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Project Title: **Title: Investigating the potential of Neuregulin-1 in optimizing myelin repair following spinal cord injury**

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Summary (250 words max single spaced):

Spinal cord injury (SCI) is a devastating event which causes lifelong disability and results in significant emotional and financial burden for patients, their families and the healthcare system. At the tissue level, SCI causes loss of oligodendrocytes, demyelination and concomitant axon degeneration. Moreover, activation of glial cells leads to the formation of glial scar. These pathologic changes are progressive and permanent, and cause neurological disability with little or no functional recovery. There are currently no therapies which can effectively restore motor function in the chronic stage of SCI. Our lab has shown for the first time that a growth factor named neuregulin-1 (Nrg-1) is severely depleted following SCI. Here we show that replacement of lost Nrg-1 improves myelination, attenuates axon loss, and reduces glial scar formation in a rat model of chronic traumatic SCI. Nrg-1 treatment improved myelin thickness at the injury epicentre, increased the number of mature oligodendrocytes in the area surrounding the injury site, and caused maturation of oligodendrocyte precursor cells in vitro. Furthermore, Nrg-1 reduced axon degeneration, preserved neurons around the injury site, and reduced glial scar. For the first time, we have demonstrated positive modulatory effects of Nrg-1 on the post-SCI microenvironment at the chronic stage of injury. These results suggest the potential of Nrg-1 to promote tissue repair following SCI, and as a part of combinatorial approaches to regenerative therapies including stem cell transplantation.

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Background and Rationale

Outlining the challenge: Spinal cord injury (SCI) is a devastating event associated with high mortality and significant, often permanent disability in survivors. SCI occurs with an annual incidence between 11.5 and 53.4 per million in developed countries, and the majority of new cases occur in individuals less than 40 years old[1]. There are currently over 85,000 Canadians living with chronic spinal cord injury. The most common cause of SCI is motor vehicle accidents, with sports injuries, work injuries, violence and falls also contributing significantly[2]**.**

SCI leads to profound loss of motor and sensory function at and below the site of injury, as well as significant bowel, bladder and musculoskeletal complications[1]. Rates of anxiety and depression are also high in SCI patients and their family members[3]. In addition to the functional, physical and emotional costs associated with SCI, there are also significant financial costs to the healthcare system. The cumulative lifetime cost of care for an individual patient of SCI is estimated between 1.5 and 3 million Canadian dollars, with an annual economic burden of 2.67 billion Canadian dollars nationally for SCI patients[4].The high morbidity and economic burden of SCI have led to considerable research into the mechanisms that underlie its pathology and developing effective therapeutic strategies for SCI. Currently, there is no viable treatment option for chronic SCI patients.

Spinal cord injury: The pathophysiology of SCI can be explained by dividing into primary and secondary injury processes. Primary injury results from the initial impact to the spinal cord, often accompanied by persisting mechanical compression[1]. Secondary injury encompasses numerous biochemical and cellular processes that contribute to progressive acute and chronic tissue damage. Mechanisms postulated to undergo secondary injury include but are not limited to ischemia, vascular injury and systemic hypotension[5], glutamate[6] and calcium[7] cytotoxicity, neuroinflammation, cell death, myelin damage, astrogliosis and glial scar formation[8].

Among the injury mechanisms, activation of astrocytes has a major impact on the repair mechanisms with both detrimental and beneficial effects[8]. Astrocytes are the most abundant glial cells, and perform numerous critical functions in the central nervous system (CNS). These include regulation of the blood-brain barrier[9], metabolism of neurotransmitters[10], modulating synaptic plasticity[11] and regulation of cerebral blood flow[12]. In SCI, however, astrocytes undergo significant morphological and functional changes. These changes, including hypertrophy of their cell processes, upregulation of intermediate filaments, and production of cytokines and chemokines, are collectively referred to as reactive astrogliosis[8]. This process is believed to be a defensive response to injury intended to maintain blood-brain barrier function and protect against glutamate excitotoxicity and immune cell infiltartion[6]. Unfortunately, reactive astrogliosis ultimately leads to the production of inhibitory extracellular matrix (ECM) compounds which form glial scar and limits the potential for repair and tissue regeneration.

Another major consequence of secondary injury from SCI is loss of myelin at the injury site. Myelin is a proteolipid that ensheaths axons. In the CNS, myelin is an extension of the cell membrane of specialized glial cells – oligodendrocytes – which surrounds axons in a spiral fashion[13]. SCI results in the death of oligodendrocytes and loss of myelin (demyelination) which consequently result in loss of axons and functional impairment[14,15].

The adult spinal cord contains two populations of endogenous precursor cells with the potential to replace lost myelinating oligodendrocytes. Neural precursor cells (NPCs) are stem cells with the multipotential capacity to differentiate to all neural and glial cell lineages. There are also resident oligodendrocyte precursor cells (OPCs) that are committed progenitors of the oligodendroglial lineage. Following SCI, both NPCs and OPCs are activated[16]. However, under the injury environment, these cells show limited ability for oligodendrocyte differentiation[17]. It is therefore evident that the post-SCI microenvironment is not conducive to the replacement of oligodendrocytes and remyelination of injured axons. As such, optimization of the injured spinal cord tissue will be critical for successful repair following SCI.

One of the contributors to the hostile microenvironment following SCI are chondroitin sulfate proteoglycans (CSPGs). CSPGs are critical ECM components of the glial scar which are a result of reactive astrogliosis. They limit axon regeneration[18] and negatively modulate NPCs following SCI[19]. Our lab was the first to show that CSPGs limit effective migration of NPCs to the injury site and their ability for oligodendrocyte differentiation, thus limiting regenerative processes[20,21]. Disruption of CSPGs with Chondroitinase ABC (ChABC) improves the regenerative response of NPCs and promotes functional recovery following SCI in rats[20,22]. This highlights the importance of modulating CSPGs as a mechanism of repair, and also the importance of optimizing the microenvironment following SCI for successful regenerative therapies. Recent studies in our lab has shown the potential of Neuregulin-1 (Nrg-1) therapy in modulating astrogliosis in SCI (manuscript under review).

