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Cortical Stimulation Induces White Matter Plasticity Following Spinal Cord Injury

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2019

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Neuroscience Graduate Program

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Abstract

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This thesis describes experiments conducted to elucidate the potential for neuromodulation to induce white matter plasticity in the spinal cord injured rat. In addition, it highlights experiments designed to address the effect of neuromodulation on additional sequalae of spinal cord injury. Potential experiments are proposed to address the mechanisms by which the injured spinal cord responds to neuromodulation and the contribution of white matter plasticity to other facets of spinal cord injury and recovery.

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ACKNOWLEDGEMENTS

One humbling and accidental discovery during the past six years was how many amazing people I am lucky to have in my life. To them I am eternally grateful.

An unconditionally supportive family: Mom, Dad, George, Ariel, and Smurf.

The civilians: DSOH. Delipta Wisconsin. *Lapine bombacio*. Dizzy. A Birb. An Owl.

Folks on the other side: Dr. Buck Foss. Dr. Lesley Chaboub. Dr. Caroline Cvetkovic. Dr. Allie Widman. Dr. Maluttsy Horjano. Dr. Stephanie Seeman. Dr. Jason Silver. The Drs Kuhar. The Drs Baxter-Danaher.

Above and beyond lab support from: Rob Robinson. Amanda Fischedick. Darrin Howell.

The MBMBaM Book Club.

DEDICATION

For the people I knew and worked with who suffered traumatic brain injuries, who inspired me to pursue my graduate studies and a career dedicated to improving treatments and outcomes for those with neural injuries.

Chapter 1. **MYELIN PLASTICITY, NEURAL ACTIVITY AND TRAUMATIC NEURAL INJURY**

This chapter was published in its entirety, including text and figures, as "Myelin plasticity, neural activity, and traumatic neural injury" in Developmental Neurobiology (2018) 78 (2), 108-122. BR Kondiles, PJ Horner. Copyright 2017 Wiley Periodicals, Inc.
Acknowledgements: The authors would like to acknowledge Matthew Landry for creating the schematic in figure 1. We would also like to thank Dr. Lesley Chaboub and Dr. Matt Hogan for their critical review of this article. We also acknowledge John Boom for his invaluable assistance in manuscript preparation.

1.1 Abstract

The possibility that adult organisms exhibit myelin plasticity has recently become a topic of great interest. Many researchers are exploring the role of myelin growth and adaptation in daily functions such as memory and motor learning. Here we consider evidence for three different potential categories of myelin plasticity: the myelination of previously bare axons, remodeling of existing sheaths, and the removal of a sheath with replacement by a new internode. We also review evidence that points to the importance of neural activity as a mechanism by which oligodendrocyte precursor cells (OPCs) are cued to differentiate into myelinating oligodendrocytes, which may potentially be an important component of myelin plasticity. Finally, we discuss demyelination in the context of traumatic neural injury and present an argument for altering neural activity as a potential therapeutic target for remyelination following injury.

1.2 INTRODUCTION

Oligodendrocytes form myelin sheaths by extending long processes that wrap around axons to affect action potential conduction. Pio del Rio Hortega first described these cell types in 1928 [1]. For decades, myelin and oligodendrocytes were considered a static player in the nervous system. In 1966, Yakovlev and Lecours described the "maturation" and formation of myelination in the human nervous system over decades of life, concluding once myelin was formed it was static [2]. Work in the 1980s examined the possibility of oligodendrocyte turnover in adult organisms in both health and disease, though oligodendrocytes were thought incapable of full remyelination following demyelination (reviewed by [3]). Only recently have scientists begun to consider that the formation of myelin is a dynamic and ongoing process throughout an organism's lifetime. This review summarizes recent studies that show that myelin growth and refinement occur during development, but also that myelin plasticity is required for motor or cognitive learning, memory, and recovery from injury or disease. Following insult, pathological demyelination and aberrant spared myelin structures can lead to abnormal signal conduction, nerve fiber vulnerability and even degeneration. As a result, many investigators are interested in improving remyelination as a potential therapeutic target to improve recoveries and outcomes.

The term "myelin plasticity" can encompass a number of different cellular processes. As researchers continue to probe this recently discovered form of central nervous system (CNS) plasticity, new questions continue to emerge with each new finding. What are the proper methods to empirically measure myelin plasticity? What is the role of neural activity in modulating myelin sheath adaptation? How do discrete changes in myelin morphology contribute to better signal conduction following injury or disease? This review focuses on the state and limitations of our understanding of CNS myelin plasticity in a variety of circumstances

from development to recovery from traumatic injury. Further, we review current myelin measurement techniques for what they can and cannot tell us about myelin plasticity. For the purposes of limiting the focus of this review, we omit oligodendrocyte precursor biology and inflammatory demyelination models. For further consideration on myelin precursor biology the reader is referred to one of the many excellent reviews on the topic (for example, [4], [5]). For an excellent review of the regulation of myelin regeneration in inflammatory disease, please see [6]. In addition, we discuss the exciting opportunities for experimentation around the role of neural activity and myelin growth and present an argument that influencing myelin plasticity through neural activity may be a target for restoration of the function of injured neural circuits.

1.3 DEFINING MYELIN PLASTICITY

1.3.1 *Role of Myelin in the CNS*

In the CNS of vertebrates, action potential speed is controlled in part by the myelination of axons. Myelination increases conduction velocity by reducing the capacitance and increasing membrane resistance of axons, such that action potentials regenerate at Nodes of Ranvier via saltatory conduction. Many features of myelin can affect its function, and different measures used to describe myelin are listed in Table 1. Longer myelin sheaths, or internodes, and thicker myelin increase signal velocity [7]. Modeling has suggested that axon diameter and internode length are of greater importance than myelin thickness in affecting conduction velocity [7], [8]. The size of the node is another important determinant in conjunction with internode length, and thus can affect conduction velocity, as well [9], [10]. Other features reflect more subtle myelin characteristics, such as the level of compaction between layers, the number of wraps, and its attachment to the axon. Myelin adheres to proteins in the axon at the paranodes, regions flanking the node on each side (for a review on nodal architecture, please see [11]. More subtle features of myelination are more rarely measured because they require tedious, unbiased analysis of electron microscopy (EM). Nonetheless, decompaction, aberrant laminar structure and blebbing of myelin membranes has been described, particularly as a function of normal aging process (reviewed by Peters in 2002 [12]). The effect of these abnormalities on conduction velocities has not been fully modeled. However, Guiterez and colleagues have taken advantage of a genetic model of myelin decompaction to show that loss of laminar structure leads to a significantly reduced conduction velocity of the optic nerve [13].

In the CNS myelin sheaths are formed during the maturation of oligodendrocytes from glial progenitor cells (for a review on cues of progenitor maturation and differentiation, see [14]).

A recent model of zebrafish and mouse developmental myelination proposes that oligodendrocytes wrap via a leading cellular process that makes initial contact with the axon [15]. Each new wrap evolves from this internal lip as new membrane is generated. In the mouse, this process maintains cytoplasmic continuity with the soma until post-natal day 60 (P60), even after compaction has begun. The continuity may be re-established, potentially allowing for some degree of refinement [15]. In an alternate model formed from an examination of developing bovine and avian tissue, myelin is proposed to form via fusion of membranes and proteins around the axon to be ensheathed [16]. These exemplars are seemingly incompatible and future work is needed to determine whether one model is correct or even if a hybrid of these two mechanisms occurs. Furthermore, it has not been shown which, if either, of these models contributes to myelin plasticity and development in the adult.

Although many CNS axons are empirically found to be myelinated for maximal conduction velocity, many are myelinated for sub-maximal conduction, implying the appropriate conduction velocity for a given axon is not necessarily the fastest, but rather the velocity that allows for action potentials to arrive at axon terminals at the appropriate time [7]. For example, the auditory system has emerged as a prime model for studying variable signal timing (reviewed by [17]). Signals from each ear must arrive in auditory processing nuclei with temporal precision in order to localize sound. Auditory axons display a spectrum of myelination patterns in order to accomplish this precision. Indeed, a recent study has shown that temporal precision of the auditory system in rodents is established in part via myelination differences, and that neural activity plays a role [18]. Furthermore, axons in the mouse cortex have been observed to be irregularly myelinated along their lengths [19], and in the zebrafish different CNS tracts are reliant to differing degrees on neuronal activity to dictate internode lengths [20]. These studies

all point to the need for precise signal timing and suggest an inherent capacity of new oligodendrocytes to modify sheath morphology and thereby conduction times throughout life.

In addition to affecting action potential conduction velocity, recent reviews have highlighted how myelination may also provide metabolic [21], structural, and functional [22] support that contributes to axon survival. Thus, it is important to consider that myelin plasticity likely plays a role in multiple axonal functions beyond conduction speed.

1.3.2 *Three Models of Myelin Plasticity*

As oligodendrocytes and myelin serve such important and varied functions, it is logical that changes in neural circuitry would dictate an alteration of myelination. For the purpose of this review, we distinguish between three possible cellular processes that structurally alter myelin, described in Figure 1: 1) formation of new myelin sheaths around previously unmyelinated axons 2) remodeling of an existing myelin sheath and 3) the removal and replacement of a myelin sheath with a structurally different internode. In Table 1 we classify some measures of myelin changes, the category of myelin plasticity each may represent, and the appropriate research techniques to demonstrate these processes. However, it is important to recognize the limits and assumptions of each technique. For example, each histological approach reveals only a part of the morphological features of myelin and cannot be confidently interpreted as affecting the function of conduction velocity. Furthermore, many of these techniques are mutually exclusive, making the assessment of a full panel of myelin characteristics a great challenge that has slowed progress in the field. Here we consider the uses and limitations of current methods but also consider emerging technologies that will help investigators probe some of the most exciting models of myelin plasticity.

1) Myelination of Bare Axons by New Internodes

In the adult CNS, an axon may not be uniformly myelinated along its length [19], [23], providing a potential site for future myelination and signal refinement. Thus, one source of myelin plasticity in adults may be the coverage of completely or partially unmyelinated axon segments; this concept is illustrated in Fig 1A. The logical question then follows: are mature oligodendrocytes capable of division to form new internodes, or are progenitor cells responsible for forming new myelin sheaths?

In the 1980s some studies suggested that mature oligodendrocytes were capable of dividing [24] often based on little more than the morphology of nuclei which Ramon y Cajal had suggested implied mitosis (reviewed by [3]). A few studies did suggest long-term non-pathological turnover of oligodendrocytes and myelin (e.g. [25], [26]; reviewed by [3]) but many studies focused on demyelination models to determine the source of new myelin sheaths (again, see [3]). Distinguishing between previously developed and newly generated oligodendrocytes or myelin was made possible with the advent of proliferation reporters, such as bromo-deoxyuridine (BrdU). For example, tritiated thymidine was used in a study that concluded oligodendrocytes proliferate in response to injury [27], with the determination of oligodendrocytes based solely on cellular morphology. More recently, genetic or viral reporters of the oligodendrocyte lineage and membrane-targeting reporters that visualize myelin have been developed [28]–[30]. Though these reporters need not be restricted to injury, they have proved useful to isolate the source of newly generated oligodendrocytes and myelin following injury (e.g. [29]). Indeed, studies show that oligodendrocyte precursor cells (OPCs), not oligodendrocytes, are the source of remyelination, and that OPCs divide and differentiate in response to injury [31]. Recent studies have shown more directly that, at least in injury, mature oligodendrocytes do not contribute to

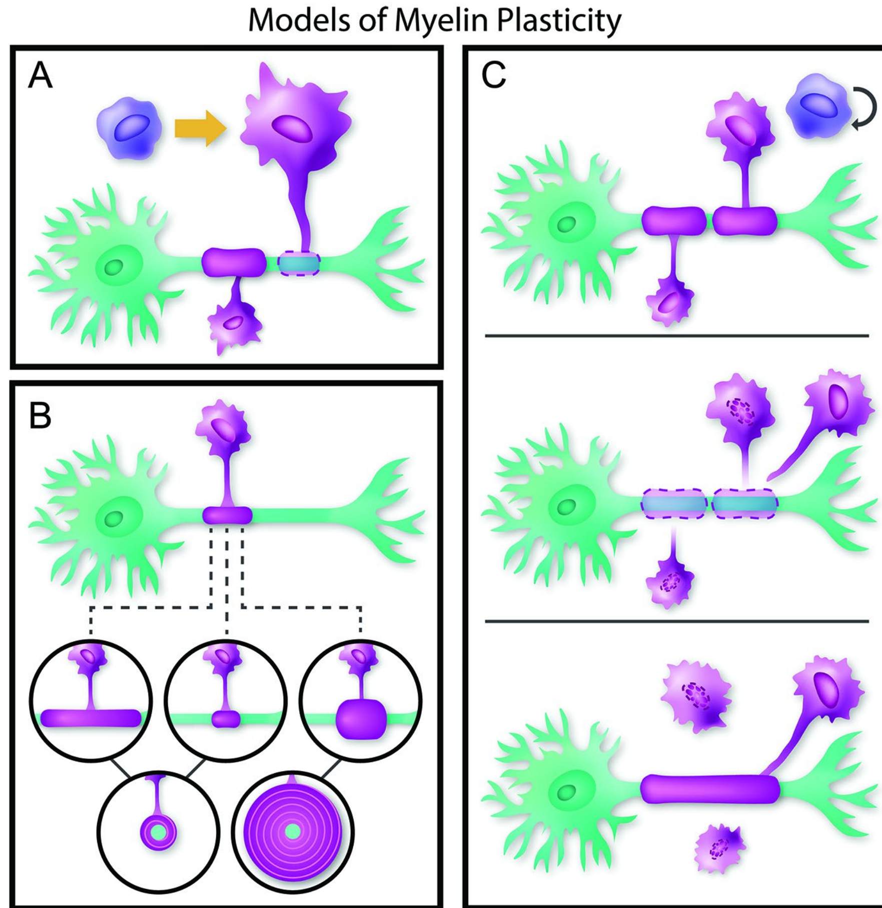

Figure 1.1 Three Models of Myelin Plasticity

A) Myelination of Bare Axons by New Internodes: An oligodendrocyte precursor cell (OPC) differentiates to form a new oligodendrocyte in order to myelinate an unmyelinated section of axon B) Remodeling or Refinement of Existing Myelin: An existing sheath may lengthen, shorten, or thicken. In cross section, thickening of an internode reflects an increase in the number of myelin wraps C) Replacement of Myelin Sheaths by New Internodes: Two existing, short internodes retract from the axon, while an OPC leaves the cell cycle to differentiate and form a single longer sheath. Currently, no data exist regarding the fate of oligodendrocytes with retracted sheaths.

remyelination [32]. To our knowledge, researchers have not yet conducted similar studies in non-injured models to show that mature oligodendrocyte do not proliferate or form new myelin. Such studies are needed to definitively show that OPCs are the source of new sheaths in non-pathological conditions in a manner similar to the post-insult environment.

If the majority of new myelin is formed by newly differentiated oligodendrocytes, forming new myelin is ultimately about signaling OPC populations to differentiate into myelinating oligodendrocytes. A number of studies have shown that OPC populations are sensitive to their surroundings and respond to multiple migration, proliferation, and differentiation cues in a variety of species, locations, and conditions (for just a few examples and reviews, see [33]–[36]). Consequently, myelin plasticity may ultimately require the initiation of signals that affect the OPC cell cycle to induce migration, division, or differentiation. In Fig. 1A, a previously bare segment of axon is myelinated by a newly matured oligodendrocyte. An intriguing possibility is that this "plasticity" may actually represent late developing myelination, which has been suggested to occur in humans in cortical white matter [2]. The distinguishing characteristic between late developing myelin and adult myelin plasticity would be the degree to which these myelination patterns are pre-determined, in contrast with a reliance on external influences.

2) Remodeling or Refinement of Existing Myelin

There are several potential mechanisms by which existing myelin sheaths may be remodeled, as illustrated in Fig. 1B. Remodeling may involve altering the length of the myelin sheath, which affects conduction velocity via altering the number of "jumps" action potentials make down the length of an axon. Adding or subtracting wraps of myelin affects the sheath thickness, which directly relates to the resistance and capacitance of the axonal membrane, thus

affecting conduction velocity. There is as yet little direct evidence regarding the potential for remodeling of existing myelin, although the model proposed by Snaidero and colleagues allows for the re-establishment of cytoplasmic channels, potentially allowing for refinement [15][1]. Many of the commonly used techniques for measuring myelin are listed in Table 1; unfortunately, EM and immuno-labeling are temporally limited and can only describe the current state of a myelin sheath. Immuno-labeling and EM cannot describe the history of a sheath (e.g., established or regenerating), and cannot confirm that an observed change in myelin morphology is the result of remodeling or degeneration. More recently developed tools, such as genetic or viral reporters and live imaging, provide greater insight into OPC and myelin dynamics (for a review, please see [37]). The further development of these tools, particularly in mammalian systems, will allow researchers to discriminate between old and new myelin sheaths and track the fates of oligodendrocytes over time. Furthermore, these techniques will allow scientists to address which of the types of remodeling occur and probe into the mechanisms by which these changes take place. Tracking the history and changes of individual sheaths and cells will definitively prove or rule out the possibility of remodeling, and this line of inquiry will likely yield exciting findings.

3) Replacement of Myelin Sheaths by New Internodes

Due to the compacted layers of mature myelin sheaths, instead of remodeling it may be more energetically and structurally feasible to replace non-optimal or dysfunctional sheaths. New sheaths would be more structurally suited to new signaling requirements. Thus, one final mechanism of myelin plasticity may be myelin replacement, which is shown in Fig. 1C: two existing sheaths are broken down, and an OPC exits the proliferative cycle to differentiate into a

[1] Since the publication of this paper, several studies demonstrating myelin remodeling have been published. These papers are discussed in subsequent chapters.

myelinating oligodendrocyte. This mechanism would necessitate a complex pathway, wherein the oligodendrocyte detects the inadequacy of a myelin sheath and subsequently removes it. A new OPC then matures and extends a process to form a myelin sheath structurally different from the previous one. The fate of the oligodendrocyte once a sheath is retracted is another intriguing component of this pathway, as the oligodendrocyte may myelinate multiple other axons. Furthermore, studies in adult mice show that a sudden loss of oligodendrocytes leads to axonal deterioration and long-term disruption of myelination leads to axonal degeneration [38]–[40]. Therefore, if oligodendrocytes purposefully retract myelin sheaths, an axon must be able to survive the transient demyelinated period. These studies indicate that myelin sheath removal in the healthy nervous system would rely on molecular stabilization of the axon through a process that is not yet understood.

Table 1-1 Myelin Morphological Measures

Change in	**Observed via**	**Evidence for Model of Plasticity**
G-ratio	Cross-sectional electron microscopy (EM); Immunohistochemistry (IHC) (less accurate)	Remodeling; Replacement
Myelin Compaction	Longitudinal or horizontal EM	Remodeling
Number of Myelin Wraps	Cross-sectional EM (combined with metabolic or membrane label and immune-EM)	Remodeling; Replacement
Number of Myelinated Axons	EM; IHC; live imaging	New Internodes; Remodeling
OPC / Mature Oligodendrocyte Number	IHC; live imaging combined with a genetic/viral reporter system	New Internodes; Replacement
Myelin or Axon Protein Expression	IHC; Western blotting	New Internodes; Remodeling; Replacement
Myelin Sheath Length	IHC; live imaging & genetic/viral membrane reporter	Remodeling; Replacement

Measures of myelin morphology and requisite experimental and/or imaging techniques used to demonstrate myelin changes. Each measure may add evidence to one of the three models of myelin plasticity. Advances in *in vivo* live imaging have allowed scientists to observe many of these measures in single cells and myelin over time and in response to stimuli.

1.4 THE CASE FOR NEURAL ACTIVITY AS A MODIFIER OF MYELINATION

1.4.1 *Neural Activity Modulates Myelin Development*

Myelination occurs both during prenatal and postnatal development (reviewed by [41]). While individual myelin profiles are variable both within a fiber tract and even within a single axon, there is an evolving literature that suggests myelin growth and adaptation is strongly influenced by the activity of discrete axonal pathways. Indeed, a number of studies both *in vitro* and *in vivo* illustrate that neural activity may be a key regulator of myelin morphology, in addition to its known effects on neural and axon morphology.

To determine the extent to which neuronal activity changes myelin structure, researchers used pharmacological agents to increase (via scorpion toxin, a sodium channel agonist) or decrease (via tetrodotoxin (TTX)) neuronal firing in an *in vitro* prep from dissociated embryonic mouse cerebral neurons [42]. Blocking action potentials with TTX halted myelination, as measured by the number of myelinated axons. The most significant effect occurred when TTX was added transiently just before myelination began. Inducing higher firing rates with scorpion toxin caused more fibers to be myelinated than in control cultures without affecting the number of oligodendrocytes. This finding implies that increased neural activity didn't cause OPC proliferation, but did encourage individual oligodendrocytes to myelinate more axons. Downregulation of neural activity in mouse optic nerves *in vivo* by TTX decreased the number of myelin sheaths when administered in the post-natal critical period [42]. These data were further confirmed in developing zebrafish, where blocking synaptic vesicle release decreased the number of myelin sheaths, while increasing vesicle release increased sheath number [43]. Taken together, these studies imply that neuronal activity affects myelination not by increasing proliferation, but by influencing the number of sheaths produced. Further *in vivo* work has

suggested that while neuronal firing may induce preferential wrapping of active axons over neighboring axons, neuronal firing is also important to maintain and encourage continued development of the myelin sheath [44], implying again that activity affects the behavior of already myelinating cells, rather than proliferation. Finally, the studies discussed above do not show a direct effect of neuronal activity on oligodendrocyte sheath production. It is important to note that neural activity also influences the location and size of excitatory segments of the axon [45], which may indirectly affect the location and attachment of myelin sheaths.

If neural activity does signal certain axons for myelination or myelin plasticity, what is the mechanism by which neuronal activity induces changes in oligodendrocytes and myelin? The *in vitro* study above examined oligodendrocyte numbers [42], but what is the effect of increases in neuronal firing or a change in rate on OPC populations and subsequent differentiation into myelinating oligodendrocytes? A study in developing rat optic nerves found that blocking neuronal firing via TTX decreased the number of new OPCs, an effect that could be rescued by applying platelet derived growth factor [46]. Wake, Lee, and Fields showed that in cultured dorsal root ganglion neurons, glutamate vesicle release along axons increased myelination and also caused an increase in oligodendrocyte protein production, though electrical activity did not seem to influence OPC proliferation [47]. A later study showed that non-synaptic neurotransmitter released from axonal varicosities increased OPC internal calcium levels, which led to myelin protein production and preferential myelination of electrically active axons [48]. Together, these studies suggest that OPCs are capable of detecting changes in neural activity, and respond by differentiation and the initiation of myelination, but they failed to definitively and quantitatively measure new myelin sheaths.

Most of the studies reviewed above utilized pharmacological approaches, but there is an increasing interest in how changes in the environment or exposure to a physical or cognitive challenge can modulate myelination. For example, two laboratories have recently shown the effect of developmental deprivations on myelin formation. Surprisingly, monocular deprivation during mouse development led to an increase in oligodendrogenesis without increasing OPC numbers, though myelin sheaths are shorter [49]. These data imply that, contrary to the findings outlined above, decreased neuronal activity causes more new myelinating oligodendrocytes, though variations between the experiments make direct comparisons difficult. For example, it is possible that the pharmacological approaches used in the studies above caused a greater overall decrease in activity. On the contrary, the 2 weeks of deprivation used in this study might have initiated a compensatory mechanism not seen in shorter term experiments. The authors also showed that glutamate might be a key signal involved in regulating developmental myelination, as knocking glutamate transmission down caused similar effects [49]. A study published in 2012 showed that depriving mice of social interactions during a critical period from P21-P35 reduced oligodendrocyte complexity and decreased oligodendrocyte protein levels in the prefrontal cortex without affecting axon structure. Furthermore, these animals showed decreased performance on cognitive tasks [50]. Together, these studies point to the requirement of neuronal activity, rates of neural firing, and circuit afferent activity for formation of myelin in development. While the studies reviewed above focus on the critical periods when a distinct axonal tract is formed and develops activity, more recent work has begun to examine modifiers of myelin growth and plasticity in the mature nervous system.

Although these studies provide strong evidence both *in vitro* and *in vivo* for the importance of activity as a modifier of myelination, we must also consider *in vitro* work that

shows OPCs differentiating and myelinating in the absence of axons. As early as 1990, cultures showed the initiation of wrapping on glass fibers [51]. Further work *in vitro* work showed the addition of factors deposited on the fibers affected myelination in culture [52]. In 2012, the Chan group further explored the importance of nanofiber type and diameter in differentiation and myelination by cultured rat OPCs [53]. More recently, work has shown that oligodendrocytes cultured with microfibers may have intrinsic properties, which dictate their myelinating capacity to some degree, regardless of axonal cues [54]. Though evidence strongly suggests neural activity is an important regulator, these culture systems provide useful tools for exploring additional cues that may affect myelination.

1.4.2 *Myelin Plasticity in the Adult*

As we have detailed, for many years the question of myelin plasticity only arose in the context of injury or disease. Recently scientists began to explore the possibility that under non-pathological conditions, myelin and white matter remodeling are essential to normal function. White matter plasticity in adult humans and animal models is reviewed in great detail elsewhere [55]–[59]. Here we highlight select studies and relevant concepts and begin to consider where the opportunities and remaining questions lay for the role of myelin plasticity in the function of the adult nervous system.

Much of the literature regarding adult white matter remodeling revolves around learning induced changes. Many of these studies use diffusion tensor imaging (DTI) and fractional anisotropy (FA) to visualize changes in the flow of water in CNS tracts over time. These metrics can serve as an indirect measure of myelination changes through the mathematical assessment of white matter volume and direction. Learning induced changes in white matter is reviewed elsewhere [60]. For example, DTI has shown that participants that learn a new motor skill (in this

case, juggling) exhibited increased FA in the intraparietal sulcus not see in those that did not learn a new task [61]. Similar work in rodents revealed an increase in FA in the sub-sensorimotor cortex white matter following motor skill learning, which correlated with an increase in myelin basic protein staining [62]. Although FA is not a direct measurement of myelin changes, it does show an effect of learning on CNS tracts in living organisms. Further refinement of MRI scans and processing may provide more in-depth and exciting information regarding myelin-specific changes.

