Sequential therapy of anti-Nogo-A antibody treatment and treadmill training leads to cumulative improvements after spinal cord injury in rats.

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Abstract

Intense training is the most clinically successful treatment modality following incomplete spinal cord injuries (SCIs). With the advent of plasticity enhancing treatments, understanding how treatments might interact when delivered in combination becomes critical. Here, we investigated a rational approach to sequentially combine treadmill locomotor training with antibody mediated suppression of the fiber growth inhibitory protein Nogo-A. Following a large but incomplete thoracic lesion, rats were immediately treated with either anti-Nogo-A or control antibody (2 weeks) and then either left untrained or step-trained starting 3 weeks after injury for 8 weeks. It was found that sequentially combined therapy improved step consistency and reduced toe dragging and climbing errors, as seen with training and anti-Nogo-A individually. Animals with sequential therapy also adopted a more parallel paw position during bipedal walking and showed greater overall quadrupedal locomotor recovery than individual treatments. Histologically, sequential therapy induced the greatest corticospinal tract sprouting caudally into the lumbar region and increased the number of serotonergic synapses onto lumbar motoneurons. Increased primary afferent sprouting and synapse formation onto lumbar motoneurons observed with anti-Nogo-A antibody were reduced by training. Animals with sequential therapy also showed the highest reduction of lumbar interneuronal activity associated with walking (c-fos expression). No treatment effects for thermal nociception, mechanical allodynia, or lesion volume were observed. The results demonstrate that sequential administration of anti-Nogo-A antibody followed in time with intensive locomotor training leads to superior recovery of lost locomotor functions, which is probably mediated by changes in the interaction between descending sprouting and local segmental networks after SCI.

Key words: locomotor training, axonal sprouting, corticospinal, 5-HT, Ia afferents, exercise, c-fos, kinematics.

Abbreviations: SCI, spinal cord injury; ASIA, American Spinal Injury Association; NgR1, Nogo Receptor 1; CST, corticospinal tract; ACsA, anti-cyclosporin A, UT, untrained; ST, step-trained; BWS, body-weight support; BDA, biotin dextran amine; PBS, phosphate-buffered saline; VGluT1,vesicular glutamate transport 1; GM, gray matter; CC, central canal; LDCom, dorsal commissural nucleus; D, the dorsal nucleus.

1. Introduction

Despite numerous treatments being tested in animal experiments and clinical trials, no cure has been found for spinal cord injury (SCI) (Thuret et al., 2006). Individual treatments may result in some functional improvements, but they will unlikely emerge as panaceas for SCI; rather, tailored combinations of treatments with a scientific basis are proposed to be potential therapies that will lead to cumulative improvements in SCI outcomes (Thuret et al., 2006; Edgerton et al., 2008; van Hedel and Dietz, 2010; Fouad and Tetzlaff, 2012; Starkey and Schwab, 2012). Particularly, locomotor training is presently the most clinically successful treatment to promote functional recovery after different central nervous system (CNS) injury types, and it is becoming routine for incomplete SCI patients in rehabilitation centers worldwide (Raineteau and Schwab, 2001; Dietz and Harkema, 2004; Thuret et al., 2006; van Hedel and Dietz, 2010; Marsh et al., 2011; Fouad and Tetzlaff, 2012; Starkey and Schwab, 2012). Moreover, suppression of Nogo-A, one of the most restrictive growth inhibitory molecules in the adult CNS which is up-regulated upon neuronal damage, is a promising pharmacological treatment to promote axonal regeneration, sprouting, and functional recovery after a variety of CNS injuries in preclinical tests (Raineteau and Schwab, 2001; Schwab, 2004; Thuret et al., 2006; Pernet and Schwab, 2012; Starkey and Schwab, 2012; Schwab and Strittmatter, 2014). Human antibodies against Nogo-A have been tested in Phase I clinical trials in ASIA category A patients and are being prepared for a placebo-controlled Phase II clinical trial (Pernet and Schwab, 2012; Starkey and Schwab, 2012). It is hoped that a rational strategy can be developed to combine these treatments to maximize behavioral recovery after SCI (Thuret et al., 2006; Fouad and Tetzlaff, 2012; Starkey and Schwab, 2012).

We previously examined the effects of anti-Nogo-A antibody treatment and treadmill training in rats with incomplete thoracic SCI; individual treatments improved locomotor performance compared to no treatment, but their simultaneous application showed no locomotor improvement (Maier et al., 2009). Similarly, Nogo Receptor 1 (NgR1) gene deletion and multimodal training alone were shown to improve functional recovery in a mouse model of mild cervical SCI, but their combined effect caused no functional improvement (Harel et al., 2010). Since the recovery mechanisms of Nogo-A suppression and training were not only different but also possibly competitive, their simultaneous application might have negatively interfered with each other's pathological and functional development after SCI; therefore, timing between interventions may be critical for combination therapies to reach their full potential (Maier et al., 2009; Starkey and Schwab, 2012). This notion is supported by our recent study in a rat model of large forebrain cortex stroke demonstrating that sequential application of anti-Nogo-A treatment followed by intensive forelimb reaching training resulted in almost completely restored skilled forelimb functions associated with organized fiber sprouting patterns, but their simultaneous application led to poorer functional outcomes compared to individual treatments and no treatment associated with aberrant fiber sprouting patterns (Wahl et al., 2014).

Based on these findings, we hypothesized that sequential combination of Nogo-A suppression and rehabilitative training would lead to cumulative improvements in SCI outcomes when compared to either treatment alone. To test this hypothesis, the present study investigated the individual and combined sequential effects of anti-Nogo-A antibody treatment for the first 2 weeks and treadmill training starting 3 weeks for 8 weeks after incomplete thoracic SCI in rats.