Neuregulin 1:Nrg-1 is an important growth factor for oligodendrocyte differentiation and myelination in the nervous system[23]. Nrg-1 contains an Epidermal Growth Factor (EGF)-like domain and activates ErbB receptor tyrosine kinases[24]. The gene is evolutionarily conserved in both structure and function, present in vertebrates and invertebrates[25]. In humans, it is encoded by the NRG1 gene on chromosome 8p[26] and the open reading frame is highly conserved in rat and mouse[27]. Nrg-1 activates ErbB dimerization resulting in phosphorylation and activation of the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways[24]. Nrg-1 is essential for life as mouse embryos with homozygous Nrg-1 deletion (Nrg-1 -/-) develop significant cardiac abnormalities and die. In the nervous system, Nrg-1 knock down or deficiency of its ErbB receptors results in impaired Schwann cell development and loss of peripheral motor and sensory neurons[28]. Additionally, oligodendrocytes fail to develop in the spinal cord of Nrg-1-/- null mice, an effect that is reversed with administration of recombinant Nrg[29].

The NRG1 gene has also been implicated in human disease such as psychiatric and cardiovascular conditions. NRG1 was identified as a candidate gene for schizophrenia in familial genetic analysis, and mice hypomorphic for Nrg-1 or the ErbB4 receptor show schizophrenia-like behavioral abnormalities[26]. Nrg-1/ErbB signaling also regulates cardiac function. Trastuzumab, a monoclonal antibody that blocks the ErbB2 receptor (HER2), was noted to increase the risk for advanced heart failure when combined with an anthracycline agent in breast cancer patients[30]. Recombinant human Nrg-1 (rhNrg-1) has been shown to improve cardiac function and survival in rat models of ischemic and viral-induced cardiomyopathy[31]. rhNrg-1 improved cardiac function in heart failure patients in phase II clinical trials[32]. Critically, these clinical trials have also established the safety of rhNrg-1 for clinical use. In summary, signaling through the Nrg-1/ErbB pathway is critical for development of the cardiac and nervous

systems, particularly for cells involved with myelination in the central and peripheral nervous systems.

Neuregulin-1 in SCI: Previous work in our lab demonstrated that Nrg-1 is significantly depleted after SCI and does not recover to pre-injury levels even at the chronic stage[33]. Other studies also show that conditional knockout of Nrg-1 expression in adult mice with SCI prevents endogenous remyelination and recovery from injury compared to wild-type injured mice[34]. Our lab demonstrated that restoration of Nrg-1 levels after SCI with local delivery of rhNrg-1 to the injury site increased survival and replacement of oligodendrocytes and enhanced axonal preservation at the acute stage of injury[33].

Hypothesis and objectives: In this study, we sought to test the **hypothesis** that Nrg-1 enhances remyelination and tissue repair in chronic SCI. Our aim was to study the effect of Nrg-1 therapy specifically on scar formation, axon preservation, oligodendrocyte maturation and remyelination in the chronically injured spinal cord.

Materials and Methods

Animals, SCI model, and treatments

All experimental protocols and animal procedures in this study were approved by the University of Manitoba in accordance with the guidelines and policies established by the Canadian Council on Animal Care. A well-established and clinically relevant model of incomplete compressive SCI at mid-thoracic level was induced in adult (8-10 weeks) female Sprague Dawley rats of 230-250g weight [20,35]. Under deep Isoflurane (4%)/O2 (2%) anesthesia, rats received laminectomy at thoracic levels T6–T8. A 35g modified aneurysm clip was applied for 1 min at T7 of the spinal cord extradurally inducing a compressive injury. Immediately after SCI surgeries, each animal received a combination of buprenorphine (Temgesic®) (0.05 mg/kg) and meloxicam (Metacam® Boehringer Ingelheim GmbH.) (2 mg/kg) followed by three additional doses of buprenorphine (0.05 mg/kg) with 8-hour intervals for pain management. Rats were housed in standard plastic cages at 22° C before SCI and at 26°C afterwards in a 12:12 h light/dark cycle with soft paper bedding to prevent skin erosions and urine scalding. Pelleted food and drinking water were available *ad libitum*. SCI rats were examined daily and their bladder was expressed manually three times a day until the return of reflexive bladder control. This model results in significant motor and sensory impairments below the injury level.

At the time of SCI, rats were randomly assigned to receive either vehicle (0.1% bovine serum albumin, BSA, in 0.9% saline) or rhNrg-1β1 (1µg/day) administered intrathecally through the subarachnoid space using indwelling catheters connected to osmotic minipumps (Alzet ®, 2006) for 6 weeks. Animal surgeries and animal care were all done by senior graduate students in the Karimi lab.

Tissue Processing

At 10 weeks post-SCI, animals were deeply anesthetized and perfused transcardially with cold phosphate-buffered saline (PBS) (0.1M) followed by 3% paraformaldehyde in 0.1M PBS for tissue fixation. Two (2) cm sections of the spinal cord centered at the injury epicenter were dissected, embedded in Optimal Cutting Temperature (OCT) material, and stored at -80°C. Spinal cord segments were cut into 35µm serial cross-sections using a cryotome and

mounted onto Superfrost Plus Micro Slides (Fisher Scientific). The precise epicenter was determined by staining for demyelination with luxol fast blue (LFB) and hematoxylin and eosin (H&E). Animal perfusion and dissection of spinal cords was done by senior lab members; I contributed to embedding, sectioning and staining procedures.

