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A re-assessment of the effects of a Nogo-66 receptor antagonist on regenerative growth of axons and locomotor recovery after spinal cord injury in mice

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Abstract

This study was undertaken as part of the NIH “Facilities of Research-Spinal Cord Injury” project to support independent replication of published studies. Here, we repeated a study reporting that treatment with the NgR antagonist peptide NEP1-40 results in enhanced growth of corticospinal and serotonergic axons and enhanced locomotor recovery after thoracic spinal cord injury. Mice received dorsal hemisection injuries at T8 and then received either NEP1-40, Vehicle, or a Control Peptide beginning 4–5 hours (early treatment) or 7 days (delayed treatment) post-injury. CST axons were traced by injecting BDA into the sensorimotor cortex. Serotonergic axons were assessed by immunocytochemistry. Hindlimb motor function was assessed using the BBB and BMS scales, kinematic and footprint analyses, and a grid climbing task. There were no significant differences between groups in the density of CST axon arbors in the gray matter rostral to the injury or in the density of serotonergic axons caudal to the injury. Tract tracing revealed that a small number of CST axons extended past the lesion in the ventral column in some mice in all treatment groups. The proportion of mice with such axons was higher in the NEP1-40 groups that received early treatment. In one experiment, mice treated with either NEP1-40 or a Control Peptide (reverse sequence) had higher BBB and BMS scores than Vehicle-treated controls at the early post-injury testing intervals, but scores converged at later intervals. There were no statistically significant differences between groups on other functional outcome measures. In a second experiment comparing NEP-treated and Vehicle controls, there were no statistically significant differences on any of the functional outcome measures. Together, our results suggest that treatment with NEP1-40 created a situation that was slightly more conducive to axon regeneration or sprouting. Enhanced functional recovery was not seen consistently with the different functional assessments, however.

Keywords

corticospinal tract; Nogo; Nogo-66; axon regeneration; sprouting; motor system; mice; biotinylated-dextran amine; BDA; tract tracing; locomotor recovery

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Introduction

Achieving a high level of recovery of function after spinal cord injury is likely to require the regeneration of the axons of the long ascending sensory and descending motor and autonomic tracts of the spinal cord. Accordingly, there is great interest in identifying the reasons for regeneration failure, and in developing interventions to promote regeneration that would otherwise not occur. In this regard, there has been considerable interest in the evidence for various inhibitory molecules in myelin, including Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OmGP). These proteins are expressed by oligodendrocytes, and are thought to interact with the Nogo receptor on axons to trigger signaling events that lead to axon growth inhibition. These different molecular pathways that inhibit axon regeneration present reasonable targets for therapeutic interventions, for example by using antibodies to block Nogo or synthetic peptides to block the interaction between Nogo, OmGP, and MAG and the Nogo receptor (Lee et al., 2003; Xu et al., 2004).

Although a number of strategies show promise for enhancing regeneration, a barrier to translation is that promising findings are often not re-evaluated in independent replications to assess the robustness and reproducibility of the effects. To meet this need, the NINDS launched the “Facilities of Research-Spinal Cord Injury” (FOR-SCI) replication project, in which promising published studies are independently replicated. Here, we repeat an experiment reporting that delayed subcutaneous treatment with the NgR antagonist peptide NEP1-40 (Nogo extracellular protein, residue 1–40) results in enhanced growth of corticospinal (CST) axons, sprouting of serotonergic (5HT) fibers and enhanced locomotor recovery after thoracic spinal cord injury.

Materials and Methods

Consistent with the requirements of the FOR-SCI replication project, our goal here was to repeat as closely as possible the key experiments reported in Li and Strittmatter (2003). All experimental procedures were as described in the original report. Where necessary, the original authors were contacted for clarification of procedural details. The original report described results from a total of eight different groups of animals (10–12 animals per group) treated in one of 3 ways: 1) Subcutaneous delivery of NEP1-40 peptide or Vehicle via implanted osmotic minipump beginning at the time of the initial injury; 2) Intraperitoneal delivery of NEP1-40 peptide or Vehicle starting 3–4 hours post-injury with daily injections continuing for 14 days; and 3) Subcutaneous delivery of NEP1-40 peptide or Vehicle via implanted osmotic minipump beginning 7 days post-injury. Here we focus on the second and third treatment protocols because these are initiated within a time frame that could be feasible for human therapeutic interventions.

Discussions with the original authors revealed that the overall study was carried out as several separate experiments, and we elected to follow this practice because of the numerous groups and different techniques involved. Accordingly, we first replicated the portion of the overall study that involved the delivery of the NEP1-40 peptide 3–4 hours after the spinal cord injury (called Experiment 1A). The portion of the overall experiment in which treatment with the peptide was initiated beginning 7 days post-injury (called Experiment 2) was done separately. The surgeries for Experiment 1A were carried out on June 24, 2005. The surgeries for the 7 day delayed treatment experiment, Experiment 2, were carried out on November 21, 2005.

In addition to the groups included in the original experiment, a new group that received a control peptide was included here. Accordingly, in the experiment involving treatment within 4hrs after injury, a separate control group received a peptide that had the reverse sequence to the NEP1-40 (called the Reverse Peptide group). In the experiment involving treatment beginning

7 days post injury, a separate control group received a peptide in which the NEP1-40 amino acid sequence was scrambled (called the Scrambled Peptide group).

We also carried out a follow-up experiment (called Experiment 1B), which was undertaken because in Experiment 1A animals treated with NEP1-40 exhibited higher BBB scores during the early post-injury period in comparison to Vehicle-treated controls. Accordingly, we carried out a follow-up experiment to determine whether the difference was reliable. The surgeries for Experiment 1B were carried out on November 21, 2005.

Peptides

The NEP1-40 peptide sequence was: acetyl-RIYKGVIAIQKSDEGHPFRAYLESEVAISEELVQKYSNS-amide. The reverse peptide was exactly the reverse sequence. The scrambled peptide used in Experiment 2 was acetyl-SYVKEYAPIFAGKSRGEIKYQSIEIHEAQVRSDELVQSLN-amide. Peptides were prepared by Dr. Stephen Strittmatter, who generously provided them to us for the purpose of the replication.

EXPERIMENT 1: EARLY TREATMENT

All experimental procedures were as described in Li et al, 2003. Dr. X. Li, the first author of the original paper, traveled to the Reeve Irvine Research Center and performed all spinal cord surgeries in order to ensure that the lesions were done in a way that was comparable to what was done in the original study. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Spinal Cord Surgical Procedures—Mice were anesthetized with Avertin (0.5ml/20g). It should be noted that Li, et. al used ketamine and xylazine as an anesthetic. Our IACUC prefers Avertin, as ketamine and xylazine have been found to result in adverse effects, including seizures, in some mouse strains. We felt that this modification in experimental protocol would be inconsequential for the primary functional and anatomical outcome measures. When supplemental anesthesia was required, one fourth of the original dose was given. Body temperature was maintained by placing mice on a water-circulating jacketed heating pad at $37 \pm 0.5^\circ\text{C}$. The skin over the upper thoracic area was shaved and cleaned with a Betadine solution. The skin was incised, and then the connective and muscle tissue were bluntly dissected to expose T6 and T7 as described (GrandPre et al., 2002). A T6 laminectomy was completed taking care not to damage the spinal cord during the dorsal lamina removal. A dorsal hemisection was performed at T6 with a 30 gauge needle making sure to sever the dorsal and dorso-lateral corticospinal tracts (CSTs). In order to insure that the lesion was complete laterally, the needle was passed through the dorsal part of the spinal cord several times. The goal was to create a lesion that extended down to the central canal.

Reeve-Irvine Research Center personnel performed all laminectomies and closures and Dr. Li performed all dorsal hemisections. After completion of the dorsal hemisections, muscles were sutured with 5-0 chromic gut, and the skin was closed with surgical staples. A total of 31 female C57BL/6 mice (Charles River), 7–8 weeks of age, received dorsal hemisections in the early treatment Replication 1A. A total of 18 female C57BL/6 mice (Charles River), 7–8 weeks of age, received dorsal hemisections in the early treatment Experiment 1B.

Tract tracing—In order to trace the corticospinal tract, tracer injections were made into the right sensorimotor cortex 30–60 minutes after the completion of the spinal cord injuries. For this purpose, mice that had just received spinal cord injuries and were still under anesthesia were passed to a second surgical station, placed in a stereotaxic device, the fur was removed by shaving, the scalp was incised and the skull overlying the sensorimotor cortex was carefully

removed with a dental drill. Miniruby BDA [dextran, tetramethylrhodamine, and biotin (mini ruby): Molecular weight 10,000; 10% in DH₂O (Molecular Probes, Eugene, OR)] was injected into a total of 4 sites (0.4 μ l per site over a 3–5 minute time period) using a 10 μ l Hamilton microsyringe tipped with a pulled glass micropipette. Coordinates were 1.0 lateral, 0.5mm deep to the cortical surface, and +0.5, -0.2, -0.7, and 1.0mm with respect to Bregma. After the injections were completed, the skin overlying the skull was sutured with 4-0 silk.

