Nogo-A Antibody Improves Regeneration and Locomotion of Spinal Cord–Injured Rats

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Spinal cord trauma leads to loss of motor, sensory and autonomic functions below the lesion. Recovery is very restricted, due in part to neurite growth inhibitory myelin proteins, in particular Nogo-A. Two neutralizing antibodies against Nogo-A were used to study recovery and axonal regeneration after spinal cord lesions. Three months old Lewis rats were tested in sensory-motor tasks (open field locomotion, crossing of ladder rungs and narrow beams, the CatWalk® runway, reactions to heat and von Frey hairs). A T-shaped lesion was made at T8, and an intrathecal catheter delivered highly purified anti-Nogo-A monoclonal IgGs or unspecific IgGs for 2 weeks. A better outcome in motor behavior was obtained as early as two weeks after lesion in the animals receiving the Nogo-A antibodies. Withdrawal responses to heat and mechanical stimuli were not different between the groups. Histology showed enhanced regeneration of corticospinal axons in the anti-Nogo-A antibody groups. fMRI revealed significant cortical responses to stimulation of the hindpaw exclusively in anti-Nogo-A animals. These results demonstrate that neutralization of the neurite growth inhibitor Nogo-A by intrathecal antibodies leads to enhanced regeneration and reorganization of the injured CNS, resulting in improved recovery of compromised functions in the absence of dysfunctions.

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The growth inhibitory properties of central nervous system (CNS) myelin and, in particular, of the membrane protein Nogo-A play an important role in the restricted capability of adult mammalian CNS axons to sprout and regenerate after large CNS injuries.[1,2] As a consequence, only very limited functional recovery is achieved after traumata-like stroke or lesions of the spinal cord. Nogo-A is a high-molecular-weight membrane protein that is enriched in myelin of the adult CNS.[3] It induces neurite growth arrest by at least two inhibitory domains: one in the C-terminal domain ("Nogo-66"[4]) and one in the Nogo-A–specific domain.[5–7] Although the binding site for Nogo-66, NgR, has been characterized and shown to bind also other myelin proteins such as MAG or OMgp,[8–10] the high-affinity binding site for the Nogo-A–specific domain[6] remains to be molecularly identified.

Soon after the growth inhibitory properties of CNS myelin were discovered,[11,12] a monoclonal antibody (IN-1) was produced against a high-molecular-weight inhibitory membrane constituent (NI-250) that was later renamed Nogo-A.[5,13,14] The monoclonal antibody IN-1 had a strong neutralizing activity for neurite growth inhibition exerted by Nogo-A and CNS myelin.[5,13,15] In vivo application of the antibody IN-1, an IgM that had to be applied to the experimental animals mostly through grafting of antibody-producing hybridoma cells, resulted in long-distance regeneration of lesioned fibers in the spinal cord of adult rats; it also enhanced compensatory fiber growth from unlesioned tract systems.[2,16–18] These anatomical findings were paralleled by improved functional recovery after spinal cord injury, brainstem lesions, or stroke.[17,19–22]

In this study, we analyzed a much more clinical setting by studying the effects of two new monospecific monoclonal antibodies directed against defined regions of Nogo-A, the antibodies 11C7 and 7B12,[6] on adult spinal cord injured rats anatomically for corticospinal

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tract (CST) regeneration and behaviorally for improvement of sensory-motor functions. Both antibodies neutralize the inhibitory activity of Nogo-A and CNS myelin for neurite outgrowth (O. Weinmann et al., submitted). They were acutely applied for 2 weeks as highly purified IgGs into the subdural space over the spinal cord by fine catheters connected to osmotic minipumps, a procedure that can be applied to higher mammals and also humans. Our results demonstrate long-distance regeneration of corticospinal tract axons and enhanced recovery of locomotion, swimming, beam and ladder-rung crossing, in the absence of hyperalgesia after 9 weeks of survival.

In addition, functional magnetic resonance image (fMRI) was used to evaluate potential treatment-induced improvements in the sensory system.

Materials and Methods

Animals

All experiments were performed according to the guidelines of the Veterinary Office of the Canton of Zürich, Switzerland. A total of 69 adult female Lewis rats (160–190gm) were obtained from a Specific Pathogen Free (SPF) breeding colony (R. Janvier, Le Genest-St-Isle, France) and kept as groups of four to six animals in standardized cages (type 4, Macrolon) on a 12-hour-light 12-hour-dark cycle on a standard regimen with food and water ad libitum.

Antibodies

The mouse monoclonal antibody 11C7 was raised against an 18–amino acid Nogo-A peptide corresponding to the rat sequence amino acids 623 to 640,[5,6] whereas the monoclonal antibody 7B12 was produced against the recombinant prokaryotically produced Nogo-A fragment amino acids 1 to 979.[6] Both antibodies are monospecific for Nogo-A on Western blots; 7B12 recognizes the C-terminal part of the Nogo-A–specific region.[6] IgGs were purified on protein A–Sepharose columns and concentrated to 3mg/ml in phosphate-buffered saline. A monoclonal mouse IgG directed against wheat auxin was purified in the same way and used as control antibody. The use of antibodies against other myelin or CNS binding proteins (eg, myelin basic protein) as control proteins was avoided because of known autoimmune reactions.

Study Design

Animals were operated on in three mixed (anti–Nogo and control antibody) batches and underwent an identical surgical and behavioral procedure except for the fMRI study where only the third batch of animals was screened. The experiment was performed in a fully double-blind manner: the rats were coded with random numbers and the groups were mixed in the cages. All experimenters were blind to the treatments throughout all phases of the experiment, which included operation, health care, behavioral and fMRI analysis, and evaluation of regeneration, sprouting, and lesion size.

Before surgery, all animals were handled and trained for the behavioral tests for 4 weeks before baseline measurements were taken. For pump implantation, rats were randomly divided into the experimental groups: lesion + antibody 7B12, lesion + antibody 11C7, lesion + control antibody. Antibody treatment started immediately after the lesion by rinsing the wound with 1μl of the corresponding coded antibody, followed by the coded pump implantation. After 2 weeks, the pumps and catheters were removed and cerebrospinal fluid (CSF) was obtained to measure the antibody concentration. Behavioral assessments were taken at weekly intervals. After 5 weeks, the CST was unilaterally traced. Nine weeks after surgery, at the end of the behavioral protocol, an fMRI study was performed under anesthesia and the rats were subsequently perfused (Fig 1A).

Surgical Procedures

Animals were anesthetized with a subcutaneous injection of Hypnorm (120μl/200gm body weight; Janssen Pharmaceutics, Beerse, Belgium) and Dormicum (0.75mg in 150μl per 200gm body weight; Roche Pharmaceuticals, Basel, Switzerland). Vitamin A–containing eye ointment was applied to protect the eyes from dehydration during the relatively long procedure.

A T-shaped lesion that included the dorsal half of the spinal cord with the main CST as well as the dorsolateral and ventromedial parts of the CST was made at thoracic level T8 with iridectomy scissors and a sharp, pointed blade (Fig 3C).

A fine intrathecal catheter (32 gauge; Recathco, Allison Park, PA) was inserted from the lumbar level L2/L3 and pushed up to T10, delivering the antibodies from an osmotic minipump (5μl/h, 3.1μg IgG/μl, Alzet 2ML2; Charles River Laboratories, Les Oncins, France) into the CSF for 2 weeks. Analgesics (Rimadyl; Pfizer AG, Zürich, Switzerland) were given perioperatively by subcutaneous injection. The antibiotic Baytril (5mg/kg body weight, SC; Bayer AG, Leverkusen, Germany) was administered once a day for 7 days, starting at 1 day before operation, to prevent bladder infections.