Immunohistochemistry

Frozen tissues sections used for immunohistochemistry (IHC) were warmed, hydrated with PBS, and blocked for 1 hour at room temperature in 1% BSA, 5% non-fat milk and 0.3% Triton X in PBS. Primary antibody solutions were prepared in the blocking solution described above and incubated overnight at 4°C. Primary antibodies and their concentrations are listed in Table 1. Myelin basic protein (MBP) was used to label myelin, neurofilament 200 (NF200) was used to label neurons, CS56 was used to label CSPGs, amyloid precursor protein (APP) was used to label degenerating axons, and adenomatous polyposis coli (APC) was used to label mature oligodendrocytes (see Table 1). Primary antibodies from separate species were used for double immunolabelling. Following primary antibodies, tissues were then washed 3 times in PBS and incubated with appropriate secondary antibodies (see Table 1) for 1 hour at room temperature. Tissues were then washed 3 times in PBS, and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:5000) for 20 minutes at room temperature. Finally, tissues were washed 3 times in PBS and cover-slipped with Mowiol mounting medium.

Imaging and tissue quantifications: All cross-sections were imaged according to established protocols in our lab using fluorescence microscopy. MBP, APP, NF200 and CS56 were quantified by immunointensity measurements using unbiased methodologies. The cross-sectional area was traced and integrated density was measured for each cross-section using ImageJ software (version 1.50, developed by National Institute for Health). The integrated density was then normalized to the area measured. For APP images, only lateral columns were included in the experiment. APC-positive cells were quantified by cell in counting in lateral column white matter areas in Adobe Photoshop CS6 v13.0. A grid with standardized spacing of 500 pixels was used to randomly select quantifiable area in lateral columns. Fifteen (15) fields in each lateral column of each section were selected and traced. After selection of boxes for inclusion in quantification, the APC channel image was overlayed. APC+ and APC+DAPI+ cells falling in the selected boxes were counted for each image, and the ratio of APC+/DAPI+ cells were calculated. I was responsible for all IHC staining, imaging, and cell quantifications.

Assessment of Myelin Thickness

For assessment of myelin thickness, spinal cords from vehicle and Nrg-1 treated animals (n=3/group) underwent fixation in 4% paraformaldehyde and osmification in 1% OsO4. Semi-thin sections (0.5µm) were cut from the injury epicenter, 1mm rostral and 1mm caudal, and stained with Toluidine Blue. Images were taken of right lateral column, left lateral column and dorsal column areas with 630x magnification under oil immersion. Axons were randomly selected for assessment of myelin thickness using a previously described method[15]. A grid with 10 equally spaced vertical lines was overlaid onto each image, and only axons which were superimposed by the vertical lines were quantified. Axons were measured for their myelin ratio (MR). This was defined as the distance from the outer surface of the myelin on both sides (fiber diameter, D), divided by the distance across the axon itself (axon diameter, d). A completely demyelinated axon would have an MR (D/d) of 1, and a myelinated axon would have an MR >

1, with higher MR corresponding to thicker myelin. A total of 1133 axons were analyzed using this method using ImageJ software. Animal perfusion, tissue harvesting and sectioning were done by a graduate student in the Karimi lab, and I conducted imaging and MR quantification independently.

Isolation and culture of oligodendrocyte precursor cells (OPCs)

Mixed glial culture were obtained from rat cortex (postnatal day 1-3). Mixed cultures were plated and incubated until confluent (~14 days) at 37°C in astroglia media (DMEM+ 20%FBS). The mixed glial culture was shaken for 1 hour at 200rpm at 37°C to remove microglia. The media was removed and cells were shaken at 200rpm at 37°C overnight to allow OPCs to detach from the astrocyte layer. The cell suspension was then collected and transferred to an untreated petri dish and incubated for 30 minutes at 37°C. The cell suspension was then passed through a sieve (40µm pore size) into conical tubes and centrifuged for 10min at 100g. The pellet was resuspended in media and counted with a hemocytometer. OPCs were seeded in 3 separate PDL-coated multichambers. Each multichamber was treated individually with 200ng/ml Nrg-1 and incubated at 37°C for 7 days in OPC media containing 2% serum. OPC isolation was done by a senior member of the Karimi lab; I contributed to cell plating, seeding and treatments.

Immunocytochemistry

We examined the effect of Nrg-1 on the maturation of OPCs by assessment of morphological complexity. Immunocytochemistry (ICC) was performed with primary antibodies against O4 to label OPCs (Table 1). Wells were washed with PBS and then fixed with 3% PFA for 20 minutes at room temperature. Blocking was done for 60 minutes with 1% BSA, 5% non-fat Milk at room temperature. Primary antibody against O4 was incubated for 90 minutes at room temperature followed by secondary antibodies for 60 minutes at room temperature. Slides were then washed 2 times in PBS and then incubated with DAPI (1:5000) for 20 minutes at room temperature. Finally, slides were washed 3 times in PBS and coverslipped with Mowiol mounting medium.

Slides were imaged at 200x magnification and images were captured from cells which were adequately isolated to permit individual morphological analysis. OPC maturation was assessed using Fractal dimension (FD) analysis which renders a value between 1 and 2 depending on morphological complexity[36]. Cells with near-bipolar morphology result in an FD value near 1, and cells with higher morphological complexity result in higher FD values. Fluorescence images were cropped to show only one cell, and converted to 8-bit (greyscale) format in Image J. The threshold was adjusted in order to visualize the entire cell (Image: Threshold), and an outline of the cell was produced. The Fractal Box Count tool was used with the default box sizes (2, 3, 4, 6, 8, 12, 16, 32, 64) and the resulting FD values were recorded for each cell. I performed all the immunocytochemistry staining, imaging and quantification independently.

