I, John J Vennemeyer, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering.

It is entitled:
Investigation of Magnesium-based Interventions for Central and Peripheral Nervous Tissue Regeneration

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Abstract

Damage to tissues in the central nervous system (CNS) and peripheral nervous system (PNS) is of serious concern since they possess a limited capacity to regenerate. A new generation of interventions centered on the essential element magnesium (Mg) offers a promising proposition to improve treatment options for nervous tissue trauma.

The long-term goal of our research is to understand how ionic and metallic Mg can be used to therapeutically interact with CNS and PNS tissues. Initial experiments focused on studying the interaction between Mg ion (Mg$^{2+}$) solutions and CNS-derived neural stem/progenitor cells (NSCs) in vitro. Latter studies focused on using Mg metal as a novel biomaterial in peripheral nerve (PN) tissue engineering.

The use of Mg$^{2+}$ solutions to treat traumatic brain injury (TBI) and stroke is well documented and shows tremendous promise in animal models; however, there are still obstacles to translating these findings into effective treatments for humans. When administered systemically, Mg$^{2+}$ can attenuate a host of complex, harmful biochemical cascades that occur in CNS tissue in the hours and days after injury, though the exact mechanisms are still unclear. With this in mind, we sought to develop a culture system that would be responsive to changes in Mg$^{2+}$ in an effort to better understand the interactions between Mg$^{2+}$ and CNS tissue (in the form of NSCs). The central hypothesis of this work was that neural cells (NSCs) would show altered biological responses, including beneficial effects, to moderately elevated Mg$^{2+}$ concentrations in vitro.

Severe injuries to the PNS are also resistant to current treatment methods. There is a great clinical need for an engineered alternative to autografts, the current standard of treatment for management of nerve defects greater than 3 cm. It has been hypothesized that one of the principal reasons that nerve conduits are ineffective at bridging defects greater than 3 cm is that there is inadequate mechanical support for tissue regeneration in the lumen of the conduit. We proposed that metallic Mg, which has been shown to
be biocompatible and biodegradable in orthopedic and vascular applications, could serve as a physical support, a scaffold, within a nerve conduit. The central hypothesis of this work was that metallic Mg, placed in the lumen of a nerve conduit, would improve nerve regeneration by serving as a scaffold that would safely resorb into the body over time, after mechanical support was no longer required by regenerating tissue.

The results from the research in this dissertation identify a culture system for studying Mg$^{2+}$ supplementation using primary CNS-derived stem cells and present a novel use for metallic Mg as a biodegradable scaffold in PN tissue engineering. New methods for monitoring neural cell health in vitro are described, as are methods for monitoring the response of nerve tissue in the presence of Mg metal and the resorption of Mg metal surrounded by nerve tissue in situ. The findings of this research will contribute toward the development of more sophisticated culture systems for studying the interaction between neural cells and Mg$^{2+}$ and also toward the further development of biodegradable metals as tissue engineering scaffolds for the improvement of PN tissue repair.
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Figure 22 Effects of Mg$^{2+}$ supplementation/ plating substrate on neurite characteristics. Mg$^{2+}$ supplementation had no effect on #neurites/neuron, though plating on laminin did. The total length of neurites present was increased by plating on laminin though 10 mM Mg$^{2+}$ supplementation also increase neurite length in without laminin. Laminin also increased the average #neurites and avarage maximum neurite length per neuron while Mg$^{2+}$ supplementation increased the maximum neurite length without laminin. Asterisks mark significant differences from the (NL -0) control by Kruskal-Wallis one-way ANOVA with Dunn’s post hoc. Error bars = standard deviation.

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Figure 24 Colored composite micrograph storyboard. A composite micrograph of an IHC stained nerve bundle from an uncrushed nerve without Mg (-Mg/-Cr) is shown (A) along with each isolated IHC stain used to create the composite (B-D). Grayscale composites of individual stains (B-D) were created in Photoshop by stitching together high magnification digital photographs taken under fluorescence. Grayscale composites were then colored and combined in Photoshop (D) to create a single, colored representation of the tissue in select slides (‘B’ (S100) corresponds to the red channel, ‘C’ (ED1) corresponds to the green channel and ‘D’ (DAPI) corresponds to the blue channel).

Figure 25 Overview of images without Mg (nerve tissue only) subjected to random sampling. Full composites of each image examined are shown to give the reader perspective on the size of the images being sampled. Both Uncrushed (A, B) and Crushed (C, D) nerves were examined. Two different staining protocols were used so that several histochemical stains could be evaluated. In the ESD protocol, slides were stained for the presence of the ED1 antibody (A, C – green), a marker for macrophages (an indicator of inflammation), S100 (A, C – red), a marker for Schwann cells, and DAPI (A, C – blue), a marker for cell nuclei. In the GND protocol, slides consecutive with the ESD slides were stained for GLUT1 (B, D – green), a marker for Glucose1 transporter protein (an indicator of the integrity of the nerve/blood barrier), NF200 (B, D – red), a marker for axons, and DAPI (B, D – blue). Scale bars = 250 µm.

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Figure 28 Overview of images with Mg (nerve tissue only) subjected to random sampling. Full composites of each image examined are shown to give the reader perspective on the size of the images being sampled. Both Uncrushed (A, B) and Crushed (C, D) nerves were examined for gradient effects due to a Mg filament. Two different staining protocols were used so that several histochemical stains could be evaluated. In the ESD protocol, slides were stained for the presence of the ED1 antibody (A, C – green), a marker for macrophages (an indicator of inflammation), S100 (A, C – red), a marker for Schwann cells, and DAPI (A, C – blue), a marker for cell nuclei. In the GND protocol, slides consecutive with the ESD slides were stained for GLUT1 (B, D – green), a marker for Glucose1 transporter protein (an indicator of the integrity of the nerve/blood barrier), NF200 (B, D – red), a marker for axons, and DAPI (B, D – blue). Yellow stars indicate the cavity left by a Mg filament. Scale bars = 250 µm. ................................ ................................ ................................ .......... 104

Figure 29 Representative IHC staining and epineurial implantation of a Mg filament. IHC staining in control and crushed tissue with Mg filament implant in connective tissue at 1 week. Yellow stars mark the cavity left by the Mg filament (which was displaced in sectioning). There is a concentrated population of macrophages encapsulating the space occupied by the magnesium needle as seen in Mg/crush condition (A). Nerves that had been crushed (A,B) were clearly distinguishable from those that had not (C,D) due to the lack of GLUT1 staining around the nerve bundles in the crush conditions (B) as opposed to the control (D). GLUT1 also positively stained blood vessels, visible as tiny green circles within the nerve fascicle (B,D). Scale bar = 250 µm. ................................ .......... 107

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1.0 Introduction and Specific Aims

Damage to tissues in the CNS and PNS is of serious concern since they possess a limited capacity to regenerate. 3% of all visits to the ER include trauma to peripheral nerves (5% including nerve root and brachial plexus injuries), representing over 300,000 injuries each year \(^{(16,17)}\), and over 1.4 million visits to the ER involve trauma to the brain or spinal cord \(^{(18)}\). Current methods of treating traumatic brain injuries (TBI) and peripheral nerve (PN) injuries rarely result in full functional recovery \(^{(7,19)}\). A new generation of interventions centered on the essential element magnesium (Mg) offers a promising proposition to improve treatment options for nervous tissue trauma. The long-term goal of our research is to better understand how ionic and metallic Mg can be used to therapeutically interact with CNS and PNS tissues. The studies outlined in this document represent the initial steps toward this goal.

Aim 1:

The long-term goal of the research in Aim 1 is to use a neural cell culture preparation to advance our understanding of how ionic Mg (Mg\(^{2+}\)) therapeutically interacts with CNS tissues. For this work, we chose a primary cell culture system in which murine brain cells are disaggregated into a single cell suspension and plated in a culture medium that allows survival and then selective growth of neural stem/progenitor cells (NSCs). These cells can then be put into a differentiation medium that allows analysis of their differentiation into neurons. The purpose of the work herein is to characterize the effects of elevating external Mg\(^{2+}\), because this mimics the situation that might ensue if elevated Mg\(^{2+}\) were to be used as a neuroprotective treatment for brain tissue damage. The central hypothesis of this work is that, because Mg\(^{2+}\) solutions are neuroprotective in vivo, CNS-derived murine neural stem/progenitor cells (NSCs) grown in vitro will tolerate, but will also show altered biological responses (including beneficial effects) to a certain range of Mg\(^{2+}\) concentrations above normal physiological levels.
Mg is an essential element in the body and is involved in hundreds of cellular processes, including DNA replication, energy production and consuming reactions i.e. oxidative phosphorylation and the TCA cycle. There is substantial evidence that lowered Mg$^{2+}$ is involved in the pathophysiological consequences following traumatic brain injury (TBI) and stroke, and that Mg$^{2+}$ supplementation after brain damage improves functional recovery in a variety of experimental animal models. However, there are still obstacles to translating these findings into effective treatments for humans. To understand the various challenges to human translation, it will be necessary to better understand issues like the exact mechanisms of Mg$^{2+}$ actions and Mg$^{2+}$ cellular transport and homeostatic systems, none of which are well characterized. We sought to use a relatively novel culture system to determine how neural cells responded to Mg$^{2+}$ and to determine if this would be useful for further studies of the cellular mechanisms responsible for this therapeutic effect. The hope is that this will guide future animal experiments. The first step toward this goal is to study the interactions between elevated Mg$^{2+}$ and NSCs.

Aim 1: Test the hypothesis that neural cells will show altered biological responses, including beneficial effects, to moderately elevated Mg$^{2+}$ concentrations in vitro. In particular, identify the type of response and identify the tolerated and/or potentially beneficial range. This information is crucial to understanding what happens to nervous tissues in vivo when exposed to elevated levels of Mg$^{2+}$. To address this, we characterized the dose-response of NSC numbers in the presence of elevated exogenous concentrations of Mg$^{2+}$, using different time periods of Mg$^{2+}$ exposure. NSC number was quantified by digital image analysis and plate reader assays. We also monitored NSC ‘behavior’ in the presence of elevated Mg$^{2+}$ concentrations, by measuring effects on the differentiation of NSCs.

Hypothesis 1: Numbers of NSCs will increase in response to moderate increases in local Mg$^{2+}$ concentration.
Rationale: Based on the fact that Mg\textsuperscript{2+} can biphasically stimulate cell division in other cell types, such as chondrocytes\textsuperscript{24} and endothelial cells\textsuperscript{25}, we first measured absolute numbers of NSCs at two different time periods after raising external levels of Mg\textsuperscript{2+} in vitro over normal.

