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The Effects of Nervous System Injury on Sensory and Motor Function

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The Effects of Nervous System Injury on Sensory and Motor Function

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Nao Ishiko

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2015
The Thesis of Nao Ishiko 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

2015
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ABSTRACT OF THE THESIS

The Effects of Nervous System Injury on Sensory and Motor Function

by

Nao Ishiko

Master of Science in Biology
University of California, San Diego, 2015
Professor Yimin Zou, Chair
Professor Takaki Komiyama, Co-Chair

In this thesis, I will discuss three studies in relation to the recovery of function after nervous system injury in hopes of answering a vital question. How do manipulations of specific inhibitors of regeneration affect the functional recovery of rodents after SCI, and what circuit-level change(s) might underlie these functional changes?

Our first study investigates the mechanism of peripheral conditioning by using a demyelinating reagent, ethidium bromide (EtBr), to test whether
demyelination, rather than mechanical nerve crush of axons is sufficient to induce the regeneration-promoting, conditioning effect. After assessing regeneration, as well as functional recovery through behavioral studies, we found that demyelination is a likely contributor to the conditioning effect. These studies provide the framework for further investigation into the mechanisms of the conditioning effect.

The second study combines our novel EtBr-mediated conditioning paradigm with inhibition of the repulsive axon guidance molecule Wnt. In these studies, bone marrow stromal cells (BMSCs) secreting the Wnt inhibitor frizzled-related peptide 2 (SFRP2), were grafted at a cervical level 1 (C1) lesion site caudal to main body of dorsal funiculi, second order, sensory neurons. In our studies, better sensory functional recovery was observed in animals with SFRP2 treatment compared to naive animals. Circuit level analysis demonstrated that axons of conditioned sensory neurons sprouted and made new connections with a small subset of dorsal column neurons, caudal to the main body of dorsal column nuclei. Transient silencing or re-transection of this remodeled circuit at thoracic level 10 (T10) resulted in the loss of recovered sensory function, indicating that this remodeled circuit is sufficient to confer the observed recovery of sensory function.

Our third study aims to minimize the effect of Wnt signaling in a newly generated line of related to receptor tyrosine kinase (Ryk) conditional knockout (cKO) mice. Ryk is a repulsive receptor of Wnt, and we studied corticospinal axon plasticity in this mouse model. After C5 dorsal column lesion, better motor-related function recovery was observed in Ryk cKO mice. Circuit level analysis
showed increased collateral sprouting with Ryk deletion in motor cortex. Secondary lesion at C3 resulted in a loss of both the enhanced recovery mediated by Ryk deletion, as well as a loss of corticospinal axon collaterals between levels C3 and C5. Whereas after lesioning of the corticospinal motor axons at the level of the pyramid (pyramidotomy), functional recovery was lost completely, implicating a role for the remodeled corticospinal tract in the recovery of motor function.

These studies indicate that, while regeneration is not easily achieved, local sprouting and axon plasticity might offer more attainable circuit changes after spinal injury. We have found that this plasticity leads to significant motor and sensory functional recovery in rodents, and may be a potential avenue for mediating recovery for approximately 280,000 patients affected by SCI in the United States.
Chapter 1: Introduction

1.1 Spinal Cord as a Relay Center

Few would contest that the brain is the functional command center to our bodies, residing in ever-changing physiological conditions. A different region of the nervous system is the Peripheral Nervous System (PNS), which consists of nerve fibers directly in contact with various body regions such as skeletal muscles, skin, and viscera (organs). In order for the vertebrate to have a properly functioning, highly regulated body in a developing environment, signals initiated at the brain must reach their targets and the body must constantly report its status to the brain.

The connector in essence, and what allows this flow of signals throughout the lifetime of vertebrates to take place within the nervous system, is the spinal cord. With exceptions such as five senses of perception, which do not require intense, spinal participation, the majority of the information transmission is done through the spinal cord. Without the spinal cord, our legs would not move (bend) as we intend, our skin will not respond to noxious stimulus, our hearts will not pump properly, and we will not breath effortlessly, even if the brain and the rest of the body are completely intact.

Together with the brain, the spinal cord is the other part of the central nervous system (CNS) and its main job is to relay information between the PNS and the brain. The spinal cord originates at the caudal boundary of the medulla oblongata, part of the brainstem. It runs along the longitudinal body axis and...
tapers into conus medullaris, the lower extremity of the cord located slightly above the waist (Marieb et al., 2010). It is enclosed by three layers of membranes called spinal meninges; pia mater as the innermost layer, wrapped within arachnoid and finally the outermost layer, dura mater which altogether provide mechanical protection of the cord (Marieb et al., 2010).

The spinal cord passes through the vertebral cavity of the spinal column, a segmented skeletal structure providing longitudinal supports to vertebrates. Each segment of the spinal column, or vertebra, together with the corresponding spinal cord is divided into 4 regions: from rostral to caudal, cervical, thoracic, lumbar, and sacral. Each contain a set number of spinal segments; 7, 12, 5, and 5 (fused vertebrae), respectively.

Each spinal section is designated by a specific vertebra along the cord. There is a bilateral pair of spinal nerves that reside peripherally. They diverge into two nerve roots, dorsal and ventral, before they enter the spinal cord separately (Bear et al., 2006). The dorsal roots contain both somatic and visceral sensory information coming from corresponding dermatomes and enter the dorsal side of the cord (Krassioukov et al., 2012, Marieb et al., 2010). Within each root, there is a cluster of sensory cell bodies called dorsal root ganglia (DRG) and their unique bifurcating axons that send one axon branch into the PNS and the other into the spinal cord. This unique architecture of the DRG has provided some informative studies within a realm of neuronal regeneration and will be further discussed. Ventral roots, extending from the ventral side of the
spinal cord, contain both somatic and visceral motor information (voluntary motor and autonomic information).

Within the spinal cord, there are two distinct regions; inner gray matter surrounded by outer white matter. White matter is defined by its whitish color of long myelinated axons. It contains both myelinated and unmyelinated axons that pass through the cord to transmit and relay signals to their targets. White matter is subdivided into three regions depending on the location within the cord: dorsal funiculus, ventral funiculus, and lateral funiculus. Each funiculus contains various fiber tracts of axons with similar functions and targets.

In contrast, gray matter, defined by a unique butterfly shape when cut transversely, consists of the cell bodies of neurons and shorter neuronal processes of axons and dendrites. Gray matter is subdivided into two regions: dorsal horns, the upper part of the “butterfly wing” and ventral horns, found in the “wing's” lower section. The dorsal horn interneurons receive nociceptive and tactile sensory input (from dorsal root) and the ventral horn contains the alpha and gamma motor neurons that mediate movement. A small bump between the dorsal and ventral horns is called lateral horns where neurons of the autonomic nervous system reside (Krassioukov et al., 2012, Marieb et al., 2010). Due to its architecture, each spinal cord segment relays different types and amounts of sensory, motor and autonomic information. For instance, the nerves in the fifth cervical vertebrae (C5) relay sensory information from biceps brachii and conversely send motor commands to the elbow flexor and shoulders for their flexion and abduction, respectively (American Spinal Injury Association). There is
no Autonomic Nervous System (ANS) corresponding to C5. As it happens, the ANS has more complex spinal/brainstem innervation when compared to motor and sensory regulation and control. Organs and glands are regulated by two, not completely opposing, antagonistic systems; the sympathetic system (fight or flight), and parasympathetic system (rest and digest). The former sends signals through thoracic vertebrae T1-T12 and lumbar vertebrae L1-L2, while the latter do so through sacral vertebrae S2-S4. Losing either one at any level, if not both, means losing homeostasis. The major systems regulated by the ANS include the cardiovascular system, respiratory system, lower urinary tract, distal gastrointestinal tract, pelvic organs and male genitalia. Each spinal segment relays information between the brain and the rest of the body below the spinal level.