Early Treatment Experiment 1A: Animals (10–11 per group) received the first intraperitoneal (IP) injection of NEP1-40 Peptide, Reverse Peptide, or Vehicle 2–6 hours after the spinal cord injury. NEP1-40 and the reverse peptide were dissolved in Vehicle (83% PBS plus 17% DMSO) at a concentration of 2.088mg/ml and mice received 100 μ l of the solution. Given that animals weighed approximately 18g, this represents an approximate dose of 11.6 mg/kg. The average times at which mice in the different groups received the first injection were NEP1-40: 5hrs 23 minutes; Reverse Peptide: 5hrs and 2 minutes; Vehicle: 5hrs and 21 minutes. This is slightly more time delay than in Li et al., where the delay was reported as 3–4hrs. The same dose of the peptides or Vehicle was then given once daily for 14 days (100 μ l of solution per injection).

Early Treatment Experiment 1B: The procedure was identical to that in early treatment Experiment 1A, although only NEP1-40 and Vehicle groups were included. Animals received the first dose of NEP1-40 Peptide or Vehicle 3 hours and 51 min on average after the spinal cord injury, range 2 hrs 37 min – 5 hrs 17 min. Average times for the different groups were NEP1-40: 3 hrs 52 minutes (2 hrs, 45 min – 5 hrs); Vehicle: 3 hrs and 51 minutes (2 hrs 37 min – 5 hrs 10 min).

Post Operative Care—Following the surgeries, the mice were immediately placed on a water-circulating jacketed heating pad. After recovering from the anesthetic, animals were housed 4–5 per cage on Alpha-Dri bedding. For 10–14 days after surgery, animals received lactated ringers (5mg/100g, sub-cutaneously) for hydration, the analgesic Buprenex (Buprenorphine, 0.01 mg/kg), and Baytril (Enrofloxacin 2.5mg/kg, sub-cutaneously) for prophylactic treatment against urinary tract infections (UTIs).

Animals were monitored twice daily for general health, coat quality (indicative of normal grooming activity) and mobility within the cage. Injured mice typically resume these activities the day following injury. In addition, signs of paralysis were monitored, including lack of hind limb movement, tail flaccidity, and instability/ uncoordinated movement. Animals were also monitored for signs of skin lesions on the paralyzed limbs or autophagia of the toes. None of the animals exhibited skin lesions or autophagia throughout the experiment. Bladders were manually expressed twice daily for the entire length of the study. Animals were monitored for urinary tract infections (UTIs) for the entire duration of the experiment and no UTIs were observed.

Our animal care protocol calls for the following: if an animal failed to resume normal activities, showed evidence of skin lesions or autophagia, or had symptoms of UTI, the veterinarian was consulted and, if distress continued, the animal was euthanized. In this study no animals were euthanized due to poor health, and no animals exhibited autophagia. We did, however, have an overall mortality rate for the Early Treatment Experiment 1A of 19.4%, with 36% (4 out of 11) of NEP1-40 and 20% (2 out of 9) of the Vehicle control group dying. There were no deaths in the Reverse Peptide group. Mortality rate for Early Treatment Experiment 1B was 17%, with 22% (2 of 9) in the NEP1-40 group and 13% (1 of 9) in the Vehicle group dying. This mortality rate is in line with that reported in the original Li et al study (2003) in which the overall mortality rate was 19.8%. For more details, see Results.

Behavioral Assessment—Functional assessments began on post-operative day 2. Hind limb motor function was assessed with three different types of motor measures: 1) open field locomotion, assessed with the Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB) (Basso et al., 1996) and the Basso Mouse Scale (BMS) (Basso et al., 2006), 2) inclined grid walking, and 3) kinematics, as assessed with ink foot print analysis as well as video kinematic measures.

The BBB is a 21-point scale designed to assess hind limb locomotor recovery after injury to the thoracic spinal cord. This scale provides a measure of hindlimb function ranging from complete paralysis to normal locomotion by assessing hind limb joint movements, stepping, trunk position and stability, forelimb-hindlimb coordination, paw placement, toe clearance, and tail position. All animals had their bladders manually evacuated 5–10 minutes prior to being placed in the open field (150 × 100 cm) for the prescribed 4 minute time period. Rating of hindlimb movement and locomotion was scored simultaneously by two observers who were blind to the treatment groups. BBB assessments for Experiment 1A were made on days 2, 7, 10, 14, 17 and 20 post-injury. BBB assessments for Experiment 1B were made on days 2, 4, 8, 15, 18 and 22 post-injury.

The BMS is a 9-point scale, designed to assess hind limb locomotor recovery after spinal cord injury to the thoracic spinal cord in mice. This scale categorizes combinations of hind limb joint movements, trunk position and stability, stepping coordination, paw placement, toe clearance, and tail position. All animals had their bladders manually evacuated 5–10 minutes prior to being placed in the open field (150 × 100 cm) for the prescribed 4 minute time period. Rating of locomotion was scored simultaneously by two observers who were blind to treatment group. BMS assessments for Experiment 1A were made on days 2, 7, 10, 14, 17 and 20 post-injury. BMS assessments for Experiment 1B were made on days 2, 4, 8, 15, 18 and 22 post-injury.

The inclined grid walking task requires animals to climb a wire grid (35cm long with 2.54 cm squares) at a 45° slope. The number of instances in which the hind paws slipped and fell below the grid (errors) were scored for each of 3 trials in which the animal climbed from the bottom to the top of the grid. All animals were pre-trained for 10 days prior to surgery, and for Experiment 1A were tested on days 10, 13, and 18, 3 trials per time point, post operatively. Animals were tested on days 9, 16, and 21 in the Experiment 1B again for 3 trials per time point. Errors were averaged across the 3 trials at each time point. All researchers involved in data collection were blind to the treatment group.

Kinematic assessments were done in 2 ways. The animals were first tested with the foot print analysis, where the animal's paws were dipped in ink (front paws in red and hind paws in blue) and animals were allowed to walk across a 50 cm runway with paper placed on the bottom. Stride length and stride width for the left and right hind paws were calculated. The animals were tested on post-lesion days 14 and 18 in Experiment 1A (5 trails per time point) and on post-lesion days 7, 14, and 21 in Experiment 1B. A second kinematic measure was made by video taping the animals from underneath while they ambulated across a plexiglass runway. This allows for post-hoc measurements of stride length, stride width, and paw rotation of the hind limbs. The animals were tested on days 13 and 19 in Experiment 1A, and on days 10, 17, and 22 in Experiment 1B.

Euthanasia and tissue collection—Animals were euthanized by anesthetic overdose and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde on post operative day 20 in Experiment 1A and day 23 in Experiment 1B. Spinal cords and brains were removed and immersed in 20% sucrose for cryoprotection. Histological procedures are described below.

EXPERIMENT 2: DELAYED TREATMENT

A total of 29 female C57BL/6 mice (Charles River), 8 weeks of age, received dorsal hemisections, resulting in 8–9 animals per group. The spinal cord surgeries for animals in the delayed treatment group were identical to what is described above for the early treatment Experiments 1A and B, except that mice did not receive BDA injections on the day of the spinal cord surgery.

Seven days post injury, mice were anesthetized and osmotic minipumps (Alzet model 2002) were implanted into the subcutaneous space. The animals received pumps with Vehicle (83% PBS plus 17% DMSO), NEP1-40 in vehicle (0.193mg/ul), or Scrambled Peptide in vehicle (0.193mg/ul). The reagents were delivered at a rate 0.6ul/hr for 14 day at a dose of 11.6 mg•kg⁻¹ • d⁻¹.

Post Operative Care—Post operative care was the same as outlined in Experiment 1 A and B. In this study no animals were euthanized due to poor health, and only one animal exhibited autophagia during the first few days post injury. There was an overall mortality rate in Experiment 2 of 14% pre-treatment (4 of 29) and 12% post-treatment (3 of 24). Thirteen % (1 of 8) of the animals in the Scrambled Peptide group and 22% (2 of 9) of the animals in the Vehicle control group died. No animals in the NEP1-40 group died.

Behavioral Assessment—Assessment of functional recovery began on post operative day 2. Hind limb motor function was assessed using the BBB and BMS scales, inclined grid walking, and video kinematics as in Experiment 1 A, B. All researchers involved in data collection were blind to the treatment group. Animals were assessed for open field locomotor abilities using the BBB and BMS scales on days 2, 4, 8, 15, 18, 22, 28, 37, and 44 post injury. Animals were tested on the grid-climbing task on days 9, 16, 21, 30, and 46 post injury. Foot print kinematics were measured on days 7, 14, 21, 29, and 43 post injury. Video kinematics were measured on days 10, 17, 22, 30, and 43 post injury.

