UNIVERSITY OF CALIFORNIA, SAN DIEGO

Blocking Wnt Signaling in the Corticospinal Tract and Proprioceptive Sensory Axons

A Thesis submitted in partial satisfaction of the requirements of the
Degree Master of Science

in

Biology

by

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2013

The Thesis of Maysam M. Pessian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2013

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Acknowledgements

I would like to acknowledge Dr. Yimin Zou. Not only did his previous research establish the basis of my studies, but his direct mentorship and oversight of my research had a great effect on my development in the field.

I would also like to acknowledge Dr. Edmund Hollis II whom directly supervised the entirety of my research. Figures 1-4 were created by and donated by Dr. Hollis and he assisted in forming the graphs used in this thesis using the data I had produced.

ABSTRACT OF THE THESIS

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Professor Yimin Zou

Spinal cord injury (SCI) can lead to physical dysfunction or paralysis. These features of SCI have been documented to relate to damage to the corticospinal tract of axons in the spinal cord. Wnt is a morphogen credited, amongst many other developmental tracts, with the anterior-posterior directional growth of the developing spinal cord. This gradient is mediated by Wnt's interaction with the Ryk receptor. It has been recently documented that in the mature spinal cord, following injury, Ryk-mediated Wnt repulsion of spared or sprouting axons restricts plasticity. This study sought out to block the Wnt signaling pathway via ligand –binding inhibitors via bone marrow stromal cell grafts and Ryk receptor silencing via monoclonal antibody infusion to increase corticospinal tract axon plasticity and to see if an increase in plasticity correlates with

increased fine motor functional recovery. Most of the parameters used in the experiments involiving the cell grafts were duplicated in an experiment utilizing the peripheral nervous system conditioning paradigm by injecting ethidium bromide into the sciatic nerve. We found discrepancies in behavioral studies correlated more strongly with lesion cavity size as opposed to axon plasticity after injury, opening avenues of research looking at the mechanisms behind the glial response to SCI and the potential role of Wnt signaling associated with the glial response to SCI.

INTRODUCTION

Spinal cord injury (SCI) is debilitating injury that effects a significant amount of the world's population today. Up to 20,000 patients annually suffer from SCI in the United States alone [1]. SCI induces a multitude of problems and difficulties for the victims ranging from physiological to psychological. One of the most significant consequences of SCI is loss of motor function, paralysis. My research sets out to test for axon regeneration specifically in the rodent central nervous system (CNS) and behavioral recovery after spinal cord injury by disrupting a developmental signaling pathway via bone marrow cell grafts secreting specific inhibitors and a receptor knockout antibody infusion.

The muscular atrophy and consequent deficits in motor function after SCI can be attributed to damage to the corticospinal tract (CST) [2]. The CST is defined as the numerous (roughly 1,000,000) axon fibers descending from the motor neurons in the primary motor cortex [2]. These axons travel down the spinal cord with most of the fibers in human and non-human primates terminating onto the motor neurons that directly result in muscle activity. In primates, the CST terminations onto individual motor neurons in the spinal cord result fine motor control, while connections with interneurons in the spinal cord lead to the organization and execution of multi-muscular movements such as running or climbing. In rodents, although the CST still governs voluntary "fine" motor control [3], it is well documented that the axons that make up the CST primarily synapse

onto interneurons in the dorsal horn of the grey matter of the spinal cord [4].

To understand the mechanisms linking the primary motor cortex neurons and motor function, whole-cell recordings of the individual neurons in the motor cortex have been used. In the 1960's, Edward Evarts was able to correlate the activity of individual neurons with specific motor behaviors in primates [2]. Further research was conducted that linked varying motor cortex activity with not only the initiation of movement, but also the directionality and the force exerted by the movement [2]. These studies showed that the activity in the neurons in the primary motor cortex not only correlates with the activity in individual muscles, but also "higher-level" functions. This fact is strong evidence for defining the role of the CST in voluntary movement. A behavior experiment looking at the firing patterns of motor cortex neurons while a subject initiates a "precision" grip calling for the specific placement of digits on a handle as opposed to a "power" grip where the subject was to merely exert as much force as possible on handle showed varying firing patterns between the tests [2]. This experiment confirms the primary motor cortex's, along with the CST's, role in "fine" motor control. Loss of motor function is attributed to seemingly irreversible damage to these axons. Current research is looking at the reversal or prevention of the denervation atrophy depends on the capacity for surviving motor neurons to sprout and re-innervate as many muscle fibers as possible [5]. This emphasis on the regeneration of surviving motor neurons opened the door to many avenues of research including the topic of my studies, which look at the effect of the morphogen family of Wnts on axon plasticity.

Plasticity can be defined as a neuron's ability to remodel its projections in response to activity or injury. Motor function recovery following SCI is attributed to

plasticity and consequent compensation of uninjured motor systems, however, a significant proportion of functional recovery is also associated with the enhancement of original firing patterns [5]. In a primate study, the investigators found the dorsal component of the spinal cord contains about 95% of all the CST fibers while the ventral component only contains the remaining 5% [5]. Following the SCI, regression analysis showed that the total number of CST contacts with motor neurons in the spinal cord significantly correlated with the subjects performance on skilled motor tasks like the Whishaw forelimb reach task [3, 5]. Over the course of a motor activity period via behavior tasks, the investigators found a three-fold increase in CST axon contacts on motor neurons as compared to an uninjured system [5]. The study did not define any mechanisms of the action, but clearly established that neural plasticity is not only active in the spinal cord following injury but also positively correlates with recovery of motor function. These results call for further study into the actual mechanisms of this plasticity and the process's enhancement.

More recent studies on rodents also showed a substantial increase in CST collaterals after SCI as well. *Bareyre et al.* found a 2-fold increase in dorsolateral and ventral CST collaterals 4 weeks after SCI in mice [6]. In their studies, the investigators utilized the presynaptic marker SV2 in order to see if these new CST collaterals made direct contacts with motor neurons and they found that after 4 weeks the number of motor neurons contacted by CST axons had also doubled [6]. This group concluded that axons in the lateral portions of the CST rewire themselves after injury and form new direct synapses on motor neurons whose previous descending input had been

derived indirectly from dorsal CST axons, compensating for the loss of dorsal CST axons resulting from the dorsal lesion [6].

These well-documented studies pointing out axon plasticity in the CST following spinal cord injury drove the Zou lab's studies looking at the development of axons and finding ways to manipulate their growth. This drive led to the study of the role of Wnt morphogens, which also act as axon guidance molecules, after SCI and research into the mechanisms of their actions after SCI.

Wnts and axon guidance

Many facets of Wnt signaling have been defined. During development, the organization of the nervous system is based on the gradients of many guidance cues that direct sprouting axons to their appropriate targets along the appropriate axes. The role of Wnts in axon guidance was discovered utilizing the classical model of axon guidance, the commissural neurons of the spinal cord [7]. The canonical Wnt pathway is documented as resulting in the formation of the secondary axis during development highlighted by an accumulation of B-catenin in the cytoplasm; a "noncanonical" Wnt pathway was also discovered and is credited with activating planar cell polarity, which guides cellular movement during development [8]. During the development of the nervous system, upon reaching the midline, commissural axons decussate and post-crossing commissural axons are repelled by Slits and Semaphorin 3B secreted from the floor plate [9]. A global anterior–posterior guidance mechanism ensures the precise 90-degree turn of commissural axons immediately after midline crossing. A decreasing anterior-to-posterior mRNA gradient of the Wnts (Wnt4, Wnt7b, Wnt5a, and Wnt7a) dictates the

appropriate anterior turning of post-crossing commissural axons via the Frizzled3 receptor [7]. Disruption of the Wnt gradient leads to guidance defects of post-crossing commissural axons [7]. The logical target for Wnt signaling manipulation would be the family of receptors that are activated by Wnts.

In *Drosophila,* it was found that the receptor protein Derailed (Drl) mediates Wnt repulsion in the CST [10]. Wnt5, expressed in the posterior commissure, repels anterior commissural axons that express Drl, while the posterior commissural axons do not express Drl and mis-expression of Drl results in repulsion from the posterior commissure and aberrant projection through the anterior commissure [10]. In the vertebrate nervous system, the Drl homolog, Ryk, is expressed in cortical neurons. Ryk in the descending corticospinal motor axons mediates Wnt repulsion and is required for the descending growth of CST axons down the spinal cord [11].

Liu et al. used an *in situ* probe for Ryk and mapped out the varying locations of Ryk expression during the development of a mouse embryo (P0-E18.5) [12]. Their *in situ* results showed that Ryk is expressed in CST axons at the appropriate times to mediate Wnt repulsion of the developing spinal cord posteriorly [12]. In order to confirm that this Ryk-mediated Wnt repulsion is necessary for axon guidance, the group performed collagen gel assays with CST axons. They found that addition of purified anti-Ryk in the collagen gel assays blocked the repulsive effects of Wnt proteins, whereas the preimmune control did not. In the presence of preimmune serum, motor cortical axons tended to grow away from the point source of Wnt1 and Wnt5a. When anti-Ryk was included, motor cortical axons were no longer repelled, indicating that Ryk is required for mediating Wnt repulsion of CST axons [12]. The same study also found that neonatal spinal cord

secretes diffusible repellents that repel CST axons. Motor cortical axon cultures showed substantial radial growth in the presence of anti-Ryk, while in the preimmune control, CST axons were repelled by neonatal spinal cord in an anterior-posterior fashion. The group concluded that the graded repulsive effect is likely due to the graded distribution of the Wnt proteins, which they also conclude may provide directional information for cortical motor axons to grow posteriorly along the spinal cord [12]. *Liu et al.* finalized their study with an *in vivo* study on the effects of silencing Ryk function with their anti-Ryk antibody to test the role of Ryk in mice. They found that mice injected with anti-Ryk showed a marked reduction in CST fibers posterior to the injection site but an increase in CST areas anterior to the injection sites, whereas mice injected with the vehicle control or with the preimmune serum showed normal CST areas.

