UNIVERSITY OF CALIFORNIA, SAN DIEGO

Axonal regeneration following complete spinal cord transection in *NgR* and *NgR/Nogo/MAG* knockout mice

A Thesis submitted in partial satisfaction of the requirement for the degree Master of Science

in

Biology

by

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The Thesis of Renee Chow is approved and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

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DEDICATION

To my family for their support,

my labmates and advisors for their guidance,

and my friends for their encouragement.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
BBB	Basso, Beattie, and Bresnahan scale
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
CST	corticospinal tract
DAPI	4',6-diamidino-2-phenylindole
DRG	dorsal root ganglion
ER	endoplasmic reticulum
GFAP	glial fibrillary acidic protein
HSB	high salt buffer
KO	knockout
MAG	myelin-associated glycoprotein
NF	neurofilament
NGS	normal goat serum
NgR	Nogo Receptor
NNRG	NgR/Nogo/MAG
OMgp	oligodendrocyte myelin glycoprotein
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PNS	peripheral nervous system
ROCK	RhoA kinase

ACKNOWLEDGMENTS

I would like to thank all the members of the Zheng lab for guiding me through my project: Binhai Zheng for welcoming me into his lab and the world of research; Fang Xie for instructing me in assessing locomotor recovery of the mice and supervising me through this project; Jae Lee for training me in lab techniques critical to the completion of this project such as immunostaining, fluorescent microscopy and data analysis; Julia Herrmann for her invaluable insight and advice in lab and life; Sen Luu, Sharon Chow, Tim Batangan, Dwayne Chang, Michael Crawford, Andrea Chan and Katherine Ruby for their company and support throughout my stay with the lab.

ABSTRACT OF THE THESIS

Axonal regeneration following complete spinal cord transection in *NgR* and *NgR/Nogo/MAG* knockout mice

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Functional recovery following central nervous system (CNS) trauma is often restricted by stagnant growth of severed axons. A number of studies indicate that the presence of endogenous inhibitory molecules from astroglial scarring and myelin debris contribute to the diminished regenerative capacity of CNS axons. To assess the roles of two specific myelin-derived inhibitors – Nogo and myelin-associated glycoprotein (MAG) – and their common receptor, NgR, in the regrowth of mature

axons, we evaluated complete spinal transection models of single *NgR* and triple *NgR/Nogo/MAG* knockout (KO) mice. Previous studies of single *NgR* mutants using a dorsal hemisection model have shown that despite possible displays of improved behavioral recovery, there is no detectable regeneration of the corticospinal tract (CST). We focused our analysis on serotonergic (5-HT) fibers because previous studies suggest that these axons have a high intrinsic regenerative capability. Our results indicate that though there is an increase in 5-HT fiber sprouting within the injury site of single *NgR* KO mice, there is no significant enhancement in 5-HT fiber regeneration beyond the injury site in either NgR single mutants or NgR/Nogo/MAG triple mutant mice. These data suggest that disrupting two major myelin-derived axon growth inhibitors together with their common receptor NgR remains insufficient to elicit enhanced axon regeneration in the adult mammalian CNS. This calls for a re-examination of this molecular pathway for therapeutic development to treat spinal cord injury.

I. Introduction

Failure of axonal regeneration within the injured mature CNS is a multifactorial consequence of a decrease in positive environmental cues, a decline in the innate capacity for axonal growth, and the presence of active inhibitors of axonal regeneration (Filbin, 2003). Severed axons form dystrophic growth cones that were initially thought to be dormant structures incapable of further growth (Ramon y Cajal, 1928). However, later studies showed that when placed in a permissive setting such as that provided by a peripheral nerve graft, injured axons are able to project extensions over long distances (David and Aguayo, 1981). Along with other studies, this led to the hypothesis that the adult mammalian CNS contains inhibitory factors that actively block axon regeneration following injuries.

The extent to which CNS inhibitors suppress axonal growth has yet to be determined, as an increasing number of active inhibitors have been discovered, many of which are found in CNS myelin or the scar tissue. Immediately following injury, severed axons are inhibited by inhibitory molecules from surrounding myelin debris and intact oligodendrocytes (Yiu, G. & He, Z., 2006; Jones et al., 2003). Axonal growth is further limited upon formation of the glial scar, which is associated with upregulation of inhibitory chondroitin sulfate proteoglycans (CSPGs) (Yiu, 2006). Myelin-derived inhibitors were first recognized as non-permissive elements in the adult mammalian CNS environment when in vitro assays revealed that axonal growth was stifled by CNS myelin, but not by peripheral nervous system (PNS) myelin (Schwab et al., 1985; Yiu, G. & He, Z., 2006). Further studies stemming from this

observation led to the identification of several myelin-derived inhibitors, including Nogo and MAG, and their receptors (Fig. 1).

Nogo is a membrane protein with three known isoforms (Nogo-A, -B and -C) arising from the same gene through alternative splicing and promoter usage. Common to these three isoforms is an inhibitory 66-amino acid loop (Nogo-66) found on the extracellular surface of myelin. The isoform most highly expressed by oligodendrocytes is Nogo-A, which contains both Nogo-66 and another unique amino terminal (amino-Nogo) inhibitory domain (Yiu et al., 2006). Much of Nogo-A is found on the cytoplasmic surface of the endoplasmic reticulum (ER) where it has been shown to have a role in shaping the tubular ER (Voeltz et al., 2006). Low levels of Nogo-66 are detected on the plasmalemmal surface of cultured oligodendrocytes (Sandvig et al., 2004). The membrane topology of Nogo remains controversial, but it seems that Nogo-A is largely confined to the ER in the intact CNS and functions as an active in vivo inhibitor of axonal growth mainly after cellular lysis following injury (Sandvig et al,. 2004). The function of Nogo varies depending on CNS maturity. Recent studies have shown that Nogo-A induction coincides with CNS neural plate formation and has been implicated in neuronal differentiation and neuritogenesis (Caltharp et al., 2007). Following development, Nogo-A, which in the fetal stage is expressed throughout the brain, is primarily localized to oligodendrocytes where it inhibits neurite outgrowth.

Unlike Nogo, MAG, a transmembrane protein with five immunoglobulin-like domains in its extracellular region and a short intracellular domain, is present and

capable of inhibiting growth in both CNS oligodendrocytes and PNS Schwann cells (Yiu et al., 2006; Sandvig et al., 2004). MAG has long been recognized as a bifunctional molecule. Outgrowth assays support a role of axonal growth promotion for MAG in neonatal dorsal root ganglion (DRG) neurons that switches to inhibition in adult DRG neurons (Shen, Y.J. et al., 1998). This change in response to MAG is generally attributed to a developmentally regulated decrease in endogenous neuronal cAMP levels that occurs sometime around birth (Domeniconi et al., 2002; Cai et al., 2001). Additionally, studies indicate that, prior to injury, low affinity MAG interaction with axonal gangliosides GD1a and GT1b confers long-term axon stability and regulates axon cytoarchitecture (Pan, B. et al., 2005). MAG's inhibitory activity following CNS injury is largely ascribed to a soluble proteolytic fragment of MAG, termed dMAG, released by myelin debris surrounding the injury site (Tang et al, 1997; Sandvig et al., 2004).

Both Nogo and MAG exert their inhibitory influences through the Nogo receptor, NgR (Sandvig et al., 2004; Yiu et al., 2006). NgR is a glycosylphosphatidylinositol (GPI)-linked protein that binds with high affinity to Nogo-66, MAG and oligodendrocyte myelin glycoprotein (OMgp), another myelin-derived inhibitor, despite structural differences among these ligands (Yiu et al., 2006). Because NgR lacks an intracellular component, it requires coreceptor activity for signal transduction (Yiu et al., 2006; Sandvig et al., 2004). Two members of the tumor necrosis factor receptor family, p75 and TROY, have been found to complex with NgR. In neuronal populations that do not express p75, TROY has been found to

functionally substitute for p75. Studies have indicated that a third receptor component, LINGO1, complexes with NgR and p75/TROY and that co-expression of these three elements confers sensitivity to myelin inhibitors and subsequent RhoA/ROCK activation in non-neuronal cells (Yiu et al, 2006). Activation of RhoA, a small GTPase, is a convergence point in the intracellular signaling pathway of growth cone collapse mediated by CNS inhibitory molecules following injury (Yiu et al., 2006; Tang et al., 2003, Sandvig et al., 2004). Inhibitory ligand binding with NgR leads to activation of RhoA by guanine nucleotide exchange factors (GEF), which in turn begins an activation cascade of the Rho-associated kinase ROCK and LIM kinase that results in phosphorylation of the actin depolymerization cofactor cofilin, restricting axonal outgrowth by stabilizing the actin cytoskeleton (Yiu et al., 2006). Another Rho GTPase influenced by NgR signaling is Rac1, which when activated leads to growth cone mobility. Upon injury, it is thought that inhibitory molecules shift the balance of RhoA/Rac1 signaling in favor of RhoA-mediated growth cone collapse (Sandvig et al., 2004). Interestingly, NgR expression is absent in the fetal brain, which may explain the functional changes that both Nogo and MAG undergo from development through maturity (Caltharp et al., 2007).