The rule of thumb, with a few exceptions, is that more information flows at higher levels of the cord, as a larger ratio of white to gray matter can be viewed on the rostral side of the spinal cord. In addition to neurons, there are glial cells, which are the supporting cells of neurons, in both CNS and PNS. They include Astrocytes, Microglia, Ependymal cells, and myelinating Oligodendrocytes in the CNS and Satellite cells and myelinating Schwann cells in the PNS (Marieb et al., 2010). These are a focus in spinal cord injury studies due to their responses to the event of injury, which will be further discussed.

1.2 Spinal Cord Injury Patient Overview

According to Mayo Clinic, spinal cord injury (SCI) is an insult to any part(s) of the spinal cord, vertebrae, or ligaments, which results in temporal or
permanent deficit in perception (sensation), motor functions (movement), and/or autonomic functions (Mayo Clinic). Approximately 12,500 new patients are diagnosed with SCI every year in the United States, and approximately 276,000 people are living with SCI (NSCISC). The average age at the time of injury is around 42 years old, which increased approximately 9 years from the same data, compiled in the 1970’s. 80% of the SCI occurs among males. Vehicle crashes are listed as the leading cause of the injury, followed by falls, acts of violence, and sports (NSCISC).

The severity of the SCI is broadly determined by two factors; the level of the injury and completeness. When higher levels of the cord are damaged, more functions will be affected compared to the same damage at a lower cord level. Tetraplegia is the loss of motor and sensory functions due to spinal injury at the cervical level, affecting forelimbs (arms), in addition to the trunk and hindlimbs (legs). Contrasting to tetraplegia, paraplegia is a deficit anywhere below the cervical levels and therefore forelimb functions must be spared (Sisto et al., 2008).

The second major factor in determining the severity of the symptoms is “completeness” vs. “incompleteness”. The former refers to conditions when almost all sensory and motor functions below the injury site is lost due to “complete” damage to the cord, while the latter refers to injury with some spared sensory or motor functions below the injury site (Mayo Clinic). When these two factors are combined, complete tetraplegia generally results in most severe symptoms and individuals with incomplete paraplegia have shown the best prognosis (Sisto et al., 2008).
The American Spinal Injury Association (ASIA) provides an assessment scale termed, International Standards for Neurological Classification for SCI (ISNCSCI). This assessment scale focuses on types and severity of motor and sensory loss to evaluate the deficit in a systematic way. The scale, A through E, represents the different deficits by letter. A complete loss (no motor or sensory function preserved at S4-S5) has an A designation, B represents incomplete sensory loss, C and D as incomplete motor loss with higher motor functional loss for C, and E represents normal for someone who experienced SCI. In addition to ISNCSCI, there is another scale termed International Standards to document remaining Autonomic Function after Spinal Cord Injury (ISAFSCI). It was established in 2012, to help health professionals systematically evaluate ANS functional loss after SCI (Krassioukov et al., 2012).

In addition to the previously listed physical symptoms, the psychological burdens of those affected by SCI are also important factors in patient health. 1 out of 5 SCI patients are reported to be suffering from depression (Christopher and Dana Reeve Foundation). Currently, medical treatments after SCI are limited and broadly divided into early initial emergency care and later long-term treatment including rehabilitation (Christopher and Dana Reeve Foundation). Emergency care focuses on stabilizing essential physiological functions including breathing and blood pressure, as well as to protect the spinal cord from further damage. Once the extent of damage is assessed by X-rays, CT scans, MRIs, etc, and following neurological examinations of motor, sensory, and ANS functional loss, the focus shifts to manage secondary complications including irregular blood pressure, various infections, pressure sores and muscle spasms. The concept is to minimize secondary complications, while attempting to restore
some of the spared function through rehabilitation instead of completely fixing it as it was before (Ramel et al., 2014). This limitation of SCI treatment is due to the nature of adult CNS; The CNS does not regenerate once it is severed.

1.3 Lack of CNS regeneration after SCI

If the damaged spinal cord was a demolished freeway, sooner or later, debris would be removed, damage would be measured and necessary materials would be brought to the site. A new road would be constructed and re-connected to spared routes eventually allowing traffic to flow as it had before. Unfortunately, this is not the sequence of events one expects to happen after SCI. The central dogma of neurobiology, by the father of neuroscience, Santiago Ramón y Cajal, states “Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.” (1928). Presently in 2015, this holds true to a certain extent and there are two injury responses to share the brunt of the issue, 1) lack of growth capacity and 2) inhibitory environment after SCI.

Although their definitions are fluid to some extent, in this thesis regeneration refers to injured axon re-growth (possibly forming novel connections), whereas sprouting refers to the local, short range growth of either injured or uninjured axons. Axonal plasticity in SCI studies refers to any change in axonal arborization including regeneration and sprouting.
1.4 Lack of Intrinsic Growth Capacity

In order to elucidate the mystery of deficient intrinsic growth capacity of adult CNS axons after injury, PNS axons have been serving as a model with their pro-regenerative responses after injury (Richardson and Issa 1984, Kordower and Tuszynski, 2008). Genetic screening (microarray) has identified thousands of transcriptional changes after injury of peripheral axons. Studies of individual molecules have identified many regeneration-associated genes (RAGs) although not all of them have shown causal regenerative activities (Ma and Willis, 2015, Huebner and Strittmatter, 2009). RAGs include cytoskeletal proteins such as growth-associated protein-43 (GAP-43), cytoskeleton-associated protein-23 (CAP-23), cell adhesion molecules such as CD44, neuropeptides, and transcription factors such as activating transcription factor-3 (ATF-3) and c-Jun (Kordower and Tuszynski, 2008, Ma and Willis, 2015, Yoon and Tuszynski, 2012). While there is no single RAG manipulation that mimics the regenerative effect alone, when modified together, some RAGs have shown regenerative responses to some extent. For instance, co-overexpression of GAP43 and CAP-23 results in regeneration of CNS branch of DRG neurons into peripheral nerve graft (Buffo et al., 1997, Bomze et al., 2001). While some RAGs act in a more specialized role, others are transcription factors with “hub” like features that tend to have more broad influences when manipulated (Ma and Willis, 2015). One of these is ATF-3, the overexpression of which results in increased peripheral axon regeneration (Seijffers et al., 2006, 2007). Including these transcription factors, expression of a number of RAGs is regulated by cyclic adenosine monophosphate (cAMP). Therefore manipulation of cAMP has shown positive regenerative responses after SCI (Lu et al., 2014).
Another molecular manipulation that increases intrinsic growth capacity is the upregulation of mammalian target of rapamycin (mTOR) signaling, which promotes axonal growth during development (Park et al., 2009). The mTOR pathway is suggested to be involved in growth cone formation after injury in vitro (Verma et al., 2005). This pathway is negatively regulated by phosphatase and tensin homolog (PTEN), which is one of the major targets to upregulate the pathway for pro-regenerative responses. PTEN deletion has shown dramatic increase of regeneration, including promotion of regrowth of corticospinal tract (CST) (Liu et al., 2010).