Cerebrospinal Fluid Collection and Antibody Measurements

CSF probes were obtained from cisterna magna punctures and the concentration of the different antibodies was measured with a sandwich enzyme-linked immunosorbent assay.

Tracing

After 36 days, animals were anesthetized with Isoflurane anesthesia. The CST was unilaterally traced with 10% biotin dextran amine (BDA, MW 10,000; Molecular Probes, Eugene, OR) in 0.01M phosphate-buffered saline using a Hamilton syringe to inject a total volume of 2.0μl at 4 sites of the sensory-motor cortex.

Animal Care

BODY WEIGHT. Changes in body weight were monitored daily. If the animals lost more then 5% of body weight during 1 day, a subcutaneous injection of Glucose 5%-NaCl 0.9% 2:1 Solution (Fresenius Kabi AG, Stans, Switzerland) was given.

BLADDER. During the first 3 weeks, bladder function was controlled by the same person at three defined times per day.

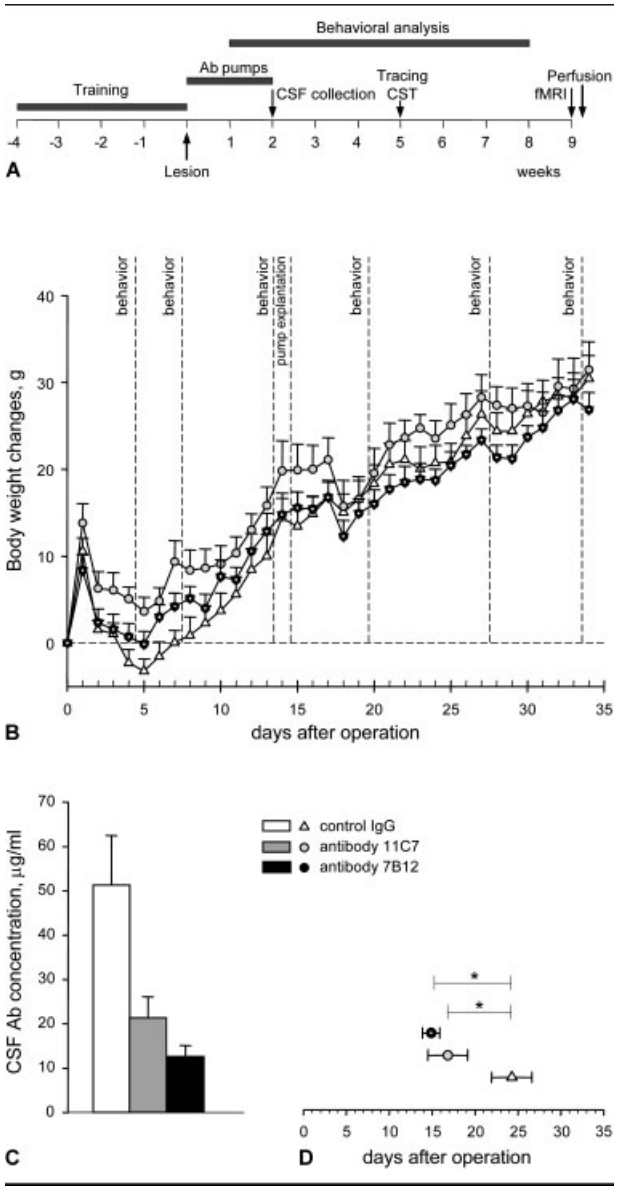

*Fig 1. Study design, body weight changes, antibody concentrations in cerebrospinal fluid (CSF) and recovery of bladder function. (A) Study design for the 13 weeks duration time of the experiment. (B) Mean values of individual treatment groups showing daily weight changes over a time period of 7 weeks. Stress-inducing interventions (behavioral tests, removal of pumps and catheters) are indicated with vertical lines. The initial gain in weight reflects the Ringer solution injections after the main surgery. (C) Mean values of antibody concentrations (µg/ml) measured in the CSF after 2 weeks of continuous delivery by Alzet pumps. (D) Time point of recovery of autonomous bladder voiding. Anti–Nogo antibody–treated rats regained autonomous bladder function 7 to 9 days earlier than control antibody–treated rats. *$p < 0.01$. CST = corticospinal tract; fMRI = functional magnetic resonance imaging.*

Full bladders were emptied. Bladder infections were rare. Bladder function scoring was as follows: dysfunction was defined as full bladder, medium to high pressure required at manual voiding of the bladder; normal function was empty to half full bladder, voiding after slight touch.

Behavioral Analysis

All tests were monitored by a digital video camera and analyzed in a double-blind manner. Before the surgery, after 4 weeks of pretraining, baseline measurements were taken. After the operation, behavioral assessments were taken at weekly intervals.

BBB LOCOMOTOR SCORE. Rats were allowed to move freely and were scored during 4 minutes by two observers for their ability to use the hindlimbs. Joint movements, paw placement, weight support, and fore/hindlimb coordination were judged according to the 21-point BBB locomotion scale.[23]

SWIM TEST. The setup for the Swim Test consisted of a rectangular Plexiglas basin (150 × 40 × 13cm). The level of the water (23–25°C) was high enough to prevent the rats from touching the bottom of the basin. Intact animals swim by paddling with their hindlimbs and the tail, holding their forelimbs immobile under the chin.[24] A total of five runs per rat were monitored using a mirror at 45 degrees at the bottom of the pool to film the rats from the side and the bottom simultaneously. The swimming performance was analyzed by scoring their movements according to the following criteria: forelimb usage: 2 points = no use (normal), 1 point = 1 arm for the whole distance or both for half the distance, 0 points = both arms used all the time; hindpaw distance (base of support): 2 points = small distance, hindlegs are underneath the body, 1 point = legs are outside the body, but feet still remain underneath, 0 points = large distance, legs and feet are outside the body; hindlimb stroke: 2 points = powerful strokes, 1 point = moderate strokes, 0 points = weak or no strokes; tail movement: 2 points = regular strong movements of the whole tail; 1 point = partial movements; 0 points = no or only very weak movements. Normal swimming thus resulted in seven to eight score points, a value that was routinely reached by well-trained rats.

LADDER-RUNG WALKING TEST. The animals had to cross a 1-meter horizontal ladder elevated to 1 meter from the ground. A defined stretch of 60cm was chosen for filming and analysis. To prevent habituation to a fixed bar distance, we placed the bars irregularly (1–4cm spacing). The animals crossed the ladder twice in the same and once in the opposite direction. The number of foot slips or total misses was counted and divided by the total number of steps in each crossing.