Our main hypothesis stems from previous studies of myelin inhibitory ligand and receptor knockouts (Kim et al., 2003; Kim et al., 2004; Simonen et al., 2003; Zheng et al., 2003; Zheng et al., 2005) that have shown less consistent regeneration than one would expect, which suggests that there is much overlap and redundancy in the system. We hypothesized that disrupting multiple components of the myelin

inhibitory pathway, in particular, Nogo-A,B, NgR and MAG, would remove most of the redundancy in the system and would therefore be sufficient to elicit enhanced axon regeneration in the CNS. We tested this hypothesis by analyzing the regeneration phenotype of mice lacking all these three components. If our hypothesis is correct, by knocking out multiple components of the pathway including two inhibitors and a common receptor we will be able to see much more consistent regeneration in these mutants. The serotonergic (5-HT) fibers of the raphespinal tract were chosen for analysis because they have been shown to be more prone to regenerative sprouting than the corticospinal tract fibers that our lab has been studying. In fact, a study by the Strittmatter lab has shown regeneration of 5-HT fibers in the single *NgR* knockout (Kim et al., 2004). We have included an NgR mutant in our study as a reference point to examine the degree of any axon regeneration in our triple knockout.

The results obtained from our single *NgR* study contradict with published data, showing no significant difference in either behavioral recovery or 5-HT fiber regeneration from that of the wild type controls. In contrast, our NgR/Nogo/MAG (*NNRG) triple* KO mice show increased behavioral recovery; however, there is no significant difference in 5-HT fiber regeneration between the *NNRG* KO and WT sets, suggesting that the behavioral improvement is not dependent on raphespinal tract regeneration. Interestingly, our data with the triple KO mice indicate that there is a correlation between 5-HT and glial fibrillary astrocytic protein (GFAP) immunoreactivity, supporting arguments that astrocytes may be permissive, rather than inhibitory, to 5-HT fiber regeneration

II. Methods

Surgery and Care of Animals

All analyzed animals were females. *NNRG* mutants and controls were in 129S5×C57BL/6 mixed background, while *NgR* mutants and controls were in C57BL/6 background. These two studies were performed independently of each other but the results are presented here together for comparison purposes. Injuries were induced once the mice reached 6-8 weeks of age. They were first anesthetized with Avertin (1.3% tribromoethanol and 0.8% amyl alcohol; Sigma-Aldrich, St. Louis, MO), then shaved of back hair and swabbed with 70% isopropyl alcohol. A midline incision was made over the thoracic vertebrae, the paravertebral muscles were separated from the vertebral column and retracted, and a laminectomy was performed at T7-8. This was followed by complete transection of the spinal cord at this level using irridectomy scissors followed by a microknife. After hemostasis was achieved, the muscle layers and the skin were sutured.

Behavior Analysis

For six weeks following spinal cord injury, open-field locomotor deficits and recovery were assessed by weekly four-minute observations using the Basso, Beattie, and Bresnahan (BBB) scale modified for mice (Basso et al., 1996). During this observation period, BBB raters were blind to the mouse genotypes.

Histology

Animals were euthanized at specified post-injury times by anesthetic overdose with Nembutal and perfused transcardially with 4% paraformaldehyde (PFA) in 1X PBS (phosphate-buffered saline). Brains and spinal cords were removed and post-fixed by immersion in the perfusing solution for 2 h at 4°C, then cryoprotected overnight in 30% sucrose in PBS. The spinal cord tissue was then embedded in Tissue Tek O.C.T. compound (Sakura Finetek, Torrance, CA), frozen, and sectioned in two ways. First, an 8 mm block surrounding the injury site was sectioned in the sagittal plane, collecting every section on slides. Second, the remaining segments rostral and caudal to the injury site were sectioned in the transverse plane. All sections were cut at 20 μm.

Immunohistochemistry

Immunohistological detection of GFAP and either serotonergic fibers or neurofilament (NF) was performed on spinal cord sections derived from the animals of the complete transection experiments. The following primary antibodies were used: rat monoclonal GFAP antibody (1:500; Invitrogen, Carlsbad, CA), rabbit polyclonal serotonin antibody (1:20,000; Immunostar, Hudson, WI) and rabbit polyclonal NF antibody (1:500; Chemicon, Billerica, MA). Sections were washed with high salt buffer (HSB; 0.5 mM sodium chloride, 15 mM sodium phosphate, 0.3% Triton-X 100), then incubated overnight at room temperature in primary antibody diluted in 5% normal goat serum (NGS) in HSB, washed with HSB, and then incubated with Alexa

fluor 488 and 546 secondary antibodies (all used at 1:500; Invitrogen, Carlsbad, CA) for 90 m at room temperature. After washing with HSB and staining with 4',6-diamidino-2-phenylindole (DAPI), sections were mounted with Fluoromount G and imaged with a Nikon Eclipse E800 fluorescence microscope.

Quantification

Serotonin fiber infiltration into the injury site was determined using NIH ImageJ software version 1.37. For the *NgR* knockout study, the injury site, defined by GFAP staining, was traced and the injury area measured. Immunoreactive serotonergic fibers within this area were selected by thresholding and measured by area. In the sections where fibers were seen caudal to the injury site, the areas where these fibers reside were traced, thresholded and the highlighted area measured. For the triple knockout study, serotonergic fibers were selected by immunoreactive area as described for the NgR study caudal to and within the injury site, as well as in the 200 μm region rostral to the injury; neurofilament was measured by immunoreactive area 200 μm rostral to, within and 200 μm caudal to the injury site, as defined by GFAP staining, and measuring the percent immunoreactive area.

III. Results

Locomotor Recovery

To assess locomotor deficits and recovery following complete transection of the spinal cord at the thoracic level (T8), we used the standardized open field BBB scoring (Basso et al., 1996). The original scoring scale has a range of 0 to 21; we used a mouse-modified BBB scale (Zheng et al., 2003) which starts at 0 for complete hindlimb paralysis and goes up to 18 for normal hindlimb movement (Basso et al., 1996).

On post-injury Day 1, all *NgR* KO and control mice scored in the 0 to 1 range. Throughout the six-week observation period, both sets of mice averaged scores in the 0 to 5 range, corresponding to varying degrees of hindlimb joint movement without sweeping, plantar placement or stepping motions. Though the NgR-deficient mice show a trend of increased locomotor activity, the difference in the BBB scores between the two sets is not statistically significant (Fig. 2).

In contrast, the *NNRG* KO mice score significantly higher on the BBB scale than the control mice (Fig. 3). Importantly, on post-injury Day 1, all *NNRG* KO and control mice exhibited complete hindlimb paralysis with scores of 0, indicating lesion completeness. Over the course of the observation period, the recovery of the control mice fell within the same range as that observed with the NgR-deficient and control mice, while the *NNRG* KO mice recovered far more hindlimb movement and averaged scores of about 8, which corresponds to not only movement of the hindlimb joints but also sweeping or stepping without weight support, by six weeks post-injury.

Lack of Detectable Regeneration of Serotonergic Fibers in NgR-Deficient and *NNRG* KO Mice

To determine the effect of knocking out components of myelin-derived inhibitory pathways on axonal regeneration, we performed immunohistochemical analysis of serotonergic fibers of the raphespinal tract in our *NgR* and *NNRG* KO mice together with the control mice. These fibers were chosen for analysis because they partake in locomotor networking and have been shown to be more prone to regenerative growth than the corticospinal tract fibers, which the lab has been studying.