While these molecular manipulations are informative, cellular level manipulations have been conducted through DRG studies to induce intrinsic regenerative responses. As one expects, peripheral axons regenerate when the sciatic nerve of DRG sensory axons are mechanically compressed. Conversely, there is no significant regeneration response of central branch of DRG axons arising from the same neurons after injury. A fascinating point of interest is that when spinal cord injury is performed after a peripheral injury of DRG neurons, the central axons in the spinal cord show elevated regeneration capacities. Research has shown that upregulation of intrinsic growth capacity of the DRG neurons promotes regeneration of both peripheral and central axons. This phenomena, described by Richardson and Issa (1984), is termed “peripheral conditioning,” due to its “conditioning effects” of CNS into pro-regenerative states after PNS crush. Although upregulation of known RAGs have been confirmed after conditioning treatment, overexpressing these RAGs is not enough to induce
regeneration effects and therefore unveiling the molecular/cellular mechanism of this conditioning effect is crucial (Kordower and Tuszynski, 2008).

1.5. Inhibitory Environment (extrinsic) Followed by SCI

Research published in *Science* (1981), conducted by Aguayo and David, demonstrated that axons in the damaged spinal cord and the medulla, both elongated into the “bridge” of growth permissive peripheral environment in rat, approximately 30 millimeters. This research suggested that CNS axons possess the ability to regenerate, caused by the hostile anti-regenerative environment of the injured axons. As long as they are in the right environment, CNS axons should regenerate, and therefore the search for extrinsic limiting factors began. A few decades of studies have shown two broad classes of extrinsic factors in the CNS; physical barriers and chemical cues (present inhibitory cues and lack of growth promoting cues) (Kordower and Tuszynski, 2008).

The major physical impediment to regeneration is a “glial scar,” which forms at the injury site to prevent the damage from spreading (Kordower and Tuszynski, 2008). Along with microglia and meningeal fibroblast, the major component of the glial scar is a hypertrophic reactive astrocytes characterized by its dense, fibrous filaments (Silver and Miller, 2004, Kordower and Tuszynski, 2008). Another physical “barrier” is the formation of a cyst at the site of injury, which results in missing a “bridge” of growth permissive tissues for axons. When the lesion cavity is filled with permissive matrix of collagen, fibronectin, Schwann cells, etc, peripheral axons regrow (Williams, 1983, Dubovy, 2004, Chernousov and Carey, 2000).
There are three classes of chemical inhibitors which are found to prevent damaged axons from regeneration and those include myelin associated inhibitors (MAIs), chondroitin sulphate proteoglycan (CSPGs), and axon guidance molecules (Kordower and Tuszynski, 2008). MAIs are secreted from myelin and myelin debris, represented by Nogo, myelin-associated glycoproteins (MAGs), and oligodendrocyte-myelin glycoprotein (OMgp) (Schnell and Schwab, 1990, Giger et al., 2010). These three types of molecules bind to shared receptors, Nogo-66 receptor 1(NgR1) and PirB (Lee et al., 2010), but exact downstream pathways are still under investigation. Each of them are identified as regeneration inhibitors (Buchli and Schwab, 2005, Mckerracher et al., 1994, Wang et al., 2002), and Nogo deletion showed increased sprouting of intact CST after pyramidotomy. However, there were no synergic effects after genetic deletion of all 3 inhibitors and they may not play a central role in preventing regeneration in vivo (Lee et al., 2010).

CSPGs are molecules bound to extracellular matrix (ECM) of glial scar, are rapidly upregulated within 24 hours after injury, and inhibit neurite growth (Silver and Miller, 2004, Dou and Levine, 1994). One of the main inhibitory molecules secreted by the glial scar, enzymatic digestion of CSPGs’ gylcosaminoglycan (GAG) side chains by chondriotinase ABC (ChABC) promotes axon regeneration (Kordower and Tuszynski, 2008). A third extrinsic inhibitory cue is the expression of repulsive axon guidance molecules such as semaphorins, ephrins, and Wnts (Pasterkamp et al., Fabes, et al., 2007, Liu, et al., 2008, 2006, Giger et al., 2010). Wnt signaling after spinal cord injury is further discussed in chapter 3 and 4.
In addition to inhibitory cues, a lack of growth-stimulating cues is thought to be one reason why damaged CNS axons do not regenerate. These cues include neurotrophic factors such as BDNF, NGF, and IGF. The production of neurotrophic factors by Schwann cells after PNS injury is thought to be key to the successful regeneration that occurs after PNS injury (Giger et al., 2010, Kordower and Tuszynski, 2008, Bhatheja and Field, 2006). Although both intrinsic and extrinsic factors offer various areas to investigate, there is no single cue that promotes CNS regeneration. A combination of multiple cues appears to be required.
Chapter 2: Chemical Demyelinating Reagent-Induced Peripheral Conditioning

Our first study investigated the peripheral conditioning in DRGs by comparing the existing conditioning paradigm of mechanical nerve crush to a novel conditioning treatment of chemical demyelination. This was an attempt to explore what brings the damaged sensory neurons into the intrinsic growth states. We used two known demyelinating reagents, ethidium bromide (EtBr) and lysolecithin (LPC) to induce chemical demyelination (Riet-Correa et al., 2002).

When sciatic nerves are crushed, or in other words, mechanically compressed, the axons undergo demyelination followed by remyelination (Gupta et al., 2004). The events correlate with apoptosis and proliferation of Schwann cells (forming the myelin sheath around axons), upregulation of RAGs, and eventually peripheral regeneration. It is also observed that, following sciatic nerve demyelination by a chemical demyelinating reagent, injured axons undergo sprouting as Schwann cells undergo proliferation (Hall, 1973). These studies provide us the opportunity to test the hypothesis; whether peripheral demyelination is a necessary component of the conditioning phenomena that promote regeneration of injured CNS axons. In order to test the hypothesis, rats were separated into two groups and each received a different conditioning treatment; one with existing sciatic nerve crush and the other with sciatic nerve injection of EtBr. In this study, we compared the conditioning effect at three levels; the molecular level - quantification of RAG expression including ATF-3 and c-Jun, at the cellular level - measuring the changes in scale of demyelination
of sciatic nerve over time, and finally at the functional level - assessment of functional recovery by accelerating rotarod, beam crossing, and hindpaw toe spread test.

2.1 Results

Adult female rats received, sham operation, bilateral sciatic nerve crush, bilateral sciatic nerve PBS injection, bilateral sciatic nerve injection of EtBr at different concentrations (0.01%, 0.05%, or 0.1% wt/vol EtBr), or bilateral sciatic injection of LPC (1% wt/vol). After one week, C4 dorsal column lesion was conducted and bone marrow stromal cells (BMSCs) were grafted to fill the cavity, as a permissive “bridge” for regeneration (Wu et al., 2003). Animals were sacrificed at various time points and immunohistochemistry was conducted to assess RAG expression in the sciatic nerves (Fig. 1-1). Similar ATF-3 and c-Jun expression was induced by both nerve crush and EtBr treatment [0.05% wt/vol EtBr] and more robust expression was seen with the higher EtBr concentration [0.1% wt/vol EtBr] (Fig. 1-1). LPC injection resulted in a slight elevation of RAGs, but significantly less than nerve crush or EtBr (Fig. 1-1). This upregulation of ATF-3 and c-Jun persisted for at least two weeks after injury. We also observed a strong correlation between decreased myelin density and increased ATF-3 and c-Jun expression that was independent of treatment (Fig. 1-1).

At the cellular level, Schmued gold (III) chloride staining was used to assess myelin density of the sciatic nerves (Fig. 1-2). While nerve crush resulted in more severe demyelination locally (within 1000um) both distal and proximal to the crush site with the lowest myelin density at the lesion epicenter, EtBr [0.1% wt/vol] treatment resulted in a significantly wider range of the demyelination
proximal to the injection site (> 8000um) and persisted even after 2 weeks of injection. LPC injection resulted in similar levels of demyelination as EtBr initially (at 3 and 7 days post-injury), however by 14 days, significant remyelination had begun to occur (Fig. 1-2). After 16 weeks, levels of myelin density had recovered to pre-treatment levels in all groups.

