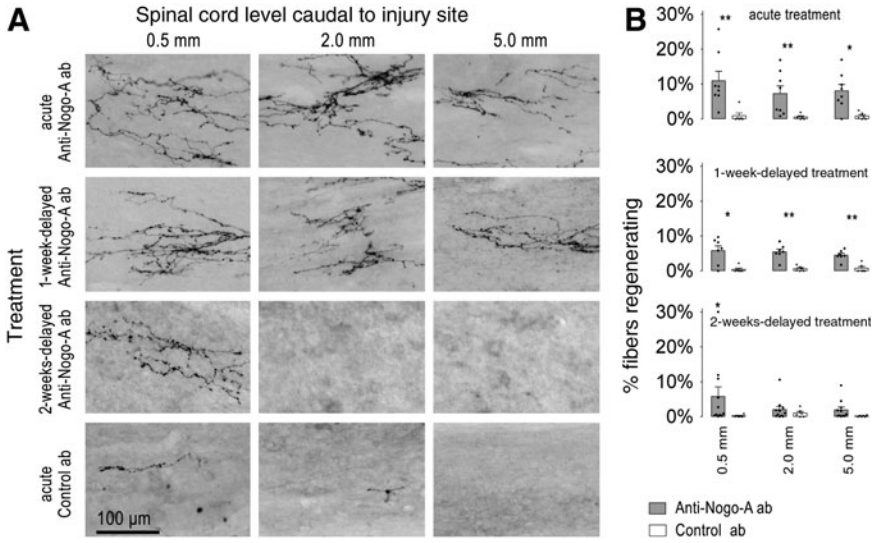

FIG. 4. Corticospinal tract (CST) axons caudal to the lesion after acute and 1- or 2-weeks-delayed anti-Nogo-A or control antibody treatment. (**A**) Photographs of BDA-traced CST fibers in the lower thoracic spinal cord taken at increasing distances caudal to the lesion site. Regenerating fibers are typically fine, follow an irregular course, and often arborize 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-labeled axons caudal to the lesion site were divided by the total number of labeled CST fibers for each animal in the dorsal CST and reported as percentage of regenerating fibers. Black dots and crosses represent the single animal values of anti-Nogo-A antibody- and control antibody-treated animals, respectively. Bars indicate means ± standard error of the mean (*$p < 0.05$, **$p < 0.01$ by Mann-Whitney U tests).

around the lesion site and did not enter the lesion site, as described previously (Liebscher et al., 2005). Labeled CST fibers observed below the lesion site were consistently of thin caliber and followed a tortuous course, mostly within the grey matter (Figs. 4A and 5). Most fibers arborized extensively within the grey matter forming numerous collaterals. In control antibody-treated rats, regardless of the antibody infusion onset, very low numbers of labeled CST fibers were observed below the injury site, as indicated by a low percentage of 0.2–1% regenerating fibers (Fig. 4A, bottom row; Fig. 4B; Fig. 5, top row). In contrast, anti-Nogo-A antibody-treated rats showed significantly higher numbers of labeled CST fibers at all analyzed levels below the injury site, if the treatment was started acutely or with a delay of 1 week after injury (Figs. 4 and 5). The percentage of regenerated fibers was highest after acute treatment, reaching means of 7.2–10.9% ($p < 0.01$ at 0.5 mm and 2 mm, $p < 0.05$ at 5 mm). In the 1-week-delayed 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 fibers 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 caudal to the injury (Fig. 4B).

Chondroitin sulfate proteoglycan immunoreactivity at the lesion site

To look for molecular mechanisms behind 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 injury. Staining with the CS-56 antibody, which recognizes chondroitin-4- and chondroitin-6-sulfate 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 neighboring spared tissue (Fig. 6B). In contrast, at 1 and 2 weeks after injury, the immunohistochemical signals for CS-56 had augmented substantially and had spread to the neighboring spared tissue (Fig. 6C and D). This time course of CSPS 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 fibers (e.g., of the CST), and enhanced recovery of locomotor function. Delaying the start of intrathecal anti-Nogo-A antibody infusion after the 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 1-week-delayed anti-Nogo-A antibody treatment, 2-weeks-delayed treatment led to a minimal increase in fiber 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.