Tract tracing—In order to trace the corticospinal tract, mice were anesthetized 28 days post injury and 10% miniruby BDA was injected into the right sensorimotor cortex, as in Experiment 1 A, B.

Euthanasia and tissue collection—Mice were euthanized at 46 days post injury (18 days post miniruby BDA injection) by anesthetic overdose and were perfused with 4% paraformaldehyde. Spinal cords and brains were collected and immersed in 20% sucrose for cryoprotection.

Histological Assessment—Histological procedures from all experiments, Experiment 1A and B and Experiment 2, were identical. Three tissue blocks were prepared from the spinal cords: 1) a tissue block extending from 5mm above to 5mm below the lesion and containing the injury site; 2) the portion of the spinal cord rostral to the tissue block containing the lesion, extending to the spino-medullary junction; and 3) the portion of the spinal cord caudal to the tissue block containing the lesion, extending to caudal-most segment. The blocks were sectioned on a cryostat at 20μm. The main block containing the lesion was sectioned in the sagittal plane, collecting every section, and maintaining serial order during histological processing. All sections from the block containing the lesion were stained for BDA. The rostral end of the spinal cord above the injury block and the caudal end below the injury block were sectioned transversely. Sets of sections were stained for BDA and 5HT as described below. The brains were sectioned in the coronal plane, and sections taken every 500μm were stained for BDA.

It should be noted that in the original study, tissue blocks were embedded in a glutaraldehyde-polymerized albumin matrix and sectioned on a Vibratome®. Sagittal sections were cut at a thickness of 30µm and transverse sections were cut at a thickness of 50µm. We consider it highly unlikely that these differences in histological procedures could account for the differences in results, but the possibility cannot be completely excluded.

BDA staining: Sections were washed in PBS and 0.1% Triton X-100, incubated for 1 hour with avidin and biotinylated HRP (Vectastain ABC Kit, Vector Labs, Burlingame, CA), washed in PBS, and then reacted with DAB in 50 mM Tris buffer pH7.6, 0.024% hydrogen peroxide and 0.5% nickel chloride.

Cross-sections from the rostral-most block were used to determine the extent of CST labeling above the lesion and the number of BDA-labeled axon arbors that enter the gray matter of the thoracic spinal cord above the lesion (see below). Cross-sections through the caudal segments were used to count axons that extend past the lesion site. The brainstem was sectioned in the coronal plane and stained for BDA allowing counts of BDA-labeled axons in the medullary pyramid.

Quantitative assessments of CST axons—The actual methods used to quantify CST sprouting rostral to the lesion are not explained in the Li and Strittmatter paper. Discussions with Dr. Strittmatter revealed that in their study images were taken at 20X 100 µm lateral and 100 µm ventral to the base of the dCST. Within this field, the image was thresholded so that BDA-labeled fibers were above threshold, and the percent area occupied by BDA labeled fibers was determined. We carried out our analysis in the same way, capturing images that were 150µm in width and 100µm in height. Using image J, the digital image was thresholded so that BDA labeled axon arbors were above threshold, and then the % area occupied by BDA labeled axon arbors was determined.

It is critical in any analysis of this sort to correct measures of axon density (in this case OD values) for the overall amount of CST labeling in order to control for differences in tract tracing efficiency. In our previous studies, we have done this by counting the number of labeled CST axons above the injury in either the medullary pyramid or in the dorsal column. Counting axons in the medullary pyramid is easier because they are not tightly packed, and is advantageous because the pyramid contains the entire CST. In contrast CST axons in the spinal cord distribute into separate tracts, the dorsal CST in the base of the dorsal column and the dorsolateral CST (we have not detected BDA labeled axons in the ventral column in mice (Steward et al., 2004). Consequently, we counted the total number of labeled CST axons in the medullary pyramid in each animal, and then calculated a ratio of the raw OD value (indicative of the number of BDA labeled collaterals)/the total number of CST axons in the medullary pyramid. Thus, the higher the ratio, the more BDA-labeled axon arbors extend from the main tract in segments rostral to the injury.

A potential disadvantage of counting axons in the medullary pyramid is that BDA travels down the axon in a coherent bolus, and there is at least the theoretical possibility that the BDA could actually pass through proximal axons leaving them unlabeled. Therefore, we also took the alternate approach of counting BDA labeled CST axons in segments above the injury. This approach has the advantage that axon counts are made in sections very near the ones in which OD values were taken. For this purpose, thin (1–3µm thick) sections were taken and mounted on microscope slides prior to BDA staining. In these thin sections, individual BDA labeled axons can be distinguished even when packing density is high (as is the case in the main tract). BDA labeled axons in the main CST in the dorsal column were counted under high magnification, and measures of the density of terminal arbors were then normalized based on these counts.

Assessment of serotonergic (5HT) axons—To assess the distribution of serotonergic (5HT) axons, cross-sections were taken from the blocks above and below the block containing the lesion. Free floating sections were washed in PBS. After blocking in 5% normal goat serum in PBS, sections were incubated in primary antibody at 4°C overnight (rabbit anti-serotonin, Sigma S-5545, 1:5000 in PBS) with 5% normal goat serum. The following day, sections were washed in PBS and incubated with the fluorescent secondary antibody (goat-anti-rabbit IgG, AlexaFluor 488, 1:250 in PBS) with 5% normal goat serum and 0.1% or 0.5% Triton X-100 for 2 hours at room temperature. The sections were then rinsed three times in PBS and coverslipped with Kaiser's glycerol jelly.

To quantify 5HT labeled axons, transverse sections from each animal were selected that did not have folds, tears, or other histological or immunostaining artifacts. Digital images of one section rostral and caudal to the injury were captured. Using Image J, the threshold was set so that 5HT positive axons were above threshold, and the percent area occupied by 5HT positive axons was determined in 3 measuring sites on each side of the spinal cord: 1) the gray matter of the dorsal horn; 2) the gray matter of the ventral horn; and 3) the gray matter at the level of the central canal, which at thoracic levels includes the intermediolateral column (the area with the highest density of 5HT positive axons). For these measurements, the gray matter in the dorsal and ventral horns was outlined and a rectangle was placed over the gray matter in the intermediate zone. The sampling area was determined and the percent area occupied by 5HT positive axons on the both sides was averaged to obtain a single value for each site for that section.

Results

Animal attrition

Experiment 1A, Early Treatment—A total of 6 out of 31 animals died over the course of the experiment. Four of these were in the group that was receiving NEP1-40; these animals were found dead in the morning 2, 3, 6, and 11 days post-injury without having shown signs of illness or debilitation the day prior. The other two were receiving Vehicle. One of the mice died as the animal was receiving the Vehicle injection on day 3 post-injury. The other was found dead on the morning of day 2 without showing signs of illness or debilitation on the day prior. Thus, after attrition due to all causes, the group sizes in Experiment 1A were: NEP1-40, n=7; Reverse Peptide; n=9; Vehicle, n=8.

Experiment 1B, Early Treatment—A total of 3 out of 17 animals were found dead in the morning without showing signs of illness or debilitation on the day prior. Two mice in the NEP1-40 died on the night before days 2 and day 4, and one mouse in the Vehicle group, died on the night before day 3. Thus, after attrition due to all causes, the group sizes for Experiment 1B were: NEP1-40, n=7; Vehicle, n=7.

Experiment 2, Delayed Treatment—A total of 8 animals died over the course of Experiment 2. Four died in the 7 day period before osmotic minipumps were implanted. One animal exhibited autophagia during the first days post injury. Autophagia did not continue and euthanasia was not required, but damage to 3 toes prevented inclusion in the behavioral analysis. After minipump implantation, two mice in the Vehicle group and one in the Scrambled Peptide group were found dead in the morning without showing signs of illness on the day prior to death. The two mice in the Vehicle group were found dead on day 10 and 42 post injury. The mouse in the Scrambled peptide group was found dead on day 20. Thus, after attrition due to all causes, total animal numbers at the end of the experiment were: NEP1-40, n=7; Scrambled Peptide; n=7; Vehicle, n=7.

Lesion Extent

Our goal in creating the dorsal hemisection injuries was to transect the spinal cord at a level just below the central canal, extending the lesion laterally at the same level so as to interrupt the dorsal part of the lateral column, and thus the dorsolateral cortical spinal tract (dICST), but sparing the ventral column. This lesion transects all components of the cortical spinal tract (CST) that are labeled by BDA injections into the sensorimotor cortex (because there is no evidence of ventral CST in mice, see below) but spares a large region of ventral white matter. This provides a reasonable setting in which to assess whether blockade of Nogo would enhance axon growth through surviving bridges of white matter or enhance sprouting in gray matter.