Behavioral studies of accelerating rotarod, beam crossing, and hindpaw toe spread were conducted pre-injury followed by 16 weeks of weekly tests (Fig. 1-3). There were significant differences between animals with nerve crush and EtBr treatment on each test (rotarod: repeated measures ANOVA P < 0.05, beam crossing: repeated measures ANOVA P < 0.005, and hindpaw toe spread: repeated measures ANOVA P > 0.0001). LPC injected animals recovered similarly to sciatic nerve crush animals. While crush and LPC-injection lead to recovery of pre-injury levels on all three tests, EtBr treatment resulted in full recovery only on beam crossing, but not rotarod or toe spread. Over the 16 week course of the experiment, only 50% of bilaterally EtBr-injected rats reached or surpassed peak pre-injury performance on rotarod, whereas 100% of rats with bilateral nerve crush reached pre-injury performance. On the toe spread test, a measure of sciatic nerve function (de Medinaceli et al., 1982), EtBr-injected rats recovered to 16.5±0.2mm compared to 18.8±0.3mm or 18.1±0.1mm in bilateral nerve crush or age-matched uninjured controls, respectively.

Prior to transcardial perfusion, ascending sensory axons of the dorsal column were labeled with the transganglionic tracer cholera toxin B subunit (CTB). CTB labeled axons that had grown beyond the host-graft interface,
demarcated by astrocyte-derived glial fibrillary protein (GFAP) immunoreactive scar, were counted as regenerating axons. Rats injected with EtBr exhibited a 2.7-fold increase of regenerating sensory axons in vivo compared to nerve crush (Hollis et al., 2015b).

2.2 Discussion

These results show that axotomy is not a necessary component of the conditioning lesion and that injection of the demyelinating agent EtBr is sufficient to induce peripheral conditioning effects including the regeneration of CNS axons. Application of chemical demyelinating reagent resulted in more severe demyelination, which is associated with slower functional recovery, higher upregulation of RAGs, and more robust regeneration. Although leg function is not optimal for a longer time period, it may be worth promoting a more robust intrinsic growth state via chemical demyelinating reagent, as a means to promote more robust spinal nerve regeneration. The behavioral studies that were conducted specifically examined the recovery of sensory and motor function, which reflected the more severe demyelination induced by EtBr. EtBr promoted more robust regeneration in the spinal cord compared to nerve crush and, while slightly limited in recovery of sciatic nerve function, might allow for greater CNS regeneration and therefore better functional recovery after SCI. Additionally, the discovery of a second method for inducing peripheral conditioning will allow for future studies to examine the similarities and differences in the response of sensory neurons to both EtBr and nerve crush.
Chapter 3: Remodeling of Ascending Dorsal Column-Medial Lemniscus Sensory System and Functional Recovery

Our second study investigated the effect of manipulating, both intrinsic and extrinsic factors in order to promote sensory axon plasticity and recovery of proprioceptive function in rats. The correlation between circuit level changes of sensory axons with functional recovery was investigated. The identified circuit was temporarily silenced, then permanently transected as a means of confirming that the identified circuit change was sufficient to promote the functional recovery we observed.

Based on the study from chapter 2, EtBr was chosen as a chemical demyelinating reagent to “condition” DRG neurons. In addition to using EtBr to activate intrinsic growth programs, we decreased an extrinsic inhibitory cue by inhibiting the repulsive guidance molecule signaling of Wnt. Wnt morphogens are expressed in a rostral high to caudal low gradient during spinal cord development that serves to guide axonal growth along the rostro-caudal axis of the spinal cord (Onishi et al., 2014). Wnt expression is undetectable in adult animals but is rapidly upregulated after spinal cord injury (Liu et al. 2008). Re-induced Wnts serve as a repulsive cue for damaged/regenerating axons after spinal cord injury (Liu et al. 2008, Hollis and Zou, 2012). The Zou lab has previously shown that inhibition of Wnts after spinal cord injury, by secreted frizzled-related peptide 2 (SFRP2), promotes axonal plasticity and regeneration of conditioned sensory neurons. Therefore we utilized SFRP2 grafts after injury as a mean to induce greater axonal plasticity.
Large diameter, primary, mechanoreceptive, sensory neurons that respond to the peripheral conditioning paradigm ascend within the dorsal funiculus and constitute the first stage of the dorsal column-medial lemniscus pathway (Fig. 1-1) (Marieb et al., 2010). This pathway contains proprioceptive and mechanoreceptive sensory information from both hindlimb and forelimb to the brainstem nuclei via gracilis fasciculus and cuneatus fasciculus, respectively. Both nuclei are located at the medial-dorsal part of the medulla. *Nucleus gracilis*, which receives input from spinal levels caudal to T6 (predominantly hindlimb mechanoreceptive information), is located more medial and caudal than *nucleus cuneatus*, which receives input from T6 and above. The dorsal column nuclei receive information from the first order sensory axons, and extend second-order sensory axons rostral and ventral in the medial lemniscus to reach their thalamic target, the ventroposterolateral thalamic nucleus (VPL). One benefit of studying the gracile fasciculus is that it can be lesioned with minimal damage to other tracts, including the descending corticospinal motor tract that is located ventral to the ascending sensory fibers. The second benefit is the ability to use peripheral conditioning of the sciatic nerve to increase the intrinsic growth state of ascending sensory axons. While the majority of first order sensory neurons reach their target of *nucleus gracilis* in the medulla, there are small number of *nucleus gracilis* neurons also located at the cervical level of the spinal cord, where they receive sensory input and relay it to VPL. Our dorsal column lesion at high cervical level was located at, or above, a subset of *nucleus gracilis* neurons, approximately 5% of the total population (by stereological quantification). The C1 wire-knife lesion severed all of the sensory axons extending into the main body of *nucleus gracilis*.
In the study, we asked whether peripheral conditioning combined with Wnt pathway inhibition via SFRP2 graft would result in better proprioceptive recovery after a C1 dorsal column lesion. We investigated changes in circuit connectivity and investigated whether they correlated with behavioral recovery.

3.1 Results

Rats received bilateral EtBr injection in the sciatic nerves. One week later, bilateral C1 dorsal column lesion was performed in order to severe the ascending sensory pathway while sparing the descending corticospinal motor tract (Fig. 2-1). At the injury site, naïve syngeneic bone marrow stromal cells (BMSCs) or BMSCs secreting SFRP2 were grafted in order to inhibit Wnt signaling, as well as to provide a permissive substrate for regeneration. After 16 weeks of weekly behavioral tests including accelerating rotarod, beam crossing, and grid walk were conducted, animals were injected with the transganglionic tracer cholera toxin B subunit (CTB, 1% wt/vol in dH2O) into sciatic nerves bilaterally as well as the retrograde tracer florogold (FG, 4% wt/vol in PBS) into bilateral VPL for circuit investigation. In order to assess the survival of dorsal column *nucleus gracilis* neurons caudal to the injury, a cohort of intact and a cohort acutely injured rats with naïve BMSC grafts were injected with CTB and FG as described above. Animals were transcardially perfused 5 days after tracer injection. After injury, there was a 50% reduction of FG-labeled dorsal column neurons in the cervical spinal cord caudal to the lesion site at C1, compared to intact animals (Hollis et al., 2015), leaving roughly 2.5% of the total *nucleus gracilis* circuitry intact.