The results of this study showed that there was a biphasic dose-response of NSCs numbers to an increase in Mg\textsuperscript{2+} concentration; cell numbers increased in response to concentrations up to 10 mM above physiologic levels (0.81 mM in normal culture medium) and decreased in response to higher concentrations. Cell numbers were measured by the Crystal Violet assay, and confirmed by manual cell counts. A traditional cell-counting assay, the Alamar Blue assay, was inaccurate in the presence of increased Mg\textsuperscript{2+} and was not a useful assay for NSCs.

Hypothesis 2: Normal NSC behavior, as measured by the ratio of cells that differentiate into neurons and the elaboration of neurites, will not be harmed and may be increased by moderate increases in local Mg\textsuperscript{2+} concentration.

Rationale: One measure of NSC health and normal functioning is the ability of the stem/progenitor cells to differentiate into neurons in vitro when placed in a specific differentiating medium. We examined whether raising external levels of Mg\textsuperscript{2+} in this medium would influence the amount of neurons produced and/or the appearance of the neurons (number/length of neurites).

We found that the ratio of differentiated to non-differentiated cells did not change in response to increased Mg\textsuperscript{2+}, but other measures (length of neurites) did, in the same dose range that gave positive effects on cell numbers.

The results from Aim 1, described in Chapter 3, provide a baseline of NSC behavior in response to Mg\textsuperscript{2+} supplementation and also describe inaccuracies of a popular cell counting assay in the presence of
elevated Mg$^{2+}$. These results set the stage for future development of more sophisticated culture systems for studies of the interaction between neural cells and Mg$^{2+}$.

**Aim 2**

Severe injuries to the PNS are also resistant to current treatment methods. There is a great clinical need for an engineered alternative to autografts, the current standard of treatment for management of severe nerve injuries. This is a major focus of tissue engineering; restoring or replacing injured or pathological nerve tissue with scaffolds that may support new, healthy tissue growth. Successful biologic function of such a scaffold is dependent on the action of growth factors, extracellular matrix and surrounding cells\textsuperscript{26}. Ideally, the biomaterials used to construct these scaffolds should possess sufficient mechanical integrity and porosity to support the migration of cells and vasculature into the scaffold and gradually disappear as they are replaced by functional tissue\textsuperscript{27}.

The nerve conduit, a hollow tube used to connect the ends of a severed nerve, has been investigated as an alternative to autografts for over 100 years, although more intensive, sophisticated efforts began after the 1970s\textsuperscript{28}. However, despite intensive research efforts, nerve conduits are rarely used in the clinic and are reserved for nerve gaps of less than 3 cm. It has been hypothesized that one of the principle reasons nerve conduits are ineffective at bridging larger defects greater is that there is inadequate mechanical support in the lumen of the conduit for tissue regeneration to occur. The long-term goal of the research in Aim 2 is to develop a novel nerve scaffold consisting of a conduit containing a Mg filament. The **central hypothesis** of this section of the work was that metallic Mg, placed in the lumen of a nerve conduit, would improve nerve regeneration by serving as a physical support for cells to cross the injury gap, and then this scaffold would safely resorb into the body over time, after mechanical support was no longer required by regenerating tissue.
Aim 2: Design and begin characterization of a novel use of Mg metal for repair of traumatized peripheral nerves.

The first step toward achieving this aim was to ensure that Mg metal was biocompatible with nerve tissue. Based on previous research in the field, we formulated the following Hypothesis:

**Hypothesis 3:** Mg filaments will be biocompatible when implanted immediately adjacent to healthy and crushed peripheral nerve tissue.

*Rationale:* We proposed that Mg filaments would be biocompatible within peripheral nerve tissue based on the numerous studies of Mg implantation in non-nervous tissues\(^29\) and more specifically based on the single reference we could find for implantation in nervous tissue in the literature\(^30\). Because of the lack of data on nervous tissue placement, a significant goal for these studies was to determine basic biocompatibility when Mg was implanted into the connective tissue immediately adjacent to both healthy and damaged peripheral nerves (PNs). We also wished to assess whether corrosion products from the Mg might be released into neighboring tissues and influence adjacent damaged nerve tissues.

In order for a Mg filament to perform the desired scaffolding function, the rate of Mg resorption in the body must be tailored so the filament can provide initial mechanical support while the nerve is regenerating and then resorb as the tissue remodels itself. Therefore, we proposed the following.

**Hypothesis 4:** Untreated Mg filaments will resorb at a rate appropriate for nerve regeneration.

*Rationale:* Peripheral nerve (PN) regeneration in humans occurs at the rate of approximately 1 mm day, followed by tissue remodeling over one to two years\(^31\). In rats, it has been shown that when silicone conduits were used to repair a gap of 1 cm, all animals had tissue present at the
midpoint of the conduits after 4 weeks (Williams and Longo, 80s). Thus, a Mg filament must remain intact for four or more weeks for a relatively short gap of up to 1 cm. When Mg filaments of the diameter we proposed using (~200 µm) were placed in cell culture medium, they were corroded almost completely in 24 hours. However, it is known that the in vivo rates of corrosion can be significantly slower than rates in vitro. It is also known that rates of corrosion in vivo can vary widely from tissue to tissue, based on subtle differences in the microenvironment around the implanted Mg. Therefore, it was important that our first experiments document the rate of Mg corrosion in our specific microenvironment, healthy, injured and regenerating peripheral nerves.

To test Hypothesis 3 and to start testing Hypothesis 4, we implanted Mg filaments adjacent to healthy and injured PN tissue for 1 week in a rat model. Biocompatibility and Mg resorption were grossly assessed by histology. A digital analysis technique was also developed for quantifying immunohistochemical staining, the accuracy of which was confirmed by qualitative observations. Histological analyses revealed minimal inflammation around the Mg filament or corrosion of the Mg filament. These results suggested good biocompatibility and initial corrosion resistance. This study is provided in Chapter 4.

Because of the positive results from this first in vivo study with Mg filaments, we began testing further hypotheses regarding the actual use of a Mg-containing nerve conduit in nerve repair.

**Hypothesis 5:** Because the Mg filaments will remain intact and will provide the appropriate physical support (a scaffold), a Mg-containing nerve conduit will support regeneration of PN tissues across a short nerve injury gap, and this repair will be as good as or better than the regeneration through an empty conduit.

**Rationale:** To test our hypothesis that our Mg-containing scaffold would be compatible with regenerating nerve tissues and will hopefully even improve regeneration repair, we first tested its
use in a non-critical, or ‘short’, PN gap (an injury that would heal with an empty conduit). Our reasoning was that this would provide a good control to determine if the Mg metal would interfere with the normal processes of nerve regeneration. Because there would be regeneration within an empty conduit, we would be able to compare the histology of the regenerating tissues with and without Mg present. Criteria for successful nerve regeneration were histological measures e.g. presence of appropriate cells/cell structures, and also the size of the leg muscles innervated by the damaged sciatic nerve at the end of the experiment, a measurement of functional outcome.

A short gap that would heal quickly would also allow us to assess Mg resorption (which required animals to be sacrificed) at a relatively early timepoint.

In our initial experiment implanting Mg filaments in proximity to PNs, Mg filaments showed good biocompatibility in proximity to healthy and injured mature PN tissue, although we did not know if the regenerating PN tissue might react differently to the Mg filament, prompting Hypothesis 6:

**Hypothesis 6:** When used inside a nerve conduit, a Mg filament will be biocompatible with regenerating nerve tissue.

*Rationale:* Based on our initial experiment and the evidence from Mg implants in other tissues, we proposed that the regenerating PN tissue would not be harmed from contact with a Mg filament and would not be harmed when/if the Mg began to corrode significantly.

Since we expected nerve tissue to regenerate across our experimental nerve defect, even in empty conduits, we wanted to compare the regenerative response to a Mg-containing conduit to another type of positive control. We elected to use a keratin hydrogel, which when used to fill nerve conduits, has been
shown to improve PN regeneration. We then tested the combination of both adding a Mg filament and adding the hydrogel, to determine if there might be a synergism between these two conditions that we predicted would both stimulate/enhance nerve regeneration or regeneration rate.

**Hypothesis 7:** Keratin hydrogel filler in a nerve conduit will enhance nerve regeneration above that seen with a saline-filled conduit, and when combined with a centrally located Mg filament, would synergistically improve nerve regeneration.

**Rationale:** Keratin hydrogel, placed within a nerve conduit, has been shown to improve nerve regeneration in mice 32, rats 33 and rabbits 34. We hypothesized that a Mg filament might have greater mechanical stability than the keratin hydrogel within the lumen of a conduit. While the hydrogel creates filamentous structures that might guide cell growth, they are aligned randomly and not axially, as is the Mg filament. Thus, we proposed that the Mg filament might offer a more direct and physically intact guidance structure than the hydrogel, but the hydrogel would provide other beneficial attributes, like a chemical environment more conducive to PN regeneration. Thus, we proposed that the combination of the two might be synergistic in improving the rate of nerve regeneration.

To test Hypotheses 5-7, and to continue testing Hypothesis 4, we repaired gaps in adult rat sciatic nerves with nerve conduits with or without an axially placed single Mg filament, and filled with saline or keratin hydrogel after the conduit was in place. These were left in the animals for 6 weeks. During this 6 weeks, behavioral assays were done on the animals weekly. After 6 weeks in vivo, PN tissue was examined by histology, Mg resorption was assessed by micro computed tomography and functional recovery was assessed by measuring the size of the leg muscles innervated by the damaged sciatic nerve. Regenerating nerve tissue successfully grew through each conduit; including all of those containing Mg. Mg filaments showed good biocompatibility, assessed by histology, and each Mg filament was engulfed by regenerated
nerve tissue, strongly supporting our hypothesis that Mg is biocompatible with these tissues. Mg filaments had begun to resorb by 6 weeks and the degree of resorption was increased in the presence of keratin hydrogel. In terms of muscle size, Mg-containing conduits and conduits filled with keratin (without Mg) performed better than empty control conduits. This study is provided in Chapter 5.