Overall the lesions in Experiment 1A were somewhat larger than intended, in that there was damage to the ventral white matter in most of the animals. All of the lesions extended past the central canal, and ballooning of the central canal was evident as is typical when the central canal is damaged. The lesion site was filled with connective tissue, as is typical for spinal cord lesions in mice, and there was minimal if any cavitation. The lesion site was highly compact, so that the intact parenchyma rostral and caudal to the injury was separated by less than 100µm in some mice (see for example, figure 1).

Two animals, #11 (Reverse Peptide group) and #13 (NEP1-40 group) had lesions that were virtually complete transections rather than dorsal hemisections, and so were eliminated from analyses. The predicted effect of inhibiting Nogo is enhanced axon growth through areas containing myelin, especially white matter tracts and areas of gray matter containing myelin. Growth through the lesion site itself was not expected, and was not seen. The lesion in animal #31 (a Vehicle control) also extended into the ventral column, and a cyst was present leaving a reduced white matter bridge for potential regenerating axons. Given the small size of the potential bridge of white matter, this animal is not included in the quantitative comparisons of CST axon distribution in the different treatment groups.

The lesions in Experiment 1B were for the most part consistent with our intentions. The lesions extended just past the central canal and transected the dorsal part of the lateral column on both sides, but spared almost all of the ventral columns. The slight difference in the extent of the lesion in Experiment 1A and 1B probably accounts for the differences in average BBB and BMS scores during the early post-lesion intervals and may also account for the greater degree of CST extension past the lesion site in the animals of Experiment 1B (see below).

Most of the lesions in Experiment 2 were consistent with our intentions, and overall, were similar to the lesions in Experiment 1B. In three animals, the lesions were slightly smaller than intended in that the central canal was spared. The dorsal column and especially the dCST were completely transected in these animals.

Assessment of regeneration and/or sprouting of corticospinal tract (CST) axons

Of the 25 mice in Experiment 1A (early treatment), 23 show excellent labeling of the CST; two other mice show moderate labeling, which was nevertheless sufficient for assessing CST sprouting/regeneration. Of the 15 mice in Experiment 1B (early treatment), 13 show excellent labeling of the CST; one mouse showed moderate labeling, which was sufficient for assessing CST sprouting/regeneration. Of the 23 mice in Experiment 2 (7 day delayed treatment), 20 show excellent labeling of the CST; 3 show moderate labeling, which was sufficient for assessing CST sprouting/regeneration.

Discerning bona fide from artifactual BDA labeling

As we were undertaking this study, we made a discovery that complicates the interpretation of the tract tracing data here. Specifically, we discovered a BDA labeling artifact that can lead to

axonal labeling suggestive of long distance regeneration after spinal cord injury. The artifact is seen when tracer injections are made at the same time as a spinal cord injury, and occurs as the result of leakage of BDA into the cerebrospinal fluid followed by diffusion to the injury site in the spinal cord where axons take up the tracer. As discussed further below, the most obvious manifestation of the artifact is a pattern of labeling that has previously been misinterpreted as evidence of robust regeneration of the CST in NOGO knockout mice (Kim et al., 2003). The discovery of the BDA labeling artifact is of critical importance for the present study because the same tract tracing procedures were used for Experiment 1A and B (BDA injections at the time of the lesion). Indeed, some of the animals in both treated and control groups exhibited what we now recognize as artifactual labeling.

As described elsewhere (Steward et al., 2007) the signs indicating artifactual labeling include: 1) labeling of axons in ectopic locations rostral and caudal to the lesion especially in the lateral column; 2) labeled axons that appear hollow, and that are less reflective in dark field illumination than axons labeled by orthograde transport; 3) a higher level of background labeling around the injury site, labeling of axons on both sides of the lesion, and labeling of neurons and glia at the injury site; and 4) indications that the injection penetrated the cerebral ventricle. In what follows, we first consider the distribution of BDA labeled CST axons in a case that shows none of these signs of artifactual labeling. This case (a Vehicle-treated control) also illustrates the pattern of CST labeling that is characteristic of normal untreated mice with dorsal hemisection injury. We then discuss the artifact and its impact in the present study.

The organization of the CST in mice as revealed by BDA tracing has been described elsewhere (Steward et al., 2004). Following BDA injections into the sensorimotor cortex, the main component of BDA labeled CST axons is in the ventral part of the dorsal column contralateral to the injection, the dCST. A second contingent of axons descends in the dorsal portion of the lateral column (dlCST). In some mice, BDA labeled axons are seen ipsilateral to the injection in the dorsal column and/or the dlCST representing an un-crossed contingent of CST axons.

In experiments involving BDA injections into the sensorimotor cortex in un-injured mice, we have not detected BDA-labeled axons traveling longitudinally in the ventral column ipsilateral to the injection in the position of the ventral CST that is seen in rats. BDA labeled axons can be seen, however extending down into the ventral column from the ventral horn along fingers of gray matter that contain dendrites of motoneurons (Steward et al., 2004).

The distribution of CST axons in a Vehicle-injected control mouse from Experiment 1A is illustrated in Figure 1. In a cross section taken at a high thoracic level rostral to the injury (Fig. 1A&C), BDA labeled axons are prominent in the dCST and dlCST contralateral to the injection (the left side of the spinal cord). There is also a dense plexus of BDA labeled axon arbors throughout the gray matter on the side of the labeled tract. Some labeled collaterals extend to the contralateral side (Fig. 1C). No BDA labeled axons are seen in a cross-section taken below the level of the injury in either the dorsal column, lateral column, or ventral column (Fig. 1B&D) indicating that the CST was completely transected. Sagittal sections of the block containing the lesion site reveal a highly compact lesion extending from the dorsal surface of the spinal cord through the central canal but sparing the ventral columns (Fig. 1E–G). The sagittal section illustrated in Fig. E is taken through the main CST in the dorsal column; Fig. 1F is taken through the gray matter lateral to the main tract, and Fig. 1G illustrates BDA labeled axons in the dlCST. BDA labeled axons in both the main CST in the dorsal column and the dlCST end near the lesion site in swollen balls characteristic of axons that have been amputated. The overall level of background staining around the lesion site is low in the mouse illustrated in Fig. 1, in contrast to what is seen in animals with artifactual labeling. Consistent with the absence of artifactual labeling in this mouse, there was no indication that the tip of the microsyringe penetrated the cerebral ventricle at the cortical injection site (Figure 1G).

Some mice in the present study clearly exhibited labeling that we now recognize as artifactual; the most striking example is illustrated in Figure 2. In cross-sections taken at a high thoracic level rostral to the injury (Fig. 2A&C), the CST and its terminal arbors are heavily labeled, but there are also large numbers of BDA stained axons in the ventral part of the lateral column on both sides (an ectopic location). The axons in ectopic locations were larger than the axons in the main or dL CST (Fig. 2C) and appeared as hollow tubes when viewed at high magnification. In cross-sections taken caudal to the injury, there were no axons that were darkly stained for BDA in either the main tract or the dL CST and no BDA labeled arbors in the gray matter, but there were large numbers of hollow appearing axons in the ventral portion of the lateral column on both sides (Fig. 2B). This is exactly the pattern that is seen when BDA is injected directly into the ventricle soon after a spinal cord injury (Steward et al., 2007).

Sagittal sections containing the lesion revealed prominent labeling of the main CST in the dorsal column and extensive collaterals in the gray matter rostral to the injury (Fig. 2D). These darkly stained axons ended just rostral to the injury site. In addition to the labeling of the CST, there were also BDA labeled axons in the dorsal column caudal to the lesion and in the ventral column beneath the lesion. The axons in the dorsal column caudal to the lesion and in the ventral column appeared more lightly stained than the axons in the dorsal column and the axonal arbors in the gray matter rostral to the injury. There was also a higher level of staining around the injury site including stained cells with a morphology suggestive of both neurons and glia (Fig. 2E). Consistent with the interpretation that some of the labeling is due to uptake of BDA that was present in the cerebrospinal fluid, inspection of the injection site in the cortex revealed evidence that the tip of the microsyringe had penetrated the cerebral ventricle at the time of the cortical injection (Fig. 2F).