These studies show the required function of the Ryk receptor in CST development and provide more avenues for research into axon growth and guidance.

In a healthy adult spinal cord, Wnt mRNA expression is undetectable, however after spinal cord injury, re-induction of Wnt1, Wnt4, and Wnt5a occurs as evidenced by expression of mRNA in the cells immediately surrounding the lesion [12]The re-induction of Wnts after CNS injury is coupled with the recurrence of Ryk as well. The re-induced Wnts form gradients with the areas of highest concentration at the lesion sites and decrease both anteriorly and posteriorly relative to the lesion sites. Because Wnt signaling is prevalent during development and also after injury, it provides a potential avenue of further research. Manipulation of the Wnt gradient after SCI may promote CST regeneration, just as blocking Wnt-Ryk signaling during development results in defects in guidance of CST axons.

Previous work conducted in the Zou lab has implemented two strategies to observe the affects of Wnt-Ryk signaling manipulation: Ryk antibody infusion and cell grafts using bone marrow stromal cells (BMSC) secreting Wnt inhibitors, secreted frizzled-related protein 2 (SFRP2) or Wnt inhibitory factor 1 (WIF1) [8].

SFRPs are antagonists that directly bind to the Wnt protein and prevent binding with the Frizzled3 receptor [8]. Disruption of the Wnt-Frizzled3 signaling pathway results in dysfunction in both the canonical and non-canonical Wnt signaling pathways [8]. WIF-1 was discovered to bind to the extracellular domain of Wnts and inhibit Xwnt-8–Dfz2 interactions. The unique and highly conserved WIF domain is homologous with the Wnt-binding domain of Ryk [8].

Previous studies manipulating Wnts in the injured spinal cord

In the experiments utilizing cell grafts, syngeneic BMSCs were isolated from adult female rats and transduced *ex vivo* to secrete Wnt4, WIF1 or SFRP2 [13]. These cells were grafted at a cervical level 4 (C4) site of spinal cord injury. In order to label ascending sensory axons within the dorsal columns, the transganglionic tracer cholera toxin B subunit (CTB) was injected bilaterally into the sciatic nerves 3 days before sacrifice. Axon regeneration was assessed by the growth of CTB-labeled axons beyond the caudal host-graft interface and into BMSC grafts. The animals grafted with WIF1 or SFRP2 secreting BMSCs showed higher proportions of regenerating CTB-labeled axons while the animals grafted with the Wnt4 secreting BMSCs not only showed significantly fewer numbers of regenerated sensory axons, but also experienced repulsion of conditioned sensory axons from the cell grafts. These studies confirmed the role of Wnt-

mediated repulsion after SCI [13] . These results make up a significant part of the basis of my current studies.

Studies involving function-blocking Ryk antibody infusion conducted by *Liu et al.* also laid many foundations down for my research. Immediately following a C4 lesion, four injections of polyclonal Ryk antibodies were made into the spinal cord along the dorsal midline at 1.5 mm rostral, 0.5 mm rostral, 0.5 caudal, and 1.5 mm caudal to the lesion site [12]. 5 weeks after the initial lesion, the group found that in animals injected with the control treatment (Artificial CSF), many corticospinal tract axons retracted up to 1 mm away from the lesion border whereas the animals given the antibody treatment showed the presence of many CST axons close to the lesion border as defined through GFAP immunohistochemistry. Histological observation also revealed sprouting of CST collateral branches after the injury only in the antibody infused animals. Immunohistochemistry allows the investigator to visualize neurons and other cellular components *in vivo*, however, neither of the previously mentioned studies relate their findings to actual functional recovery of distal skeletal muscles after SCI. My experiments implemented several behavior studies coupled with Wnt-Ryk signaling inhibitors following SCI to assess functional recovery.

Peripheral Conditioning Lesion

The previously mentioned studies involving the BMSC grafts also utilized another paradigm in stimulating axon plasticity. It has been well documented that injury to the peripheral nervous system conditions sensory neurons in the central nervous system to regenerate [14]. A peripheral injury enhances the intrinsic growth capabilities of sensory

neurons in the CNS via gene regulation for transcriptional factors associated with axonal growth [15]. These previous studies point out that neural growth is based on the expression and transport of many proteins specifically required for neurite outgrowth and that early activation of the cascade of events resulting in this regulatory network greatly enhances this growth.

In the previous studies conducted by the Zou Lab, it was found that up regulation of Ryk in the primary sensory neurons in the dorsal root ganglia occurred in addition to the up regulation of growth-stimulating genes after peripheral conditioning [13]. In this study the investigators proceeded to manipulating Wnt-Ryk signaling via BMSC grafts as stated before. The results of this study showed only a 60% increase in regeneration with Wnt inhibition, however this increase does open the door to further study in combinational therapies to optimize a growth-stimulating environment. The current study on sensory axon regeneration will utilize sciatic nerve demyelination via 0.1% ethidium bromide injection followed one week by a C1 wire knife lesion as opposed to the sciatic nerve crush peripheral conditioning lesion and subsequent C4 wire-knife lesion.

Ethidium bromide is a well-documented demyelinating agent. Previous studies show that ethidium bromide induces Schwann cell intoxication leading to myelin condensation and subsequent demyelination [16]. These studies also demonstrated that rats do not displace robust clinical changes in behavior following sciatic nerve demyelination allowing for more implications to be made on CNS regeneration based on behavior studies.

The dorsal column nuclei, nucleus cuneatus and nucleus gracilis, are credited with receiving most of the ascending dorsal column sensory axons and relaying that

information to the thalamus [17]. My study utilizes injection of tracer into the ventral posterolateral nucleus of the thalamus to visualize sensory axon density in the dorsal column.

Analysis of behavioral function

The behavioral studies that were used in my experiments are defined extensively in Ian Whishaw's *Analysis of Behavior in Laboratory Rodents*. He starts his analysis with the statement: "The nervous system is designed to produce behavior, and so behavioral analysis is the ultimate assay of neural function"[3]. For my studies I implemented the entire set of behavioral studies Whishaw categorizes as "skilled movement". The cohesive feature of the "skilled movements" is that they seem much more disrupted by cortical lesions than are species-typical movements or movements of locomotion on a flat surface [3]. The distinguishing feature of the movements is that they require rotatory movements, irregular patterns movement, selective movements of a limb, and movements that break up the patterns of normal antigravity support [3]. Skilled movements in rodents and primates are quite comparable, which makes rodent models translatable to humans [18]. The analysis suggests using a full complement of behavior studies in order to neutralize as many variables as possible. For example, animals may display functional recovery in tasks such as the skilled forelimb reach over the course of a period of time, however, there is no way to determine whether this functional recovery is attributed to actual regeneration of neurons in the affected region of the nervous system or the recovery may be due to behavioral changes the animal makes in order to compensate for the injury to perform the task. In this case, skilled forelimb movement tasks spontaneous food pellet retrieval and the "grid-walk" task, which requires the animal to implement

gross motor movement to transverse a 1 meter long grid, would be implemented. A "beam cross" task will also be administered to the animals involved with the sensory axon regeneration experiment, this task is very similar to the gird walk task in design however a limited amount of tactile feedback is required in order to successfully position limbs on the beam as to not fall off. The utilization of sensory axon pathways warranted this test to look for possible trends between behavioral recovery and axon regeneration. . These defined behavior studies coupled with immunohistochemistry analysis of the lesion and presence of CST projecting neurons proximal to the border of the legion make up the analytical basis for my research.

I propose that disrupting Wnt-Ryk signaling after SCI will decrease CST axon retraction, induce sprouting of axon collaterals and promote functional recovery of fine and gross motor skills.

MATERIAL AND METHODS

Behavior Testing: All animal work in this research was approved by the UCSD Institutional Animal Care and Use Committee. Experimenters were kept blinded to the conditions of the subjects conducted all behavior testing and histological analysis. Adult female Fischer 344 rats were food restricted and trained on both skilled and unskilled forelimb behavioral tasks. There were 13 animals total used in this experiment: 6 animals, which received the monoclonal Ryk antibody and 7 control animals, which received an IgG antibody. All animals were trained on both the skilled movement forelimb reach task and the non-skilled gird walk task a week prior to injury. Following the injury, the animals were scored weekly for 16 weeks on both tasks and this data was analyzed at the completion of the experiment. The animals were sacrificed at week 18.

