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De-Novo Myelination of Grafted Neural Progenitor Cells In the Adult CNS

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by

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ABSTRACT OF THE THESIS

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While spinal cord injury (SCI) affects as many as 1 in every 250 people in the US, there are currently no clinically available therapies capable of improving functional recovery. SCI Patients experience debilitating paralysis,

increased pain, and decreased quality of life. As such developing an effective therapeutic technique is vital.

One recent therapeutic advance utilizes stem cell transplantation as a means to relay information across a spinal cord lesion. Embryonic day 14 (E14) spinal cord cells, constitutively expressing enhanced green fluorescent protein (EGFP), grafted into adult rat spinal-lesions; differentiate into neurons, grow long distances, become myelinated, and create functional neural relays.

3 months after E-14 hemisection grafts, GFP stained spinal cord cross sections caudal to the lesion, were viewed using transmission electron microscopy (TEM), in order better characterize the graft derived axons in the host spinal cord.

In total 104 Graft derived axons were examined. Axon diameter ranged from 0.15μm to 1.7μm. It was found that 23% of graft-derived axons were myelinated by host Oligodendrocytes. The average diameter of myelinated axons was significantly larger than that of non-myelinated axons ($p<0.05$). Interestingly the G-ratio of myelinated axons was equal to that of developmentally myelinated axons, potentially overturning previous notions (0.766μm ± 0.045μm). Finally it was established that 2/3^{rds} of graft-derived axons came into direct physical contact with host myelin, laying credence to the possibility that myelin may be a growth permissive substrate for early stage neurons.

Chapter 1: Introduction

1.1 Spinal Cord Injury: Patient Overview

Spinal cord injury (SCI) is an extremely debilitating affliction for which, currently, there are no effective therapies available. SCI can broadly be described as an insult to the spinal cord resulting in a loss of sensory or motor function below the site of injury. It affects as many as 1 in every 250 people, and it's estimated there are anywhere from 238,000 to 1,275,000 people living with SCI in the US alone with 12,000 new cases each year (NSCISC)(Reeve Foundation). Since 2010 motor vehicle accidents account for the largest proportion of reported spinal cord injuries (36.5%); followed by: falls (28.5%), violence (14.3%) usually from gunshot or knife wounds, and finally sport related incidents (9.2%). The remainder of SCI cases (11.4%) can be attributed to birth and genetic defects, natural disasters, or unknown/unreported causes (NSCISC). The average age at injury is around 43 years old with males reported to be 2-4 times more likely than females to incur an SCI (NSCISC)(Reeve Foundation).

In recent decades medical advances and care practices have substantially increased the life expectancy of SCI patients. From the 1950's to the 1970's the average survival time following SCI was estimated to increase from 4.5 years to just over 9 years, with most deaths occurring in the first year post injury (Deitric and Russi 1958) (Le and Price 1982)(DeVivo et al. 1989).

The current estimated survival period following injuries has continued to increase with the average for some populations nearing 40 years (NSCISC). However, life expectancy among SCI patients is only 70%-80% of the general populations, with Pneumonia and Septicemia being the leading causes of death; both are exacerbated by immobility (McColl et al. 1997)(NSCISC).

Following SCI, patients' quality of life (QoL) significantly decreases while their cost of living increases. QoL is a multifaceted measurement of ones overall satisfaction as determined through self reported patient questionnaires, which often separate life satisfaction into discrete categories (Tate et al. 2002). Leading causes of dissatisfaction among patients are often attributed to; bowel and bladder control, pain, spasticity, sexual life, self-care, and lack of motor function (Noonan et al. 2007)(Van Koppenhagen et al. 2007). Currently bowel and bladder control, and to some degree pain and spasticity, can be modulated through surgical or pharmaceutical therapies. However, intervention is not always effective, can have unwanted side effects, and can be incredibly expensive. In fact, specialized care costs often exceed government-aided funds for SCI individuals unable to work, placing the monetary burden, and often strain on family members (Unalan et al. 2001). In fact, one study on the QoL of primary caregivers to SCI patients, often a spouse, reported scores significantly lower than age match controls in all QoL categories, except bodily pain (Unalan et al. 2001). All expenses considered the first year post spinal cord injury is estimated to cost an individual upwards of $1 million dollars, with subsequent

years averaging around $150,000 dollars, depending on the severity and level of injury (NSICSC).

Since the causes of SCI are diverse, the neurological deficits and symptoms among patients vary greatly. As such the spinal cord level and the severity of injury are generally used to better classify patients. The spinal cord is divided into cervical, thoracic, lumbar and sacral regions (the sacral region classically is not described as spinal cord.) Each spinal cord region is composed of segments containing only one pair of nerve-roots, which act as the only means of entry and exit for central and peripheral nervous systems. The nerve roots extend into the periphery in between vertebral bodies, bones that surround and protect the spinal cord. While the cervical region has 8 nerve roots and 7 vertebral bodies, the other regions have equal numbers of roots and vertebrae: 12, 5 and 5 respectively from rostral to caudal (Kirshblum et al. 2012). The superior most spinal cord segment damaged describes the level or site of injury (Sekhon and Fehlings 2001). Sensory neurons send afferent projections of environmental perception into the CNS through dorsal roots nearest their receptor location. For the somatosensory system, skin regions near one another send projections into the same nerve root, and are called dermatomes. Once in the CNS sensory signals may then ascend to the brain where they can be interpreted. Motor neurons send efferent projections that exit the CNS through the ventral roots nearest their muscle target. Groups of muscles near one another are innervated by the same nerve root, and are called myotomes (Purves et al. 2012) (Kirshblum et al. 2012). As such,

damaging a particular spinal cord segment results in loss of sensory perception and or motor function, paralysis, below the level of injury. Approximately 55% of spinal cord injuries occur at the cervical level, which corresponds to quadriplegia, paralysis of all four limbs. Injuries occurring at cervical segment 5 through cervical segment 1 (C5-C1) are particularly detrimental as they result in paralysis of breathing muscles, resulting in patients being dependent upon mechanical ventilation for the duration of the lives. Its estimated that the remaining 45% of spinal cord injuries are evenly spread among the other spinal levels and lead to varying degrees paraplegia, paralysis of the lower extremities (Sekhon 2001). CT scans and MRIs are used to observe the damaged spinal segment, also known as the lesion site, however the resulting images lack the sufficient resolution needed to infer the neurological deficit.

Within groups of patients with the same injury level there can still be a vast difference in physiological symptoms. The spinal cord is organized into gray matter, the location of neuronal cell bodies, surrounded by white matter, ascending and descending axonal tracts (Purves 2012). The severity of neurological deficit sustained following SCI corresponds to the extent of damage done to the cord at the injury site, often described in terms of ‘completeness’. An incomplete injury is due to sparing of some sensory or motor tracts across the lesion, resulting in a patient’s retention of that particular function. On the other hand, a complete injury would be one where all ascending sensory and descending motor tracts are damaged, resulting in ‘complete’ paralysis below the level of injury. Since current imaging techniques

lack sufficient resolution, physicians must diagnose the severity of injury based on neurological tests. Most commonly used is the American Spinal Injury Association (ASIA) Impairment Scale (AIS), which independently assesses dermatomes and myotomes, associated with each spinal nerve segment. The AIS defines injury completeness as loss of all sensory and motor function below the last sacral segments (S4-S5). Following assessment an ASIA grade is determined of (A-E), which can distinguish between; complete lesions (A), complete motor lesions with mild sensory tract sparing (B), complete sensory lesions with mild motor tract sparing (C), moderate motor tract sparing (D) and intact spinal cords (E) (Kirshblum et al. 2012).

Many aspects of the initial insult, and medical care following, shape the morphology of the lesion, and by extension the resulting severity of the injury. SCI's most often occur from a high force impact on the spine causing vertebrae to either dislocate, fracture, or both depending on the direction and torsion of the force (Holdsworth 1963). If the force applied to the vertebral column causes the spine to bend forward (from back to front) and hyperflex, then the vertebrae taking the blunt of the force can compress and fracture, causing bone fragments to embed in the spinal cord (Davis 1929). Alternatively injuries that do not shatter the vertebrae may have a force that causes ligaments supporting the vertebral column to tear or loosen, allowing the vertebrae to dislocate and slide past one another trapping the spinal cord in between. A force applied longitudinally along the vertebral column can result in a compression, or burst fracture, where the vertebral body taking the blunt of the force will completely

shatter (Holdsowrth 1963). Further the time it takes to decompress and stabilize the spine may have a major effect on a patient's neurological outcome, as compression may lead to secondary ischemic injury as well (Delamarter et al. 1995) (Fehlings and Perrin 2006).

With a loss of sensory and motor function individuals suffering from SCI are extremely susceptible to infections from unseen cuts, bedsores from lack of mobility, and urinary tract infections from self-catheterization; all of which can cause illness or death if untreated. This means patients must constantly monitor their day to day activities. Aside from sensory and motor function the autonomic nervous system can also be drastically damaged following SCI. Normally the sympathetic and parasympathetic nervous systems act in conjunction with one another to maintain a homeostatic state. However in SCIs occurring above C6-C7 there can be a disconnection between the sympathetic and parasympathetic nervous systems, which can allow for autonomic dysreflexia (AD). AD is an extremely detrimental hyperreflexia where sensory neurons near the level of injury stimulate sympathetic ganglia, which then constrict blood vessels below the lesion. Parasympathetic innervation from above the lesion is unable to mediate the reflex response, and in response a potentially deadly rapid increase in blood pressure and slowing of heart rate ensues. Many stimuli can illicit the AD response including an over full bladder, tight clothing, male erection, or even an ingrown toenail. In most cases removal from the initial stimulus should stop the reflex, but if AD persists seizure and death may occur. (Karlson et al.1999).

Damaged CNS pathways also result in inappropriate signal transmission. Neuropathic pain, a non-nociceptive signal initiated by neurons, is thought to be the perception of pain in response to signals sent by damaged neurons (D'Angelo et al. 2013). Unfortunately, this type of pain is often not alleviated by the common pharmacotherapeutics. Neuropathic pain is often treated with anti-convulsants, anti-depressants, and Gabapentin, a drug designed to mimic the neurotransmitter GABA, all with limited success for SCI patients (McCarberg et al. 2012). While there are currently no effective therapies for SCI, finding one is extremely necessary due to the high prevalence and incidence rates, stringent monetary burden, and effects on patient quality of life.

1.2 Central and Peripheral Regeneration

The earliest known patient description of SCI is thought to be writings discovered from an Egyptian physician dating back to 2500BC; the author described SCI as “an ailment not to be treated” (Hughes 1988). Santiago Ramon y Cajal, the father of neuroscience, even proclaimed, “In the adult [CNS], the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree” (1912). While our understanding of why the CNS has such abysmal regenerative capacity has increased vastly, we do not currently have any effective therapy; as such, Santiago Ramon y Cajal’s description still holds true as the central dogma of CNS regeneration.