2. Materials and methods

2.1 Animals

All procedures were approved by the UK Home Office and performed under the Animals (Scientific Procedures) Act 1986. Twenty-eight female Sprague-Dawley rats (200-250 g) were obtained from Charles River (Margate, UK). Animals were housed individually on a 12-hour light-dark cycle at 19-21°C with 45-65% humidity and fed standard chow and water *ad libitum*. They were acclimated to the facility and handled for 7 days before surgery. No animals were excluded from final analysis.

2.2 SCI induction and antibody treatment

Animals were anesthetized with isoflurane (5% induction and 1.5% maintenance in O_2) and maintained on a heat pad throughout the surgical procedure. Their backs were shaved and cleaned with povidone-iodine. Ophthalmic ointment was applied to the eyes to prevent drying.

Under aseptic conditions, a dorsal midline incision was made from approximately T8 to L3. The skin and muscles were retracted to expose the vertebral column. A laminectomy was performed between T9 and T10, and the dorsomedial, dorsolateral, and ventromedial parts of the corticospinal tract (CST) were transected at T9 with iridectomy scissors (i.e. a T-shaped lesion) (Liebscher et al., 2005; Maier et al., 2009).

Animals were blindly and randomly treated with either mouse monoclonal IgG1 anti-Nogo-A antibody 11C7 (kindly supplied by Novartis, Basel, Switzerland) or control antibody mouse monoclonal IgG1 anti-cyclosporin A (ACsA, Novartis) (n = 14 for each antibody) (Oertle et al., 2003; Zhao et al., 2013). Osmotic pumps (Alzet

2ML2, Durect Co., Cupertino, USA) were filled with 11C7 or ACsA, attached to custom-made catheters (0.702 mm inner diameter, 00702, Detakta, Norderstedt, Germany and 0.108 mm inner diameter, 0041, ReCathCo, Allison Park, USA and), and placed in bottles with sterile saline in a 37°C water bath on the day before surgery. Following SCI, a partial laminectomy was performed to open a hole at L2. The large/proximal part of the catheter was anchored to the paravertebral muscles caudal to the hole with sutures, and the small/distal part of the catheter was inserted into the subdural space through the hole, and pushed rostrally to T10. The pump body was implanted subcutaneously and continuously delivered the antibody into the cerebrospinal fluid through the catheter for 2 weeks (2 ml, 5 μl/hr, 3 mg IgG/ml). For each day of surgery the same number of 11C7 and ACsA pumps were implanted in a blinded and random fashion.

The muscles and skin were sutured in layers. Animals were placed in an incubator until they regained consciousness and then were returned to their cages. Baytril (2.5 mg/kg), buprenorphine (0.015 mg/kg), and saline (10 ml) were administered subcutaneously at 0, 24, and 48 hours after surgery. Bladders were manually expressed twice daily until reflex bladder emptying returned; thereafter, animals were checked at least once daily. The osmotic pumps were removed after 2 weeks.

2.3 Basso, Beattie, Bresnahan (BBB) scale

General locomotor activity of the animals in an open field was measured weekly after SCI using the BBB locomotor rating scale (Basso et al., 1995).

2.4 Locomotor training

Before training began, the two antibody treatment groups were further divided into four counterbalanced groups: ACsA+ untrained (UT), ACsA+ step-trained (ST), 11C7+UT, and 11C7+ST (n = 7 in each group, BBB scores three weeks after SCI were around 12 for both ACsA+UT and ACsA+ST groups and around 14 for both 11C7+UT and 11C7+ST groups, Fig. 1a).

For the step-trained groups, training started 3 weeks after injury (i.e., 1 week following the end of antibody treatment) and consisted of bipedal walking on a body-weight support (BWS) treadmill system (Rodent Robot 3000, Robomedica Inc., Irvine, USA) for 20 minutes followed by quadrupedal walking on a rat dual lane treadmill (760303, Harvard Apparatus, Cambridge, UK) for 20 minutes (Maier et al., 2009). During bipedal walking, the head and upper body of the animals were placed in the cloth tube that was attached to the computer-controlled BWS device, and the amount of BWS was adjusted according to individual ability. Their hindlimbs stepped on the treadmill at 7 cm/s first and then gradually increased to 21 cm/s by week 4 of training. Similarly, quadrupedal walking started at 7 cm/s and 0% grade and reached 21 cm/s and 10% grade by week 4 of training. Training was conducted 5 days per week for 8 weeks. The untrained animals were handled as often as the step-trained animals.

2.5 Walking pattern

Bipedal walking of the animals on the BWS treadmill system was measured 11 weeks after SCI. Their lateral hindlimb areas were shaved, and 3-mm-diameter retro-reflective markers were placed on the skin overlaying the anatomical landmarks of iliac crest, greater trochanter, lateral epicondyle of the femur, lateral malleolus, and fifth digit distal phalange. The three-dimensional position of the markers was recorded using a seven-camera motion capture system (Nexus software and Bonita

cameras, Vicon, Oxford, UK) at 100 Hz, while animals walked bipedally at 14 cm/s for 10 consecutive steps provided with individually adjusted BWS. Collected data were transformed to Cartesian coordinates and analyzed using custom software in MATLAB (MathWorks, Natick, USA). Toe drag, paw position, and step consistency were measured to assess functional recovery of the animals.

Toe drag normally does not occur during walking in rats, but they often drag their toes after SCI (Basso et al., 1995; Maier et al., 2009). Toe drag of the animals was quantified by the percent time that their toes were in contact with the treadmill belt (toe raise $\leq$ 1 mm) in the swing phase, and the beginning and end of the stance and swing phases of the step cycle were indicated by the extremes of forward and backward motion (i.e. change of position) of the toes. Values were averaged over the 10 step cycles and between left and right sides.

