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Assessment of NgR1 Function *In Vivo* After Spinal Cord Injury

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Abstract

Background: Neuronal Nogo-66 receptor 1 (NgR1) has attracted attention as a converging point for mediating the effects of myelin-associate inhibitory ligands in the CNS, establishing the growth restrictive environment, and limiting axon regeneration following traumatic injury.

Objective: The importance of NgR1 has been undermined by several studies that have shown the lack of substantial axon regeneration following spinal cord injury (SCI) in NgR1 knockout or knockdown animal models. This study aims to investigate the factors that may be contributing to the discrepancy.

Methods: We used mice carrying either a homozygous or heterozygous null mutation in the NgR1 gene and subjected them to either a moderate or severe SCI.

Results: Locomotor function assessments revealed that the level of functional recovery is affected by the degree of injury suffered. NgR1 ablation enhanced local collateral sprouting in the mutant mice. Reactive astrocytes and chondroitin sulfate proteoglycans (CSPGs) are upregulated surrounding the injury site. MMP-9, which has been shown to degrade CSPGs, was significantly upregulated in the homozygous mutant mice compared to the heterozygous or wild-type mice. However, CSPG levels remained higher in the homozygous compared to the heterozygous mice, suggesting that CSPG-degrading activity of MMP-9 may require the presence of NgR1.

Conclusion: Genetic ablation of NgR1 may lead to significant recovery in locomotor function following SCI. The difference in locomotor recovery we observed between the groups that suffered varying degrees of injury suggests that injury severity may be a confounding factor in functional recovery following SCI.

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Keywords

Nogo receptors; spinal cord injury; axonal regeneration; animal studies

INTRODUCTION

Each year in the United States, over 10,000 new cases of spinal cord injury (SCI) are reported.¹ Despite new treatment regimens, the functional recovery of patients is far from ideal. One of the physiological hurdles for promoting axon regrowth is the presence of growth inhibitors in the CNS myelin sheath, including Nogo, MAG (myelin-associated glycoprotein), and OMgp (oligodendrocyte myelin glycoprotein).² Although there are multiple pathways that lead to the inhibition of axon regeneration, neuronal NgR1 has been identified as the converging point for these endogenous myelin-associated inhibitors (MAIs).³ NgR1 is a glycosylphosphatidylinositol-linked leucine rich repeat protein that signals through multiple transducers such as the low-affinity neurotrophin receptor p75, TNF α receptor superfamily member 19 (TROY), and LINGO-1,^{4,5} as well as integrins, EGF receptor, B-lymphocyte stimulator (BLYS), and leucine-rich glioma-inactivated protein 1 (LGI1).^{6,7,8,9,10}

Many attempts have been made to disrupt the interaction between NgR1 and its ligands. Enhanced axon outgrowth and functional recovery following SCI have been shown with Nogo-A antibodies,¹¹ the NgR1 antagonist peptide NEP 1-40,¹² and soluble NgR ectodomains.¹³ Surprisingly, some studies have shown contradictory evidence. NgR1 was not required for mediating MAI signaling *in vitro*.¹⁴ CST regeneration was not improved in NgR1-null mice or Nogo ligand-deficient mice.¹⁵ These results suggest that removing NgR1 alone may be insufficient to enhance axon regeneration and functional recovery.

To further elucidate the *in vivo* role of NgR1, we studied functional recovery and axon regeneration following SCI in NgR1-deficient mice. We hypothesized that differences in the injury model and the method of behavioral analysis used may underlie the inconsistent findings. Thus, we examined whether the severity of injury affects the functional outcome. Functional recovery was assessed using the Basso Mouse Scale (BMS).¹⁶ We further investigated whether behavioral differences are correlated with axon regeneration, collateral sprouting, and alterations in expression of related proteins. Our data support the idea that NgR1 is one of the players in a number of redundant pathways that mediate the growth inhibitory effects of the CNS.