The “harsh decree,” however, does not hold true for the peripheral nervous system, in which regeneration following nerve injury often occurs. Countless studies have been performed in order to elucidate the mechanisms behind the morphological and physiological changes that occur in the PNS and CNS following injury, and how they relate to the difference in regenerative capacity. The disparity between central and peripheral nervous system regeneration can be attributed to three major factors; the intrinsic growth potential of resident neurons, the system architecture, and the systems environmental composition. Regeneration depends on a synergistic interaction between each of these factors, which only occurs naturally in the peripheral nervous system.

Following injury peripheral nerves are able to change their intrinsic growth state and send out projections in the absence of extrinsic or therapeutic intervention. In order for this to occur a set of regeneration-associated genes (RAGs) must be expressed, and endogenous growth suppressive genes must not. RAGs represent a wide-ranging category of molecules including: transcription factors, kinases, cell adhesion molecules, and proteins of unknown function (Ferguson and son 2011). Potential RAGs have been discovered through gene expression analyses of peripheral neurons following injury (Costigan 2002)(Stam et al. 2007). Specifically the dorsal root ganglion (DRG) neurons have been instrumental in understanding the power of RAG expression, as they have both central and peripheral projections.

While the mechanisms of injury induced RAG expression are still mostly unknown, recent work has increased our understanding greatly. In brief, following axotomy there is an influx of calcium ions at the site of injury, which sends retrograde action potential to the neuronal soma (Mandolesi 2004). In some instances the axotomy may be too deleterious to the neuron causing it to initiate apoptosis. For example, DRG sensory neurons, which are particularly resistant to cell death following axotomy, will undergo apoptosis around 25% of the time following axotomy (Lewis et al. 99). If the neuron isn't killed by the injury itself, then RAG expression may be initiated (Ferguson and Son 2011). Longer-term injury signals may also be involved in changing the internal growth state of the neuron and may be due to axonal transport deficits, or immune signaling pathways (Hanz et al 2005) (Lai et al. 2008)(Yin et al. 2003).

Research into how RAGs induce a growth state in mature peripheral neurons could potentially translate into a therapy for SCI

The peripheral architecture, containing layer upon layer of fibrous and connective tissue protecting the nerve, has long been understood to be conducive towards growth. The thick layers surrounding nerves even give adequate cross sectional area for suturing, which has been successfully performed on severed nerves since the late 16th century (Ferrara 1596) (Belen et al. 2009). The smallest structural unit of peripheral nerves is the fiber, which may be composed of a single axon, if myelinated, or multiple axons if not myelinated. Myelin is a lipid rich insulation membrane made by schwann cells in the PNS and oligodendrocytes in the CNS (discussed in detail in chapter 1.3) Whether myelinated or not a schwann cell surrounds each peripheral fiber. Three layers of fibrous and connective tissue then create the structural support of the nerve. The endoneurium, a thin layer surrounding and connecting individual fibers, plays an important role in regeneration, as it is able to remain intact following nerve injury. The combination of the endoneurium and the fibers it connects, is called a fascicle. Each Fascicle is surrounded by the perineurium; a striated layer composed of alternating polygonal cells and collagen. A nerve may contain one or more fasciculi, which are surrounded and connected via the epineurium, a strong connective layer that makes up 30-70% of a nerves total cross-sectional area; the epineurium is what Gabriele Ferarra realized he could suture in 1596 (Geuna et al. 2009). Because peripheral nerves innervate nearly every part of the body, they are highly susceptible to

damage during movement or trauma, which creates the necessity for such extreme structural support and organization. Being spread throughout the body also affords the PNS a large spatial allocation and the added volume from its layered organization does not impact its function (Guena et al. 2009).

The structure not only protects and stabilizes the PNS, but also directly aids in regeneration. Following nerve injury the axon proximal to the site of injury may die back a bit, but the endoneurial tubes of both the proximal and distal nerve segments remain intact (Cajal 1928). Within hours following injury the proximal segment may begin sprouting, and within days the injured axon may start to regenerate towards the distal segment (Czaja et al. 2008). In order for regeneration to occur the intact endoneurial tubes must be cleared of debris and coated in a growth permissive substrate; both of these conditions are mediated and organized by schwann cells, and do not occur in the CNS (Cornbrooks 1983)(Stoll et al. 1989).

In the PNS the endoneurial tubes are cleared in the distal nerve via Wallerian Degeneration, segmental clearance of myelin and cellular debris (Waller 1850). When the distal axonal segment no longer receives inputs from its cell body, microtubule depolymerization and axolemema degradation occur; this is often referred to simply as axonal death or as granular disintegration of the axonal cytoskeleton (GDC) (George and griffin 1994) (Vargas and Barres 2007). In response the degradation of axons, schwann cells and Fibroblasts release high levels of Neuregulin (Nrg), fibroblast growth factor 2 (FGF-2), and an array of other neurotrophic factors. These signaling molecules act on

myelinating schwann cells in contact with damaged axons, which in response stop production of myelin related proteins and lipids. The neurotrophic factors also cause myelinating and non-myelinating schwann cells in the distal segment to dedifferentiate into a proliferative, progenitor like state (Yang et al. 2012)(Syed et al. 2010). In response myelin becomes destabilized; it unravels and separates from the axon (Trapp et al. 1988). The degenerating myelin debris acts as both a physical barrier for regeneration and a chemical barrier, as it contains myelin associated glycoprotein (MAG) and other known inhibitors of axon growth (Myelin will be discussed in detail in chapter 1.3) (Shen et al. 1998)(David et al. 1995). In the first few days following peripheral injury an immune response is initiated, during which the nerve-blood barrier breaks down; Schwann cells then recruit an influx of macrophages into the lesion site (Griffin et al. 1992)(Shamash et al. 2002). In the PNS both invading macrophages and activated schwann cells can quickly clear the obtrusive myelin and axonal debris, creating a clear pathway that regenerating peripheral axons can follow (Stoll et al. 1989).

While Wallerian Degeneration is completed within 1-2 weeks following peripheral nerve injury, debris clearance can take years to occur in the CNS (Griffin et al. 1994)(Becerra et al. 1995). One major reason for this disparity is that oligodendrocytes, the myelinating cells of the CNS, do not aid in WD. Though oligodendrocytes and schwann cells both myelinate axons, they are two very different cell types. Schwann cells will only myelinate one axonal segment, while oligodendrocytes will send out many projections and myelinate

up to 15 segments (Peters and Proskauer 1969). The composition and thickness of the myelin produced, as well as the signals for myelination are also different (discussed in chapter 1.3). A major difference of central WD is that oligodendrocytes undergo apoptosis or become quiescent in the absence of electrical signals from their axons (Barres et al. 1993a) (Barres et al. 1993b) (Frei et al. 2000). Oligodendrocytes are also unable to engulf myelin and axonal debris. While schwann cells increase in number and help clear myelin, oligodendrocytes decrease in number and become non-functional, causing myelin and axonal debris to remain as a barrier for extended periods of time in the CNS.

In order for injured peripheral axons to regenerate towards their original targets, they must cross the site of injury and grow into the distal nerve segment. Schwann cells play a major role in this process as well; they do so by attracting the regenerating axons to the injury site, creating structural support necessary for crossing the injury site, and creating a growth permissive environment in the endoneurial tubes. The neurotrophic factors released by schwann cells, macrophages, and fibroblasts, specifically nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) among others, act as chemo-attractants for regenerating axons; coaxing them towards the site of injury and into the distal nerve segment (Lindholm et al. 1987)(Zhang et al. 2000). Activated schwann cells will even adhere to one another within the endoneurial tubes, creating long support rods known as bands of bunger (BoB). Schwann cells in the BoB secrete Laminin, an extra-cellular matrix (ECM)

component and highly permissive substrate for axon growth (Cornbrooks et al. 1983).

Neither the hierarchical architecture, which provides structural guidance, nor the permissive environment provided by schwann cells are present in the CNS, and the CNS lesion site becomes a strong physical known as the glial scar While only a handful of axons may reside within an endoneurial tube, all of the axonal tracts of the CNS lie within the meninges with no physical boundaries between tracts. Peripheral nerves are able to grow along endoneurial tubes, lined with Laminin, where they are guided and nourished by schwann cells, to their original targets. However, in the CNS there are no remnant structural guidance cues following injury. Instead there are abundant amounts of myelin debris, containing the prototypical myelin inhibitors. All of the above differences are environmental factors, and understanding how they limit the CNS regenerative capacity can and has led to the development of potential therapeutic strategies (discussed in chapters 1.3 and 1.4).

In order for functional recovery to occur, large-diameter regenerating axons need to be remyelinated. Demyelinated axons have severe blocks in conduction, which may make them non-functional (Felts et al. 1997). Remyelination restores saltatory conduction and signal transduction. In the PNS regenerating axons are given trophic support and guidance through adhesion to the Schwann cells making up the BoB (Guena et al. 2009). It's currently hypothesized that reciprocal signaling between the axon and Schwann cells via cellular adhesion molecules, cadherins, integrins, neurotrophic factors, and

Neuregulin, cause Schwann cell differentiation into myelinating Schwann cells (Ficker and Bennett 2011) (Anderson et al. 2005) (Stassart et al. 2013). Axons release size dependent concentrations of Neuregulin 1 type III, which then acts as a signal for the differentiated Schwann cells to remyelinated (Brinkman et al. 2008) (Michailov et al. 2004). Although the CNS does not readily regenerate axons, oligodendrocytes have been shown to remyelinated axons in the adult mammalian CNS, but atypically with thinner myelin sheaths (Blakemore 1974) (Ludwin and Maitland 1974).

1.3 Myelin

1.3.1 Physics of myelin

An axons conduction velocity, the speed (m/s) at which action potentials propagate is determined by various physical properties of the axon. During an action potential an influx of positive charge opens voltage gated ion channels in the axonal membrane. The passive distance that the influx of charge can propagate before decaying is known as the length constant, and a longer length constant equates to a faster conduction velocity. The resistance or, “leakiness” of the membrane, axon diameter, and resistance of ions to flow within the axoplasm, all have an effect on the conduction velocity.

Myelination is a means of increasing conduction velocity without increasing axon diameter (Goldman and Albus 1963). Along myelinated segments charge is insulated within the cytoplasm and the increased membrane resistance prevents the charge from leaking out, which increases the distance that an influx of ions could passively flow, and thus the length constant increases (Kandel et al. 2000). Myelin also acts to increase conduction velocity by creating a greater distance between the cytoplasm and the extracellular fluid. The cytoplasm of a neuron and the extracellular fluid represent two electrolytic solutions separated by a thin plasma membrane. Opposite ionic charges across the membrane are attracted to one another, and accumulate on either side of the membrane. The membrane then acts as a capacitor where membrane capacitance represents the amount of ionic charge that can accumulate across the plasma membrane. Coulomb’s law states that

the attractive force of two point charges is dependent on the inverse square of the distance between them. Ultimately, a greater distance between the cytoplasm and the extra cellular fluid causes a decrease in membrane capacitance, which means that ions can flow with less resistance within the axoplasm and conduction velocity is increased (Chomiak and Hu 2009).