The pattern of labeling seen in Figure 2 is clearly artifactual, but there were also cases where distinguishing between artifactual and bona fide labeling was less straightforward. For example, Figure 3 illustrates the BDA labeling pattern in a mouse that received NEP1-40 in which the microsyringe clearly penetrated the cerebral ventricle during the injection (Fig. 3H). The main tract and dL CST are heavily labeled in cross sections rostral to the injury (Fig. 3A) and a few hollow appearing axons are evident in ectopic locations (the ventral column). No BDA labeled axons are evident in cross sections caudal to the injury (Fig. 3B and G). Sagittal sections through the injury site reveal excellent labeling of the CST in the dorsal column and labeled axon arbors in the gray matter rostral to the injury (Fig. 3C). There was minimal labeling of axons in ectopic locations except for a small number of axons in the ventral column that appeared to extend from the gray matter rostral to the injury and continue past the injury in the ventral column (Fig. 3E arrows). These axons appeared more lightly stained than the bona fide CST arbors in the gray matter rostral to the injury, and were less reflective in dark field illumination (Fig. 3D and F), which is characteristic of artifactually labeled axons. Nevertheless, the axons also had exactly the trajectory that would be expected if axons grew from the gray matter rostral to the injury into the ventral column and then past the lesion into caudal segments (see below). Given the presence of some axons that appear to be artifactually labeled and the fact that the microsyringe penetrated the ventricle (Fig. 3H), it cannot be determined whether the BDA labeling of axons in the ventral column is artifactual or real. In the end, we considered the axons that passed ventrally as being artifactually labeled because no BDA labeled axon arbors were detected in segments caudal to the injury, in contrast to other cases described further below.

Overall, in Experiment 1A, artifactual labeling was seen in 4/8 animals in the Vehicle group, 4/10 animals in the Reverse Peptide group, and 3/14 animals in the NEP1-40 group. In Experiment 1B, the incidence of artifactual labeling was much lower because by the time the lesions were made, we had begun to suspect the cause of the artifactual labeling, and took care

to avoid penetrating the ventricle during the injection procedure. Artifactual labeling of a few axons was seen in two Vehicle-treated animals in Experiment 1B.

No artifactual labeling was seen in Experiment 2. This is consistent with other data indicating that BDA injections do not produce artifactual labeling if they are made a few weeks after the spinal cord injuries (Steward et al., 2007) as was the case in Experiment 2.

Comparison of CST distribution across treatment groups

In the original paper by Li et al (2003), the major difference between treatment groups was in the density of axon arbors extending from the main CST into the gray matter in segments rostral to the injury. In their untreated control mice, there were few axons extending from the main CST laterally whereas abundant collaterals were seen in mice that received NEP1-40.

In the present experiment, mice in all of the treatment groups had large numbers of axons arbors extending into the gray matter from the main dCST. That is, all mice exhibited the pattern that Li et al saw exclusively in mice treated with the NEP1-40 peptide. For example, Figure 4 illustrates examples of the pattern of BDA labeling in segments rostral to the injury in 4 Vehicle-injected control mice and 4 mice that received NEP1-40 in Experiment 2 (chosen because of the absence of artifactual labeling).

As in Li et al, we compared the density of axon arbors in the gray matter using raw OD values. In Experiment 1A, OD values were lowest in the Vehicle treated group, slightly higher in the NEP1-40 group, and highest in the Reverse Peptide group (Figure 5A). Analysis by ANOVA revealed no significant differences between groups, but also that variances differed significantly. Thus, the data were analyzed by Kruskal-Wallis, which also revealed no significant differences between groups. In Experiment 1B, OD values were nearly identical in the Vehicle treated group and NEP1-40 groups (Fig. 5B). In Experiment 2, OD values were essentially identical in the Vehicle treated and NEP1-40 groups and slightly higher in the Scrambled Peptide treated group (Fig 5C). Analysis by ANOVA revealed no significant differences between groups but also revealed that variances differed significantly. Thus, the data were analyzed by Kruskal-Wallis, which again revealed no significant differences between groups.

Assessing CST arbor density by raw OD values has the disadvantage that the values depend on the overall level of labeling of the CST. To control for differences in overall labeling efficiency, we normalized the OD values by counting the number of labeled CST axons in two locations, the medullary pyramid and the dorsal column rostral to the injury site. The raw OD values were then divided by these axon counts to obtain labeling ratios.

The labeling ratios obtained by dividing by the number of labeled axons in the medullary pyramid are illustrated in Fig. 5D–F. In Experiment 1A, the labeling ratio was lowest in the Vehicle treated group, slightly higher in the Scrambled Peptide group, and highest in the NEP1-40 group (Figure 5D). In Experiment 1B, the labeling ratio was lowest in the Vehicle treated group, and highest in the NEP1-40 group. In Experiment 2, however, the labeling ratio was highest in the Vehicle control group. Analysis by ANOVA and Kruskal-Wallis again revealed no significant differences between treatment groups.

The labeling ratios obtained by dividing by the number of labeled axons in the dCST are illustrated in Fig. 5G–I. In Experiment 1A and B, the labeling ratios were essentially identical across groups. In Experiment 2, labeling ratios were lowest in the Vehicle group, slightly higher in the NEP1-40 group, and highest in the Reverse Peptide group. Analysis by ANOVA and Kruskal-Wallis again revealed no significant differences between treatment groups.

In the original paper, Li et al. used student's t-tests to compare CST arbor density in Vehicle-treated vs. NEP1-40 treated groups. They do not report using a Bonferroni correction to control for multiple t-tests. There were 3 groups in our studies, and so the student's t-test is not appropriate. Nevertheless, to fully replicate the details of the original study, including the statistic used, we compared Vehicle-treated vs. NEP1-40 groups using the student's t-test. Again, there were no significant differences between Vehicle-treated and NEP1-40-treated groups in any of the experiments.

Given that the conditions of Experiments 1A and B were comparable, we also combined the data from the two experiments to increase statistical power. Using these data, the labeling ratios obtained by dividing by the number of labeled axons in the medullary pyramid were significantly different by two tailed student's t-test that assumed equal variance ($p=.04$), but not by a student's t-test that took into account the significant difference in variance between groups ($p=.06$).

Extension of CST axons past the lesion

In the course of these experiments, we made the surprising discovery that control mice exhibit a capacity for CST regeneration that has not previously been appreciated. Specifically, following lesions of the dorsal column, axons extend past the lesion via the ventral column and form elaborate terminal arbors in segments caudal to the injury (Steward et al., submitted). We made this discovery in our analysis of the control (vehicle injected) mice from the present study and of heterozygous littermate controls for Nogo knockout mice in another series of experiments. Given the hypothesis that elimination of inhibition by Nogo would enhance axon growth in myelin containing regions, it was of interest to evaluate whether this regenerative growth response was enhanced in mice treated with NEP1-40.

In Experiment 1 A and B, only two mice exhibited BDA labeled axons extending beyond the lesion in the ventral column and/or BDA labeled axonal arbors caudal to the lesion. The most dramatic was a mouse treated with NEP1-40 (Figure 6). Overall labeling of the CST was excellent in this mouse (Fig. 6A&D). There were no labeled axons in the dCST, dlCST or ventral column caudal to the injury documenting that the lesion completely transected descending CST axons (Fig. 6B). Nevertheless, inspection of serial sagittal sections revealed that ten BDA labeled axons extended past the lesion site in the medial part of the ventral column (Fig. 6E, arrows). The axons that bypassed the lesion via the ventral column could be traced back into the gray matter rostral to the injury, but it was not possible to determine whether they originated from cut axons in the dCST or from terminal arbors in the gray matter. Most of the axons extended ventrally and caudally from the gray matter, turned caudally, and then re-entered the gray matter caudal to the lesion. Striking BDA labeled axon arbors were also seen in the gray matter caudal to the injury in this mouse (Fig. 6F and G), and 2 BDA labeled axons were seen in cross sections taken about 4mm below the injury (Fig. 6C). A complete reconstruction of the axon arbors that extend beyond the lesion is illustrated in the drawing in Fig. 6H, which is a composite of the axons seen in the collection of sagittal sections.

The only other mouse in Experiment 1A with axons extending beyond the injury was one of the mice treated with the Reverse Peptide, in which six axons were seen extending past the lesion. In this mouse, there were fewer BDA labeled axon arbors in the gray matter caudal to the lesion, but the overall level of BDA labeling was also less extensive. In both of these cases, the lesion did not extend into the ventral column, and there was no evidence of scar tissue or cysts in the ventral column ventral to the lesion site.

In Experiment 1B, BDA labeled axons were seen extending past the lesion in the ventral column in 1/7 Vehicle treated controls and 4/6 NEP1-40 treated mice. Thus, the overall incidence of BDA labeled axons extending past the lesion in Experiments 1A and B was 1/12 in the Vehicle-

treated control group, 5/12 in the NEP1-40 treated group, and 1/6 in the Reverse Peptide treated group.

In Experiment 2, BDA labeled axons were seen extending beyond the lesion in the ventral column in 4/7 Vehicle treated control mice, 5/6 NEP1-40 treated mice and 4/7 mice that received the Scrambled Peptide. As in Experiment 1A and B, only a small number of axons extended past the lesion, however, despite the extensive labeling of the CST rostral to the lesion. It was noteworthy that the BDA labeled arbors in the gray matter in caudal segments were considerably more extensive than in the mice in Experiment 1 A and B. Examples from each of the 3 treatment groups are shown in Figure 7, Figure 8, and Figure 9. In each of these cases, the lesions completely transected the dCST and lCST.