Hind paws are normally placed parallel to the body at lift off and initial contact during walking in rats, but after SCI they are frequently rotated inward or outward (Basso et al., 1995). Paw position of the animals at lift off and initial contact and throughout swing were measured by the plane angle between the direction of the treadmill and a straight line connecting the ankle and toe markers. Values were averaged over the 10 step cycles and between left and right sides.

Walking pattern is normally very consistent in rats, but their steps often become less consistent after SCI (Maier et al., 2009). Spatial stepping consistency of the animals was quantified using principal component analysis. Cartesian coordinate data from an anatomical landmark were evenly resampled to remove temporal variation among the 10 step cycles, and the data for each of the three axes were arranged into a matrix with columns and rows representing the steps and resampled time series, respectively. The matrices were transformed into principal components,

and the percentage of variance explained by the first principal components was averaged and used to assess step consistency; the higher the average variance explained, the more consistent the walking pattern of that landmark. Step consistency was analyzed with all the anatomical landmarks, and the same analysis was previously performed with toe movement in two and three dimensions (Fong et al., 2005; Maier et al., 2009). Values were averaged between left and right sides.

2.6 Inclined climbing

Climbing ability of the animals was measured on a custom inclined climbing apparatus 11 weeks after SCI (Maier et al., 2009). The climbing apparatus consists of two rows of eight individual platforms alternating between left and right with an incline of 10°. The design allows normal rats to climb the apparatus platform-by-platform on the left and right sides by placing one paw on a platform at a time consistently. The platforms are movable, and distances between the platforms were randomly ordered at the beginning of each trial to prevent the animals from learning the task. To encourage them to reach the top of the apparatus from the bottom, their cages were ported to the top of the apparatus with the presence of food rewards (Froot Loops, Kellogg's, Battle Creek, USA).

Animals were habituated to the climbing apparatus and testing procedure 1 week before measurement. On the day of measurement, they were placed at the bottom of the apparatus to climb to the top in three trials, and cameras were placed on one side of the apparatus to record the trials at 60 f/s. Errors were counted from the video recordings and averaged over trials, and an error was scored when a platform was skipped by a paw, multiple attempts were made to place a paw on a platform, placement was made on a platform by any part of the body other than the plantar

surface of a paw, or a paw slipped off a platform at lift off. Values were averaged over the three trials and between left and right sides.

2.7 Nociception assays

Response of the animals to noxious stimuli was tested after the other behavioral tests were completed. Thermal nociception was measured using the Hargreaves method (Hargreaves et al., 1988). Animals were acclimated to a Plantar Test Apparatus (Ugo Basile, Varese, Italy) for at least 30 minutes before testing. The radiant heat source was positioned under the glass floor directly beneath the plantar surface of the hind paw, and the infrared intensity was adjusted with a constant voltage power supply to obtain a baseline response time of approximately 12 seconds in normal animals during the test. The latency of hind paw withdrawal was automatically recorded by the apparatus. Values were averaged between left and right sides.

Mechanical (tactile) allodynia was measured using calibrated von Frey filaments (Stoelting, Wood Dale, USA). Animals were placed in a plastic cage with a wire mesh bottom and allowed to acclimate for at least 30 minutes before testing. The filaments were applied to the mid-plantar surface of the hind paw through the mesh floor during the test. The 50% withdrawal threshold was determined using the up-down method (Chaplan et al., 1994). Values were averaged between left and right sides.

2.8 BDATracing

After behavioral data collection was completed, the CST was traced by injections of the anterograde tracer biotin dextran amine (BDA; 10000 MW;

Invitrogen, Carlsbad, USA) into the sensorimotor cortex. The procedure was performed under aseptic conditions while animals were anesthetized with isoflurane. A small hole was drilled into the skull 1 mm lateral and 1 mm posterior to bregma. A total of 3 µl of 10% BDA in 0.01 M phosphate-buffered saline (PBS) was injected unilaterally at four sites of the sensorimotor cortex using a Hamilton syringe (Maier et al., 2009).

2.9 Tissue preparation

Animals were euthanized 12 weeks after SCI. They walked on the treadmill (quadrupedal, 21 cm/s, 0% grade) for 1 hour and then returned to their cages for 1 hour before euthanasia. Animals were killed with an overdose of sodium pentobarbital (100 mg/kg ip) and transcardially perfused with 4% paraformaldehyde in phosphate buffer.

Spinal cords were removed, postfixed in the same fixative overnight at 4°C, cryoprotected in 30% sucrose in PBS for at least 48 hours at 4°C, embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, USA), frozen, and kept at -80°C. Sagittal sections of the thoracic spinal cord containing the lesion site were cut at 25 µm in a cryostat, transferred to adhesive-coated slides using the CryoJane Tape-Transfer System (Leica, Buffalo Grove, USA), and stored at -80°C. Coronal sections of the spinal cord at C7 and from T13 to L5 were cut at 25 µm in the cryostat, collected as free-floating sections in PBS, and stored in cryoprotectant at -20°C.

2.10 Lesion size

Every spinal cord section containing the lesion site was photographed under a microscope in bright-field mode (EC Plan-Neofluar 2.5x/0.075; LSM 510, Carl Zeiss,

Jena, Germany). The lesion was manually outlined on the images using Reconstruct (Fiala, 2005). The software then reconstructed the lesion and calculated the lesion volume.