The forelimb reach task was designed as previously described by Miklyaeva, Castaneda, and Whishaw [19]. Food restricted animals were taught to reach across a 1cm gap and retrieve a 45mg sucrose pellet. A "success" was only rewarded when the animal successfully grasped and ingested a food pellet.

The grid walk task was also implemented in this experiment to test non-skilled limb movement. The tract was 5 feet long and 10 inches wide; the grids were separated by 2.25 $inch^2$ squares. Animals were required to complete a total of 3 lengths on the track in either direction during each weekly behavioral testing period. The number of forelimb "miss-steps" where the animal misses the grid with either

their left or right forelimb are calculated relative to the total number of steps it took that animal to complete the 3 lengths. As described in Whishaw's original description of behavioral tasks looking at the movement of limbs, videotaping was implemented so that the videos could be watched at slower rates to correctly categorize the miss-steps [3].

The beam cross test was administered to the animals receiving the peripheral conditioning lesion. This test is similar to the grid walk task however its requires tactile feedback from the curvatures of the beam to the subject in order to successfully position limbs during movement as to not fall off the side. This behavioral task can potentially show trends in sensory axon density after SCI based on behavior.

Video Analysis: The gird walk task was videotaped from the underside of the grid so that miss-steps are more clearly visible during video analysis. The video was analyzed on Macintosh QuickTime software, where the clips were viewed at slow frame rates to maximize the accuracy of the categorization of the animal's movement.

Sciatic nerve ethidium bromide injection: 0.15 EB (ethidium bromide) sciatic nerve injections were administered to adult female Fischer 344 rats (150-165g) unresponsive to toe or tail pinch under isoflurane anaesthetic. An area over the hindlimb was shaved and cleaned with povidone-iodine before incision caudal and parallel to the femur. The sciatic nerve was exposed and injected with EB. After injection, the skin was closed with surgical staples.

C1 and C5 dorsal column lesion: Following a training period for the various tasks, the animals were injured and implanted with an intrathecal catheter that was then connected to an osmotic mini-pump (Durect Corp., 0.25ul/hr infusion) at week 0 and then

tested for 16 weeks. The injury was conducted as described previously. [13]Animals were deeply anaesthetized with 2ml/kg of ketamine cocktail (25mg/ml ketamine, 1.3mg/ml xylazine and 0.25mg/ml acepromazine). Spinal level C1 was exposed by laminectomy and the dura was punctured over the dorsal horn, approximately 1.2mm lateral to midline in the animals involved in the sensory axon study. Spinal level C5 was lesioned in the animals involved in the CST study. A Scouten wire-knife (David Kopf Instruments, Tujunga, CA) was lowered to a depth of 1mm from the surface of the spinal cord and extruded. The dorsal columns were lesioned bilaterally with two passes of the wire-knife.

Isolation and transduction of syngeneic BMSCs: Primary BMSCs were isolated from adult female Fischer 344 rats by flushing cells out of the femur with standard media (DMEM with 10% FBS and Pen/Strep/Glu). Cells were cultured in standard media and passaged at 80% confluence. BMSCs were transduced *in vitro* with filtered supernatant from PhoenixA cells containing retrovirus encoding either myc-tagged Wnt4, HA-tagged WIF1 or HA-tagged SFRP2 (cloned from mouse cDNA library) and polybrene [10μg/ml]. Transduced BMSCs were selected for with G418 [1mg/ml] and the production of Wnt4-myc, WIF1-HA and SFRP2-HA were confirmed via Western blot. Prior to transplantation, BMSCs were cultured in standard media for 48hrs, trypsinized [0.05% vol/vol] and resuspended in PBS at 100,000 cells/μl.

Osmotic Mini-Pump Implantation and Explanation: Intrathecal catheters attached the pumps were threaded through magna cisterna, under the dura to the cervical spinal cord so that the antibody could be delivered directly to the injured tissue. Control rabbit IgG or Ryk monoclonal antibody [1mg/ml in Artificial CSF] was released 0.25uL/ day for 28 days. The pumps were removed at week 4.

Biotinylated Dextran Amine (BDA) Tracing at Week 16: Upon the completion of the behavior tests at week 16, 10% BDA tracer was injected directly into the motor cortex to visualize CST axons as previously described. [12] The animals were anesthetized and small "burr" holes were made in the skull, over the sensorimotor cortex, BDA (10,000 molecular weight; Invitrogen), an anterograde tracer, was then injected into motor cortex via picospritzer.[20]. BDA injections were made bilaterally to visualize the longitudinal growth of CST axons in sagittal sections in both directions.

Retrograde Labeling of *Nucleus Gracilis* Cells via VPL Injection: Five days before perfusion, 4 months after injury, 4% fluorogold (in PBS) was injected bilaterally into the VPL in the thalamus.

Tissue Processing and Immunohistochemistry: After allowing 2 weeks for BDA tracer transport, at week 18, the animals were deeply anaesthetized with ketamine cocktail and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde in PBS. Spinal columns were post-fixed overnight at 4°C in 4% paraformaldehyde. Tissue was transferred to 30% sucrose in PBS for cryoprotection and sectioned on a cryostat (Leica, Buffalo Grove, IL) at 40μm thick and collected as free-floating sections. Sections were washed three times with TBS, blocked for one hour in TBS with 0.25% triton-X100 (TBST) and 5% donkey serum, then incubated overnight at 4°C with primary antibodies in TBST plus 5% donkey serum. On the second day of staining, sections were washed three times, incubated with Alexa Fluor conjugated secondary antibodies (Life Technologies, Grand Island, NY) for two and a half hours at room temperature (RT) The following primary antibody was used in this study: rabbit anti-GFAP (1:750; Dako) to detect glial fibrillary acidic protein. Histology was performed using the program Stereo

Investigator Version 9 (MBF Science, Williston, VT) By visualizing GFAP- secreting cells, investigators can discern the boundaries of the lesion and also overlay the presence of BDA labeled CST axons and note the proximity of these axons to the lesion.

For BDA reaction, sections were incubated sequentially with ABC Elite Reagent Kit (Vector Labs) and Alexa Fluor 488 conjugated streptavidin (Life Technologies)

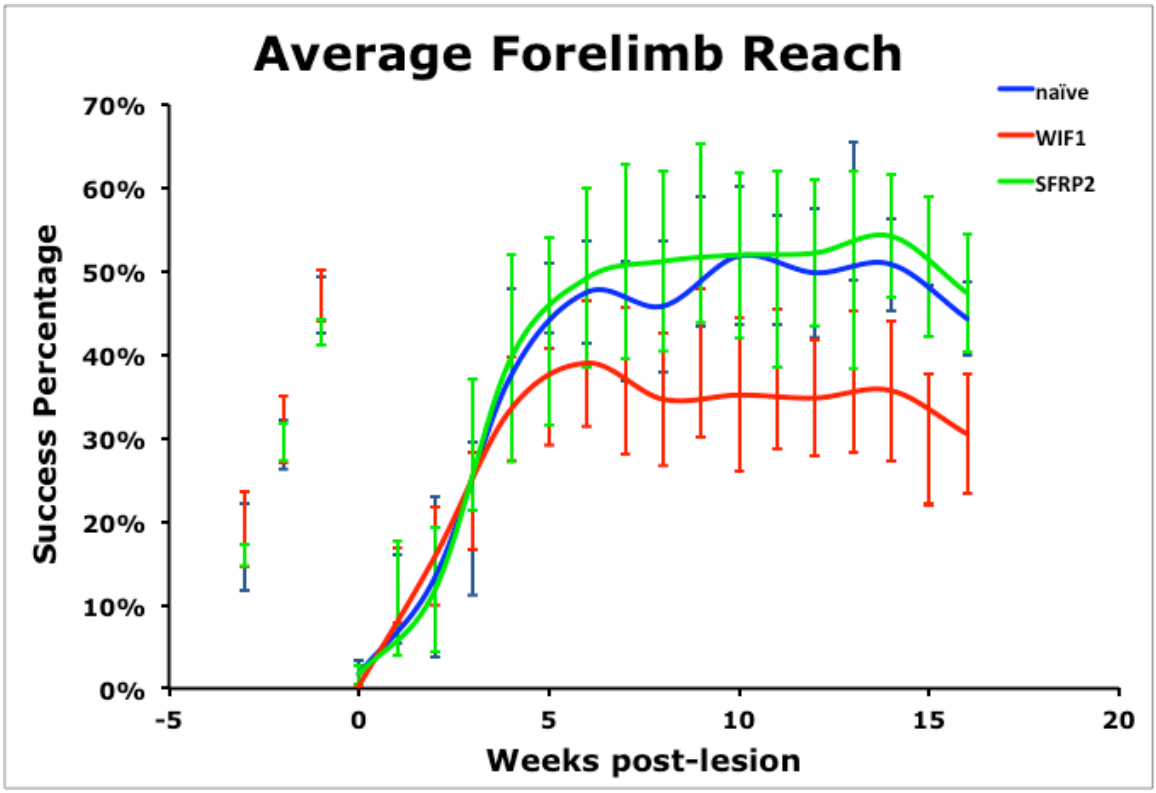
Quantification of BDA-Labeled and Fluorogold-Labeled Axons: Images were taken on an inverted Zeiss LSM510 confocal microscope with LSM acquisition software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Image density quantification was done on thresholded fluorescent images using ImageJ (NIH, Bethesda, MD). Statistical figures were created using Microsoft Excel. An investigator blinded to the experimental group performed all analyses.