It is again important to note that in un-injured mice, we have not seen evidence of a ventral CST (that is, BDA labeled axons extending longitudinally in the ventral column). Thus, the profiles illustrated in Fig. 7, Fig. 8, and Fig. 9 are unique to injured animals. In some cases, it was possible to follow BDA labeled axons through serial sections from the gray matter rostral to the lesion into the ventral column and past the lesion (Fig. 7A–D) and then back into the gray matter caudal to the injury (Fig. 7E&F) where they formed complex arbors (Fig. 7F). Importantly, all of the axons that passed the lesion traveled in the ventral column ipsilateral to the labeled dCST. This is of considerable importance because the ventral CST seen in rats and other species is an un-crossed pathway whereas the main tract in the dorsal column (the dCST) is crossed. Thus the axons that pass the lesion in the ventral column are on the wrong side to be ventral CST axons.

There were no apparent differences in the number or distribution of axons that passed the lesion in the different treatment groups. Indeed, the most elaborate arbors caudal to the lesion were seen in a mouse that received the Scrambled Peptide (Fig. 9).

Assessment of 5HT axons

The distribution of 5HT labeled axons in segments rostral to the injury was similar to what is seen in un-injured control animals (Figure 10A). A dense plexus of axons was present in the gray matter of the intermediolateral column (IML), and labeled axons were distributed more diffusely throughout the ventral horn. There were few labeled axons in the dorsal horn. In segments caudal to the injury, the overall density of 5HT labeled axons was much less, and labeled axons were seen only in the ventral horn (Figure 10B).

Quantitative analyses confirmed the reduction in the density of 5HT labeled axons in caudal segments consistent with depletion as a consequence of the injury (Figure 10, note differences in scale between graphs of data from rostral and caudal sites). The graphs show the density of 5HT axons in the dorsal horn, intermediate zone (which at thoracic levels is the IML) and ventral horn. There were no statistically significant differences in the density of 5HT labeling in caudal segments in the different treatment groups in any of the experiments (Figure 10).

Functional assessments

Experiment 1A—As noted above, two animals, #11 (Reverse Peptide group) and #13 (NEP1-40 group) had lesions that were too large, being virtually complete transections rather than dorsal hemisections. The BBB scores of these mice on the final day of testing were 1 and 0.5, consistent with complete or near complete transections. The lesion in mouse #31 was also too large, and there was a large cyst in the ventral column. Interestingly, however, the BBB score for animal #31 on the final day of testing was 10, which is identical to the group average. Data from all three of these animals are excluded from the averages in the quantitative analyses with the result that the average BBB scores for the Reverse Peptide group and NEP1-40 groups

are slightly higher than if all animals are included whereas the average score for the Vehicle control group is un-changed.

Hindlimb locomotor function as measured by the BBB scale: Figure 11 illustrates BBB scores for animals in the 3 treatment groups in Experiment 1A across post-injury days. BBB scores for animals in the NEP1-40 peptide group were higher than the scores for the Vehicle-treated controls on testing days 7, 10, and 13, but not on days 17 and 20. Surprisingly, BBB scores for animals that received the control Reverse Peptide were also higher on these days.

A repeated measures ANOVA revealed a main effect of group ($F=5.1(2,95)$, $p<0.05$), a main effect of days post injury ($F=237(5, 95)$, $p<0.0001$) and a group by days post injury interaction ($F=3.1(10, 95)$, $p<0.005$). Fisher's PLSD revealed a significant difference between the NEP1-40 and Vehicle groups, $p<0.01$, and between the Reverse Peptide and Vehicles groups, $p<0.05$, with the NEP1-40 and Reverse Peptide groups showing higher BBB scores. Day-by-day comparisons revealed a significant difference between groups on all days post injury except day 17 and day 20 ($p<0.01$). The Vehicle group exhibited lower BBB scores across days post injury than either the NEP1-40 or Reverse Peptide groups.

Hindlimb locomotor function as measured by the BMS scale: The same general pattern of results was evident for the scores from the BMS assessment (Figure 11). Scores for animals in the NEP1-40 peptide group were higher than the scores for the Vehicle-treated controls on testing days 7, 10, and 13, but not on days 17 and 20; the same was true of the scores for the control Reverse Peptide group. A repeated measures ANOVA revealed a main effect of days post injury ($F=95.9(5,70)$, $p<0.0001$) and a group by days post injury interaction ($F=2.2(10, 70)$, $p<0.05$). Day-by-day comparisons revealed a significant difference between groups on all days post injury except days 10 and 13 and days 17 and 20 using Fisher's PLSD, $p<0.01$. The Vehicle group demonstrated lower BMS scores across days post injury than either the NEP1-40 or Reverse Peptide groups.

Inclined grid walking, footprint, and video kinematic analyses: There were no significant differences between groups at any time point in the inclined grid test (Figure 12A) footprint analysis (Fig. 12B&C). For the video kinematic analysis (Fig 12D&F), there were no significant differences between groups on day 14, but the vehicle group had a significantly wider stance on day 19. A wider stance reflects abnormal foot placement, and so the two treated groups showed better function than the Vehicle control.

Experiment 1B

Hindlimb Locomotor function as measured by the BBB scale: BBB scores across days were comparable in the NEP1-40 and Vehicle-treated control groups (Figure 11). A repeated measures ANOVA revealed no difference between groups and no group by days post injury interaction. There was the expected difference between days post injury, $F=38(5, 55)$, $p<0.0001$, as the animals recover locomotor abilities.

Hindlimb locomotor function as measured by the BMS scale: As with the BBB, BMS scores across days were comparable in the NEP1-40 and Vehicle-treated control groups. A repeated measures ANOVA revealed no difference between groups and no group by days post injury interaction. There was the expected difference between days post injury, $F=25.9(5, 55)$, $p<0.0001$.

Inclined grid walking, footprint, and video kinematic analyses: There were no significant differences between groups in the inclined grid walking task, footprint analysis, or kinematic video analysis.

Comparison of Experiment 1A and B—Functional assessments were carried out on different post-injury days in the two replications. BBB assessments in Experiment 1A were made on days 2, 7, 10, 14, 17 and 20 post-injury, and on days 2, 4, 8, 15, 18 and 22 post-injury in Experiment 1B. Nevertheless, it is reasonable to compare scores for the two replication experiments on particular days (day 2) and between time points that differ by only 1 day (days 7 vs. 8; days 14 vs. 15; and days 17 vs. 18). These pair-wise comparisons revealed substantial differences between BBB scores for Experiment 1A vs. 1B ($p < 0.05$) for both NEP1-40 and Vehicle groups, as well as when the data were collapsed across the group for each experiment, $p < 0.01$. The reason for the discrepancy is not clear because the same surgeon produced the lesions for both groups and the same individuals carried out the BBB analysis. Given this disparity, it is our opinion that the data from the two groups cannot be combined, and that experiment 1A and 1B should be considered separate and independent replications.

While there was no significant difference on the second to last days tested, Day 17 Experiment 1A vs. day 18 Experiment 1B, there was a difference between the Vehicle groups from Experiment 1A and Experiment 1B on the last day of testing, day 20 Experiment 1A vs. Day 22 Experiment 1B, $p < 0.01$. Moreover, when the groups from each experiment are collapsed, there is a difference between replications, $p < 0.05$.

The BMS analysis demonstrated similar difference between Experiment 1A and 1B. On Day 2, the first testing day, when the data is collapsed across groups for each replication, BMS scores for animals in Experiment 1B were higher than the scores in Experiment 1A, $p < 0.01$. The two Vehicle groups also differ significantly, $p < 0.05$. On the last day of testing, day 20 Experiment 1A vs. Day 22 Experiment 1B, there was a significant difference between Vehicle groups, $p < 0.01$, and a significant difference when the data is collapsed across group for each replication, $p < 0.05$, although there was no difference between NEP1-40 groups.

Experiment 2, Delayed Treatment

Hindlimb locomotor function as measured by the BBB scale (Figure 11): A repeated measures ANOVA revealed no effect of group and no group by days post injury interaction. There was the expected difference between days post injury, $F = 17.2$ (8,144), $p < 0.0001$, as the animals recover some locomotor abilities.

Hindlimb locomotor function as measured by the BMS scale (Figure 11): A repeated measures ANOVA revealed no effect of group and no group by days post injury interaction. There was the expected difference between days post injury, $F = 19.9$ (8,136), $p < 0.0001$, as the animals recover some locomotor abilities.

Inclined grid walking, footprint, and video kinematic analyses (Figure 12): There were no significant differences between groups on the inclined grid walking task, footprint analysis, or video kinematic analysis.