NARROW BEAM CROSSING. Three different 1-meter wooden beams (two with rectangular cross-sections [2 × 2cm; 1.2 × 1.2 cm] and one round beam of 2.5cm diameter) elevated 50cm over the ground had to be crossed three times each. Crossing the beam by placing both hindlimbs correctly was scored as 2 points; 1.5 points were assigned when an animal placed both paws only sometimes in cor-

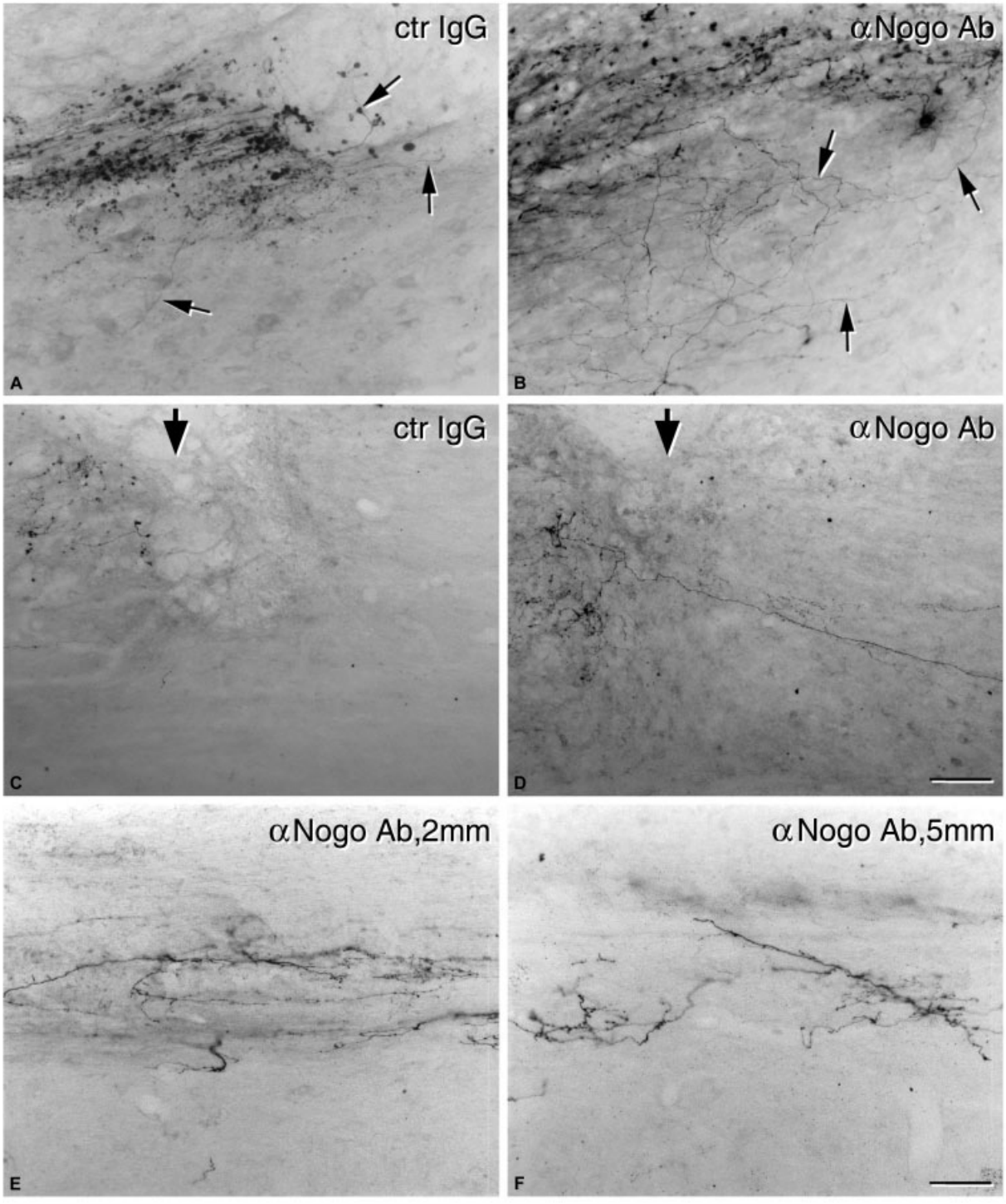

*Fig 2. Regenerative sprouting and long-distance regeneration of the transected corticospinal tract (CST). (A, B) Transected CST rostral to the lesion sprouts less in a control IgG-treated rat (*arrows, *A) than in an anti–Nogo-A antibody–treated animal (B). (C, D) CST fibers with irregular course cross the lesion site* (thick arrow) *through a ventral gray matter bridge in an 11C7 anti–Nogo antibody rat (D, see also reconstruction in Fig 4) but are absent in a control IgG animal (C). (E, F) CST fibers arborize in the caudal spinal cord at 2mm (E) and 5mm (F) from the lesion in an anti–Nogo antibody–treated rat. Calibration bar = 48μm (A, B, E, F) and 96μm (C, D)*

rectly plantar position or touched the beam on the side. One point was given if the rat was able to cross the whole beam and both hindpaws contacted the beam actively; 0.5 points were given if the rat could only traverse half of the beam. The scores of all three beams were added to a maximum score of 18 points.

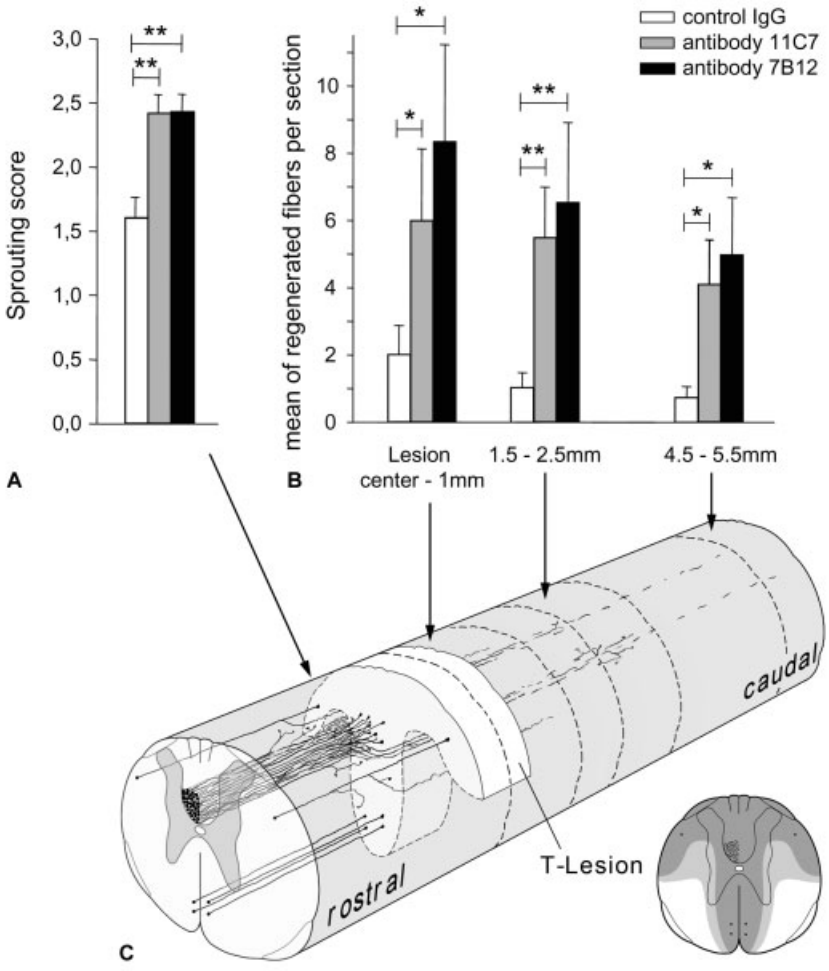

*Fig 3. Quantification of sprouting and regeneration of the lesioned corticospinal tract (CST) in antibody-infused spinal cords. (A) Sprouting of the CST rostral to the lesion is higher in anti–-Nogo-A antibody–treated groups than in the control IgG group. **p < 0.001. (B) Mean number of CST fibers per section, counted at three levels caudal to the lesion: from the center to 1mm, from 1.5 to 2.5mm, and from 4.5 to 5.5mm. Anti–Nogo antibody–treated rats show higher fiber numbers than controls. *p < 0.01, **p < 0.001. N = 12–19 animals per group. (C) Scheme of the spinal cord with T-lesion (cross-section: dark gray indicates extent of primary lesion; light gray shows secondary tissue loss), rostral sprouting zone, CST (main, dorsolateral, and ventromedial parts), and regenerating fibers growing on lateral and ventral tissue bridges. Areas of fiber counts are indicated.*

CATWALK® RUNWAY. The CatWalk® was developed as a semiautomated evaluation system to study differences in gait after injury.[25] The walkway consists of a dark Plexiglas runway with a glass floor where the animal's paws, tail, or abdomen induces light reflections at the contact points. The more pressure is exerted, the larger the total contact area and the brighter the pixels. We analyzed the weight distribution on forelimbs and hindlimbs.