RESULTS

Behavioral Testing

Animals treated with BMSC grafts secreting WIF1 tended to exhibit impaired behavioral recovery in fine skilled movement following SCI.

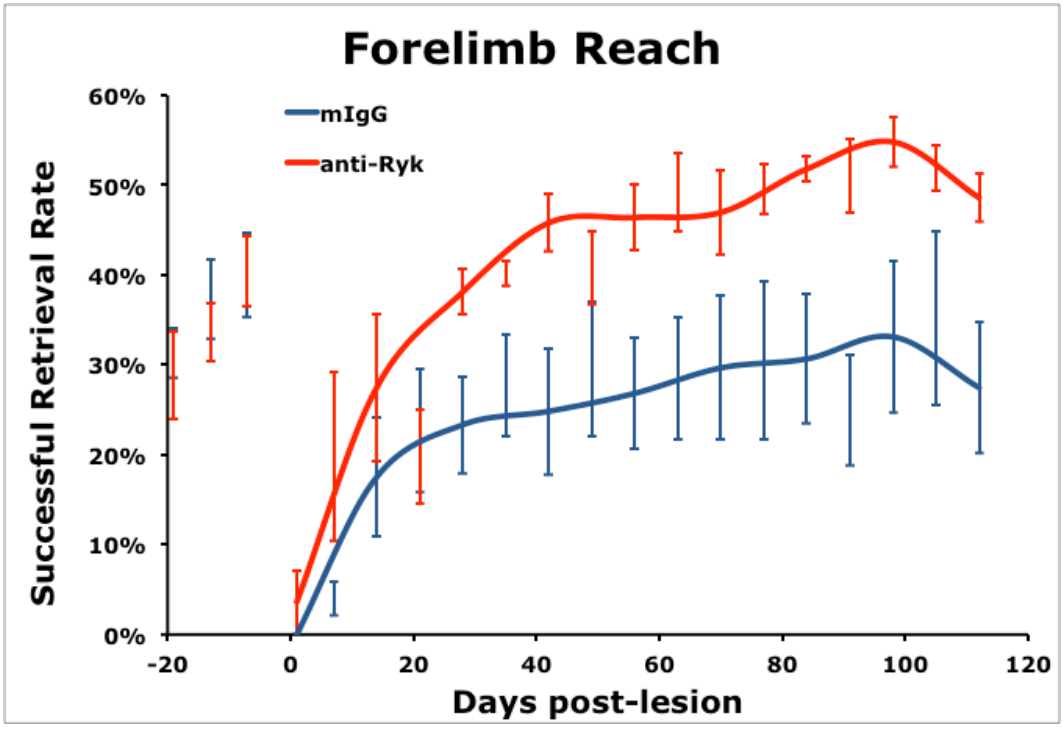
Graph 1. Recovery of fine motor control assayed by forelimb reach is mildly impaired when BMSC grafts secrete WIF1. Repeated measures ANOVA P=0.33



Recovery of fine motor function after a C5 dorsal column lesion is similar when animals are grafted with either control naïve BMSCs or SFRP2-secreting BMSCs. Animals grafted with WIF1-secreting BMSCs, however, exhibit a trend towards impaired behavioral recovery

Animals treated monoclonal Ryk silencing antibody show significantly high gains in fine skilled behavioral recovery

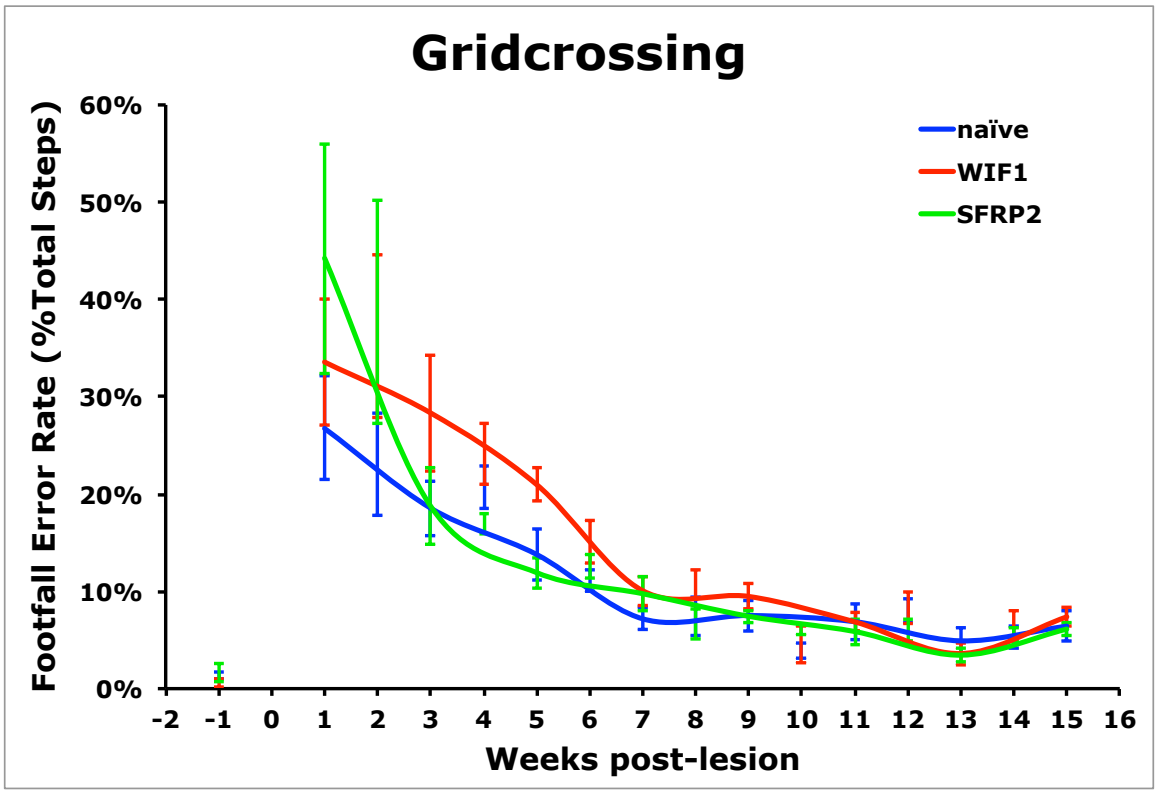
Graph 2. Continuous infusion of monoclonal Ryk antibody for 28 days promotes recovery of skilled forelimb function after SCI (n=6 mIgG, n=5 anti-Ryk, Repeated measures ANOVA P<0.05)



Animals that did not receive BMSC grafts, exhibited impaired recovery when infused with control mouse IgG by osmotic minipump for 28 days. Those infused with function blocking Ryk antibody show significantly more recovery compared to the IgG control group.

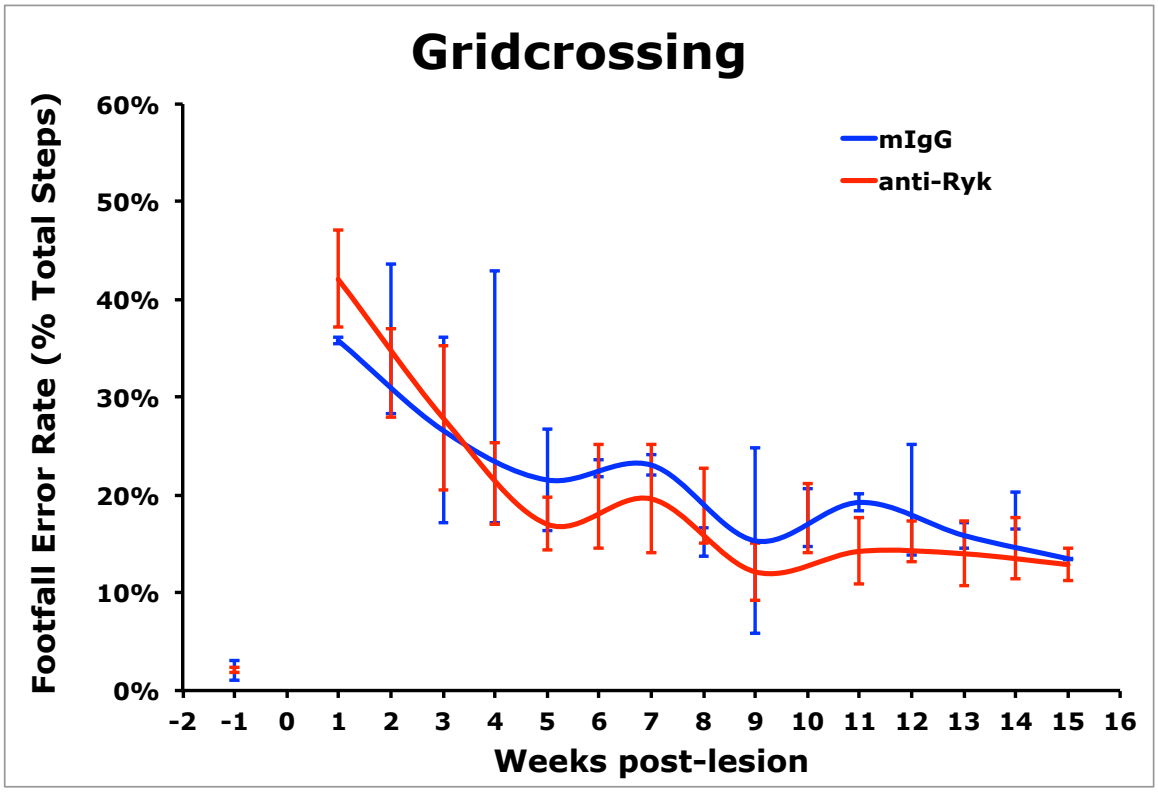
Recovery of animals on non-skilled motor tasks showed no differences between treatment groups

Graph 3. Non-skilled motor function recovered similarly, independent of Wnt inhibition by BMSC grafts.