Discussion

The present study assessed the reproducibility and robustness of the findings of Li et al (2003) that treatment with the NgR antagonist peptide NEP1-40 results in enhanced growth of corticospinal and serotonergic axons and enhanced locomotor recovery after thoracic spinal cord injury in mice. Our anatomical analyses did not support the interpretation that treatment with NEP1-40 robustly enhanced regenerative growth of CST axons or 5HT axons. Similarly, the various functional assessments also did not support the interpretation that treatment with NEP1-40 consistently enhanced recovery of hindlimb motor function. Mice in all treatment groups exhibited a form of regenerative growth involving extension of CST axons around the lesion via the ventral column. This form of regenerative growth appears to be slightly enhanced

(or at least accelerated) in mice that received NEP1-40. In what follows, we begin by considering critical technical issues regarding the study, and then the implications.

Impact of the BDA labeling artifact

During the course of this study, we discovered a BDA labeling artifact that can occur in the experimental paradigm used for Experiment 1 in which mice received intracortical BDA injections at the time of a spinal cord injury (Steward et al., 2007). The artifactual labeling occurs when BDA leaks into the cerebral ventricle and diffuses in the CSF to the injury site. Axons near the injury site take up the tracer, and then appear to be labeled via orthograde transport.

In its most obvious form, the artifact involves labeling of large axons in the lateral column, which is an ectopic location for CST axons. A number of mice in our experiments did exhibit this artifactual labeling, and were excluded from the quantitative histological analyses. None of the mice illustrated in Li et al (2003) showed the obvious form of the artifact, however.

Importantly, we cannot exclude the possibility of artifactual labeling of axons in the normal location of the CST (for example the gray matter or the ventral column near the lesion site as in Figure 3). For example, we took the conservative view that the labeling seen in the mouse in Figure 3 was artifactual because there was labeling of a few large axons in ectopic locations (the lateral column), the micropipette clearly penetrated the ventricle during the intracortical injection, and the thin axons in the ventral column were less reflective in dark field than bona fide labeled axons in other locations. We cannot exclude the possibility, however, that the labeled axons seen in the ventral column were in fact CST axons labeled by orthograde transport. Clearly, the strategy of making BDA injections at the time of a spinal cord injury is problematic because it is impossible to be certain that labeling is not artifactual, including the labeling of CST arbors in the gray matter. For this reason, the measures of the density of CST arbors in the gray matter may also be problematic in animals where there is artifactual labeling. Importantly, the artifact was not seen in experiments in which BDA injections were made several weeks after the spinal cord injury (Experiment 2), so that the data from these experiments can be interpreted with greater confidence.

Assessment of CST sprouting in segments rostral to the injury

Li et al. (2003) reported that mice treated with NEP1-40 exhibit striking sprouting of CST axons in segments rostral to the injury. This conclusion was based on differences in the density of CST arbors in the gray matter. In un-treated mice, few arbors extended from the main CST in the dorsal column whereas profuse arbors were seen in mice treated with NEP1-40. A major difference between our results and theirs was that in the present study, both control and treated mice exhibited profuse arbors in the gray matter. The reasons for this discrepancy are not clear. The pattern of labeling in the control mice in Li et al. is puzzling, because in a previous paper from Strittmatter's group that used the same tracing procedures, there were substantial numbers of arbors extending from the main tract in mice that were the wildtype littermate controls for Nogo knockout mice (Kim et al, 2003). In fact, the pattern of labeling shown in Kim et al in control mice was more similar to the pattern interpreted as extensive sprouting in mice treated with the NEP1-40 peptide in Li et al (2003). This discrepancy was discussed with Dr. Strittmatter, who indicated that in other experiments from his lab, arbors are seen extending from the dCST in control mice when injections are made more laterally into the cortex (that is, more lateral than 1mm). We used the same coordinates for the injections that Li et al reported (1mm lateral), but it is possible that the injected BDA spread further laterally in our experiments.

Quantitative assessments of the density of CST arbors revealed trends that did not reach statistical significance. Direct comparisons of CST axon density revealed no significant differences between groups. When raw density values were expressed as a ratio of the number of labeled axons in the CST, and results from different experiments were combined, one of the pair-wise comparisons by the Student's t-test revealed differences that were significant at $p=.04$ assuming equal variances. Variances were not equivalent, however, and comparisons with the Student t-test taking unequal variance into account just missed significance $p=.06$. Li et al. (2003) compared values with the Student t-test (there is no statement about whether variance is equal between groups). The fairest conclusion is that we see a trend similar to theirs (94% probability of a difference between groups), but we certainly do not see the robust difference in CST arbor density that they report.

One final point regarding the quantitative analyses and the comparison between studies should be noted. Li et al (2003) carry out the quantitative analyses of CST axons using "...sections from the first five to seven animals processed". We analyzed all of the animals that had appropriate lesions that did not exhibit artifactual BDA labeling. There was no explanation of why the analysis in Li et al. was limited to only some of the animals. There is no obvious reason why this should have resulted in a sampling bias, but in seeking reasons for the differences in results, all differences in procedure are important to note.

Li et al. also report a higher density of 5HT axons in caudal segments in NEP1-40 treated mice. The actual method used to assess 5HT axons was not explained in the original report, but discussions with the authors revealed that digital images were thresholded, and then the "skeletonize" function of the NIH image was used to calculate total fiber length. The values reported here are raw 5HT axon density (that is, the skeletonize function was not applied). It is unlikely that this accounts for our failure to see any hint of increased 5HT axon density in NEP1-40 mice, but this possibility cannot be completely excluded.

Regenerative growth of CST axons via the ventral column

The conclusion that CST axons have actually regenerated past the lesion via the ventral column requires that the possibility of axon sparing be rigorously excluded. In this case, it is very unlikely that these are spared axons. First, we have not seen CST axons extending through the ventral column in normal mice. Second, the axons in injured animals are ipsilateral to the main tract, whereas axons in a ventral CST (if it was present) should be contralateral to the main tract (and ipsilateral to the cortex of origin). Third, the axons that extend past the lesion take a meandering course. Finally, the number of axons passing the lesion in the ventral column is higher in the mice that were killed at longer intervals after the lesion (Experiment 2). Thus, these axons meet most of the criteria for regenerating axons (Steward et al., 2003).

The form of regenerative growth involving extension of CST axons past the lesion via the ventral column occurs in control mice (Steward et al., submitted), but the proportion of mice with CST axons extending past the lesion via the ventral column was higher in the NEP1-40 groups that received early treatment. This is consistent with the interpretation that treatment with NEP1-40 enhances regenerative axon growth through white matter tracts. In experiment 2, however, mice in all treatment groups exhibited CST axons passing the lesion via the ventral column. In this regard, our findings differ from those of Li et al (2003). Thus, the effect of treatment with NEP1-40 may be to accelerate a form of growth that normally occurs rather than enabling growth that would otherwise be impossible. Interestingly, recent studies have shown that acute application of myelin-derived ligands are less effective in causing growth cone collapse in neurons deficient in Nogo-66 receptor 1 whereas chronic presentation of substrate bound ligand (OmGP) is effective in blocking neurite outgrowth regardless of the presence or absence of Nogo-66 receptor 1 (Chivatakarn et al., 2007). One interesting speculation is that activation of the Nogo-66 receptor 1 might play a role in causing growth

cone collapse during the acute post-injury period when myelin proteins are exposed as myelin degenerates, and play a minimal role if any in chronic axon growth inhibition.

The conclusion that CST axons have actually regenerated past the lesion via the ventral column requires that the possibility of axon sparing be rigorously excluded. In this case, it is very unlikely that these are spared axons. First, we have not seen CST axons extending through the ventral column in normal mice. Second, the axons in injured animals are ipsilateral to the main tract, whereas axons in a ventral CST (if it was present) should be contralateral to the main tract (and ipsilateral to the cortex of origin). Third, the axons that extend past the lesion take a meandering course. Finally, the number of axons passing the lesion in the ventral column is higher in the mice that were killed at longer intervals after the lesion (Experiment 2). Thus, these axons meet most of the criteria for regenerating axons (Steward et al., 2003). Taken together, these facts make a very strong case that CST axons do regenerate past the lesion.

Treatment with NEP1-40 does not reliably and robustly enhance functional recovery

In one of the two experiments involving early treatment, mice treated with either NEP1-40 had higher BBB and BMS scores than vehicle-treated controls at the early post-injury testing intervals, but scores converged at later intervals. Interestingly, mice treated with the reverse peptide also had higher locomotor scores. Differences between NEP1-40 and vehicle-treated controls were not seen in Experiment 1B, however. Similarly, there was no difference in locomotor scores between treated and control groups in Experiment 2, in which treatment was initiated 1 week post-injury. Thus, our results fail to replicate the findings of Li et al in terms of the beneficial effect of delayed treatment with NEP1-40 on locomotor recovery.