With the *NgR* study, rostral serotonergic fibers run along the dorsal side and spread ventrally as they approach the injury site in both the control (Fig. 4A) and KO (Fig. 4C). Fibers penetrating through the injury site and into the caudal region are observed in six mice, three from the control set (Fig. 4B) and three from the KO set (Fig. 4D). In all six of these mice, visible caudal fibers appear to travel through GFAP-positive ventral tissue bridges. In contrast to published data (Kim et al., 2004?), the caudal fibers seen here are few in number and do not extend far past the injury site. Quantification of 5-HT-positive fibers within (Fig. 5A) and caudal (Fig. 5B) to the injury area indicate that removing the Nogo receptor does not confer enhanced serotonergic fiber regeneration. GFAP immunoreactivity within the injury site (Fig. 5C) and total injury area (Fig. 5D) are comparable between control and NgR KO. Though 5-HT-positive fibers are seen only in GFAP-positive regions, we found

no correlation between the amount of 5-HT and GFAP immunoreactivity within the injury area (Fig. 6) of our *NgR* knockouts and controls.

Serotonergic fiber staining of the *NNRG* study displayed little to no 5-HT-positive fibers within and caudal to the injury site in both the control (Fig. 7A) and KO (Fig. 7C) samples. One mouse each from the control (Fig. 7B) and the KO (Fig. 7D) groups exhibit a single short 5-HT positive fiber caudal to the injury site. In both cases, these fibers do not travel through the injury site by way of GFAP-positive tissue bridges; further experimentation is required to determine the method by which these single fibers traverse the injury site. Quantification of the serotonergic fibers rostral to, within and caudal to the injury site (Fig. 8A) indicate that knocking out Nogo, MAG and their common NgR receptor is insufficient to permit serotonergic fiber regeneration through a complete transection spinal cord injury. However, quantification of GFAP in the same regions reveals significantly increased GFAP immunoreactivity in the rostral area of the *NNRG* mutants (Fig. 8B). The injury area between the controls and mutants are comparable (Fig. 8C). As seen in the *NgR* study, 5-HT-positive fibers rostral to the injury site are found exclusively in GFAP-positive tissue. In the *NNRG* controls and mutants, the data suggests a correlation between the amount of 5-HT and GFAP immunoreactivity both rostral to (Fig. 9A) and within the injury site (Fig. 9B).

No significant difference in neurofilament immunoreactivity within an *NNRG* subset

Since the *NNRG* KO mice do not exhibit regenerative serotonergic fibers, we stained a subset of *NNRG* mutants (Fig. 10A) and controls (Fig. 10B) for neurofilament to assess general axonal growth following injury. Though there is a trend of increased NF immunoreactivity rostral to the injury site of *NNRG* mutants, there is no statistically significant difference between the control and *NNRG* KO NF data from the regions rostral to, within or caudal to the injury site (Fig. 11A). Within this subset of mice, GFAP immunoreactivity within these three regions (Fig. 11B) and the size of the injury area (Fig. 11C) were comparable between the control and mutant sets. In contrast to the characteristic restriction of 5-HT immunoreactivity to the rostral side of the injury site, NF immunoreactivity is visible throughout the sagittal spinal cord section. Analysis of NF with respect to GFAP immunoreactivity (Fig. 12) indicates that there is no correlation between the two measures within the *NNRG* subset.

IV. Discussion

Limited plasticity within the CNS environment remains a major barrier along the path to functional recovery following injury. While it is known that a variety of inhibitory molecules exist within this system, the extent to which each exerts its inhibitory effect is unknown. Single knockout studies of myelin-derived inhibitors have thus far been unable to consistently yield improved regeneration in Nogo (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003) or NgR knockout mice (Kim et al., 2004; Steward et al., 2007; Zheng et al., 2005). Our triple knockout model sought to determine the efficacy of removing multiple sources of inhibition. Despite a marked increase of locomotor recovery in our triple knockout mice, histological analysis revealed no serotonergic fiber regeneration beyond the lesion. The results of this study indicate that deleting the three inhibitory molecules NgR, Nogo and MAG is insufficient to permit axonal regeneration, suggesting that growth inhibition by these molecules is not the only growth-limiting factor. Therefore, we shall explore other possible mechanisms that may account for the improved behavioral recovery of the triple knockout mice such as axonal sprouting around the injury site, axon regeneration by other tracts and an increase of local plasticity within the lumbar spinal cord.

The results of our single *NgR* knockout, which we used as a point of reference for assessing regeneration in our triple knockout, conflict with published data of improved locomotor recovery and increased fiber regeneration of the mutant. This discrepancy with the *NgR* results can be attributed to many factors, the most likely of

which include the different genetic backgrounds and inherent surgical variation such as in the severity of inflicted injury. A study of Nogo-A deficient mice suggested that the C57BL/6 strain, with which our mice were more heavily backcrossed, is less prone to regeneration than the 129/SvJ strain (Dimou et al., 2006).

Though we did not observe serotonergic fiber regeneration in either of our models, the triple knockout model shows a correlation between 5-HT and GFAP immunoreactivity, which supports the burgeoning argument that astrocytes may be permissive to axon regrowth, in particular from serotonergic fibers. There is much literature on the various roles of reactive astrocytes in response to CNS insult, fueling the ongoing debate as to whether post-injury cellular hypertrophy and gene expression changes of astrocytes are detrimental or beneficial to recovery (Sofroniew, 2005). Our mutants display elevated GFAP immunoreactivity rostral to the injury site that may potentially be associated not only with permission of serotonergic fiber growth but also the growth of other as of yet untested fiber tracts.

We have also shown that our *NNRG* mutants exhibit increased hindlimb locomotor recovery despite a lack of serotonergic fiber growth at the thoracic level, suggesting that this behavioral improvement is not attributable to the fibers of the raphespinal tract. In a subset of our *NNRG* samples comprised of three control and five mutant samples, we assayed for neurofilament at the thoracic level to determine general axonal growth following injury. While statistical analysis of NF reports no significant difference, we do observe a trend of increased NF rostral to the injury site that may become significant with the analysis of more mice. Further experimentation

is required to draw a conclusion on general axonal growth in the absence of Nogo, MAG and NgR, as well as the overall significance of myelin-derived inhibitors in hindering regeneration.

Our findings of increased NF-positive staining rostral to the injury site indicate that while there is no observable axonal regeneration, there exists some level of plasticity within this region. Future experiments should focus on assessing other forms of plasticity such as sprouting that may account for the enhanced behavior observed in the *NNRG* mutants as well as identifying the cell types from which the NF-positive axons arise. Methods such as reticulospinal tract tracing and neurotransmitter-specific stains for various neuron cell types will bring us closer to identifying fiber tracts whose regenerative capacity may be elevated by eliminating Nogo/MAG/NgR mediated inhibition, thus clarifying the efficacy of removing multiple components of CNS inhibition in permitting axonal regeneration and improving locomotor recovery.