Animals grafted with BMSCs exhibited no differences in the rates of recovery from SCI on the grid crossing task, a test of gross motor control (Graph 3). Those infused with Ryk monoclonal antibody or mouse IgG control showed similar recovery on the grid crossing task (Graph 4).

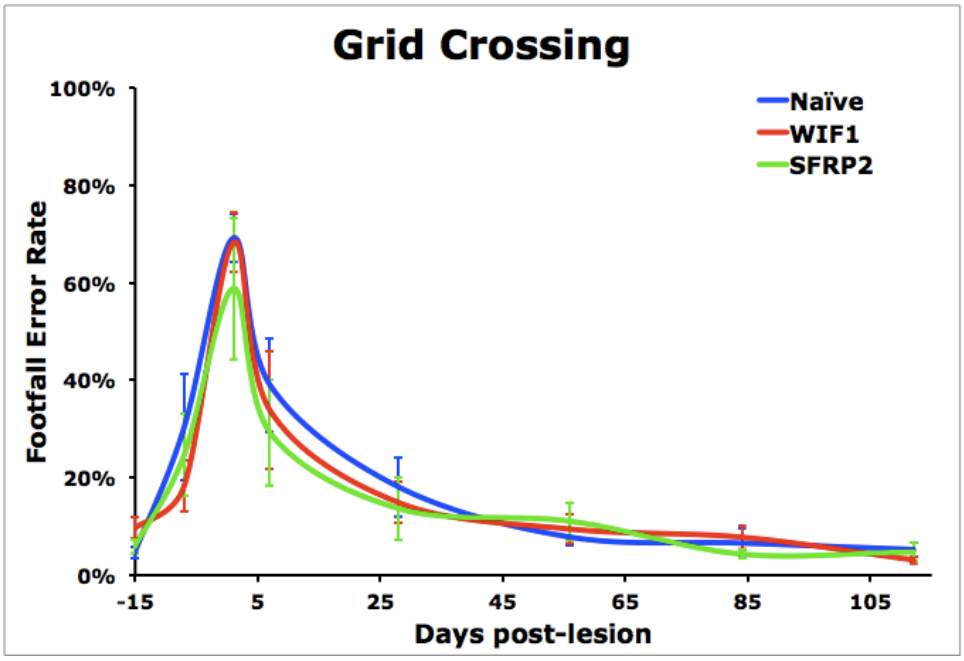
Graph 4. Non-skilled motor function recovered similarly, independent of Wnt-Ryk inhibition by monoclonal antibody infusion.



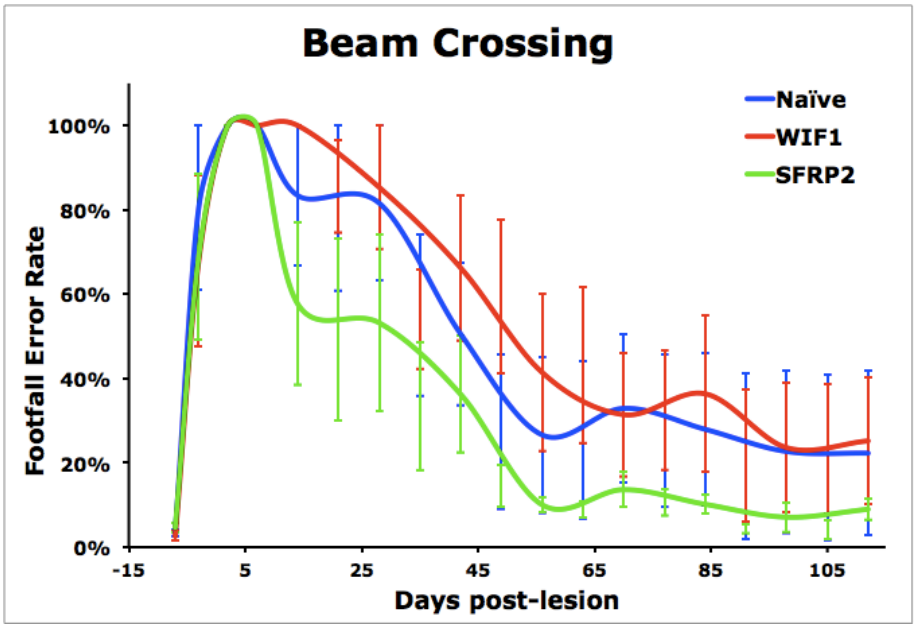
Non-skilled motor function recovered similarly in animals with peripheral conditioning lesion, independent of Wnt inhibition by BMSC grafts.

Animals peripherally condition lesioned and grafted with BMSCs exhibited no differences in the rates of recovery from SCI on the grid crossing task, a test of gross motor control (Graph 5). Similar recovery rates were also observed in the beam cross task, despite the task's reluctance on sensory input (Graph 6).

Graph 5. Non-skilled motor function recovered similarly, independent of Wnt inhibition by BMSC grafts.



Graph 6. Non-skilled motor function coupled with tactile response also recovered similarly to previous experiments, independent of Wnt inhibition by BMSC grafts.



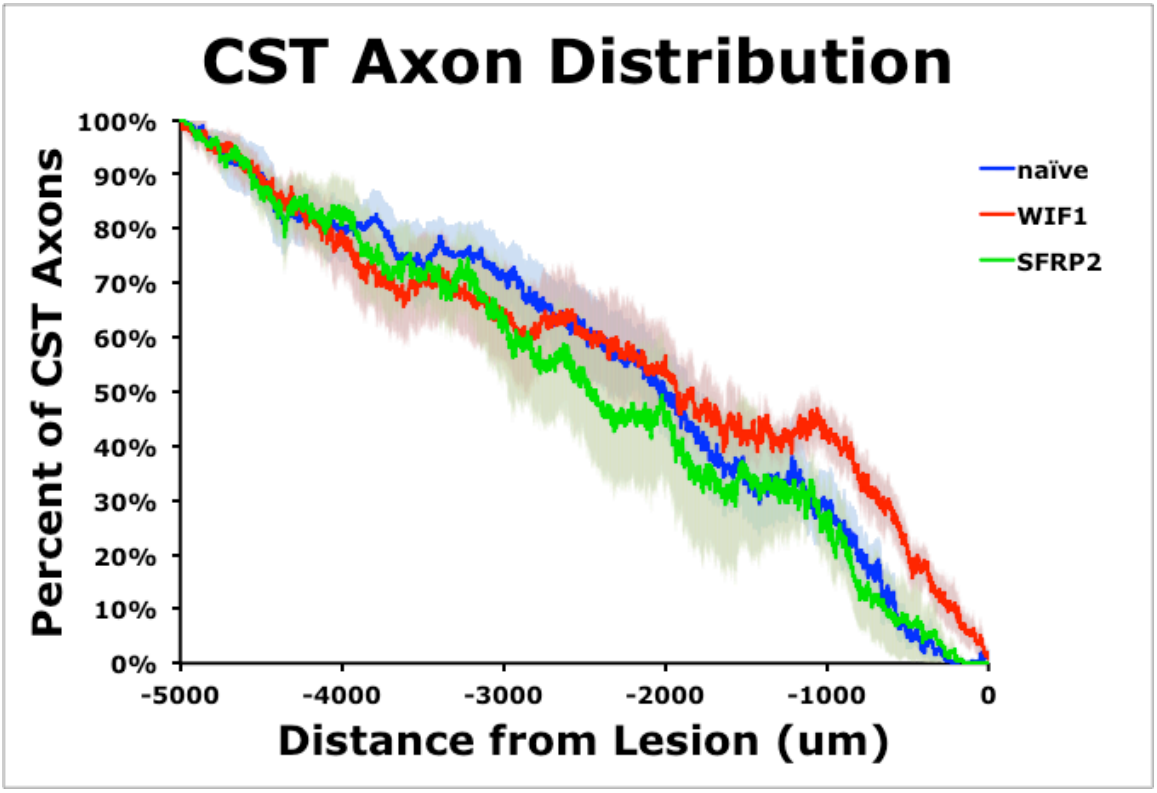
Quantification of CST Axons

Inhibition of Wnt signaling was not able to reduce CST axon retraction at 4 months post-SCI