2.11 BDA staining

Six replicate sections of the spinal cord at the cervical (C7), thoracic (T13), and lumbar (L1) levels were processed for BDA immunohistochemistry. Sections were treated with 0.3% H_2O_2 in TNT buffer (distilled water, 0.1 M Tris-HCI, 0.15 M NaCl, 0.3% Triton X-100; Sigma-Aldrich, St. Louis, USA) for 20 minutes to quench endogenous peroxidase activity and blocked in TNB buffer (distilled water, 0.1 M Tris-HCI, 0.15 M NaCl, 0.5% blocking reagent; TSA Biotin Kit, PerkinElmer, Waltham, USA) for 30 minutes. They were sequentially incubated with avidin-biotin complex reagent (1:100, 30 min; Vectastain Elite ABC Kit Standard, Vector Laboratories, Burlingame, USA) in TNT buffer, Streptavidin-HRP (1:100, 30 min; TSA Biotin Kit) in TNT buffer, biotinyl tyramide in amplification diluent (1:75, 10 min; TSA Biotin Kit), and ExtrAvidin−FITC (1:500, 30 min; Sigma-Aldrich) in TNT buffer, including three 5-minute washes in TNT buffer after each step. Sections were mounted on slides and coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, USA).

BDA-labeled fibers were manually located and counted at T13 and L1 under a confocal laser scanning microscope (Plan-Apochromat 40x/1.4; LSM 880, Carl Zeiss), and they were photographed at C7 and automatically measured using particle analysis in ImageJ (NIH, Bethesda, USA). To compensate for tracing variability, the number of fibers at T13 and L1 were normalized to the number of fibers at C7.

2.12 Analysis of 5-hydroxytryptamine (5-HT)

Six replicate sections of the spinal cord segment L4 were processed for 5-HT immunohistochemistry. Sections were washed three times for 10 minutes each in PBS, blocked with 10% normal donkey serum (Sigma-Aldrich) in PBST (PBS containing 0.2% Triton X-100) for 1 hour, and incubated with primary antibodies against 5-HT (rabbit, 1:18000; Immunostar, Hudson, USA) for 24 hours and choline acetyltransferase (ChAT) (goat, 1:500; Millipore, Watford, UK) for 48 hours in blocking solution at 4°C. They were washed (3 x 10 min), incubated with secondary antibodies conjugated to Alexa Fluor 555 (donkey anti-rabbit, 1:500; Life Technologies) and Alexa Fluor 488 (donkey anti-goat, 1:500; Life Technologies, Paisley, UK) for 2 hours in blocking solution, washed again (3 x 10 min), and mounted for microscopy.

Images of the ventral horn were captured using the tile scan function of the confocal microscope (40x). 5-HT varicosities in close apposition to motoneurons relative to the cell perimeter were measured manually using ImageJ (Maier et al., 2009). Cells with a minor axis of less than 25 μm were not measured, to substantially exclude the gamma motoneurons.

2.13 Analysis of vesicular glutamate transport 1 (VGluT1)

The same procedure was carried out for VGluT1 staining on six replicate sections of the spinal cord segment L2. Primary antibodies were anti-VGluT1 antibody (guinea pig, 1:10000, 24 hr; Millipore) and the anti-ChAT antibody. Secondary antibodies were biotin-SP-conjugated IgG (donkey anti-guinea pig, 1:500, 2 hr; Jackson ImmunoResearch, West Grove, USA) and the Alexa Fluor 488 dye.

Binding of biotinylated reagents was revealed by Alexa Fluor 555-conjugated streptavidin (1:500, 2 hr; Life Technologies).

Sections were photographed under the confocal microscope (40x). The density of VGluT1 puncta was obtained by measuring the number of VGluT1 positive dots in the regions of interest (ROIs) using MATLAB (Fig. 3a). In addition, VGluT1 positive boutons in close apposition to large motoneurons (minor axis $\geq$ 25 μm) relative to the perimeter of the soma and proximal dendrites were measured manually using ImageJ. The puncta density of all the treatment groups was normalized to the puncta density of the ACsA+UT group in the intermediate region.

2.14 c-fos expression

The same procedure was also carried out for c-fos staining on six replicate sections of the spinal cord segments L2-L5. Primary antibody was anti-c-fos antibody (rabbit, 1:500, 24 hr; Biotechnology, Santa Cruz, USA). Secondary antibody was the Alexa Fluor 555 dye (donkey anti-rabbit). Images of the sections were captured using the tile scan function of the confocal microscope (Plan-Apochromat 20x/0.8), and c-fos positive neurons were counted manually in each lamina according to the cytoarchitectonic organization of the spinal cord using Image-Pro-Plus (Media Cybernetics, Silver Springs, USA) (Molander et al., 1984).

2.15 Statistical analysis

All data (i.e. functional assessment and histological analysis) were obtained by observers blinded to treatment allocation. For all kinematics, climbing test, sensory test, lesion volume, CST and 5-HT counts a two-way factorial ANOVAwas used to test the differences between training (trained vs non-trained) and antibody (11C7 vs

IgG). When a significant main effect was found, Duncan's multiple range post-hoc test was used for all statistical tests. . For cfos analysis, a two-way ANOVA was performed to test differences between training (trained vs non-trained) and antibody (11C7 vs IgG) for each lamina. Because trends were similar between segments, data from all lumbar enlargement segments were combined (L2 to L5). Finally, for the BBB results a one-waymixed-mode ANOVA (treatments) with repeated measures (weeks) was used to determine differences between groups across all time points (ACsA+UT, ACsA+ST, 11C7+UT, 11C7+ST). Statistical analysis was performed using SPSS (IBM, Chicago, USA). Data are presented as mean ± SEM. Significances are indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1 BBB score

Training and antibody had a significant main effect on open-field locomotor activity (training week 8/9/10/11: p = 0.043/0.017/0.015/0.030, F = 4.6/6.7/7.0/5.4; antibody week 8/9/10/11: p = 0.011/0.039/0.029/0.011, F = 7.8/4.8/5.5/7.8). The 11C7+ST group continuously showed significantly better recovery than the ACsA+UT, ACsA+ST, and 11C7+UT groups after 8 weeks post-injury (Fig. 1a).