Appendix

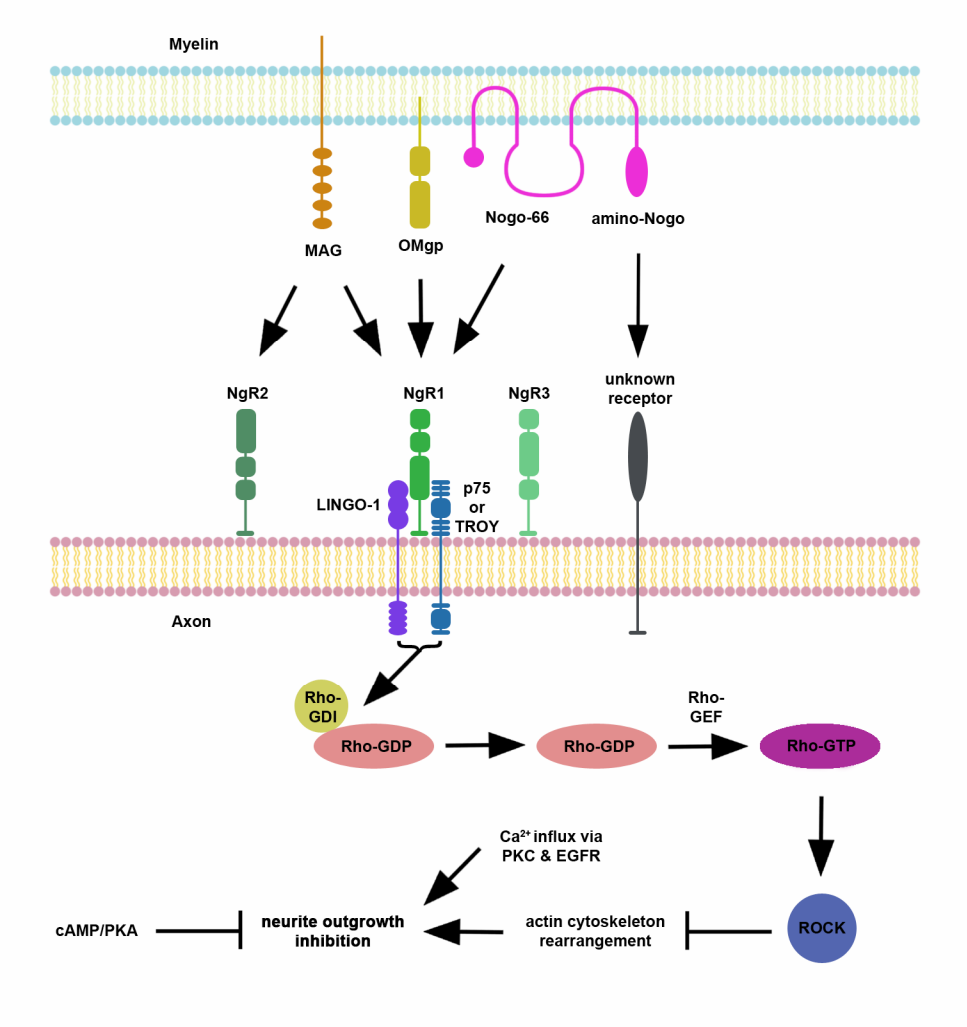

Figure 1. Schematic overview of myelin-associated inhibitors of neurite outgrowth and their downstream effectors. The proteins MAG, OMgp and Nogo, three known inhibitors of axonal regeneration expressed by oligodendrocytes of the central nervous system, commonly bind the GPI-anchored NgR1 protein. NgR1 transduces the inhibitory signal by forming a receptor complex with its transmembrane co-receptors LINGO-1 and either p75 or TROY. MAG also interacts with NgR2, a Nogo receptor protein whos signal transducing co-receptors have not yet been identified. The inhibitory signal triggers an activation cascade in the RhoA/ROCK signaling pathway leading to actin cytoskeleton rearrangement, growth cone collapse and ultimately inhibition of neurite outgrowth. Other downstream effectors of neurite outgrowth include PKC and EGFR, which are outgrowth inhibitors linked to calcium influx, and cAMP/PKA, which alleviates outgrowth inhibition.

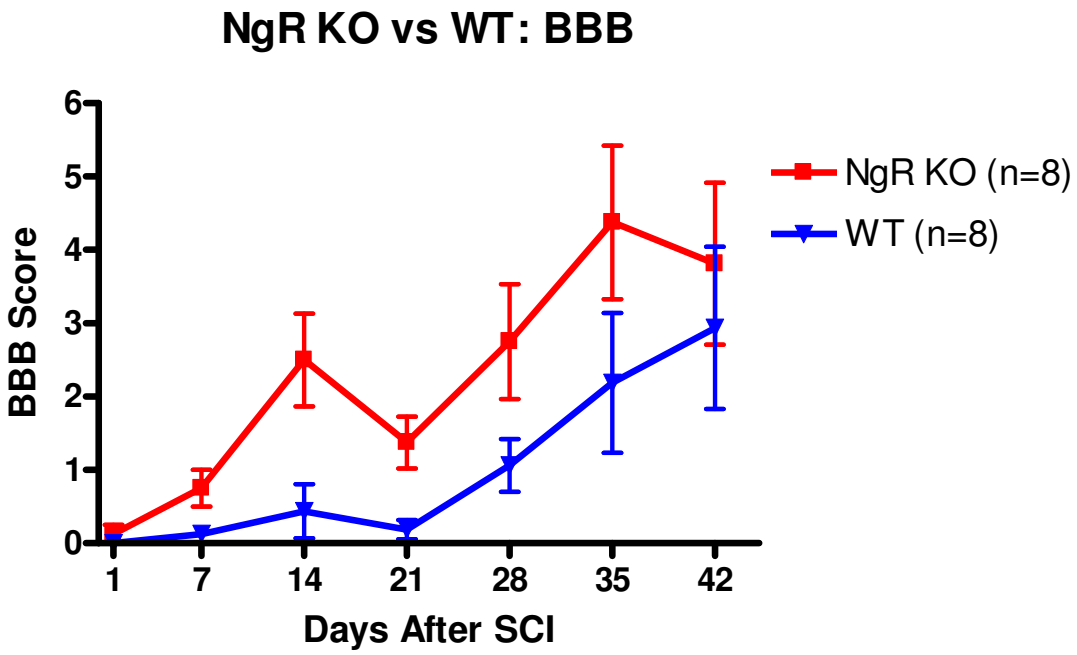

Figure 2. BBB scores of NgR knockout and wildtype mice over a six-week observation period following a complete thoracic level transection of the spinal cord. There is no significant difference in locomotor recovery between the NgR-deficient and the wildtype mice ($P > 0.05$; Two-way ANOVA).

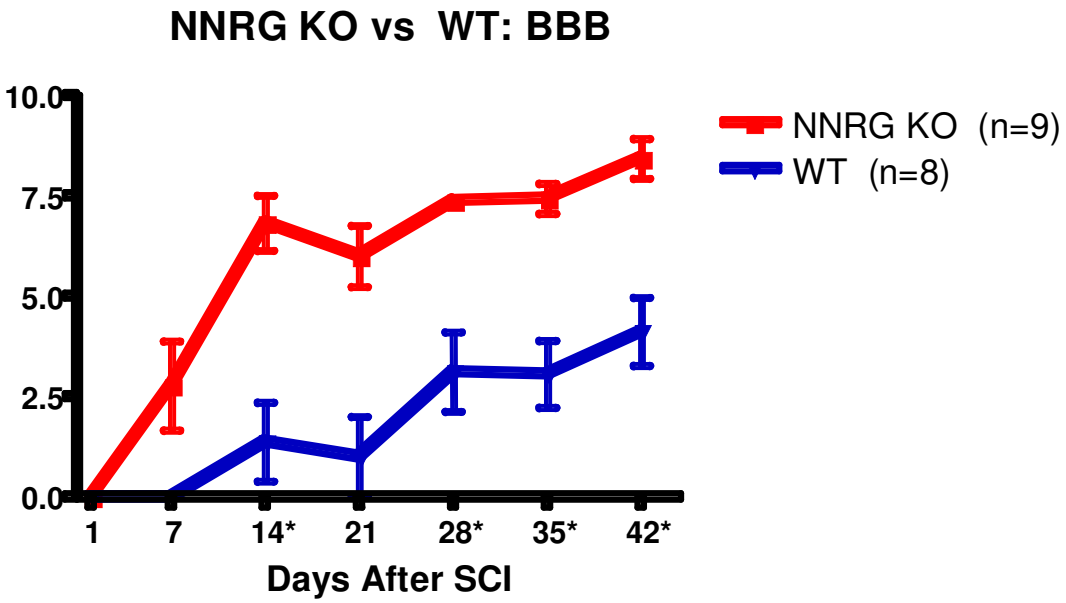

Figure 3. BBB scores of *NNRG* knockout and wildtype mice over a six-week observation period following a complete thoracic level transection of the spinal cord. The knockout mice show improved locomotor recovery compared to the controls on days 14, 28, 35 and 42 (P = 0.0002; Two-way ANOVA).