MATERIALS AND METHODS

Spinal cord injury

All experiments were performed in 10- to 12-week-old mice. All surgical procedures were performed in accordance with the University of Rochester Committee on Animal Resource guidelines. 29 twelve-week old mice, which include wild-type (n = 9) and those heterozygous (NgR1 +/-; n = 11) or homozygous (NgR1 -/-; n = 9) for a null mutation in the NgR1 gene,¹⁵ underwent either nearly complete dorsal transection surgery (1.5 mm

deep; Severe SCI group) or dorsal hemisection surgery (0.8 mm deep; Moderate SCI group) at T8 level. For immunoblotting experiments, additional wild-type animals (n=4) and homozygous mutant animals (n=4) were subject to the same anesthesia but underwent sham surgeries (laminectomy only without spinal cord injury) to serve as control groups. Mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg). Hair on the back was shaved and the surgical site was disinfected with betadine and 70% ethyl alcohol. Laminectomies were performed at T8 and spinal cord was exposed. A dorsal transection was performed at T8 using a microknife. To ensure complete interruption of the dorsal and lateral CST, the cut lesion was repeated twice. The paravertebral muscle layer was approximated with interrupted 4-0 vicryl and skin was closed with non-absorbable sutures in interrupted pattern. All procedures were performed under a surgical microscope. The mice were kept in a heating chamber to recover until they regained consciousness. Postoperative pain was relieved by the administration of Fluxinin Meglumine (2.5 mg/kg S.Q. every 12 hours) for three days. Baytril, at a dose of 5 mg/kg, was injected subcutaneously every 24 hours up to one week after the surgery. Bladders were pressed three times a day until voluntary control was regained.

Behavioral Test

The Basso Mouse Scale (BMS)¹⁶ was used for assessing the hindlimb locomotor function recovery at 1, 7, 14, 21 and 35 days after surgery. All animals had their bladders manually evacuated 5 minutes prior to being placed in the open field for a 4-minute time period. Rating of locomotion was scored simultaneously by two observers who were blind to the treatment groups.

Axon tracing

For tracing the CST, 1ul neuronal tracer biotin-conjugated dextran amine (BDA) (10%, 10000MW, Molecular Probes) was stereotactically injected into two sites of the right sensorimotor cortex at three weeks post-surgery using a Micro Syringe Pump Controller (World Precision Instruments). Injection was performed at the following coordinates: Site 1, 1.0 mm lateral, 0.5mm anterior to bregma, 0.5 mm deep into the cortical surface; Site 2, 1.0 mm lateral, 0.5mm posterior to bregma, 0.5 mm deep into the cortical surface. The infusion rate was 100 nl/min. The needle was kept in the injection position for an additional 5 minutes before withdrawal.

Histology and CST axon analysis

Mice were euthanized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde on day 35 post-surgery. Spinal cords were removed and post-fixed in 4% paraformaldehyde for two hours. The tissue samples were then transferred to 15% sucrose in 4°C overnight and to 30% sucrose for another overnight before freezing in -80°C. The spinal cords were cut into five segments: 1) 10 mm segment centered around the injury site; 2) 5 mm segment rostral to segment 1 or “segment R”; 3) 5 mm segment rostral to segment R or “segment R1”; 4) 5mm segment rostral to segment R1 or “segment R2”; 5) 5 mm segment caudal to segment 1 or “segment C”. Segment 1 was cut into parasagittal sections and segments C, R, R1, and R2 into transverse sections using a cryostat (Thermo Shandon