Results

Nrg-1 treatment enhanced remyelination in chronic SCI

Our lab had previously identified a positive role for Nrg-1 in enhancing oligodendrocyte differentiation in acute SCI[33]. In this study we sought to determine the effects of Nrg-1 treatment on remyelination in chronic SCI. In order to assess myelin thickness, we performed myelin ratio on individual axons at the injury epicenter and 1mm rostral and caudal to the injury. This analysis revealed significantly higher myelin thickness at the injury epicenter in Nrg-1 treated animals compared to vehicle treated group (Figure 1; $p < 0.05$, two-way ANOVA, n=3/group). There was no significant difference between these groups in segments adjacent to the epicenter. Next, we assessed total myelin using immunointensity measurement of myelin basic protein (MBP) on sections spanning 3mm caudal to 3mm rostral to the epicenter. Although these data showed a higher expression of MBP under Nrg-1 treatment in sections 3mm caudal to 1mm rostral of the epicenter, this difference was not statistically significant (two-way ANOVA, n = 3/group, Figure 2).

We next investigated whether Nrg-1 enhances oligodendrocyte preservation and maturation. We assessed the number of mature oligodendrocytes in lateral column white matter areas using immunohistochemistry for APC on sections spanning 4mm caudal to 4mm rostral from the epicenter. Interestingly, we found an increase in the proportion of APC+ oligodendrocytes in areas rostral to the injury epicenter in Nrg-1 treated SCI animals compared to vehicle SCI rats. These differences were significant at 1mm, 2mm and 4mm points ($p < 0.01$, two-way ANOVA, n=3/group, Figure 3). These data suggest that Nrg-1 treatment has a positive effect on preservation of mature oligodendrocytes in the injured spinal cord which persists to the chronic stage of injury.

We complemented our *in vivo* SCI studies and investigated the effects of Nrg-1 on the maturation of OPCs using direct *in* vitro assays. Morphological analysis of OPC showed a modest yet significant increase in the complexity of OPCs in the Nrg-1 compared to vehicle treated conditions, indicating their maturation into myelinating oligodendrocytes, ($p < 0.01$, n=15 cells/group/experiment from 3 different experiments, Figure 4).

Taken together, these results indicate that restoration of Nrg-1 following SCI has a beneficial effect on myelination and myelin preservation. This benefit was demonstrated through a variety of *in vitro* and *in* vivo paradigms. While short-term benefits of Nrg-1 replacement in the acute stage of SCI have been previously shown in our lab, the data presented here show for the first time that these benefits persist even at the chronic stage of injury.

Nrg-1 attenuates axon degeneration

Given that myelin loss is one of the underlying causes of axonal degeneration, and we found beneficial effects of Nrg-1 on myelin preservation following injury, we sought to study the effect of Nrg-1 on axonal preservation in chronic SCI. Immunohistochemical analysis was performed for APP as a marker for degenerating axons[37] in the lateral column of white matter at various distances spanning 3mm caudal to 3mm rostral of the SCI epicenter. There was significantly reduced expression of APP in the Nrg-1 treated group 1mm caudal to the epicenter (see Figure 5A; $p < 0.05$, two-way ANOVA, n=3/group). We also performed similar analysis for the neuronal marker NF200 to assess preservation of neurons. We found significantly higher NF200 expression in sections 1mm caudal to the epicenter (see Figure 5C; $p < 0.05$, two-way ANOVA, n=3/group). Although there was a trend towards lower APP and higher NF200 expression at the epicenter of Nrg-1 treated spinal cords, this difference did not reach statistical significance.

Taken together, these data demonstrate that Nrg-1 treatment attenuates neural degeneration in chronic SCI.

Nrg-1 treatment attenuates the formation of chronic glial scar following SCI

Lastly, we assessed the production of chondroitin sulfate proteoglycans (CSPGs), critical components of the pathological glial scar which inhibit regeneration and contribute to degenerative processes following injury[38]. Studies in our lab has shown that CSPGs inhibit NPCs survival and their ability for oligodendrocyte differentiation[19–21]. Immunohistochemistry for CSPGs was performed at the epicenter as well as 1mm caudal and rostral. There was a significant reduction in the production of CSPGs in the Nrg-1 treated animals compared to vehicle group at the lesion ($p < 0.05$, n=5/group, Figure 6). No significant difference in CSPG production was detected in perilesional regions. Our findings show the ability of Nrg-1 in reducing the formation of inhibitory CSPGs and thereby promoting repair mechanisms following SCI.

Discussion

In my thesis, I have shown that Nrg-1 has the potential to improve myelination of axons at the site of injury, attenuate neural degeneration, and reduce glial scar formation. Significantly, these benefits were demonstrated, for the first time, at the chronic stage of injury. This has important implications for the potential of Nrg-1 therapy to promote long-term improvement in SCI patients.

The majority of SCI cases do not represent complete spinal cord transection[1]. Typically some axons are preserved through the injury site and, at least initially, maintain innervation of their target tissue. However, secondary injury processes result in profound inflammatory changes that cause death of oligodendrocytes, loss of the myelin sheath which supports axons, and further axon degeneration as a result[39,40]. Therefore, maintenance and/or restoration of the myelin sheath represents a crucial therapeutic target for improving outcomes for SCI patients.

In addition to activation of resident precursor cells, the use of NPC and OPC transplants to achieve this goal has been proposed[20,41–43]. Clinical trials have been undertaken in Canada to investigate the potential of NPC transplantation, however such trials using stem cell transplant as single therapy have encountered difficulties demonstrating benefit[44]. A recent international trial using neural stem cell transplantation showed modest improvement in sensory function, but no improvement in motor function[45,46]. These results were predicted since preclinical studies, including work published by our lab, have clearly demonstrated that NPC transplantion alone is insufficient to promote effective tissue repair and functional recovery, and that a combinatorial approach is necessary to synergistically optimize the post-SCI microenvironment[20,41]. To date, no effective combinatorial treatment with a proven safety and efficacy profile in humans has been discovered.