Axons are myelinated in segments that are separated by Nodes of Ranvier. At the nodes voltage gated sodium channels are highly concentrated. Since myelin acts to insulate the internodal segments, action potentials jump from one node to the next. This jumping fashion of signal propagation is known as saltatory conduction and greatly increases conduction velocity (Aggarwal et al. 2011).

Myelination, however, does not significantly increase the conduction velocity of all axons. The length of the internode is a property of axon diameter, thus larger diameter myelinated axons conduct signals faster than smaller diameter myelinated axons (Rushton 1951). In 1951 William Rushton mathematically modeled the relationship between axon diameter and conduction velocity of both myelinated and non-myelinated fibers. In order to do so Rushton had to make some assumptions on the properties of axons and myelin, which did not always hold true. Nevertheless Rushton's equations worked very well with most observed phenomena. One particular result of his model was that it theorized that myelinated fibers with a diameter less than 1μm would have slower conduction velocities than non-myelinated axons of the same diameter. This critical value of 1μm fits well with observations in the PNS,

but in the CNS there are many axons myelinated that are smaller than 1μm in diameter (Waxman and Bennett 1972). By revising the parameters of Rushton's model, Waxman and Bennett came up with 0.2μm as the critical fiber diameter at which myelination becomes favorable (1972).

One of the major parameters Waxman and Bennett changed was the G-ratio, the ratio of axon diameter to the total fiber diameter. Rushton set the G-ratio as a constant equal to 0.6, which is very near what is observed in the PNS. However, the G-ratio within the CNS often ranges from around 0.6-0.9 (Waxman and Bennett 1972). The reason behind differences in CNS and PNS G-ratios isn't known, but recent models of myelin optimization have suggested some clues. While Rushton assumed conduction velocity as the driving force of the G-ratio, he neglected to consider volume consumption into his model. While the total volume in the body is finite, the PNS volume far exceeds that of the CNS (discussed in chapter 1.2), as such smaller axon diameter would be important and favorable in the CNS. In order to formulate a global optimum G-ratio for the CNS, Chomiak and Hu weighed the benefits to conduction efficiency gained through myelination, against the volume taken up by increasing myelin thickness; in doing so they came up with a theoretical optimum G-ratio of 0.77 for the CNS.

1.3.2 Myelination

Myelin is essentially modified plasma membrane with an extremely high lipid content (70-85%)(Baumann and Dihn 2001)(Quarles et al. 2006). It is

synthesized by Oligodendrocytes in the CNS and Schwann cells in the PNS. While the signals mediating central myelination are not well understood, much progress has been made in understanding peripheral myelination, which may shed light onto central myelination as well. In the PNS, neurons have been shown to be in constant contact with, and direct the differentiation of, developing Schwann cells (Jennsen and Mirsky 2005). Neuregulin 1 type III (NRG1-III), expressed by neurons, regulates nearly all steps of Schwann cell development and myelination by binding Erb-B receptors present on Schwann cells. The expression level of NRG1-III in neurons is directly proportional to the axons diameter via currently unknown mechanisms.

Mice lacking NRG-1-ErbB signaling in early development show a severe absence in Schwann cell progenitors (Meyer and Birchmeister 1995). Postnatally Schwann cells either become myelinating and ensheath one axon segment, or become non-myelinating and ensheath multiple small diameter axons (Remak bundle). NRG-1-ErbB signaling activates genes associated with different Schwann cell fates and thus acts as a master regulator for differentiation (Taveggia et al. 2005). Lastly the G-ratio in the PNS is also mediated by NRG1-II-ErbB signaling., and NRG1-III over expression in neurons causes to hyper-myelination and loss of function results in hypo-myelination (Michailov et al. 2004).

Signals of survival and differentiation are not unidirectional between neurons and Scwhann cell but rather reciprocal. Scwhann cells have been shown to release trophic factors such as Brain Derived Neurotrophic Factor

(BDNF), Nerve Growth Factor (NGF) and Ciliary Neurotropic factor (CNTF) among others (Acheson et al. 1991)(Bandtlow et al. 1987) (Bunge et al. 1993). Mice developmentally lacking Schwann cells, showed a market decrease in the survival of peripheral neurons (Reithmacher 1997). Schwann cells mediate the structural reorganization of an axons membrane channels, causing them to localize at the nodes of Ranvier (Salzer 1997). Finally Schwann cells play a major role in eliciting axon sprouting and regeneration following peripheral nerve injury as discussed in chapter 1.2.

Oligodendrocytes also find their target locations by contact-mediated guidance involving various cell adhesion molecules present on axons (Bradl and Lassman 2010). The differentiation of oligodendrocyte precursors into myelinating oligodendrocytes has is dependent on Notch-Jagged signaling, but only within a tightly regulated temporal period (Genuod et al. 2002). NRG1-III also effects myelination in the CNS, but to a lesser degree, as mice lacking NRG1-III in the CNS do not show hypo-myelination, but neurons overexpressing NRG1-III do show hyper-myelination (Brinkman et al. 2008). This most likely means that multiple and potentially redundant mechanisms of inducing and controlling myelination in the CNS exist, but currently these mechanisms represent a large gap in our knowledge, which may prove beneficial for not only SCI, but also for demyelinating diseases such as multiple sclerosis.

Remyelination and myelin maintenance are also major topics of interest as many detrimental diseases involve loss of myelin sheaths, including the

leukodystrophies, multiple sclerosis (MS), and spinal cord injury. In the CNS there exists pools of quiescent oligodendrocyte precursor cells (OPCs) that can differentiate into oligodendrocytes in response to astrocyte or macrophage activation (Carroll et al. 1998)(Glezer et al. 2006). Newly formed Oligodendrocytes migrate towards the injury where they can remyelinate damaged axons (Franklin et al. 2008). However, remyelinate axons have been shown to have larger G-ratios, meaning less layers of myelin and sub-optimal conduction abilities (Ludwin and Maitland 1984). The observation of hypomyelinating phenotype has not only been used to describe remyelination, but has been hypothesized to be a property of adult Oligodendrocytes (Young et al 2013). Animal models of remyelination often use toxins such as lycolecithin and ethydium bromide to dissolve myelin sheaths, and use G-ratio analysis as the primary means of determining remyelinated axons (Blakemore and Franklin 2008).

1.4 Environmental Inhibition of Growth

Experiments from the early 1980's illustrated that a peripheral nerve graft acted as a permissive substrate for the growth of CNS axons distances up to 3.5cm (Agouyo et al. 1981). This finding had two interpretations; either the lack of growth capacity within the CNS was due to the local inhibitory environment or that the PNS contained growth promoting factors. In a seminal experiment, Caroni and Schwab plated fibroblasts on top of live spinal cord coronal slices and observed the fibroblasts implanting exclusively on the grey matter, non-

myelinated cell bodies, and not on the white matter, myelinated axon tracts (Caroni et al. 1988). Caroni et al. hypothesized that this might be do to an inhibitory molecule present on myelin itself, which led to the discovery of Nogo, the first myelin bound inhibitory protein. Nogo was named because of its negative effect on growth, no growth became abbreviated Nogo, and has since been shown to inhibit axon regeneration and growth (GrandPre et al. 2000). Since then more myelin bound inhibitory molecules, such as myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) were discovered along with their receptors, all of which have similar inhibition of axon growth (Giger et al. 2010).

Because of the prototypic myelin inhibitors effects on outgrowth in-vitro it was proposed that using antibodies to block the myelin bound inhibitory proteins would aid in regeneration by making the local environment of the spinal cord more permissive of growth. In 2003 three separate groups independently knocked out the Nogo gene in mice, predicting to find axonal regeneration after injury. In-vitro, the Nogo knockouts were shown to be effective for neurite outgrowth, however the in-vivo results were conflicting. Kim et al. reported axonal sprouting, a growth of spared axons into the injured area, rostral to the lesion, and an extensive amount of regeneration, growth of damaged axons, below the injury site in Nogo mutant mice (2003). Concurrently, Simonen et al. saw a trend of sprouting in the Nogo mutants; however, the quantification of the sprouting compared to that of control animals and was not statistically significant (2003). Finally, Zheng et al. failed to notice any degree of

regeneration or sprouting in their Nogo mutants (2003). In order to further test the effect of the prototypic myelin inhibitors a Nogo, MAG, and OMgp triple knockout was produced. While some sprouting was seen, there was no difference in axon regeneration, and no functional difference, as measured by weight bearing, range of motion, and strength behavioral tests, between the triple knockout, individual gene knockouts, and control animals (Lee et al. 2010). There are however, other inhibitory molecules present in the CNS, which may act as redundant inhibitors of axon growth and account for the lack of regeneration observed from knocking out the major myelin inhibitory molecules. For example netrins, ephrins, semaphorins, and wnt's are all known to be inhibitory to some axonal tracts present in the CNS, and have been shown to be upregulate following spinal cord injury (Giger et. al. 2010). While the myelin inhibitory molecules may inhibit growth, the excision of them from the local environment may not, in itself, be substantial enough to induce axon regeneration. Blocking the myelin inhibitors may still be a potential therapeutic but it must be accompanied with other techniques in order for axon regeneration and functional recovery.

The physical environment of the CNS following injury also acts a major barrier towards regeneration. Following CNS injury there is infiltration of macrophages, and upregulation of microglia. The onset of the immune response causes astorcytes to become activated and upregulate GFAP. Actiaved astrocytes will migrate towards the lesion site and form a tight barrier around the site of injury known as the glial scar. The astorcytic glial scar

formation is reminiscent of to their response in forming the blood brain barrier, and may have beneficial effects in blocking infective agents from entering the CNS. However, the glial scar becomes a major physical barrier for axon regeneration. One reason for this is the active astrocytes begin to release Chondroitin sulfate proteoglycans, which have been shown to inhibit axon regeneration all over the CNS. One therapy revolves around degrading the harmful CSPGS, using the enzyme chondroitinase abc, but only modest regeneration has been seen following treatment.

1.5 Cell Transplantation Therapies

1.5.1 Bridge Experiments

One of the main goals of cell transplantation based therapies has been to create a more growth-permissive environment at the lesion site in order to enable axon regeneration across it. This model of regeneration is often referred to as a bridge because the more permissive environment supports the growth of the injured CNS across it. The earliest bridge experiments were done using peripheral nerve grafts and showed that some CNS axons have an innate potential to regenerate in favorable environments (David and Aguayo 1981). These early PNS grafting studies, however, had severe limitations in the types and numbers of axons entering.