If learning requires (or causes) changes in white matter in the CNS, it then becomes necessary to ask what role OPCs and oligodendrocytes may play, and how myelination may change. If new oligodendrocytes are the source of new myelination, what would the effect on learning be if new oligodendrocytes could not form? McKenzie and colleagues asked this question by conditionally knocking out a transcription factor, MyrF, essential for oligodendrocyte differentiation [63]. The loss of MyrF did not cause demyelination when it was knocked out in OPCs at P60. However, the mature animals had deficits in learning a skilled motor task (complex wheel running) despite no other observed behavioral deficits. The authors concluded that the production of new oligodendrocytes and new myelin was essential to learn a new motor task [63]. More recent data suggests that the maturation of new oligodendrocytes is essential to learning on a time scale of mere hours [64], providing support for the idea that oligodendrocytes may have developed mechanisms of rapid deployment or adaptation of myelin structure.

Providing evidence that new myelin in the adult CNS must be formed by new oligodendrocytes, a recent study has shown that knocking out new oligodendrocyte formation in the adult brain leads to motor deficits and conduction deficits, which can be rescued by

transplanting OPCs [65]. As social interaction may be necessary for the development of appropriate myelination [50], so too may isolating adult animals cause thinner myelin in the prefrontal cortex, which can be rescued by reintroduction of social interaction [66]. When enriched environments are combined with skilled reaching training, more oligodendrocytes are generated in the adult cortex [67].

These studies demonstrate myelin plasticity occurs in the adult CNS as a response to non-pathological fluctuations in nerve or organism activity. They also provide strong evidence for the formation of new myelinating oligodendrocytes as the source of rapid and long-lasting sheath plasticity, though they have not completely ruled out the possibility of remodeling of existing myelin sheaths. It is noteworthy that most of these studies fail to directly measure changes in myelin morphology, but instead rely on indirect measures such as increases in OPC differentiation. Technological advances will allow scientists to probe more directly into the precise morphological changes that accompany myelin changes associated with learning or daily functioning. The effect of comparable changes of myelin morphology on conduction velocity have been mathematically modeled [68]. However, given the challenge of measuring multiple myelin and axon indices at once, we have incomplete data to mathematically model the physiological impact of myelin plasticity. Hence, further quantitative studies of myelin and axon parameters that definitively delineate new from old myelin are needed. A final question with an equal shortage of empirical data is how newly matured oligodendrocytes are integrated into existing or remodeled circuits to affect function and behavior.

1.4.3 *Dependence of Myelin Plasticity on Neural Activity*

Whether myelin plasticity relies on the myelination of bare axons, the remodeling of existing sheaths, or sheath replacement, researchers need to determine the signaling mechanisms

to prompt these changes. Studies have pointed to the possibility that neural activity may influence adult myelin plasticity, potentially in a manner comparable to developmental myelination.

In 2014, Gibson and colleagues published a study regarding the effects of short-term optogenetic stimulation on myelination [69]. Stimulating cortical areas induced circular running behavior and subsequently increased OPC proliferation. Weeks later, there were more oligodendrocytes and thicker myelin sheaths in the cortical and white matter areas of stimulated mice than in non-stimulated animals. The implication of this finding is that new oligodendrocytes are responsible for these thicker myelin sheaths, though this was not directly tested. Future work may elucidate whether such axons were previously unmyelinated and definitively show that thicker myelin was formed exclusively by newly oligodendrocytes. One final component of Gibson et al's work that is worth noting is that stimulated animals also showed an altered gait, showing a functional consequence of altered myelination [69].

Another recent study has shown the potential importance of physical activity on brain myelination. Alvarez-Saavedra and colleagues showed that voluntary running induced OPC and oligodendrocyte proliferation and myelination of the cerebellum [70]. Though the direct effect of neuronal activity on myelin changes was not measured, this study provides compelling evidence for increasing activity as a way to affect myelin plasticity. Indeed, it has been known for some time (as reviewed by Cotman and Berchtold [71]) that physical activity may increase plasticity, likely through increased expression of brain derived neurotrophic factor (BDNF). In support of the idea that BDNF and exercise alter oligodendrocyte behavior, studies have found that exercise after traumatic injury affects the levels of oligodendrocyte proteins associated with myelination and repelling neural growth [72], [73]. However, other studies have shown that

exercise in and of itself is not sufficient to increase OPC differentiation [64]. One other important consideration is changes in neuronal activity induced through increased physical activity may actually reflect a significant alteration in the pattern of neural activity, rather than the overall amount of firing. Teasing out the global effects of increased physical activity and individual effects of an alteration of an axon's signaling remains an important question.

Many researchers have begun to probe into the mechanisms by which active axons signal OPC differentiation and myelination. A full commentary on the exciting work being conducted on the signaling cascades and molecules between axons and OPCs/oligodendrocytes is beyond the scope of this review. Several recent reviews have touched on this evolving field (for example, [55], [56]), including the potential for signaling via non-synaptic neurotransmitter release. Future exciting work will further elucidate how this complex signaling cascade targets axons for myelination.

1.5 TRAUMATIC NEURAL INJURY, DEMYELINATION, AND REMYELINATION

Acute injury cascades have been characterized in a number of species, and follow a similar pattern across many models, including rodents, cats, nonhuman primates (for just a few examples, see [74]–[77], for a review see [78]). Following injury, oligodendrocytes and their myelin sheaths are susceptible to damage and cell death, which leads to morphological and functional consequences. Types of myelin abnormalities and the experimental methods used to detect them are listed in Table 2. Typical morphological changes of the spared tissue in the chronic SCI environment include the retraction of axons from their post-synaptic contacts, axonal degeneration and death, and the formation of a physical and chemical barrier to encapsulate the injury, the glial scar (reviewed by [79]). Demyelination, and myelin abnormalities can occur at both the internode and the paranodal attachments [75], and abnormal myelination is predicted to affect conduction [80] and also affects behavior [81]. Channel spreading is a phenomenon wherein sodium and potassium channels "leak out" of the normally highly constrained nodal area [82], [83], which is related to demyelination and subsequent changes in action potential conduction velocity. Blast injuries have been found to decrease axon initial segment size which may affect excitability [84]. All of these factors combine to yield a disruption of neuronal signaling which can lead to loss of motor and sensory function below the injury.

Table 1-2 Myelin Pathology

Pathology	Observed via	Potentially repaired via
Blebbing/Loss of Compaction	EM, genetic/viral labeling	Remodeling; Replacement
Demyelination	IHC; EM	Replacement
Channel Spreading	IHC	Replacement
Paranodal Attachment Abnormalities	EM	Remodeling; Replacement

Commonly occurring myelin abnormalities observed following SCI, and potential mechanisms that may contribute to re-establishment of proper myelination and signal conduction.

Even in the absence of an intervention after neural injury, spared nerve fibers undergo remyelination. Older studies showed very thin myelin observed by EM (less than 20% of normal thickness) following a compressive injury [85], [86], which was thought to represent failed remyelination. More recent studies have also suggested chronic demyelination in a rat contusion SCI model [87]. However, rigorous new methods showed that in rodents with chronic SCI spared axons are remyelinated, and regenerated myelin is nearly indistinguishable from pre-injury myelin [8], [29], [88]. Surprisingly, this is a recent epiphany in the field that formerly associated very thin myelin sheaths with new myelin. New rigorous methods indicate abnormally thin profiles are likely myelin on degenerating or dystrophic axons [8]. It may be that a dystrophic axon creates a relationship of aberrant communication that causes a series of myelin sheath retractions and replacements, but this is a new and important area of future research. Work by Lasiene et al was the first to definitely discriminate between cut fibers and spared fibers while simultaneously quantifying myelin regeneration, showing that on spared axons, myelin is near normal thickness [8]. Further studies in rats confirmed these findings and also demonstrated that regenerating myelin sheaths are near normal thickness but initially shorter than normal (less than 50 μm) [88].

Future studies need to employ complementary techniques to establish if these findings hold true in other models, but the implication is that myelin regeneration is more efficient than previously thought. More recently, Powers et al used a retroviral-driven membrane-bound reporter to isolate new from old myelin sheaths after neural injury [29]. These data show that regenerating myelin sheaths are short and thick at 1-month post-injury, but the sheath expands to near normal lengths over several months. These data indicate that myelin sheath plasticity exists in the adult injured nervous system and is largely reliant on the formation of new myelin sheaths. These findings were corroborated by Hesp et al using a membrane reporter to visualize new myelin and oligodendrocytes, which showed that new oligodendrocyte maturation continues for several months post-SCI [89]. These data indicate a surprisingly long window of myelin replacement and refinement after neural injury. Extended periods of oligodendrocyte death shown to occur in SCI [90] may be related to the removal of damaged sheaths and oligodendrocytes, which would correlate with late oligodendrocyte regeneration.

Though these data support a replacement model of myelin plasticity, they do not rule out the remodeling model. Thus, each of the forms of myelin plasticity outlined in Figure 1 may occur following traumatic neural injury: demyelinated or previously unmyelinated axons may be myelinated, spared or damaged myelin may be remodeled, and damaged or old myelin may be replaced. It will be important to identify the signals that call for myelin regeneration or refinement after injury and more important to define the functional effects of this protracted myelin plasticity.

Two main issues arise when considering evidence of efficient long-term remyelination following injury. The first is to reconcile these findings with many years of contradicting work. Since remyelination of an injured CNS was first described in 1961 [91], many studies have been

conducted in a variety of animal models of various types of injury. Frequently, these studies were limited by experimental design and insufficient techniques, as well as biases towards assumptions of chronic demyelination. An early study of weight-drop induced injury in cats described thin regenerated myelin, but only had one animal in the 3 month and 16 month time points [92]. Our intention is not to refute seminal and pioneering work in remyelination, but merely to call attention to the models and methods available at the time. In a long term model of cuprizone induced demyelination, Ludwin and Maitland described thin myelin following injury but they observed a trend towards normal myelin at six months [93]. This result could reflect only a slight variation that caused slower remyelination, which was ultimately observed by Lasiene et al and Powers et al. Thus arises another important point when reconciling multiple studies: the need to consider the specifics of the demyelinating insult. The extent to which remyelination in a multiple sclerosis (MS) model, such as cuprizone induced demyelination, can be directly compared with a compression or contusion SCI model has not been established. In some MS models, regenerated myelin can re-establish function, but in human patients there is variable regeneration (reviewed by [94]). Further work is necessary to determine the factors which dictate poor remyelination in these patients, and what, if any, correlates can be made to the SCI literature for the betterment of both patient populations.

The topic of patient populations leads to the second matter, the necessity of establishing the relevance of rodent models of post-SCI remyelination to human patients. Studies detailing the cellular changes in human SCI patients are exclusively post-mortem studies with multiple changing variables (e.g. [95]–[97]). The differences in methods make it difficult to directly compare rodent and human studies. Future research techniques may describe the patterns of myelination in chronic SCI patients with more precision and controls, which may determine

whether findings in rodents are relevant in humans. Larger models, such as porcine and non-human primates present another potential avenue for describing post-injury remyelination as it may be relevant to humans.

1.6 ACTIVITY BASED THERAPIES FOR REMYELINATION

The importance of myelin for structural support, trophic support, and maintenance of axonal electrical profiles all indicate the importance of re-establishing sufficient myelination following SCI. Indeed, studies have shown that encouraging remyelination in disease models contributes to better axonal survival as well as functional recovery (for example, see [98]). Researchers and clinicians have proposed a variety of approaches aimed at myelin preservation or remyelination after SCI; for a review please see [99]. Proposed pharmacological approaches target multiple pathways including the inflammatory response, decreasing oligodendrocyte apoptosis, and increasing OPC proliferation and differentiation. Biomaterials have been proposed to bridge glial scars and improve regeneration. Cell transplants of neural precursors and OPCs have been proposed as well.

Activity based therapies for SCI are used to improve neural regeneration and promote synaptic plasticity. Physical therapy for SCI increases neural activity in descending and ascending tracts. Increasing neural activity via electrical stimulation has also been used following SCI. For example, certain patterns of intraspinal microstimulation (ISMS) have been shown to improve functional outcomes in rodent SCI models [100], [101], epidural stimulation may raise neuronal excitability below the lesion (reviewed by [102]), and cortical stimulation of the uninjured hemisphere may lead to better behavioral recoveries and axonal growth [103], [104]. Furthermore, many stimulation paradigms also involve a degree of retraining on a physical task, similar to physical therapy. As outlined previously, retraining may be important for remyelination as training on a task affects oligodendrocyte development [67] and white matter tracts [60]. The mechanisms of these stimulation paradigms rely on directly causing or increasing the likelihood of action potential firing. However, until recently the effect of altering

neural activity on glia following SCI had not been explored. A recent paper has shown that following SCI inducing neuronal activity via cortical stimulation causes an increase in OPCs, mature oligodendrocytes, and myelin protein expression [105]. This exciting finding, in conjunction with the evidence for activity based myelination, and the evidence in support of the use of activity therapies for peripheral nerve injuries [106] provides a strong argument for a possible new target for treating demyelination following SCI which may also help induce neuronal recovery. Furthermore, though the demyelination process in MS is different than SCI, both conditions require remyelination efforts, and recent work has shown that in a model of MS, neuronal activity may induce OPC populations to differentiate via glutamate signaling [107].

There is reason to believe that comparable to developmental and non-pathological conditions, neural activity may also regulate myelin plasticity in the post-SCI environment. The model of myelination proposed by Snaidero and colleagues [15] would allow for extant internodes to elongate for some time following their initial formation. Findings that sheaths are initially shorter may actually be indicative of the phases of sheath remodeling and the need for axonal or local signals to refine axonal coverage and myelin sheath morphology [8], [29], [88]. Axo-myelinic synapses provide a mechanism for neuronal activity based signaling directly from axons to myelin sheaths [108]; they may be capable of signaling for alterations in internode characteristics.

Although refinement of surviving myelin is a possibility, the majority of research thus far points to newly generated myelinating cells as the main source of myelin plasticity after SCI. Given the importance of neuronal activity in developmental myelination, it is logical to look to activity as a driver for post-injury remyelination. The ability to fire action potentials is an important signal that an axon is still viable; remyelination of axons that are cut off from their cell

bodies and are no longer able to conduct signals would serve no functional purpose. Following demyelination in the adult mouse brain, newly born OPCs form temporary synapses with corpus callosum neurons as they are remyelinated [109]. Axon-OPC synapses are seen in mouse development [48]. It is possible that a similar mechanism of communication occurs in the remyelinating spinal cord. Indeed, the remyelination of surviving axons that has been shown to occur in chronic rodent SCI models [29], [89] may be a result of neural activity in those axons. Further research will elucidate the effect of increasing or decreasing activity levels in individual axons or tracts to determine the effect on remyelination.

A new frontier for researchers is to rigorously model and assess how the amount and patterns of neural activity encode changes in axonal and myelin structure. Not all neural activity is created equal. Research has shown that stimulation frequencies that mimic developmental oscillations are most effective at inducing myelination *in vitro* [110]. Higher activity levels are not necessarily better at inducing myelination, as stimulating neurons to seizure-like levels did not induce myelination changes [69]. Similarly, physiologically relevant stimulation paradigms may induce better functional recoveries in SCI [101]. An approach that addresses both neural and glial recovery will be necessary to improve outcomes after SCI; neither component in isolation is sufficient. Acutely, the lack of trophic and metabolic support caused by demyelination may contribute to neuronal death. By the chronic phase, when remyelination can occur, meaningful neural connections may be beyond repair. Future research will elucidate the patterns of activity most conducive to both neural and myelin recovery, which will ultimately lead to better functional outcomes following SCI.

1.7 CONCLUSIONS AND FUTURE QUESTIONS

Future work will determine which of the three potential forms of myelin plasticity outlined in Figure 1 occurs in both the intact and injured nervous system. One form of plasticity may dominate or multiple mechanisms may coincide. In turn, there may be mechanisms of plasticity that are induced by distinct insults such as pathology. It bears mentioning that the Nodes of Ranvier are also capable of undergoing structural changes, and the relative location and size of the nodal regions also contribute to the conduction of action potentials. Recent studies showed that these parameters may also be adjusted to alter signal timing [9], [10].

For example, if a partially myelinated axon requires longer internodes, all three plasticity forms may co-occur as some existing internodes lengthen, newly matured oligodendrocytes simultaneously myelinate previously bare portions of the axon and replace older shorter internodes. Many questions arise from such a hypothetical. The complexity of signaling necessary to govern such elaborate processes across axons, OPCs, and oligodendrocytes must be great. An oligodendrocyte can produce a 300 μm sheath which lies 30 μm away from its cell body [111]; what are the energetic constraints of remodeling vs. replacement over such large spatial domains? The advent of long-term live imaging via cranial and spinal windows, high-resolution microscopy, and reporter strains will help to answer these and other exciting questions.

Our awareness and appreciation of myelin plasticity has undergone a renaissance driven by exciting new discoveries of remarkable myelin regeneration and plasticity. Scientists are applying new research techniques to explore myelination as a vital component of adult neural plasticity. Many fundamental questions regarding myelin plasticity remain, especially in the context of neural injuries. New technology, such as live imaging and novel genetic/viral

reporters, will continue to allow researchers to establish the extent to which existing myelin degenerates, regenerates, or remodels as a function of aging or injury. A key question is which cellular populations signal myelin growth and which models best represent the biology of myelin segment replacement, regrowth and adaptation. If the theory that increasing or altering neural activity alters myelin morphology continues to gain momentum, it will be necessary to empirically establish the patterns and levels of axonal activity that drive glia to myelin sheath growth, remodeling, or replacement. Further, while MRI and DTI are powerful imaging tools for evaluating myelin in humans that have led to exciting hypotheses, there is much work to be done. A reconciliation of MRI signals with those of rigorous genetic models is needed to develop a deeper understanding of how myelin plasticity is manifest in humans compared with animal models. As future studies answer many of the exciting questions outlined in this review, we are likely to see new treatments that induce both glial and neural recovery to provide better outcomes for individuals with neural injuries.

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Chapter 2. PATTERNED ELECTRICAL ACTIVITY OF AXONS INCREASES NODAL FREQUENCY AND MYELIN THICKNESS AFTER INJURY

2.1 ABSTRACT

An exciting nascent concept in neuroscience is myelin sheath plasticity in response to neural activity and learning. This study tested if patterned electrical activity modulates myelin plasticity in the injured adult nervous system. The motor cortex of spinal cord injured rats was electrically stimulated for three weeks and myelin sheath thickness, internode length, and axon morphology were quantified in the cord. Reconstruction of long-tract fibers revealed that stimulation induced significant myelin and node rearrangement outside of the lesion zone where remyelination has not previously been thought to occur. Specifically, myelin is thicker and internodes are shorter than in unstimulated controls. These data reveal a wide-scale alteration of nodal and myelin patterns in response to electrical activity and lay the groundwork for a new model of white matter plasticity.

2.2 INTRODUCTION

In the central nervous system (CNS), oligodendrocytes form myelin, which insulates axons to adjust the speed at which they conduct action potentials [1]. Axonal conduction velocity is directly related to axonal diameter, the thickness of myelin and the length of the myelin sheath [2]. Recent research in the intact nervous system has highlighted the importance of myelin and white matter plasticity for neural functioning and learning [3][4]. One potential regulator of myelin morphology during development and in the young adult is levels of neural activity [5]–[7]. Increasing neural activity via optogenetic stimulation of the CNS results in thicker myelin [5], [6] while sheath length is reduced when neural activity is inhibited via sensory deprivation during development [7]. These studies demonstrate myelin structural plasticity that correlates with neuronal activity during development and adolescence, and additional work highlights the importance of myelin alteration during motor learning [3]. Recent studies have revealed that the potential for myelin plasticity might be reduced in older animals [8]. We sought for the first time to examine whether activity dependent myelin plasticity could occur in the injured adult CNS. If so, driving neural activity to affect myelin structure might afford therapeutic benefit.

Demyelination following spinal cord injury (SCI) disrupts signal conduction and contributes to a loss of motor and sensory function, and thus is an important experimental model for the study of adult myelin repair [9], [10]. Previously, we showed that remyelination occurs extensively following SCI [11]–[13], but that full remyelination and sheath plasticity can take months to achieve. Furthermore, only spared axons undergo extensive and complete remyelination while severed fibers that are degenerating exhibit limited myelin repair [12], [13]. These results drove us to hypothesize that patterned neural activity could facilitate remyelination and directly affect myelin structure of spared axons. The goals of this study were to determine i)

if the adult injured environment retains the potential for activity dependent white matter plasticity observed in intact models and ii) if increasing activity in spared axons accelerates nodal and myelin remodeling and remyelination. To test the hypothesis that action potential conduction in spared axons is an important signal to induce remyelination following SCI, we electrically stimulated the corticospinal tract (CST) in rats following cervical level 4 (C4) injury. We chose to apply low frequency stimulation, as it has been shown to increase myelin thickness *in vivo* [6] and augment myelin production in *in vitro* experiments [14]. Axon and myelin plasticity were quantified in spared CST axons. We have previously shown spared axons of the lesion epicenter are extensively remyelinated with thinner myelin and shorter sheaths that expand over time [13], but that remyelination is confined to within several millimeters of the lesion [12]. With electrical stimulation, we observed myelin sheath remodeling outside of the lesion zone, where demyelination and myelin repair has not been thought to occur. These data imply that neuromodulation signals a requirement for altered conduction within a fiber tract at distance from the lesion, and they are the first to show extensive myelin remodeling over long-distances of descending axons induced by neuromodulation. A better understanding of activity-induced nodal and myelin plasticity is likely to have significant impact on our understanding of the potential for myelin repair in the injured nervous system.

2.3 Methods

2.3.1 *Animals*

The University of Washington Institutional Animal Care and Use Committee approved all procedures involving animals. A total of 14 adult female Long-Evans rats weighing between 275-350 grams were used in this study. Handedness for each rat was determined via a reaching task. Animals were kept on a 12-hour light/dark cycle, with *ad libitum* access to food and water.

2.3.2 *Surgical Procedures*

Animals were anesthetized with ketamine/xylazine (50 mg/kg and 1 mg/kg) and given injections of a local anesthetic (lidocaine at 1 mg/kg and bupivacaine HCl at 1 mg/kg) prior to an incision made at midline over the cervical spinal column. The cervical spinal cord on the side of their preferred paw was exposed under aseptic surgical conditions, and animals sustained a C4 hemi-contusion injury using an Ohio State Impact Device [15]. The probe was calibrated to cause a displacement of 0.7 mm, and injuries were verified by the appearance of a bruise on the surface of the cord. The injury site was covered with gel foam, the muscles were closed in layers with absorbable suture, and the skin was closed with nylon suture. Subcutaneous injections of lactated Ringers, Baytril injectable antibiotic (5 mg/kg), and buprenorphine slow release (1.2 mg/kg) were given, and the animals woke up in a warmed recovery cage. Animals were administered Baytril antibiotic in their water bottles (25 mg/kg) for 14 days. All animals exhibited a deficit of weight bearing and usage of the affected forelimb.

After two weeks of recovery, a sub-cutaneous injection of dexamethasone (0.2 mg/kg) was given to prevent brain swelling, and 24 hours later animals were anesthetized with 2% isoflurane in oxygen. Under aseptic surgical conditions the skull contralateral to the injury was

exposed. A craniotomy was performed at coordinates corresponding to the caudal forelimb motor area (Fig. 2.1A). Two injections of 1 µl of 10% solution of biotinylated dextran amines (BDA) were given at 1 mm depth in motor cortex via a Hamilton syringe. A stimulating electrode array (described below) was then implanted into cortex with the tips of the electrodes penetrating 1 mm. The craniotomy was filled with gel foam and the array secured with dental acrylic, creating a cap which sealed the incision. Stainless steel wires for electromyographic recordings (EMG) were implanted into the dominant forelimb's wrist flexors and extensors, triceps, and deltoid. Animals were given lactated Ringers and buprenorphine subcutaneously and recovered in a warmed cage. Antibiotics were delivered as described above.

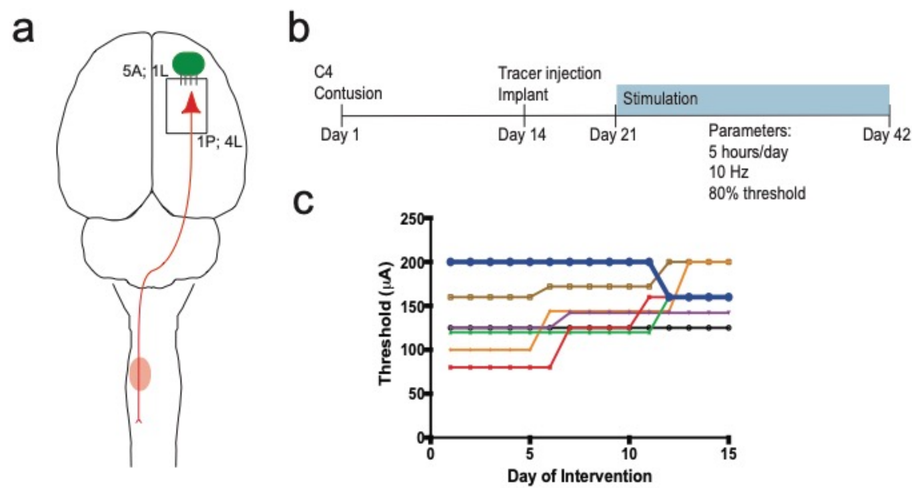

Figure 2.1 Experimental Design and Timeline.

A) Schematic of craniotomy coordinates and electrode placement, injury location, and CST. B) Timeline for experimental paradigm. C) Current required to evoke movement or evoked potential over 15 days of stimulation intervention; each trace represents an individual stimulated animal. Thresholds were assessed weekly, and daily stimulation currents were 80% of most recent threshold measurement.

2.3.3 *Stimulation*

Stimulating electrodes consisted of a 2x4 array of tungsten wires, with impedances less than 30 kOhm. The array was mounted onto a printed circuit board with a connector at the distal end. One week after electrode implantation, each rat underwent motor threshold testing, wherein each channel was tested for evoked forelimb movements and/or motor evoked potential (MEP) by delivering biphasic pulses at 10 Hz at currents ranging from 50-250 μAmps to each electrode independently. If visual confirmation of movement was equivocal, MEPs provided confirmation of muscle contraction. The channel that elicited the clearest movement or MEP at the lowest threshold was chosen as the stimulation channel for that animal for the remainder of the study; no other channels were stimulated. Threshold at the stimulating channel was tracked over the remainder of the study (Fig. 2.1C).