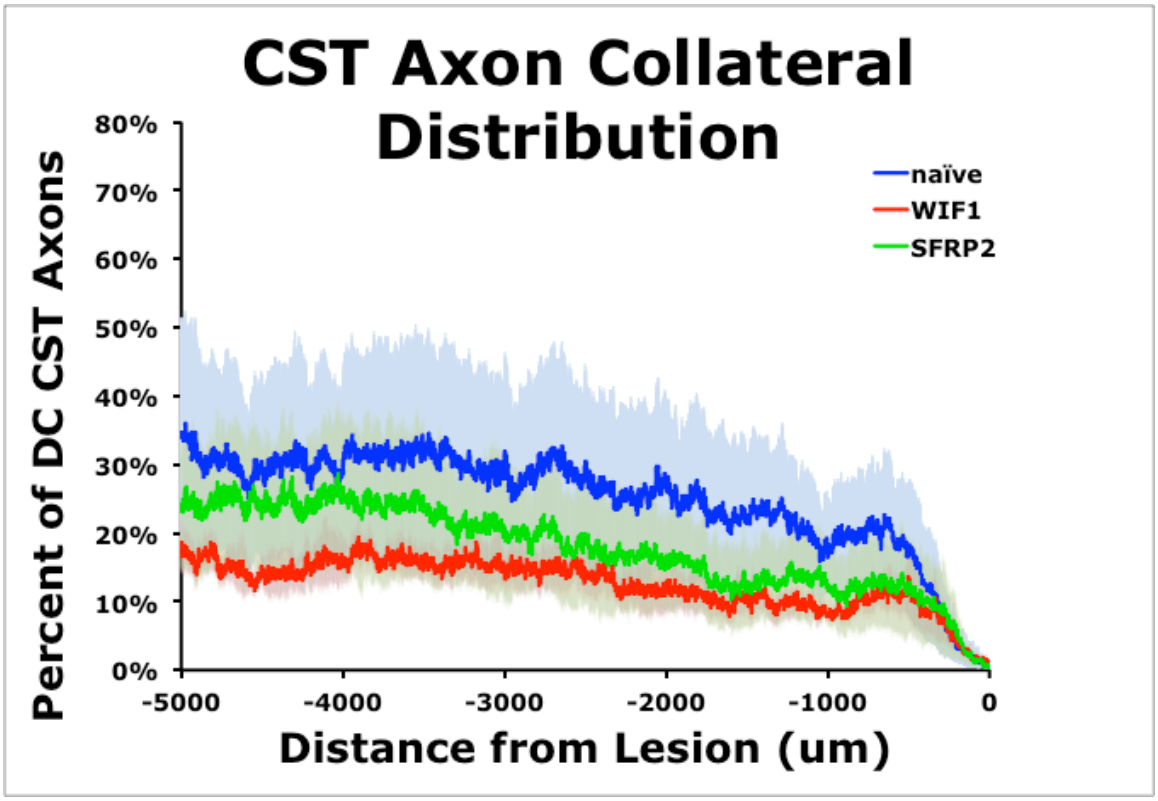
The density of BDA-labeled axons was measured over 5mm rostral to the rostral lesion boundary. Animals grafted with BMSCs exhibited no significant reduction in retraction of either dorsal column CST (dCST) axons or grey matter collaterals at 4 months post-SCI (Graph 7,8). In the absence of BMSC grafts, animals infused with

control mouse IgG or Ryk monoclonal antibody showed similar levels of retraction of BDA-labeled dCST axons and grey matter collaterals at 4 months post-SCI (Graph 9,10).

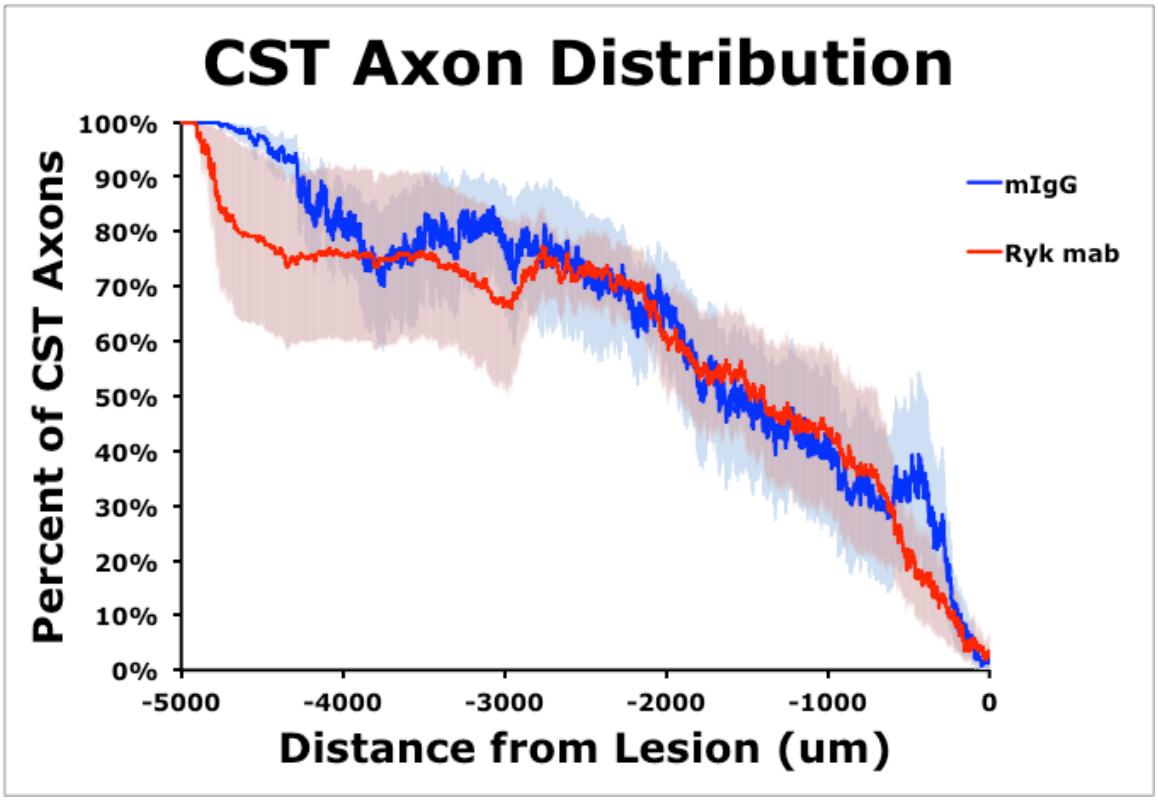
Graph 7. Normalized density of dorsal column CST axons show no significant reduction in dCST axon retraction.



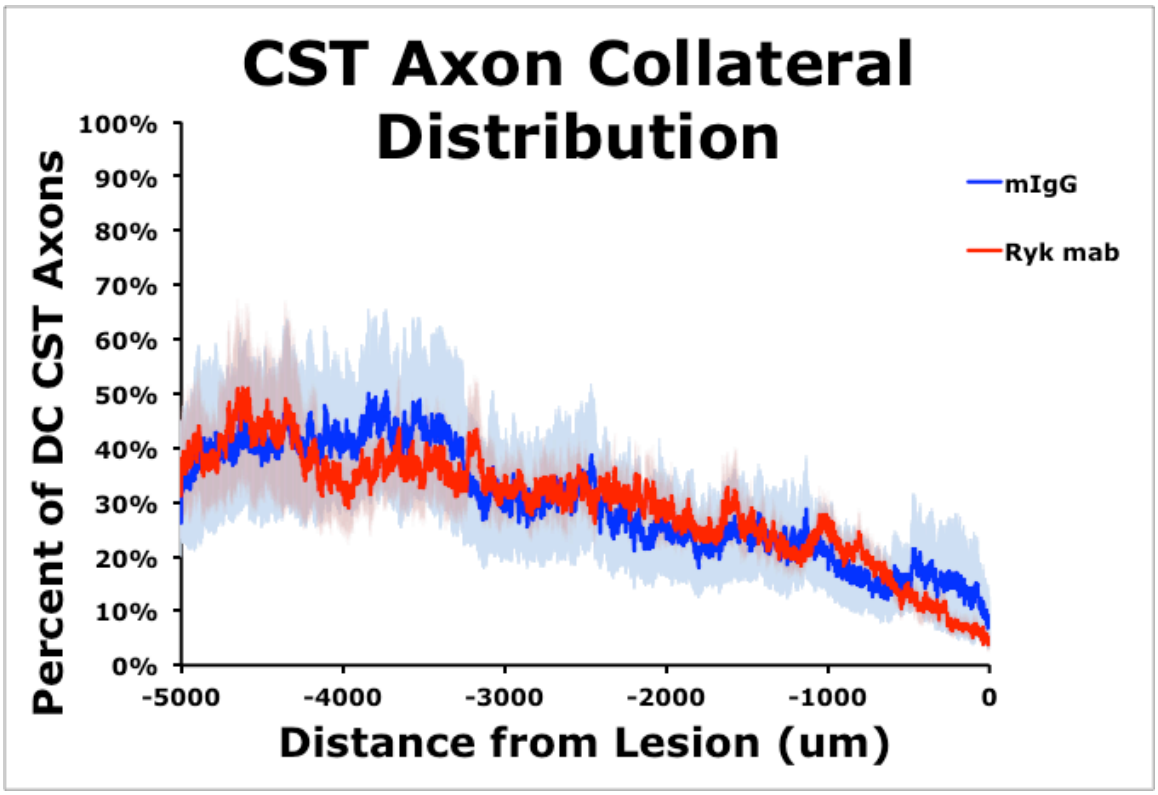
Graph 8. BDA-labeled axon collaterals in spinal cord gray matter normalized to dCST density exhibit similar densities at 4 months post-SCI, independent of the type of BMSC graft.