3.2 Dragging

Antibody had a significant main effect on toe drag during walking (p = 0.003, F = 11). The 11C7+UT and 11C7+ST groups spent less percent time dragging during the swing phase than the ACsA+UT and ACsA+ST groups (Fig. 1b). Noticeably, the 11C7+UT and 11C7+ST groups generally lifted their toes off the treadmill belt earlier and raised the toes to a maximum height lower than the ACsA+UT and ACsA+ST

groups, making no statistical difference in the average toe height during the swing phase between the groups (ACsA+UT 12.3 ± 1.9 mm, ACsA+ST 10.3 ± 0.9 mm, 11C7+UT 13.3 ± 1.8 mm, 11C7+ST 16.8 ± 2.4 mm) (Fig. 1c).

3.3 Paw position

Training and antibody had a significant main effect on paw position during the swing phase (training $p = 0.024$, $F = 5.9$; antibody $p = 0.037$, $F = 5.0$), and training also had a significant main effect on paw position at lift off ($p = 0.027$, $F = 5.7$) and initial contact ($p = 0.017$, $F = 6.8$). The 11C7+ST group adopted a more parallel paw position than the ACsA+UT group during the swing phase and at lift off and initial contact, the 11C7+ST group also adopted a more parallel paw position than the 11C7+UT group at initial contact, and the ACsA+ST group adopted a more parallel paw position than the ACsA+UT group at lift off (Fig. 1d). Noticeably, the 11C7+ST group generally kept the paw more parallel to the body throughout swing than the other groups (Fig. 1e). Overall, the 11C7+ST group maintained a more parallel paw position than the ACsA+UT group in 10 consecutive steps ($p < 0.05$, ACsA+UT 41.7 ± 4.6°, ACsA+ST 35.9 ± 4.5°, 11C7+UT 38.1 ± 11.3°, 11C7+ST 29.4 ± 8.5°).

3.4 Step consistency

Training had a significant main effect on stepping consistency of the crest ($p = 0.006$, $F = 9.5$), hip ($p = 0.003$, $F = 11$), knee ($p = 0.030$, $F = 5.4$), ankle ($p = 0.037$, $F = 5.0$), toe ($p = 0.045$, $F = 4.5$) and overall hindlimb ($p = 0.014$, $F = 7.3$) movement. The ACsA+ST group showed more consistent proximal joint (crest and hip) and overall hindlimb movement than the ACsA+UT group, and the 11C7+ST group showed more consistent proximal joint, distal joint (knee, ankle, and toe), and overall

movement than the ACsA+UT group (Fig. 1f). The differences were evident by comparing the repeatability of the step trajectories between the groups (Fig. 1g).

3.5 Climbing error

Antibody had a significant main effect on climbing error ($p = 0.022$, $F = 6.1$). The 11C7+UT and 11C7+ST groups showed a significant 45 and 42% reduction of climbing errors compared to the ACsA+UT group respectively, and there was no statistical difference in the ACsA+ST group (Fig. 1h).

3.6 Pain

Training and antibody had no effect on pain threshold in nociception assays. The latency of hind paw withdrawal from the heat source (ACsA+UT 13.6 ± 1.2 s, ACsA+ST 12.4 ± 1.1 s, 11C7+UT 12.8 ± 0.7 s, 11C7+ST 15.0 ± 1.3 s) and the 50% withdrawal threshold to von Frey filaments (ACsA+UT 11.3 ± 2.3 g, ACsA+ST 10.0 ± 2.4 g, 11C7+UT 12.0 ± 1.7 g, 11C7+ST 14.9 ± 0.1 g) were similar between the groups.

3.7 Injury severity

All animals were confirmed to have a T-shaped lesion in reconstruction. The lesion volume was similar between the groups (ACsA+UT 4.4 ± 0.3 mm^3, ACsA+ST 5.0 ± 0.7 mm^3, 11C7+UT 4.5 ± 0.6 mm^3, 11C7+ST 5.4 ± 0.6 mm^3).

3.8 Supraspinal axons

BDA labeled CST axons projected caudally mainly in the dorsal funiculus with minor components in the lateral and ventral funiculi above the lesion at the

cervical level (Fig. 2a). The CST was completely transected at T9. Sprouting CST fibers were assessed at T13 and L1; growth appeared caudal and transverse in the white matter (WM) (Fig. 2b), it became mainly transverse in and across the gray matter (GM) (Fig. 2c), and decussation across the midline near the central canal (CC) was occasionally seen in the ACsA+ST group at T13 and the 11C7+ST group at L1 (Fig. 2d).

Training and antibody had a significant main effect (training T13/L1: $p = 0.001/0.001$, $F = 43/550$; antibody T13/L1: $p = 0.001/0.001$, $F = 1100/150$) and interaction (T13/L1: $p = 0.001/0.001$, $F = 15/170$) on sprouting CST fibers. At T13, the ACsA+ST group had more fibers than the ACsA+UT group, and the 11C7+UT and 11C7+ST groups had more fibers than the ACsA+UT and ACsA+ST groups; however at L1, the ACsA+ST group had more fibers than the ACsA+UT and 11C7+UT groups, and the 11C7+ST group had more fibers than the other groups (Fig. 2e). The 11C7+UT group revealed a greater reduction of fiber growth between T13 and L1 than the other groups (Fig. 2f). In addition, fibers appeared to be distributed more widely in the ACsA+ST, 11C7+UT, and 11C7+ST groups than the ACsA+UT group at T13, and from T13 to L1, they appeared to spread further transversely in the ACsA+UT, ACsA+ST, and 11C7+ST groups but not in the 11C7+UT group (Fig. 2g).