Each animal was kept in a Plexiglass arena for 5 hours each weekday, regardless of condition (stimulated or unstimulated). Animals in the stimulation group were connected with a long cable to the Neurochip, a brain-computer interface that delivered biphasic pulses to the chosen stimulation channel at an average rate of 10 Hz for 5 minutes on/2 minutes off cycles at 80% of movement/MEP current threshold. Frequency was modulated such that the average rate was 10 Hz as it mimics physiological conditions, as opposed to a precise rate of 10 Hz. Control animals were tethered without stimulation. For consistency and to avoid circadian effects, stimulation was always begun in the morning. Stimulation was administered for 5 days per week for 3 weeks (Fig. 2.1B).

2.3.4 *Behavioral Assessments*

Animals were trained on behavioral tasks to establish handedness and baseline scores of performance. Handedness was established primarily via the pasta-matrix reaching task, shown to

be sensitive to SCI deficits [16]. Animals also learned the horizontal irregular ladder walk to test gross locomotor and CST deficits [17]. Tasks are described in more detail in Chapter 3. Animals were assessed while untethered to the Neurochip three times per week on the ladder walk, and daily for the pasta matrix during cortical stimulation.

2.3.5 *Electron Microscopy Imaging*

A subset of animals (n = 6) were each given a lethal injection of Beuthanasia and perfused transcardially first with 1x PBS, and then with 2% paraformaldehyde and 2.5% glutaraldehyde. Spinal cords were extracted, and sections of the corticospinal tract rostral and caudal to the injury were removed and post-fixed in 3% glutaraldehyde / 1% paraformaldehyde in cacodylate buffer. These were sliced coronally on a vibrating microtome at 60 µm. The BDA was developed according to the manufacturer's recommendations (NeuroTrace BDA-10,000 Tracer Kit, ThermoFisher) and CST cross sections were imaged via transmission electron microscopy (EM).

G-ratio, the ratio of axon diameter to total fiber diameter, was determined using FIJI software and a specialized G-Ratio plugin [18]. To ensure unbiased sampling of axons in the image, the plugin randomly generates crosshairs on an image. If the crosshairs fell on an intact and healthy axon the user traced the axon border and the outer myelin border, and the plugin recorded the measurements and generated the G-ratio for the fiber. Personnel blinded to treatment group assessed the G-ratios in each image, and to ensure data quality and inter-rater reliability, on occasion two personnel would assess the same image, and comparisons between their measurements were made.

2.3.6 *Immunohistochemistry*

Animals used for immunohistochemistry (IHC) (n = 8) were perfused with 4% paraformaldehyde in PBS after Beuthanasia injection, and the spinal cords were extracted and post-fixed in 4% PFA. Tissue was serially substituted over several days in 10%, 20% and 30% sucrose in PBS. Spinal cords were trimmed to 1 cm rostral and 1 cm caudal to the injury and sliced horizontally on a sliding microtome at 80 μm.

Two slices per animal were used for IHC. The first section was identified by determining the most ventral section in which the CST could be anatomically verified by eye. The second slice used for IHC was the fifth section (400 μm) dorsal from the first. Thus, the CST was sampled at two locations in the dorsal-ventral axis. All IHC was conducted on free-floating sections. Spinal cord sections were blocked with 10% goat serum and 0.1% triton-X-100, and then they were incubated in 1:100 concentrations rabbit anti-CASPR (abcam) and Alexa-flour568 conjugated streptavidin (ThermoFisher) for 3 days at 4 °C, and in secondary antibodies (goat anti-rabbit Alexa-fluor488, ThermoFisher) overnight at 4 °C. Mosaic z-stacks taken rostral to the lesion, at the lesion, and caudal to the lesion were acquired on a Leica DMI8 confocal microscope running LASX. Axons and nodes were reconstructed in 3D using Neurolucida360 (MBF Bioscience) by investigators blinded to stimulation condition.

2.3.7 *Analysis and Statistics*

Measurements of axon and sheath length reconstruction were obtained with Neurolucida360 (MBF Bioscience). G-ratio measurements were conducted using Fiji. Statistical analyses, described in Results, were conducted on Prism7.

2.4 RESULTS

2.4.1 *Electrical Stimulation of Motor Cortex Evokes Movements in Forelimb Affected by SCI*

After undergoing C4 hemi-contusion injuries, all animals demonstrated a deficit of weight bearing on the dominant forelimb, as well as deficits on behavioral assays (Fig. 3.8 & Fig. 3.9). Animals were monitored on behavioral assays throughout the stimulation paradigm to assess functional recovery, with no difference between groups over the course of the experiment (Fig. 3.8 & Fig. 3.9). This deficit confirmed disruption of the CST affecting the dominant forelimb. Stimulation of single channels in the array implanted across caudal and rostral forelimb motor cortex (coordinates in Fig. 2.1A) evoked forelimb movements (either wrist flexion/extension, forelimb extension/retraction, or shoulder retraction) or stimulus evoked potentials of forelimb muscle EMG. The channel with the lowest current threshold to evoke movement was chosen as the stimulation channel for the remainder of the study, and thresholds to evoke movement on that channel were re-measured once a week. Stimulation currents during therapy were 80% of threshold and required nominal increases over the duration of the experiment for each animal (Fig. 2.1C).

2.4.2 *BDA+ Axons are Spared Axons of the CST and Have Multiple Nodes of Ranvier*

Spared axons visualized by BDA+ signal are found in the CST of both stimulated and control animals (example tiled image of ~2cm longitudinal section in Fig. 2.2A). Previously, we have shown that in the absence of an intervention spared axons following injury have shorter myelin sheaths only at the lesion [12]. To measure the effect of stimulation on white matter and myelination patterns distant from the lesion, axons were examined over 1000 µm tiled z-stacks 8-10 mm rostral (Fig. 2.2B) and 8-10mm caudal to the injury (Fig. 2.2D). In addition, axons

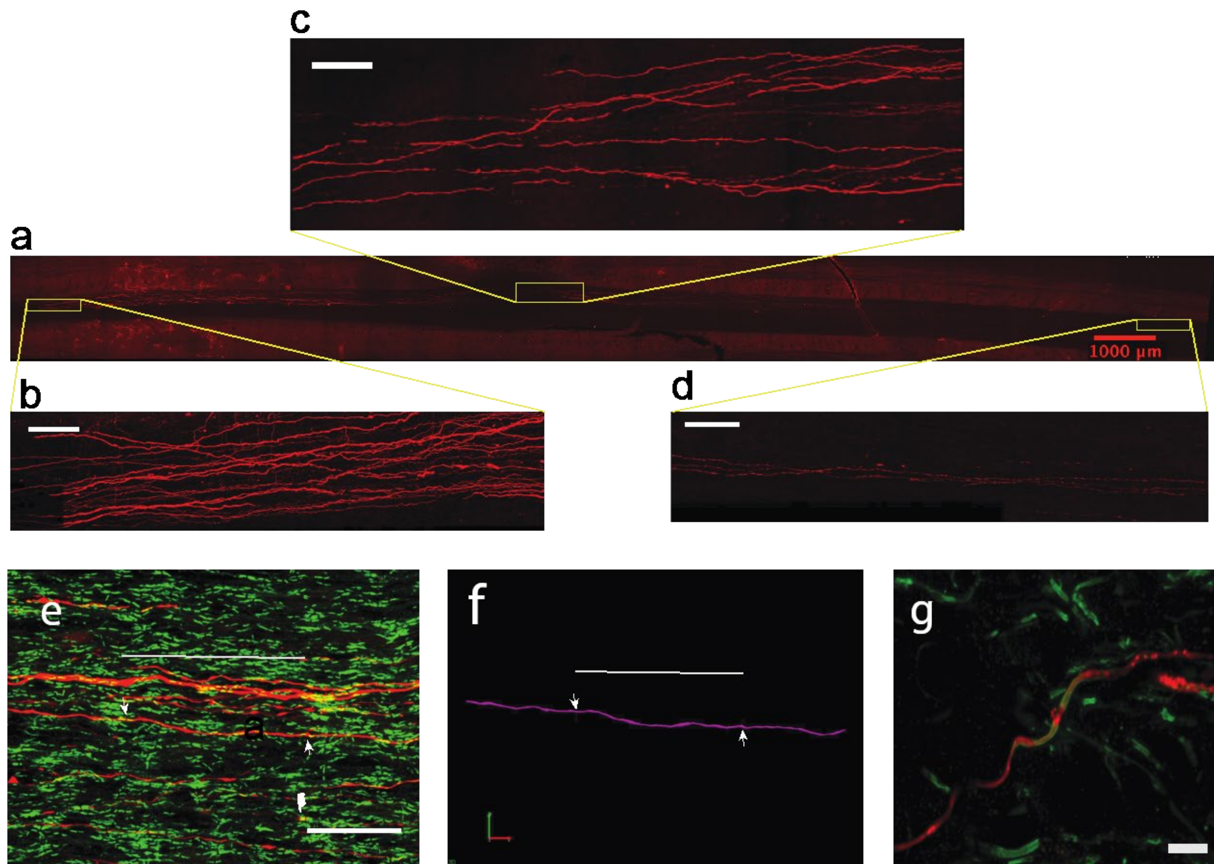

Figure 2.2 Example of BDA+ Axons and Nodes of Ranvier Within the CST Visualized in Longitudinal Sections

A) BDA+ axons are more abundant in the rostral compartment but are also present in the lesion epicenter and caudal compartment. B-D show higher magnification views in rostral, lesion, and caudal regions. E) Maximum intensity projection to visualize the protein CASPR (green) marking nodes of Ranvier (arrows) on BDA+ axons (red). An additional node is highlighted by pointing finger. F) Example tracing of axon and nodes highlighted by arrows in panel E for "node-to-node" assessment of internode length as well as axon diameter. G) Maximum intensity projection of CASPR (green) and BDA (red). Scale bars in A = 1000 µm, B-D = 100 µm, E = 50 µm, F = 5 µm

were examined at the lesion epicenter (Fig. 2.2C). Axons were only included in this analysis if they were BDA+ and did not end in retraction bulbs or dystrophic endings. Axons were traced until they left the imaging plane or could not be discerned due to proximity with adjacent axons.

In both stimulated and control animals nodes of Ranvier were identified by staining for CASPR, a paranodal protein which flanks the node of Ranvier (example tracing of BDA+ axons and nodes in Fig. 2.2E). Co-localization of CASPR and BDA allowed for identification of nodes on spared axons of the CST. Using 3D reconstructions of traced axons, the distances between node markers was determined to give a measure of myelin sheath length (Fig. 2.2F). To avoid biasing towards shorter sheath lengths, only axons that could be traced for > 350 µm were included in the analysis.

2.4.3 *Stimulated Animals Have Shorter Myelin Sheath Lengths Rostral and Caudal to the Lesion*

Previous studies in our lab showed that in the absence of an intervention following injury spared axons exhibit shorter sheath lengths at the lesion, but not rostral or caudal to the injury [12]. We expected that neuromodulation would affect myelin sheath length following injury, so we quantified sheath length rostral and caudal to the injury. We found significantly more short internodes 8-10mm rostral to the injury with stimulation (Fig. 2.3A control median = 197.9 µm n = 63 sheaths; stimulated median = 153.4 µm n = 111; Mann-Whitney $p = 0.0059$; $U = 2620$). Frequency distributions show an increase in the number of shorter sheaths in stimulated animals, and fewer sheaths over 200 µm (Fig. 2.3B). Though these populations were not normally distributed (both failed D'Agostino & Pearson normality test; control $p = 0.0205$, stimulated $p = 0.0027$), we were interested in the comparison of the means measured with and without stimulation to historical values previously published in our lab [12]. The mean sheath length in

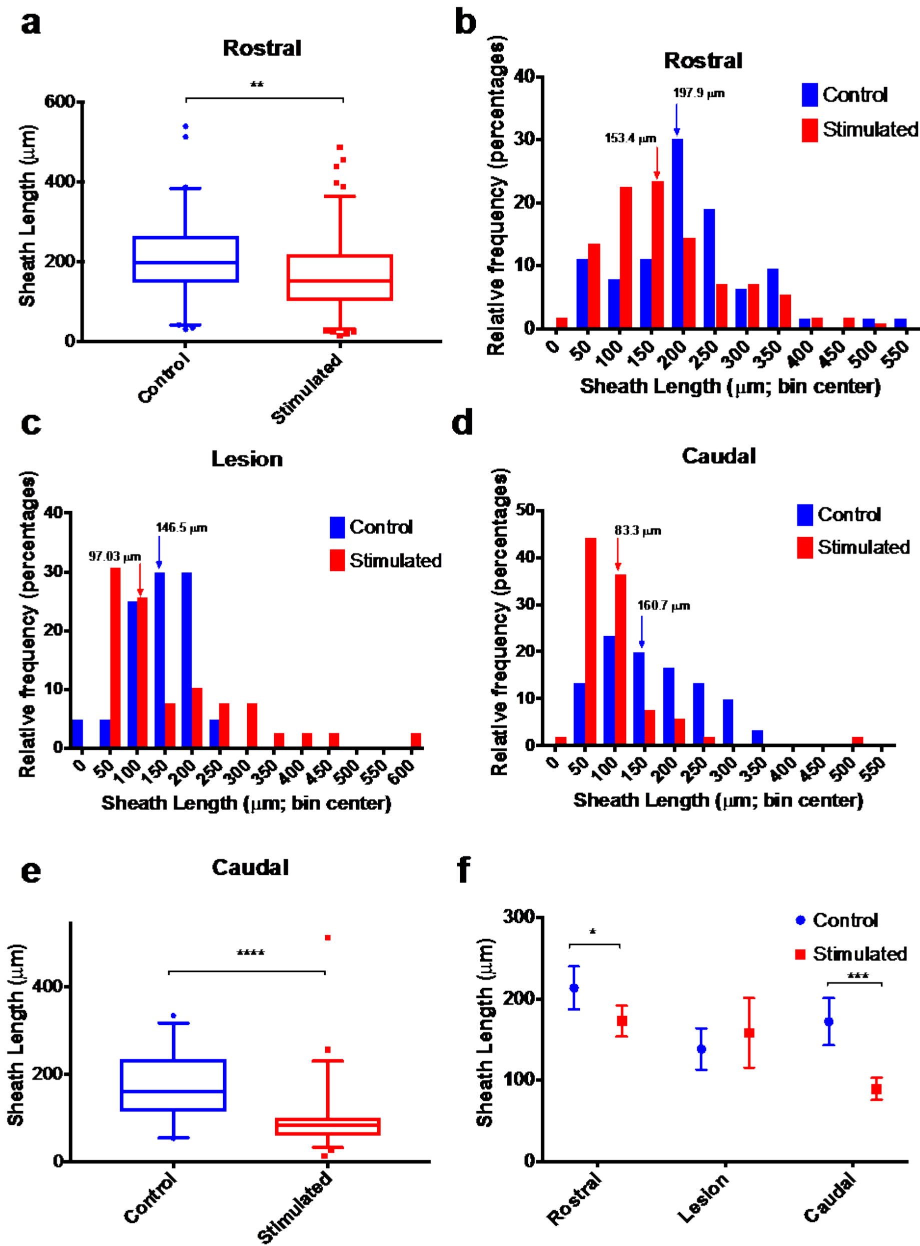

Figure 2.3 Myelin Sheaths are Shorter Rostral and Caudal to the Lesion with Stimulation

A) Boxplot of measured sheath lengths in rostral compartment shows shorter median length in stimulated animals; ** $p = 0.0059$. B) There is an increased incidence of shorter myelin sheaths rostral to the lesion epicenter. C) No clear difference in the median sheath length observed for the population of spared axons at the lesion epicenter. D) An increased incidence of shorter sheaths is seen caudal to the lesion epicenter, though there are fewer BDA+ axons present. E) Boxplot of sheath lengths caudal to the injury shows a shorter median sheath in stimulated animals; **** $p < 0.0001$. F) Mean sheath length at each compartment; stimulated animals display shorter sheaths on BDA+ axons in both the rostral and caudal compartment. Error bars represent 5-95% percentile. * $p = 0.0215$ *** $p = 0.0006$. In 3A and 3E box plots represent medians and quartiles and error bars represent 5-95% percentile intervals. Colored arrows in 3B, 3C and 3D illustrate median for control (blue) and stimulated (red) animals.

stimulated animals rostral to the injury was significantly shorter than control animals (Fig. 2.3F; 2-way ANOVA with Sidak's multiple comparisons test; stimulated mean = 172.5 µm vs. 213.4 µm in control; p = 0.0215). These current measurements of 213.4 µm in injured unstimulated control animals corroborate previous work from our lab that showed that the mean sheath length in uninjured CST axons was 207.2 µm [12]. These similar measurements support the findings that in the absence of an intervention, farther than 3mm from the injury there are no differences between injured animals and control animals [12].

We targeted the BDA injection to coordinates of forelimb motor cortex (Fig. 2.1A), and forelimb motor circuits in the spinal cord are mainly in the cervical cord [19]. In addition, we utilized an injury paradigm known to disrupt function of the CST [20]. Thus, fewer BDA+ axons were found caudal to the lesion than rostral, as can be seen in Figure 2.2A. We assessed sheath length in the same manner as in the rostral compartment on spared axons through the lesion epicenter. We found that stimulation did not affect myelin sheath length on spared axons through the lesion (Fig. 2.3C, F); there was no statistically significant difference between the two populations (stimulated population failed D'Agostino & Pearson normality test p = 0.0004 while control population passed p = 0.4853; control median = 146.5 µm vs. stimulated median = 97.03 µm; non-significant by Mann-Whitney p = 0.7091; control mean = 138.2 µm for 20 sheaths vs. stimulated mean length = 158.1µm for 39 sheaths). Though it is important to consider the relatively small numbers of sheaths, these results imply stimulation does not affect the remyelination effort at the lesion epicenter, though there does appear to be a trend of an increased frequency of longer sheaths in stimulated animals (Fig. 2.3C).

To assess the effect of stimulation on myelination patterns in more distal sections of the cord we quantified sheath length 8-10mm caudal to the lesion epicenter, and found that similar to

the rostral compartment, there were more shorter sheaths with stimulation (Fig. 2.3D). The median length was statistically significantly lower in stimulated animals than control (Fig. 2.3E; stimulated distribution failed D'Agostino & Pearson normality test $p < 0.0001$, control passed normality test $p = 0.4749$; Mann-Whitney $p < 0.0001$ for median of 160.7 µm for 30 sheaths in control vs. median of 83.33 µm for 52 sheaths in stimulated; $U = 298$). Though the distributions were not all normal, we wanted to compare the mean sheath length to historical values, and found the stimulated mean was significantly shorter (Fig. 2.3F; control mean sheath = 172 µm vs stimulated mean = 97.48 µm, $p = 0.0006$, 2way ANOVA with Sidak's multiple comparison test). The value of 172 µm for the mean sheath length seems lower than our historical value for the mean sheath length of 207.2 µm.

Distributions of sheath lengths seemed consistently skewed towards shorter sheaths for all compartments in the stimulated animals (Fig. 2.3B, C & D; skewness = 0.817 for rostral; skewness = 1.434 for lesion; skewness = 3.576 for caudal). These values provide support for the argument that stimulation induces a bias in the population toward shorter sheaths.

2.4.4 *Cortical Stimulation and Myelin Thickness*

To quantify the effect of stimulation on myelin thickness, we assessed cross-sectional EM images to measure the G-ratio of healthy fibers in the CST. The G-ratio is a measure of myelin thickness which accounts for axon diameter; it is the axon diameter divided by the total fiber diameter. Only healthy-appearing axons without irregular borders were included (example Fig. 2.4A). G-ratios below 0.45 were excluded from the analysis, as they were most often found caudal to the injury and likely represent a pathological fiber, even if the axon seemed intact (example Fig. 2.4B). In the rostral compartment, two values were omitted in the control groups and five values in the stimulated group for falling below the 0.45 threshold. In the caudal

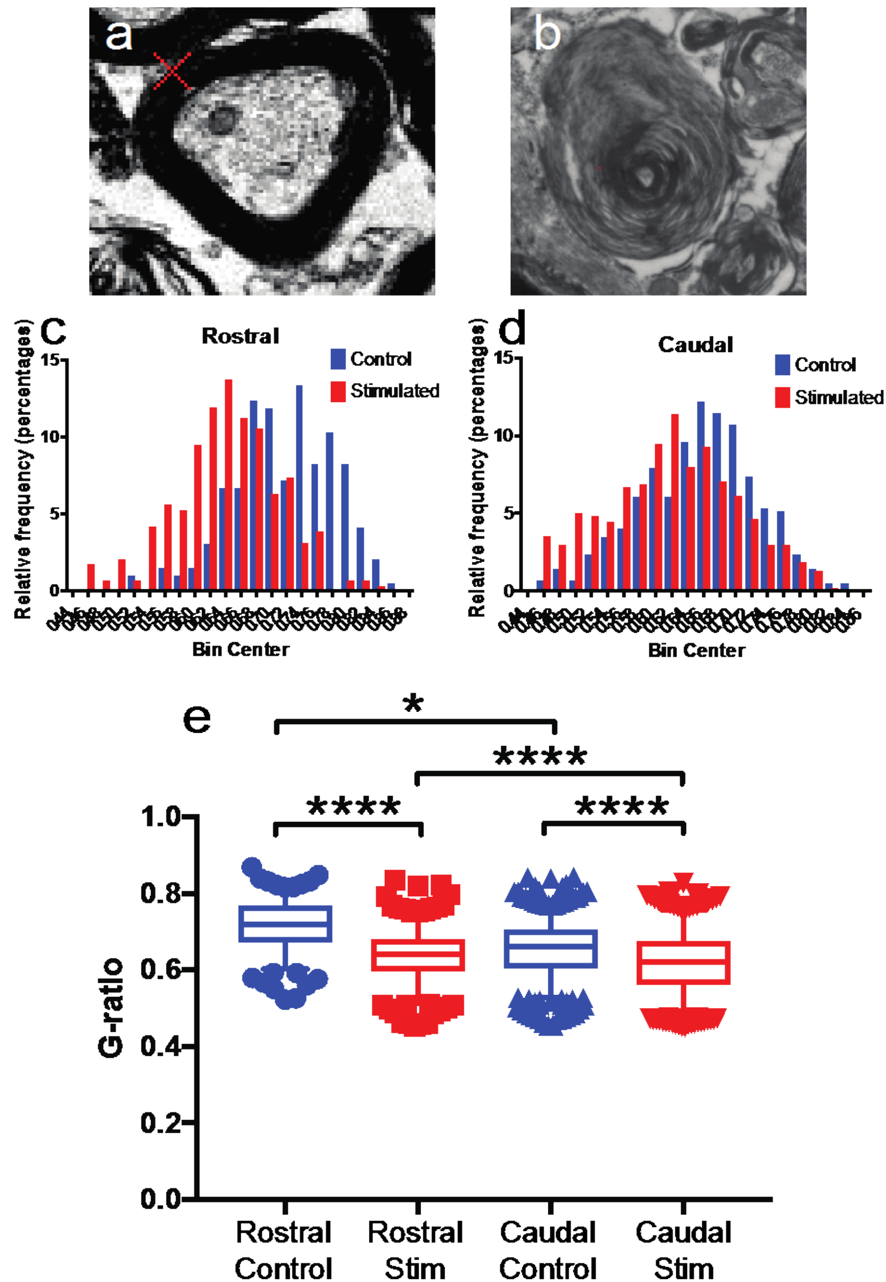

Figure 2.4 Lower G-ratios in Stimulated Animals

A) Example of a healthy myelinated axon used for G-ratio; not to scale with B) Example of fiber excluded for G-ratio below threshold of 0.45. Frequency distribution of G-ratios in control and stimulated animals C) one centimeter rostral to the lesion and D) one centimeter caudal to the injury. E) Comparison of medians between stimulated and control shows lower G-ratios in stimulated animals in both the rostral and caudal compartment. In addition, within each group G-ratios were lower in the caudal compartment than the rostral. Box-plots represent medians and quartiles and error bars represent 5-95% intervals; * $p = 0.0118$; **** $p < 0.0001$.

compartments, 27 values fell below the 0.45 threshold in the control animals, and 72 values were below the threshold in the stimulated animals.

One centimeter rostral to the lesion stimulated animals had lower G-ratios, which corresponds to thicker myelin for a given axon diameter (Fig. 2.4C, 2.4E; control median = 0.7185 for 194 fibers vs. stimulated median = 0.6399 for 284 fibers; Kruskal-Wallis with Dunn's multiple comparisons test $p < 0.0001$). In the rostral compartment the populations were normal (D'Agostino & Pearson normality test, $p = 0.1178$ for control, $p = 0.5067$ for stimulated), but we used non-parametric analyses because in the caudal compartment the stimulated population was non-normal (D'Agostino & Pearson normality $p = 0.0007$) though the control population was normal (D'Agostino & Pearson normality $p = 0.0722$). The same phenomenon of a lower G-ratio in stimulated animals was observed one centimeter caudal to the lesion (Fig. 2.4D, 2.4E, control median = 0.661 for 541 fibers vs stimulated median of 0.622 for 536 fibers Kruskal-Wallis with Dunn's multiple comparisons test; $p < 0.0001$).

Within each group (stimulated or control) there were a lower G-ratio in the caudal compartment than the rostral compartment (Fig. 2.4E; Kruskal-Wallis with Dunn's multiple comparisons test; stimulated rostral vs. caudal $p = 0.0118$; control rostral vs. caudal $p < 0.0001$).

Though animals used for EM were also injected with BDA, there were few axons that were BDA+. Since EM allows for visualization of every axon the G-ratio analysis reported above was computed on axons regardless of BDA status. Repeating the analysis on only BDA+ axons with G-ratios greater than 0.45 revealed there was no statistically significant difference in the rostral compartment with stimulation (data not shown; median for control = 0.601 for 21 fibers; median for stimulated = 0.67 for 10 fibers; Mann-Whitney $p = 0.3126$). There was also no difference between control and stimulated BDA+ axons in the caudal compartment (data not

shown; control median = 0.609 for 49 fibers vs. stimulated median = 0.596 for 141 fibers; Mann-Whitney p = 0.3219).