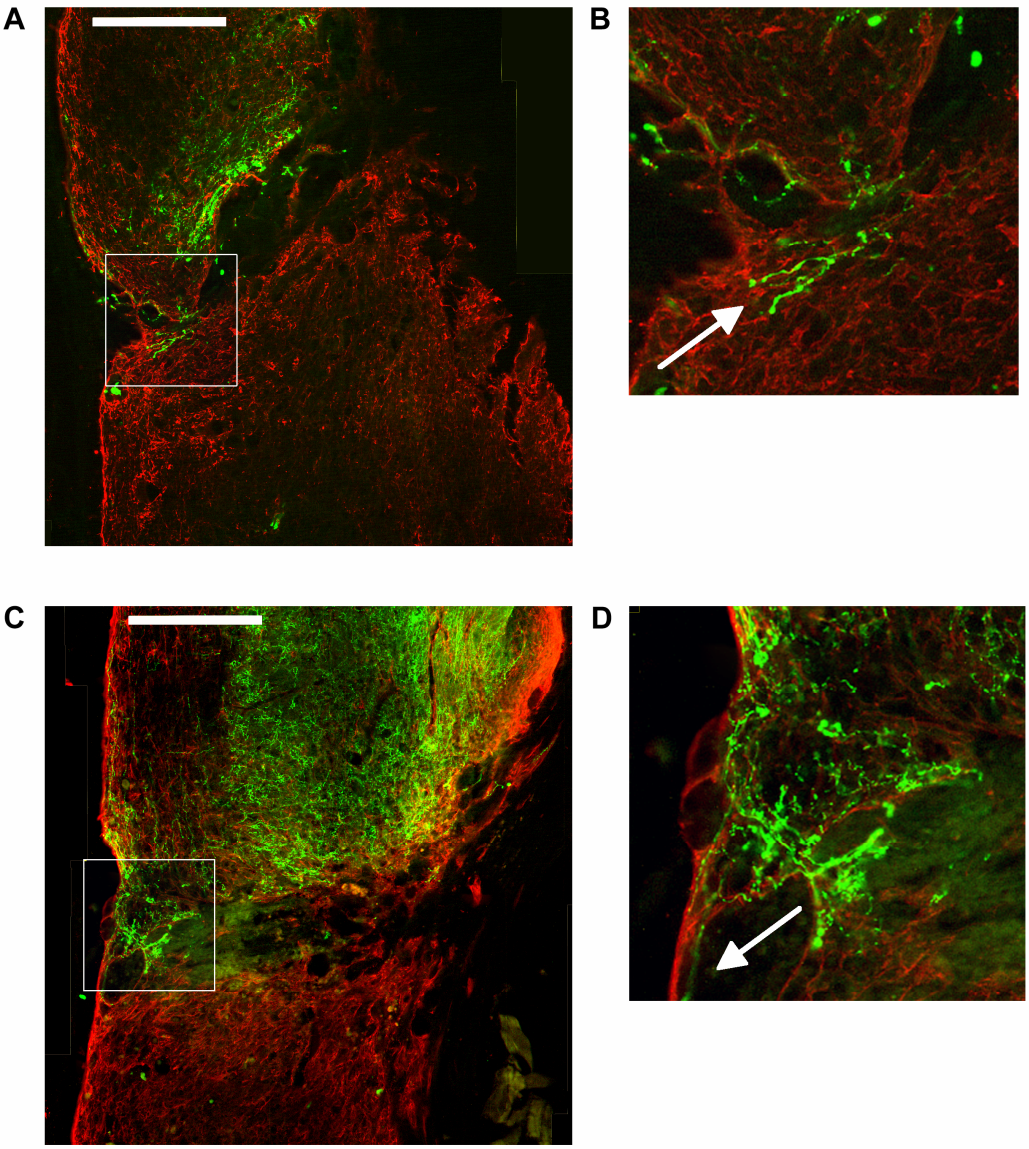

Figure 4. Serotonergic fibers detected caudal to injury site in NgR study. Representative images of caudal fibers seen in control (A) and NgR-deficient (C) mice. Close-up view of control (B) and KO (D), with arrows denoting caudal fibers. Sprouting fibers appear to travel through the injury site and into the caudal region by way of GFAP-positive ventral tissue bridges. Scale bar represents 250 um.

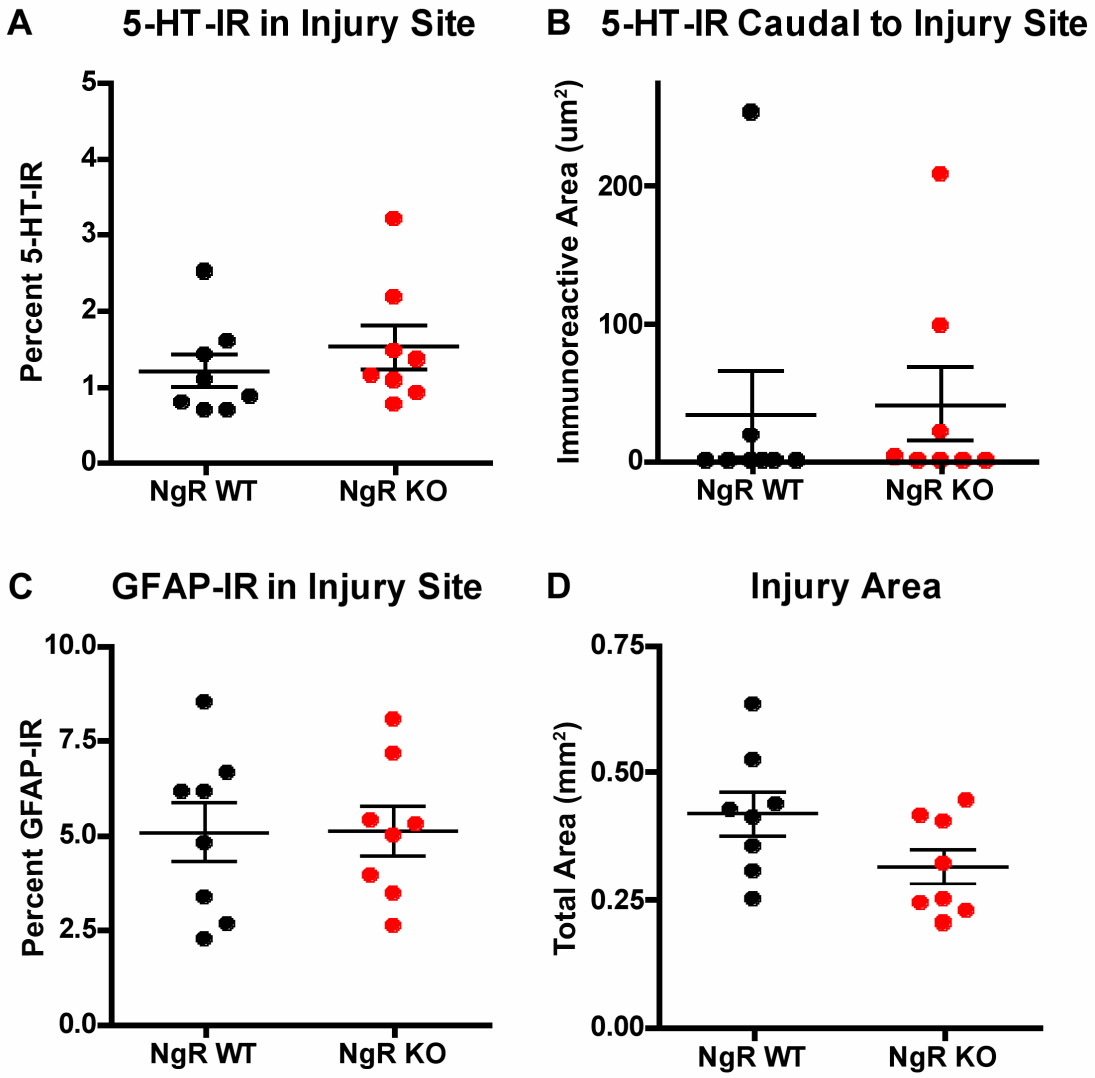

Figure 5. Histological analysis reveals no significant difference in 5-HT or GFAP immunoreactivity between NgR KO and WT sets. 5-HT fibers within (A) and caudal to (B) the injury site, as well as that of GFAP within the injury site (C) are plotted as the percent of immunoreactive area within the total area defined for analysis. The injury area (D) was consistent between the NgR KO and WT mice (for A-D, $P > 0.05$; Two-way ANOVA).