One of the fundamental challenges of transplanting NPCs to the injured spinal cord is that the majority of transplanted cells differentiate to an astrocytic lineage[20]. Previous work from our lab has demonstrated that Nrg-1 promotes NPC differentiation preferentially toward oligodendroglial lineage *in vitro*, and promotes preservation of mature oligodendrocytes in acute SCI[33]. Here we have shown that Nrg-1 promotes maturation of OPCs *in vitro*, and importantly

supports preservation and formation of mature oligodendrocytes in the chronic stage of SCI *in vivo.* These findings provide compelling evidence that Nrg-1 has the potential to address one of the major challenges of NPC transplantation, and promote the replacement of mature myelinating oligodendrocytes following SCI.

NPC transplantation is additionally challenged by inhibitory effects of CSPGs on NPCs properties[20]. As mentioned earlier, these ECM components have the potential to mechanically inhibit the migration of cells to the injury site. Therefore, a combinatorial approach to NPC transplant should include reduction of CSPG expression to allow for effective migration of transplanted NPCs into the injury site. Our data presented here suggest that positive effects of Nrg-1 therapy on oligodendrocyte differentiation may be in part due to attenuation of CSPGs expression in SCI lesion. This indicates the multifaceted role of Nrg-1 that makes it a compelling candidate therapy in combinatorial approaches for SCI.

Given the significant length that spinal cord axons travel between their cell body and their innervation target, it will be extremely challenging to use transplanted cells to regrow lost axons *de novo*. Therefore, preservation of existing axons is an important target for improving outcomes following SCI. Our results show a relative preservation of axons caudal to the injury epicenter. Additional data from our lab have shown the efficacy of Nrg-1 in improving locomotion in rats with chronic SCI (manuscript under review). Our findings showing axon preservation, combined with improved myelination of the preserved axons provides some mechanistic insights to explain the underlying cause of observed functional benefits.

While our findings are exciting and encouraging, it should be noted that there are also limitations to the methodologies employed in this project. Sample sizes for *in vivo* experiments were limited due to the time constraints of my BSc Med project (n=3-5). As such, we were not able to show statistically significant differences in the immunodensity of MBP, although a clear trend was observed. Higher sample size will be added to the data set in the near future to demonstrate the significance of these results. Additionally, our findings of improved myelination resulting from Nrg-1 treatment do not specify whether this is due to preservation of existing myelin or the recruitment of new myelinating cells. The inclusion of additional time points together with use of proliferation markers could help delineate the underlying mechanism(s).

In conclusion, we were able to show that Nrg-1 treatment improves myelination, preserves axons, and reduces glial scar in a rat model of chronic contusive SCI. These findings show that Nrg-1 therapy constitutes a promising therapeutic strategy for SCI which could be applied to a combinatorial strategy with NPC transplantation. Despite decades of research into the mechanisms underlying SCI, prognosis remains poor and patients have little hope for meaningful functional improvement. Our data provide compelling evidence for the potential of Nrg-1 to improve long-term outcomes of SCI, and give hope to patients who suffer from SCI in the future.

Figures and Tables

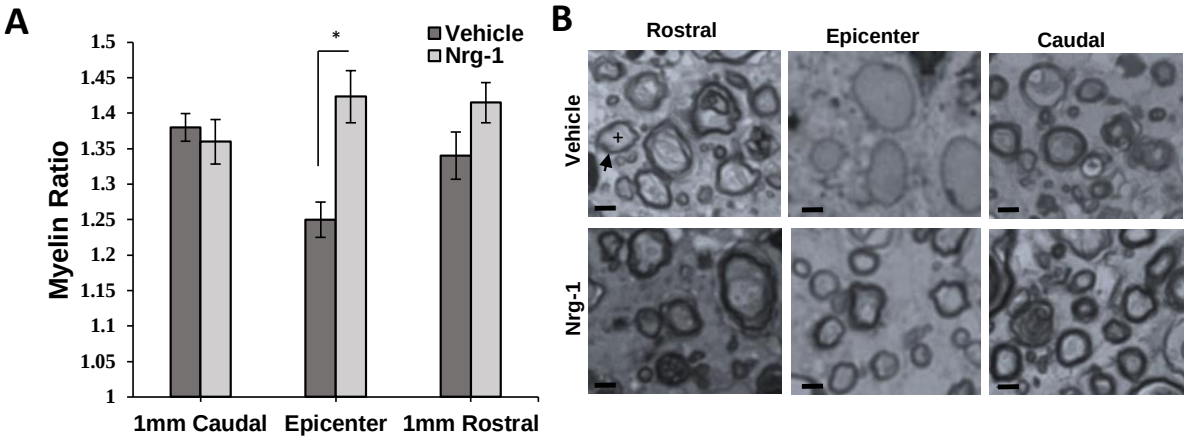

Figure 1. Nrg-1 increased myelin thickness at the injury epicenter. Myelin ratio calculations were performed on lateral column axons from the epicenter and perilesion areas at 70 day post-SCI representing chronic stage of injury. **(A)** Comparison between vehicle and Nrg-1 treated rats showed a significant increase in myelin thickness compared to vehicle treated rats. **(B)** Representative images of spinal cord lateral column at the epicenter and 1mm rostral and caudal, stained with Toluidine Blue imaged at 630x magnification in oil immersion. An axon (+) and surrounding myelin (arrow) are labelled as an example. The data show mean ± SEM. *P<0.05, one-way ANOVA followed by Holm-Sidak post-hoc test, N=3 animals/group; 1133 total axons included. Scale bar = 1µm.