In the 1980’s cell culture techniques came about, which enabled researchers to culture purified cell types (Bunge and Wood 2012). Schwann cells were known for their ability to induce PNS regeneration, and thus were a

logical cell type to graft into the CNS. Schwann cell transplants showed modest regeneration with both ascending and descending axons growing into the graft, but none were able to exit the graft (Xu et al. 1997). This may have been due to Schwann cell's limited ability to penetrate the astrocyte border surrounding the graft, and in effect, making the bridge non-functional (Williams and Bunge 2012). In order to increase their regenerative potential, Tuszynski et al., grafted Schwann cells transgenically expressing human NGF (1998). These cell transplants had moderate success in increasing cell types entering the graft, but again lacked axons exiting.

Other adult cell types have also been isolated and transplanted as potential therapeutics for spinal cord injury. Olfactory Ensheathing Cells (OEC's) were a logical choice as they natively reside within both the central and peripheral nervous systems and display similar properties to Schwann cells and Astrocytes (Ramon-Cueto and Avila 1998). Reports of regeneration following OEC grafts have had mixed and contradictory findings. Some studies have shown a lack of axons growing past the lesion site as well as no functional benefit (Lu, P et al. 2006), while other studies have reported regeneration of sensory and motor tract regeneration (Lu, J et al. 2002). Recent publications have even shown functional improvements across the most severe model of spinal cord injury, a complete transection (Ziegler et al. 2011). Other recent studies have proclaimed OEC grafts helped negate the life threatening effects of Autonomic Dysreflexia (Alinik et al. 2010). While OEC grafts may be a

promising therapy for SCI there are still many experiments and clarifications necessary before it can be implemented clinically.

1.5.2 Stem Cell Transplantation Therapies

In the 1950's it was discovered that intravenous infusions of donor bone marrow could replenish bone marrow depleted during radiation therapy in cancer patients (Thomas et al. 1957). These early procedures were the first successful stem cell transplants and illustrate the tissue regenerative potential stem cell therapies may have. Stem cells are a wide-ranging group defined by long-term self-renewal potential and an ability to differentiate into different cell types. They are self-renewing in that division can occur unequally with one resulting daughter cell remaining an identical stem cell, and the other having proliferation and lineage restricted fates (Rishi et al. 2008). Stem cells are organized into groups determined by the cell types they are capable of differentiating into. They can differentiate into; any specialized cell from all three embryonic germ layers (Totipotent), any cell type from within 1 primary germ layer (pluripotent), any cell type within a cell specific lineage (multipotent), or only 1 specific cell type (unipotent) (Rishi et al 2008). Embryonic stem (ES) cells, found in the inner cell mass of developing embryos, are pluripotent and can have an unlimited proliferative potential in-vitro (Rishi et al 2008). Early during development stem cells may divide and become un-differentiated progenitor cells, which may retain a limited proliferative potential. Neural

progenitor cells (NPCs) are one such cell type, whose lineage is restricted to glia, and neurons.

Early ES cell transplantation studies attempted to avoid tumorigenicity by driving the pluripotent stem cells towards neural specific lineages in-vitro prior to grafting. This was achieved by McDonald et al. by culturing the ES cells in media containing retinoic acid. The resulting neutralized cells were grafted into rat spinal cord lesions and were shown to differentiated in-vivo into oligodendrocytes, astrocytes, and to a smaller degree neurons (1999). McDonald et al. showed cell survival and modest functional benefits from the cell grafts 30 days after injection, however, visualization techniques at the time limited the ability to view the grafts using histology and imunohistochemistry (1999). The functional benefit of grafted stem cells was further corroborated when Lu et al. showed that neural stem cells elicit neuroprotective effects, and promote host axon regeneration following spinal cord injury through production and release of neurotrophic factors (2003). In essence using neural stem cells is a combinatorial treatment that both changes the environmental and intrinsic growth potential of host neurons, and has added benefit, as there is the potential for cell replacement as well. While the self-renewing ability of stem cells creates the necessity for caution due to potential tumorigenicity, their potential therapeutic use may prove beneficial towards overcoming multiple aspects affecting CNS regeneration.

Because ES cell lines are most often created through blastocysts donations from fertility clinics, the use of human ES cell therapies and research

have been a hotly debated ethical dilemma. However, In 2006 Shinya Yamanaka was able to revert adult somatic cells into a pluripotent state using just 4 transcription factors (Takahashi and Yamanaka 2006). This finding reduced the ethical dilemmas associated with stem cell therapies, and also created a means of avoiding immune system rejection of transplants because IPS cells could be made from a patients own fibroblasts. IPS cells, while very promising, are still relatively young and the potential for a cell retaining memory of past, along with tumorigenicity, remain major concerns in moving forward with IPS cell therapies.

1.5.3 The Neural Relay

Cell transplantation therapies aiming towards regeneration of injured host neurons have come a long way, but significant regeneration past the lesion site remains elusive. An alternative approach is to create a neural relay in which transplanted cells induce host regeneration into the graft and relay the incoming signals beyond the lesion. Paul Reier first proposed the neural relay in the 1980's as a potential therapy for SCI (Reier 1985b). He hypothesized that the loss of gray matter neurons at the lesion site was as important as creating a permissive environment for functional recovery, and work at the time had shown that embryonic tissue implanted into the brain could form new circuits with host neurons (Bjorklund and Stenevi 1984). Reier et al. grafted embryonic day 14 (E-14) rat spinal cord tissue into surgical hemi-sections. The grafted tissue differentiated into neurons, which sent projections out of the graft, and induced

host axon regeneration into the graft (1985b). However, Reier et al. encountered major problems: Firstly the visualization and characterization of the grafted cells were limited by the techniques available at the time and the tracing methods used were not reliable; Secondly only 80% of grafts survived, this may have been due to host immuno-rejection of the allogeneic grafts; Finally, the grafts that did survive had incomplete filling of the lesion site, which most likely was due to cells exiting the graft either from the injection or CSF flow in the days following (Lu et al. 2014).

Recent innovations by Dr. Paul Lu enabled him to overcome previous hurdles and the result was unprecedented success in creating a functional neural relay (Lu et al. 2012). Complete visualization of graft-derived cells was achieved using transgenic donor embryos ubiquitously expressing enhanced green fluorescent protein (EGFP) under the ubiquitin C promoter. The EGFP construct enabled the power to visualize all graft-derived axons exiting the graft, and observe potential cell migration. In order to address the potential immune response Dr. Lu and colleagues used an inbred strain of rat for both the donor tissue and host, effectively making the grafts more syngeneic then allogeneic. Later studies with human (h) hES and hIPS cells targeted potential immune rejection by either using immunosuppression drugs like cyclosporine or grafting into athymic (nude) rats. Finally, in order for a neural relay to be functional both rostral and caudal borders of the graft must fully integrate with host tissue. Dr. Lu and colleagues were able to achieve high levels of cell survival and complete filling of the lesion site by dissociating the E-14 cells into separate

solutions of fibrinogen and thrombin along with a cocktail of growth factors. When they were mixed in the lesion site, the two solutions gelled and formed a matrix that tightly held the cells and growth factor cocktail in place.

Dr. Lu used a complete thoracic transection model in which all ascending and descending axonal tracts are cut within entire cross section of spinal cord, as functional improvement would be due to the graft rather then any sort of sprouting. Dr. Lu and colleagues techniques resulted in the formation of the first neural relay to successfully improve motor function. It was observed that grafted cells were able to differentiate into neurons, oligodendrocytes and astrocytes at relatively equal numbers. Both ascending and descending axons regenerated into the graft and synapsed on graft-derived neurons. An estimated 27,000 axonal projections were sent out of the grafts, where they grew for long distances in both rostral and caudal directions, and some became myelinated. The neural stem cell derived axons were shown to synapse with host neurons. Increased motor function observed via behavioral testing and electrophysiology, indicated the neural relay was functional, and when re-transected above the lesion site, the motor function and electrical transmission disappeared. Additionally there was a major attenuation of the glial scar observed following grafting.

While this work is exciting there are many aspects that must be further characterized in order to assess the efficacy of moving this therapy into humans. For instance, pain needs to be assessed following grafting as these newly created circuits cause the potential to elicit pain or spasticity. The

uncontrolled growth of so many new axons may also prove detrimental in of itself. One study has shown that growing axons in the adult CNS can be guided towards specific locations using neurotrophic factors, which may prove as an efficacious means to guide the graft derived axons (Alto et. Al. 2009).

The observation of myelinated graft-derived axons presents a unique situation, in which, the interaction between neural stem cell derived axons and adult oligodendrocytes may be studied. Based on the properties of myelination discussed in chapter 1.3, it was hypothesized that larger diameter E-14 graft derived axons would be preferentially myelinated by oligodendrocytes. Based on the properties attributed to remyelination following injury, it was hypothesized that the G-ratio of graft derived axons would higher then the average CNS G-ratio of 0.77.

In doing Immunohistochemistry (IHC) and Immunofluorochemstry (IFC) of Dr. Lu's work it was noticed that axons appeared to preferentially exit the graft and extend long distances in the white matter. This observation was in conflict with the present dogma that asserts myelin is inhibitory towards axonal growth, but light microscopy of grafted axons displayed potential contacts with myelin. It was thus hypothesized that, myelin may act as growth permissive substrate for axonal growth.

Chapter 2 Materials and Methods

2.1 Animals

This study was done in strict accordance with the laboratory animal care and safety guidelines set by the National Institutes of Health. All protocols were approved by the United States Veterans Administration. Animals were given food and water ad libitum throughout the duration of this study. Animal surgery, embryonic spinal cord dissociation and grafting were performed as summarized below (described previously by Lu et al. 2012). For all surgeries and perfusions animals were deeply anesthetized using a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 g/ml), and acepromazine (0.25 mg/ml).

2.2 C-5 Lateral Hemi-section Surgery

Adult female Fischer 344 rats weighing 150-200g (n=10) were deeply anesthetized. Rats were fixed in place with the help of stereotaxic equipment. A small dorsal incision was made between the C-2 and T-2 processes using a medical scalpel fitted with a #15 blade. The vertebral column was exposed through cutting the dorsal muscles and separating the underlying layers of fat. The surgical area was kept accessible throughout the duration of surgery using retractors. In order to expose the spinal cord a laminectomy on the cervical level 5 vertebrae was performed using rongeur forceps. A small incision of the dura was made along the midline using a scalpel fitted with a #11 blade. A 1-2mm block of the right hemi-section of the spinal cord was removed with iridectomy scissors accompanied by microaspiration. A visual inspection of the

lesion verified that the entire right hemi-section was removed. The muscles above the wound were sutured and the skin was secured with wound clips. Animals were given post operative injections of ringers, Banamine (mg/kg) for pain, and Ampicillin (mg/kg) to avoid infection, for 3 days following surgery. Animals were placed back in their home cages and let rest for two weeks following surgery.