Graph 9. Normalized density of dCST axons show no significant reduction in axon retraction.



Graph 10. BDA-labeled axon collaterals in spinal cord gray matter normalized to dCST density exhibit similar densities at 4 months post-SCI, independent of the type of Ryk monoclonal antibody infusion.



Quantification of Fluorogold Labeled Neurons Projecting to *Nucleus Gracilis* Below Lesion Site

All animals that were given the C1 lesion experienced about a 50% reduction in ascending sensory neurons below the injury site.

Fluorogold was injected bilaterally into the VPL on the thalamus, which receives input from *nucleus gracilis*. Any visualization of fluorogold below the injury site is representative of an intact path from the thalamus to the cervical level of the spinal cord. All of the animals that were given the cervical lesion lost about half of their dorsal column sensory axons regardless of BMSC graft (Graph 11, 12)

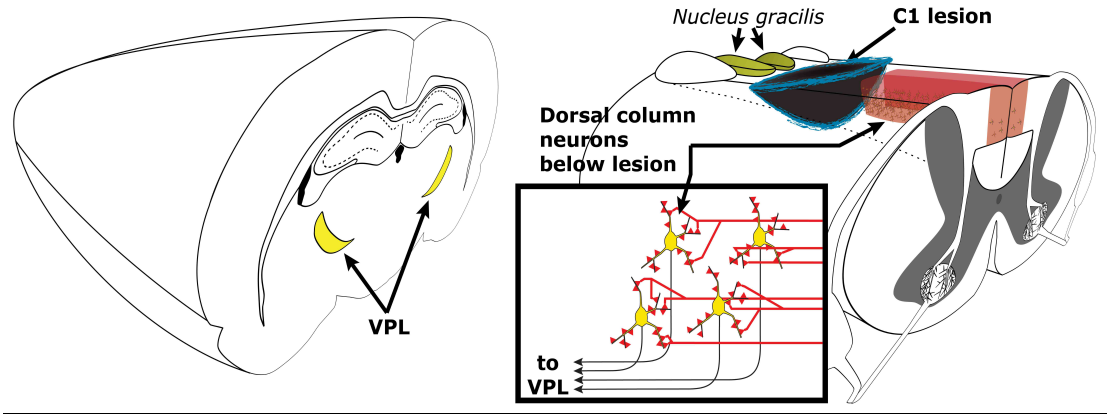
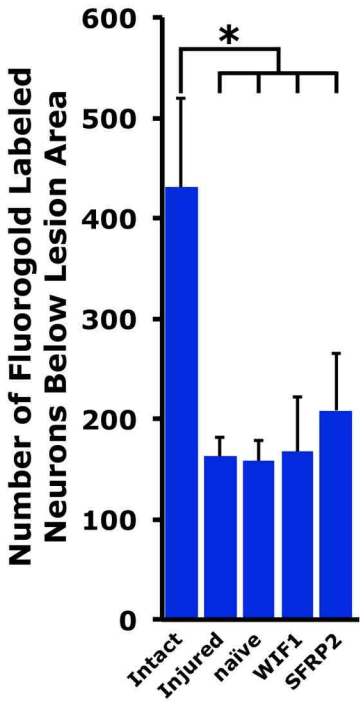


Figure 1. Fluorogold injected bilaterally into VPL of thalamus, which receives input from *Nucleus gracilis*.

Graph 11. The fluorogold neurons below the injury site were reduced by about half compared to intact animals (Dunnett's t-test $P<0.05$).



Characterization of Lesion

Inhibition of Wnt signaling may have altered scar formation in response to injury.

Histological staining of the injured spinal cord revealed a reduction in immunostaining of GFAP-immunoreactive astrocytes immediately surrounding the grafted BMSCs (Graph 13). There was a slight, non-significant increase in lesion volumes, calculated by Cavalieri estimator, in animals grafted with WIF1-secreting BMSCs (ANOVA $P=0.45$).

Graph 12. Wnt inhibition by WIF1- and SFRP2-secreting BMSC grafts attenuates astrogliosis surrounding the injury.
Graph 13. WIF1-grafted animals show a slight, non-significant trend towards increased lesion size (ANOVA P=0.45). Scale bar = 1mm

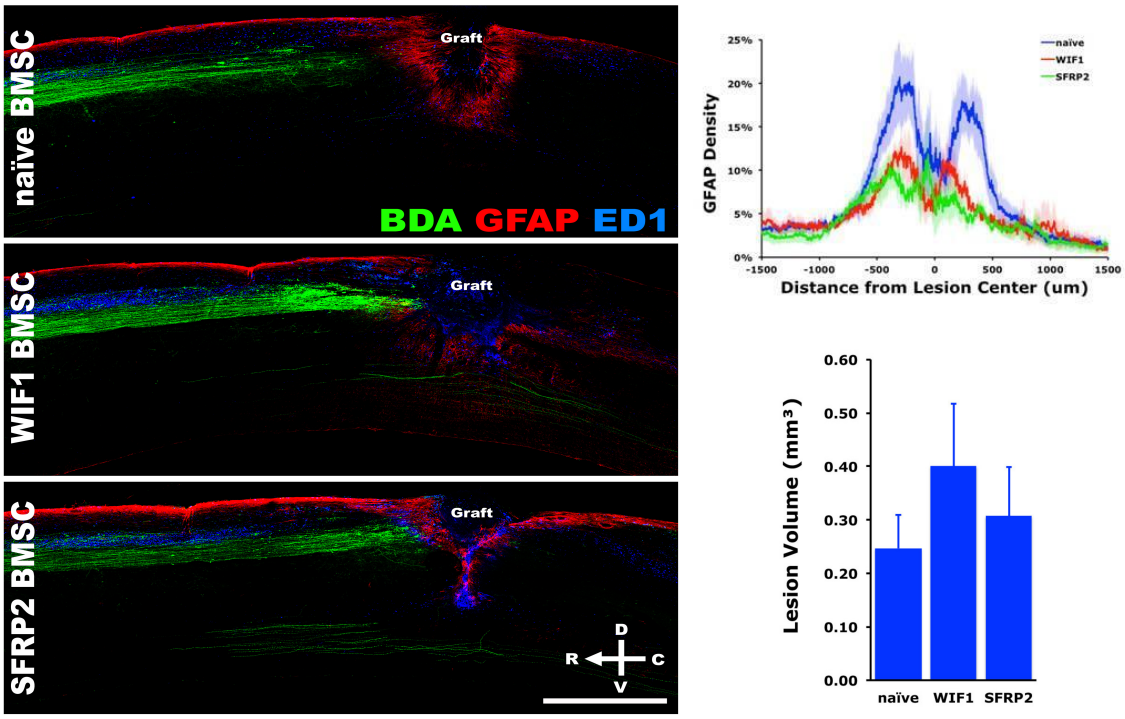


Figure 2. Host spinal cord around BMSC grafts in a C5 dorsal column lesion at 4 months post-SCI.

Animals treated with the Ryk monoclonal antibody showed slightly reduced lesion volumes compared to IgG controls.

Graph 14. Animals infused with Ryk monoclonal antibody for 28 days after C5 dorsal column wire-knife lesion show a non-significant trend towards decreased lesion size (two-tailed t-test P=0.16)

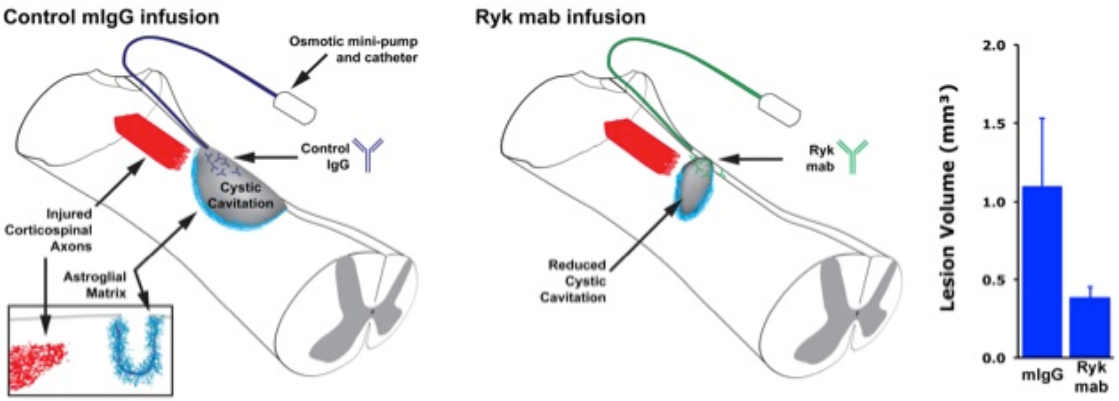
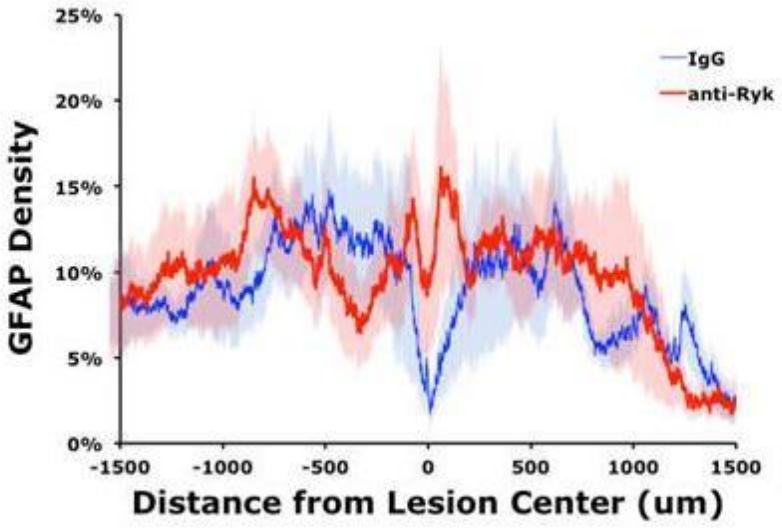


Figure 3. Cystic cavitation is slightly attenuated by Ryk monoclonal antibody infusion at 4 months after a C5 dorsal column lesion.