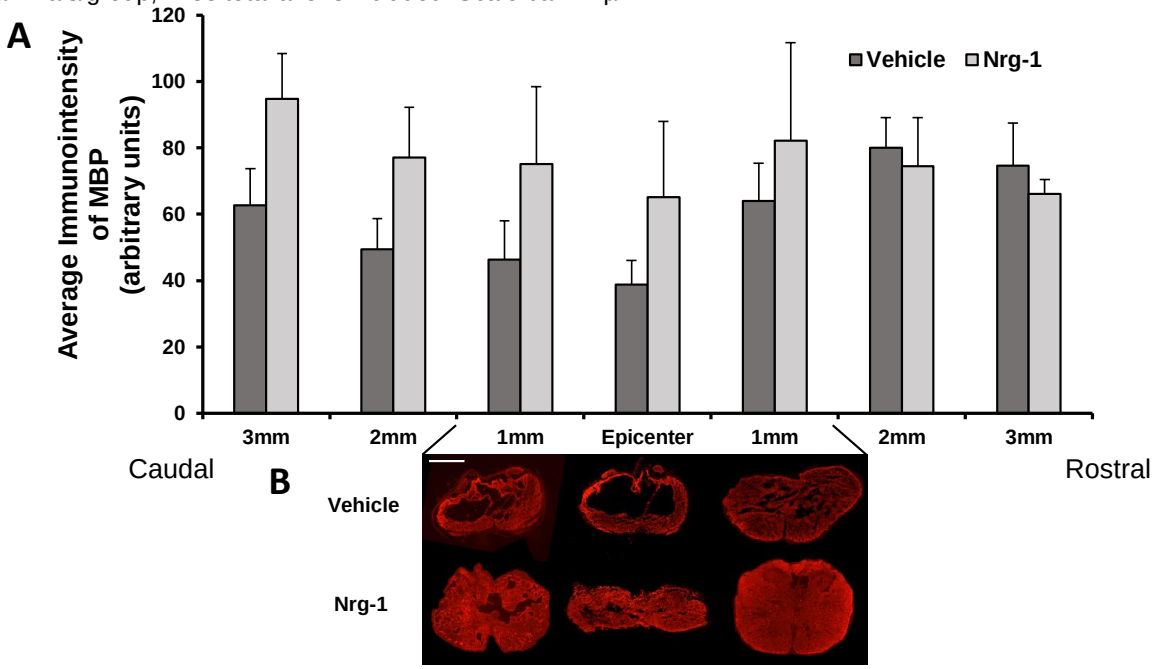

Figure 2. **Nrg-1 slightly increased MBP expression.** Assessment of MBP immunointensity was performed on the epicenter and perilesion areas spanning 3mm caudal and rostral at 70 day post-SCI representing chronic stages of injury. **(A)** Comparison between vehicle and Nrg-1 treated SCI rats showed a trend toward preservation of MBP immunointensity in Nrg-1 treated group, but this difference did not reach statistical significance. **(B)** Representative images of spinal cord cross-sections immunostained for MBP at 70d in sections at the epicenter and 1mm rostral and caudal. The data show mean ± SEM. *P<0.05, two-way ANOVA followed by Holm-Sidak post-hoc test, N=3/group. Scale bar = 1mm.