2.4.5 *Cortical Stimulation and Axon Morphology*

Myelin sheath length correlates positively with axon diameter in both injured and control animals [11]. Stimulation may also induce thinning of axons [6]. Consequently, we were interested in exploring the effect of cortical stimulation on CST axon diameters following injury, particularly in light of our finding of shorter myelin sheaths. As mentioned above, only BDA+ axons that remained in plane and did not end in dystrophic bulbs were used for this analysis. As a consequence of applying these criteria for axon selection for sheath analysis, not all axons in an image were measured, creating a sub-population of axons. In this sub-population of axons in the rostral compartment there were more thinner caliber axons with stimulation (Fig 2.5A) and the shift in the distribution towards more frequent occurrences of small caliber axons can be seen in Fig. 2.5B (Kolmogorov-Smirnov test to compare distributions, p = 0.0219; control median for 55 axons = 1.24 µm vs. stimulated median for 78 axons = 0.995 µm). Previously we reported the average uninjured CST axon as 1.379 µm [12]. This value in the uninjured animal is in agreement with the average diameter measured in the control injured unstimulated axons in this study (mean = 1.305 µm) while the average axon diameter in stimulated axons was 1.139 µm. There was a small number of axons at the lesion, but within this small sample there was no difference in the distribution or median axon diameter (Fig. 2.5C, Fig. 2.5D, stimulated median = 1.34 for 19 axons vs. control median of 1.076 for 7 axons; Kolmogorov-Smirnov p = 0.2850). The axons measured for the sheath analysis in the caudal compartment were also statistically significantly smaller in stimulated animals (Fig. 2.5E; Kolmogorov-Smirnov test, p = 0.0004; control median = 0.98 µm for 12 axons vs. stimulated median = 0.735 µm for 16 axons). The

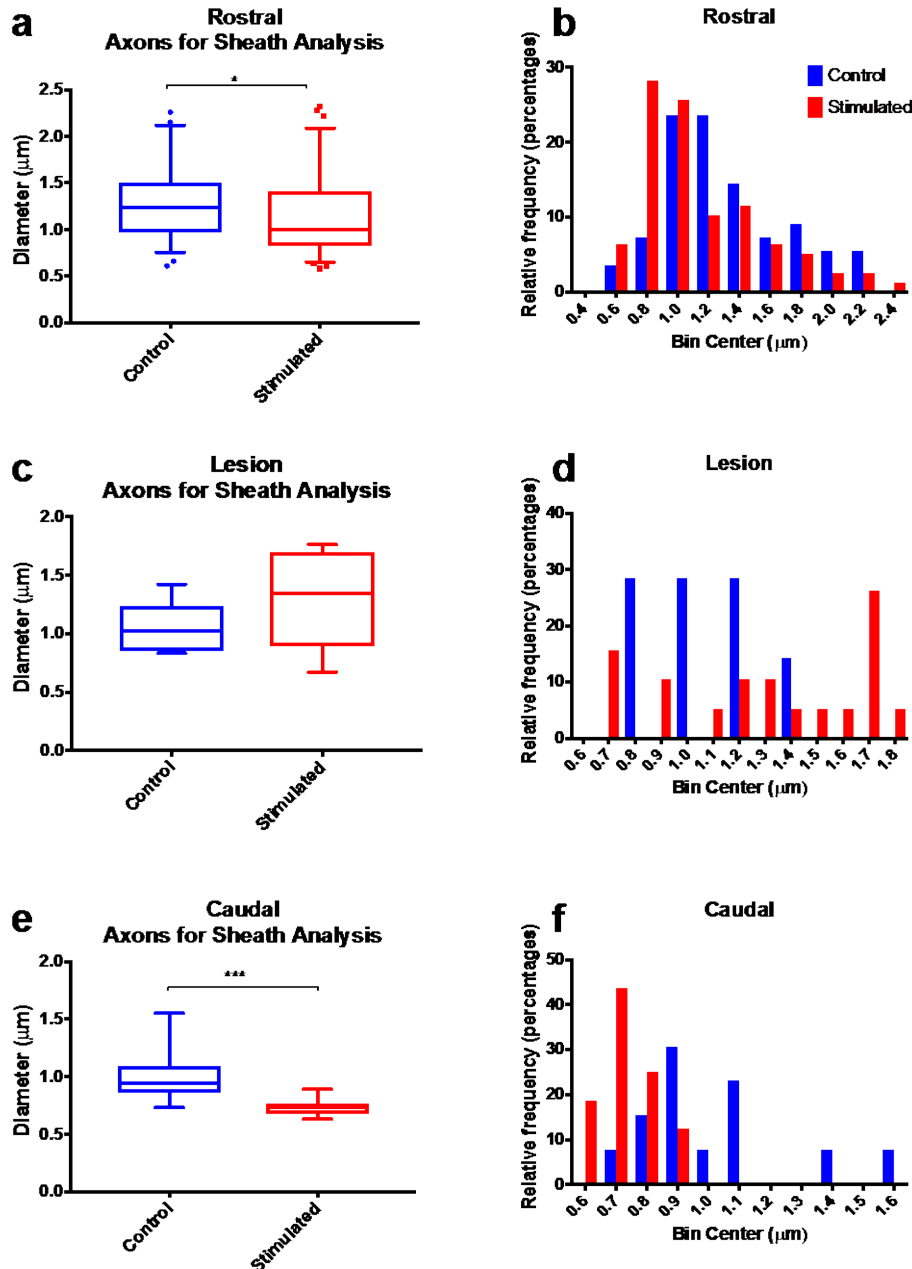

Figure 2.5 BDA+ CST Axons from Sheath Analysis are Thinner with Stimulation

A) The sub-sample of BDA+ axons used for sheath length analysis in the rostral compartment demonstrate a shift towards smaller caliber axons * p = 0.0219. B) A shift in the frequency distribution towards lower caliber axons. C) There is no difference between the control and stimulated axons at the lesion. D) A slight and non-significant trend towards higher caliber axons in stimulated animals at the lesion. E) In the caudal compartment axons used for the myelin sheath analysis are thinner in stimulated animals than control *** p = 0.0004. F) The frequency distribution of axons measured for sheath analysis in the caudal compartment shows an increase in the occurrence of low-caliber axons with stimulation. Boxplots represent medians, quartiles, and 5-95 percentiles.

frequency distribution illustrates the shift towards more small caliber axons in stimulated animals and more high caliber axons in control animals (Fig. 2.5F).

Electrically stimulating the injured CNS can invoke neural plasticity and axon sprouting [21]. We were curious to see if the stimulation paradigm invokes axon plasticity so we assessed whether the axons used for the sheath analysis branched, and whether stimulation might influence this outcome. Each axon used in the sheath analysis was classified as branched (example green and yellow traces in Fig. 2.6A) or unbranched (rose trace in Fig. 2.6A). To test if the shorter sheaths described in Fig. 2.3 were in part due to shorter sheaths found on branches, we assessed the sheath lengths only found on primary axons of passage (as opposed to secondary branches, seen in green and yellow traces in Fig. 2.6A). The median sheath on primary branches in the rostral compartment of stimulated animals was significantly shorter than in control animals (median stimulated sheath length = 171.8 µm for 89 sheaths vs. median control sheath length = 209.7 µm; Mann-Whitney $p = 0.0071$ $U = 1795$). We found that in the rostral compartment only, stimulation was related to the likelihood of branching (Fig. 2.6C; Fisher's exact test; variable stimulation, outcome branched or unbranched, $p = 0.0490$). Stimulation did not affect branching at the lesion (Fig. 2.6D) or in the caudal compartment (Fig. 2.6E).

2.4.6 *Diameter Confirmatory Analysis*

We were curious if the shift in the distribution of axon diameters reflected in the axons used for sheath analysis (Fig. 2.5) was reflected in the entire population of axons, including those that did not meet our criteria for analysis. For this reason, we measured the diameters of all discernible BDA+ CST axons in the rostral compartment only, because the caudal compartment sheath analysis typically included all the axons discernible in an image. To ensure an accurate

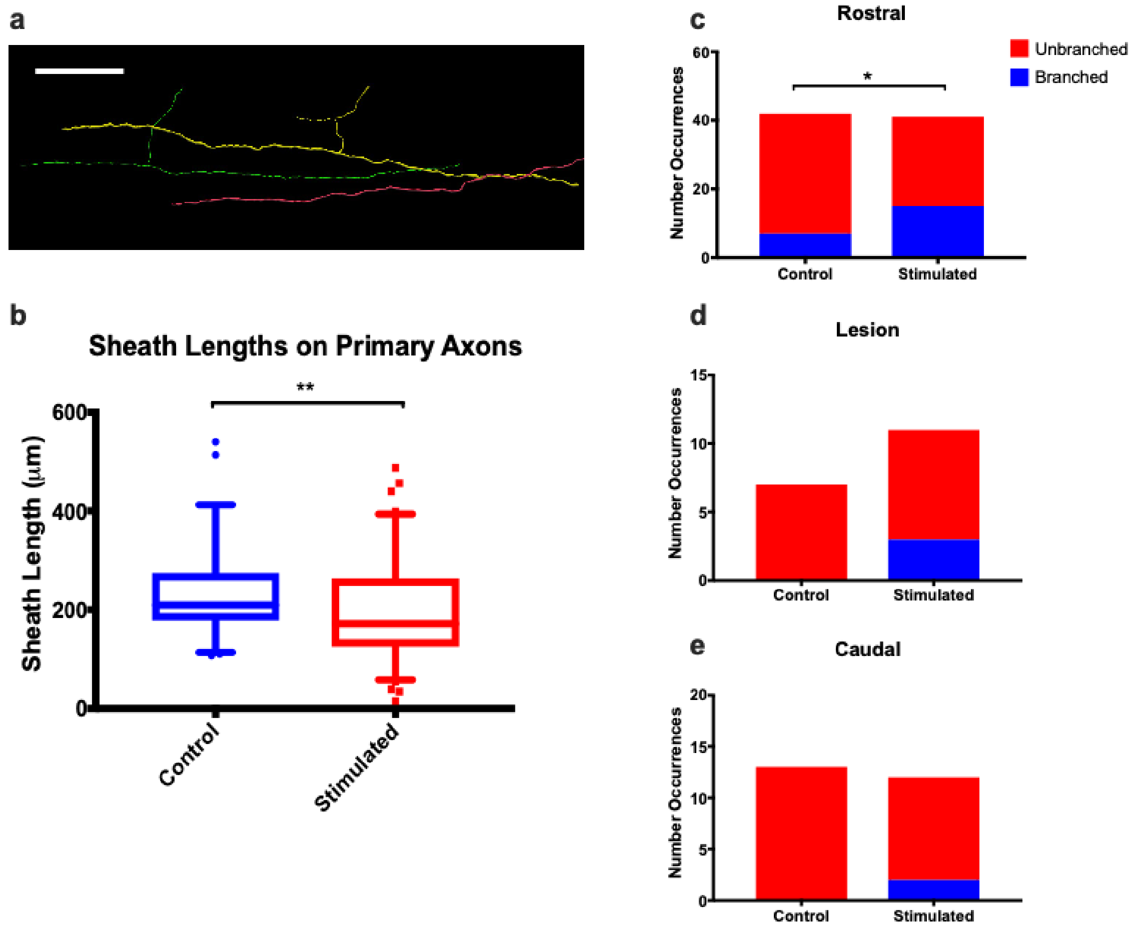

Figure 2.6 Electrical Stimulation Affects Axonal Branching Rostral to the Lesion

A) A sample trace exhibiting three axons; yellow and green traces illustrate axons with branches off the primary axon of passage, while rose axon does not branch. Scale bar = 100 µm. B) Sheath lengths on all primary branches of axons measured in the rostral compartment are shorter with stimulation than unstimulated animals ** $p = 0.0071$. Boxplots represent medians, quartiles, and 5-95% percentiles. C) Stimulated animals were more likely to exhibit branched axons than control animals in the rostral compartment * $p = 0.0490$. D) There was no effect of stimulation on branching at the lesion or E) caudal to the lesion.

measurement, we conducted repeated measurements in each animal's rostral compartment by measuring every axon intersected by an orthogonal plane at three locations (example of a single plane in Fig. 2.7A). The result of this analysis was three discrete measurements of axon diameter for each image, each reflecting a sub-population in the rostral-caudal axis. It is possible that axons exiting and entering the image might cause these sub-populations to be different. However, we found that there was no difference within each animal between the three populations' diameters (data not shown), confirming we had accurately assessed the diameters of the whole population of BDA+ axons. The sub-sample with the most axons was used to compare the diameters of the whole population of BDA+ axons. In this whole population there was a slight but statistically significant thinning of axons following stimulation (Fig. 2.7B; control median = 0.98 μm for 570 axons vs. stimulated median = 0.92 μm for 289 axons; Kolmogorov-Smirnov test, $p = 0.0473$). Thus, both the sub-population used for sheath analysis and the whole population of BDA+ axons seemed in agreement that stimulated animals exhibited thinner BDA+ axons than control.

The final method we used to quantify the diameter of axons of the CST was to extrapolate from the values for axon area obtained from the cross-sectional G-ratio analysis. Within the rostral compartment in the EM analysis, in contrast to the longitudinal analysis, stimulated axons were lower caliber than in control animals (Fig. 2.7C; control median = 0.6942 μm for 196 axons vs. stimulated median = 0.8586 for 289 axons; Kolmogorov-Smirnov test $p < 0.0001$). However, within the BDA+ population there was no statistically significant difference (Fig. 2.7D; Kolmogorov-Smirnov test $p = 0.0966$; median for 22 axons in control = 0.6118 μm; 11 axons in stimulated median = 0.8899 μm). The same analysis in the caudal compartment corroborated the findings from the fluorescence longitudinal analysis; the median diameter for

the stimulated animals was lower than the control (Fig. 2.7E; control median = 0.9319µm for 537 axons vs. stimulated median = 0.7408 µm for 555 axons; Kolmogorov-Smirnov $p < 0.0001$) though this effect was not present when considering only BDA+ axons (Fig. 2.7F; median for 60 control axons = 0.6382 µm; median for 165 stimulated axons = 0.6333 µm; Kolmogorov-Smirnov test $p = 0.8232$).

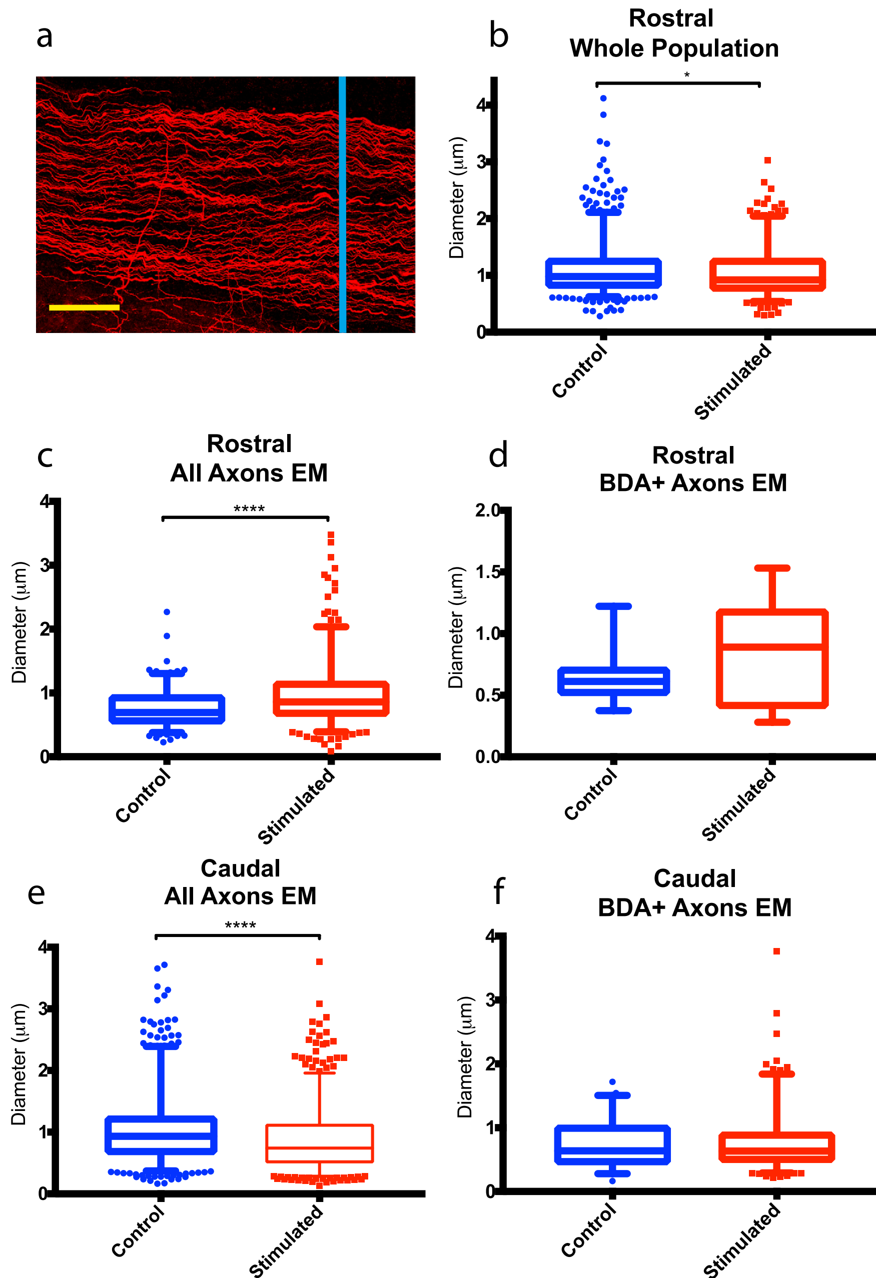

Figure 2.7 Repeated Measurements of Axon Diameter are Not Consistent

A) Example of plane intersection analysis repeated for assessing diameter throughout the compartment. Blue bar represents the plane dissecting the tiled z-stack. The diameter of each axon intersected by the plane is recorded to give an assessment of the diameters of the entire BDA+ population. Yellow scale bar = 100 μm B) The whole population of BDA+ axons assessed via plane analysis in the rostral compartment. There is a slight but statistically significant shift towards smaller caliber axons in the stimulated population; * $p = 0.0473$. C) Diameter of all axons used for the G-ratio analysis in the rostral compartment; **** $p < 0.0001$. D) BDA+ axons in the rostral compartment used for G-ratio analysis; not significant $p = 0.0966$. E) Diameter of all axons in the caudal compartment used for the G-ratio analysis; **** $p < 0.0001$. F) Diameters of BDA+ axons in the caudal compartment; not significant $p = 0.8323$.

2.5 DISCUSSION

The present study investigated neural activity dependent myelin plasticity following injury. Though there is evidence that altered neural activity patterns can alter myelination in an intact system [5] [6], this is the first in depth analysis of the effects of altered activity on myelin in an SCI model. Furthermore, this study used mature rats, building on studies conducted in developmental or young adult animals studying myelin changes following sensory deficits [7], altered social environments [22], or increased neural activity [5]. We show that the injured adult nervous system retains the capacity for activity dependent myelin plasticity and that local stimulation has the power to impact myelination patterns at distances centimeters away.

Cortical Stimulation Alters Post-SCI Myelin Morphology Rostral and Caudal to the Lesion

Animals that experienced CST stimulation demonstrated shorter myelin sheaths in the CST than those with injury alone. This finding was observed above and below the lesion, but not at the lesion epicenter (Fig. 2.3). In the absence of stimulation, shorter myelin sheaths are confined to the lesion epicenter [12]. Here, we show that stimulation induces remodeling of the entire tract, as regions rostral and caudal to the epicenter exhibit altered sheath length. Previous work in intact animals has shown relatively short-range morphological effects of stimulation on white matter morphology by stimulating and measuring within the brain (e.g. [5], [6]); the results reported here provide the first evidence of activity induced large-scale myelin modifications.

There is some disagreement in the literature about the effect of neuromodulation on sheath length. For example, depriving optic nerve axons of sensory stimuli during development caused shorter sheath lengths [7]. Intriguingly, in the peripheral nervous system, electrical stimulation induced phosphorylation of axonal neurofilaments [23], and phosphorylated neurofilaments lead to decreased internode length [24], which corroborate the present findings. It

remains to be seen whether the same mechanisms that govern internode length in the periphery will apply in the CNS. In developing zebrafish, the rate of calcium currents in oligodendrocytes dictates sheath length, and sheaths shortened with low frequency currents [25]. It is unclear whether developmental myelination is a similar process to remyelination, nor whether remyelination following injury is a process akin to normal myelin plasticity. Future work is necessary to determine exactly which mechanisms govern myelination under different circumstances.

The phenomenon of increased activity inducing thicker myelin has been documented before [5], [6]. Our finding of activity dependent thickening of myelin on stimulated axons both above and below the injury (Fig. 2.4C and Fig. 2.4D) confirms that activity dependent thickening of myelin occurs in the injured system. Interpreting this finding becomes more nuanced when we consider axon diameter. For two axons with the same diameter, the G-ratio will be lower on the axon with more myelin (i.e. the thicker sheath). However, if the same number of myelin wraps are deposited on two axons with different diameters, the G-ratio will be lower on the thinner axon. Thus, we must consider G-ratios in light of the axon diameter data (described in more detail below). It is worth noting that the median G-ratio in the rostral compartment (0.6399) is comparable to the value of 0.65 reported in the intact CST [26]. Further work will elucidate how modifications of myelin thickness and axon diameter interact with other mechanisms of fine-tuning conduction (such as node size and sheath length) to re-establish appropriate conduction following injury and stimulation.

CST Stimulation and Axon Morphology

Our results regarding axon diameter were discordant in the rostral compartment depending on method of assessment (fluorescent BDA signal; Fig. 2.5A vs. EM 2.7C, 2.7D). It

is important to examine these results in light of the strengths and limitations of each method. The BDA signal in the longitudinal fluorescence images is not actually a measurement of axon diameter; rather, it conveys fluorescence signal emitted by BDA within the axon. EM images allow for precise measurements of the axon membrane itself. However, the diameters were derived from axon area using the equation for the area of a circle, and thus are less accurate in axons that are not circular in cross section. Thus, diameter derived from EM is not directly comparable to diameter derived from fluorescence images, and both must be interpreted carefully. Nonetheless, the overall trend (i.e. increase or decrease in diameter) should be consistent in each method. Our results showed that BDA+ axons in the rostral compartment had smaller diameters with stimulation when measured by fluorescence imaging (Fig. 2.5A and Fig. 2.7B) though BDA+ axons as measured by EM did not differ in diameter with stimulation (Fig. 2.7D). An analysis of axons found in the CST on EM, regardless of BDA status, found axons in the rostral compartment were larger in stimulated animals (Fig. 2.7C). There are several important caveats to consider when interpreting these results. The axons in the EM images were not as strongly labeled with BDA as in the fluorescence images, resulting in a much smaller number of axons in the EM BDA+ diameter analysis (22 axons in control vs. 11 in stimulated). In addition, it may be that the population of axons captured by BDA in fluorescence images is not representative of the whole population. The EM analysis may have captured an additional BDA- population of axons, which might not have been stimulated, and were higher caliber. There were also technical issues with the rostral EM analysis, resulting in fewer fibers being measured (196 in control and 289 in stimulated). Thus, the diameter analysis should be interpreted carefully, and future work is needed to elucidate the effect of CST stimulation on

axon diameter following injury. Of note, the caudal compartment showed smaller caliber axons in the stimulated animals on both EM and fluorescence analysis.

Given these caveats and limitations, we must interpret the diameter results in the present analysis carefully, but a recent study establishes a precedent for thinning of axons following stimulation [6]. Furthermore, axons in the injured cord are thinner than in uninjured animals [12]. It may be that the stimulation and injury had a compounding effect, causing the thinning of axons throughout the CST (Fig. 2.5). In addition, there may also be an effect on axon caliber related to myelin or myelin plasticity. In the peripheral nervous system myelination or dysmyelination can affect axonal structure and morphology [27]; it may be that more short internodes exerts a constricting effect, or that axon caliber is altered as another means to control the signal conduction speed. In support of that latter possibility, axons of hippocampal neurons in culture increase in diameter over the course of an hour following a brief burst of 100 Hz frequency stimulation [28]. It may be that low frequency stimulation had an inverse effect in CST axons. Altogether, the literature supports the concept of thinned axons with shorter myelin sheaths with injury and stimulation, but future work is needed to confirm the phenomenon and explore the mechanisms affecting axon diameter.

The other intriguing finding related to axon morphology following stimulation was the induction of branching in the rostral compartment (Fig. 2.6C). In the uninjured mouse stimulation induced branching in interneurons [29] and electrical stimulation is used to invoke axonal sprouting following injury [21]. This finding raises an interesting concept both in terms of myelin plasticity and regeneration: to what extent is myelin plasticity also reliant on plasticity of the underlying axonal circuitry? This question is of particular importance in the regenerating

nervous system, where axonal sprouting may form new synapses to re-form circuits for motor and sensory recovery.

Remodeling vs Replacement

We cannot definitively ascertain whether the shorter sheaths observed in this study were a result of full demyelination followed by remyelination or extant myelin that was remodeled. However, short internodes are thought to be hallmarks of new myelin following injury [12], [30]. OPCs form new myelin sheaths following injury, and surviving oligodendrocytes do not contribute to remyelination efforts [31]. Work showing that pyramidal tract stimulation induces OPC proliferation provides evidence for a population of new oligodendrocytes to form new myelin [32]. Thus, there is evidence to support the conjecture that the short myelin observed distant to the lesion is new sheaths formed by new oligodendrocytes. One contrary possibility to note, though, is that remodeling of extant nodes has been described in the CNS [33]. Work in zebrafish has shown that oligodendrocyte calcium currents may be necessary to maintain sheath length, and calcium current oscillation at lower frequencies can cause sheaths to shorten [25]. Furthermore, a recent study described astrocytic regulated shortening and thinning of extant myelin sheaths [34], providing a mechanism by which existing myelin might be remodeled. These two studies reveal potential mechanisms by which the morphology of extant sheaths is altered based on external influences. The tenet that post-mitotic oligodendrocytes do not contribute to remyelination was derived from studies that focused on the lesion; myelin dynamics have not been observed or studied several millimeters away from the epicenter. Future work will need to definitively identify whether extant oligodendrocytes and sheaths are fully replaced or undergo remodeling in response to neuromodulation in a region distant from insult.