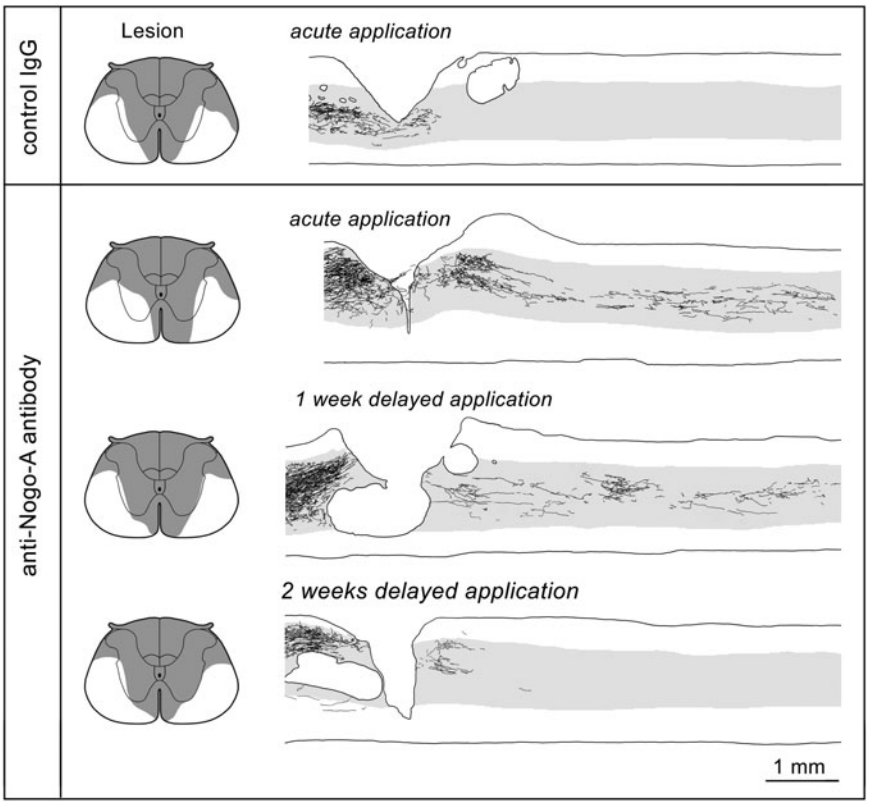

FIG. 5. Camera lucida reconstructions of paramedian sagittal sections through the spinal cord lesion site of rats that received acute or 1- or 2-weeks-delayed anti-Nogo-A antibody treatment, or acute control IgG treatment. Three adjacent sections were superimposed. Left: Reconstructed coronal spinal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

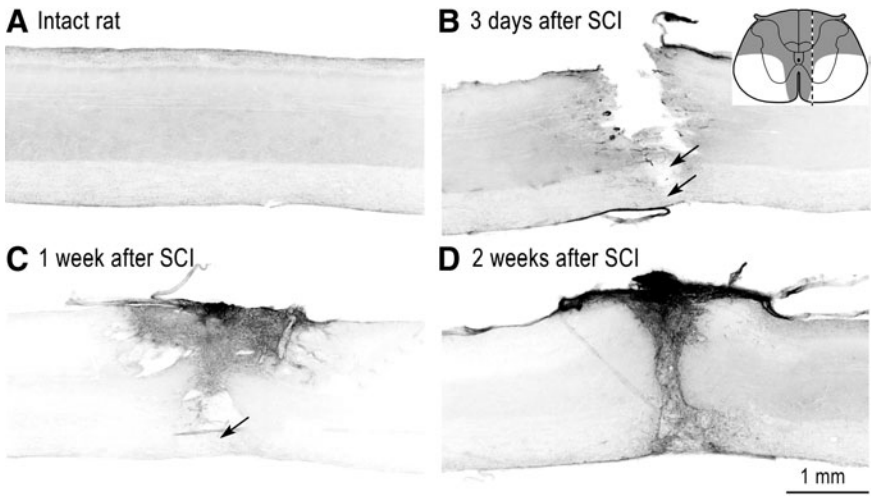

FIG. 6. Photomicrographs of CS-56 immunoreactivity of paramedian sagittal spinal cord sections at different time points after injury. (**A**) In intact animals the chondroitin sulfate proteoglycan (CSPG) levels were low. (**B**) At 3 days after injury the CSPG levels were slightly increased at the lesion site. Bridges of intact tissue with low levels of CS-56 are indicated by black arrows. (**C** and **D**) At 1 and 2 weeks after injury, CS-56 levels were substantially increased and had 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** (SCI, spinal cord injury).

In contrast to the findings of Liebscher and associates we did 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 propiospinal connections that run in the lateral tracts. Our lesions were larger, (i.e., 40–65% in the acute treatment groups, compared to the 40–50% used by Liebscher and colleagues [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, for which 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 fewer supraspinal commands, and depends to a larger extent on the spinal central pattern generator. The lower percentage of regenerating CST fibers 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 fibers may still be sufficient to control swimming, but is not adequate for the more demanding beam crossing task.

In the 2-weeks-delayed groups the lesion size tended to be slightly smaller than in the other groups, which explains the slightly better motor performance of these groups. One could argue that this slightly smaller lesion size in the 2-weeks-delayed 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 and colleagues (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-delayed 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 with 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-labeled CST fibers with the irregular morphology typical of regenerating fibers 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 fibers arborized extensively in the grey matter and formed numerous varicosities, suggesting that they formed synaptic connections and integrated into the spinal circuitry. The 2-weeks-delayed treatment led to a minimal increase in the number of labeled CST fibers in the area immediately caudal to the injury site, and no increase further below, indicating that the regeneration-enhancing effect on CST fibers was substantially decreased.

Several factors may explain why 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 fibers 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 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.

The 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 and 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 fibers of interrupted tracts and compensatory growth of unlesioned axons have to form meaningful connections with 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 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, nonfunctional spinal cord with increasing time after injury. It is noteworthy, however, that enhancement of compensatory fiber growth with formation of functionally meaningful connections by anti-Nogo-A antibodies can occur after

stroke, even if the antibody application is delayed by 1 or 4 weeks (Markus et al., 2005; Seymour et al., 2005; Tsai et al., 2007,2010). In contrast to the recovery from spinal cord lesions, stroke recovery may depend less on axon tract regeneration and on fibers 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 with delayed treatment might be due to increased plasticity of intraspinal neuronal circuits, and not due to long-distance regeneration of transected axons.

An increase of intraspinal plasticity (e.g., increased numbers of midline-crossing fibers 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 usefulness of 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 the man and the 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; Hiersemenzel 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 greater if it is started early after injury. The 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 that 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 paraplegic and tetraplegic patients.

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Author Disclosure Statement

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