The results of Aim 2 studies demonstrate that Mg metal is a viable scaffolding material for nerve tissue engineering. There was minimal inflammation in proximity to a Mg filament when implanted in mature or surrounded by regenerating nerve tissue. We did not detect any negative affects of corrosion products in tissue immediately adjacent to a resorbing Mg filament in either case. The rate of corrosion of untreated Mg placed in a nerve conduit was adequate for PN regeneration over a short gap and accelerated in the presence of a keratin hydrogel. There were detectable differences in muscle size in animals receiving conduits with and without Mg filaments, suggesting that the Mg filament improved functional recovery. These results set the stage for future studies to examine Mg filaments as a nerve scaffold in larger nerve gaps, moving this technology one step closer to clinical impact.

The format of this dissertation is as follows: Chapter 2 provides background information closely related to the research summarized in this Chapter. Chapter 3 outlines and discusses the findings from Aim 1. Chapters 4 and 5 outline and discuss the findings from Aim 2. Chapter 6 provides discussion on the impact of current results and suggestions for future studies.
2.0 Background

The content of this chapter is intended to provide the reader with background information relevant to the studies presented in later chapters. The anatomy and organization of the central and peripheral nervous systems are outlined followed by a description of injury classification in those systems, current standards of treatment, a brief overview of biodegradable metals for medical applications and limitations of current treatment methods for nerve tissue injury.

2.1 Anatomy of the nervous system

The nervous system functions to collect, process and respond to external stimuli in addition to regulating many of the fundamental processes essential for life e.g. breathing, digestion, hormone regulation etc. The nervous system can broadly be divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The PNS consists of nerves, specialized electrically excitable cells, which connect the CNS, comprised of the brain and spinal cord, to the rest of the body. A functional overview of the nervous system is shown in Figure 1.
Figure 1 Functional organization of the nervous system. Receptors in peripheral nerves send sensory information to the central nervous system via the afferent division of the peripheral nervous system. After processing in the central nervous system, motor commands travel out to distal target through the efferent division of the peripheral nervous system.

2.1.1 Anatomy of the central nervous system

The CNS is housed in the cranial cavity and vertebral column. Composed of a complex network of neural, vascular and connective tissues, the CNS functions to coordinate incoming sensory data and outgoing motor commands, regulate physiological processes and serve as the location for the higher functions of memory and learning.

2.1.1.1 Cellular structure

The operational cellular unit of the CNS is the neuron, an electrically excitable cell, which is mechanically and trophically supported by other cells called glia. There are several different types of
neurons and glia and they typically exist in a ratio of 10 glial cells / 1 neuron in CNS tissues. Generally, neurons consist of a cell body (or soma), which contains the cells organelles, and filamentous extensions of the cell body, referred to as axons (one per cell) and dendrites (may be several per cell). A general term that is used for both types of processes is neurites. Dendrites generally function to receive input from other neurons while axons serve to carry information away from the cell. Dendrites possess an arboreal structure and may branch several times to connect to dendrites from multiple cells, potentially resulting in hundreds of functional connections. Axons may send out collateral processes to interconnect with other neurons and they usually branch at their termini.

Several neuron-specific structural proteins, referred to as neurofilament proteins, polymerize to form filamentous structures (neurofilaments) that provide mechanical support of the neuronal. In other words, these act as the cytoskeleton in both axons and dendrites. There are three main neurofilament proteins, divided by mass into low, medium and high molecular weights, weighing in at 68-70 kDa, 145-160 kDa and 200-220 kDa respectively. Antibodies to the neurofilament proteins are useful tools to identify neurons and distinguish them from surrounding cells like glia.

Neurons contain the same organelles as traditional eukaryotic cells e.g. a nucleus, mitochondria, Golgi apparatus, etc. though mature neurons are generally terminally differentiated and do not divide. An overview of the general structure of a neuron is shown in Figure 2.
Figure 2 Neuron structure. The neuron is a complex, electrically excitable cell that is the principle functional building block of nervous tissues. Neurons communicate with other neurons and all other organ systems in the body to regulate the processes of life. Image from 2.

2.1.1.2 Physiological function

Neurons function by sending electrical and chemical signals to other neurons via specialized structures at the terminal branches of dendrites and axons called synapses. Chemicals contained in synaptic vesicles, called neurotransmitters, are released into the synaptic cleft where they bind to receptors present on the surface of other cells, resulting in electrical or chemical changes in the receiving cell, thus transmitting information from one cell to the next. The two most prevalent neurotransmitters in the brain are the amino acid glutamate, which is largely excitatory, and γ-Aminobutyric acid (GABA), which is inhibitory. Several other neurotransmitters exist and are classified into the following categories: monoaminergic (biogenic amines, containing one amine group including dopamine, norepinephrine, epinephrine,
serotonin and histamine); cholinergic (acetylcholine); peptidergic (neuroactive peptides); and other amino acids (aspartate, glycine).

Electrical signals are conducted along the membrane of a cell through a phenomenon called an action potential, the rapid depolarization of the cell membrane that traverses from the axon hillock to the axon terminus where it stimulates the release of neurotransmitter. The conduction speed along an axon may be increased by the addition of an insulating sheath composed of myelin, a lipoprotein supplied by specialized glial cells called oligodendrocytes. Myelination increases conduction velocity but also increases the volume of the axon. Unmyelinated axons are sufficient if the response to a stimulus may be slow or the action potential only needs to travel a short distance.

2.1.1.3 Organization and characteristics

To function, the CNS depends on the interactions between billions of cells that are highly organized into several distinct structures. These include: a center, a collection of neuronal cell bodies with a common function; a nucleus, a center with a discrete anatomical boundary; a cortex, a sheet of neuronal cell bodies; tracts, found in the white matter (specialized tissue for connecting different regions of the brain) of the CNS and consist of bundles of axons that share common origins, destinations, and functions.1 These structures interact with every other system of the body to regulate function.

The vasculature that supplies the CNS is completely separated from the neural tissue by specialized endothelial cells surrounded by a basal lamina, collectively referred to as the blood brain barrier (BBB). The BBB is selectively permeable to tightly control the passage of nutrients from the blood to the parenchyma while protecting the brain from undesirable exogenous material and to restrict various neurotransmitters to brain tissue. The brain and the spinal cord continuously circulate a specialized fluid called cerebrospinal fluid (CSF) through the four ventricles of the brain and all along the spinal cord,
separate from the blood. CSF mainly functions to mechanically cushion the brain and spinal cord and to remove metabolic waste products.

2.1.2 Anatomy of the peripheral nervous system

The PNS functions to receive external stimuli and innervate smooth and striated muscles for motor control. It consists of two subsets: the autonomic nervous system and the somatic PNS system. The autonomic nervous system consists of the sympathetic and parasympathetic subsystems. These sets of neurons and nerves (outside of the brain and spinal cord, but controlled by them) control the function of glands and blood vessels. The autonomic nervous system is not discussed in this work. The somatic PNS consists primarily of 31 pairs of spinal nerves. Each spinal nerve contains 1) efferent fibers that carry information from the CNS to muscles and 2) afferent fibers that carry sensory information from the periphery into the CNS. For each spinal nerve, the cell bodies for the efferent fibers reside primarily in the ventral horn of the spinal cord and travel to the periphery via the ventral roots, which are essentially “nerves” that connect the spinal cord to each spinal nerve. These are primarily motor fibers that control the action of muscles. Each spinal nerve also contains autonomic nerve fibers that control the actions of glands. Each spinal nerve also contains afferent sensory fibers. Many of the fibers that are transmitting the sense of touch have their cell bodies in clusters within the dorsal roots that are called dorsal root ganglia (DRGs) (Figure 3). The sensory information carried includes touch, vibration, pain, and temperature that are being transmitted to the spinal cord and brain.
Figure 3 Anatomy of the spinal cord. The spinal cord resides in, and is protected by, the vertebral column (A – Superior transverse view). Nerves project from the left and right sides to innervate the body (B). The ventral root carries motor commands from the CNS to the periphery whereas the dorsal root carries sensory information from the periphery to the spinal cord. These views are in an upside-down position because that is the way that information comes in from an MRI machine scan. So ‘dorsal’ (toward the back) is actually down in this picture and ‘ventral’ (the belly side) is up.\(^3,256\).
2.1.2.1 Cellular Structure

Similar to the CNS, nerve cells in the PNS consist of a cell body (soma) and extensions called axons and dendrites (collectively referred to as nerve fibers or neurites). The soma contains the cell’s organelles and nucleus while dendrites function to receive information from the periphery or other neurons and axons function to relay information to other neurons. As stated above, the PNS neurons that are involved in detecting senses such as touch, pain and temperature, have their cell bodies in the DRGs. One difference between the PNS and the CNS is that the dendrites of these DRG sensory neurons can be as long as axons. For example, fibers that bring information on touch from the toes must travel all the way to the dorsal root of the spinal cord in the lower back, before they connect with their cell body. A large majority of these afferent fibers (aka peripheral processes) are transmitting action potentials like axons (in contrast, the majority of CNS dendrites do not transmit action potentials). From that cell body, the efferent fiber, the axon proper, (aka the central process) extends into the spinal cord and goes to the neck area before it synapses onto another neuron. So cells exist that extend from the toes to the neck. Not all of either the efferent or afferent fibers are myelinated. So the spinal nerves contain both myelinated and unmyelinated fibers.

The efferent neurons of the somatic PNS system, which controls primarily the voluntary muscles of our body, are located in the ventral spinal cord. Their efferent axons travel from the spinal cord, through the ventral roots, through the spinal cords and eventually terminate at their targets, primarily skeletal muscles under voluntary control. The majority of these fibers are myelinated. The autonomic nervous system (mentioned to be complete) has its cell bodies in another set of ganglia outside the spinal cord and those axons travel via spinal nerves and blood vessels to smooth muscle (involuntary control), primarily in organs or around blood vessels. Portions of these fibers are not myelinated. So, again, as for the afferent system, the efferent system in spinal nerves contains both myelinated and non-myelinated fibers.
The support cells in the spinal nerves of the PNS are different from those in the brain. These are called Schwann cells. Several layers of Schwann cells will encapsulate mature axons, separating each nerve from all others. Many Schwann cells form myelin by concentrically encapsulating nerve fibers with sheets of cell membrane that are composed of proteins, lipids and lipoproteins. Myelin basically serves to insulate the electrical activity traveling down the length of a nerve fiber, and preserves the strength of the signal that otherwise would be significantly diminished over that long distance. Myelination drastically increases the speed at which electrical impulses may be carried by each fiber. The myelin formed by a Schwann cell in the PNS is basically identical to that formed by an oligodendrocytes in the CNS. As stated above, both myelinated and unmyelinated fibers are present in both sensory and motor nerves, with approximately 4 myelinated fibers for every unmyelinated fiber.