The ventral lateral funiculi were spared by the transection, and serotonergic innervation of motoneurons was quantified by 5-HT positive varicosities in close apposition to the motoneurons in the lumbar spinal cord (Maier et al., 2009). Training and antibody had a significant interaction on serotonergic synaptic density around motoneurons ($p = 0.012$, $F = 7.6$). The 11C7+ST group had 49, 86, and 70% more synapses on motoneurons than the ACsA+UT, ACsA+ST, and 11C7+UT groups, respectively (Fig. 2h).

3.9 Sensory afferents

After CST transection, VGluT1 is almost exclusively localized in primary afferent fibers and their terminals on spinal neurons (Oliveira et al., 2003; Du Beau et al., 2012). Training and antibody had a significant main effect on VGluT1 positive fiber density in the dorsal, intermediate, and ventral gray matter of the lumbar spinal cord (training dorsal/intermediate/ventral: $p = 0.001/0.001/0.014$, $F = 23/15/7.2$; antibody dorsal/intermediate/ventral: $p = 0.001/0.001/0.001$, $F = 27/24/28$). In the dorsal and intermediate regions, the ACsA+ST group had fewer fibers than the other groups, and the 11C7+UT group had more fibers than the ACsA+UT group (Fig. 3b). In the ventral region, the 11C7+UT group had more fibers than the other groups, and the 11C7+ST group had more fibers than the ACsA+ST group. The differences were evident when comparing the VGluT1 positive area and intensity in the ROIs between the groups (Fig. 3c).

Furthermore, antibody had a significant main effect on number of VGluT1 positive terminals in close apposition to motoneuron somata and dendrites (somata/dendrites: $p = 0.001/0.001$, $F = 37/14$), and training and antibody had a significant interaction on VGluT1 positive terminal density around motoneuron dendrites ($p = 0.009$, $F = 8.3$). The 11C7+UT and 11C7+ST groups had more terminals on motoneuron somata than the ACsA+UT and ACsA+ST groups, and the 11C7+UT group also had more terminals on motoneuron dendrites than the other groups (Fig. 3d).

3.10 Activation of c-fos

Elevated c-fos levels in neurons as detected by immunohistochemistry reflect neuronal activity in the lumbar spinal cord, and the number and pattern of c-fos positive neurons change over time after incomplete SCIs, from a large number of diffusely activated cells to a smaller number of specific locomotion circuit neurons (Ichiyama et al., 2008; Courtine et al., 2009). Training had a significant main effect on neuronal activity in laminae I-V and total (lamina I/II/III/IV/V/total: $p = 0.001/0.011/0.046/0.003/0.034/0.013$, $F = 14.0/7.4/4.2/11/5.0/7.0$), and antibody had a significant main effect on neuronal activity in laminae III-V, the lumbar dorsal commissural nucleus (LDCom), the dorsal nucleus (D), and total (lamina III/IV/V/LDCom/D/total: $p = 0.043/0.005/0.047/0.011/0.045/0.042$, $F = 4.5/9.1/4.3/7.4/4.4/4.6$) between L2 and L5. The ACsA+ST group showed less activity than the ACsA+UT group in lamina IV, the 11C7+UT group showed less activity than the ACsA+UT group in lamina IV and LDCom and more activity than the 11C7+ST group in laminae I-II, and the 11C7+ST group showed less activity than the ACsA+UT group in laminae I-V, the LDCom, the D, and total between L2 and L5 (Fig. 4a). There was a clear trend toward less neuronal activity with training and anti-Nogo-A antibody in laminae I-IX, the D, the intermediolateral cell group (IML), the LDCom, and throughout the spinal cord at L2-L5 (Fig. 4b).

4. Discussion

This study examined the effects of anti-Nogo-A antibody treatment applied for 2 weeks immediately following a large incomplete spinal cord transection and intensive training starting 3 weeks post-injury for 8 weeks. The results support the hypothesis that sequential combination of Nogo-A suppression and rehabilitative training would lead to cumulative improvements in SCI outcomes.

4.1 Effects of step training

Three-week post-injury onset of treadmill training improved step consistency and paw position during walking after SCI. It is not surprising that stepping was more consistent after animals were trained to walk in stereotyped patterns repeatedly for 8 weeks, and they probably learned to achieve a more parallel paw position in the process. The trained walking ability only marginally transferred to climbing ability, and generally transfer of learning from one task to another task requires these tasks to be very similar (Raineteau and Schwab, 2001; Harel et al., 2010). Three-week post-injury onset of training alone did not reduce dragging during the swing phase, which we also observed in one-week post-injury onset of training previously (Maier et al., 2009). Forelimb-hindlimb coordination in locomotor activity was frequent with training (BBB score of 13). No detrimental effect on recovery was noticed in one- and three-week post-injury onsets of treadmill training (Maier et al., 2009). Our results are consistent with previous reports and reinforce the findings that repetitive activation of motor pathways leads to beneficial changes in behavior in animals as well as humans (Dietz and Harkema, 2004; Edgerton et al., 2008; Fouad and Tetzlaff, 2012; Starkey and Schwab, 2012), suggesting that delaying the initiation of rehabilitation up to three weeks after lesion is still effective in improving stepping ability.

Treadmill training promoted CST fiber sprouting that grew caudal to the lesion, although less so than anti-Nogo-A antibody treatment in the thoracic region. Previous studies have shown that rehabilitative training can increase sprouting of CST fibers, which may be induced by up-regulation of growth/plasticity associated factors (Ying et al., 2005; Fouad and Tetzlaff, 2012; Joseph et al., 2012). Other descending tracts may also contribute to training-induced recovery (Engesser-Cesar et al., 2007;

Fouad and Tetzlaff, 2012), although serotonergic pathways did not seem to be affected by training alone based on this and our previous study (Maier et al., 2009).