Behavioral tests show that animals’ proprioceptive circuit was impaired immediately after injury, but gradually recovered over the course of 16 weeks.
(Fig. 2-2). Compared to naïve BMSC grafted animals, animals with SFRP2-secreting grafts showed faster and more optimal recovery on the rotarod task (repeated measures ANOVA P<0.05). On beam crossing task, while SFRP2 treatment resulted in slightly better functional recovery, there was no significant difference between SFRP2-secreting and naïve BMSC grafted animals (repeated measures ANOVA P<0.08). Grid crossing task showed no difference between the two groups and it is plausibly attributed to the task; among all these three studies, grid cross requires less supraspinal processing and probably relies more on central pattern generators (intraspinal circuits).

Next we assessed the circuit plasticity at and around the injury site. Although axon regeneration was not observed beyond the graft, there was a 2.5-fold increase in axon regeneration into SFRP2-secreting grafts over naïve BMSC grafts (Fig. 2-3). Caudal to the injury, there was no change in the number of FG-labeled between 5 days after acute injury to 16 weeks after injury. In SFRP2 BMSC grafted animals, there was an increased density of primary sensory CTB-labeled synapses on the spared, FG-labeled, *nucleus gracilis* neurons (Fig. 2-3).

This data was obtained as follows: the proportion of CTB-labeled boutons labeled with vesicular glutamate transporter 1 (vGlut1) that were directly apposed to FG-labeling in the dorsal columns out of the total number of CTB and vGlut1 immunoreactive puncta. Compared to intact and acutely injured animals, after 16 weeks of weekly behavioral testing, naïve BMSC grafted animals show a 50% increase in innervation of the spared dorsal column neurons, while SFRP2 grafted animals had an increase of more than 100%. This showed that SFRP2 treatment significantly increased the plasticity of axons and also correlated with recovery on the accelerating rotarod (P>0.05).
In order to test the necessity of the remodeled circuitry on functional recovery, the circuit was temporarily silenced by injection of the sodium channel blocker lidocaine (400mg/ml in artificial cerebrospinal fluid [ACSF]) into the dorsal columns. After injection, animals showed impaired performance on rotarod compared to ACSF injected controls. This impairment was transient as the lidocaine washed out. One week after lidocaine or ACSF injection, some animals underwent a T10 dorsal column lesion in order to fully transect the ascending dorsal column-medial lemniscus pathway. T10 lesion fully ablated animals’ performance on the accelerating rotarod and beam crossing task and reduced c-fos induction in nucleus gracilis neurons after sciatic nerve stimulation (Fig. 2-4). Sham operated rats were unaffected by the procedure.

3.2 Discussion

This study exhibited that combination of peripheral conditioning and inhibition of an extrinsic repulsive cue can promote neural circuit plasticity and promote greater functional recovery. Since different behavioral studies are used to assess different aspects of sensory and motor function, using the correct test set is essential.

This study is also informative, since after injury, there is an increased number of input connecting to new targets of “minority neurons” (~2.5% of total nucleus gracilis neurons) due to the loss of access to their original target. This new connection was sufficient in inducing robust functional recovery. This may imply that a small number of appropriate connections could be robust enough to overcome impairments after SCI and result in the return of some function.
Chapter 4: Remodeling of the Descending Corticospinal Motor System and Functional Recovery

In the third study, we investigated the effect of minimizing the Wnt signaling specifically in mouse corticospinal axons and assessed the recovery of skilled motor function due to local plasticity at and around the spinal injury site. Manipulation of Wnt signaling was conducted through conditional gene deletion of related to receptor tyrosine kinase (Ryk). Ryk is a repulsive receptor of Wnt and is upregulated after spinal cord injury, which limits plasticity of both motor and sensory axons following injury (Hollis et al., 2015, Hollis and Zou, 2012, Liu et al., 2008, Miyashita et al., 2009). Ryk conditional knockout (Ryk cKO) mice generated in the Zou lab were used in this study. Dorsal column injury was performed at C5 and was included the entire dorsal column, containing descending motor CST axons, required for skilled motor control, as well as ascending sensory axons. Other areas in the spinal cord including gray matter, lateral and ventral funiculus were spared injury (Fig. 3-1). Caudal to the C5 injury, CST axons connect to motor units whose target muscles include triceps brachii and distal forelimb muscles mediating wrist and digit movements. In contrast, rostral to C5, CST axons connect to motor units for biceps brachii and shoulders. The C5 dorsal column lesion specifically severs CST connection to triceps (as well as distal forelimb muscles) while connections to biceps (and shoulders) are largely intact (Fig. 3-1). In order to assess skilled functional recovery of forelimb in this mouse model, the forelimb reaching-retrieval task was employed (Fig. 3-2). This task consists of two phases; the first phase, a “reaching” phase in which an animal extends their forelimb between a thin slit and reaches for a sugar
pellet. The following phase is a “grasping/retrieval” phase in which the animal grasps the pellet, retrieves it, and puts it to their mouth. The “reaching” phase is defined as any part of the forepaw touching the sugar pellet, documented as one reach. The “grasping/retrieval” is defined as bringing the pellet back to their mouth without dropping it. Success rate was counted as the total number of grasping/retrieval events divided by the total number of reaches. Animals which didn’t reach a success rate of 30% during the 2 week training period were excluded from the study.

4.1 Results

Mice received 2 weeks of training for the skilled motor task prior to spinal cord injury. During this time period, the animals also received AAV2/6-Cre injection into bilateral motor cortices, in order to conditionally knock out Ryk (Cre recombinase expression was under the control of cytomegalovirus (CMV) promoter) (Fig. 3-1). Once animals were trained for the task and received the viral injection (age 6.1±0.1w), bilateral C5 dorsal column lesion was conducted (age 8.4±0.1w), followed by weekly behavioral tests for 12 weeks.

The forelimb reach/retrieval task showed enhanced behavioral recovery in Ryk cKO mice compared to controls. Their success rate plateaued after 6 weeks of testing and 59% of Ryk cKO mice recovered to pre-injury levels, whereas only 40% of control mice recovered to pre-injury performance levels (Fig. 3-1). Circuit level analysis revealed that there was robust collateralization of CST axons, both rostral and caudal to the C5 injury site in Ryk cKO mice, whereas control animals showed more modest collateralization (one tailed t-test
P<0.05). While Ryk cKO increased axonal plasticity locally, regeneration was not observed, as axons were unable to pass through the injury site.

We assumed that this enhanced local plasticity might explain the enhanced functional recovery in Ryk cKO mice. In order to test this hypothesis, a subset of animals, after 12 weeks of weekly behavioral testing, underwent a subsequent C3 dorsal column lesion, following which motor function and cellular changes were examined (Fig. 3-3). C3 lesion eliminated the enhanced recovery in Ryk cKO mice (ANOVA P<0.05, Bonferroni corrected t-test *P<0.05). One week after secondary C3 injury, both Ryk cKO and control mice performed similarly. Cellular analysis of cervical cord exhibited that there were few CST axon collaterals between C3 and C5 lesions in both Ryk cKO and controls, supporting the hypothesis that Ryk cKO enhanced local plasticity around C5 lesion resulted in greater functional recovery. Following recovery from C3 lesion, mice underwent a unilateral pyramidotomy of the dominant forelimb CST in order to fully sever the CST. This was done to see if animals’ partial recovery was due to CST axonal plasticity instead of other circuit changes outside CST. Pyramidotomy completely eliminated the animals’ ability to perform the reaching task in both groups.