2.1.2.2 Physiological Function

Both efferent and afferent fibers exchange information through action potentials, as in the CNS. This spike of activity is initiated at one end of the axon, typically the hillock, located near the nucleus, and is propagated along the membrane to the distal portion of the cell. Neurotransmitters are released at the terminus of an axon that stimulate either muscle action or alter the electrical potential in another neuron.

2.1.2.3 Organization and characteristics of peripheral nerves

In the work described herein, we examine the microscopic and cellular structure of normal spinal (also called peripheral) nerves. This includes not only the nerve fibers, but also the connective tissue coatings that surround them. In each PN, myelinated and unmyelinated fibers efferent and afferent fibers, are surrounded by organized layers of connective tissue to form a framework for structural stability and to provide for vascularization. In PNs, this consists of three layers. Going from the most innermost to outermost, these are the endoneurium, the perineurium and the epineurium.
The endoneurium is a thin layer of connective tissue that consists of fibroblasts and the fibers (composed of primarily collagen, but also elastin) that they secrete. The endoneurium surrounds all the individual myelinated and unmyelinated nerve fibers. All nerve fibers are first ensheathed by Schwann cells so they do not actually contact the endoneurium. The endoneurial connective tissue allows nerves to resist elongation under tension and serves as a scaffold for blood vessels and also blood elements that have left the blood vessels, like mast cells and macrophages, whose function is to deal with inflammation and infection in any tissue.

The perineurium is composed of a layer of specialized, flattened fibroblasts that produce layers of collagen and elastin fibers. These are unusual fibroblasts because, unlike any other fibroblast, they actually make close contact with each other, touching to form a watertight barrier between the nerve bundles and the outside tissues around the nerves. When the perineurium is intact, the cells become specialized so that nutrients must be taken into the cells and transported through the fibroblast cytoplasm and then pumped out of the cell in order to get to the nerve fibers. One molecule that is expressed by the cells in order to serve this function is the glucose transporter protein 1 (GLUT1). We will use antibodies to this protein, to determine if the perineurium is present, intact and serving its transport and barrier function. The perineurium encapsulates the endoneurial connective tissue and the nerve fibers to form defined bundles called fascicles. In addition to serving as a water-tight barrier around the nerve fibers, it is also the main source of tensile strength of the nerve, it provides a physical protection for nerve fibers in the endoneurium, and it maintains an intrafascicular pressure gradient that encourages cytoplasm to flow toward distal (away from the cell body) sections of the nerve. The epineurium is the outermost connective tissue structure, outside the perineurium. It is a highly vascularized sheath of loose collagen-based connective tissue that encapsulates several nerve fascicles to form the nerve proper and also serves as residence for blood vessels, mast cells and macrophages (Figure 4).
Figure 4 Nerve architecture. Nerve fibers (axons) are supported and encased in myelin by Schwann cells within the endoneurium. The endoneurium is encased by the highly vascular perineurium and is the outer layer of each fascicle. Fascicles are held together in the collagenous and highly vascularized internal epineurium. The nerve is then encased by the outer epineurium. This configuration allows bundles of several different nerve fibers to be protected from compression, tension and traction as they navigate toward their distal targets.

2.2 Injuries to the central nervous system

Treating injuries to the nervous system remain a major challenge in modern medicine as nerve tissues in both the CNS and PNS possess a limited capacity to regenerate. As such, nerve injuries are a major source of chronic disability worldwide and the subject of intensive medical research.

2.2.1 Injuries to the brain (CNS injury)

Injury to the brain is often difficult to treat for a variety of reasons including the physical fragility of the organ itself, its high vulnerability to vascular insult, sensitivity to pressure changes and selective permeability of the blood-brain barrier to many drugs. Brain injuries are especially difficult due to the
tremendous heterogeneity of disorders, which may combine variable degrees of contusion, hematomas, axonal injury, hypoxia and ischemia, all of which can influence patient outcome. Damage to CNS tissue may be the result of cellular pathology e.g. tumors, vascular insults e.g. hemorrhage, stoke or mechanical trauma, often caused by car accidents, falls or violence. Traumatic brain injury (TBI) is the most relevant brain injury modality to the work presented in this document although many of the physiologic processes and clinical interventions described are also applicable to specific varieties of stroke, as explained in the next sections.

2.2.1.1 Incidence and impact of CNS injuries

TBI occurs when an external mechanical force damages the brain and is a leading cause of death and disability in children and young adults worldwide. Each year, approximately 1.4 million people visit the ER in the United States due to TBI where 52,000 people die and 235,000 are hospitalized. The most common source of trauma is falls with the very young (<5 years) and the very old (>85 years) being the most susceptible. Older individuals generally experience poorer outcomes compared to younger populations. Car accidents are the responsible for the majority of severe cases of TBI with violence contributing to a much smaller, though still significant percentage. Survivors often suffer from chronic physical and cognitive disabilities. As the population ages, the number of people affected by TBI is expected to rise.

While advances in treatment over the past few decades have stabilized the mortality rate of those hospitalized because of TBI from 36% in 1987 to 20-24% in 1997 to current rates around 22%, many of the fundamental processes in the pathophysiology of TBI and dynamics of recovery are still poorly understood.

2.2.1.2 Native response to TBI
The mechanisms that contribute to CNS injury of any modality can be broadly categorized into primary and secondary effects. Primary injury refers the initial physical damage experienced by the tissue from mechanical trauma, increased intracranial pressure (ICP) due to the compression of brain tissue in the cranial cavity from released blood (mass effect \(^{45, 46}\)), or possible acute exposure to blood products. Secondary injury occurs in the days and weeks after primary injury and comprises edema (accumulation of fluid leading to ICP increases), accumulation of toxic blood products (in the case of hemorrhage secondary to mechanical trauma) e.g. heme and degradation products, activation of matrix metalloproteinases (endopeptidases that can cleave components of the extracellular matrix), which may disrupt the BBB, increased free radicals, proteolysis, excitotoxicity, initiation of apoptosis, and inflammation at the site of primary injury \(^{44-46, 46-49}\). If left unchecked in the hours and days after initial injury, these effects generally result in additional, and often severe, neurological damage (Figure 5). Hypotension and hypoxemia have been observed to occur in one third of patients with severe TBI \(^{50}\) and are strong predictors of poor outcomes \(^{51}\).

These same symptoms and mechanisms of injury may also be present in hemorrhagic stroke, another serious condition that affects 44,000 people each year in the United States \(^{45}\). Hemorrhagic stroke refers to bleeding in the brain, as opposed to ischemic stroke, which involves occlusion of vasculature supplying the brain, and can be broadly divided into intracerebral hemorrhage (bleeding into the brain parenchyma) and subarachnoid hemorrhage (bleeding into the subarachnoid space at the surface of the cerebrum). In TBI and hemorrhagic stroke, ischemic conditions may also be present due to disruption of vasculature. Like severe TBI, victims of hemorrhagic stroke experience high rates of mortality (only 38% of people with intracerebral hemorrhage survive one year after the incident) and survivors may experience long-term disability \(^{45}\). Because of the similarities in the physiologic mechanisms of action after TBI and hemorrhagic stroke, clinical management and potential interventions may be similar in either case. For that reason, the therapies described in this document to manage TBI, and its mechanisms of action, could also be used to manage and generally describe ‘stroke’ (especially, hemorrhagic stroke).
Figure 5 Sequence of events following traumatic brain injury. A significant factor in inappropriate cell death following TBI is secondary injury induced by ischemia and mechanical tissue disruption. One therapeutic target in this cascade is excitotoxicity. Excitotoxicity occurs when excessive glutamate, the principle excitatory neurotransmitter in the brain, is released into the extracellular space by neurons that are unable to maintain ionic gradients due to energy store depletion as a result of hypoxia or direct mechanical trauma. NMDA receptors on surrounding neurons are over-activated by the extracellular glutamate, leading to an elevation of intracellular Ca$^{2+}$. This rise in Ca$^{2+}$ precipitates the formation of NO, lipases, proteases and endonucleases that ultimately result in apoptosis or irreversible cell damage.

2.2.1.3 Magnesium in TBI

Intracellular Mg is essential in maintaining cell health and plays a major role in the processes in the CNS. The presence of Mg is essential in all energy producing and consuming reactions i.e. oxidative phosphorylation and the TCA cycle. The human body contains approximately 24 grams of Mg with about 60% of that stored in the bones, 39% present in muscle and other soft tissue and 1% present in the blood. Decreases in intracellular Mg are linked to DNA fragmentation and elevated levels of
apoptosis. In ischemic conditions, which may be present after TBI, Mg reduces cellular damage by regulating cell metabolism, maintaining mitochondrial membrane potential while preserving glucose and pyruvate levels. Immediately after TBI, intracellular Mg levels decrease in the injured tissue, leaving cells susceptible to a host of complications, including excitotoxicity and others.

2.2.1.4 Current methods for CNS injury assessment and management

Current assessment of CNS injury is done through a combination of behavioral assessment, neurophysiological evaluation, and non-invasive imaging e.g. Magnetic Resonance Imaging (MRI) and Computed Tomography (CT). There are no known biomarkers in humans that can be used to rapidly and definitively diagnose the severity or extent of an injury. Conventional primary care focuses on supporting various body functions e.g. breathing, and management of increased intracranial pressure, after which, a variety of pharmacologic and non-pharmacologic interventions may be administered in an attempt to avoid secondary complications. Care in this stage is referred to as neuroprotection. Physical therapy and neurological rehabilitation may be necessary to manage late onset sequelae e.g. seizures, behavioral disorders.
Figure 6 Sample CT images. Lighter shades of grey indicate higher density e.g. bone, while darker shades of grey indicate lower density e.g. liquid/air. See figure in 7 for heterogeneous modalities of TBI, visualized by CT. (EDH) – epidural hematoma (a collection of blood outside the dura, a protective tissue layer that surrounds the exterior of the brain), (DIA) – diffuse axonal injury (damage to white matter tracts that connect different portions of the brain), SDH – subdural hematoma (a collection of blood underneath the dura), (SAH/IVH) – Subarachnoid hemorrhage/Intraventricular hemorrhage (bleeding into the ventricles). Image modified from 25.