Potential Functional Consequences of Altered Myelin and Axon Dynamics Following Injury and Stimulation

We anticipated that electrical stimulation would induce the formation of longer internodes at the lesion epicenter, as activity dependent myelin plasticity might accelerate remyelination that typically takes months [13]. Consequently, our findings of shortened internodes at distance from the lesion and no difference in sheath length at the epicenter were surprising. Instead, these data imply that stimulation affected the tract as a whole, rather than the lesion, as reflected by thicker and shorter sheaths, potentially thinner axons, and increased axonal branching 8-10mm from the lesion. The overall effect of such morphological modifications might be the slowing of action potential conduction velocity. If stimulation post-injury induces remodeling and formation of circuits, the circuit might require slower conduction times than the intact or unstimulated injured cord. A model which explains a potential mechanism by which remodeling of white matter might contribute to functional recovery following injury is shown in Fig. 2.8.

This possibility brings up the interesting and yet unanswered conundrum of how improper action potential timing at the terminal is detected and conveys a requirement for a shorter myelin sheath millimeters away? This line of questioning is sure to yield exciting studies in the future. In addition, given our previous work showing that myelin continues to remodel over time after injury [13] it will be interesting to track activity dependent white matter plasticity for longer. Will the sheaths stay short several months after injury or persist if stimulation is terminated? Will the branches be pruned, or form functional synapses and affect recovery? The question of persistent morphological effects is relevant for functional recovery and rehabilitation from injury.

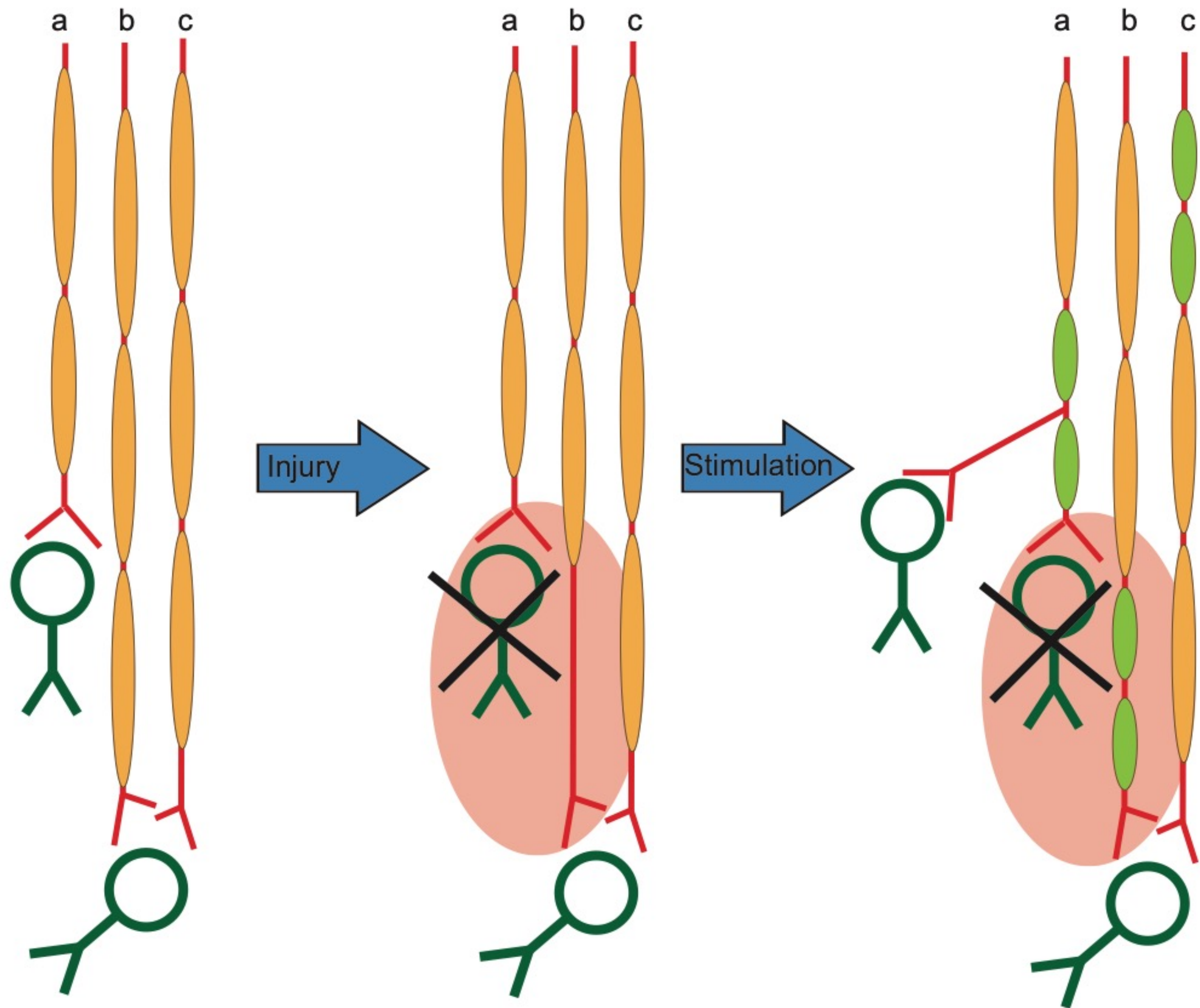

Figure 2.8 Model of Potential Functional Adaptation Induced by Stimulating Injured Axons

Myelinated axons A, B, and C are CST axons affecting motor outputs. Following injury, axon A is spared, but its post-synaptic contact has been killed. Axon B and C are both spared, but axon B is partially demyelinated. Consequently, signal from axon B arrives later than signal from axon C, which is still fully myelinated. Stimulation induced morphological changes demonstrated in the current study, including thinner axons and shorter sheaths, could slow conduction, which might serve to re-establish appropriate timing in a remodeling or regenerating circuit. Stimulation induced branching is seen in axon A, as it targets a viable post-synaptic partner; new short myelin sheaths (in green) might enable appropriate timing of signal to the new target. As axon B begins to remyelinate at the lesion (new short myelin sheaths in green), axon C might adjust its myelination profile to match the new signaling speed of axon B to re-achieve coincident timing on the post-synaptic partner.

The low number of axons and sheaths measured in the caudal compartment means that any interpretation of those results must be done with caution. However, it is worth noting that the white matter above the injury appears different from below the injury. For example, both stimulated and control animals exhibited smaller G-ratios caudal to the injury than rostral (Fig. 2.4E) and a trend towards shorter myelin sheaths (Fig. 2.3F). Though outside the scope of this analysis, it is intriguing to consider how the different populations of axons below and above the injury might affect the potential for activity dependent white matter plasticity. For example, axons caudal to the injury may be spared, but they may exhibit different morphology or physiology due to their passage through the lesion.

Furthermore, the non-significant trend for longer internodes at the lesion epicenter (Fig. 2.3C) is intriguing. An additional study designed to increase axon labeling at the lesion epicenter (by injecting higher volumes of BDA to label more axons and using a more moderate injury) with the same stimulation paradigm would enable a higher powered description of activity dependent myelin dynamics at the lesion.

Caveats and Future Work

This study was designed to test the potential for neural activity induced myelin plasticity in an injured environment. Our confirmation of such plasticity yields a great number of intriguing new research questions, which can in part address some of the limitations of the current study. We used the presence of BDA signal as a proxy for stimulated axons. We used low volume injections at the implant site to limit the spread of BDA to neurons outside of the forelimb motor cortex. The ability for stimulation to invoke forelimb movement and the anatomical confinement of BDA+ axons to the CST in the cervical cord support the assertion that the injection effectively targeted stimulated axons. However, it is possible that there were

stimulated axons not captured by BDA signal, or vice versa. A recent study that increased activity using pharmacogenetic stimulation was able to definitively show altered myelination on axons positive for cFos, an activity marker, while nearby axons negative for cFos did not exhibit changes [35]. This pharmacogenetic study provides direct evidence for one-to-one signaling from electrically altered axons to myelin modifications, suggesting the effect seen in the current analysis was directly driven by activity, and BDA signal was a sufficient proxy for stimulated axons.

It is also possible that the effects of stimulation extended beyond direct communication between axons and oligodendrocytes or myelin sheaths. The CST also innervates sensory processing circuits in the spinal cord (reviewed by [36]), and stimulus evoked forelimb contraction also produced sensory feedback. Thus, effects on myelin and axon morphology might have been induced by circuit-wide changes in activity or signals.

We chose low frequency stimulation for its ability to induce myelination [6][14], but higher frequency may be more effective at targeting progenitor proliferation [32], or functional recovery following SCI [37]. Future work that investigates differential patterns of stimulation, both in frequency and duration, will give insight into the range of effects for neuromodulation on white matter plasticity. Furthermore, the extent to which an altered myelination pattern contributes to functional or behavioral changes remains to be seen. Future work can address longer stimulation patterns and behavioral consequences.

In this study, we provide the first evidence for neuromodulation to affect white matter remodeling throughout a long tract following injury. We showed that low frequency stimulation induced shorter myelin sheaths and lower G-ratios in regions of the spinal cord normally unaltered by injury. The intriguing possibility of activity dependent white matter plasticity in

injury provides an abundance of new questions to probe the full potential and extent of activity dependent myelin plasticity, both in injury and intact systems.

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Chapter 3. **MOLECULAR AND SYSTEMS LEVEL EFFECTS OF ALTERED ELECTRICAL ACTIVITY OF MOTOR TRACTS FOLLOWING SPINAL CORD INJURY**

Acknowledgements: Several collaborators contributed to data acquisition for this chapter. Transcription data were processed by Dr. Haichao Wei in Dr. Jia Qin Wu's laboratory at the University of Texas Health Science Center at Houston. Microbiota data were processed by Nazli Yalcinkaya and Dr. Qinglong Wu in Dr. Tor Savidge's laboratory at the Baylor College of Medicine, Houston, TX. We are grateful for their expertise and assistance in collecting and interpreting these results.

3.1 ABSTRACT

The consequences of cell death and degeneration following spinal cord injury (SCI) extend beyond the central nervous system (CNS). Motor and sensory deficits below the injury can decrease the quality of life for those living with chronic SCI. In addition, there are other comorbidities that can occur with SCI, including disruption of other organ systems, such as the gastrointestinal tract. To further elucidate the disease process in SCI and the effects of neuromodulation, we conducted RNA sequencing following SCI and stimulation in mature rats to identify potential candidate genes that might have contributed to morphological changes described in the previous chapter. We also assessed the effects of stimulation on microflora populations and signaling in the gut. Finally, we assessed animals' performances on behavior tasks to track any recovery from SCI. Altogether, this chapter focuses on a multi-systems level analysis of SCI and stimulation, transcription, the gut, and behavior.

3.2 INTRODUCTION

The previous chapter focused on cortical stimulation induced morphological changes of myelin and axons in injured white matter. However, spinal cord injury (SCI) affects more than the cellular structure of neurons and glia of the spinal cord; ramifications can be seen at multiple levels, from molecular to physiological, and outside of the central nervous system (CNS) in organ systems throughout the body. A holistic study of SCI necessitates analysis of the effects of injury and interventions on the CNS and the whole organism. To further investigate the effects of cortical electrical stimulation following SCI beyond the analysis outlined in Chapter 2, we undertook additional analyses to determine i) the effects of cortical stimulation in the injured CNS on transcription ii) the consequences of stimulation on the gut microbiota and iii) functional recovery associated with the stimulation paradigm.

3.2.1 *Transcription, Myelination, Stimulation, and SCI*

Protein production and cellular function are modulated by the transcription of genetic material into ribonucleic acids (RNA). Transcription can be impacted by both external stimuli, such as insult or disease, and internal signaling, such as a developmental cue. The result of upregulated or downregulated transcription of specific genes can be an increase or decrease in their protein products. In the present study we are interested in how two external cues, SCI and electrical stimulation, might affect transcription in the nervous system. Specifically, we anticipated that any morphological effects induced by altered activity patterns in the CST would be preceded by a shift in transcriptional profile. In particular, because the present study was designed to assess the effect of neuromodulation on myelination, we anticipated that we would see an upregulation of oligodendrocyte and myelin specific transcripts. Studies have identified genes and networks affected by SCI, following electrical stimulation, and during myelination.

However, to our knowledge the influence of the combination of these three factors on transcription has not been examined.

Whether or not remyelination in SCI or demyelinating diseases requires reactivation of developmental myelin programs or is a distinct process is an ongoing debate [1]. Nonetheless, recently published transcriptional profile of developmental myelin identifies a number of upregulated genes as candidates for key players in the formation of myelin [2]. Several candidate genes identified in that study whose products are known to play a role in myelin formation or maintenance are listed in Table 1. Genes specific to myelination have also been analyzed in the context of SCI. For example, myelin basic protein (MBP) and proteolipid protein (PLP) are reported to be downregulated at multiple time points following injury [3]. The final factor that might impact the transcriptome in the present study is the application of electrical stimulation to the CNS. A recent study examined differential electrical stimulation patterns and subsequent transcriptional profiles in dorsal root ganglion neurons [4], which yields several more candidates to explore in the present study. In addition to the candidates cited in the literature, we predicted that stimulation would induce differential transcription of axonal and glial nodal genes which would correspond to altered nodal frequency. Altogether, Table 3.1 summarizes a number of genes of interest to examine following SCI and electrical stimulation.

Table 3-1 Candidates Genes

Classification	Gene	Notes / Up or Down Regulation
Myelin genes	Myelin basic protein (Mbp)	Upregulated during myelination [2] Downregulated following SCI [3]
	Myelin associated oligodendrocyte basic protein (Mobp)	Upregulated during myelination [2]
	Myelin associated glycoprotein (Mag)	Upregulated during myelination [2]
	Myelin oligodendrocyte glycoprotein (Mog)	Upregulated during myelination [2]
	Cyclic nucleotide phosphodiesterase (Cnp)	Upregulated during myelination [2]
	Proteolipid protein 1 (Plp1)	Upregulated during myelination [2]
	Ferritin heavy chain 1 (Fth1)	Upregulated during myelination [2]
	Myelin transcription factor 1 (Myt1)	Upregulated following SCI [3] Upregulated with stimulation [4]
	Tubulin polymerase promoting protein (Tppp)	Upregulated during myelination [2]
	Tubulin polymerase promoting protein 3 (Tppp3)	Upregulated during myelination [2]
Cytoskeletal / Nodal	Neurofascin (Nfasc)	Upregulated during myelination [2] Axonal and glial nodal protein [5]
	Contactin (Cntn1)	Axonal nodal protein [5]
	Contactin associated protein 1 (Cntnap1) (CASPR)	Axonal nodal protein [5] Protein product used to detect nodes (Ch. 2)
	Beta-spectrin 4 (Sptbn4)	Axonal nodal protein [5]
	Ankyrin 3 (Ank3)	Axonal nodal protein [5]
Ion Channels	Potassium voltage gated channel member 3 (Kcnq3)	Node of Ranvier ion channel [6]
	Voltage gated potassium channel subunit 2 (Kcna2)	Node of Ranvier ion channel [6]
	Voltage gated sodium channel subunit 2 (Scn2a)	Sodium channel; may be a sign of immature nodes [7]
	Voltage gated sodium channel alpha subunit (Scn8a)	Sodium channel; may be a sign of mature nodes [7]
Motor	Kinesin family member 1b (Kif1b)	Traffics Mbp mRNA during myelination [8]
Additional genes	Neuregulin 1 (Nrg1)	Regulates myelin sheath thickness [9]
	Fgfr2	Stimulation-induced transcription [4] Upregulated in myelination (reviewed by [10])
	Fgf1	Upregulated in myelination (reviewed by [10])
	Cxcl2	Inflammatory factor; contributes to remyelination (reviewed by [11])

3.2.2 *Disruption of the Gut Bacteria Following SCI*

Recent research has highlighted the importance of gut microbiota, defined as the bacterial population of the intestinal tract, in the functioning of the digestive tract as well as neural/glial development and CNS function (reviewed by [12][13]). A complex and delicate balance exists between the gut and its resident bacteria; bidirectional messaging between gut bacteria and the rest of the body can affect the body's immune reactions [14][15]. Metabolites and neurotransmitters (NTs) released by gut bacteria can act as messengers in the enteric nervous system and the autonomic nervous system neurons and glia [14]. Furthermore, altered NT signaling in the gut-brain axis (the microbiota, gut, and CNS) has been implicated in both gastrointestinal and CNS pathology [16]. Furthermore, dysbiosis, the disruption of bacterial populations, has been implicated in morphological pathology and a number of neurological and psychiatric diseases [12][13].

Disruption of the gut bacterial population has been implicated in worse functional outcomes following SCI [17]. Given the contributing role of the gut and its bacterial population to inflammatory response/host immunity and functional recovery following SCI, we sought to examine if similar disruptions of the microbiota would be found in our injured animals, as compared with naïve uninjured animals. Furthermore, we explored whether the cortical stimulation paradigm employed to target white matter remodeling would contribute to or ameliorate disruption of the microbiota following SCI. In particular, we were intrigued by the emerging importance of the gut microbiota and regulation of myelination. Dysbiosis in development has led to hypermyelination in mouse models (reviewed in [12]). Furthermore, a study recently found that inoculating mice with microbiota sampled from patients with multiple sclerosis, a demyelinating disease, induced a multiple sclerosis-like phenotype [18]. Thus, we

sought to determine if any of our measures of remyelination correlated with measures of gut/microbiota function and dysbiosis. If changes in the microbiota population or signaling is altered by CST stimulation, it would deepen our understanding of the relationship between the gut and the CNS in injury.

3.2.3 *Functional Recovery and Stimulation Following SCI*

Regaining sensory and motor function is the ultimate goal of any intervention following SCI. Although we did not expect to see a significant increase in recovery in our stimulated animals, given the relatively short time scale of our intervention, we still assessed animals on behavioral tasks to ascertain if there was any functional recovery. An improvement in recovery with stimulation could imply that the putative white matter plasticity might have contributed to a better outcome. A worse functional outcome would imply the stimulation paradigm induced pathology, or that the putative white matter plasticity had a deleterious consequence. Thus, it was important to determine what, if any, functional recovery was induced by stimulation.

3.2.4 *Towards a Holistic Analysis of SCI and Stimulation*

As outlined above, there is strong evidence to look beyond local cellular and morphological effects following SCI and stimulation paradigms. This chapter will outline additional assays conducted on the animals described in chapter two in an attempt to identify global effects of injury and stimulation.

3.3 METHODS

The 14 animals described in Chapter 2 underwent the additional assays outlined below in sections 3.3.2 and 3.3.3. Four additional animals were added which followed the protocol for injury and implant as outlined in Chapter 2, then underwent the methods as described in 3.3.1 below.

3.3.1 *RNA Isolation and Sequencing*

SCI animals underwent one week of stimulation (n=2), or one week of sham treatment (referred to as controls; n=2). Animals were perfused with ice cold saline and neural tissue was quickly isolated and snap frozen in liquid nitrogen from three regions of the spinal cord: rostral to the injury, the lesion epicenter, and caudal to the injury (Fig. 3.1). Tissue was kept at -80 °C until RNA isolation and purification. Tissue was homogenized and complete RNA was isolated using a Qiagen RNAeasy Mini Kit according to manufacturer's protocol. RNA was sequenced at Weill Cornell Epigenomics Core. The cDNA-libraries were sequenced by Illumina Hiseq 2500 sequencer (paired-end, 2x51 base pairs). Processing of libraries was completed by the Wu lab at University of Texas Health Science Center. The quality of the reads was verified using FastQC. The assessment results were summarized and visualized using MultiQC 1.6 [19]. Read mapping, transcript assembly, and expression estimation were performed as described previously [20], [21]. The rat reference genome Rnor6 was downloaded from Ensembl. The annotated file for the rat genome was from previous studies [21]. The 51-bp paired-end reads were aligned to the reference genome using TopHat v2.1.0 with default parameters [22]. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were obtained for genes and transcripts using Cufflinks v2.2.1 [23]. Read counts for genes were obtained by HTSeq-count [24]. Differential expression genes (DEGs) were performed with DESeq2 [25]. Genes with FPKM > 1

in at least one sample were retained for the analysis of DEGs. Genes were classified as DEGs if they met the criteria: 1) at least one sample's FPKM >1; 2) |log2(fold-change)| >1; 3) P-value < 0.05.

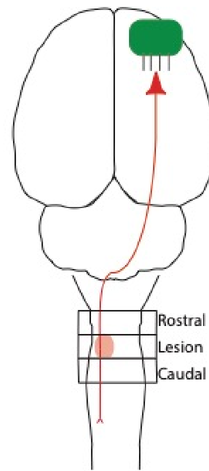


Figure 3.1 Schematic of Stimulating Electrode, CST, and Tissue Isolated for RNA Purification

3.3.2 *Gut and Microbiota Harvesting and Processing*

SCI animals from the morphology study were singly housed starting at the time of injury until euthanasia, in order to avoid transmission of bacteria between rats (stimulated n = 6; injured unstimulated n = 5). An additional three uninjured naïve animals without implants were singly housed and kept in similar conditions to be used as naïve controls. In addition, animals were kept isolated from fecal matter from other animals to avoid introducing foreign bacteria populations. Following three weeks of stimulation (or sham), animals were perfused with phosphate buffered saline and the cecum collected and snap frozen in liquid nitrogen. Samples were stored at -80°C until processing.

The Savidge lab at Baylor College of Medicine processed cecal contents for microbiome sequencing and mass spectrometry (MS). The content was lyophilized and then reconstituted with double distilled water to normalize across samples. Samples were centrifuged and supernatants were collected and stored at -80°C until MS was performed.

Amplicon data of 16S rDNA variable region of V4 or V1-V3 was generated from MiSeq platform with paired-end sequencing protocol (effective read length: 250 bp for V4 and 300 bp for V1-V3). DADA2 package (version 1.8) [26] was used for processing de-multiplexed raw sequencing reads following the default parameter settings with minor modifications. Specifically, raw sequencing reads were trimmed while maintaining the overlap regions for merging paired-end reads; sequencing primers retained in forward and reverse reads were stripped by the length of primers. IDTAXA function in DECIPHER package (version 2.6.0) [27] and its pre-built SILVA database (release 132) [28] as the training set was used for taxonomy assignment of amplicon sequence variants (ASVs) from DADA2 output. ASVs that were not classified as bacteria domain and chimeras were removed. The phylogeny of ASVs was generated with DECIPHER for sequence alignment and FastTree (version 2.1.3) [29] for tree construction. Samples with less than 1000 reads in the ASV matrix were excluded and proportional transformation was applied to normalize feature data prior to downstream analysis. Alpha-diversity indices including Shannon and Inverse-Simpson metric were calculated by Phyloseq package (version 1.24.2) [30]. Beta-diversity analysis was performed with non-metric multidimensional scaling of weighted UniFrac [31] distance in the Vegan package (version 2.5-5) [32]. Benjamini-Hochberg (BH) correction was applied for statistical analysis (Mann-Whitney-Wilcoxon test or Kruskal-Wallis test) of comparison of specific ASVs or collapsed taxa

i.e. genus and family rank between or among groups. Significance of Spearman correlation was also corrected with BH procedure.

One animal was omitted from the sequencing analysis due to issues with read number.

3.3.3 *Behavioral Assessment*

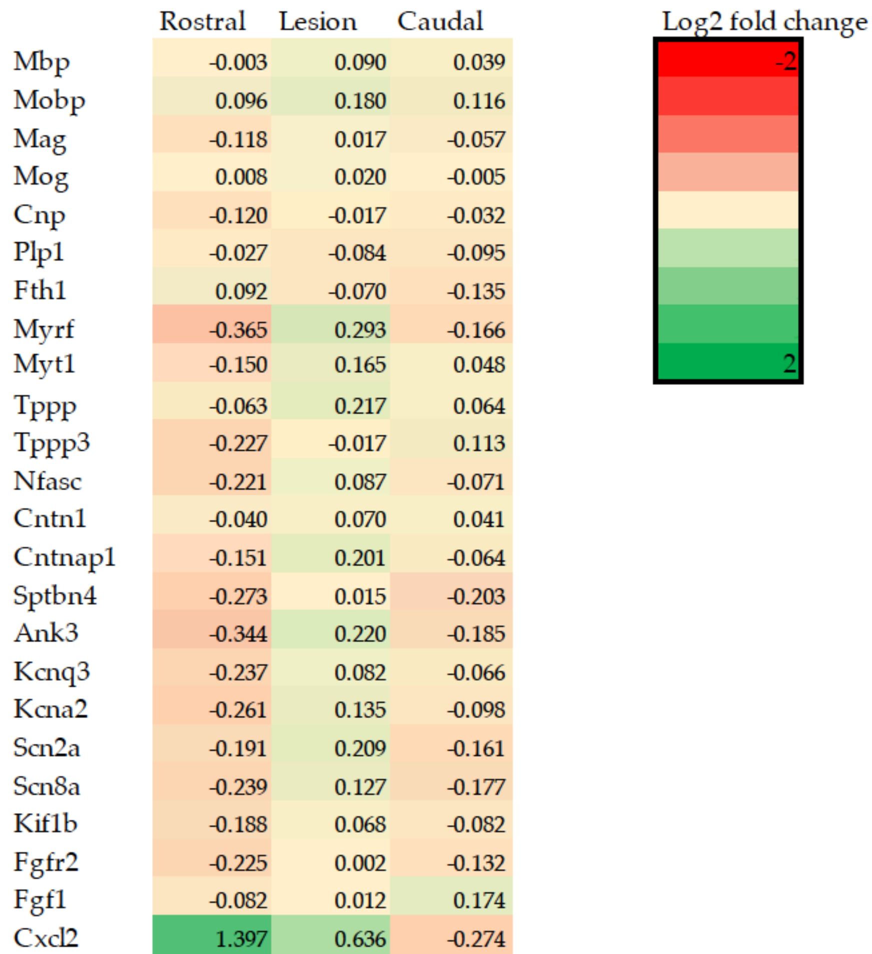
The 14 animals from Ch. 2 were trained on two motor tasks prior to injury: the pasta matrix reaching and grabbing task and the irregular horizontal ladder walk. Both tasks are sensitive to deficits induced by SCI [33][34]. The pasta matrix requires the animal to reach through a slot in the behavior arena to grab for pasta pieces with their dominant (affected) forelimb. The number of pieces broken by the affected forelimb is the score. Animals were assessed five times per week during the stimulation period, and scores were averaged for the week. The irregular horizontal ladder walk task requires animals to cross from one arena to another through a plexiglass tunnel with irregularly spaced rungs. Each step for each limb is recorded as a hit (the animal placed the paw on the rung and made forward progress) or a miss (the paw passed between the rungs or the limb did not make contact with the rung). Animals were assessed three times per week on the ladder and number of misses per crossing was assessed for each limb.