2.3 E-14 Dissection and Dissociation

Spinal cords from transgenic Fischer 344 rats constitutively expressing GFP, in all cells, under the ubiquitin C promoter were dissected using iridectomy scissors and jewelers forceps. Cells were dissociated by the following protocol described by Harris et al. (2007). Dissected cords were placed in a15 ml conical tube containing 1ml of ice cold Hank's Balanced Salt Solution (HBSS). Cells were dissociated chemically with a 0.125% trypsin solution and mechanically by centrifugation followed trituration with fire polished glass pipettes. Dissociated E-14 spinal cord cells were then resuspended in solutions of 25 mg/ml fibrinogen or 25 U/ml thrombin with a growth factor cocktail at a concentration of 200,000 cells/µl. The growth factor cocktail was added to support graft survival and contained BDNF (50 µg/ml), neurotrophin-3 (NT-3; 50 µg/ml), platelet-derived growth factor (PDGF-AA; 10 µg/ml), insulin-like growth factor 1 (IGF-1; 10 µg/ml), epidermal growth factor (EGF; 10 µg/ml), basic fibroblast growth factor (bFGF; 10 µg/ml), acidic fibroblast growth factor (aFGF; 10 µg/ml), glial-cell-line-derived neurotrophic factor (GDNF; 10 µg/ml),

hepatocyte growth factor (HGF; 10 µg/ml), and calpain inhibitor (MDL28170, 50 µM).

2.4 E-14 Transplantation Surgery

Animals were placed into stereotax to fix their position during the grafting procedure. The spine was straightened and kept horizontal by clamping a small curved hemostat onto the T2 process. The lesion site was carefully reopened in the same manner as the hemi-section surgery. A glass pipette puller was used to make microinjection needles. Needles were filled with either 2.5µL of fibrinogen or 2.5 µL thrombin cell suspension solutions. A small hole was punctured in the dura above the lesion center using a 27-gauge insulin syringe. The glass needle was fixed into a picospritzer stereotax attachment, and the needles tip was positioned into the lesion center. The picospritzer was set to short duration bursts, and cells were slowly injected until the entire lesion volume was filled. In the lesion the fibrinogen and thrombin solutions mixed in equal parts forming a fibrin matrix. Animals received the same post-operative care as described above and subjects survived for 12 weeks post-grafting.

2.5 Fluorescent Microscopy

Four weeks after grafting, animals (n=6) were heavily anesthetized, and transcardially perfused first with ice cold saline, and then 4% paraformaldehyde (PFA) and dissected. Tissue was post fixed in 4% PFA overnight at 4° C and then placed in a 30% sucrose solution for 48 hours at 4°C. Blocks of tissue 2-4

cm around the graft were cut using razor blades, and placed into tissue freezing medium on dry ice. Sections of 30μm were cut using a cryostat.

Free-floating sections were washed in Tris-Buffered Saline (TBS) then incubated for 1 hour in TBS containing 5% donkey/goat serum, and 0.25% Triton. Sections were then incubated overnight at 4°C in TBS containing 5% donkey/goat serum, 0.25% Triton and primary antibodies against; jellyfish green fluorescent protein indicating graft origin (GFP, rabbit, 1:1,500) adenomatous polyposis coli, a marker of Oligodendroctyes, (APC, mouse monoclonal, 1:400); myelin-associated glycoprotein, indicating myelin (MAG; mouse, 1:200). Sections were washed, and then incubated at room temperature for 2.5 hours with Alexa 488, 594, or 647 conjugated donkey/goat secondary antibodies (1:250) along with nuclear stain DAPI (1:1000).

2.6 Immuno Electron Microscopy

EM was done with the help of the UC San Diego Cellular and Molecular Medicine Electron Microscopy Facility (CMMEMF), and following their protocols. Animals (n = 4) were transcardially perfused with a 1.5% glutaraldehyde, 3% paraformaldehyde, and 5% sucrose solution (pH 7.4) and post-fixed over night in the same solution at 4°C. A coronal block of spinal cord, 1-2 segments caudal to the graft, was cut with a razor blade, and 50-100μm sections were sliced using a vibratome.

Free-floating sections were freeze fractured with liquid nitrogen, in order to help permeate the membrane, quenched in a 0.6% hydrogen peroxide

solution, to block endogenous immunoreactivity, and incubated with an Invitrogen rabbit anti-GFP primary antibody, diluted 1:3,000, over night at 4°C. Sections were then washed and incubated for 1 hour with a Vector Laboratories horseradish peroxidase (HRP) conjugated secondary antibody, diluted 1:50, at room temperature. Finally the sections were placed into a 0.05% diaminobenzidine (DAB) solution, with 0.04% nickelchloride to darken the stain from brown to black. The ensuing reaction was carefully monitored for dark staining with low background, which usually occurred within 2-10 minutes. Samples were left in tris-bufered saline (TBS) and brought to the CMMEMF for EM preparation and ultra-thin sectioning according to their protocols. In brief sections were post-fixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Durcupan epoxy resin. 60nm thick sections were then cut with a diamond knife using a Leica UCT ultramicrotome. The ultra-thin sections were then stained with 2% uranyl acetate and Sato's lead stain, and placed on a copper support grid. All samples were observed using a JEOL 1200EX II transmission electron microscope (TEM) and photographs were taken using a Gatan digital camera. Sections were scoured systematically and all observed GFP (+) axons were photographed at 1,500x to 10,000x magnifications

2.7 Quantification of Graft Derived Axons

All images were assessed and quantified using Adobe Photoshop CS6. For distance measurements, the scale bar generated by Gatan's digital camera software was converted into a 1 pixel thick horizontal bar. A custom measurement scale was created in Photoshop where the number of horizantal pixels in the scale bar was set to equal the actual distance of the scale bar; this meant every pixel was converted to the actual area in micrometers. Custom measurements were set up as described for each image magnification used and saved for future use. Photoshop's image analysis ruler was then used. Clicking on any two points of the image gave displayed an output function of the height, width, and distance between the two points in micrometers. The area of irregular shapes could also be displayed in micrometers by outlining the perimeter of the shape using the lasso, quick selection, or magic wand tools.

Axon Diameter was measured for relatively regular shaped axons as the average diameter of the longest and shortest distance across the center (figure 1a). For irregular shaped axons the area was found as described above and diameter was described by the following: $axon\ diameter = 2\sqrt{\pi(area)}$. The fiber diameter of myelinated axons was calculated as the axon diameter added to two times the average distance across two or more well-preserved areas of myelin. The G-Ratio was quantified as the axon diameter divided by the total fiber diameter (figure 1B).

Chapter 3: Results

Our labs recent success with grafting neural precursors has brought up many questions as to the morphology and function of the vast number of axons observed growing out of our neural stem/progenitor cell grafts. In Dr. Lu's publication it was shown that graft derived axons could be myelinated, however, the amount of graft derived axons myelinated and the quality of myelination were not characterized (2012). The present study aimed to better characterize graft derived axons, and gain incite as to the interactions of these axons with the adult CNS environment.

In order to better characterize E-14 graft derived axons, transmission electron microscopy (TEM) was chosen because of its ability to resolve fine structural details and make accurate quantifications. Immuno-EM labeling of coronal sections, 1-2 segments caudal to the C-5 hemi-section graft, was accomplished using Diaminobenzidine (DAB). When using TEM, DAB labeled axons in ultra-thin (50nm) coronal sections show up as black granular structures that can be distinguished from the surrounding tissue. Sections were systematically looked over and any DAB labeled structure was imaged at various magnifications (ranging from 1,500x to 10,000x).

3.1 Graft Derived Axon Diameter

The first aim of this project was to observe and quantify the diameter of graft derived axons. In total 104 GFP positive axons were characterized in the white matter 1-2 segments caudal to an E-14 hemi-section graft (figure 1b,1c). The diameter of graft-derived axons ranged from 0.15μm to 1.7μm. The mean diameter of all sampled GFP positive axons was 0.64μm with a standard deviation of 0.26μm (Figure 3a,b). Axon size was distributed normally about the mean (figure 3b). Overall the observed axons are within the lower-medium sized spectrum of normal adult CNS white matter. Only three axons were found to have diameters smaller than 0.2μm the theoretical diameter below which myelination is no longer beneficial (discussed in chapter 1.3).

3.2 Myelination of Grafted Neural Progenitor Cells

The process of myelination in the CNS involves interactions between oligodendrocytes and axons, which, at the present, are poorly characterized. It has been shown that reciprocal chemical and electrically signaling between oligodendrocytes and neurons is involved, and that larger diameter axons have higher amounts of both (Barres et al. 1999). In order to test if larger diameter axons were preferentially myelinated axons were first grouped into myelinated and non-myelinated categories using a visual assessment. In order for a myelinated axon to be quantified, the tissue preservation had to be quality enough to obtain the quantifications displayed in figure 2. Although data is not shown, a number of animals (n=12), were assessed in the same region of white

matter as presented in this study, for graft derived Oligodenrocytes through IFC of free floating sections for GFP and APC, and zero observed GFP/APC positive cells were found. Based on this we can make the assumption that myelination was done by host Oligodendrocytes.

Of the 104 axons quantified a substantial 23 percent were observed to be myelinated (Figure 4a). The smallest diameter myelinated axon was 0.355μm and the largest was 1.7μm with a mean diameter 0.72μm (Figure 4b,c,d). By grouping myelinated and non-myelinated axons into 0.1μm bins (figure 4b) there becomes a trend that larger diameter axons have an increased likelihood of being myelinated. This trend becomes more apparent by dividing the number of myelinated axons in each bin by the total number of axons (Figure 4c). Running a Lavene's test on myelinated and non-myelinated axon diameter showed that variance was equal and that an independent samples T-test could be run. Setting confidence limits at 95% the one-tailed hypothesis was tested that graft derived myelinated axons have larger diameters than non-myelinated axons (Figure 4d). The observed axon diameter of graft-derived axons was significantly larger for myelinated axons than non-myelinated ($p = 0.0335$), thus confirming the hypothesis that larger diameter axons would be preferentially myelinated.

3.4 G-Ratio

Numerous studies have stated that adult Oligodendrocytes lack the ability to myelinate axons like developmental Oligodendrocytes can, resulting in increased G-ratios—thinner myelination (described in chapter 1.3). Researching the

literature on the myelination of neural tissue and stem cell grafts proved fruitless, however, it was hypothesized that the dogma of adult Oligodendrocyte hypomyelination would hold true in the present study. In quantifying the 24 myelinated axons it was found that the spread of G-ratios was very tightly bound around a mean of 0.766 (standard deviation 0.046) (Figure 5a,b). The effects of hypomyelination are said to be most pronounced in axons with fiber diameters (diameter of axon + myelin) greater than 1μm (Blakemore and Franklin 2008). However, there were no apparent trends in axon diameter affecting the G-ratio and even the largest diameter axon (1.7μm axon diameter and 2.3μm fiber diameter) had a G-ratio of 0.74. A one sample T test comparing the graft derived G-ratios to the developmental population average of 0.77 confirmed that there was no statistical significance ($p=0.634$).