Although brain injuries have always been a source of death and disability in humans, the mechanisms behind cell death and dysfunction have only recently begun to be extrapolated over the past 20-25 years 56. Several systems for the classification of injuries have been explored including by etiology, symptom, prognosis, and anatomic location 7. Identifying the best mode of care for individual patients is currently one of the biggest challenges in the field due to the vast heterogeneity and severity of brain injury presentation 57. The Glasgow Coma Scale, a metric for quantifying the severity of TBIs, has been widely adopted since its inception in 1974 58. However, despite its widespread acceptance, there are unresolved issues with inter-rater reliability and poor prognostic utility 59.
As our understanding behind the basic mechanisms of brain injuries has increased, more detailed clinical guidelines have started to emerge though there is still not a clinical ‘gold standard’ in TBI treatment or injury classification.

While damage caused by initial brain trauma cannot be prevented, there is hope that secondary injury that occurs afterwards can. This is the motivation behind mechanistic studies of TBI and stroke and the subsequent development of therapies to target those mechanisms. This class of interventions is referred to as neuroprotective therapies.

2.3 Neuroprotective strategies for the management of brain injury

A number of researchers and clinicians have theorized that some, if not all, of the damaging processes that occur post-TBI and stroke can be arrested with appropriate intervention. The ultimate goal of these strategies is to preserve tissue that would otherwise be compromised due to primary and secondary injury, thus providing neuroprotection.

2.3.1 Neuroprotection state of the art

Neuroprotection is still a relatively nascent field and there is not currently a clinical ‘gold-standard’ for providing neuroprotection treatment in the days and weeks after injury. Candidate neuroprotective therapies can broadly be categorized into pharmacologic (drug-based) and non-pharmacologic. Drug-based neuroprotective therapies aim to halt the cascade of secondary injury in the hours post-TBI by targeting the mechanisms that control the pathways of excitotoxicity, inflammation, apoptosis, and other events that cause inappropriate cell death or lead to a loss of function. As each pathway that controls one of these mechanisms has been elucidated, researchers have begun investigating drugs to target them. Non-pharmacologic, physiologic interventions, such as induced hypothermia or hyperoxia, are also of interest.
Currently, of the approximately 40 large-scale, Phase III clinical trials of all neuroprotective drugs in humans performed up to date, none have been shown to be a significantly better treatment option than controls for TBI or stroke\(^{60-63}\), although, some treatments have shown trends toward a beneficial effect\(^{61}\). While positive results have been elusive thus far, the enormous potential impact of developing a successful therapy continues to attract intense research interest.

### 2.3.2 Mg as a neuroprotectant

The Mg ion, which is vital in hundreds of normal physiological processes, has shown great potential as neuroprotectant.

#### 2.3.2.1 Historical Perspective

In the early 1980’s, researchers discovered that Mg\(^{2+}\) is a non-competitive inhibitor of the NMDA receptor in normal physiological processes\(^{64, 65}\). Around the same time, techniques for accurately measuring intracellular and extracellular concentrations of Mg via NMR imaging\(^{66, 67}\) were also being realized. In the late 1980s, Vink and colleagues showed for the first time that intracellular Mg in the brain decreased by 40\%-60\% in the injured tissue after TBI in rats\(^{68}\) and that this decrease was associated with tissue injury\(^{69}\). These findings established the notion that administering Mg\(^{2+}\) to patients, post-TBI, could have a therapeutic effect. Although the mechanism of this decrease is still unclear\(^{70}\), a large body of knowledge exists to justify why Mg is needed for cells to function correctly.

Aside from its role as an NMDA receptor antagonist, extracellular Mg\(^{2+}\) can modulate Ca\(^{2+}\) and K\(^{+}\) ionic fluxes\(^{71}\), directly affect Ca\(^{2+}\) uptake\(^{72}\), increase cerebral blood flow by reversing vasoconstriction\(^{73}\) and reduce depolarization time of active neurons\(^{71}\).
Normal serum levels of Mg are between 0.75mmol/L and 0.9mmol/L \(^{74}\) with toxicity occurring at 1.74mmol/L - 2.61mmol/L. The kidneys tightly regulate Mg levels, so much so that patients with Mg deficiencies often have normal serum levels. Parenterally administered Mg has been shown to enter the CSF \(^{75}\) and increase intracellular levels \(^{76}\), though the rate of this increase can be slow \(^{74}\).

2.3.2.2 Initial animal studies

The discovery by Vink et al that intracellular Mg\(^{2+}\) levels decreased after TBI, and this decrease was associated with more pronounced secondary injury, provided the impetus for a wave of studies that sought to investigate the effects of Mg\(^{2+}\) replacement therapies in TBI and stroke. Since the late 1980s, numerous models of TBI, both in vitro and in vivo, have been developed and refined \(^{77,79}\). Mg ions, delivered parenterally, usually in the form of a Mg salt such as MgSO\(_4\) or MgCl\(_2\), have been shown to provide a neuroprotective effect, in a number of CNS pathologies in animals, including models of focal \(^{80,81}\) and diffuse \(^{82-84}\) TBI. MgSO\(_4\) and MgCl\(_2\) have been shown to have equal neuroprotective properties in animal models \(^{85}\).

2.3.2.3 Clinical trials in humans

The success of these animal trials and several others \(^{86-89}\) in the 1990s and early 2000s prompted a rush of clinical trials in humans through the late 1990s and mid 2000s \(^{90-92}\). Unexpectedly, of the few randomized controlled studies comparing magnesium therapy after TBI, none have shown magnesium administration to be effective in improving patient outcome, though, a recent smaller study conducted in India \(^{93}\) reported positive results. There is currently an ongoing NIH-NINDS-sponsored Phase III clinical trial in Los Angeles, CA whose goal is to evaluate the effectiveness and safety of field-initiated magnesium sulfate in improving the long-term functional outcome of patients with acute stroke \(^{94}\).

2.3.2.4 Limitations of Mg-based neuroprotective therapies
Due to the limited quantity of clinical trials studying Mg therapies in TBI, the methods and data from some of the most significant clinical trials \(^{90-92}\) have been critiqued and reanalyzed by many researchers. In 2008, Arango et al. \(^{95}\) found the methods of both Zhou studies \(^{91, 92}\) inadequate in randomization, blinding, allocation and data reporting, making it difficult to draw any sound conclusions about the effectiveness of the treatment. While the Temkin study \(^{90}\) was well designed, Mg was administered for a far longer period than was indicated by animal models; serum Mg level was increased to double normal plasma levels, 1.25 mM - 2.5 mM, which increased mortality in the treatment groups.

2.3.2.5 *Theories for the limitations of Mg-based neuroprotective therapies*

While altered Mg homeostasis has been associated with TBI, the correlation between ionized Mg levels in serum and CSF after injury is still not well established \(^{96, 97}\). In addition, recent studies have shown that systemic hypermagnesemia produces only marginal increases in total and ionized Mg in the cerebral spinal fluid (CSF) of humans \(^{98}\) and mice \(^{99}\), putting into question whether current methods of intravenous administration are adequate for restoring Mg deficiencies at the site of injury. It has been speculated that this was at least one possible explanation for the ineffectiveness of human clinical trials. It may be that either the brain access or the homeostatic regulation of Mg\(^{2+}\) could be more tightly controlled in humans than in rodents. Thus, magnesium therapies that combine other pharmacologic neuroprotectants and non-pharmacologic interventions are being explored \(^{23}\). In addition, methods are being explored to facilitate Mg ion transport into the CNS \(^{100}\).

2.3.3 Testing the effectiveness of neuroprotective interventions

In vitro and in vivo animal models are critical to elucidating the mechanisms of TBI and stroke and testing candidate interventions. In vitro models have proven essential to understanding the pathobiology of TBI while in vivo models offer a closer approximation of clinical presentation \(^{78, 79}\). In addition, several in vitro models of TBI have been developed in an attempt to rapidly screen neuroprotective interventions before they are tested in animal models \(^{101}\).
2.3.3.1 *In vitro models*

An in vitro model that can closely predict in vivo results provides a cost-effective tool to screen a wide variety of neuroprotective interventions. A recent review by Morrison found that out of 26 studies investigating neuroprotective agents that used in vitro and in vivo testing, in vitro models predicted in vivo results in animal testing in 23 studies (~88% of the time)\(^{101}\), supporting the relevance of these models.

Several models have been developed to recapitulate, in vitro, the principle modes of TBI including axonal transection\(^ {102}\), tissue compression\(^ {103}\), hydrostatic pressure\(^ {104}\), fluid shear stress\(^ {105}\) and strain\(^ {106}\) and tissue stretch\(^ {107}\). Typical experimental setups involve an apparatus for delivering a controlled, adjustable, injurious impact to cells or tissue in culture. Popular cell preparations include organotype cultures (thin tissue slices), dissociated primary cultures (cells taken directly from an animal), and immortalized cell lines (modified cells that will divide indefinitely). In our work discussed in Chapter 3, we used a specialized culture system to grow neural stem/progenitor cells called *neurospheres* (detailed in the following section).

Although in vitro models are useful, they must constantly be validated with in vivo models as tissues and cells isolated from the rest of the body may behave differently ex vivo and the process of disaggregating tissue to put in culture subjects the cells to injury by default\(^ {101}\).

2.3.3.1.1 *Neurosphere cultures*

The results described in Chapter 3 of this document was generated using an in vitro system of cultured neurospheres, neural stem/progenitor cells derived from the brains of newborn mice. Using this culture system, which was developed in the 1990’s, it is possible to culture stem cells from brains that could differentiate into neurons and both forms of glia: oligodendrocytes and astrocytes. In the adult
mammalian brain, stem cells capable of creating new neurons sit in a region immediately adjacent to the ventricles of the brain (the internal space that is filled with cerebrospinal fluid), called the subventricular zone. These stem cells do not normally neurons produce in situ. However, they can be manipulated to make neurons when placed into cell culture. Growth factors and other conditions in the media can be adjusted to control how these cells differentiate and proliferate.

Because these cells do not quite fit the definitions used in other cell systems, we call them stem/progenitor cells rather than strictly calling them one or the other. In this thesis, we will term these cells neural stem/progenitor cells (NSCs).

2.3.3.2 In vivo (animal) models

In vivo modeling is critical in establishing the effectiveness of a neuroprotective therapy. The use of animal models allows researchers to tightly regulate the modality and severity of TBI in an attempt to mimic the physiology in observed in the human case. Models utilizing consistent moderate to severe injury give researchers the best chance of observing a drug effect 108.