WITHDRAWAL REFLEX: PLANTAR HEATER The level of nociception for both hindpaws was evaluated by performing a standardized Plantar Heater Test (Ugo Basile; Biological Research Apparatus, Comerio, Italy) with an infrared source producing a calibrated heating beam (diameter 1mm). After one initial trial, the reflex time for the hindlimb withdrawal was determined in four successive measurements.

WITHDRAWAL REFLEX: VON FREY HAIRS. Von Frey hairs (Semmes-Weinstein Monofilaments; Stoelting, Wooddale, IL) with target force ranges from 0.008 to 300N were used. Rats were placed in a Plexiglas box with a fine-grid bottom, and the filament was pressed against the plantar surface of the foot at a 90-degree angle until it bowed and held in place for 1 to 2 seconds. This stimulation was repeated up to three times in the same location. The test was performed by using increasing filament calibers until the first positive reflex responses were noted.

Functional Magnetic Resonance Imaging

All experiments were performed on a PharmaScan 70/16 system (Bruker, Germany) operating at 7 tesla. For functional imaging, axial RARE–images were acquired with the following parameters: matrix size =

128 × 128, three slices; FOV = 40 × 40mm; slice thickness, 1mm; slice spacing, 1mm; TR, 2,350 milliseconds; TE, 76.8 milliseconds; RARE factor, 32; number of excitations = 4. Acquisition time was 20 seconds per multislice volume. The measurements were conducted as described in Reese and colleagues.[26]

FUNCTIONAL MAGNETIC RESONANCE IMAGING PROTOCOL. For somatosensory stimulation of forelimbs and hindlimbs, a categorical design consisting of 10 alternating blocks of rest and activation was used each comprising two volumes. Sensory activation was induced by electrical stimulation by application of a constant current pulse-train (I = 10mA, 2Hz; pulse duration, 1 millisecond). This design was applied to the right forepaw first, followed by the hindpaw.

fMRI studies were conducted on a control group (control IgG treated, N = 7) and a 11C7 antibody–treated group, N = 10), comprising all animals of one of the three experimental batches. In addition, healthy animals (N = 3) were studied with the same protocol. Post hoc analysis for lesion size (see below) required the removal of some rats from the analysis, leading to the following group sizes: control IgG-treated group: N = 5; 11C7 antibody–treated group: N = 7.

IMAGE ANALYSIS. For data analysis, BioMap software (in-house developed software for image analysis and visualization) was used. Before statistical analysis data from all animals was coregistered. For statistical analysis of the effect of peripheral stimulation on brain activity, parametric maps were calculated by using the general linear model. A threshold of p value greater than 0.01 was applied to the statistical maps. In addition, activation clusters had to be larger than 10 voxels.

TISSUE PREPARATION, LESION SIZE. Transcardiac perfusions were carried out with 4% phosphate-buffered paraformaldehyde containing 5% sucrose. After dissection, the tissue was embedded in Tissue Tek OCT and frozen in isopentane at −40°C. Parasagittal sections were cut on a cryostat and processed by the avidin biotin method to show the BDA tracing of the corticospinal tract fibers as described earlier.[27]

Lesions were reconstructed for each animal as cross-section projection from the sagittal section series, and the extent of the lesion was determined as percentage of the cross-section. Inflammation was scored by the amount of residual macrophages present at the end of the experiment. These cells often react with the diaminobenzidine tetrahydrochloride necessary for showing the traced CST fibers and/or are recognized by their yellowish lipid-loaded phagocytic vacuoles.

FIBER COUNTS. Interindividual tracing variability was low. The number of regenerating fibers originating from the main CST was counted on complete series of sagittal sections at a final magnification of 400× in three defined areas of 0.25mm rostrocaudal width, at 0.5mm, 2mm, and 5mm caudal to the lesion site (see Fig 3).

SPROUTING SCORES. Scores (0 = absence of sprouting, 3 = very strong sprouting) were assigned by experienced, blinded observers judging the density, abnormal course, curving toward and around the lesion, length, and arborization of CST sprouts immediately rostral to the lesion.

EXCLUSION CRITERIA. Even well-standardized operation procedures lead to relatively large variability in the lesion size, for example, by bleeding and different extent of secondary tissue loss. Rats with extremely large, subtotal lesions and animals with too small lesions therefore had to be excluded according to the following criteria:

1. Performance in the BBB test at day 4 after the operation: animals with a BBB less than 8 (no plantar position and therefore no chance of performing more challenging tests like ladder-rung walking test, etc.) and animals with a BBB greater than 12 (known to be correlated with small initial lesions) were excluded from the study.
2. Post hoc evaluation at the light microscope level: animals with an incomplete lesion of the main CST were excluded.
3. Recurrent bladder infections were considered as the third exclusion criterion.

Exclusions (26 of 69 animals were excluded) were done blindly on the number-coded, mixed pool of animals.

STATISTICAL EVALUATION. Values of different treatment groups were statistically evaluated using the Mann–Whitney U test, comparing the variation of recovery within the groups on different time points measured. The 11C7 and the 7B11 antibody–treated groups were compared separately to the control IgG group.

Statistical comparison for the BBB locomotor score within the different groups over time was done using Pearson's χ^2 test.

Results

A modified partial transection lesion (T-lesion, see Fig 3) was used. The dorsal funiculi and the CST with its lateral and ventromedial parts were completely sectioned. The primary damage left the ventrolateral fiber tracts intact, whereas the secondary lesion expanded mainly within the gray matter. The fine subdural infu-

sion catheters did not cause any additional damage. The three antibodies were infused subdurally with the same concentration (3mg/ml, 5μl/hour) and amount (360μg/day) for 14 days.

Lesions were reconstructed for each animal. The percentage of tissue destruction was similar in the three treatment groups amounting to an average of 40 to 50% of the spinal cord cross-section. The degree of residual inflammation at the end of the experiment did not differ between the three treatment groups (data not shown).

Body Weight

Animals were weighed daily to monitor their health (see Fig 1B). On the first postoperative day, the increase in weight reflects the amount of liquid injected subcutaneously to support the circulation. The first week was characterized by a slight weight loss. After this initial phase, the animals gained weight constantly, except at times of enhanced stress due to removal of pumps and CSF puncture or extensive physical demand during the behavioral test sessions. Control IgG animals and anti–Nogo-A antibody–treated animals did not differ significantly in their body weight curves throughout the experiment (see Fig 1A, B).

Antibody Concentrations in Cerebrospinal Fluid

CSF was collected through the cisterna magna at the time of pump removal, 14 days after spinal cord lesion. Concentrations of 10 to 50μg/ml mouse IgG were found, whereby the control IgG values were higher than those of the two anti–Nogo-A antibodies (see Fig 1C). This difference may be caused by the binding of the Nogo-specific antibodies to the CNS tissue and their subsequent internalization and lysosomal degradation (Weinmann et al., submitted).