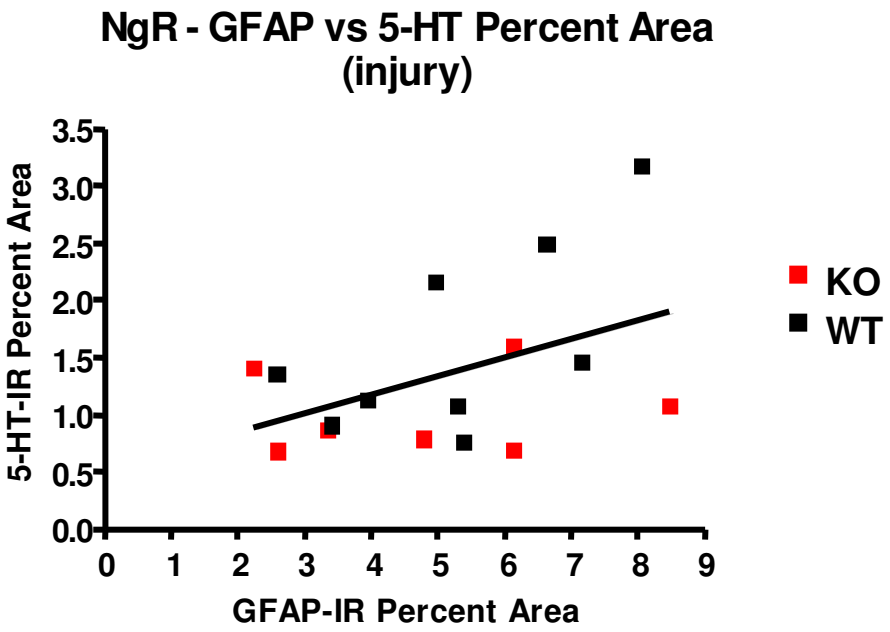

Figure 6. No correlation between GFAP and 5-HT immunoreactivity found in NgR study. GFAP and 5-HT are plotted as the percent of immunoreactive area within the lesion site. ($P > 0.05$; Two-way ANOVA).

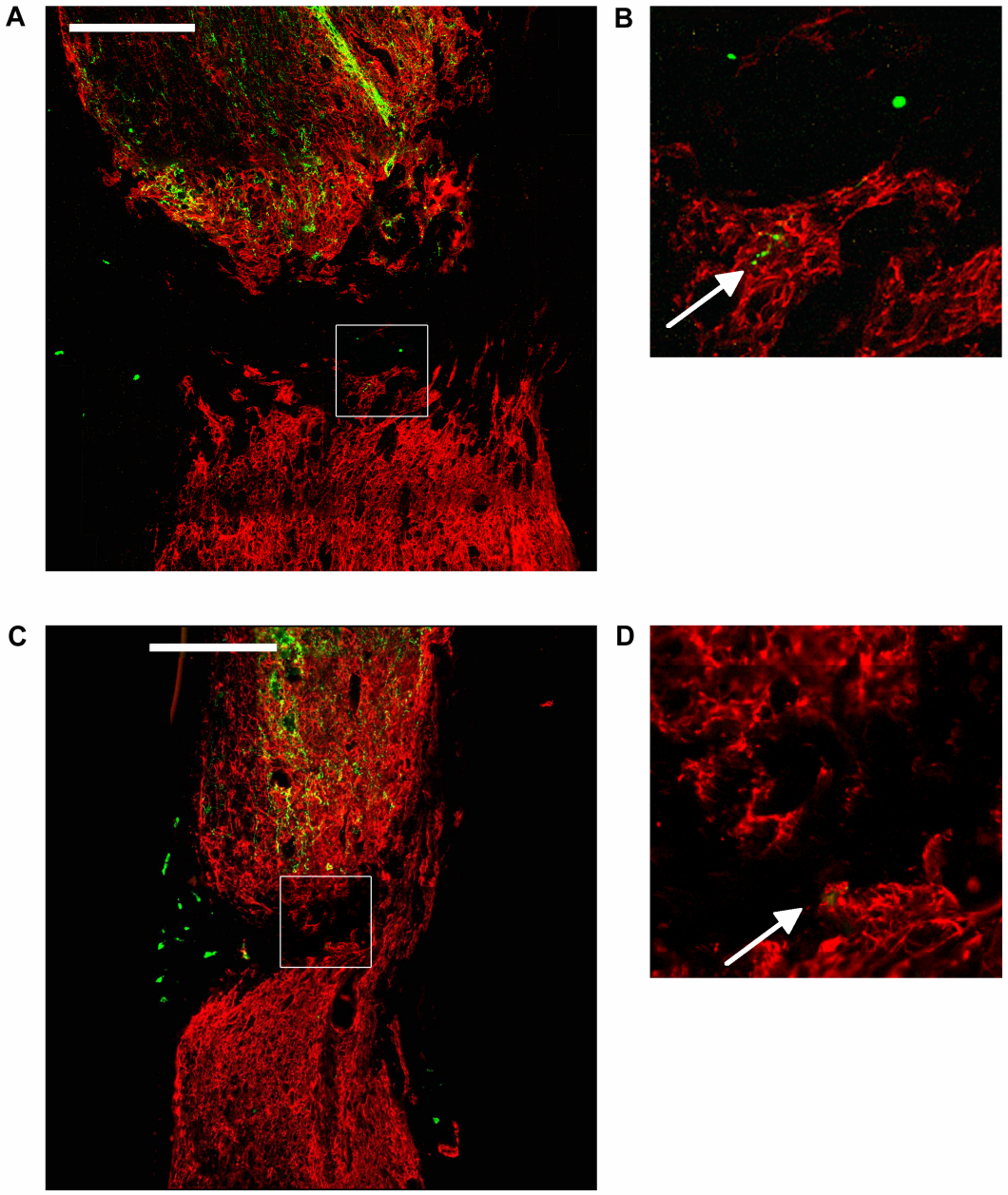

Figure 7. Serotonergic fibers detected caudal to injury site in *NNRG* study. Representative images of caudal fibers seen in control (A) and *NNRG* KO (C) mice. Close-up view of control (B) and KO (D), with arrows denoting caudal fibers. Here, sprouting fibers neither travel through GFAP-positive tissue bridges, nor are they restricted to the ventral side. Scale bar represents 250 um.