Several animal species have been used to model various aspects and modes of TBI. In the 1980’s, cats, dogs and non-human primates were all popular choices. Rodents eventually replaced these animals in most studies and are especially popular today because of their relatively low cost, ethical acceptability, simplicity of surgical procedure and ability to investigate mechanisms of injury via knockout animals 109, 110.

The four most common methods for inducing TBI are the weight-drop model (where a mass is dropped onto the head of the animal from a fixed height and orientation, the impact acceleration model (where a mass is dropped from a fixed height on to another mass that is in contact with the animal’s head), the controlled cortical impact model (where a trauma is delivered directly to the cortex via a blast of air
(where trauma is delivered directly to the cortex by a pressurized pulse of saline) \(^{111}\). A number of refined version of these basic models have been designed to mimic specific primary or secondary injuries or a mix of both \(^{112}\).

Two principal animal models for studying hemorrhagic stroke, specifically intracerebral hemorrhage, involve the infusion of either autologous blood or collagenase into the brain parenchyma \(^{49}\). Several animal species have been investigated \(^{113}\) although rats remain the most popular for the same reasons as listed previously.

### 2.3.3.3 Limitations of current models for studying Mg-based neuroprotection

Although rodent models of TBI and stroke possess many useful qualities, there continue to be difficulties in translating laboratory findings derived from these models to clinically relevant interventions. This may be due party to differences in anatomy between humans and rodents e.g. brain geometry, the ratio of white matter to grey matter and the lack of cortical gyri in rodents \(^{114}\). Also, studies examining neuroprotective drugs in animals are generally very carefully controlled, in ways that are rarely, if ever, experienced in reality. For example, the populations of rats used in many TBI studies were genetically identical, all of the same sex or unrealistically young, and studies usually exclude animals that experience ischemia or hypoxia \(^{38}\). In other studies, the effective dosage of certain drugs used in animals may be prohibitively toxic in humans \(^{39}\). Also, the administration of the drugs within the window of opportunity for effective treatment found in animals (immediately after injury) is often missed in human clinical trials \(^{60}\). Finally, there can be unexpected differences between Mg\(^{2+}\) metabolism and physiologic processing in animals and humans e.g. permeability difference in the blood brain barrier between species \(^{98}\).

### 2.4 Injuries to peripheral nerves

PN injuries often present complex cases in the clinic due to the limited regenerative potential of neural tissue and the slow rate of PN regeneration. When a peripheral nerve is damaged, axons downstream of
the injury (distal to the injury) degenerate and axons above (proximal to) the injury grow toward, and, ideally, eventually reinnervate the distal target. Regeneration is significantly superior if the regenerating axons are able to use the same cellular infrastructure (the fascicles that contain the epineurium, perineurium and endoneurium and still retain Schwann cells) that was formally inhabited by the degenerated distal axons. Because the cell bodies for most peripheral nerves reside in or near the spinal cord (up to a meter from their distal target), severe injuries may require a regenerating axon to travel an extraordinary distance with precise guidance for functional recovery to be realized. In addition, the denervated nerve fascicles and the associated muscle can undergo degenerative changes that may not be reversible if the time period is too long. Thus, full recovery is unlikely to occur in many cases without surgical intervention to speed up regeneration and assist the body in this process.

2.4.1 Incidence and impact of PN injuries

Peripheral nerve injuries occur in approximately 3% of all visits to the ER include trauma to peripheral nerves (5% including nerve root and brachial plexus injuries), representing over 300,000 injuries each year \(^{16,17}\). Severe peripheral nerve injuries typically lead to lifelong disturbances in the function mediated by the injured nerve \(^8\). As a result, peripheral nerve injury can have a significant impact on affected individuals at great cost to society.

2.4.2 Classification and characteristics of PN injuries

Nerve injuries have traditionally been classified on the Sunderland scale, graded from 1 to 5, with ‘1’ being the least severe \(^{115}\) (Figure 7). A Sunderland grade 1 injury is referred to as *neurapraxia* and may be caused exposure to heat or cold, irradiation, electrical injuries or mechanical stress (e.g. compression) and is often associated with ischemia \(^8\); conduction of signals down the axon is blocked and injury may be associated with mild myelin breakdown though the rest of the nerve anatomy is intact and recovery will occur in days to months. Sunderland grade 2 injury is more severe and is referred to as *axonotmesis*; the axon is transected, although the endoneurium surrounding it is still intact. Since only the axon is damaged
and the remaining nerve architecture is still intact, functional recovery will likely occur without intervention. Sunderland grade 3 injury is referred to as neurotmesis; the axon and endoneurium are transected, but the perineurium remains intact. Scarring may occur in the endoneurium, creating a potential barrier for regenerating axons. Still, functional recovery is likely because the damaged axons are limited to a single fascicle and surgical intervention is generally only required to relieve compression. Sunderland grade 4 injury is referred to as neurotmesis +; the axon, endoneurium and perineurium are transected, but the external epineurial sheath remains intact. This results in non-functional tissue that will likely result in scarring within the endoneurium and recovery will not occur without surgical intervention. Sunderland grade 5 injury is referred to as neurotmesis ++ and refers to complete transection of the whole nerve trunk. Surgery is required to prevent formation of a neuroma, a cyst of non-functional nerve tissue, at the proximal nerve trunk. A sixth grade of injury has also been defined as a mixed injury of all types along the damaged nerve.

A major challenge of diagnosing nerve injuries is that Sunderland grades 2 through 4 may present very similarly for up to 3 months after injury, at which point treatment is less effective than it would be immediately after injury, because of the aforementioned degeneration of distal nerve and muscle.

Figure 7 Peripheral nerve injuries graded on the Sunderland scale. The least severe grade of injury, Sunderland grade 1, refers to a crushed axon. In a Sunderland grade 2 injury, the axon is severed but the endoneurial sheath remains intact. In a Sunderland grade 3 injury, the endoneurium is compromised. In a Sunderland grade 4 injury, perineurium is disrupted and in a Sunderland grade 5 injury, the entire nerve is transected. Surgical intervention is required for grades 4 and 5, and circumstantially
for grade 3 injuries. Grade 1 and 2 injuries are left to heal on their own. A sixth grade has also been defined for damaged nerves that present with multiple grades of injury.

2.4.3 Native response to PN injury

The body responds to severe PN injury (a severed nerve) through a complex biological process referred to as Wallerian degeneration, characterized by retrograde and anterograde breakdown of damaged nerve tissue followed by new tissue growth. Immediately after injury, fibroblasts from the nerves, as well as plasma and blood-derived cells invade the space between the proximal and distal nerve stumps of the injured axon/nerve, resulting in the accumulation of a ‘clot-like’ material. The proteins, particularly those from the plasma form an oriented matrix composed primarily of the protein, fibrin. This matrix forms in cable-like fibrin strands that bridge the gap between the proximal and distal stump. This can only occur in a relatively closed space, i.e. if the two ends of the nerve are aligned and close to each other, like they would be in a nerve conduit. Non-neuronal support cells, including fibroblasts and Schwann cells, migrate from both the proximal and distal stumps and follow these fibrin cables (the matrix) and thereby are able to cross the gap. Without the fibrin cables, the cells remain attached to each nerve stump. The volume of this cable is directly related to the volume of exudate flowing from the proximal and distal stumps of the severed nerve.

During the next few days, macrophages invade the damaged tissue, breaking it down and removing it, while Schwann cells begin to rapidly divide. This initial structural support by the fibrin bridges is necessary to get the Schwann cells across the gap. This process of having a physical guidance structure (the fibrin bridges guiding the fibroblasts and Schwann cells) is referred to as contact guidance. Then, once they have crossed the gap, the Schwann cells coalesce into columns of cells that are called the Bands of Büngner. Concurrently, the neuronal cell body is rapidly producing proteins necessary for the growth of new processes and sprouts begin to form at the site of the severed axon. In the weeks to months after injury, for successful complete regeneration, it is necessary for the axonal sprouts to find their way into the Bands of Bungner, which then guide the axons across the gap. Chemotrophic and chemotropic factors
originating from both Schwann cells and the distal stump are also important for enhancing regenerating axon outgrowth and guidance. After bridging the injury gap through Bands of Büngner, axons must find their way into endoneurial tubes in the distal nerve segment (which have since been cleared of damaged nerve tissue during Wallerian degeneration).

Once axons successfully cross the gap and have entered the distal nerve stumps, axonal regeneration all the way to the distal target commences at a rate that varies by species (~1-2 mm/day for humans, 3-4 mm/day for rats, etc.)\(^\text{31}\). In the ideal case, these axons will make their way to their distal target and re-establish a functional connection. Full functional recovery, including remodeling of axonal synapses, then occurs over a slower time frame, i.e. up to 2 years in humans.\(^\text{119}\) Axons that were not successful in finding an endoneurial tube will retract back into the axonal trunk. In the mean time, the distal target muscle will begin to atrophy as a result of denervation, and after a certain time period, it will be difficult for the regenerating nerves to reverse the atrophy. However, it is speculated that it is not the muscle, but changes in the Schwann cells in the distal nerve stumps that are the time-limiting factor in nerve regeneration.\(^\text{120}\) Over time, the Schwann cells in the distal stump, which were ‘activated’ by the injury to increase production of neurotrophic and tropic factors, cease that activity if they do not encounter regenerating axons. In other words, if it takes too long for the axons to arrive, then the trophic support is not present. Thus, all efforts to enhance PN regeneration over long gaps must take into account that this needs to occur in a very timely manner.

(See\(^\text{8}\) for complete figure)

**Figure 8 Wallerian degeneration and subsequent healing processes.** After a nerve is transected (A), there is rapid retrograde and anterograde degeneration of the injured axons and supporting architecture. In the days and weeks after injury, macrophages begin to clear out debris as Schwann cells rapidly proliferate (B). The target muscle begins to atrophy, as it is no longer receiving signals from its nerve. The nucleus of the cell expands, producing the proteins needed to rebuild the axon. The proximal stump of the injured axon begins to grow out at this time. In the weeks after injury, Schwann cells begin to form specialized filaments, called bands of Büngner, which will serve the purpose of providing a physical guide for the regenerating axons toward its distal...
target (C). The regenerating axons slowly moves toward their target at approximately 1mm/day along this band of Büngner as Schwann cells re-encapsulate and myelinate the newly the regenerated tissue. The atrophy in the distal target is reversed if the axon is able to successfully reach its target and form a functional connection. However, if the axon is unable to reach its target as a result of a critical gap, physical blockade of scar tissue, or misdirection, a neuroma will form at the proximal stump and the target will not be re-innervated and will remain chronically atrophied. See 8 for complete figure

2.5 Strategies for the management of PN injuries

As stated above, PN injuries are of serious concern since severe injuries cannot heal on their own and full functional recovery is rare, even with the best treatments available to date.