Recovery of Bladder Function

During the initial days after spinal cord injury, autonomous bladder voiding was absent and the bladder had to be expressed manually two to three times a day. Autonomous voiding started around day 24 after operation in the control IgG-treated rats. In contrast, the 7B12 and 11C7 antibody–treated animals recovered spontaneous bladder function more than 1 week earlier (see Fig 1D).

Corticospinal Tract Regeneration and Sprouting

In all the animals, the typical reactions of the CST to transection were present: retraction of the fibers rostral to the lesion, formation of retraction bulbs, and, on the other hand, signs of regenerative sprouting and occasional formation of growth cones. Regeneration of the CST was quantified by counting the fibers labeled by tracer injection into the sensory-motor cortex. All the fibers growing caudally beyond the lesion center were counted in three different areas at 0.5, 2, and 5mm caudal to the lesion (see Fig 3B). The very rare unlesioned fibers could be discriminated by their straight, regular appearance; if present, they were excluded from the counts. Regenerated fibers always showed an irregular course; they could often be followed to the main tract where they took a typical course toward ventral or lateral tissue bridges on which they crossed the lesion area (see Fig 2D, Fig 4). Their trajectory was highly irregular, reflecting the growth around scarred and debris-filled territory. They were fine and often difficult to detect. The main CST was often retracted, and many retraction bulbs could be seen (see Fig 2A, C). In anti–Nogo antibody–treated rats, CST fibers were often found very close to and around the lesion and the scar, forming complex growth cones and sprouting considerably (see Fig 2B). The average number of CST fibers observed at 0.5, 2, and 5mm caudal to the lesion was significantly higher for anti–Nogo-A antibody–treated animals than for control IgG-treated ones (see Fig 2C–F, Fig 3B, Fig 4). Variations in the groups were large. In a few rats in the anti–Nogo antibody–treated groups, the lesions and scars were too large to permit growth into the caudal spinal cord; other rats showed very high fiber numbers. Rats without fibers in the caudal spinal cord were very frequent in the control IgG group. Regenerating CST fibers were often found in ectopic anatomical locations, had irregular courses, and branched in gray matter especially at the more caudal levels (see Fig 2E, F, Fig 4).

Regenerative sprouting from the lesioned CST (see Fig 2A, B) was scored using a range from 0 (absence of sprouting) to 3 (very strong sprouting); results are shown in Figure 3A. CST sprouting was significantly higher in both anti–Nogo-A antibody–treated groups than in the control IgG group.

Functional Analysis

OPEN-FIELD LOCOMOTION (BBB). At day 1, the effects of spinal shock and the operation made consistent assessments impossible. BBB values obtained from day 4 on showed recovery to the level of 12 points in the control antibody group after 3 weeks. They were increased by only one additional point during the following 4 weeks (Fig 5A). These animals never developed coordinated fore/hindlimb movements. Nogo-A antibody–treated animals showed coordinated fore/hindlimb movements around 21 days. They increased to a final level of 15 points, a value designating coordinated and weight supported walking. The proportion of rats reaching the BBB score of 14 was significantly larger in the anti–Nogo antibody groups (70–90%) than in the control IgG group (20%; Pearson's χ^2 test). Note, that the BBB scale is nonlinear: steep at 9 to 15, but much flatter at 1 to 8 and 16 to 21 points.[28]

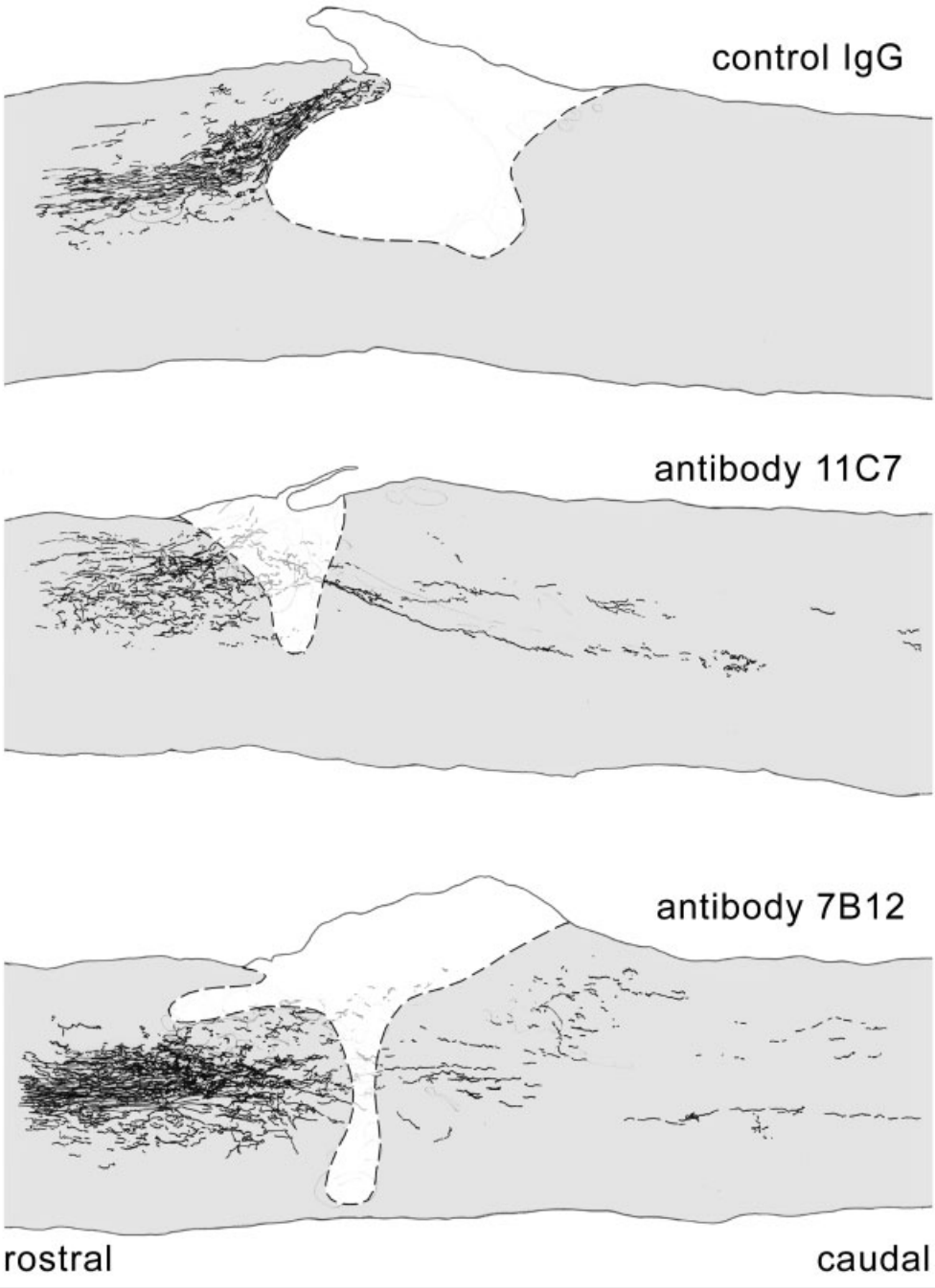

Fig 4. Camera lucida reconstructions of the spinal hemicord with labeled corticospinal tract (CST), lesion site (light area), rostral sprouting zone, and CST fibers regenerating over ventrolateral tissue bridges (gray-shaded depiction) into the caudal spinal cord. Six to eight sections were projected onto one plane. A photomicrograph of the 11C7 antibody–treated animal is shown in Fig 2D.