4.2.Discussion

This study shows that by minimizing the effect of Wnt signaling in the motor cortex, enhanced skilled motor functional recovery was achieved in mice with corticospinal lesions. Although various cellular level changes could be expected in and outside of CST, including plasticity of proprioceptive pathways, this local circuit change of CST around the injury site likely resulted in the
observed, enhanced recovery of skilled motor performance on the forelimb grasping/retrieval task. This descending motor pathway study is informative and exhibits that manipulation of one type of inhibitory guidance molecule (Wnt) achieves enhanced functional recovery.
Chapter 5: Discussions

These three studies investigated the functional and cellular changes that occur after nervous system injury. Within the spinal cord, we manipulated intrinsic and extrinsic factors in order to achieve greater functional recovery and robust plasticity, which correlated with functional recovery. When discussing chemical demyelinating agent induced peripheral conditioning, existing and novel peripheral conditioning techniques were compared at molecular, cellular, and functional levels. Based on the similar RAG expression and more robust regeneration in demyelinating reagent-induced peripheral conditioning compared to nerve crush, peripheral demyelination is identified as a central component of the conditioning phenomena, rather than axotomy. Remodeling of ascending dorsal column-medial lemniscus sensory system analyzed the effect of manipulating both intrinsic and extrinsic cues in ascending sensory pathways. Peripheral conditioning utilizing EtBr treatment of the sciatic nerve combined with Wnt inhibitor (SFRP2) secreting BMSC grafts at a C1 injury site led to better rotarod performance and more robust sprouting of injured axons. It also spared resulted in greater connectivity upon a small subset of second order sensory neurons, sufficient to induce better functional recovery. The dorsal funiculus axons entered the BMSC grafts, but failed to pass through the injury site, therefore no regeneration of the transected circuitry was observed.

Remodeling of the descending corticospinal motor system explored the effect of reducing Wnt signaling via Ryk cKO mice in CST axons after injury in a mouse model of SCI. Functional recovery was examined on skilled forelimb
reaching/retrieval tasks, as was local circuit remodeling. The results exhibited that while there was no regeneration of CST axons into or beyond the injury site, there was robust collateralization of axons in Ryk cKO mice, compared to controls.

These studies indicate that manipulation of intrinsic factors, extrinsic cues, or a combination thereof, may induce neural circuit remodeling after SCI. Therefore, seeking ways to achieve better functional recovery by promoting local plasticity of both injured and uninjured axons may be a more attainable goal in translating treatments for SCI. There are, however, problems reported with sprouting after spinal cord injury, which include neuropathic pain, autonomic dysflexia, and muscle spasms due to the generation of aberrant connections (Romelo et al., 2010). In order to address issues regarding pain, behavioral studies with animal models should be utilized when possible to evaluate pain-like behaviors, such as tactile allodynia and hyperalgesia (Chaplan et al., 1994). In our rat study of sensory plasticity after C1 injury, SFRP2 treatment did not induce pain-like behavior, and in fact appeared to ameliorate thermal sensitivity on a Hargreaves apparatus (Hollis et al. 2015). Another adverse response to aberrant plasticity after spinal cord injury is autonomic dysflexia (AD), an acute hypertension of the ANS with various homeostasis dysfunctions (Weaver et al., 2006, Rabchevskv and Kitzman, 2011). Muscle spasms are also one of the main symptoms due to unregulated neuronal plasticity (Bradbury et al., 2006). Thus, promoting functionally relevant sprouting, while preventing deleterious sprouting, is essential to patients who wish to achieve a manageable quality of life.
Chapter 6 Materials and Methods

Contents below are adapted and/or modified from the published papers, *A Novel and Robust Conditioning Lesion Induced by Ethidium Bromide* and *Remodeling of Spared Proprioceptive Circuit Involving a Small Number of Neurons Supports Functional Recovery*, in which Nao Ishiko is listed as a second author, as well as the manuscript, *Enhancing Remodeling of Corticospinal Tract for Greater Recovery of Voluntary Movement*, in which Nao Ishiko is listed as a second author.

Animals:

All rats used were adult female Fischer 344 (120-135g).

All mice used were adult female C57BL/6.

Generation of transgenic mice:

Targeting vectors were designed to contain loxP sites flanking axons 3-6 of Ryk and the PKG-neo selection cassette. They were then transfected into embryonic stem (ES) cells at the UCSD Transgenic Core facility. Southern blot and PCR confirmed the success of transformation. From these ES cells, chimeric mice were generated at the same facility, then Ryk cKO mice were selected and crossed with Ai14 B6.Cg mice containing loxP-flanked stop codon, preventing tdTomato expression. These double cKO mice were then backcrossed into
C57BL/6J for 6 generations (The Jackson Laboratory, Bar Harbor, ME, RRID:IMSR_JAX:007914).

**Surgical procedure:**

Prior to any surgical procedure, all animals were fully anaesthetized with isoflurane and lack of response to toe or tail pinch was confirmed.

**Sciatic nerve crush:**

Skin over the hindlimb was shaved and cleaned with povidone-iodine, followed by an incision caudal and parallel to the femur. Once the sciatic nerve was exposed, unilateral or bilateral nerve crush was given for 10 seconds with a pair of fine (#55) forceps. All nerve crush was conducted by a single investigator to keep injuries consistent across the animals. Skin was closed with surgical staples.

**Sciatic nerve injection:**

Sciatic nerve was exposed as described above. 36ga NanoFil needle was used to inject 2ul (1ul/branch) of 0.01%, 0.05%, or 0.1% wt/vol EtBr in PBS or PBS alone, longitudinally towards the DRG at a rate of 2ul/min. After injection, the skin was closed with surgical staples.

**T10 lidocaine injection:**

T10 was exposed by partial laminectomy and 1 ml lidocaine-HCl (400 mg/ml 1, Sigma-Aldrich, St Louis, MO) in ACSF, or ACSF alone, was injected bilaterally between two sites, 250mm deep in the dorsal funiculus. After the
injection, dorsal musculature was sutured and the skin was closed with surgical staples. 10 min after injection, animals were tested on an accelerating rotarod.

Dorsal Column Injury:

2ml/kg of ketamine cocktail (25mg/ml ketamine, 1.3mg/ml xylazine and 0.25 mg/ml acepromazine) was used to anesthetize the animals prior to injury. Laminectomy was conducted to expose C4 (C10, C5, C3, or C1, for corresponding experiments), which was followed by puncture of dura over the dorsal horn, approximately 1.2mm lateral to the midline. Scouten wire-knife (David Kopf Instruments, Tujunga, CA) was used to cut the dorsal column twice with a depth of 1mm to ensure the complete transection of dorsal column axons. The lesion cavity was filled with approximately 200,000 syngeneic bone marrow stromal cells (BMSCs) in PBS [100,000 cells/ul] via micropipette injection connected to a picospritzer (General Valve, Fairfield, NJ). Dorsal musculature was sutured and the skin was closed with surgical staples.

- Pyramidotomy:

Animals were anaesthetized with ketamine [120mg/kg] and xylazine [12mg/kg] during the surgical procedure. After pyramids were exposed, ipsilateral side of the pyramid was lesioned (cut) by 15° microscalpel (Electron Microscopy Sciences, Hatfield, PA).

- AAV injection:

Animals were fully anaesthetized with isoflurane and the skin over the skull was shaved and cleaned with povidone-iodine prior to incision. The skull
was thinned bilaterally around the area of motor cortex, followed by the injection of self-complementary AAV2/6 Cre-HA [1.49x10^{11} genome copies/ml] (Salk Institute for Biological Studies Gene Transfer, Targeting and Therapeutics Core, La Jolla, CA) into 10 sites per hemisphere (250nl/site) with the use of 36ga NanoFil needle (World Precision Instruments Inc., Sarasota, FL).