3.4 RESULTS

3.4.1 *Transcription of Candidate Myelin and Oligodendrocyte Genes is Not Modified with One Week of Stimulation*

A variety of candidate genes involved in myelination, SCI, and electrical stimulation are highlighted in Table 3.1. Differential expression analyses comparing stimulated animals and non-stimulated animals found very small and not statistically significant changes in every candidate genes' transcription at all three spinal cord regions ($p > 0.05$ for comparisons for all genes in Fig. 3.2, Wald's test). We had hypothesized that myelin specific genes would be upregulated to support new myelin formation, and cytoskeletal proteins and ion channels associated with the node of Ranvier would also be upregulated, given greater nodal frequency with stimulation (see Chapter 2) The lack of any drastic up- or down- regulation was surprising.

Less than 100 genes met the criteria for differential expression with stimulation (Fig. 3.3). Very few genes were differentially expressed in more than one region. In the rostral compartment there were 10 upregulated genes with stimulation, 8 of which were unique to that region (Fig. 3.3A). There were four downregulated genes, one of which, Fjkx1, was also found to be downregulated at the lesion and caudal to the lesion (Fig. 3.3B). The lesion had 12 upregulated genes, two of which, Cidea and Igkc, were also upregulated in the rostral compartment. In addition to the common downregulated gene, Fjk1, there were 26 unique statistically significantly downregulated genes at the lesion (Fig. 3.3B). Caudal to the lesion there were the most statistically significantly different genes between stimulated and control animals: there were 46 upregulated genes, one of which was the commonly upregulated Cidea. There were only two downregulated genes, Gm8444 (which was upregulated at the lesion) and Fjx1, which was universally downregulated. (Fig. 3.3B).



	Rostral	Lesion	Caudal
Mbp	-0.003	0.090	0.039
Mobp	0.096	0.180	0.116
Mag	-0.118	0.017	-0.057
Mog	0.008	0.020	-0.005
Cnp	-0.120	-0.017	-0.032
Plp1	-0.027	-0.084	-0.095
Fth1	0.092	-0.070	-0.135
Myrf	-0.365	0.293	-0.166
Myt1	-0.150	0.165	0.048
Tppp	-0.063	0.217	0.064
Tppp3	-0.227	-0.017	0.113
Nfasc	-0.221	0.087	-0.071
Cntn1	-0.040	0.070	0.041
Cntnap1	-0.151	0.201	-0.064
Sptbn4	-0.273	0.015	-0.203
Ank3	-0.344	0.220	-0.185
Kcnq3	-0.237	0.082	-0.066
Kcna2	-0.261	0.135	-0.098
Scn2a	-0.191	0.209	-0.161
Scn8a	-0.239	0.127	-0.177
Kif1b	-0.188	0.068	-0.082
Fgfr2	-0.225	0.002	-0.132
Fgf1	-0.082	0.012	0.174
Cxcl2	1.397	0.636	-0.274

Figure 3.2 Differential Expression of Candidate Genes

Differential expression between control and stimulated animals of the candidate genes highlighted in Table 3.1. In addition to failing to fall above/below criteria for 1.5 fold change, none of these differential expression patterns were statistically significantly different between the two groups.

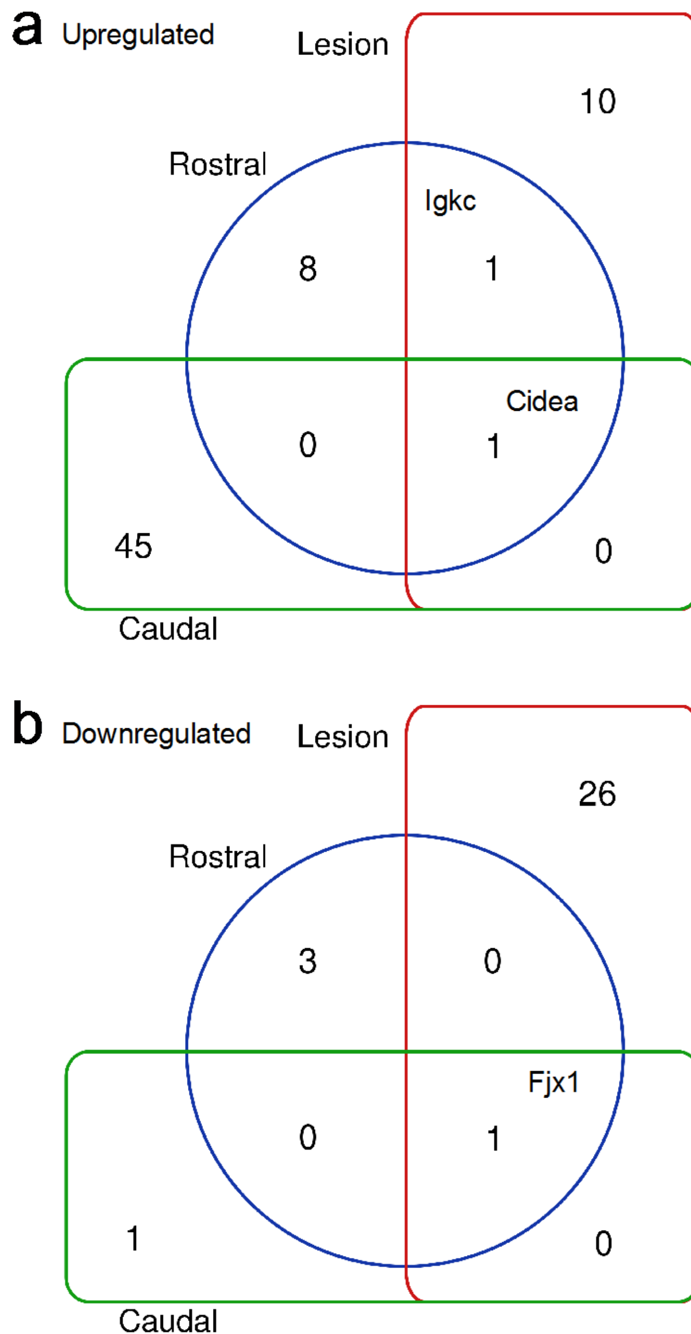


Figure 3.3 Numbers of Statistically Significantly Differentially Expressed Genes

A) Upregulated (>1.5 log2fold) and B) Downregulated (<-1.5 log2fold) genes for each of the three compartments of the spinal cord (Wald's test, $p < 0.05$). Genes identified in multiple compartments are labeled. In addition, one downregulated gene in the caudal compartment, Gm8444, was upregulated in the lesion compartment. Figure generated at http://bioinformatics.psb.ugent.be/webtools/Venn/

3.4.2 *Effects of Cortical Stimulation on Gut Bacteria Populations and Neurotransmitter Signaling*

Cecum contents taken at the end point of the experiment were analyzed via MS to determine NT levels produced by the gut bacteria. Levels of GABA, glutamate (glu), glycine (gln), tryptophan, and serotonin were measured in stimulated, control (injured/implanted without stimulation) and naïve (uninjured and unimplanted) animals (Fig. 3.4). In addition, the cecum weights were compared as a measure of gut function and motility.

There were no statistically significant differences between the weights of the cecum for any of the groups (one-way ANOVA with Tukey's test for multiple comparisons; $p = 0.1530$) or for levels of NT between any of the groups (one-way ANOVA with Tukey's test for multiple comparisons; tryptophan $p = 0.6832$; serotonin $p = 0.3076$; GABA $p = 0.8366$; glu $p = 0.5974$; gln $p = 0.9254$), though some different trends and patterns emerged in several of the NTs. Glycine levels seemed fairly stable in all the groups (Fig 3.4F), while stimulation seemed to bring levels of tryptophan, serotonin, and glutamate closer to naïve animals, while control animals trended towards lower levels (Fig 3.4 B, C, and E, respectively). Control and stimulated animals were more similar in their GABA levels than naïve animals (Fig. 3.4D). However, none of these trends were statistically significant.

Given recent findings relating gut and microbiota health, inflammation, and recovery following SCI, we examined NT levels in all groups as a function of performance on a behavioral task that assesses functional recovery (see section 3.3.3). Specifically, we were interested in GABA, as it may play an important role as a signaling molecule in both systemic and gut inflammation [35]. We compared NT levels in the cecum of each animal (regardless of condition) as a function of their final scores on a pasta matrix reach and grab task, where higher values indicate better performance on the task. A linear regression revealed GABA showed a

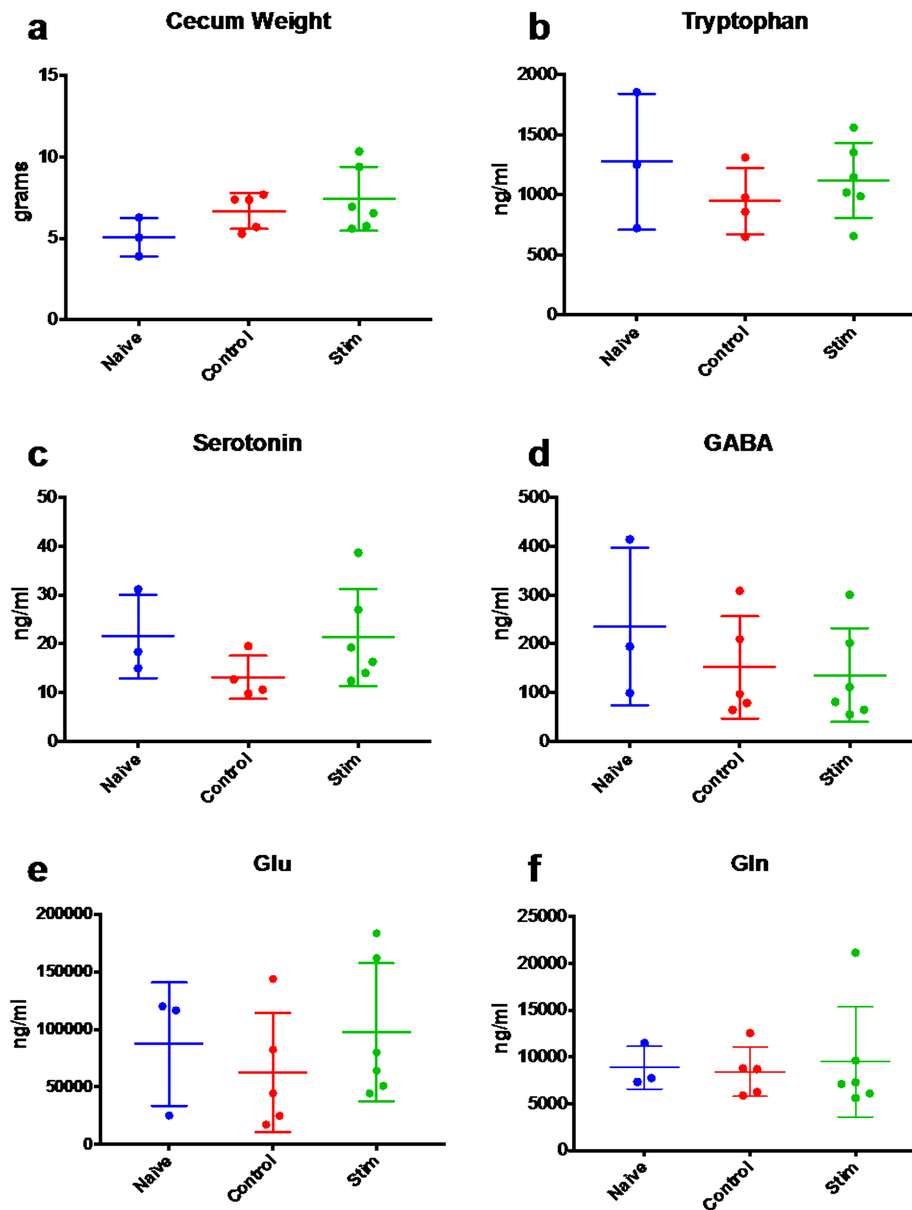


Figure 3.4 Cecum Weights and Neurotransmitter Levels

Naïve (blue) animals were singly housed and perfused at the same time point as control animals (red; injured and implanted without stimulation) and stimulated animals (green; injured and implanted with three weeks of subthreshold stimulation). The cecum from each animal was harvested and A) weighed at the end of the experiment and levels of NT B-F) were measured via mass spectrometry. There were no statistically significant differences between any of the groups for any of the weights or NT measured. Each data point represents a single animal; error bars represent mean and SD.

statistically significant non-zero slope (Fig. 3.5 $Y = 12.91*X + 82.31$; $p=0.0238$) while all other NTs did not (data not shown).

Because GABA is formed from glutamate, we analyzed the ratios of glutamate and GABA to test if increased GABA levels might decrease glutamate stores. No statistically significant relationship emerged. In naïve and stimulated animals higher GABA levels trended towards an association with lower levels of glutamate, though the opposite trend occurred with control animals (Fig. 3.6 all correlations non-significant, Pearson's correlation, naïve $p > 0.9999$; control $p = 0.1333$; stimulated $p = 0.0583$).

To explore if there was any relation between the microbiota signaling of our SCI rats and white matter remodeling, we examined NT levels as a function of average myelin sheath length in the spinal cord, with no relationships emerging (data not shown).

The populations of bacteria present in the cecum were measured via 16s sequencing in order to assess the impact of SCI and stimulation on the diversity of the microbiota. The abundance of bacterial families found in each animal's cecum do not show a drastic shift from naïve animals with either SCI or stimulation (Fig. 3.7A). To quantify the diversity in each sample, the inverse of the Simpson's diversity index was calculated (Fig. 3.7B); this index considers the number of species present as well as the relative abundance. Injured and unstimulated animals seemed to display a greater variability of diversity, and naïve animals may have a trend towards lower diversity (i.e. lower index value) though there were no statistically significant differences between any of the groups (Mann Whitney-Wilcoxon with BH multiple comparisons correction).

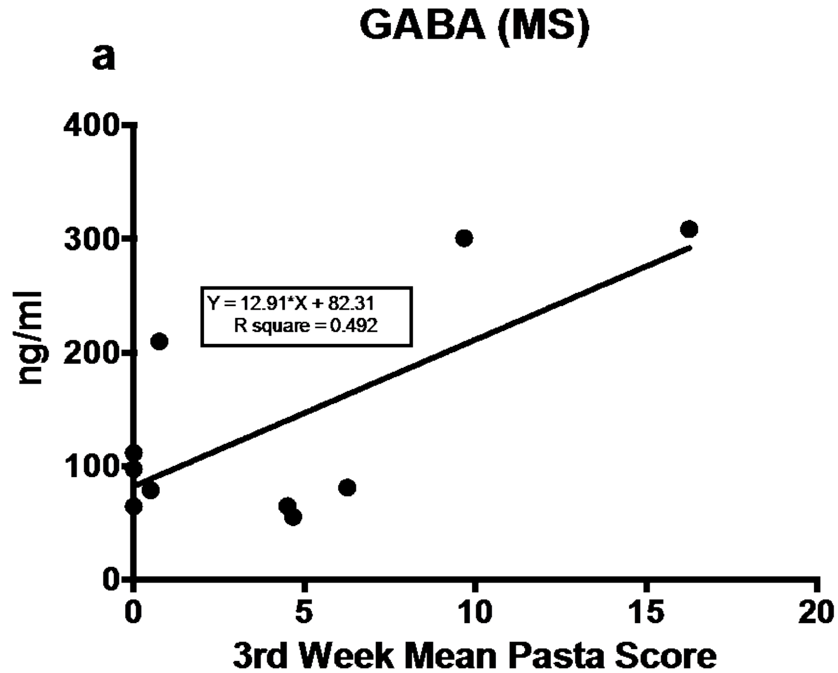


Figure 3.5 GABA Levels in the Cecum and Motor Task Performance

Performance on the pasta task is assessed via the average number of pasta pieces broken over three assessments in the final week of the stimulation paradigm for all injured and implanted animals, regardless of stimulation. Statistically significantly non-zero slope by linear regression; $p = 0.0238$.

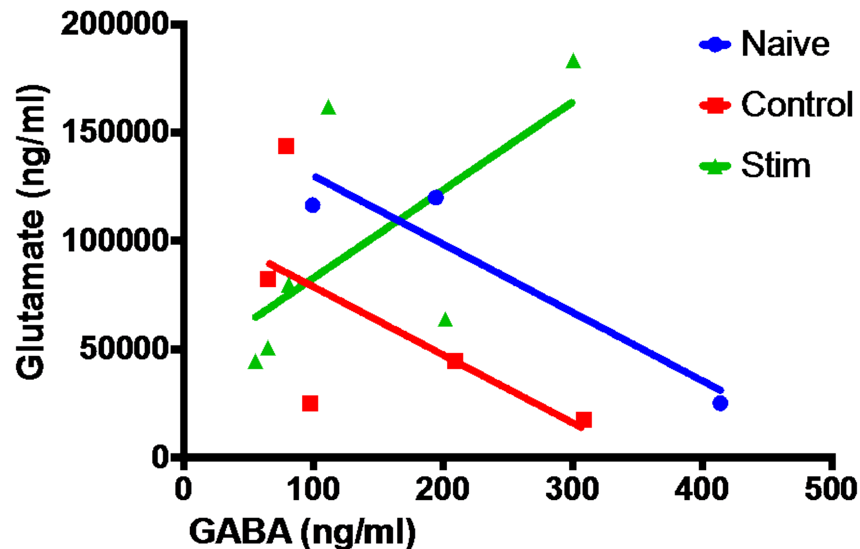


Figure 3.6 GABA and Glutamate Levels in the Cecum

A trend emerged in naïve and stimulated animals, wherein decreased glutamate levels in the cecum were associated with higher GABA levels. The opposite trend was seen in control animals. No relationships were statistically significant.

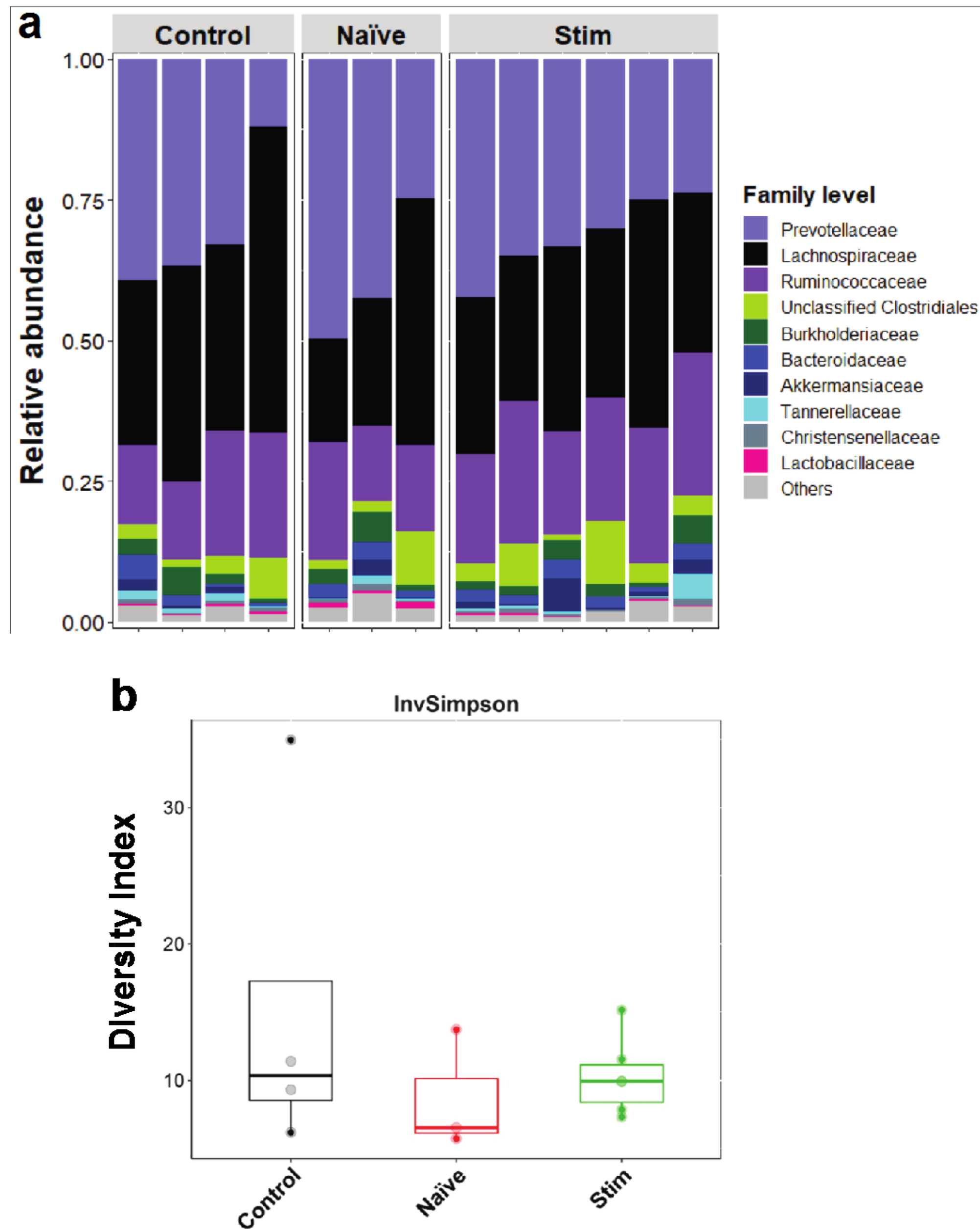


Figure 3.7 Bacteria Families and Diversity in the Cecum

A) Abundance of families present in control (injured and implanted without stimulation), naïve (uninjured and unimplanted) and stimulated (injured and implanted with stimulation). Each column represents one animal. B) Inverse-Simpson diversity index for each group; there was no statistically significant difference between any of the groups by pairwise-comparisons (Mann Whitney-Wilcoxon). Figure prepared by Dr. Qinglong Wu.

3.4.3 *Three Weeks of Cortical Stimulation Does Not Alter Functional Recovery*

Animals showed a deficit on the pasta matrix task following injury, denoted by a decrease in the average number of pieces broken (Fig. 3.8; preinjury vs. pretreatment control $p < 0.0001$; stimulated $p < 0.0001$; 2Way ANOVA with Dunnett's multiple comparisons). Neither control nor stimulated animals showed a statistically significant improvement over the three weeks of retraining as compared to pretreatment values. There was no statistically significant difference between control and stimulated animals at any time point measured (2way ANOVA with Sidak's multiple comparisons test $p > 0.99$ for all comparisons).

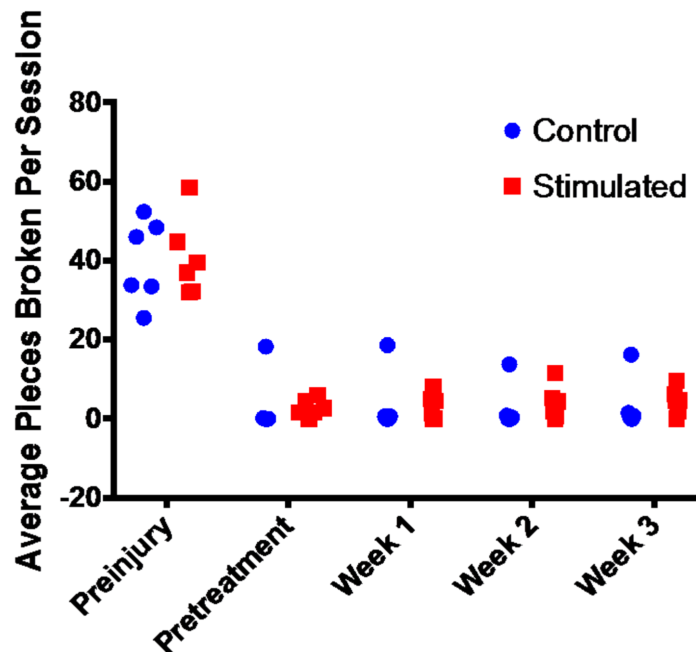


Figure 3.8 Forelimb Reaching Assessed via the Pasta Matrix Task

Both control and stimulated groups show a deficit following injury in their ability to break pasta with their dominant (affected) forepaw (Preinjury vs. Pretreatment). There were no statistically significant differences in either group in their ability to perform the task following three weeks of stimulation.

To assess overall locomotor deficits and recovery, animals were trained on the irregular horizontal ladder walk before injury and throughout the stimulation paradigm. The number of missed steps per crossing normalized to total steps per crossing averaged across each time point is shown in Fig. 3.9. Animals missed steps both with the dominant forepaw on the injured side (Fig. 3.9A) and all other paws (Fig. 3.9B) following injury. No statistically significant difference was seen in the number of steps missed over the treatment period or between groups (2way ANOVA with Sidak's multiple comparisons test $p > 0.99$).