2.5.1 Surgical intervention

Surgical intervention is the only treatment option for severe nerve injuries (Sunderland grade >2). There are three main strategies for surgically repairing damaged nerves: direct repair, graft repair and nerve transfer. In direct repair, the proximal and distal nerve ends are simply sewn back together. In graft repair, a graft is used to bridge the proximal and distal nerve ends when direct repair is not possible. In nerve transfer, the distal stump of a severed nerve is sutured to the side of a neighboring functioning nerve when direct repair or nerve grafting is not possible.

2.5.1.1 Direct repair

Direct repair is also called neuroanastomosis or end-to-end neurorrhaphy. Microsurgical techniques allow for the rejoining of individual fascicles and this has been shown to improve recovery. One of the biggest challenges of a successful direct repair is not inducing tension on the injured nerve. Elongation of more than 10% of the original length of the nerve is associated with ischemia and cell death in the repaired nerve 121. Factors attributing to this include that the perineurium may become permeable (PN tissue is isolated from the blood, similar to the CNS) and the endoneurium damaged 17. In addition, the damaged nerve tissue must be surgically excised in order to avoid scar formation 122.
2.5.1.2 Nerve grafting

In cases where a direct repair is not possible, an autograft is the preferred bridging method. An autograft may be obtained by excising a sensory nerve, often the sural nerve in the lower leg. The excised nerve is then used to bridge the gap between the injured proximal and distal stumps. Advantages of using an autograft include biocompatibility, established tissue architecture and the presence of Schwann cells. Despite these qualities, there are still several challenges to this approach and full recovery is rarely achieved.

One major issue is that there are differences in the architecture of sensory and motor nerve fibers. Using a sensory nerve autograft to repair a motor nerve is much less effective than repairing a motor nerve with a motor nerve although sensory nerves may be repaired with either type \textsuperscript{123,124}. Also, there is an inherently limited supply of autograft material and complications may arise from the surgery required to retrieve the autograft.

2.5.1.3 Nerve transfer

In cases where it is not possible or feasible to join the proximal and distal nerves after injury, using a surgical technique called nerve transfer may still preserve the distal nerve and its target. An expendable donor motor nerve is located near the distal target of the injured nerve. The distal stump of the injured nerve is sutured to the side of the donor nerve then the donor nerve is then injured, activating a regeneration response in the donor nerve causing axons to migrate into the distal stump of the injured nerve and re-enervate the target. Re-wiring then takes place at the level of the cortex (in the CNS) to enable functional operation of both the distal target of the donor nerve and that of the injured nerve. Disadvantages to using this nerve transfer include a shortage of expendable donor nerves and relatively few surgeons trained in this technique \textsuperscript{125}.

2.5.1.4 Allografts
Allografts, nerve tissue taken from another human, have been used successfully although immunosuppressant medication must be administered for up to two years after the repair. Acellular allografts have also been used successfully but are prohibitively expensive. These interventions are reserved for the most extreme cases e.g. extensive brachial plexus injuries, where there are limited options available.

These challenges, coupled with the less than ideal recovery rate, have led researchers to investigate alternative biological and synthetic grafting materials.

2.5.2 Nerve conduits

Nerve conduits are engineered alternatives to autografts and have been manufactured from a variety of materials including naturally derived e.g. venous grafts, collagen, fibrin and synthetic e.g. polyesters (PCL, PGA, PLLA etc.). They are especially attractive because they are relatively inexpensive, easy to produce, have a long shelf life, show good biocompatibility, are biodegradable and the technique for placement is simple \(^{28}\). In addition, topographical cues can be used to guide nerve outgrowth and the conduit itself can prevent ingrowth of potentially scar-causing fibrous tissue \(^3\).

2.5.2.1 Conduit state of the art

The inherent drawbacks of autografts include a second surgery required to harvest the donor nerve, donor nerve site morbidity, a limitation of the amount of donor tissue that can be harvested and a success rate of only \(\sim 80\%\) \(^{126}\). These issues have led researchers to look toward a variety of engineered alternatives. Nerve conduits are currently the focus of intense research interest \(^{15}\), with the ultimate goal of creating a bioengineered construct that can be used to guide functional regenerating nerve tissue across a defect more effectively than an autograft. In addition, and importantly, engineered conduits provide an enclosure for the accumulation of growth factors excreted from the proximal and distal stumps, they prevent fibrous tissue invasion which can lead to neuroma formation and they decrease tension across the nerve defect \(^{127}\).
Currently, there are several FDA-approved nerve conduits on the market. However, their lack of an extracellular matrix, growth factors and support cells has limited the use of these conduits in clinical practice to repairing gaps in small diameter, noncritical nerves of less than 3 cm in length or in large diameter, critical nerves less than 1 cm in length.

2.5.2.2 Historical perspective

The concept that physical guidance and enclosure are required to direct the axons of injured nerves to their distal target after injury has been around since at least the 1880’s when Austrian scientists attempted to bridge nerve defects in humans using autografts, bone, Mg foil tubes and fat sheaths. These interventions were not very effective and it was not until the 1960’s that microsurgical techniques, which aligned individual fascicles within a damaged nerve, were developed and greatly improved recovery outcomes. Since that time, much has been progress has been made in surgical research, with the autologous nerve graft emerging as the clinical ‘gold standard’ when PN injuries larger than 1-2 cm need to be repaired.

The modern quest for developing an engineered construct began in earnest in the late 1970’s when Lundborg and colleagues performed a series of experiments designed to assess the spatial temporal progress of peripheral nerve regeneration. A silicone tube, referred to as a ‘viewing chamber’, was used to bridge defects ranging in size from 6-10 mm in rat sciatic nerves. Using this model, Lundborg and colleagues were able to identify and describe the biological events in nerve regeneration at weekly intervals, including the formation of a fibrin matrix across the defect at 1 week that must be formed so that Schwann cells, fibroblasts and endothelial cells from the proximal and distal stumps could migrate and meet in the middle.

Since then, conduits made of non-resorbable materials, like silicone, have been associated with nerve compression and chronic inflammation. Absorbable conduit materials are now preferred as they allow...
for greater interaction between the regenerating tissues and the local environment while reducing the incidence of axonal compression. Researchers have continued to improve on this concept by altering a litany of design factors, including material selection, coatings and topography, addition of growth factors and various cells or designing channels into the device design.

2.5.2.3 Properties of successful conduits

Several approaches to increase the volume and quality of nerve tissue that regenerates through the conduit have been tested in animals since the late 1970’s. Through these works, a number of conduit properties have been identified that improve functional recovery (Figure 9). Many efforts involve testing different conduit materials with a variety of geometric cross-sections to provide a gross guiding function in combination with different conduit fillers to provide a microenvironment conducive to cell growth. Other efforts have included the use of several biodegradable and non-biodegradable conduit materials, filling the lumen of the conduit with a variety of hydrogels, channels or micro-patterned fibers, and seeding those fillers with Schwann cells, stem cells or growth factors.
Figure 9 Properties of an ideal nerve conduit. A number of properties have been identified to improve the regenerative response in a nerve conduit over that of an empty condiment. The conduit should be porous to allow for the exchange of nutrients and biodegradable to avoid chronic irritation. The incorporation of growth factors into the conduit can stimulate the regenerating nerve tissue. Incorporating support cells e.g. Schwann cells, various stem cells, can accelerate the regenerative process. Materials that are electrically conductive have been shown to induce stronger regenerative response and physical guidance from intraluminal channels and oriented nerve substratum has also been shown to be effective at encouraging tissue outgrowth.9, 256

2.5.2.3.1 Material Selection

A large number of natural and synthetic biomaterials have been investigated for use as nerve conduits. Material selection and processing will determine the biocompatibility of a conduit and biocompatibility will be dependent on both the micro and macro structure of the scaffold.140 The adsorption of biologic molecules, cell adhesion, migration and differentiation can all be affected by the surface chemistry of an implanted biomaterial.141 Physical properties of an engineered scaffold, such as porosity can influence cell proliferation and migration.142
Naturally derived biomaterials possess cell-binding domains that make them favorable for axonal attachment and migration. Indeed, the total protein within a typical nerve is almost half collagen types I and II. Type I collagen conduits have been used to successfully bridge 20mm nerve gaps in the human comparably to autografts. Xenogeneic acellular grafts, chitosan, silk fibroin and fibrin conduit have also been investigated. Coatings of other naturally derived molecules, including laminin and fibronectin, have also been attempted.

2.5.2.3.1.2 Synthetic Materials

Synthetic nerve guide materials have been largely confined to polyesters thus far including poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly-e-caprolactone (PCL), and poly(L-lactic acid) (PLLA). These materials are biodegradable, well characterized and already used in a number of FDA approved devices including suture materials and orthopedic screws. The degradation rate of these polymers can also be highly engineered. In current FDA-approved conduits, typical resorption occurs in 1-2 years. However, degradable polymers may also cause local inflammation at the site of implantation due to acidic corrosion products.

Several non-degradable polymers have also been examined including silicone, Teflon, polyethylene and others. These materials are generally avoided in favor of biodegradable options as non-degradable materials pose a higher risk for infection, nerve compression, and chronic inflammation.

For the study described in Chapter 5, we collaborated with a laboratory that has spent over 10 years developing nerve conduits; Dr. Kacey Marra and her students provided us with biodegradable PCL nerve conduits that had been successfully tested in animals.
There is a need for internal support within the conduit to provide contact guidance for Schwann cells and other support cells and structures i.e. the fibrin matrix bridges described by Lundborg et al. The basal lamina serves this support function in autografts though there are issues with the matrix formation within a conduit that prevents regeneration in longer gaps (>10 mm in rodents, >30 mm in humans). Oriented scaffolding within the lumen of the conduit has been shown to significantly affect the ability of regenerating tissue to overcome gaps of up to 15mm in rodents (the longest gap achievable in a rat sciatic nerve model), likely by stabilizing the formation of the naturally occurring fibrin matrix.