**CTB injection into sciatic nerve:**

Three days prior to sacrifice, the sciatic nerve was exposed as previously described and 36ga NanoFil syringe was used to give animals bilateral sciatic nerve injection with 1% wt/vol solution of the transganglionic tracer cholera toxin B (CTB; List Biological Laboratories, Campbell, CA) in dH2O. Each animal received 1ul of the solution above into tibial and common peroneal branches bilaterally (4ul total).

**FG injection into VPL:**

animals were injected into VPL (11 bilaterally, 30 unilaterally) with 1 ml of 4% wt/vol solution of fluorogold (hydroxystilbamidine, methanesulfonate; Life Technologies, Grand Island, NY) in 12 sites bilaterally as previously described.

**Sacrifice and Tissue processing:**

Animals were anaesthetized with ketamine cocktail prior to transcardial perfusion with ice-cold PBS followed by 4% wt/vol paraformaldehyde in PBS. Spinal cord, sciatic nerves, DRGs, and tibialis anterior muscles were obtained and post-fixed overnight in 4% wt/vol paraformaldehyde at 4°C. The following day, the tissue was transferred to 30% wt/vol sucrose in PBS for cryoprotection and sectioned on a cryostat (Leica, Buffalo Grove, IL).
For the peripheral conditioning experiment & ascending sensory experiment:

DRGs and sciatic nerves were sagittally sectioned at 20um and muscles were at 30um, then the tissues were directly mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Spinal cord was sagittally sectioned at 40um and free-floating sections were collected.

Spinal cords were sagittally sectioned at 40um including the injury sites and the brainstems were coronally sectioned at 40um and mounted directly on Superfrost Plus slides (Fisher, Scientific, Pittsburgh, PA).

For the descending motor experiment:

Spinal cords were sagittally sectioned at 20um including the injury sites and the brainstems were coronally sectioned at 20um and mounted directly on Superfrost Plus slides (Fisher, Scientific, Pittsburgh, PA).

Prior to staining, sections were washed with PBS three times, followed by a one hour block in PBS with 0.25% triton-X100 (PBST) and 5% donkey serum at room temperature (RT) to reduce the nonspecific antibody binding. After primary antibodies were applied at the concentration mentioned below, sections were incubated overnight at 4°C. The following day, the sections were washed three times with PBS, then incubated with Alexa Fluor conjugated secondary antibodies (Life Technologies, Grand Island, NY; Jackson ImmunoResarch, West Grove, PA) for 2.5 hours at RT. Sections were then counterstained with DAPI
[1μg/ml] (Sigma-Aldrich, St.Louis, MO) for 10 minutes followed by wash with PBS for three times.

**Antibodies used for fluorescent immunohistochemistry were as follows:**

Goat anti-CTB [1:10,000; 3-day incubation] (List Biological Laboratories, Cat# 703, RRID: AB_10013220), monoclonal N52 anti-NF200 [1:500] (Sigma-Aldrich, Cat# N0142, RRID: AB_477257), rabbit anti-GFAP [1:750] (Dako, Carpinteria, CA, Cat# Z0334, RRID: AB_10013382), rabbit anti-cJun [1:100] (Cat# 9165, RRID: AB_2130165), rabbit anti-ATF3 [1:200] (Cat# sc-188, RRID: AB_2258513), rabbit anti-Iba1 [1:1500] (Wako Chemicals USA, Inc., Richmond, VA, Cat# 019-19741, RRID: AB_839504), rabbit anti-Ryk (1:250), goat anti-CTB (1:10,000; 3-day incubation; List Biological Laboratories), monoclonal N52 anti-neurofilament 200 kD (1:500; Sigma-Aldrich), guinea pig anti-vGlut1 (1:1,000) and rabbit anti-fluorogold (1:2,000; Millipore, Billerica, MA), rabbit anti-GFAP (1:750; Dako, Carpinteria, CA), monoclonal 6G6-1C9 anti-PSD95 (1:100; Thermo Fisher Scientific, Rockford, IL), rabbit anti-c-Jun (1:100; Cell Signaling, Danvers, MA), monoclonal 219E1 anti-bassoon (Synaptic Systems, Goettingen, Germany), monoclonal GAD-6 anti-GAD65 (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-c-Fos (1:100; AbCam, Cambridge, MA).


**Myelin Staining:**

Sciatic nerve sections were washed twice in dH2O, then twice in 0.025M PB with 0.9% NaCl (working solution), followed by incubation for 1 hour at RT in
0.2% wt/vol gold (III) chloride solution with 0.0075% vol/vol H2O2 in working solution. Sections were washed twice more in working solution, then incubated for 5 min in 5% sodium thiosulfate at RT before three final washes in dH2O.

**Image acquisition and analysis:**

All images were acquired on an inverted Zeiss LSM510 confocal microscope with LSM acquisition software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Ethidium bromide fluorescence emission at 590nm was imaged on a Zeiss LSM510. An Axiovert 40 CFL with an AxioCam MRm and AxioVision software (Carl Zeiss Microscopy, LLC) was used to image gold (III) chloride myelin staining of sciatic nerves. Image density was done on thresholded images using ImageJ (NIH, Bethesda, MD). ImageJ was used to measure myelin density along a 100um thick tracing of thresholded panoramic images stitched together in Photoshop CS5 (Adobe, San Jose, CA). An investigator blinded to the experimental group performed all analyses.

**Behavioral training and studying:**

All behavioral training started two weeks prior to sciatic nerve crush or injection of EtBr as described above. Accelerating rotarod was set at an initial speed of 5 rpm, 45rpm max, and stopped when either animal reached 300 seconds or failed to keep up with speed. Beam crossing was done with a plastic rod with 1 inch diameter, and length of 60 inches. 3 passes were counted as one trial and number of footfalls were counted out of total steps. Toe spread analysis was conducted when hind paw plantar surfaces were dipped in povidone-iodine solution and animals were placed on a white paper with two “barriers” on both sides (narrow path). Based on the toe prints, the mean distance was calculated
by averaging bilaterally the distance between the first and fifth toes from three
prints. Prior to sciatic nerve crush or injection, animals were trained and also
provided “baseline” pre-injury scores. After injury, 16 weeks of weekly behavioral
testing was conducted by investigators blind to the experimental groups. Beam
crossing was done with a plastic rod with 1 inch diameter, and length of 60
inches. 3 passages were counted as one trial.

For the ascending sensory experiment:

All animals were trained for "accelerating rotarod (5 r.p.m. initial, 45 r.p.m.
max, 300s max; Stoelting, Wood Dale, IL) and beam crossing (1 inch diameter,
60 inch span, three passages per trial)" for two weeks, followed by bilateral
sciatic injection of EtBr [0.1% wt per vol]. Among 41 animals, 11 were also tested
in a third behavioral study, grid-crossing (1 inch spaced wire grid, 60 inch span,
three passages per trial), in addition to rotarod and beam crossing. After SCI, all
animals received testing for 16 weeks, twice a week by independent investigators
blind to the experimental groups.
Figures:

**Figure 1-1: Regeneration-associated gene expression in the dorsal root ganglia after sciatic nerve injury**

a-b) Similar ATF-3 and c-Jun expression was induced by both nerve crush and EtBr injection to the sciatic nerve [≥0.05% wt/vol]. c-d) There was a robust negative correlation between myelin density and both ATF-3 and c-Jun expression observed across all treatment groups. Upregulation of ATF-3 and c-Jun persisted for at least two weeks after injury in rats either injected with 0.1% EtBr into the sciatic nerve, or with crushed sciatic nerve.
Figure 1-2: Demyelination in the sciatic nerve.

a) Loss of myelin is shown by Gold (III) chloride of longitudinal sciatic three days after nerve crush or injection of demyelinating agents (myelin staining is indicated by dark purple). b) After nerve crush, lowest myelin density is seen at the lesion epicenter (two-tailed t-test: 3 days P < 0.005, 7 days P < 0.001, 14 days P < 0.05). c) (C) EtBr [0.1% wt/vol] and LPC [1% wt/vol] injected to the sciatic nerve resulted in demyelination over several millimeters proximal to the injection site (ANOVA: 3, 7 days P < 0.05, 14 days P < 0.0001). After 16 weeks, myelin density recovered pre-treatment level in all groups. Data presented as mean (n = 3/group). Scale bar represents 250 µm.
Figure 1-3: Only nerve crush interrupted innervation distal to sciatic injury.