3.5 Graft Derived Axon Host White Matter Interaction

In order to test the hypothesis that host myelin may act as a permissive substrate for axonal growth physical interactions between grafted axons and myelin were quantified. For non-myelinated axons contact was defined as any part of the axon clearly touching host myelin (figure 6a). As myelinated axons are already unsheathed contact was defined by any part of the fiber in touching another myelinated axon (figure 6b) 84% of myelinated fibers, and 60.8 % of no-myelinated axons were in physical contact with host myelin for a total 66% of all observed axons (figure 6c). While this is not proof in of itself that myelin is

permissive towards growing early stage axons, it does represent a that significant interaction does occur.

Chapter 4: Discussion

Understanding how developing neurons interact with the adult CNS may prove beneficial for spinal cord injury, as well as all potential cell transplantation therapies. As much progress has been made towards driving neural stem/progenitor cells towards specific fates, the question of what cell types might be best for regeneration therapy arises. The data presented in this study suggest that larger diameter axons may be preferentially myelinated by host Oligodendrocytes. This presents a novel therapeutic strategy of culturing and grafting populations of neural stem/progenitor cells, driven towards large diameter axonal fates.

Increasing the overall diameter of graft-derived axons may prove beneficial for multiple reasons. First, in order to produce the most effective and functional neural relay, signal transduction must be as strong and efficient at possible. As discussed in chapter 1.3 the physics of larger axon caliber alone lead to increased signal conduction velocity and efficiency, both of which are increased further upon myelination. As stated earlier the effects of creating a functional relay on pain and the potential for aberrant signal transduction need to be carefully studied, nonetheless, potentially increasing the strength and speed of signals crossing the lesion cavity may prove beneficial. The increased electrical activity and chemical expression from larger diameter axons (discussed in chapter 1.3 as

important developmental cues for OPC differentiation and migration) may also prove a benefit of grafting larger diameter axons.

Although predetermining axonal caliber at stem or progenitor cell stages is not currently feasible, transgenically expressing proteins that larger caliber neurons may express is. One future project, which has already been initiated, is transfecting cultured E-14 cells with virus containing NRG1-III (discussed in chapter 1.3). As discussed NRG1-III has been shown to induce hypermyelination and OPC differentiation in the developing CNS, but not in the adult CNS. While this could be viewed as an innate property of adult OPC's, it may instead represent a lack of signaling molecules in adult axons. This could be studied by comparing transplanting E-14 spinal cord cells overexpressing NRG1-III and observing OPC differentiation and myelination as compared to overexpression of NRG1-III in host neurons, or in non-stem cell grafts expressing NRG1-III.

Following demyelinating injuries or lesions, it has been shown that adult oligodendrocytes hypomyelinate their axonal targets, however, why this occurs still isn't understood (Bunge et. al. 1961)(Blakemore 1974)(Gledhill and McDonald 1977). It's been proposed that hypomyelination is a property of adult oligodendrocytes (Young et al. 2013), however, the data presented above indicates otherwise. Since reciprocal signaling between axons and oligodendrocytes is necessary for myelination (Barres et al. 1999), and the E-14 graft derived cells were not hypomyelinated, it can be inferred that the hypomyelination observed following injury is due to a miscommunication of axonal caliber by adult neurons. This finding may have lasting effects on future

approaches for not only spinal cord injury but also demyelinating diseases such as MS. Once the signals for CNS myelination are discovered, they could be implemented to induce normal myelination from endogenous adult OPCs.

As shown in this study the functional relay model, developed by Dr. Paul Lu, may also double as a novel in-vivo technique for studying myelination. The current in-vivo techniques use harsh that dissolve membranes, like ethydium bromide and lycolecithin. Since myelin is essentially a modified plasma membrane, then axonal membranes may be damaged as well, which could skew results and diminish interpretations. Currently, one of the main ways that remyelination is distinguished from surrounding spared tissue is by the characteristic high G-ratios. However, based on the results presented, this may not always be the case, and it is possible that normal myelination is occurring following chemical demyelination, but with no way of being observed. Grafting neural stem/progenitor cells expressing GFP, gives a unique ability to assess any and all de-novo myelination. Because cells can be cultured, and thus manipulated, prior to grafting, this model may prove effective in elucidating the mechanisms involved in myelination.

Finally the observations of growth of graft derived axons into and along host white matter has brought up many questions as to how and why these developmental neurons are unaffected by the prototypic myelin inhibitors. Further research in our lab has recently shown that CNS myelin actually acts as a growth enhancing substrate for early stage neurite outgrowth, and gene expression analyses looking into why are currently underway. Understanding how early

stage neurons are able to grow so well in the CNS creates the potential for gene therapies designed towards enabling regeneration of damaged neurons.

Figures

Figure 1: Diagram of C5 Hemisection Graft and Quantification Area of Focus.

Embryonic day 14 Rat spinal cord neurons constitutively expressing EGFP were grafted two weeks after a C5 hemisection lesion. (A) Grafts completely fill the lesion cavity and attenuate the glial scar. (B) Axons grew for long distances in white matter (WM) and were (C) myelinated. (C) Myelination was assessed in cross sections 1-2 segments caudal to the graft using TEM. Scale bars: A 350μm; B 48μm; C 0.2 μm

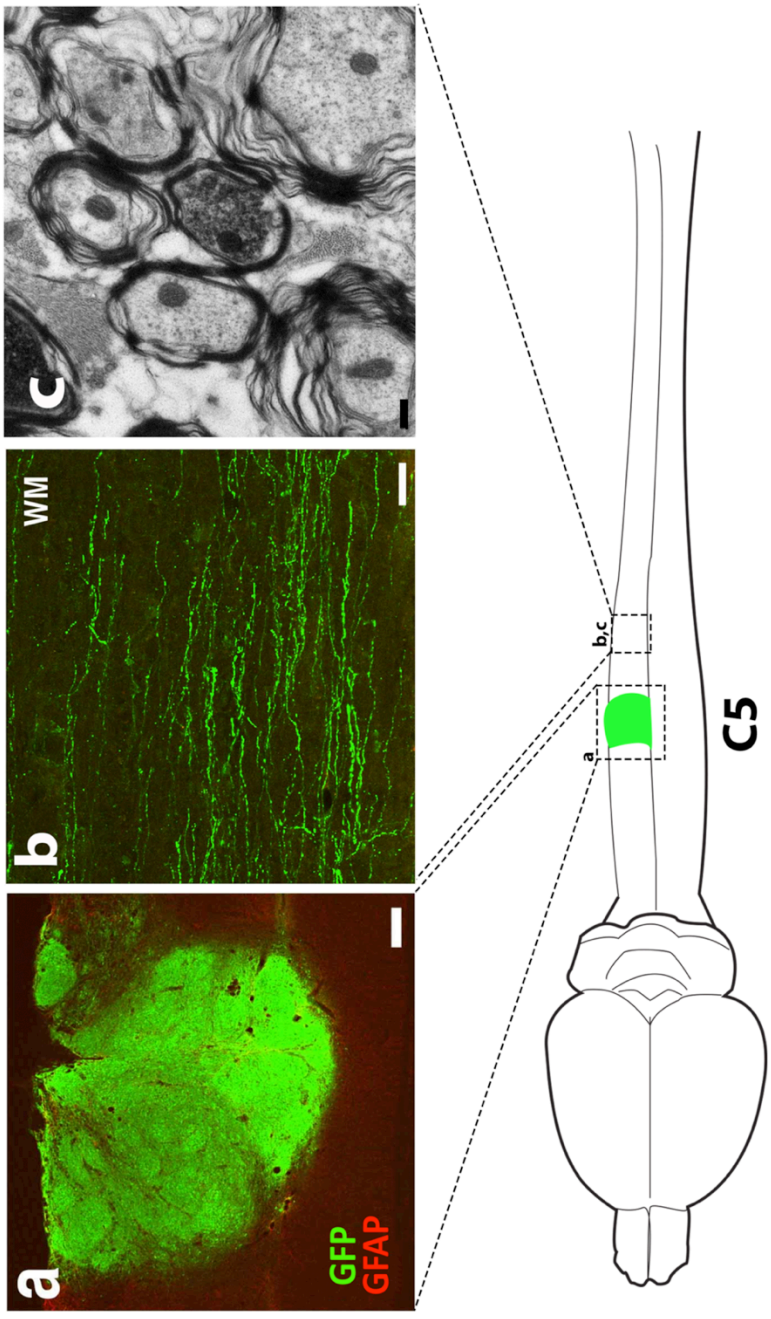
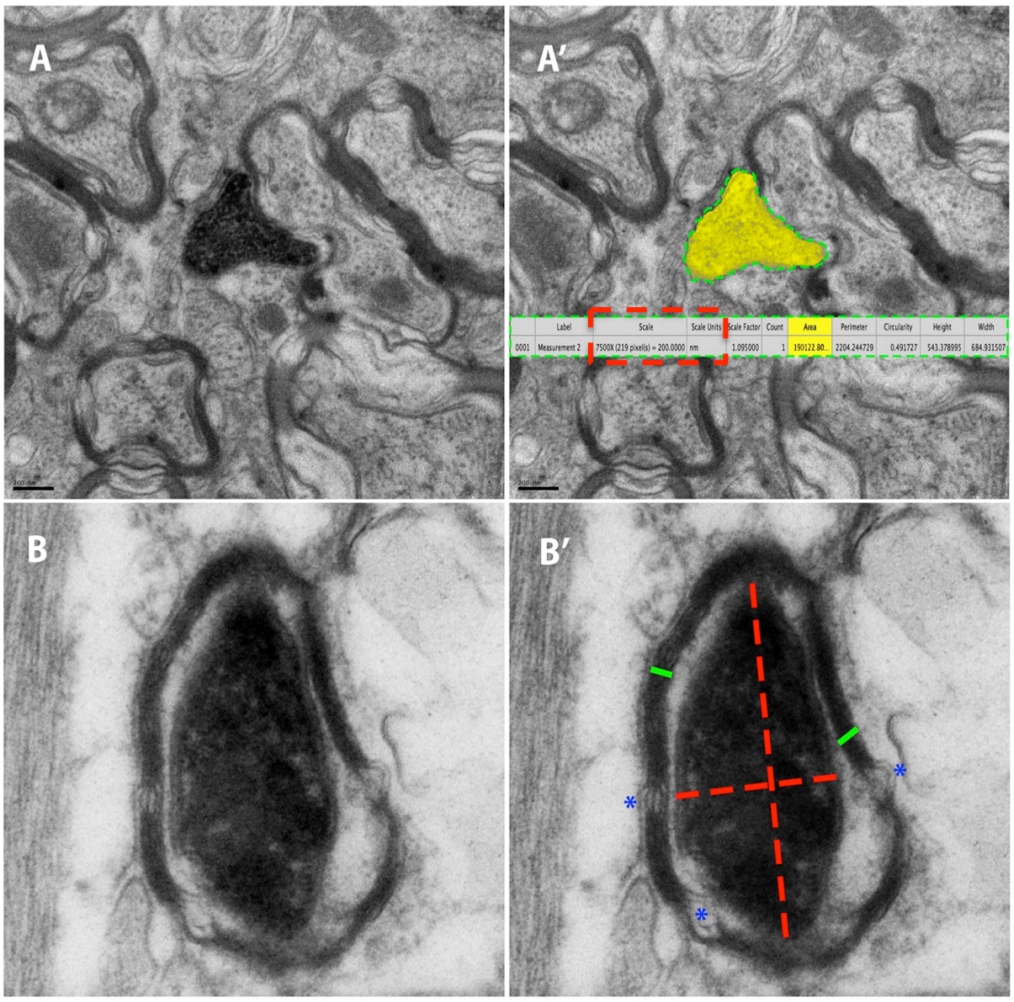

a
GFP
GFAP
b
WM
c
a
b,c
C5

Figure 2: Axon and Fiber Diameter Quantification Techniques.