SWIM TEST. Rats swim by using exclusively their hindlimbs and the tail while holding the forelimbs immobile under the chin. Buoyancy allows often even more severely lesioned animals to make distinguishable movements of hips, knees, or ankles. We scored the deviations from the normal swimming patterns, that is, use of forelimbs, abnormal or absent tail movements, and increased distance between the hindlimbs.

A maximal score of 7 to 8 was reached by all the rats before surgery (see Fig 5B). Seven days after the lesion, the swimming pattern had greatly changed: rats swam mostly with their forelimbs and with occasional hindlimb strokes (score 2–3). After 34 days, the control rats were still severely impaired; they had reached a level of 3 points and seemed to plateau thereafter (see Fig 5B). The anti–Nogo antibody groups increased their perfor-

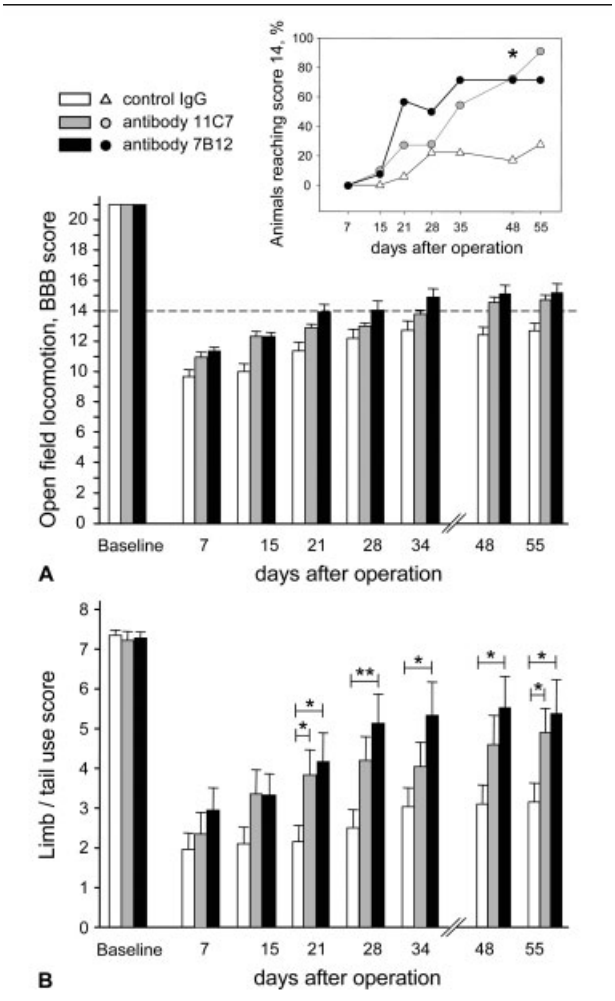

Fig 5. Open-field locomotion and swimming. (A) BBB scores of spinal cord–injured antibody-treated rats. Anti–Nogo antibody–infused animals regain coordinated, fully weight supporting forelimb/hindlimb stepping (BBB, >14) earlier (control IgG vs antibody 7B12: $p < 0.001$*; Pearson's* χ^2 *test) and in larger numbers (control IgG vs antibodies 11C7 or 7B12:* $p < 0.01$*). Consistent coordination was rare in control antibody animals. (B) Swim score shows persistent impaired swimming pattern in control rats, whereas anti–Nogo antibody–treated animals showed close to normal movement patterns.* $^*p < 0.01$, $^{**}p < 0.001$.

mance steadily. They reached 4 points at 21 days and 5 to 6 points at 34 to 55 days (see Fig 5B).

LADDER-RUNG WALKING TEST. This test assesses precise foot placement ability on an irregular ladder, a task that requires complex descending motor control with strong involvement of the CST.[29] The test therefore is suitable to obtain detailed information from mildly lesioned or well-recovered animals. The control antibody animals improved from a very high error rate (only 15–20% correct foot placements) to 40% correct steps at 4 to 8 weeks (Fig 6A). The anti–Nogo-A antibody–treated animals showed a faster and more complete recovery of performance reaching 40 to 50% of correct placements at 15 to 21 days (7B12 antibody group) and 60 to 70% at the end of the testing phase (55 days after lesion, both anti–Nogo-A antibody–treated groups; see Fig 6A).

NARROW BEAM CROSSING This test reflects the capability of the animals to maintain balance while walking on beams with increasing degrees of difficulty (smaller diameter, round vs square). The task cannot be accomplished by animals rating below 8 points on the BBB scale because it needs the capability of precise paw placement, which is dependent on the integrity of the CST. At 7 days after injury, the animals of all the treatment groups were not able to cross the beams (score of 0–1; see Fig 6B). The performances remained very low in the control antibody rats: They gained only approximately 10% of their initial ability after 4 to 8

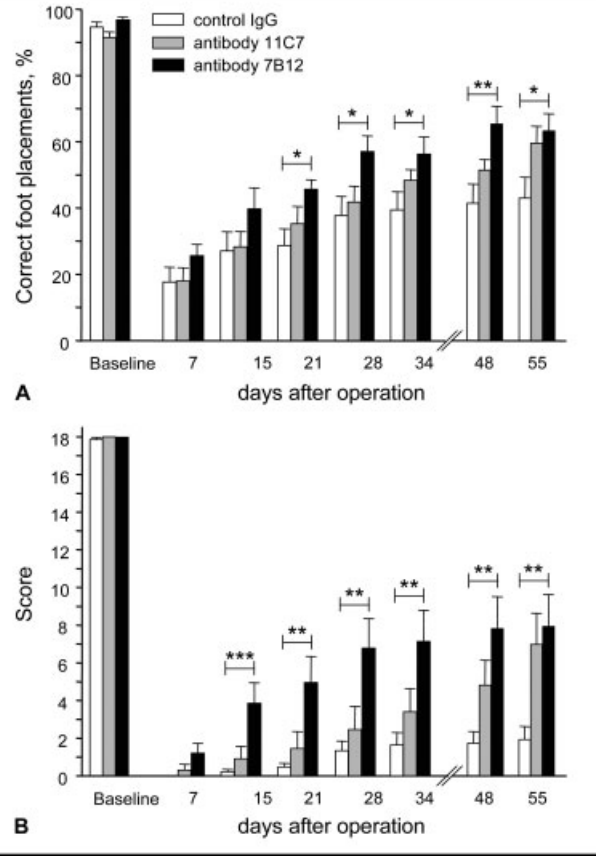

Fig 6. Ladder-rung walking test and narrow beam. (A) Percentage of correct hindfoot placements during crossing of a horizontal ladder with irregularly spaced rungs. (B) Rats sequentially crossed two rectangular beams and a round beam. Performance was very poor with minimal recovery in the control antibody rats but reached approximately 40% of baseline in the anti–Nogo antibody groups. $^*p < 0.01$, $^{**}p < 0.001$.

weeks of recovery (see Fig 6B). Very much in contrast was the progress achieved by the Nogo-A antibody–treated groups: The antibody 11C7–treated rats recovered up to 36% (6.5 points) and the antibody 7B12–treated animals reached 40% (7 points) of their baseline performance (see Fig 6B).

FOOT PRINT ANALYSIS: CATWALK®. Foot prints were recorded while the rats crossed a glass plate that was illuminated from the side. Paw contact was quantified by counting high-intensity pixels.[25] Normal adult rats distributed their weight about equally on their forelimbs and hindlimbs (Fig 7). Seven days after spinal cord lesion, most of the body weight was supported by the forelimbs. Recovery toward normal values occurred in the anti–Nogo antibody groups, but much less in the control antibody animals (see Fig 7).