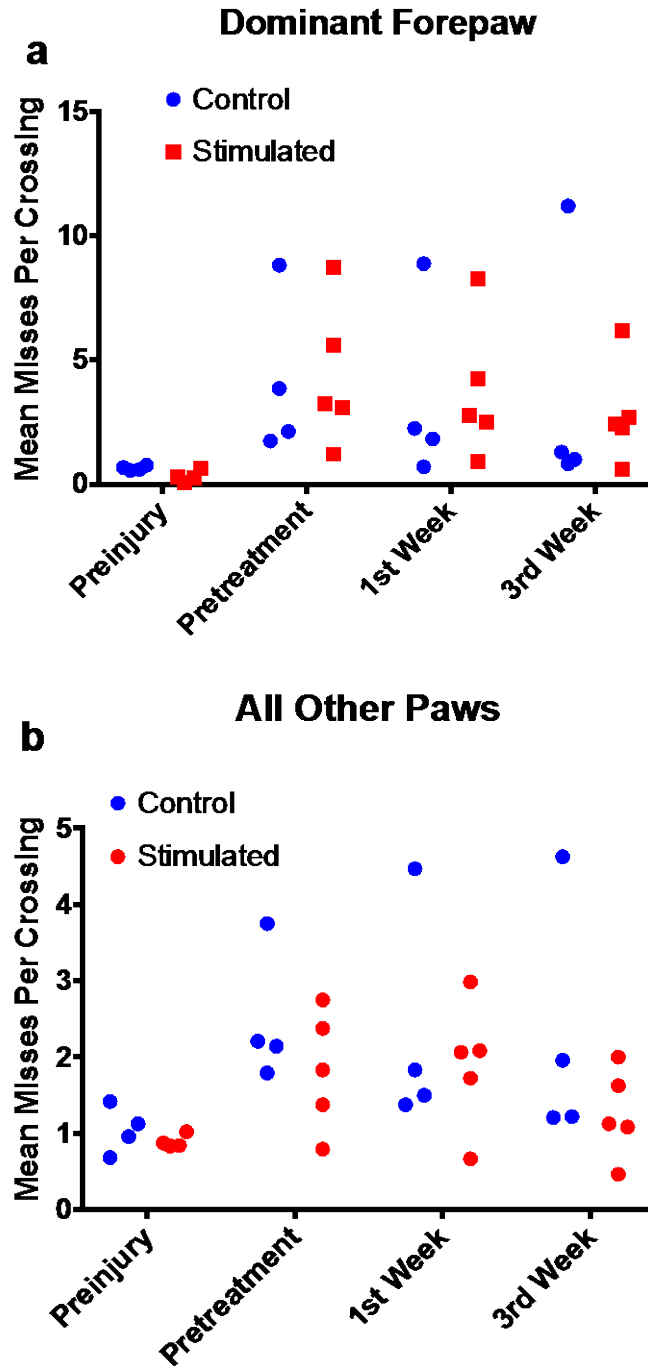


Figure 3.9 Locomotor Performance Assessed via Horizontal Irregular Ladder Walk

The number of missed steps per crossing by A) the dominant forepaw and B) all other paws over the experiment. There were no statistically significant improvements (as measured by fewer missed steps) in either group over the course of the stimulation paradigm.

3.5 DISCUSSION

3.5.1 *One Week of Stimulation Fails to Differentially Express Candidate Myelination and Nodal Genes*

We sequenced and analyzed transcripts throughout the injured CNS to ascertain if stimulation would affect any transcriptional programs involved in SCI recovery or remyelination. However, we saw no statistically significant difference in the expression any of our candidate genes (Fig. 3.2). We expected that the putative newly formed myelin (see chapter 2) would require upregulation of myelination specific genes. Furthermore, an increase in the frequency of nodes of Ranvier would require more cytoskeletal proteins and ion channels necessary for its formation. It may seem counterintuitive to search for transcripts of nodal genes in corticospinal axons, when the cell bodies of CST axons are centimeters away in cortex. While transcription of RNA occurs at the nucleus, there is evidence that axons can undergo local translation, which might have influenced the transcriptional profile in the spinal cord. Active and regenerating axons undergo local translation at the axon (reviewed by [36]), potentially to allow for adaptation to fluctuating metabolic and signaling needs. Furthermore, the transport of few mRNA molecules would allow for local translation of multiple copies of proteins needed in high abundance, reducing the burden of axonal transport [36]. Thus, in a stimulated axon in an injured environment, axons may induce local translation to supply protein to re-establish ion channels and cytoskeletal elements associated with the node of Ranvier. Additionally, oligodendrocytes and myelin transfer exosomes of mRNA to axons following NT stimulation [37]. Thus, we hypothesized we might find transcripts related to oligodendrocyte functioning, myelination, and axonal function in RNA from the cord.

Given our negative result for nodal transcripts, it is likely that protein products for building and maintaining nodes of Ranvier are translated in the soma. Thus, future work could analyze transcript levels at layer 5 of the cortex, where CST neurons originate. If differential expression of nodal genes is found there with stimulation, it would provide evidence not only for production of new protein to support nodal arrangement, but also provide evidence that glial-derived exosomes or local translation are less common in the injured stimulated cord.

One more provocative explanation for our negative results regarding myelin and oligodendrocyte gene expression may be that the change in myelin patterns observed in the cord with stimulation (Fig. 2.3) represents remodeling of existing sheaths, rather than the differentiation of progenitors to form new oligodendrocytes and new sheaths. At the lesion surviving oligodendrocytes do not participate in remyelination [38], and no difference in myelination patterns was observed there (Fig. 2.3C). If new oligodendrocytes were forming and producing myelin at the lesion, regardless of the stimulation, we would have failed to detect differential expression there. However, if rostral to the lesion existing oligodendrocytes are remodeling extant sheaths, such a process might not require as much upregulation of myelination genes. This explanation would account for the lack of differential expression. Future repeats of this stimulation paradigm can address this possibility by using a membrane reporter for myelination and thymidine analogues to identify new oligodendrocytes and myelin in conjunction with fluorescent *in situ* hybridization (FISH) probes for candidate myelin genes. The colocalization of increased FISH signal with thymidine analogue negative cells might support the remodeling hypothesis, whereas FISH signal in newly derived oligodendrocytes would imply the changed myelination patterns are the result of new oligodendrocytes and new myelin sheaths.

Despite compelling evidence in the literature for meaningful up- and down-regulation of candidate genes following stimulation and SCI, and despite a morphological phenotype induced by stimulation that presumably would require an altered transcriptional profile, we failed to see many meaningful stimulation-induced changes in transcription. None of our candidate genes (Table 3.1) had differential expression greater than or less than 1.5-fold (Fig. 3.2), nor were the differences statistically significantly. Furthermore, there were not many differentially expressed genes in the three regions of cord (Fig. 3.3). There are several potential reasons for this negative result. First, it may be that transcriptional effects induced by the injury eclipsed any effect of the stimulation. As a consequence, differential expression between stimulated and control animals would fail to distinguish any subtle stimulation induced differences. The lack of statistically significant up- or down-regulation in any of the myelination candidates was particularly surprising, given our phenotypic evidence of new myelin. However, many of the myelination candidates identified in Table 3.1 came from an analysis of myelin isolates [2], whereas our data were derived from whole cord homogenates. Thus, we may have failed to detect oligodendrocyte or myelin specific transcripts amidst the background of additional cell populations. In general, the lack of cell sorting may have contributed to a wash-out effect, leading to a lack of significant differences between the two groups. These confounding factors might also explain why none of the genes that were up- or down-regulated with one or five hours of stimulation in dorsal root ganglion neurons *in vitro* [4] failed to overlap with any of our statistically significantly regulated genes. That study used a relatively homogenous neuron population and a very short stimulation paradigm, unlike our whole cord sample and 3 weeks of stimulation. Finally, we only had 2 animals per group; a repeat with higher n might find more conclusive findings.

Despite the lack of confirmation of any of our hypotheses regarding our candidate lists, it is worthwhile to note that the gene Fjx1 was drastically downregulated in all three regions of the spinal cord following stimulation. In the mouse Fjx1 was identified as a regulator of dendritic morphology; a lack of Fjx1 led to dendrite extension *in vitro* [39]. One potential interpretation of our data might be that stimulation induces dendrite extension for the formation of new synapses. This interpretation is particularly intriguing in light of the increased branching seen BDA+ axons of the rostral compartment (Fig. 2.6A). Future elaboration of the role of Fjx1 in neural morphology and greater elucidation of the effects of stimulation on neuronal and dendritic morphology will determine if this relationship is meaningful. Cideea was upregulated in all three regions, which was very surprising; this gene is reportedly involved in the interaction between adipose tissues [40]. Further studies identifying cell-specific changes in gene expression may elucidate the key players and networks induced by SCI, stimulation, and remyelination.

3.5.2 *Cortical Stimulation Has Little Effect on Gut Bacteria Following Injury*

Given the relationship between the gut microbiota, CNS functioning, NT signaling, and inflammation [12][13][16], we undertook additional analyses in our cortically stimulated SCI animals to determine if the paradigm affected dysbiosis and bacterial NT signaling. Though some trends emerged in the levels of NT in the cecum in control, stimulated, and naïve animals (Fig. 3.4), there were no statistically significant differences between any of the groups. We anticipated that cortical stimulation would decrease inflammation, a finding that has been illustrated in non-SCI animals [41]. Decreased inflammation might be reflected in higher levels of GABA in the cecum [35]; however, this was not the case as there was no relationship between stimulation group and GABA. We also examined whether increases in GABA were associated with decreased levels of glutamate, as that might indicate conversion of glutamate to GABA

(Fig. 3.6). Though there was a trend for higher GABA to be associated with lower glutamate levels in stimulated and naïve animals, this finding failed to reach significance. Given recent findings showing a relationship between SCI induced dysbiosis with inflammation and functional recovery [17], we explored GABA levels as a possible correlate of functional recovery. Indeed, we found that increased GABA correlated with better performance on a reach and grab task in the final week of the experiment This relationship was independent of stimulation, in that both stimulated and control animals exhibited this correlation. Still, the positive correlation between recovery and GABA levels is intriguing in light of recent findings regarding SCI recovery and gut-mediated inflammation [13]. Further work may elucidate if cortical electrical stimulation may modulate systemic inflammation by measuring more direct inflammatory markers, such as cytokine production.

To elucidate whether the microbiota and gut functioning acts as a mediator of remyelination and white matter, we examined whether any of our measures of gut function/signaling and dysbiosis correlated with a measure of white matter remodeling, namely, sheath length. There was no relationship between internode length and NT.

Previous work has shown a shift in the classes of bacteria in the gut following SCI, and specifically an increased presence of the class Clostrideae [17]. We were curious to see if such a shift would be present in our SCI animals, and if stimulation would affect the relative abundance. Three families present in the guts of our animals (Lachnospiraceae, Ruminococcaceae, and other unclassified Clostridiales) were from the class Clostrideae, but there was no difference in the abundances of each family between groups (Fig. 3.7). The lack of any significant difference between the naïve animals and either injured group is surprising. One possible confound is that though great care was taken to not cross-contaminate between groups, the present study was not

conducted in a germ-free facility with specialized isolation cages, so animals may not have been sufficiently isolated from each other.

3.5.3 *Cortical Stimulation, Myelination Patterns, and Functional Recovery*

Behavioral assessments are used in rodent models to assess the possibility for SCI interventions to improve functional recovery in human patients. Targeting post-SCI demyelination and encouraging remyelination is one avenue to promote functional recovery [42][43]. To assess any functional recovery that accompanied the stimulation paradigm and subsequent morphological remodeling, we assessed behavioral outcomes on two tasks: the pasta matrix reach and grab task and the irregular horizontal ladder walk. Animals in both groups showed deficits on both tasks following injury (Fig. 3.8; Fig. 3.9) but failed to show statistically significant improvement on either task over the three weeks of the experiment. It is important to consider the lack of a significant result in light of the timescale for functional recovery and other interventions in SCI rats; many studies do not find functional improvement until 6-12 weeks following onset of an intervention [44][45]. It is possible that with a longer treatment the groups would diverge, and an increased functional recovery would be observed. In addition, stimulation interventions can display delayed effects, where functional recovery is not observed for weeks after the therapy ends [45]. Future work that extends the intervention and assessment period would address these possibilities.

Importantly, these negative results establish that thicker myelin, thinner axons, and shortened internode length induced by the stimulation paradigm are not associated with a deficit in recovery on this time scale. Even if an improvement was observed, this analysis was not conducted in a way that could determine the contribution of myelin plasticity (as opposed to synaptic or axonal plasticity) to functional recovery. We purposely chose low frequency

stimulation for its potential to alter myelination patterns [46], [47] and because high frequency stimulation beyond normal physiological ranges (e.g. seizure-like levels of activity) do not alter myelin patterns [48]. Certain frequencies of cortical stimulation induce axonal regeneration following SCI (reviewed by [49]) alter oligodendrocyte proliferation [50], and increase myelin protein production and functional recovery [51]. Future studies may explore a range of stimulation patterns and frequencies to discover which are most efficacious at targeting remyelination as well as synaptic and neural plasticity following SCI. Previous work in our lab has shown that remyelination takes months [52], while the critical window for neural regeneration is likely on a shorter time scale [53]. Given these temporal constraints, it may be that different paradigms of stimulation may be targeted to distinct cell populations at different points to maximize recovery.

3.5.4 *Conclusion*

Independent studies on SCI, transcription, inflammation, electrical stimulation, gut microbiota, and functional recovery led us to hypothesize that strong relationships would emerge between these variables in our injured and stimulated animals. As outlined above, many of these data turned out to be negative, though that does not inherently mean a relationship does not exist. In many cases, there were technical issues that prevented deeper analysis. For example, as detailed above, functional recovery might require a longer to reach significance. Transcriptional regulation associated with the observed myelin phenotype might occur in different temporal windows than the one measured in the current study. An understanding of the transcriptional profiles that drive regeneration and remyelination following SCI will contribute to our ability to target pathways that will lead to better recoveries and outcomes. Knowledge of the systemic

impacts, such as the effects on the microbiota and gut function, of both SCI and therapeutic interventions will also contribute to targeting therapies to better address sequelae and improve recovery. Future work may target these topics to elucidate the relationships between these systems and improve treatments.

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Chapter 4. SUMMARY AND FUTURE DIRECTIONS

4.1 ABSTRACT

The work presented in this thesis encompasses two independent fields: activity dependent myelin plasticity and spinal cord injury (SCI). The spinal cord is an ideal model to study long white matter tracts and the effects of neuromodulation on myelin. The contribution of demyelination to motor and sensory dysfunction following SCI makes remyelination efforts, potentially driven by altered activity patterns, a worthwhile pursuit. A seminal finding of this work is the demonstration of the injured nervous system to undergo activity dependent changes in myelination patterns. This final chapter describes mechanisms of activity dependent myelin plasticity in the context of white matter microenvironments. It will also consider systems level effects of neuromodulation and SCI. Finally, it will describe potential future experiments to explore neuromodulation, myelin plasticity, and recovery from SCI.

4.2 MYELIN PLASTICITY, ACTIVITY DEPENDENT MYELINATION, AND MODELS OF PLASTICITY

The first chapter of this thesis introduces the concept of myelin plasticity in the intact adult central nervous system (CNS) and considered evidence for myelin plasticity's role in normal functioning [1]–[3]. In addition, studies have showed the potential for neural activity to influence the formation and maintenance of myelin sheaths (e.g. [4]–[6]). The formation of new myelin is thought to be reliant on the differentiation of oligodendrocyte precursor cells (OPCs) into new myelinating oligodendrocytes, and increasing activity has been shown to induce OPC proliferation [4], [7].

If myelination is not static throughout life, what are the cues which induce alteration of myelin patterns? Post-mortem studies of humans have suggested that myelin undergoes protracted development, with deposition of new sheaths continuing for decades [8]. On the other hand, myelin plasticity might rely on external cues, as learning and exercise have been shown to be reliant on or initiate changed OPC, myelin, and white matter dynamics [1][3]. The differences between human and rodent myelination patterns throughout the lifespan makes comparisons between rodent and human studies somewhat difficult, given humans' relatively long life span. Myelination begins earlier in the mouse than in the human (reviewed by [9]) and one must take care when attempting to make direct comparisons between rodent stages of life to humans. Therefore, it is important to consider how age might affect myelination dynamics as we interpret research in this realm. The extent to which activity dependent myelin plasticity builds on a preset developmental path is unknown. Future work which tracks "set" myelination patterns and perturbs them through direct neuromodulation, such as stimulation, or external input such as exercise or sensory input may shed light on this subtle yet important interplay.

Regardless of the extent to which protracted hardwired development or external cues affect myelination patterns, there is strong evidence that organism age influences myelin plasticity. For example, a loss of sensory input during development affects optic nerve myelination [10] while depriving or enriching sensory stimulation fails to affect existing cortical myelination in mature animals [11]. Granted, the optic nerve is a more heavily myelinated tract, while cortical myelin is more variable, making direct comparisons between the two regions difficult. Still the concept that a system's response to external cues may also be reliant on the state (such as age) of the OPCs and oligodendrocytes is an intriguing one. This possibility is corroborated by studies describing a critical window for experience dependent myelin plasticity in the prefrontal cortex; the age at which mice were deprived of social interactions affected myelination in the prefrontal cortex [12]. An intriguing possibility is that younger animals (and younger OPCs) retain more capacity for externally driven myelination changes. In young adults, this potential might still be present [4], but it may be decreased with maturity. In the optic nerve, later-born OPCs differentiate and form myelin that is shorter than myelin formed by oligodendrocytes derived from OPCs born during development [13]. The data reported in this thesis describe a potential "priming" mechanism (i.e. injury) which may allow for more plasticity potential than would normally be possible in a mature animal. A recent paper describes a reduced capability for OPC populations in aged animals to differentiate and myelinate, though remyelination can be rescued pharmacologically [14]. Furthermore, OPCs "age" throughout life, exhibiting distinct genetic profiles and expressing varying receptors in both a temporal and regionally defined manner [15]. Thus, there may be an inherent potential for myelin plasticity that may decline with age, though this potential might be reactivated with an appropriate stimulus, such as injury or pharmacological treatment.

Chapter 1 describes three models of myelin plasticity: the myelination of previously bare axons, the remodeling of existing sheaths, and the replacement of old sheaths with new ones. Since Chapter 1 was published, new work utilizing live imaging in rodent models and zebra fish have revealed some exciting findings about the potential for remodeling of existing sheaths [11], [16]. Astrocytes may play a role in preventing or enabling thrombin mediated cleavage of paranodal attachments of myelin wraps, providing a mechanism by which myelin may be shortened and thinned [17]. Importantly, cortical myelin is known to be variable [18], and large sections of bare axons may provide substrates for the deposition of new myelin, a phenomenon that is observed in the optic nerve [13]. These studies imply all three models of plasticity may occur in the intact brain. In the context of the spinal cord and the corticospinal tract (CST), where myelination is more uniform (Fig. 4.1; [19]) and live imaging is more difficult given the depth of fiber tracts from the surface, similar work has not yet been done to confirm that all three models contribute to myelin plasticity. In the present study, the injured cord was fairly uniformly myelinated in both unstimulated and stimulated animals (Fig. 4.1), leaving not much space for intercalation of new sheaths. Therefore, replacement or remodeling seem the most likely mode of myelin plasticity, though further work with conditional reporters, proliferation markers, or live imaging is needed to confirm this hypothesis.

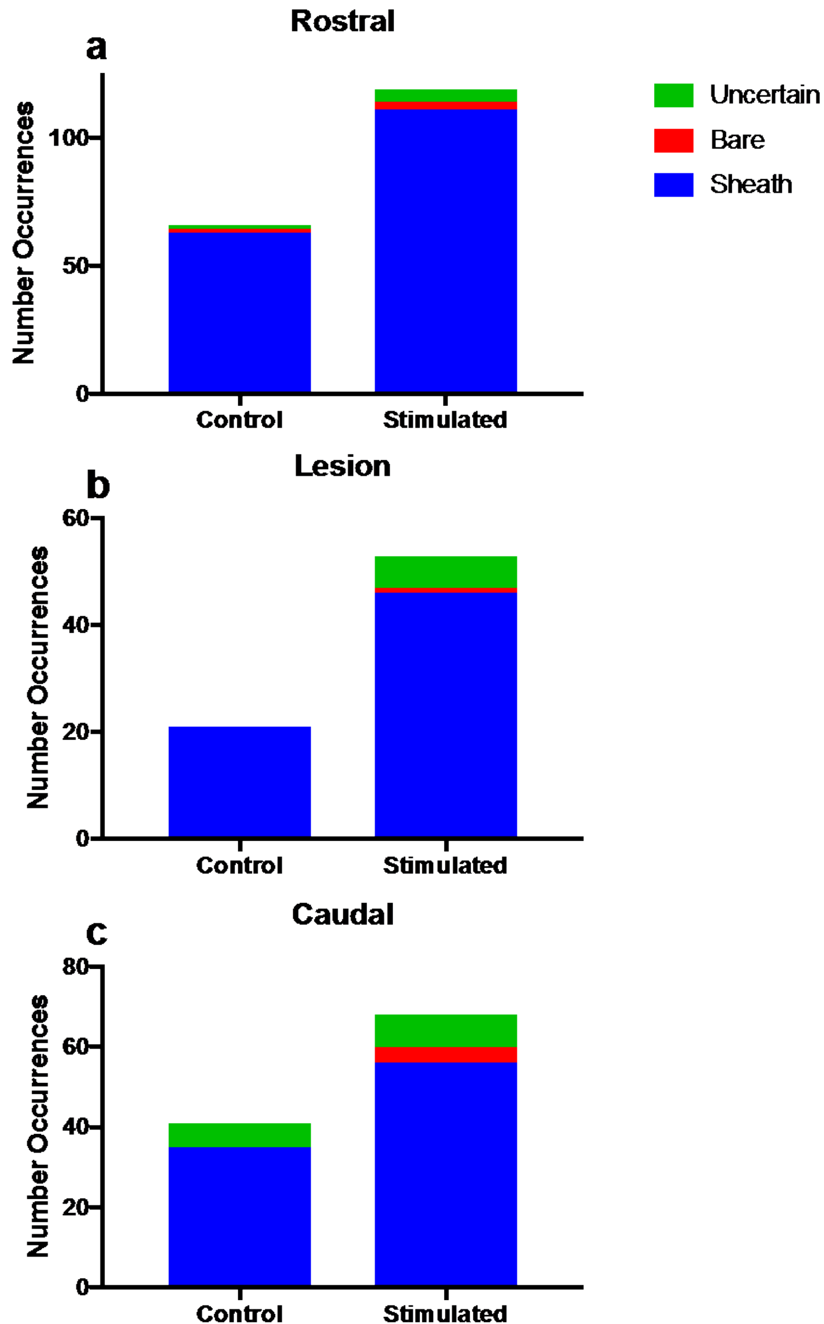

Figure 4.1 Corticospinal Tract Axons Are Heavily Myelinated

Quantification of the number of occurrences of sections of myelinated, bare, and uncertain sections of axon in the A) rostral compartment B) lesion epicenter and C) caudal compartment. There was no effect of stimulation on the occurrence of bare/myelinated/uncertain sections (Chi-square; rostral p = 0.374; invalid at lesion due to lack of occurrences of bare or uncertain; caudal p = 0.2720). A section of axon was classified as bare if two hemi-nodes were observed in succession. Sections of axon were classified as uncertain if there was any equivocal classification of nodes or hemi-nodes.

4.3 ADDITIONAL WHITE MATTER CELL TYPES IN MYELINATION AND INJURY

Long fiber tracts in the CNS consist of multiple cell types working in tandem. Though the present study was not designed to dissect the mechanisms between these additional cell types and OPC/oligodendrocyte populations, we would be remiss to leave out a discussion of the other cellular interactions that might contribute to myelin plasticity or may be disrupted in injury. A few key studies are summarized below as they pertain to white matter plasticity and injury.

4.3.1 *The Axon – More than Substrate for Myelination*

The extent to which axons are passive or active participants in the myelination process is a source of debate. Oligodendrocytes will myelinate carbon nanofibers in culture [20]. Cultured OPCs from different regions in embryonic rats showed consistency in internode lengths based on the region they were harvested from, rather than the substrate or tissue they were cultured on, implying myelin sheath morphology is an intrinsic factor of oligodendrocytes dictated by their original microenvironment [21][2]. The details of axonal machinery which may play a role in glial signaling will be discussed in greater detail below (see section entitled "Potential Mechanisms of Activity Dependent Myelin Plasticity"). The rest of this section will focus on other axonal factors relevant to the current study.

As was discussed in Chapter 2, the data regarding the diameter of CST axons must be interpreted with caution. Nonetheless, a higher incidence of small caliber spinal axons following stimulation was a surprising finding (Fig. 2.5), though a similar phenomenon has been observed following optogenetic stimulation of callosal fibers [22]. Axonal thinning occurs following injury [19], which could be linked to injury severity, but we have no reason to believe the

[2] This finding that rat embryonic OPCs already express distinct heterogenous intrinsic profiles needs to be reconciled with studies that mouse OPC heterogeneity arises with age.

stimulated animals sustained worse injuries than the control based on behavioral outcomes (Fig. 3.8 & Fig. 3.9).

Axon diameter correlates with sheath length [23], and thus there may be an inherent bidirectional effect wherein axon diameter affects sheath length and vice versa. However, in the present study a comparison of the diameter measured for each axon to the average sheath length for that axon shows there is not a clear relationship between diameter and sheath length (Fig. 4.2). There is a potential relationship in the rostral compartment for higher caliber axons to exhibit longer sheaths (Fig. 4.2A), but the variability in this plot makes drawing meaningful conclusions difficult.

Short term high frequency stimulation of hippocampal neurons induced axons to increase in diameter [24]. Our long-term and low frequency stimulation paradigm had the opposite effect, indicating a possible frequency-dependence of electrical stimulation. Very few studies of myelin plasticity consider the type or features of the axon being myelinated, so this may be an interesting line of inquiry for future work.