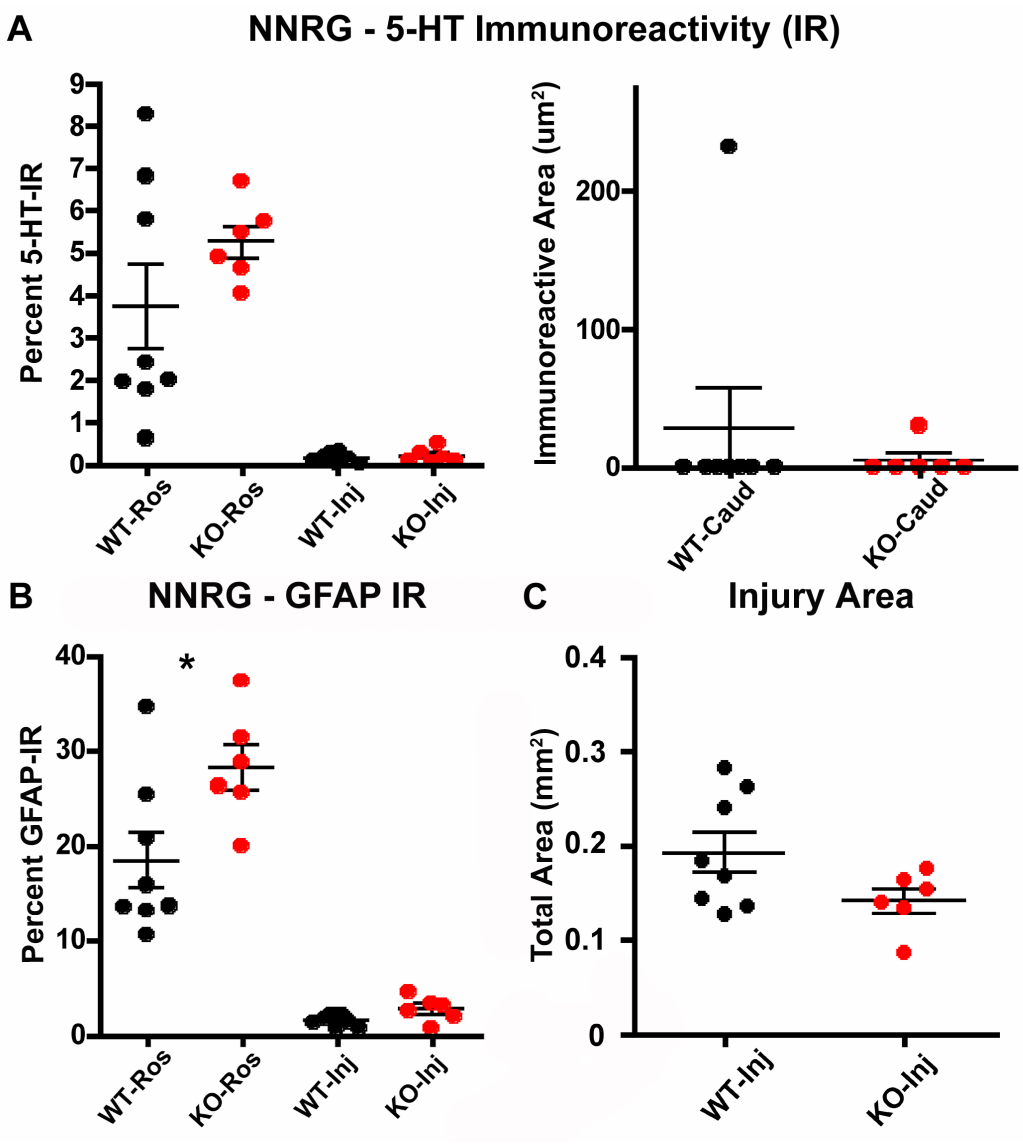

Figure 8. Histological analysis reveals increased GFAP immunoreactivity in *NNRG* KO; no significant difference in 5-HT immunoreactivity. Quantification shows elevated GFAP immunoreactivty rostral to injury in *NNRG* KO ($P<0.0001$; Two-Way ANOVA). 5-HT fibers (A) and GFAP (C) rostral to and within the injury site are plotted as the percent of immunoreactive area within the total area defined for analysis. 5-HT fibers caudal (B) to the injury site are plotted as raw immunoreactive area. The injury area (D) was consistent between the *NNRG* KO and control mice (for A-C, $P > 0.05$; Two-way ANOVA). The two mice with the greatest 5-HT immunoreactivity within the WT set (A) correspond to the two WT mice with the greatest GFAP immunoreactivity (B) rostral to injury. Despite the presence of two clusters within the WT set (A), these mice were handled and analyzed without any special treatment and thus were included in our data.

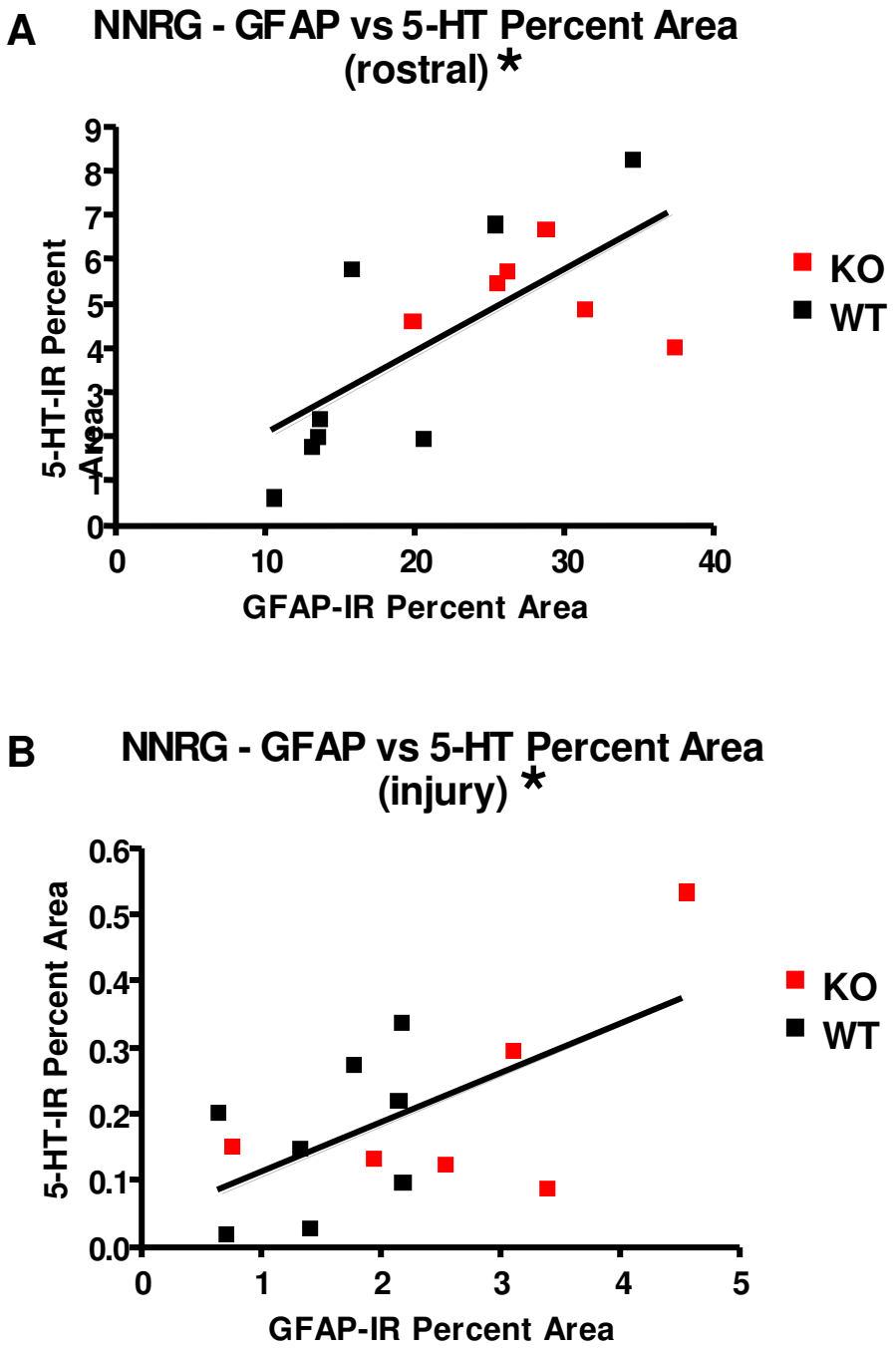

Figure 9. Correlation between GFAP and 5-HT immunoreactivity found in *NNRG* study. GFAP and 5-HT data are plotted as the percent immunoreactive area within the total defined area. In both the regions rostral to (A) and within (B) the injury site (P=0.0054 and 0.0217, respectively; Two-way ANOVA), 5-HT immunoreactivity increases with GFAP immunoreactivity.

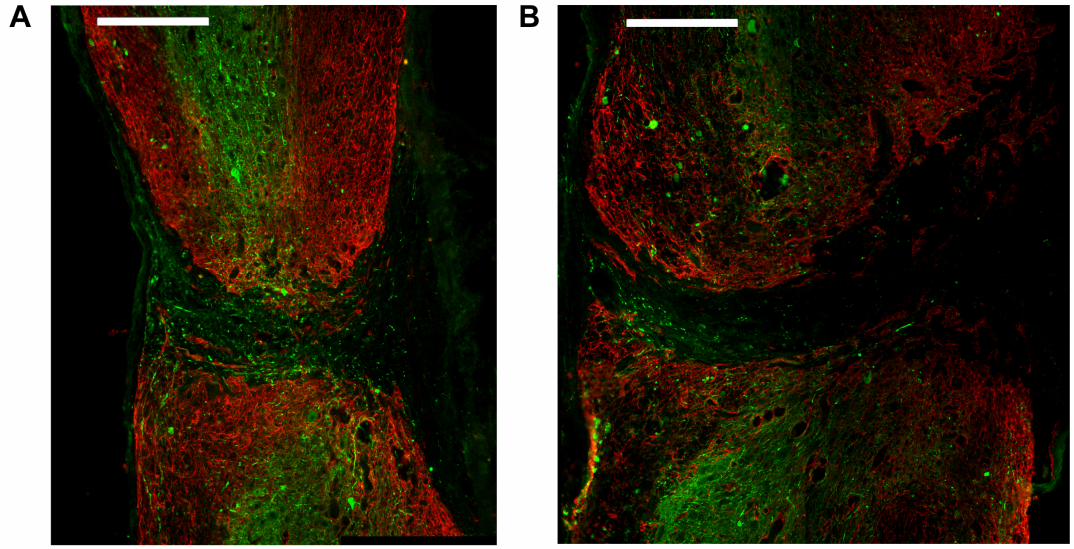

Figure 10. Neurofilament and GFAP staining images from *NNRG* study. Representative images from a *NNRG* KO (A) and a control (B). Scale bar represents 250 um.