Descending input to interneurons residing in the deep dorsal and intermediate gray is thought to modulate activity of proprioceptive and cutaneous afferents, which has been shown to occur through presynaptic inhibition in rhythmic patterns during locomotion (Eccles et al., 1962; Jankowska et al., 1981; Dubuc et al., 1988; Perreault et al., 1999; Rudomin and Schmidt, 1999). Descending modulation is disrupted along with increased afferent fibers and terminals after SCI, which is associated with hyperreflexia and spasticity (Nelson and Mendell, 1979; Skinner et al., 1996; Raineteau and Schwab, 2001; Tan et al., 2012). Our results show that training not only reduced sprouting of afferents in the dorsal and intermediate gray matter but also increased CST sprouting in regions where interneurons mediating presynaptic inhibition reside (Hughes et al., 2005). Similar to our previous study using electron microscopy, training alone did not show an increase in Ia afferent terminations apposing alpha motoneurons (Ichiyama et al., 2011). Such increase only occurred with anti-Nogo-A antibody treatment. These changes can explain the depressed H-reflexes and reduced spasticity after training in SCI that have been reported previously (Raineteau and Schwab, 2001; Trimble et al., 2001; Fouad and Tetzlaff, 2012).

In the present study, we chose to investigate a medial motor pool, which putatively contains motoneurons innervating axial musculature. This was done in order to avoid possible selection of motoneurons innervating different muscles, potentially antagonistic groups. It is likely that activity of all hindlimb motor pools (innervating axial, proximal, distal muscles) is involved in changes driven by

locomotor training, given the need to control both posture and locomotion during stepping.

Training also reduced neuronal activity in the lumbar spinal cord associated with walking, which is in agreement with our previous findings (Ichiyama et al., 2008; Courtine et al., 2009). Interestingly, this reduction is similarly observed in areas with reduced Ia afferent input after step training. It is possible that these two observations are somehow linked, but further experiments are necessary to determine such relationship.

4.2 Effects of anti-Nogo-A antibody treatment

Anti-Nogo-A antibody treatment reduced dragging during the swing phase and climbing errors after SCI, which coincides with our previous results (Maier et al., 2009). However, it only marginally improved step consistency during walking. Anti-Nogo-A antibody treatment improved paw position during the swing phase on average but not at lift off and initial contact, and while paw position was rotated at initial contact and lift off, forelimb-hindlimb coordination was consistent in locomotor activity (BBB score of 14). Our results suggest that some functions are recovered and maintained after several weeks following 2 weeks of anti-Nogo-A antibody delivery, but others are lost with time after injury and treatment.

Anti-Nogo-A antibody treatment greatly promoted CST fiber sprouting caudal to the lesion, as expected (Liebscher et al., 2005; Thuret et al., 2006; Maier et al., 2009; Starkey and Schwab, 2012); however, most of the fibers did not directly reach the lumbar spinal cord. Nevertheless, it is plausible that indirect reconnections enabled by anti-Nogo-A antibody helped to reduce dragging and climbing errors, because the CST is thought to be important for control of distal musculature necessary

to reduce dragging during the swing phase and perform skilled motor tasks(Raineteau and Schwab, 2001; Bareyre et al., 2004; Liebscher et al., 2005). Serotonergic synapses on motoneurons at L4 did not appear to be affected by anti-Nogo-A antibody treatment alone 12 weeks after SCI, whereas in our previous study it was found to increase serotonergic fibers and synapses on motoneurons at L3 in similar experimental settings 10 weeks after SCI (Maier et al., 2009). It is possible that at the specific time points investigated here a retraction of 5-HT varicosities on motoneurons was observed, suggesting a dynamic process of sprouting and pruning of terminals with treatments (i.e. use and disuse).

Anti-Nogo-A antibody treatment also increased primary afferent fibers and terminals onto motoneurons in the spinal cord caudal to the lesion, clearly demonstrating that suppression of the myelin associated neurite growth inhibitory protein Nogo-A results in sprouting of fibers and synapse formation from different systems in line with previous evidence (Wills et al., 2012; Akbik et al., 2013; Petrinovic et al., 2013). Similar observations have been made with other plasticity enhancing pharmacological combinations, such as adeno-associated virus encoding L1 and chondroitinase ABC (Chase ABC) treatment which increased excitatory glutamatergic terminals at motoneuronal somata and pre-motor interneurons (Lee et al., 2012).

Interestingly, anti-Nogo-A antibody treatment reduced neuronal activity associated with walking similar to step training, while it increased descending and sensory inputs. This regulation of neuronal activity was most prominent in laminae III-V, the LDCom, and the D suggestive of significant changes in interneuronal processing and integration of descending and afferent information. We speculate that the animals with anti-Nogo-A antibody treatment intentionally and/or unintentionally

trained themselves in the cage, which helped them to develop a unique strategy for functional compensation that was different from treadmill training.

4.3 Effects of sequential therapy

Sequentially combined therapy with anti-Nogo-A antibody treatment and treadmill training resulted in cumulative functional improvements after SCI; animals with sequential therapy showed better step consistency during walking as seen with training alone compared to no treatment, and they also showed less dragging during the swing phase and fewer climbing errors as seen with anti-Nogo-A antibody treatment alone compared to no treatment. In addition, sequential therapy improved paw position during walking better than individual treatments. Forelimb-hindlimb coordination was consistent, paw position was parallel, and toe clearance was frequent in locomotor activity with sequential therapy (BBB score of 17). The superior recovery is in contrast to our previous study where one-week post-injury onset of treadmill training, overlapping in time with the anti-Nogo-A antibody treatment, showed no functional improvement (Maier et al., 2009). It is known that the half-life of anti-Nogo-A antibody in cerebrospinal fluid is around 2 days (Liebscher et al., 2005) and therefore it is highly unlikely the antibody was still active within the spinal cord at the time locomotor training started in the present study. These observations strongly suggest that our observed additive effects are due to the chronological separation of the interventions.