Transmission electron microscopy (TEM) images of E-14 graft derived axons, stained with DAB, (A, B) are displayed alongside the technique used to quantify diameter in Photoshop (A', B'). Irregular shaped axons (A, A') were outlined (green dashed line) using the quick selection, magic wand, or lasso tools. (A) An algorithm was used to convert pixel length into distance (Red dashed lines), and area of the highlighted region was displayed (yellow). Photoshop's ruler tool was used was used for regular shaped axons (B, B'), along with the algorithm for distance, in order to average the longest and shortest lengths across the center (red dashed lines). The ruler tool was used at two well-preserved areas of myelin to find its average thickness (green solid line), while avoiding poorly preserved sections (blue asterisks). Scale bar: 0.2μm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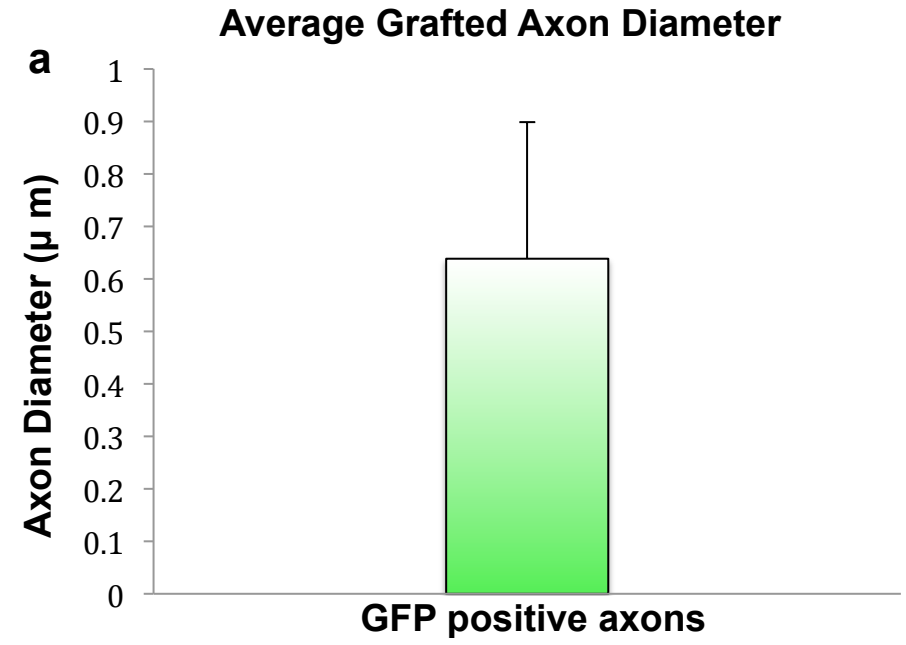
Label Scale Scale Units Scale Factor Count Area Perimeter Circularity Height Width
0001 Measurement 2 7500X (219 pixel(s) = 200.0000 nm 1.095000 1 190122.80 2204.244729 0.491727 543.378995 684.931507

Figure 3: Diameter of GFP Positive Axons Caudal to C5 Hemisection Grafts

(a,b) Overall 104 GFP positive axons were imaged and quantified. (a) The mean diameter of all quantified axons was found to be 0.64μm with standard deviation of 0.26μm (error bar represents standard deviation). (b) Axons ranged from 0.15μm to 1.7μm in diameter with a normal distribution about the mean, putting them in the small-medium sized category.


Average Grafted Axon Diameter
a
Axon Diameter (µ m)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GFP positive axons

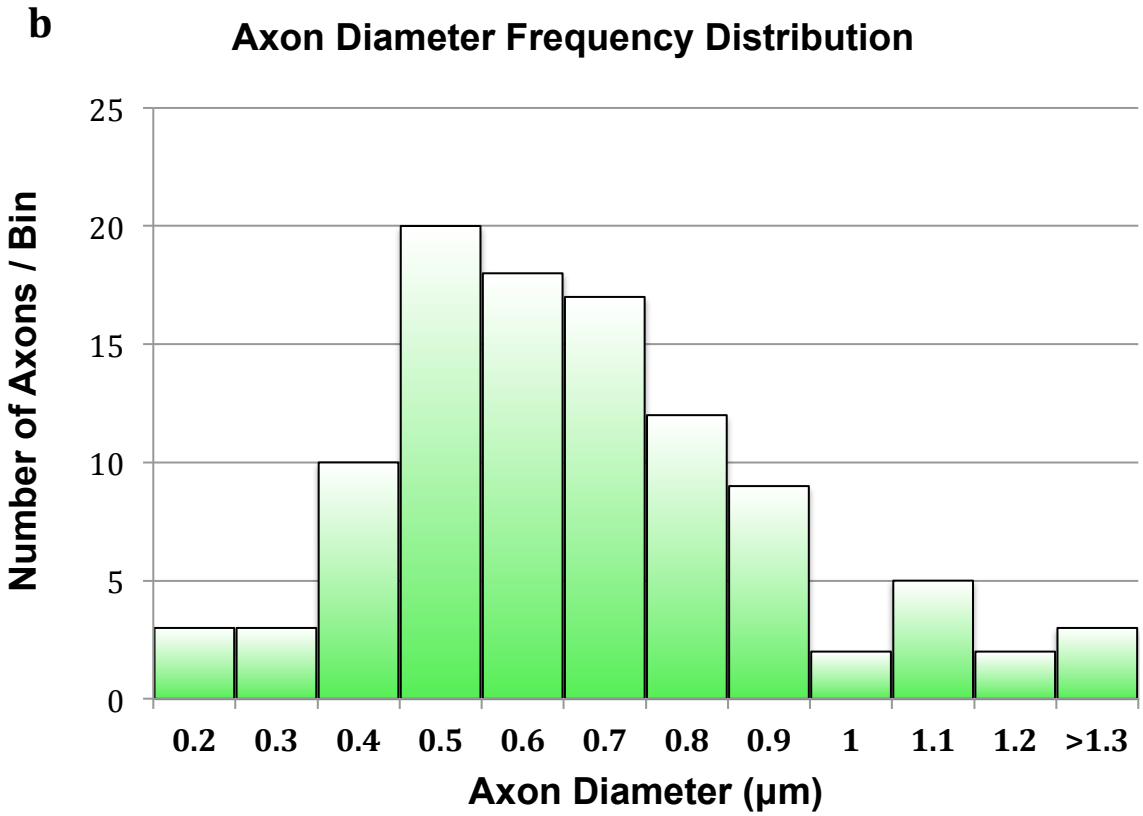

b
Axon Diameter Frequency Distribution
Number of Axons / Bin
25
20
15
10
5
0
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
>1.3
Axon Diameter (µm)

Figure 4: Larger Diameter Axons Are Preferentially Myelinated

(a) 23% of grafted axons were myelinated. (b,c) Grouping axons by myelinated and non-myelinated into bins of 0.1μm between shows a trend that larger diameter axons may be preferentially myelinated. (d) Myelinated axons have a statistically significant average diameter that is larger than non-myelinated axons. $p=0.0335$