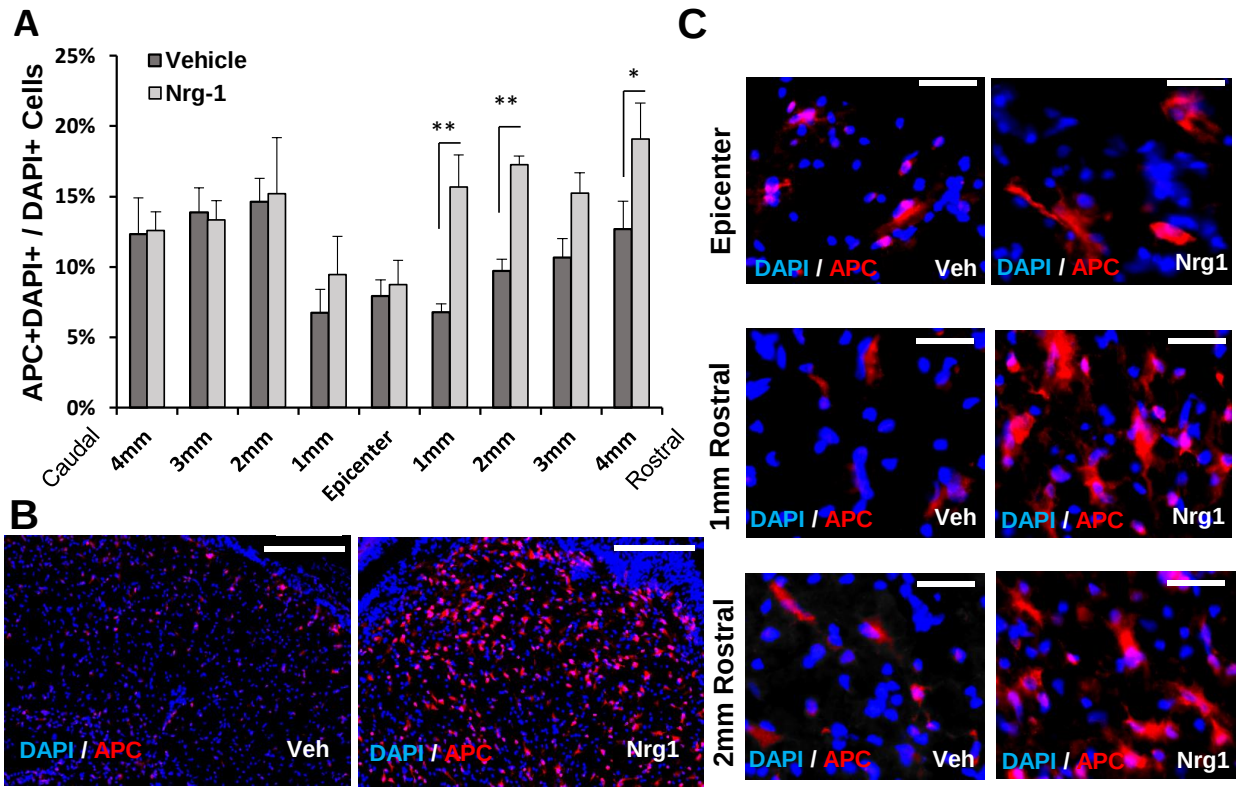

Figure 3. Nrg-1 Increased the number of mature oligodendrocytes rostral to the epicenter. (A) Comparison between vehicle and Nrg-1 treated rats showed an increase in the proportion of APC+ cells in sections rostral to the epicenter, with significant differences at 1mm, 2mm and 4mm distances. **(B)** Images from lateral column white matter tracts in sections 2mm rostral from the epicenter, demonstrating a clear increase in APC+ cells in the Nrg-1 group. **(C)** Representative images of APC+ cells (red) and DAPI stained nuclei (blue) showing an increase in the proportion of APC+ cells rostral to the epicenter. The data show mean ± SEM. *P<0.05; **P<0.01, two-way ANOVA followed by Holm-Sidak post-hoc test, N=3/group. Veh = Vehicle, Scale bar = 200 µm in **(B)**, 50µm in **(C)**.

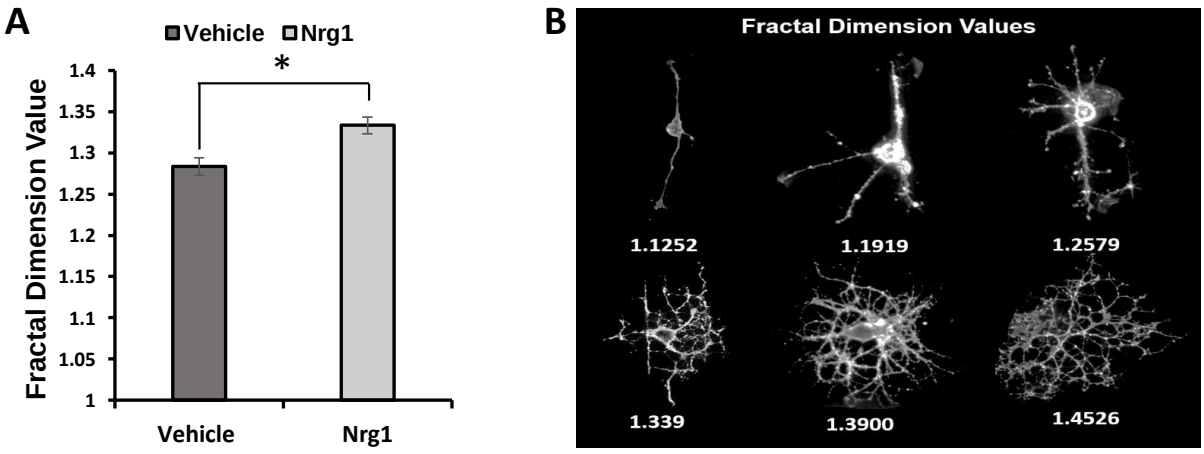

Figure 4: **Nrg-1 increased morphological complexity of OPCs. (A)** Comparison between vehicle and Nrg-1 treated OPCs showed a significant difference in FD values. **(B)** Representative images of OPCs with increasing morphological complexity and corresponding FD values. The data show mean ± SEM. *P<0.05, two-tailed t-test, N=15 cells/group/experiment, taken from 3 separate experiments.