Cryotome FSE Cryostats, Fisher Scientific). Each segment was embedded in OCT (Sakura Finetek) and cut into 35 μ m sections. Sections were washed in 1X PBS and 0.1% TritonX-100, then blocked in 10% horse serum, 0.1% TritonX-100 and 1X PBS for 1 hour at room temperature. Subsequently, the sections were incubated in streptavidin conjugated with Alexa Fluor 594 (Invitrogen, 1:10,000) in the same blocking solution at 4°C overnight. After washing in 1 $\times$ PBS for three times, sections were mounted with Mounting Medium for Fluorescence (Vector Lab). Images were taken under a BX41 Olympus Fluoview confocal microscope connected to a DP70 digital camera. Image-J software (National Institute of Health) was used to quantify the CST axons. The quantified field was taken at 250 μ m lateral and 300 μ m ventral to the base of dorsal CST. Within this field, the image was thresholded and the percent area occupied by BDA-labeled fibers was determined, as described.¹⁷ Differences in CST collateral sprouting between the groups were based on 30 transverse sections per group analyzed using a two-way ANOVA test.

Immunohistochemistry

After rinsing in PBS, sections were incubated with blocking solution (5% normal donkey serum in PBS/0.1% Triton X-100) for 1 hour at room temperature. Slides were incubated with primary antibodies specific for Glial Fibrillary Acidic Protein (anti-GFAP; Sigma; 1:250) or Chondroitin Sulfate (anti-CS-56; Sigma; 1:200) at 4°C overnight and with secondary antibody Alexa Fluor 488 Donkey anti-Mouse (Invitrogen; 1:1000) at room temperature for two hours in the dark. All antibodies were diluted with 2% donkey serum in PBS/0.1% Triton X-100. Slides were rinsed with PBS after each incubation, mounted with Mounting Medium for Fluorescence (Vector Lab), and examined under a BX41 Olympus Fluoview confocal microscope connected to a DP70 digital camera.

Immunoblotting

A 0.5 cm segment centered on the injury site was dissected from the mice 24 hours post-surgery. Tissue samples were homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail (Roche) by sonication. Tissue homogenates were centrifuged at 12,000 rpm for 20 minutes at 4°C and supernatant was saved for analysis. Samples each containing 100 μ g of total protein were separated by SDS-PAGE (10% gel) in Laemmli Sample Buffer (Biorad). PVDF blots (Immobilon-FL, Millipore) were probed for MMP-9 catalytic domain (Millipore; 1:1000), GFAP (Sigma; 1:1000), CS-56 (Sigma; 1:1000), and α -actin (Abcam; 1:1000). Blots were imaged using the LI-COR Odyssey system. Band intensity was quantified using the LI-COR Odyssey software. Relative protein expression levels were calculated by normalizing the band intensity of the target protein to its respective α -actin loading control band intensity.

Statistical Analysis

All quantitative data are expressed as mean $\pm$ SEM. All statistical tests used the Prism software (GraphPad). p-values < 0.05 were considered significant. Statistical comparisons between groups were made using the two-way ANOVA test, followed by Bonferroni's posttest.