There was a trend towards reduced lesion volumes in animals infused with Ryk monoclonal antibody for 28 days after C5 SCI (two-tailed t-test P=0.16). Lesion size in animals infused with Ryk monoclonal antibody was similar to lesion size in animals grafted with BMSCs.

Silencing Ryk via antibody infusion does not alter astrocyte density near the lesion

Graph 15. Ryk monoclonal antibody infusion did not alter reactive astrogliosis patterns at 4 months after injury when infused for the first 28 days.



PCP signaling compound and Wnt receptor Celsr3 is expressed in reactive astrocytes immediately proximal to the lesion.

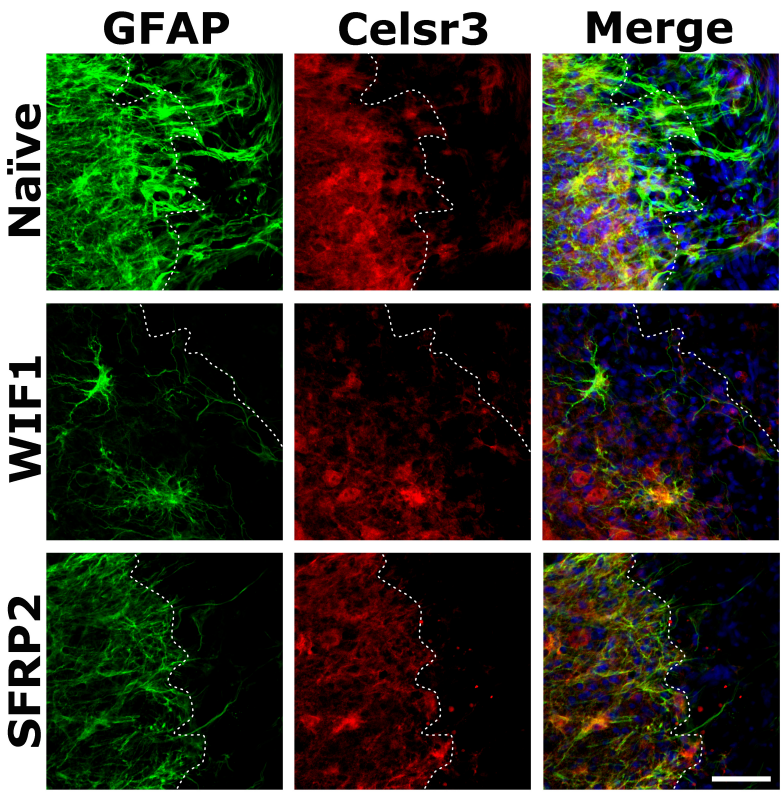


Figure 4. Reactive astrocytes express the PCP signaling component Celsr3. Scale bar = 50μm

DISCUSSION

The most significant trend drawn out from the study was a tendency for having larger lesion size correlate with more deficient behavioral recovery. At the level of detail the tissue was available to me for observation, axon density in the descending CST following injury was similar between all the control and experimental groups and clearly inhibiting Wnt signaling at this scale does not significantly enhance axon plasticity after SCI. The absence of plasticity after injury however may be directly influenced by the actual lesion size and subsequently the physical number of axons spared from the lesion. A primate study reported significant behavioral recovery coupled with 60% CST axon density recovery after C7 hemisection and no other treatment other than repeated behavioral tasks [21]. The group concluded that this high level of plasticity post cervical injury is only possible in primates and humans on an anatomical basis allowing for the sparing of many more CST axons compared to rodents. This conclusion shares many implications with the results from my studies. The trend between lesion size and functional recovery nascent in my studies may be due to a larger number of spared dorsal column axons associated with smaller lesion sizes. The group involved in the primate study also concluded that when using rodent models to look at SCI, it would be most beneficial in terms of translation to administer hemisections as opposed to full transections to better mimic the amount of spared axons that would appear in a partial human injury. They reported that rodents only recover 3% of pre-lesion CST axon density when sprouting occurs from the ventral component [21]. These findings account

for the lack of plasticity in all of my control and experimental groups despite Wnt signaling inhibition. The primate group's future studies will involve paying particular attention to the spared CST axons and potential methods of plasticity enhancement of these spared axons.

In addition to the amount of spared CST axons associated with larger lesion sizes, larger lesion sizes also more greatly physically displace descending axons from their original and importantly functional targets. Aside from the direct effects of the physical lesion size on functional recovery, my data indicates that astrogliosis at the site of injury is reduced by Wnt-inhibitory factor secreting BMSCs. Recent studies have implicated the role of Wnt and Ryk signaling in many facets of the spinal cord's response to damage [22]. Disrupting the glial response at the site of injury may affect forelimb function as the tissue damage can spread into neighboring neural substrates, originally unaffected by the lesion. It has been proposed that Ryk is expressed by astrocytes, especially astrocytes closest to the site of injury and form the glial scar [22]. In that study, the researchers concluded that because of high Ryk expression after SCI in the most active astroglia nearest to the lesion, these astrocytes are not only secreting Wnts but are also serve as targets for Wnt ligands. The researchers suggest Wnt signaling may play a role in the spinal cord's glial response.

If the astrocytes closest to the site of injury and take part in formation of the glial scar are most pivotal to astrocyte-mediated recovery then they cannot be solely dependent on the activity of Ryk receptors. The results of my experiment showed that a monoclonal anti-Ryk antibody infusion following SCI supplemented recovery. It has also been

documented earlier that silencing Ryk via antibody infusion post SCI leads to more axonal branching[12].

The expression of PCP components in reactive astroglia indicate that Wnts may be a mechanism for orienting astrocytes and directing the formation of the glial scar and also that Wnt regulation of astroglia extends beyond Wnt-Ryk interaction. Celsr3 is not involved in the canonical Wnt pathway previously described as present in astrocytes yet we found that Celsr3 is expressed in astrocytes. My findings showing more deficient astroglial responses in the animals treated with cell grafts compared to that of the animals treated with the anti-Ryk antibody are congruent with the hypothesis that astrocytes utilize multiple Wnt signaling pathways. The secreted Wnt inhibitors bind directly to the Wnt ligand and restrict the ligand from signaling. WIF1 blocks Wnt-Ryk signaling, as it shares sequence homology with the Wnt binding site of the Ryk receptor. SFRP2, meanwhile, blocks Wnt-Frizzled signaling which is required for both the canonical and planar cell polarity signaling pathways. Animals treated with anti-Ryk antibodies only lose function of their Ryk receptors, if astrocytes utilize multiple Wnt signaling pathways then the newly secreted Wnts post SCI will still have other receptors like Frizzled3 and Celsr3 to interact with and activate the astroglial response.

IgG has been proposed to reduce inflammatory cytokines after SCI and preserve neural tissue [23]. My data, however showed the greatest cystic cavitation in animals infused with mouse IgG and these animals consequently suffered very low skilled motor recovery rates.

CONCLUSION

I originally had set out to modulate Wnt-Ryk signaling to stimulate axon plasticity in the CST following SCI and assess any correlations with behavior testing, however, based on my data from these experiments I believe that in order to achieve significant behavioral recovery, the glial response to the injury and the effects of the physical size of the injury should be the areas of most study. The histology depicting the axon densities in the CST following the injury did not parallel the observations I made during the 16 weeks of behavioral testing while the lesion size and astrocyte activity correlated with a majority of the behavioral tests' results.

I conclude that individually, lesion size and not axon plasticity following injury, correlates most significantly with loss of functional control where the larger the lesion, the greater number of actual damaged axons and the more greatly displaced CST axons are from their original targets. However I believe that higher levels of functional recovery will require a combination of many factors including smaller lesions enhanced glial response and enhanced CNS axonal plasticity so that the original descending dorsal column targets are more accessible to the appropriate axons. Future studies based on these results should look at characterizing the components of the glial response, especially astrocytes, as to determine how to enhance the glial response to injury.

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