Sensory Tests

To test the animals for their ability to respond to heat or light touch within a reference range of perception and to assess a possible hyperalgesia due to aberrant fiber growth, we used two classic withdrawal reflexes.

HEAT-INDUCED WITHDRAWAL REFLEX (PLANTAR HEATER). This test is an indirect measure for the sense of pain elicited by heat: animals withdraw their hindpaw and sometimes start licking. All the animals showed a baseline reflex time of 6.5 seconds, which varied only slightly after the lesion. Five and 7 weeks after the lesion, all animals showed baseline values, and no differences between the treatment groups were seen (Fig 8A). No allodynia or hyperalgesia could, therefore, be noted.

SENSITIVITY TO LIGHT TOUCH (VON FREY HAIR TEST). None of the groups, whether control antibody or anti–Nogo-A antibody treated, showed relevant changes in sensitivity to touch by defined hair sizes 3 and 8 weeks after the lesion. Thus, also with this sensory test neither hyperalgesia nor allodynia could be observed (see Fig 8B).

Functinal Magnetic Resonance Imaging

fMRI allows localization of the cortical representation of fore and hindlimbs and their responsiveness to peripheral sensory stimulation.[30] fMRI can also be used to visualize the functional consequences of neuronal plasticity in animals with different types of CNS lesions.[26,31] Here, we applied sensory stimuli to the fore

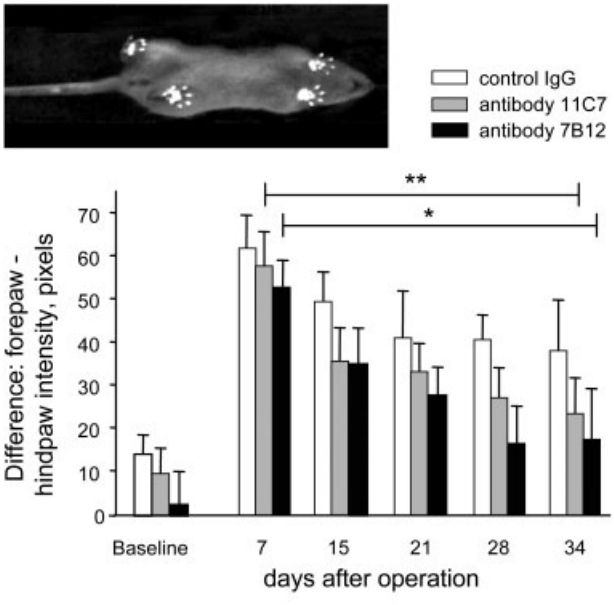

*Fig 7. Footprint analysis (Catwalk®) showing body weight distribution on forelimbs and hindlimbs. Close to equal distribution in intact rats changes massively in favor of the forelimbs after injury. Recovery close to normal weight distribution occurs in the anti–Nogo antibody rats, but much less so in the control group. *$p < 0.01$, **$p < 0.001$ for the comparison of the 5-week time point with 7 days after operation.*

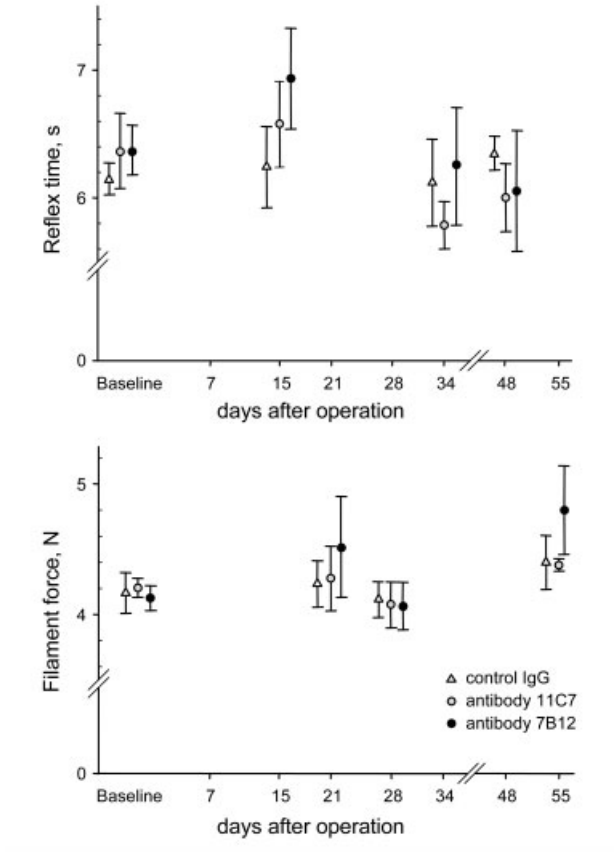

Fig 8. Sensory tests for pain and allodynia. (A) Withdrawal reflex in response to a warm light spot directed to the plantar surface of the hindpaw. The reflex time is similar in all the groups. (B) Withdrawal of hindlimb upon mechanical stimulation with filaments of increasing thickness/force.

and hindpaws, respectively, and measured the neuronal response in the somatosensory cortex by using the blood oxygenation level dependent (BOLD) effect MRI.

Electrical cutaneous stimulation of the *forepaw* led to a clear activation (BOLD response) in the contralateral primary somatosensory cortex (Fig 9). The maximum *t* value in this cluster was 11.7 corresponding to a very high significance of this response ($p < 0.0001$). On the single subject level, a clear activation could be observed in all but one animal. The center of activation was always located in the same position with minor deviations of less than 1mm.

A clear BOLD response to *hindpaw stimulation* was found for the anti–Nogo antibody (11C7)–treated group ($t_{max} = 4.58$; see Fig 9). For the IgG control group, no voxels could be detected which exceeded the threshold of *p* value less than 0.01. A significant difference in BOLD response in the hindlimb somatosensory cortex therefore could be detected between the 11C7- and IgG-treated animal group.

Discussion

Two different highly purified IgG antibodies directed against the Nogo-A–specific region of Nogo-A were infused intrathecally for 2 weeks into adult rats afflicted with partial spinal cord lesions. Anatomically, the interrupted CST showed regeneration of axons over distances of several millimeters, a reaction that was absent in the control IgG-treated animals. Recovery in open-field locomotion, swimming, and ladder and beam walking occurred earlier and to a significantly higher level in the two anti–Nogo antibody–treated groups than in the control IgG-treated animals. The behavioral improvement persisted for up to 6 weeks after the cessation of the treatment. These results resemble and extend the anatomical and behavioral observations made in several earlier studies with the monoclonal antibodies IN-1 and IN-1 Fab.[16,18–20] They also resemble the observed enhanced regeneration of CST axons and serotonergic fibers and the improved recovery of locomotor behavior after blockade of the Nogo–receptor interaction by the peptide NEP 1-40[4,32] or by a Nogo neutralizing receptor body.[33] All these findings demonstrate a crucial role of Nogo as an inhibitor of axonal regeneration and functional recovery in spinal cord–lesioned rats.[2]

Antibodies against Nogo-A were infused into the subdural space of the lower spinal cord. These antibodies reach the entire spinal cord and brain via the CSF circulation and penetrate well into gray and white matter of brain and spinal cord in the adult rat or macaque monkey (Weinmann et al., submitted). Anti–Nogo antibodies bind to the tissue, a fact that is reflected by the lower free antibody concentration in the CSF compared with control IgGs. Oligodendrocytes, the main cell type in the adult CNS expressing Nogo-A, and neurons, which express Nogo-A at a lower level, internalize the bound antibodies together with the Nogo-A antigen; both then are delivered to lysosomes (Wein-