RESULTS

Locomotor function recovery is affected by injury severity

To examine whether the severity of the injury affects the degree of functional recovery following SCI, we monitored the locomotor recovery over a period of five weeks after a severe or moderate SCI using the Basso Mouse Scale (BMS), a behavioral test specifically developed to assess locomotor function recovery in mice following SCI. In the Severe injury group, the BMS score ranged from a score of 0 to 7 over the five weeks of recovery (Fig. 1A), which functionally corresponds to an improvement from “no ankle movement” to regaining “frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and lift off, and severe trunk instability”.¹⁶ None of the wild-type mice exhibited improvement in locomotor function over the five weeks. In comparison, NgR1 heterozygous mutants improved from an average score of 0.5 to 2.5, whereas the homozygous mutants improved from an average score of 0.25 to 4.25. There were significant effects of recovery time ($F = 10.06$, $p < 0.0001$, two-way ANOVA), no significant effect of genotype ($F = 2.36$, $p = 0.1564$), and significant interaction between recovery time and genotype ($p = 0.0037$). The homozygous mutants showed significant improvement compared to the wild-type at three ($p < 0.05$, Bonferroni post-test) and five ($p < 0.01$) weeks post-SCI. The heterozygous mutant showed a similar trend of functional improvement but did not reach a statistical significance compared to the wild-type mice. For the Moderate injury group, the BMS score ranged from a score of 1 to 7 over the five weeks of recovery but no significant differences between the groups were detected (Fig. 1B). This score range corresponds to an improvement from “slight ankle movement” to regaining “frequent or consistent plantar stepping, mostly coordinated, paws parallel at initial contact and rotated at lift off”.¹⁶ There were significant effects of recovery time ($F = 18.21$, $p < 0.0001$), no significant effect of genotype ($F = 1.59$, $p = 0.2914$), and no significant interaction between recovery time and genotype ($p = 0.8133$). We further analyzed BMS subscores to examine whether there were differences in fine locomotor functions that may not have been reflected by changes in the overall BMS score.¹⁶ The mean subscore ranged from 0 to 1.8 for the Severe injury group and 0.33 to 6.67 for the Moderate injury group, with no significant differences between any of the groups (Fig. 1C, D). Thus, the degree of locomotor recovery in NgR1-deficient mice is influenced by the severity of the injury.

NgR1 ablation promotes CST collateral sprouting but not long-distance axon regeneration

To study the effects of NgR1 ablation on axon regeneration, we examined the CST axons using the anterograde tracer biotin-conjugated dextran amine (BDA) injected into the right sensorimotor cortex. Five segments from each spinal cord sample were processed histologically. Representative sections from the 10 mm segment centered on the injury site and two 5 mm segments immediately rostral and caudal to the 10 mm segment are shown in Fig. 2. We were able to detect the dorsal CST in the rostral sections in all groups (Fig. 2A – D). Comparing to the caudal sections, however, dorsal CST was labeled in none of the groups (Fig. 2F – H) except the sham-operated control (Fig. 2E), suggesting that no or very few CST fibers were able to cross the injury site following SCI regardless of the genotype of the mice. We were able to confirm this finding in the sagittal sections around the injury site, showing that labeled CST fibers terminate immediately rostral to the injury site in all groups

(Fig. 2J – L). Thus, no obvious difference in CST fiber regeneration across the injury site was found between the groups.

A closer analysis of the rostral sections, however, revealed a difference in CST fiber sprouting to the contralateral spinal cord among the groups (Fig. 3A). We calculated the percent area ratio of labeled CST fibers in the right half to the left half of the spinal cord (Fig. 3B). There was no significant effect of genotype ($F = 2.74$, $p = 0.0665$), proximity to the injury site ($F = 1.52$, $p = 0.2213$), or interaction between genotype and proximity to the injury site ($p = 0.5947$). Significantly greater sprouting to the contralateral spinal cord was found in the homozygous mutant mice compared to the wild-type mice in the segment 5-10 mm rostral to the injury site (segment R, $p < 0.05$) but not in segments located more distant from the injury site. The heterozygous mutants exhibited a level of contralateral sprouting intermediate to that seen in the wild-type and the homozygous mutants but this did not reach a statistically significant difference. The same trend is also observed in the segment 10-15 mm rostral to the injury site but, again, this was not significantly different among the groups. These data suggest that, although NgR1 ablation may not be sufficient to promote CST fiber regeneration across the injury site, collateral sprouting to the contralateral spinal cord may be enhanced.