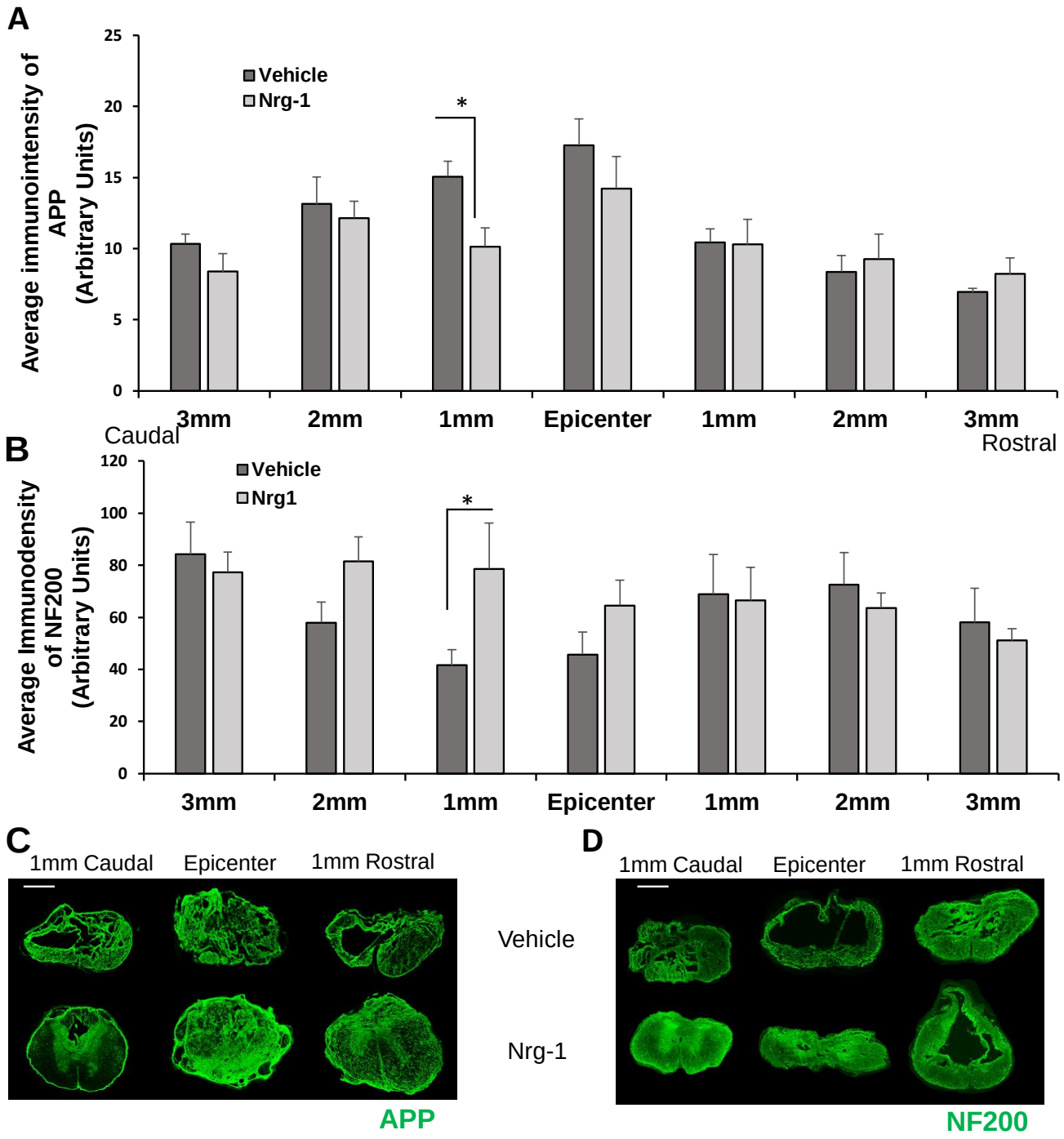

Figure 5. Nrg1 attenuated neural degeneration. Assessment of APP and NF200 immunointensity was performed on the epicenter and perilesion areas spanning 3mm caudal and rostral at 70 day post-SCI representing chronic stages of injury. **(A)** Nrg-1 treatment significantly reduced APP immunointensity, a marker for axon degeneration, 1mm caudal to the epicenter (*p < 0.05, two-way ANOVA, n=3/group). **(B)** Representative images of spinal cord cross-sections immunostained for APP at the epicenter and 1mm rostral and caudal. **(C)** Nrg-1 treatment significantly preserved NF200 expression, a neuron marker, 1mm caudal to the epicenter (*p < 0.05, two-way ANOVA, n=3/group). **(D)** Representative images of spinal cord cross-sections immunostained for NF200 at the epicenter and 1mm rostral and caudal. The data show mean ± SEM. *P<0.05, two-way ANOVA followed by Holm-Sidak post-hoc test, N=3/group. Scale bar = 1mm.

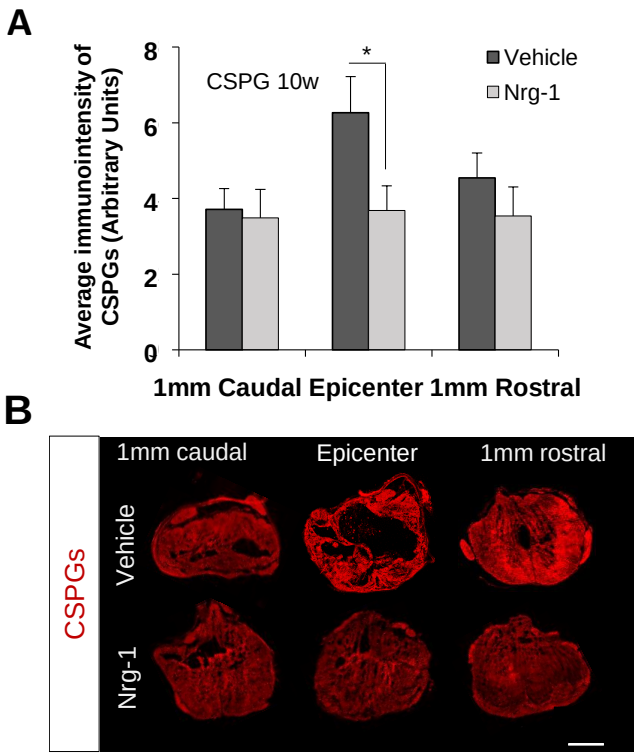

Figure 6. **Nrg-1 reduced CSPG production**. Assessment of CSPGs immunointensity was performed on the epicenter and perilesion areas at 70 day post-SCI representing chronic stages of injury. **(A)** Comparison between vehicle and Nrg-1 treated SCI rats showed a significant reduction in CSPGs immunointensity in Nrg-1 treated group at the injury epicenter compared to the vehicle treated groups. **(B)** Representative images of spinal cord cross-sections immunostained for CSPGs are depicted at epicenter and 1mm rostral and caudal to the lesion center. The data show mean ± SEM. *P<0.05, two-way ANOVA followed by Holm-Sidak post-hoc test, N=5/group. Scale bar = 1mm.

Table 1. Antibodies used for immunostaining with corresponding dilution factor.

Antibody	Specificity	Animal	Dilution Factor
Primary Antibodies			
MBP	Myelin	Mouse	1:500
APC	Oligodendrocytes	Mouse	1:50
NF200	Neurons	Rabbit	1:500
APP	Degenerating axons	Rabbit	1:800
CS56	CSPGs	Mouse	1:150
04	OPCs	Mouse	1:200
Secondary Antibodies			
Alexa488	Mouse	Goat	1:500
Alexa568	Mouse	Goat	1:500
Alexa647	Rabbit	Goat	1:500
DAPI	Nuclei	N/A	1:5000

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