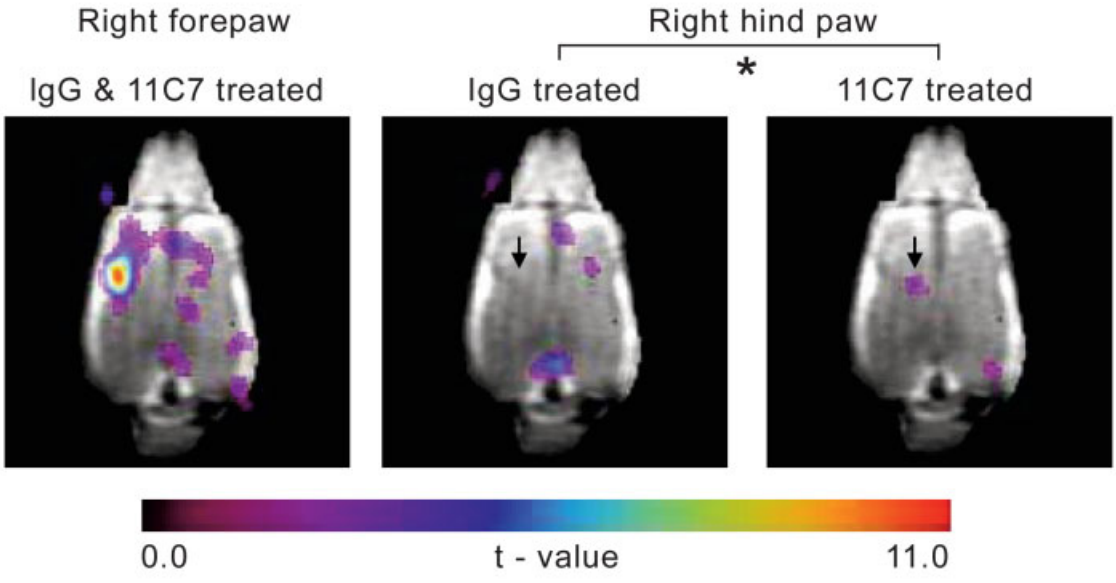

Fig 9. Blood oxygen level dependent (BOLD) signal functional magnetic resonance imaging activation maps for the right forepaw of pooled control- and anti–Nogo–treated animals (left). Maps for the hindpaw activation derived from the group analysis for the two different treatment regimens. Only those animals were included into the analysis, for which a clear signal change could be observed during forepaw stimulation. A significant differential activation of the hindlimb area (arrow) *after hindpaw stimulation is seen only in the 11C7-treated animals* ($p < 0.01$, $k > 10$). *Color bar indicates intensity of BOLD signal.*

mann et al., submitted). Downregulation of cell surface Nogo-A by antibodies, in addition to blockade of the Nogo-A active site against which the antibodies 11C7 and 7B12 are directed, is probably an important aspect of the in vivo mechanism of action of these antibodies. In contrast, lesion size and inflammation were similar in control IgG and anti–Nogo-A IgG–treated animals.

In vivo application of IN-1 antibodies into the CSF of adult rats led to an upregulation of the neuronal growth machinery as reflected by enhanced levels of cytoskeletal protein mRNAs and spontaneous, transitory sprouting of intact fibers, eg, CST or Purkinje axons.[34] We hypothesize that Nogo-A antibodies enhance the lesion-induced growth response of axotomized neurons in the injured CNS and simultaneously induce growth in noninjured neurons. The consequences of these processes are regeneration of lesioned axons and enhanced compensatory sprouting and growth of fibers from intact axonal systems.[17,35,36] Although the proportion of CST axons successfully regenerating over long distances (ie, $<$1cm) was small in the present and almost all the earlier studies, their extensive arborizations could still exert very important functional effects on the local neuronal circuitry in the caudal spinal cord segments. The small proportion of successfully regenerating axons may be caused by the massive barrier effect of the destroyed tissue and the scar that forms around it, as well as to the presence of additional neurite growth inhibitory factors in the adult spinal cord tissue. In addition to the CST, other descending tract systems (rubro-, reticulo-, vestibulospinal tract, 5-HT fibers) can be expected to show Nogo antibody–induced regeneration and enhanced compensatory sprouting[2,33,36] and to contribute importantly to the observed functional recovery.

The extent of behavioral recovery was unequal for the various behaviors tested. Thus, swimming behavior recovered to almost 75 % of its normal level in the anti–Nogo-A–treated animals. Ladder walking recovered to 60 to 65% and beam crossing to less than 50% of normal. In all these tests, however, the control antibody–treated animals plateaued at much lower levels of performance. The maximal recovery rate observed corresponds well to the degree of descending control from the brain required for these various tasks: whereas swimming can be controlled largely through spinal central pattern generating networks,[37] precise positioning of hindfeet on irregularly spaced ladder bars and balancing on narrow beams requires fine-tuned descending control for which the number of regenerating fibers may not be sufficient. Obviously, the CST is not the only system involved in these behaviors; the plastic reactions of other descending and ascending tracts to Nogo-A neutralization remain to be studied.

Functional MRI is an additional readout to assess the neurological improvement of spinal cord lesioned animals.[38] Although electrical stimulation of the intact forepaw led to a very strong BOLD response, stimulation of the impaired hindpaws induced only a significant BOLD response in animals treated with 11C7, but not in the IgG-treated group. This suggests that treatment with the Nogo-A antibody leads to increased plasticity and to circuit rearrangement not only in the motor but also in sensory systems. Interestingly, an almost identical result was recently found in rats with dorsal columns contusion lesions and implantations of neural stem cells.[38]

Fibers that are induced to grow over long distance in the adult CNS might not be able to find appropriate targets and recreate a developmental situation. It was astonishing and encouraging, therefore, that the functional consequences of anti–Nogo-A antibody treatment seemed exclusively beneficial and meaningful for the animals. Neurogenic pain has been interpreted as a consequence of intraspinal sprouting and formation of wrong connections.[39,40] In this study, neither the heat stimulus–induced retraction reflex of the hindlimb nor the withdrawal reflex induced by light touch was changed in the anti–Nogo-A antibody–treated animals, suggesting that chaotic growth did not occur to an extent creating hyperalgesia or allodynia. An earlier study using the IN-1 anti–Nogo-A antibody also showed that spasticity-like tonic activity in extensor muscles disappeared in the anti–Nogo antibody–treated animals, but not in control antibody–treated, spinal cord–injured rats.[20] Several neurotrophic and axonal guidance factors as well as ECM proteins were seen to be upregulated in response to lesions of the CST in the spinal cord; they may attract and guide regenerating axons to meaningful targets.[41] In addition, activity-dependent stabilization of meaningful connections, perhaps in parallel to pruning of nonused fibers, may be an additional important mechanism which shapes the formation of new circuits in the injured adult CNS.[35,37] Suppression of Nogo-A therefore would crucially enhance mechanisms that already exist and govern the restricted spontaneous plasticity and recovery processes after CNS lesions.

In conclusion, these observations show that two antibodies against an important active site of Nogo-A infused as highly purified IgGs subdurally over the lower spinal cord of adult rats with partial spinal transection lead to enhanced regeneration of CST axons and to significant improvements of motor recovery in the absence of detectable malfunctions. Such antibodies can now be developed for future therapeutic use in spinal cord–injured patients.

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