NgR1 ablation is correlated with changes in levels of reactive astrocytes, CSPGs, and MMP-9

To identify candidates that may be complementing the effects of NgR1 signaling *in vivo*, we examined the immunoreactivity of Glial Fibrillary Acidic Protein (GFAP) and chondroitin sulfate proteoglycans (CSPGs).⁸ CSPGs are one type of extracellular matrix molecules that are associated with glial scars, are dramatically upregulated during the neuroinflammatory phase, and are growth inhibitory.⁸ Thus, we expected increased GFAP and CSPG immunoreactivity at the injury site and examined whether changes in the levels of reactive astrocytes and CSPGs are correlated with NgR1 expression. Consistent with our predictions, we observed increased immunoreactivity of both GFAP and CSPGs surrounding the injury site (Fig. 4I – P). We could not detect any obvious differences in GFAP expression from the transverse sections between the rostral and caudal sites (Fig. 4A – H), most likely because inflammation is a fairly localized response and because the immunohistochemistry was performed five weeks post-surgery, whereas maximum astrocyte activation is known to occur within a few days to a week of the injury.¹⁸ Thus, the upregulation of CSPGs and GFAP-expressing astrocytes at the injury site likely contributed to restricting the CST fibers from regenerating across the injury site despite NgR1 ablation.

Several studies have demonstrated the ability of MMPs to proteolytically degrade CSPGs.^{19,2019,2122} We isolated a 0.5 cm segment centered around the injury site 24 hours post-surgery and analyzed the expression of MMP-9 using immunoblotting (Fig. 5A – C). Notably, both the pro- and active- forms of MMP-9 were at undetectable levels in the sham-operated homozygous mutants. However, both forms were significantly increased in homozygous mutant mice following SCI ($p < 0.05$). There was significant effect of treatment ($F = 6.25$, $p = 0.0223$ for pro MMP-9; $F = 9.87$, $p = 0.0085$ for active MMP-9), no significant effect of genotype ($F = 0.51$, $p = 0.6089$ for pro MMP-9; $F = 0.24$, $p = 0.7909$ for

active MMP-9), and no interaction between treatment and genotype ($p = 0.0789$ for pro MMP-9; $p = 0.3717$ for active MMP-9).

We next examined the expression of CSPGs. Immunoblotting experiments yielded significantly increased band intensity (Fig. 5A, D) in samples from wild-type ($p < 0.001$) and homozygous ($p < 0.05$) mutant animals compared to their respective sham-operated control animals. This upregulation, however, was not observed in heterozygous mutants. There was a significant effect of treatment ($F = 23.03$, $p = 0.0001$) and genotype ($F = 4.44$, $p = 0.0271$), and significant interaction between treatment and genotype ($p = 0.0221$). CSPG level is further reduced in the heterozygous mutants compared to the homozygous mutants even though active MMP-9 expression post-surgery in the heterozygous mutants was not significantly elevated.

We also assessed whether GFAP was differentially expressed among the groups (Fig. 5A, E). GFAP expression was significantly increased in wild-type but not in NgR1 mutant mice 24 hours following SCI compared to before SCI ($p < 0.01$). There was significant effect of treatment ($F = 8.80$, $p = 0.0083$) and genotype ($F = 3.61$, $p = 0.0481$) but no significant interaction between genotype and treatment ($p = 0.1465$). Thus, NgR1 ablation may contribute to ameliorating the inflammatory response and reducing the inhibitory effects of glial scar on the regeneration of CST fibers.

DISCUSSION

The identification of NgR1 as a converging point for the signaling of neurite outgrowth inhibition has given rise to efforts in disrupting the interaction between NgR1 and its ligands as means to enhance axon regeneration in SCI and other traumatic injury in the CNS. Despite the accumulating evidence that suggests NgR1 to be nonessential for mediating the myelin-associated inhibition,^{14,15,23} we report here that NgR1 plays important roles in restricting collateral sprouting in the damaged spinal cord. In additions to the MAIs, we also speculate that NgR1 may have additional functions *in vivo*, such as modulation of glial scar formation and the degradation of CSPGs through interactions with MMPs. Although NgR1 antagonism alone may not be sufficient to promote axon regeneration, it may have functionally important implications, such as enhancing collateral sprouting and improving locomotor function recovery. Our findings seem to suggest that multiple redundant pathways exist to mediate the growth inhibitory signaling in the CNS and that NgR1 function *in vivo* may be confounded by additional molecules.