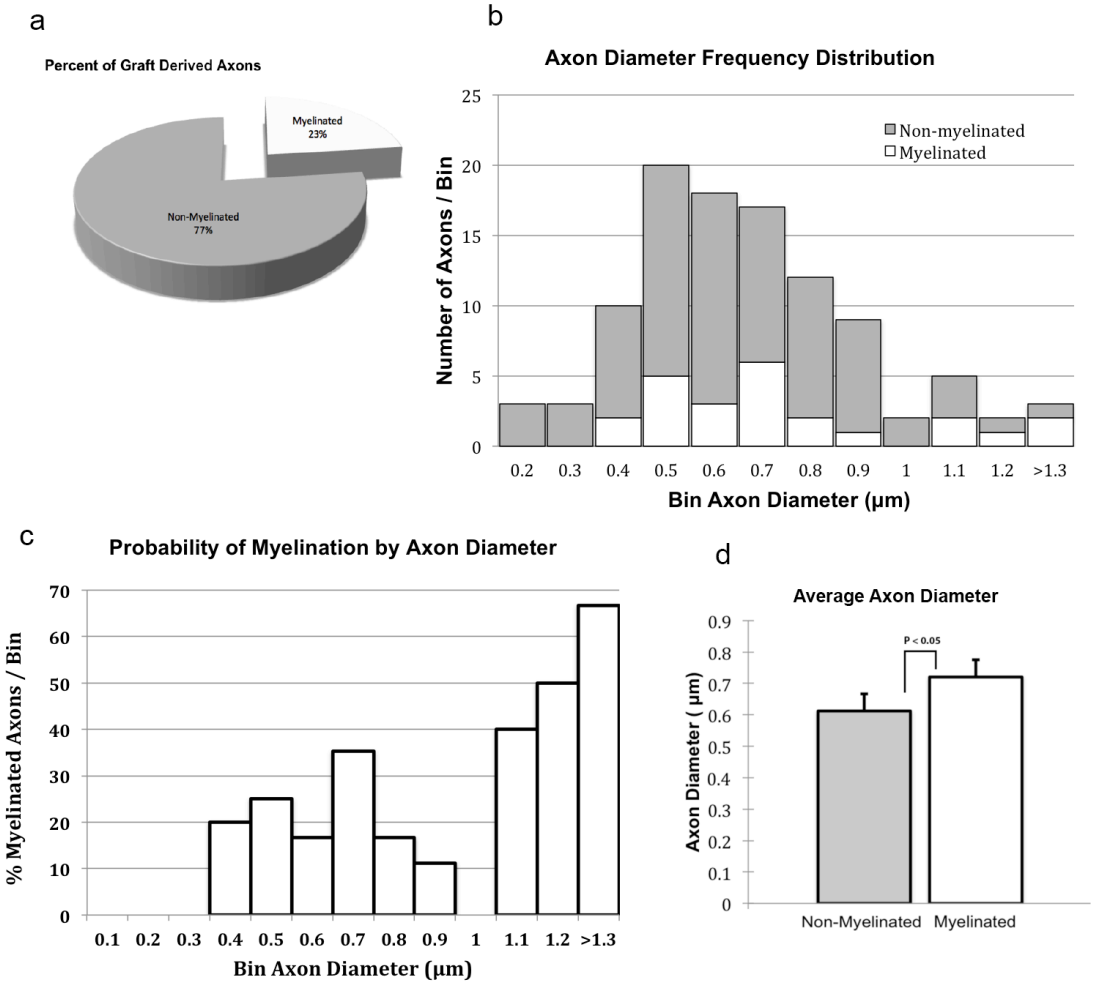
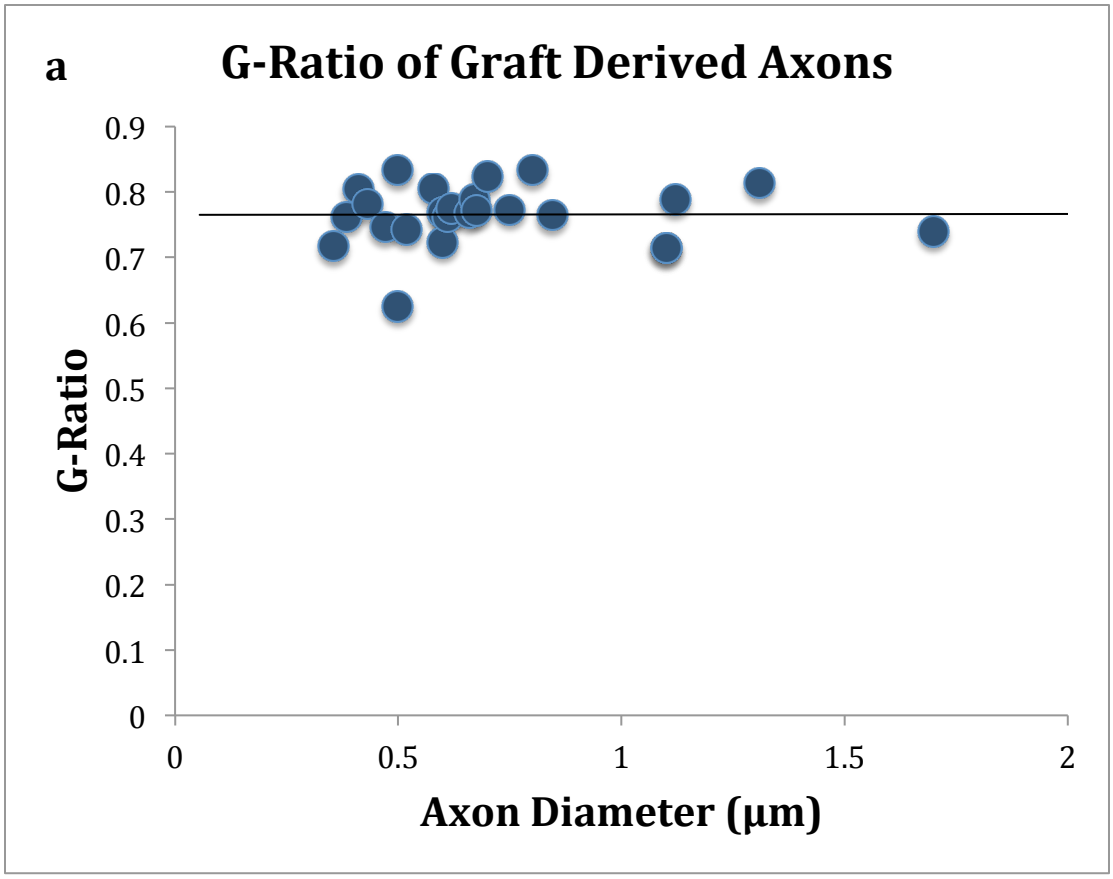

a
Percent of Graft Derived Axons
Myelinated
23%
Non-Myelinated
77%
b
Axon Diameter Frequency Distribution
Non-myelinated
Myelinated
Number of Axons / Bin
0
5
10
15
20
25
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
>1.3
Bin Axon Diameter (µm)
c
Probability of Myelination by Axon Diameter
% Myelinated Axons / Bin
0
10
20
30
40
50
60
70
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
>1.3
Bin Axon Diameter (µm)
d
Average Axon Diameter
Axon Diameter (µm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
P < 0.05
Non-Myelinated
Myelinated

Figure 5: Myelination of Graft Derived Axons is Equivalent to Developmental myelination

(a,b) G-ratios are tightly clustered near the global optimum CNS average of 0.77. (a) The smallest quantified G-ratio was 0.625 from an axon with a diameter of 0.5μm, and the largest was 0.83 also corresponding to an axon of 0.5μm diameter. (b) The average G-ratio of all graft derived myelinated axons was found to be 0.766 with a standard deviation of 0.046. The average G-ratio is equivalent to that of developmentally myelinated axons, whose


a
G-Ratio of Graft Derived Axons
G-Ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
Axon Diameter (µm)

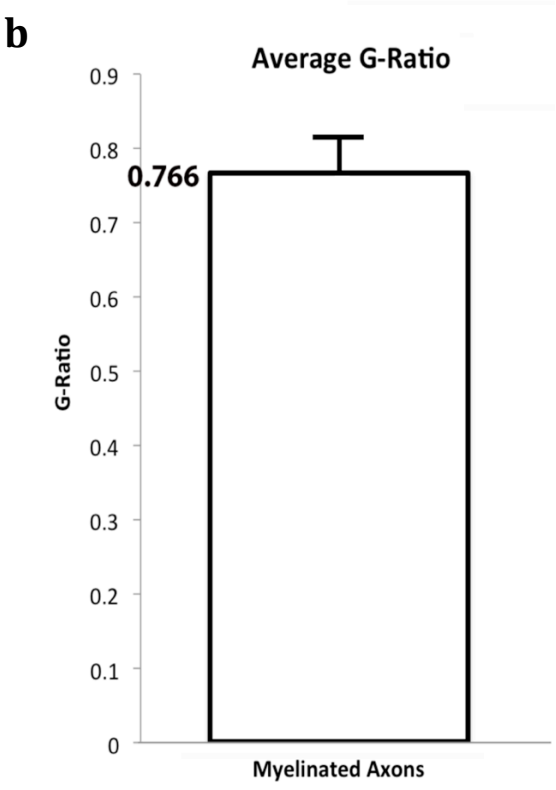
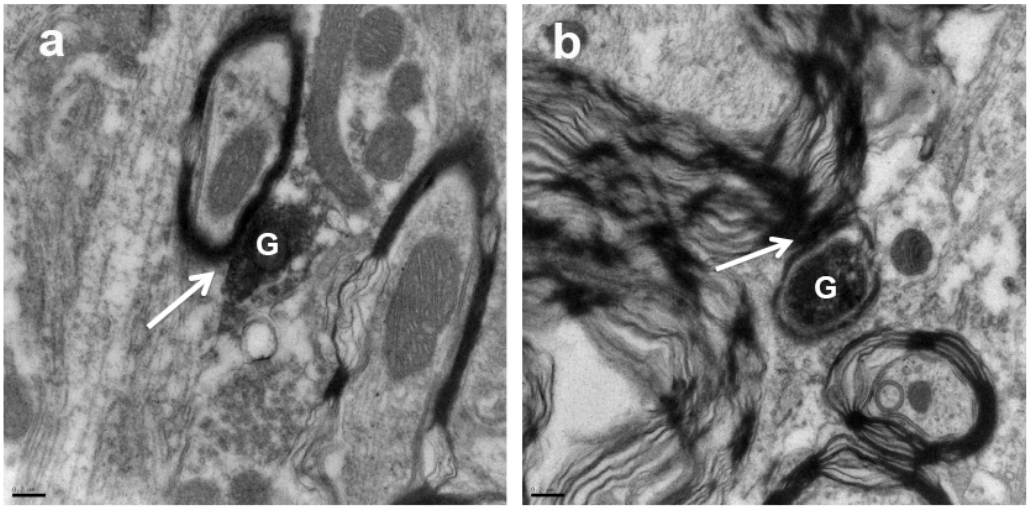
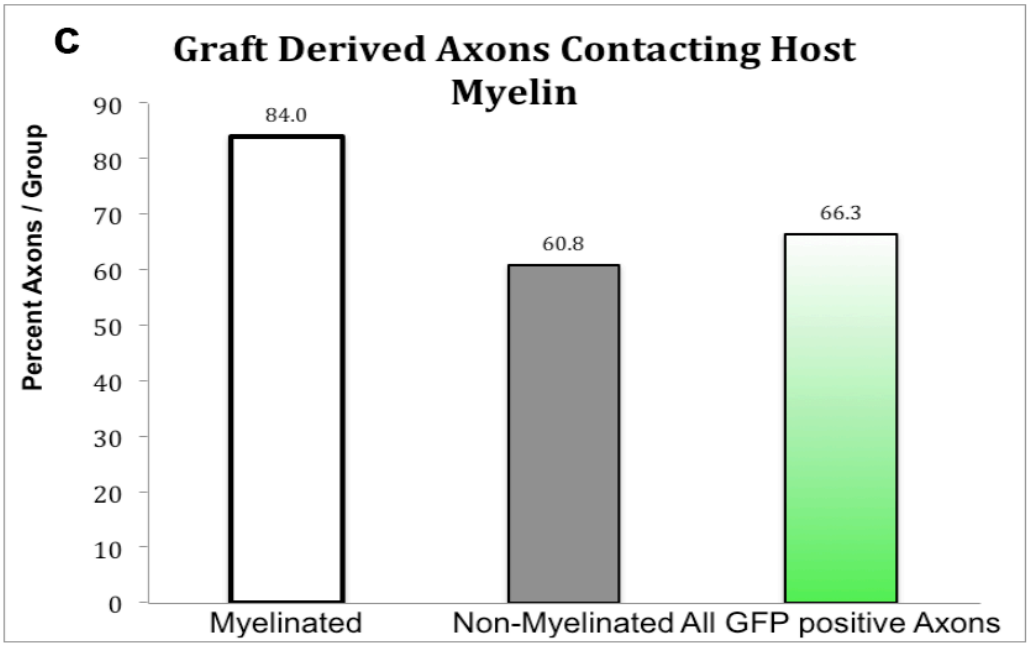

b
Average G-Ratio
0.766
G-Ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Myelinated Axons

Figure 6: Graft Derived Axons Directly Contact Host Myelin.

(a,b) TEM images showing graft derived axons (G) in contact with host myelin (arrow). (b) Myelinated axons were characterized as “in contact” if any part of the fiber was in contact with host myelin. (c) 84% of myelinated fibers, 60.8 % of non-myelinated axons, and 66.3% of all axons were in direct physical contact with host myelin. (scale bars: a,b 0.2 μm


a
G
b
G


c
Graft Derived Axons Contacting Host Myelin
Percent Axons / Group
90
80
70
60
50
40
30
20
10
0
84.0
60.8
66.3
Myelinated
Non-Myelinated
All GFP positive Axons

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