All neuromuscular junctions (stained with tetramethylrhodamine-conjugated α-bungarotoxin, α-Btx) in tibialis anterior lacked pre-synaptic synaptophysin apposition at 7 days after sciatic nerve crush, but not after demyelination. Data presented as mean ± s.e.m. Scale bars represent 50 µm.
Figure 1-4: EtBr treatment impairs recovery of behavioral performance.

EtBr [0.1% wt/vol] treated animals exhibit slower recovery on (A) accelerating rotarod (repeated measures ANOVA P < 0.05) and (C) beam crossing (repeated measures ANOVA P < 0.005) and reduced recovery of (B) hindpaw toe spread (repeated measures ANOVA P < 0.0001). Data presented as mean ± s.e.m. n = 6/group.
Figure 2-1: C1 lesion of ascending dorsal column axons.

a) Schematic of C1 dorsal column lesion. (b) (Left) Location of the C1 lesion is shown (arrowhead) relative to the main body of nucleus gracilis in intact horizontally-sectioned spinal cord. (Right) Presence of CTB-labelled puncta surrounding a VPL-projecting, fluorogold-labelled neuron is shown in transverse section of an intact spinal cord caudal to the injury site, enlarged in inset. (c) Sagittal section of spinal cord after C1 dorsal column lesion illustrating CTB-labelled sensory axons (red) lesioned at C1 and fluorogold-labelled dorsal column neurons (yellow) with a few caudal to the lesion (arrowheads) and some innervated by CTB-labelled axons (arrows). Scale bar represents 1,000 um (b) and 500 um (c).
Figure 2-2: Behavioral performance after C1 lesion.

SFRP2-secreting BMSCs graft treatment resulted in (a) improved functional recovery on an accelerating rotarod (repeated measures ANOVA P <0.05; n = 20 (naive), 21 (SFRP2)) and (b) slightly faster recovery on the beam-crossing task (repeated measures ANOVA P < 0.08; n = 20 (naive), 21 (SFRP2)). (c) There were no differences on the grid-crossing task (n = 5 (naive), 6 (SFRP2)). Baseline, pre-EtBr injection, levels shown with dashed line (grey box represents s.e.m.). Data presented as means±s.e.m., except paw withdrawal thresholds, which are presented as the median and interquartile range.
Figure 2-3: SFRP2 treatment showed enhanced ascending proprioceptive circuit plasticity after SCI.

(a) CTB-labeled vGlut1-immunoreactive puncta (white) contact fluorogold-labeled dorsal column neurons caudal to the C1 lesion (box in b). (b) Heat maps of vGlut1-immunoreactive puncta in CTB-labeled axon bouton-like structures. BMSC grafts are traced in red and the main body of n. gracilis in yellow. (c) The proportion of CTB-labeled vGlut1-immunoreactive puncta directly apposed to FG-labeled neurons, adjusted for the number of remaining FG-labeled neurons (stereological counts), was significantly increased in SFRP2–BMSC-grafted animals compared with acutely injured controls (Dunnett’s t-test, *P<0.05) and intact animals (Bonferroni-corrected post hoc t-test, *P<0.05). (d) Positive correlation between rotarod performance at week 16 and the proportion of CTB-labeled synapses on FG-labeled neurons (Pearson correlation coefficient 0.608, bivariate ANOVA P<0.05). Data presented as mean±s.e.m. Scale bars represent 500 um; n = 4 (intact), 5 (acutely injured, naive) and 6 (SFRP2).
Figure 2-4: Remodeled circuit is necessary for functional recovery.

a) Schematic of lidocaine injection and T10 transection of fasciculus gracilis. b) Transient silencing of the ascending sensory pathway impairs functional recovery on accelerating rotarod, with substantial recovery by 1h (repeated measures ANOVA $P<0.0005$; $n = 7$ (ACSF: naive, SFRP2) and 8 (lidocaine: naive, SFRP2)). Full transection of fasciculus gracilis at T10 eliminates behavioral recovery on (c) accelerating rotarod (ANOVA $P>0.0005$, Bonferroni-corrected post hoc t-test ***$P>0.0005$) and (d) beam-crossing (ANOVA $P>0.0001$; $n = 7$ (intact: naive, SFRP2) and 8 (T10 lesion: naive, SFRP2)). Data presented as mean±s.e.m. Scale bars represent 5 um.
Figure 3-1: Enhanced skilled forelimb recovery and CST collateralization after C5 dorsal column lesion.

a) Timeline of experimental procedures. b-c) Schematic of C5 dorsal column lesion in relation to motor neuron pools for distinct forelimb muscle groups (adapted from McKenna, Prusky, and Whishaw, 2000) d) Enhanced skilled forelimb performance are seen in mice with Ryk conditional deletion compared to controls. e-h) Mice with Ryk conditional deletion showed greater collateralization both rostral and caudal to the C5 injury site than controls (one-tailed t-test P<0.05). Injury site is at 0mm, positive numbers represent caudal and negative numbers represent rostral to the injury site. Axon index is thresholded pixels in sagittally sectioned spinal cord divided by thresholded pixels in transversely sectioned pyramids.
Figure 3-2: Changes of corticospinal connectivity after C3 dorsal column lesion.

a) Schematic of C3 dorsal column lesion followed by C5. b) Enhanced recovery of Ryk cKO mice is eliminated after C3 injury (ANOVA P<0.05, Bonferroni corrected t-test *P<0.05). c-e) There are few CST axon collaterals between C3 and C5 lesions in both Ryk cKO and controls. Injury site is at 0mm. Negative number represents rostral, positive represents caudal to the injury site. Axon index is thresholded pixels in sagittally sectioned spinal cord divided by thresholded pixels in transversely sectioned pyramids.
Figure 3-3: Behavioral recovery of skilled forelimb reaching/retrieval task.

Enhanced recovery is seen in Ryk cKO mice after C5 dorsal column lesion (Repeated measures ANOVA P<0.05). This enhanced recovery is eliminated after C3 lesion and completely lost after pyramidotomy.
Figure 4-1: Experimental timeline and schematic of C1 ascending dorsal column lesion
Figure 4-2: Experimental timeline and schematic of C5 dorsal column lesion
*Figures 1-1 – 1-4 and part of the legends were taken from the paper published in Experimental Neurology, *A Novel and Robust Conditioning Lesion Induced by Ethidium Bromide* (Hollis et al., 2015), in which Nao Ishiko is listed as a second author.

*Figures 2-1 – 2-4 and part of the legends were taken from the paper published in Nature Communications, *Remodeling of Spared Proprioceptive Circuit Involving a Small Number of Neurons Supports Functional Recovery* (Hollis et al., 2015), in which Nao Ishiko is listed as a second author.

*Figure 3-1 – 3-4 and part of the legends were taken from a manuscript, *Enhancing Remodeling of Corticospinal Tract for Greater Recovery of Voluntary Movement* (Hollis et al), in which Nao Ishiko is listed as a second author.
References