The difference in locomotor recovery we observed between the two groups that suffered varying degrees of injury suggests that injury severity may be a confounding factor in functional recovery following SCI. Thus, the injury models and behavioral analyses used in different studies may have yielded conflicting results. Our results demonstrate that the *in vivo* function of NgR1 needs to be studied under the appropriate context. In the moderately injured mutant mice, the animal still retains some locomotor function. Consequently, any effects due to the ablation of NgR1 alone would appear trivial in the context of other intact locomotor functions. In the severely injured mice, however, the animal has almost completely lost all locomotor function. Thus, any minor improvement in function due to

NgR1 ablation would appear significant. The severe injury model allows us to observe the *in vivo* functions of NgR1 in isolation. Thus, for patients who suffer severe loss of behavioral functions, NgR1 antagonism may produce appreciable functional recovery. These effects are still present in the case of moderate injury but are merely masked by other intact functions.

Several studies have implicated NgR1 in physiological functions such as enhancing synaptic plasticity and collateral sprouting,^{13,24} which may improve behavioral outcome without obvious changes in axon regeneration. Our finding that NgR1 ablation was sufficient to enhance collateral sprouting but not long-distance axon regeneration is consistent with these studies. 8252627282930

We propose several factors that may be masking the functional importance of NgR1. Reactive astrocytes and CSPGs, which are components of the glial scar, add to the complexity of the system because they may have both beneficial and detrimental effects, such as protecting the spared tissue surrounding the injury cavity from inflammation while causing secondary tissue damage.^{8,31} Several studies have implicated MMPs as an important link between these components and regeneration following SCI. MMP-2 and MMP-9 have been shown to facilitate the formation of glial scar and to degrade CSPGs, consequently improving functional recovery and enhancing remyelination following spinal injury.^{19,32,33} We have shown that NgR1 mutations are correlated with specific changes in the levels of MMP-9, GFAP-expressing reactive astrocytes, and CSPGs. While the mechanisms and significance of these correlated changes are yet to be investigated, these observations further reinforce the importance of taking into consideration these and other potential confounding factors in future studies of NgR1 function and in devising treatment methods for spinal cord injuries.

Other concerns that consistently arise when studying animal models with targeted gene disruption are any developmental compensatory changes in gene expression that may have occurred in the genetically altered animals, which may explain some of the differences between chronic and acute inhibition. Studies that have used an acute approach generally have observed substantial differences in regeneration and recovery^{13,34,35} while those using a chronic approach observe the contrary.^{15,23,36} Some possible explanations for this discrepancy have been proposed, such as the potential regeneration-promoting functions of acutely administered treatments and/or the possible endogenous functions of NgR1 or MAIs to enhance regeneration.³⁷ Our results are consistent with the latter as we have shown evidence which implies that NgR1 may be required for the CSPG-degrading function of MMP-9. In addition, NgR1 has been shown to undergo cleavage by MMP-3 and generates soluble N-terminal fragments that can function as antagonists of the inhibitory signaling pathway.³⁸ These results imply that, if NgR1 has endogenous functions that may enhance regeneration, the complete lack of NgR1 in germline deleted animals may deprive the injured axons of such beneficial effects, resulting in unsubstantial levels of regeneration or recovery. Thus, these potential complex interactions between NgR1 and other molecules may account for at least some of the inconsistent findings regarding the importance of NgR1. More complete characterization of genetic changes that are occurring in animal models as well as further studies that are consistent in the degrees of injury, genetic models, and methods of analysis are necessary.