Sequential therapy greatly promoted CST fiber sprouting caudal to the lesion, which coincides with simultaneous combination that showed the largest amount of CST sprouting just caudal to the lesion previously (Maier et al., 2009). Here, we further demonstrate that sequential combination allowed the sprouting fibers to

directly extend into the lumbar segments, likely associated with the effects of three-week post-injury onset of training. It is possible that CST sprouting enabled by anti-Nogo-A antibody was further enhanced by growth factors up-regulated by training. It remains to be determined whether changes in number of observed CST fibers are true regenerating axons or sprouting from spared axons because the ACsA+UT group also showed some residual CST fibers caudal to the lesion several months after the initial injury. Sequential therapy also increased serotonergic synapse numbers onto motoneurons at L4, most likely through similar mechanisms.

The fact that sequential therapy led to lower levels of Ia afferent sprouting and synapse formation onto lumbar motoneurons than anti-Nogo-A antibody treatment alone, indicates that the augmented sprouting enabled by anti-Nogo-A antibody treatment was reduced by step training, probably to stabilize functionally important and prune exuberant fibers and synapses (Wahl et al., 2014). This use-dependent mechanism may be important for functional locomotion because ectopic sprouting of Ia afferents has been shown to lead to spasticity (Liu and Chambers, 1958; Nelson and Mendell, 1979; Tan et al., 2012). It is also interesting that similar to our previous observations, no changes in allodynia and hyperalgesia were observed. As the lesion itself has been shown previously not to induce abnormal sensation, the lack of differences between groups strongly suggests our treatments had no effect on these parameters (Liebscher et al., 2005; Maier et al., 2009). Locomotor training and anti-Nogo-A antibody preferentially affected proprioceptive muscle afferents.

Modulation of neuronal activation associated with walking was most pronounced with sequential therapy, where activation of c-fos was lowest compared to individual treatments and no treatment especially in laminae III-V. Together with the increased sprouting of CST fibers into lumbar segment in deep dorsal horn and

intermediate zone of the gray matter and modulation of Ia afferent sprouting, integration of proprioceptive information with descending commands could be a key mechanism underlying the superior functional recovery in sequential therapy. The interactions and interdependence between CST terminations in spinal cord and afferent sprouting in deep dorsal horn laminae have recently been shown during development and after injury (Chakrabarty and Martin, 2011; Tan et al., 2012).

This study emphasizes that training onset should follow in time with regenerative medicine in combination therapies, which is convenient for clinical applications because early training onsets are usually unrealistic in the treatment of SCI patients (Fouad and Tetzlaff, 2012). The next steps are to seek potential treatments that can add to the present sequential therapy for further cumulative improvements, and optimize the related parameters, such as treatment timing, drug dose, and training type, frequency, duration and intensity.

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Figure legends

Figure 1 Behavioral outcomes (n = 7 per group). Data were obtained from the BBB locomotor rating scale (a), walking pattern analysis (b-g), and inclined climbing (h). BBB score (offset horizontally for clarity), toe drag, paw position, step consistency, and climbing error are presented as mean ± SEM (a, b, d, f, h). Representative examples of up-and-down movement of the toe during the swing phase, planar movement of the right hind paw during the swing phase from lift off to initial contact, and three-dimensional movement of the right hindlimb markers in 10 consecutive steps are presented in (c), (e), and (g). *P < 0.05, **P < 0.01.

Figure 2 Supraspinal axons (n = 7 per group). The CST was anterogradely traced with BDA on the right side. CST axons projected caudally in the dorsal, lateral, and ventral funiculi at C7 (a). BDA labeling is displayed in grayscale for clarity. Past the lesion site, sprouting CST fibers grew transversely from rostral (R) to caudal (C) in a preferred direction in the WM (b). The growing direction of CST fibers became mainly transverse in and across the GM (c). CST fibers could grow across the midline near the central canal (CC) in the ACsA+ST and 11C7+ST groups (d). Normalized number of BDA labeled fibers at T13 and L1 is presented in (e). The L1 to T13 CST fiber ratio is presented in (f). Location of the CST fibers is schematically represented in (g). 5-HT positive varicosity density on large motoneurons is presented in (h). Data are presented as mean ± SEM (e,f,h). *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar: 20 μm.

Figure 3 Primary afferents (n = 7 per group). Three ROIs were placed on each side of the gray matter at L2 to measure the VGluT1 positive dots (red: VGluT1, green:

ChAT); a dorsal ROI (300 x 130 μm^2) was positioned next to the medial side of the dorsal horn in laminae III-IV, an intermediate ROI (300 x 300 μm^2) was positioned near the dorsal CST covering the VGluT1 cluster, and a ventral ROI (200 x 200 μm^2) was centered on the motoneurons in the medial ventral horn (a). Normalized VGluT1 positive puncta density in the ROIs is presented in (b). Representative images of the ROIs with VGluT1 staining are displayed in grayscale for clarity in (c). Number of VGluT1 positive bouton in close apposition to large motoneuron somata and proximal dendrites is presented in (d). Data are presented as mean ± SEM (b,d). *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar: 100 μm.

Figure 4 Neuronal activity associated with walking (n = 7 per group). Number of c-fos positive neurons between L2 and L5 is presented in (a). Representative camera lucida drawings and number of c-fos positive neurons at L2, L3, L4, and L5 are presented in (b). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.