One intriguing possibility is that the thinner axons may represent newly sprouted branches of spared axons. The axon diameter and sheath length data were collected on all branches of axons that met criteria for inclusion (see Chapter 2), without any consideration for axonal branching, and indeed there was increased branching in the rostral compartment of stimulated animals (Fig. 2.6). Intriguingly, in interneurons of the mouse prefrontal cortex, stimulation dependent myelination also corresponded with increased axonal branching [25]. Though care must be taken to not over-extrapolate a finding in cortical interneurons to far-projecting CST axons, this study provides evidence for neuromodulatory co-regulation of both axon morphology and myelination. In the context of SCI and long-range tracts, cortical

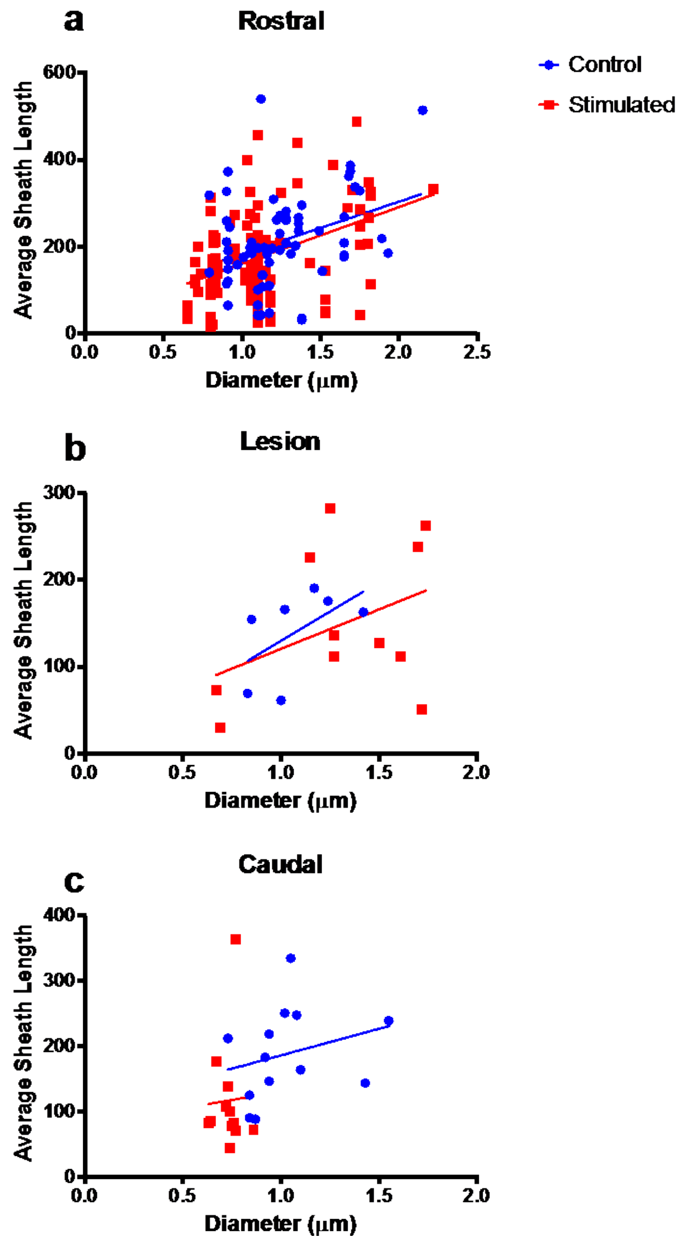

Figure 4.2 Sheath Length as a Function of Axon Diameter

Each axon diameter was plotted against the average sheath length on the axon. A) Linear regression found a positive slope to describe the relationship between axon caliber and sheath length in both conditions in the rostral compartment, though there was substantial variability not captured by the best fit lines (control slope = 120.4 +/- 41.24 r square = 0.1227 p = 0.00049; stimulated slope = 130.2 +/- 27.41 r square = 0.1756 p < 0.0001). B) In the lesion there was no clear relationship between diameter and sheath length (control r square= 0.3057; stimulated r square = 0.1549). Linear regression failed to find an accurate best fit line and did not find non-zero slopes in either condition (control p = 0.1980; stimulated p = 0.2311). C) In the caudal compartment there was no relationship between axon diameter and average sheath length (control r square = 0.07134; stimulated r square = 0.00014), and linear regression found slopes were not non-zero (control p = 0.3777; stimulated p = 0.9072).

stimulation in conjunction with spinal stimulation can affect CST branching and growth (e.g. [26]). Thus, it may be that the stimulation paradigm employed in this study affected axonal morphology. Future studies which focus more directly on tracing all projections of spared axons might answer this exciting possibility.

Myelinated axons must establish functional excitable domains to establish ion channel gradients and propagate action potentials. Inherent in the phenomenon of myelin plasticity is the corollary of nodal plasticity; the nodes of Ranvier must re-cluster to unmyelinated regions of the axon in order to be exposed to the extracellular environment. Nodes of Ranvier are tightly regulated domains, with cytoskeletal and membrane proteins that constrain ion channels and structure (reviewed by [27]). This tight regulation is disrupted following demyelination in a phenomenon called channel spreading, wherein ion channels typically constrained to the node of Ranvier "leak" outside of the node [28]. However, few remyelination studies consider the clustering of excitable domains and re-establishment of nodes of Ranvier. In optic nerve crush, injured axons that have regenerated following a multi-modality intervention can re-establish excitable domains [29]. Remyelination might be sufficient to induce appropriate clustering of axonal proteins, as it does during development.

Variable sizes of the node of Ranvier have been observed in the corpus callosum, and computational models predict increasing the excitable area of the axon might potentially have significant effects on conduction [30]. Interestingly, we found no evidence of altered nodal size in stimulated animals (Fig. 4.3), suggesting that varying node of Ranvier size might not be a mechanism of fine-tuning conduction in the injured CNS. As described above, there may also be differences in myelination patterns (uniform vs. irregularly myelinated) in different tracts of the CNS, which may influence which parameters are fine-tuned to adjust conduction speed.

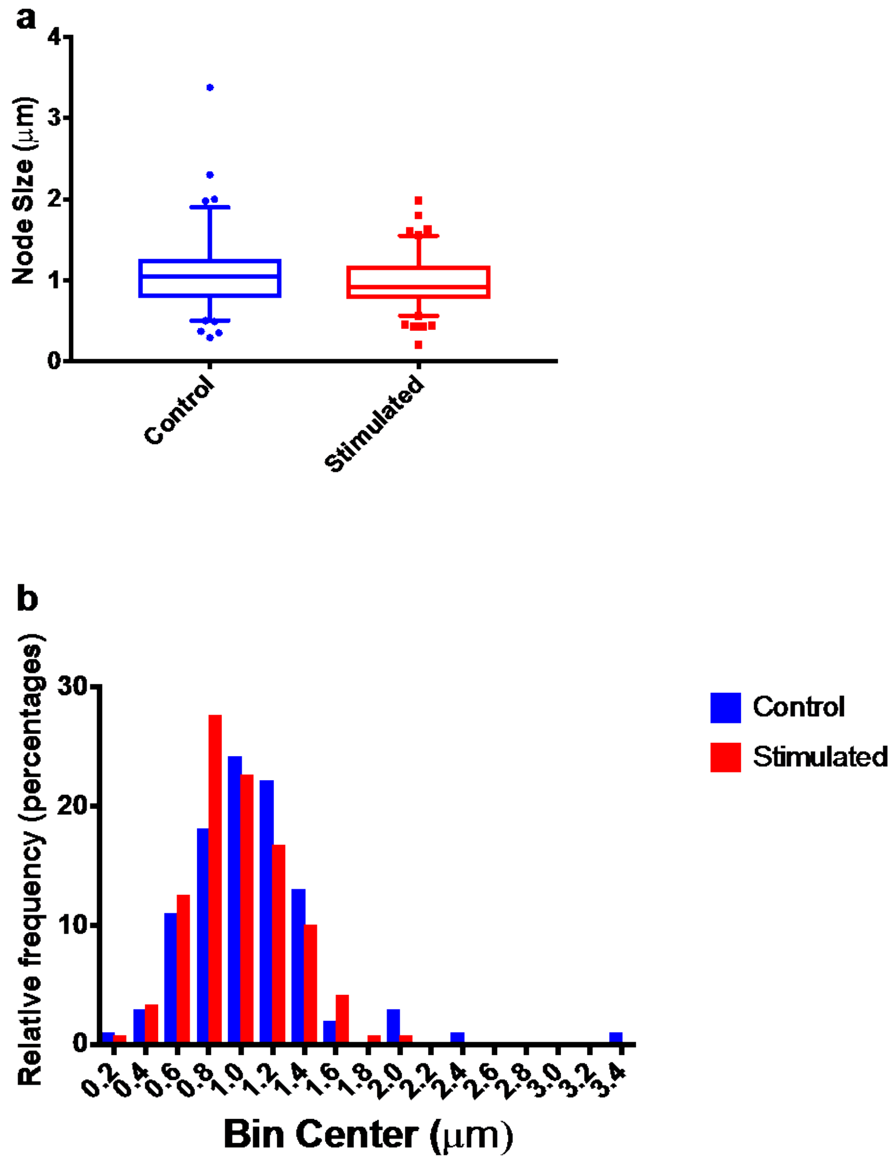

Figure 4.3 Node Size in the Rostral Compartment

The size of the node in was not statistically significantly different between stimulated and control axons in the rostral compartment A) Box plot showing no difference in the median (control = 1.04 µm for 99 nodes; stimulated = 0.92 µm for 119 nodes; Mann-Whitney $p = 0.1215$). Error bars represent 5-95% intervals. B) Frequency distribution shows considerable overlap between the two populations.

4.3.2 *Diversity of Astrocytes – Role in Myelination and Injury*

Astrocytes display regional and morphological diversity (reviewed in [31]). A full commentary on their diverse roles in function is beyond the scope of this chapter, but a few key points highlighting interactions between action potential conduction, myelination, SCI, and astrocytes are highlighted here.

Perinodal astrocytes were described in the 1970s and recognized as a potential supporter of axonal homeostasis, metabolism, and ion buffering (reviewed by [32]). Dye filled astrocytes in the optic nerve examined under electron microscopy showed delicate perinodal processes with continuity to processes that made contact with the basal lamina, implying they provided metabolic support at the node [33]. Their role in glutamate uptake, specifically, has been proposed as a mechanism by which they support axonal and oligodendrocyte metabolic and ionic integrity (reviewed by [34]).

One additional role for perinodal astrocytes that is particularly relevant to the present thesis was commented on in the previous section: thrombin mediated cleavage of anchoring proteins which attach paranodal myelin loops to the axonal membrane [17]. The authors show that extracellular thrombin can be inactivated by an astrocyte-released factor, preventing the detachment of myelin loops. Knocking down the release of this factor allowed for the cleavage of myelin attachments to the axon, subsequently removing the wrap, with the consequence of increasing the size of the node of Ranvier and decreasing conduction velocity. Earlier work from the same group showed that other factors released by astrocytes directly modulated myelination in an action potential dependent manner [35]. Other additional factors secreted by astrocytes which may support developmental myelination or remyelination have been identified (reviewed by [36]). However, these two astrocytic regulators of myelination (activity dependent

encouragement of myelination and control of remodeling) are of particular interest in the current study, as they address a potential contributory mechanism to the formation of new myelin, or a mechanism by which existing myelin might be remodeled.

The final component of astrocyte dynamics relevant to the current discussion is astrocyte responses following injury. The potential "activation" of astrocytes following insult towards a pro- or anti-myelinating phenotype is the source of a relatively new debate, and the specific contexts which dictate the phenotype are a source of ongoing research (reviewed by [36]). Beyond astrocytic effects on the oligo-lineage, injury induces astrocytes to contribute to the formation of a glial scar around the lesion, a series of chemical and physical barriers long thought to contribute to the lack of neural regeneration (reviewed by [37]). Here, glial progenitors and astrocytes contribute to repulsive cues, such as chondroitin sulfate proteoglycans (CSPGs) and myelin associated inhibitory molecules (reviewed by [38]). However, contradictory findings have emerged from experiments which show that decreasing the astrocytic scar fails to improve regeneration [39]. The lack of substantial progress in promoting regeneration through the removal or prevention of CSPGs has led some to posit that the fundamental tenet of astrocytes/scar and CSPGs as a key inhibitor of regenerations needs to be re-examined [40]. One potential reason for conflicting evidence and a lack of consensus may be the confound of lumping multiple experimental injury types, severities, and models when trying to dissect astrocyte and glial precursor dynamics following injury. No doubt, the interplay between cell types, inhibitory cues, and regenerating axons is complex; additional research will hopefully shed light on this important component of SCI treatment.

4.4 OPCs, Myelin, and Oligodendrocytes as More than Insulators

Classically, the main functional role of myelination is to insulate axons to affect signal propagation. As our appreciation for myelination as an integral component of normal functioning deepened, so too has our understanding of the additional roles it plays besides regulating the speed of action potential conduction. This section will review a few relevant studies examining additional roles the oligo-lineage may play in both the intact and injured nervous system.

To test if myelinated axons required myelin to survive, the effect of sustained demyelination on axons in the corpus callosum was examined using a cuprizone model of demyelination [41]. The authors prevented remyelination by depleting the OPC population via irradiation, which led to reduced numbers of surviving axons. This effect could be rescued by transplanting OPCs which induced rapid remyelination [41]. One potential supportive role the myelin sheath plays is to provide metabolic support for the axon, as the segregation of the axon from the extracellular space by its myelin wraps presents a unique metabolic challenge. Oligodendrocytes express machinery for lactate transport, and knocking it out leads to axonal degeneration, implying metabolic support by myelin is necessary for myelinated axon survival [42]. Furthering the evidence for myelin support of axons, oligodendrocytes and sections of non-compact myelin release exosomes upon stimulation by glutamate [43]. These exosomes prevented metabolic stress in neurons, supporting the concept of myelin as a supportive structure. The relatively rare instances of unmyelinated segments of axons in the present study (Fig. 4.1) could be interpreted to support the concept of myelin as a supportive structure: the axons of the CST are centimeters away from their cell bodies, and complete coverage of axons by myelin might be an essential support. However, the present study was not a complete analysis of all axons in the CST. Definitively describing the percentage of unmyelinated axons and a loss

of function study preventing (re)myelination would be necessary to posit if myelination is necessary for CST axon survival.

The role of the proliferating population of OPCs in the intact and injured nervous system is another important factor to consider. Do OPCs contribute to homeostasis and recovery besides as the source for myelinating oligodendrocytes? Studies describing OPCs in both the intact and injured system clearly illustrate a dynamic population capable of detecting and reacting to environmental cues (e.g. [44][45]). Following injury, cells expressing Ng2 (a marker for OPCs) react by proliferating (e.g. [46]) and do not only contribute to the oligodendrocyte pool; they display the ability to form astrocytes and contribute to the glial scar [45] and form myelinating Schwann cells [47]. Furthermore, Ng2+ cells may contribute to prevention of regeneration of axons at the glial scar border [48]. If driving neural activity also induces proliferation of the OPC population, as has been shown in multiple studies (e.g. [4], [49]) the behavior of OPCs in the injured nervous system following neuromodulation is of particular importance. Future work using lineage tracing and proliferation markers may examine how electrical stimulation affects OPC behavior and differentiation at the scar. As was discussed in previous sections, managing the glial scar following injury is an important question facing the field; detailing how interventions such as stimulation help or hinder will provide vital information for increasing regenerative efforts.

Though there was no difference in the average myelin sheath length at the lesion with stimulation (Fig. 2.3F), this section highlights several other features of OPC dynamics and myelination, besides sheath length, that might affect neural functioning at the lesion. In the same regard, activity-induced differential myelination patterns distant from the injury (i.e. shorter sheaths) may not just be a response to a necessity for altered conduction velocity but may also

contribute in other ways to axonal functioning. Additional experiments that probe the extent to which neuromodulation and putative newly proliferated OPCs contribute to axon health, remyelination, or other processes at the lesion will provide valuable information to increase our understanding of the dynamic role OPCs and myelin play in both the intact and damaged nervous system.

4.5 Potential Mechanisms of Activity Dependent Myelin Plasticity

The mechanisms of neural activity dependent initiation and maintenance of myelination are an ongoing line of research. Axons of the CST are glutamatergic [50]. Studies have investigated the potential for activity dependent axonal vesicular glutamate release as a messenger to OPC populations to induce myelination (e.g. [51][52]). Beyond the initial events of myelination, work in zebrafish has shown that vesicular release may be more important for maintaining sheaths than for their initiation [53]. Indeed, the axo-myelinic synapse formed between fully formed myelin sheaths and the axon has been described as a glutamatergic dependent mechanism to modulate oligodendrocyte calcium levels, which may govern oligodendrocyte morphology and signaling [54]. Other neurotransmitters have been shown to affect OPC or oligodendrocyte ion currents (e.g. GABA [55]), and OPCs have been shown to express different receptors at different developmental states [15] implying there may be a temporal constraint to the type of signaling that is most efficacious at affecting myelination. In addition, as highlighted above, astrocytes play a role in activity dependent initiation of myelin [35]. Clearly, activity dependent myelin plasticity is a complex process involving axon signals, proliferation of OPCs, initiation of myelination, and maintenance of nascent sheaths. The next section will look beyond local signaling between individual axons and individual OPCs to consider how neuromodulation might exert more global effects on tracts within the CNS or the whole organism, and how these global effects might in turn influence myelin plasticity.

4.6 A Holistic Approach to Neuromodulation and SCI

There is strong evidence for direct signaling between axons and OPCs and myelin [6], [51], [52], [54], [56]. However, a second component of this work was to consider neuromodulation and its effect on SCI more globally, specifically the gut microbiota and functional recovery. To that end, this final section will highlight a few studies regarding the reciprocal effects of neuromodulation on other systems in the body, and how indirect neuromodulation might also affect myelination. Furthermore, it is necessary to address the potential for neuromodulation on myelin plasticity to address sequelae of SCI and to improve functional recovery.

4.6.1 *Intended Local Targets and Unmeasured Global Effects*

The stimulation paradigm used in this study was chosen because it was tractable and physiologically relevant; the CST was activated in motor cortex, the signal propagated through the lesion, and reached forelimb muscles in the periphery. The timing of evoked potentials measured in the forelimb muscle (Fig. 4.4) ensured the CST was stimulated (as opposed to other descending motor pathways) [57]. However, though CST fibers in the rat innervate interneurons that synapse on motor neurons, they also synapse onto local motor and sensory processing circuits in the spinal cord (reviewed by [58]). Furthermore, stimulation induced muscle contraction would inevitably activate cutaneous and proprioceptive afferents to the CNS. Though stimulation was at 80% of movement threshold, all of this circuitry was likely modulated to some extent by the stimulation paradigm. Indeed, in all *in vivo* studies that modulate activity it is difficult to discriminate how off-target effects of stimulation might contribute to the variable being measured. This chapter presents many studies that provide strong evidence for local, direct

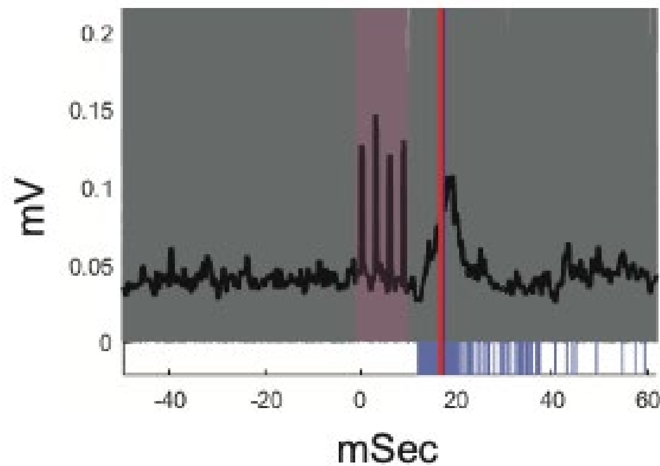

Figure 4.4 Sample Evoked Potential Measured in Forelimb Muscle in Response to Cortical Stimulation

Triggered average of electromyographic signal of 172 stimulation events. Pink box defines the stimulation artifact (4 pulses at 200 μA). The blue line illustrates the detected evoked potential at a latency of 17.05 mSec after the stimulation onset.

interactions between axons and oligo-lineage cells, but the possibility for secondary effects to affect neural activity and myelin plasticity must also be acknowledged.

Multiple studies already cited in this work have shown that neuromodulation dependent myelin plasticity might be induced by altering circuit activity without optogenetic, chemogenetic, or electrical stimulation. For example, modifying sensory input is an indirect form of neuromodulation, and multiple studies have utilized such mechanisms to alter myelination patterns (e.g. [10], [11]). Even less direct mechanisms, such as social isolation, affect myelination patterns in the prefrontal cortex [12]. Modulating neural activity through indirect mechanisms may have unintended additional consequences; for example, social isolation is likely doing more to an organism than decreasing activity in the prefrontal cortex. As was the case in the present study, the possibility that social isolation or sensory experiences have

unmeasured consequences on neuronal conduction or plasticity must be considered. "Activity dependent" myelin plasticity might also be modified by off-target effects of altering neuronal activity. Nonetheless, these studies show that directly stimulating the circuit of interest is not necessary to evoke myelin plasticity, as processing external stimuli and experiences was sufficient to alter myelination patterns.

In the context of SCI, it would certainly be preferable to use less invasive neuromodulation than intracortical stimulation as treatments for neural regeneration, myelin plasticity, or functional recovery. For example, exercise would certainly drive activity in motor circuits, and physical therapy is often a component of treatment following neural injury. In rats exercise improves cognitive performance following brain injury through brain-derived neurotrophic factor (BDNF) signaling [59]. Intriguingly, BDNF signaling onto OPCs is necessary for activity dependent myelin plasticity [60]. Together, these studies provide a mechanism by which exercise after neural injury may contribute to recovery, potentially in part through myelin plasticity. Indeed, as researchers further elucidate the role of myelin plasticity in normal functioning, we may discover many more phenomenon in which myelin plasticity plays a part. The remaining question is to determine exactly how myelin plasticity contributes to functional recovery in SCI and other neural injuries.

4.6.2 *Neuromodulation, SCI, and Non-Neural Organs*

Deficits and dysfunction following SCI are not limited to the CNS. As was discussed in chapter 3, microbiota disruption occurs following SCI, negatively influencing recovery [61]. Besides gut dysbiosis, SCI also induces "leaky gut," wherein bacteria populations exit the gut and take up residence in other organs, such as the spleen, liver, and lung [61]. Autonomic dysreflexia is a sequelae of SCI wherein autonomic control of blood pressure and heart rate is

disrupted (reviewed by [62]). As our understanding of SCI as a whole-body disease increases, so too must treatments also address multi-organ symptoms and sequelae. In addition, we must consider the effects off-target consequences of interventions, such as stimulation. For example, as spasticity and autonomic dysreflexia are thought to be caused by aberrant sprouting and neural plasticity (reviewed by [62]), care must be taken to prevent neuromodulatory interventions from worsening SCI sequelae.

4.6.3 *Activity Based Paradigms to Induce Functional Recovery*

Stimulating the injured nervous system to induce neural plasticity and functional recovery is an ongoing field of research (e.g. [26], [63], reviewed by [64]). The extent to which activity dependent myelin plasticity can contribute to functional recovery following SCI remains to be seen. The present study established that inducing myelin plasticity did not worsen functional outcomes (Fig. 3.8 & Fig. 3.9), but longitudinal experiments would be necessary to truly establish whether a stimulation paradigm that induces myelin plasticity also improves motor recovery. In addition, if improved functional recovery is observed it will be necessary to discriminate between contributions of neural plasticity and myelin plasticity.

4.7 CONCLUSION AND FUTURE DIRECTIONS

The experiments presented in this thesis probed the potential for neuromodulation of the CST to affect axon and myelin plasticity, CNS transcription, microbiota populations, and functional recovery following SCI. The results yielded the first evidence of long-range tract remodeling in response to altered neural activity, with injury as a potential primer for plasticity. Specifically, at distances 8-10mm from the lesion, where injured animals normally exhibit axons and myelin similar to uninjured controls, we found axons may be thinner (Fig. 2.5 & Fig. 2.7), myelin is thicker (Fig. 2.4), and shorter myelin sheaths (Fig. 2.3) after 3 weeks of electrical stimulation of the CST. We predicted that shifts in transcription would be required to induce altered morphology, so we conducted RNA-sequencing on a subset of animals at an early time point in the stimulation paradigm. However, none of the candidate genes identified as regulators of myelin or nodal plasticity were altered after one week of stimulation, and less than 100 genes were differentially expressed with stimulation (Fig. 3.3). After three weeks of intervention, stimulated animals did not display any statistically significant difference in motor recovery as measured by a forelimb reaching task or locomotion task (Fig. 3.8 & Fig. 3.9). There were no statistically significant differences in levels of neurotransmitter produced by cecum microflora with stimulation as compared to sham (injured and implanted without stimulation) or naïve (no injury no implant) controls (Fig. 3.4). There was an intriguing finding of increased cecum GABA levels in animals that displayed better final scores in the pasta matrix reaching task (Fig. 3.5). Altogether, these experiments provide strong evidence for activity driven myelin plasticity following SCI and inspire several potential future directions to elucidate more of the mechanisms involved and potential applications to SCI treatment.

Following injury new myelin is thought to only be derived from newly proliferated and differentiated OPCs [65]. The present study presents the first ever evidence of differential myelin patterns distant from the lesion. Thus, the next logical step is to determine the oligodendrocytes responsible for the differential myelination. It may be that extant sheaths are remodeled (Fig. 1.1B), as has been demonstrated in mouse cortex [11]. Mature oligodendrocytes may extend new myelin sheaths, though there has been no evidence published yet that this phenomenon occurs following SCI. Finally, OPCs might be induced to differentiate and replace old myelin sheaths with new, shorter ones. To address this question, future experiments might be better conducted in mouse models, where genetic tools and *in vivo* imaging [66] will allow for fate mapping and live imaging of myelin plasticity.

The goal of this study was to ascertain whether or not there was potential for activity-dependent myelin plasticity in the injured nervous system. Now that the potential has been established, future experiments are necessary to detail which patterns and durations of stimulation most affect myelin dynamics and functional recovery. There is evidence that a single stimulation event does not induce myelin plasticity [67], but the minimum number of events for the induction of myelin plasticity has not been established. Furthermore, there is little consensus across the literature regarding stimulation frequencies, and only a few experiments have used more than one type of stimulation paradigm. Thus, the field could benefit from a rigorous assessment of multiple stimulation paradigms. Such a study could address which features of myelination are most affected (e.g. OPC proliferation vs. myelination initiation vs. maintenance) by varying features of stimulation (e.g. frequency, number of events, duration of stimulation period). The present study used three weeks of stimulation to target a window of OPC proliferation and remyelination following SCI [46], but future work should examine the effects

of shorter, longer, or even intermittent periods of stimulation. One potential reason we failed to see an improvement in functional recovery in our stimulated animals may have been the short time scale of this experiment; future work which tracks recovery in stimulated animals for longer, with a variety of stimulation paradigms, may elucidate not only which patterns of stimulation target myelination plasticity but also whether those changes persist, and whether they promote functional recovery. In conjunction with the above experiments, tissue should be collected to further elucidate how these differential patterns of stimulation affect the gut microflora, particularly in regards to recovery.

This thesis explored the potential for electrical stimulation following SCI to induce activity dependent myelin plasticity and affect other sequelae of injury. The evidence for myelin plasticity has inspired additional questions to discriminate its mechanisms and potential applications. Furthermore, these data also provide evidence to seek out the extent to which white matter plasticity with neuromodulation may contribute to functional recovery in SCI.

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