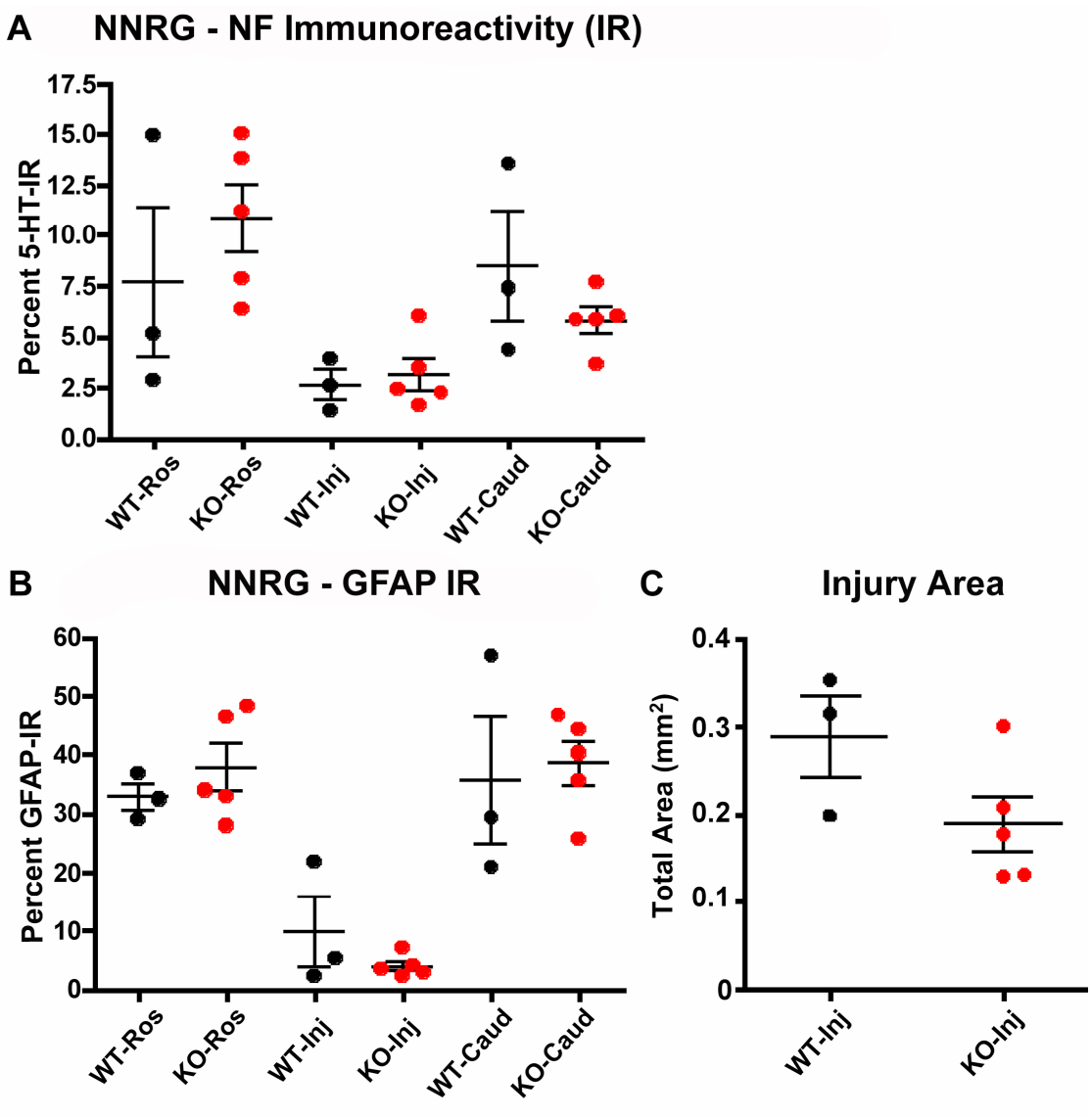

Figure 11. Histological analysis in subset of *NNRG* samples reveals no significant difference in NF or GFAP immunoreactivity between *NNRG* KO and control. NF (A) and GFAP (B) in the regions rostral to, within and caudal to the injury site are plotted as the percent of immunoreactive area within the total area defined for analysis. The injury area (C) was consistent between the *NNRG* KO and WT mice.

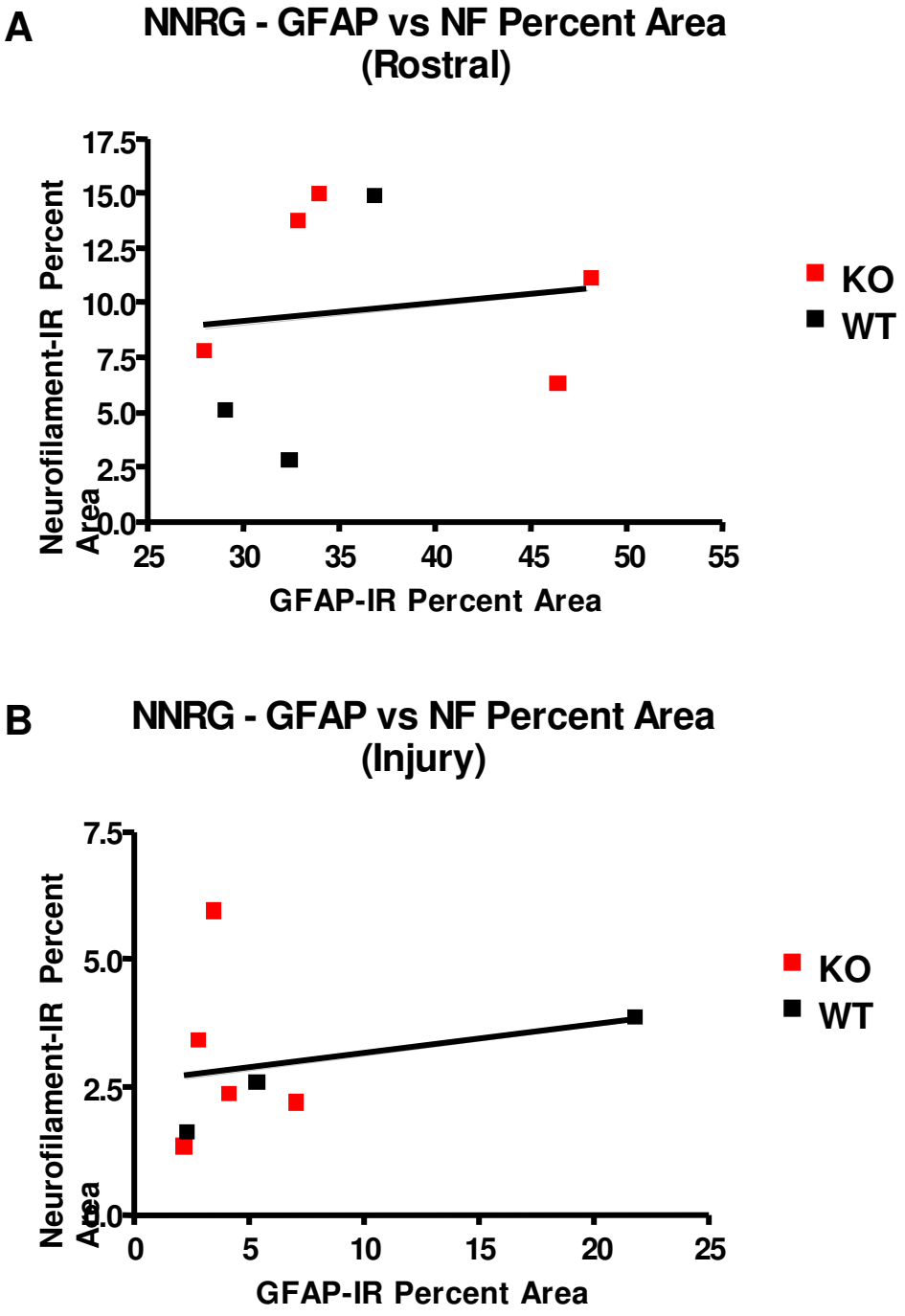

Figure 12. No correlation between GFAP and NF immunoreactivity in *NNRG* study. GFAP and NF are plotted as the percent of immunoreactive area both rostral to (A) and within the injury site (B) ($P > 0.05$; Two-way ANOVA).

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