The lesions in Experiment 1A were slightly larger than the lesions in Experiment 1B, and the impairment of locomotor function over the first few testing days was greater in Experiment 1A. It is noteworthy that the initial deficits in Experiment 1A were more comparable to the deficits of the mice in Li et al (2003). One possible explanation for the differences between Experiments 1A and 1B, and between our results and those of Li et al, is that the effect of NEP1-40 treatment is seen only with lesions of a particular level of severity. In any case, our results indicate that treatment with NEP1-40 does not robustly and reliably enhance functional recovery even when initiated early after the lesion.

Our experiments also failed to replicate the findings of Li et al on other functional outcome measures. This was true for both Experiment 1, involving early treatment, and Experiment 2 in which treatment was delayed.

Conclusion

Our experiments overall failed to replicate the findings of Li et al regarding enhanced sprouting and regeneration of the CST and the serotonergic system. Nevertheless, when taken together, our results do suggest that treatment with NEP1-40 created a situation that was slightly more conducive to a form of axon regeneration or sprouting that also occurs in un-treated mice. Similarly, we failed to replicate the findings of enhanced functional recovery, although there was a trend for enhanced locomotor recovery at early intervals in Experiment 1A. The failure to replicate is not likely to be due to major procedural differences. We made every effort to carry out the experiments in the same way as Li et al. The same surgeon created the lesions (S. Li); the same peptides were used (provided by Dr. Strittmatter); the same strain of mouse was used; and the same analyses and testing procedures were used. The only notable procedural difference was in the method of sectioning (we took frozen sections; Li et al took Vibratome sections). We feel that it is unlikely that this difference could account for the differences in results, but this possibility cannot be completely excluded. Thus, the exact treatment used here does not robustly and reliably enhance regenerative growth as measured by the techniques used

or functional improvement in the assays used. It is likely that there are unidentified experimental variables that account for the different outcomes. Identifying these will be the key to making therapeutic strategies using NEP1-40 more reliable and robust.

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Figure 1. Distribution of CST axons in a Vehicle-injected control mouse from Experiment 1A
 A) Cross section taken at a high thoracic level rostral to the injury. BDA labeled axons are prominent in the dorsal CST (dCST) and dorso-lateral CST (dlCST) contralateral to the injection (the left side of the spinal cord). B) Cross section taken at a high lumbar level caudal to the injury. Note the absence of BDA labeled axons in either the dorsal column (dc), dorsal part of the lateral column, or ventral column (vc). C) Higher magnification view of the section illustrated in A. D) Higher magnification view of the section illustrated in C. E–G) Sagittal sections of the block containing the lesion site. E&F are two adjacent sections through the main labeled tract in the dCST. G is taken through the lateral column to reveal the dlCST. H) injection site in the sensorimotor cortex.

Figure 2. Artifacts of BDA labeling of axons by BDA. The panels illustrate both bona fide orthograde labeling of CST projections and axons labeled as a result of the artifact

A) Cross section taken at a high thoracic level rostral to the injury. Bona fide BDA labeled axons are prominent in the dorsal CST (dCST) and dorso-lateral CST (dICST) contralateral to the injection (the left side of the spinal cord). Artifactually labeled axons are present in the ventral part of the lateral column on both sides and in the ventral column (arrows). B) Cross section taken at a low thoracic level caudal to the injury. Note the absence of BDA labeled axons in either the dorsal column, dorsal part of the lateral column, or ventral column. C) Higher magnification view of the section illustrated in A. Artifactually labeled axons are indicated by the unlabeled arrow. D) Sagittal section of the block containing the lesion site. This section is taken through the main labeled tract in the dCST. Artifactually labeled axons are indicated by the arrow. E) Higher magnification view of the section illustrated in D. F) Injection site in the sensorimotor cortex.

Figure 3. BDA labeling pattern in a mouse that received NEP1-40 in which the microsyringe penetrated the cerebral ventricle during the injection

A) Cross section taken at a high thoracic level rostral to the injury. Bona fide BDA labeled axons are prominent in the dorsal CST (dCST) and dorso-lateral CST (dlCST) contralateral to the injection (the left side of the spinal cord). Artfactually labeled axons are not obvious although a few hollow appearing axons are present in the ventral column (not shown). B) Cross section taken at a high lumbar level caudal to the injury. Note the absence of BDA labeled axons in either the dorsal column, dorsal part of the lateral column, or ventral column. C&D) Bright field and dark field photomicrographs of a sagittal section through the injury site. E&F) higher magnification views of the ventral column below the lesion site. Note that the BDA

stained axons in the ventral column seen in bright field illumination (E) are not seen with dark field illumination (F). H) Injection site in the sensorimotor cortex. Note BDA staining of periventricular structures (arrow) indicating that the microsyringe penetrated the ventricle.

Figure 4. Examples of the pattern of BDA labeling in segments rostral to the injury
 The panels show the labeling patterns in 4 Vehicle-injected control mice and 4 mice that received NEP1-40 from Experiment 2.

Figure 5. Quantitative assessments of the density of CST axon arbors in the gray matter rostral to the injury

A–C) illustrate comparisons of the % area occupied by BDA labeled CST axons. D–F) illustrate the percent area values normalized to the number of BDA labeled CST axons in the medullary pyramid. G–I) illustrate the percent area values normalized to the number of BDA labeled CST axons in the dorsal CST rostral to the lesion.

Figure 6. Extension of CST axons past the lesion via the ventral column in a mouse from Experiment 1A treated with NEP1-40

A) Distribution of BDA labeled CST axons in a cross section taken at a high thoracic level rostral to the injury. B) High power view of the dorsal column (dc) and ventral column (vc) from a cross section taken caudal to the injury. Note the absence of BDA labeled axons in the main tract. C) High power view of the lateral column (lc) and ventral horn (vh) from the same section shown in B. Note one BDA labeled axon in the lateral column and one axon arbor in the ventral horn. D) Sagittal section through the block containing the lesion site at the level of the labeled dorsal CST (dCST). E) Sagittal section taken from very near the midline showing BDA labeled axons extending past the lesion in the ventral column. F&G) BDA labeled axonal

arbors in the gray matter caudal to the lesion. H) Reconstruction of the axon arbors that extend beyond the lesion. This is a composite of the axons seen in the collection of sagittal sections.

Figure 7. Extension of CST axons past the lesion via the ventral column in a Vehicle-treated mouse from Experiment 2

A) Sagittal section through the block containing the lesion site at the level of the labeled dorsal CST (dCST). Arrow indicates axon extending from the gray matter into the ventral column. B) higher magnification view of the section shown in (A). Arrows indicate axon extending from the gray matter into the ventral column. C&D) Axons extending past the lesion; E&F) Axons re-entering the gray matter caudal to the lesion. G) Reconstruction of the axon arbors that extend beyond the lesion. This is a composite of the axons seen in the collection of sagittal sections. The scale bar in (B) applies to (C–F).

Figure 8. Extension of CST axons past the lesion via the ventral column in a mouse from Experiment 2 treated with NEP1-40

A) Sagittal section through the block containing the lesion site at the level of the labeled dorsal CST (dCST). Arrow indicates axon extending from the gray matter into the ventral column. B&C) Sagittal sections taken more laterally through the gray matter. Note elaborate BDA labeled arbor caudal to the lesion in (C). D) Higher magnification view of the elaborate arbor caudal to the injury from the section shown in (C). E) Reconstruction of the axon arbors that extend beyond the lesion. This is a composite of the axons seen in the collection of sagittal sections. The scale bar in (A) applies to (B–C) also.

Figure 9. Extension of CST axons past the lesion via the ventral column in a mouse from Experiment 2 treated with the scrambled peptide

A) Sagittal section through the block containing the lesion site at the level of the labeled dorsal CST. Arrow indicates axon extending from the gray matter into the ventral column. Note elaborate BDA labeled arbor caudal to the lesion; “c” indicates the area shown in panel C. B) Sagittal section taken more laterally through the gray matter; “d” indicates the arbor shown in panel D. E) Reconstruction of the axon arbors that extend beyond the lesion. This is a composite of the axons seen in the collection of sagittal sections.

Figure 10. Assessment of 5HT labeled axons in segments rostral and caudal to the injury
 A) Distribution of 5HT stained axons in a cross section taken at a high thoracic level caudal to the injury. INT= intermediate gray matter, which at thoracic levels is the location of the intermediolateral column. B) distribution of 5HT labeled axons in a cross section taken at a high lumbar level caudal to the injury. Graphs show the quantitative assessment of 5HT axon density rostral and caudal to the injury in the different experiments. Note differences in scale between graphs of data from rostral and caudal sites. There were no statistically significant differences in the density of 5HT labeling in caudal segments in the different treatment groups in any of the experiments.

Figure 11. Hindlimb locomotor function as measured by the BBB and BMS scales in the different experiments

The graphs illustrate the average BBB and BMS scores for the different groups over time after the lesion.

Figure 12. Inclined grid walking, footprint, and video kinematic analyses
The graphs illustrate the scores for the different groups in the 3 tasks.