CONCLUSION

Our data demonstrate some important functions of NgR1 that may not have been evident in previous studies due to the complexity and redundancy of the inhibitory signaling pathways in the CNS. These findings strengthen the idea that NgR1 antagonism alone may not be sufficient to induce significant improvements following SCI but may necessitate combination with other therapeutic measures such as spatial or temporal regulation of glial scar formation or CSPG degradation. Because many molecules have multiple functional roles, complete ablation of any single one may generate unexpected side effects or the lack of desirable effects. Nevertheless, NgR1 antagonism can lead to functionally significant outcomes such as enhancing collateral sprouting and improving locomotor recovery even in the absence of obvious long-distance axon regeneration. Thus, future studies may aim to elucidate the physiological role of NgR1 using appropriate injury models and to examine the effectiveness of combined therapy to complement NgR1 antagonism as means of treating spinal cord injuries.

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Figure 1. BMS locomotor function recovery

The Basso Mouse Scale (BMS) was used for assessing the hindlimb locomotor function recovery at 1, 7, 14, 21 and 35 days after SCI. (A) In the Severe injury group, the homozygous mutant mice showed significant improvement in locomotor recovery at three and five weeks post-SCI compared to the wild-type mice. (B) In the Moderate injury group, all three groups showed improvement over the five weeks but no significant differences were detected between the groups. (C, D) No significant differences were found from analyses of subscores. * $p < 0.05$; ** $p < 0.01$; $n = 3$ per group.

Figure 2. BDA tracing of CST fibers

Representative spinal cord sections from sham-operated wild-type control (A, E, I) and spinal cord injured wild-type (B, F, J), heterozygous (C, G, K), and homozygous (D, H, L) NgR1 mutant mice. All images are shown dorsal (A - H) or rostral (I - L) to the left. Transverse sections that are 5-10 mm rostral (segment R; A - D) or caudal (segment C; E - H) to the injury site are shown. BDA labeled main dorsal CST (arrowheads) were not detected in caudal sections from mice that underwent SCI (F - H). (I - L) Sagittal sections show CST fibers terminating immediately rostral to the injury site (arrows) in spinal cord injured mice.

Figure 3. Contralateral sprouting of CST fibers

(A) Representative spinal cord sections from sham-operated wild-type control and spinal cord injured wild-type, heterozygous, and homozygous NgR1 mutant mice. Images are shown dorsal to the left. Transverse sections that are 5-10 mm rostral (segment R) to the injury site are shown. The percent area of BDA labeled CST axons were compared between the left and right halves of the spinal cord section. (B) Ratio of axon counts in the right half over the counts in the left half of the spinal cord section in segments rostral to the injury site. Significant increase in contralateral sprouting was detected in the homozygous mutant compared to the wild-type mice in segment R. * $p < 0.05$.

Figure 4. Immunoreactivity of GFAP and CS-56

Representative spinal cord sections from sham-operated wild-type control (A, E, I, M) and spinal cord injured wild-type (B, F, J, N), heterozygous (C, G, K, O), and homozygous (D, H, L, P) NgR1 mutant mice. All images are shown dorsal (A - H) or rostral (I - P) to the left. Transverse sections that are 5-10 mm rostral (segment R; A - D) or caudal (segment C; E - H) to the injury site are shown. Increased GFAP (I - L) and CSPG (M - P) immunoreactivity is detected surrounding the injury site (arrows) in sagittal sections. Q shows a no-primary control for background CS-56 expression.

Figure 5. MMP-9 activation, CSPG degradation, and glial scar formation in NgR1 mutant mice
 (A) A 0.5 cm segment centered on the injury site was dissected from the mice 24 hours post-surgery and was analyzed for levels of MMP-9, CSPGs, and GFAP. Two mice per group are shown. Expression in each lane was normalized to β -actin loading control. Expression of the pro- (B) and active- (C) forms of MMP-9 was significantly elevated in the homozygous mutant mice post-surgery compared to pre-surgery. (D) CS-56 expression was significantly increased in the wild-type and homozygous mutant mice, but not in heterozygous mutant mice, post-surgery. (E) GFAP was significantly elevated in wild-type mice post-surgery. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 3$ independent experiments.