Several novel biomaterial solutions have been tested for this purpose. Clements et al attempted to use a sheet of poly(acrylonitrile-co-methylacrylate) with a microstructure resembling linearly oriented filaments. It has been shown that cells will migrate preferentially along the long axis of microfilaments and that the diameter of these filaments may also affect cell migration. Internal filaments that have been tested as part of conduit systems include suture material (~250 µm in diameter), biodegradable and non-biodegradable polyglactin, catgut (ovine intestinal submucosa) and polyamide filaments and autograft material. In the experiments performed by Arai et al, there was no difference in the performance of biodegradable and non-biodegradable filaments used to bridge a gap length of 15 mm as measured by number of myelinated axons and sensory re-innervation at three months. However, there was a difference detected, with biodegradable filaments performing better, at 6 months. This same group also showed that the macrophages have a significant role in the regenerative process and that mild inflammation caused by internal filaments may actually be beneficial to new tissue growth.

2.5.2.3.3 Incorporation of growth factors

Nerve growth factors, or neurotrophic factors, are chemicals (usually proteins) that promote cell growth. If they are produced in one place, in sufficient quantities that they create a concentration gradient, then they can be “tropic” and can guide axonal growth towards the tissue producing the factor, in this case, the correct target tissues. It was demonstrated in the early 1980’s that the distal stump of a severed nerve
would release these chemicals, guiding regenerating axons from the proximal stump $^{164}$. For this reason, several investigators have attempted to incorporate various growth factors including *nerve growth factor* (NGF), *neurotrophin-3* (NT-3), *brain-derived neurotrophic factor* (BDNF), *glial-derived neurotrophic factor* (GNDF) and others into biodegradable polymers that release the drug as they degrade $^3$. Each of these compounds has been shown to affect nerve behavior, with GNDF showing exceptional promise in improving the regeneration of peripheral nerves $^{156,165}$. The implementation of these interventions is quite challenging though as success requires precise sequence, timing, dosage, delivery, duration and synergy of multiple growth factors $^{166}$.

### 2.5.2.3.4 Incorporation of support cells

Schwann cells are critical in the regeneration process of injured nerves, since they not only form the Bands of Büngner, but also produce multiple neurotrophic factors $^{167}$. As such, they have been intensely studied as additives to nerve guides to accelerate and improve the regeneration response with some success $^{19,168}$. Recent advances in cell culture techniques have led researchers to investigate the effects of loading pluripotent stem cells, or those differentiated toward Schwann cell or neuronal lineages, into conduits as well $^{19,169,170}$.

### 2.5.2.3.5 Hydrogels

Hydrogels are composed of strongly hydrophilic polymeric chains that can form a water-based gel. These constructs may provide excellent scaffolding for cellular infiltration and, thus, have been investigated as fillers for nerve conduits. Various natural and synthetic materials have been processed into hydrogels to perform this function including collagen $^{171}$, laminin $^{171}$, keratin (derived from human hair) $^{33,34,172,173}$ and alginate $^{174,175}$. Hydrogels have also been used to construct conduits $^{176,177}$ although they have not performed as well as autografts, likely because of mechanical weakness. Keratin hydrogel was used as a nerve conduit filler in the work presented in Chapter 5 of this document.
2.5.2.3.6  Electrical activity

It has been shown that recovery of peripheral nerve injuries can be improved by applying a brief electrical stimulation in the form of both AC or DC current to the proximal nerve stump after injury. The hypothesized mechanism is that certain growth-associated genes are upregulated by the damaged nerves in response to electrical stimulation, and evidence is accumulating that support this. The timing and duration of electrical stimulation impacts the magnitude of this effect, with long term stimulation (2 weeks) not being as effective as short term stimulation of 1 hour immediately after injury. One limitation of this therapy is that long-term implantation of electrodes is associated with local inflammation and fibrosis around the electrode.

2.5.3  Testing nerve conduit effectiveness

While much work has been done to demonstrate the effectiveness of nerve conduits in various animal models, clinical use is still relatively limited. This is partly because, while animals can closely model appropriate clinical conditions, these models can only approximate the human condition. Also, when a patient has a traumatic injury, the surgeon will not know exactly what type or size of nerve injury is present until the surgery begins. So it might be relatively rare that all the nerve gaps are <3 cm. Rather than go to the trouble of purchasing conduits, which may be used only rarely, surgeons may just rely on autografts, which can be used for any size gap, up to 10 cm.

2.5.3.1  In vitro material screening

In vitro testing is used to provide information regarding the effects of a biomaterial on cytotoxicity, genotoxicity, cell proliferation and differentiation of candidate biomaterials for nerve tissue engineering. These tests are more easily standardized than in vivo models, although they can provide only limited information on potential foreign body response, vascularization or other tissue/biomaterial interactions. A number of advances in the basic science of nerve regrowth have been identified,
through the use of in vitro systems, elements of Schwann cell/axon interaction\textsuperscript{190}, how growth factors affect cell outgrowth\textsuperscript{3}, and how injury alters gene expression\textsuperscript{191}.

2.5.3.2 \textit{In vivo modeling}

In vivo models are essential for evaluating devices for clinical effectiveness. By assessing conduit performance in a variety of animal models in clinically relevant biological conditions, biocompatibility, tissue response and mechanical function of the conduit may be assessed, as well as functional recovery. These data are critical components of the FDA approval process for new medical devices.

2.5.3.2.1 \textit{Animal models}

A number of animals have been used to test the effectiveness of nerve conduits in the past 50 years (Table 1). The rat sciatic nerve defect model has emerged as a popular choice for testing conduits due to easy surgical access to the sciatic nerve and the relatively low cost of using rats. Although rodents have been shown to demonstrate superior neuroregenerative capacity compared to humans\textsuperscript{192}, they still remain the most economical choice for early conduit screening.

\textbf{Table 1} \textit{Overview of animal models used to test nerve conduits in the published literature since 1950.} Rats have emerged as the most popular animal model because they are relatively inexpensive, possess easily accessible nerves and demonstrate robust neuroregenerative capacity. Table modified from\textsuperscript{13}.

<table>
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<tr>
<th>Species</th>
<th>Rat</th>
<th>Mouse</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Cat</th>
<th>Sheep</th>
<th>Monkey</th>
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<td>14</td>
<td>4</td>
<td>10</td>
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<td>416</td>
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</tbody>
</table>

2.5.3.2.2 \textit{Assessment methodologies}

A number of modalities exist for assessing the effectiveness of the nerve conduit. Many popular methods involve histology of the regenerated tissue. Immunohistochemistry can be readily applied to serially
sectioned tissues in order to identify markers for Schwann cells, axons, or inflammation. Morphometric analyses may also be used to compare variables such as the number of axons, density of axons in regenerating tissue (N-ratio) or axon diameter compared to myelin thickness (G-ratio) in slices at various depths of the nerve/regenerated tissue. Electrophysiology may also be used to measure conduction along regenerated nerve tissue (mean conduction velocity – MCV) and the magnitude of the contraction of a muscle innervated by the regenerating tissue (compound muscle action potential – CMAP). Other popular assessment methodologies include comparing muscle weight, muscle cross sectional area, gait kinematics, retrograde labeling of sensory and motor nuclei (applying a dye to one part of a cell e.g. the axon, that is transported back to the nucleus) and, in the rat sciatic nerve model, the sciatic function index (SFI - a method for evaluating functional recovery by measuring specific features of rat footprints after injury)\textsuperscript{193}.

2.5.3.2.3  Limitations of in vivo models

A recent review, published in 2012, exhaustively examined the published literature to survey the animal models and parameters for success used in nerve conduit testing over the past fifty years \textsuperscript{13}. Over 70 materials were used in several different species. The nerve gap length, choice of nerve to damage and assessment methodology also varied widely.

Another survey of the literature published in 2007 \textsuperscript{194} found that, despite their frequent use, many of the methodologies used to assess conduit performance were not able to consistently resolve differences between animals treated with nerve grafts, autografts or untreated controls in one of the most common in vivo models, the rat sciatic nerve defect model. Methods shown to be effective in discriminating between treated and untreated nerves were nerve fiber count, N-ratio, gait analysis, histological examination (binary presence of regenerated axons in distal segments of regenerating nerves) and CMAP. Retrograde labeling and nerve fiber diameter may provide insight into the state of a regenerated nerve but are not good measures of functional recovery. The SFI and measures of MCV have not been shown to consistently discriminate between experimental groups.
In the work presented in Chapter 5, histological examination, weight of the gastrocnemius (a major muscle innervated by the sciatic nerve) and behavioral analyses (simple gait analysis, measurement of footprint parameters (similar to SFI)) were used to compare groups. Histologic examination was chosen because it has proven an effective method for resolving differences between groups in the past. Muscle weight, although not nearly as consistent as histologic analysis, was chosen because it is still an acceptable metric for evaluating differences between experimental groups and the data collection is methodologically simple and fast. Even though behavioral analyses have produced mixed results in the past, we still attempted them, as our conduit system had not been previously tested in animals.

2.6 Biodegradable metals

Biodegradable metals have become the center of intense focus in the biomedical research community in recent years and represent a departure from the paradigm of using only corrosion resistant metals in medical devices. They have become the focus of a new generation of medical devices that are implanted into the body to provide temporary mechanical support during the healing process of injured tissue and are then resorbed into the body. Mg metal, in particular, is currently being investigated as an exciting material for bioresorbable orthopedic screws and plates and vascular stents, due to the fact that it has desirable mechanical properties, is well tolerated by the body and biodegrades readily in the aqueous environment of the body. In the experiments described in Chapters 4 and 5, we present initial studies to evaluate a novel use of this metal as a biodegradable, filamentous support within the lumen of a biodegradable nerve conduit.

2.6.1 The need for biodegradable implants

There are many instances in medicine where there is a need for an implantable device with only limited performance. For example, when treating bone fracture in a pediatric patient, orthopedic hardware used to stabilize the fracture often has to be removed as soon as the bone is healed to avoid stunting future bone
growth. In adult patients, orthopedic implants are often a source of chronic pain and frequently need to be removed, necessitating an additional surgery. Stents used in vascular surgery to relieve vessel occlusion eventually cause fibrosis at the site implantation, in part because surrounding tissues become irritated by the hard metal moving against the constantly moving blood vessels. Fibrosis necessitates additional surgery that often involves placing a second stent on top of the first or removing the fibrotic vascular tissue and replacing it with a synthetic alternative. Also, tissues are constantly remodeling; an implant that eventually degrades will be remodeled into tissue that is a closer approximation of normal tissue than a static implant composed of titanium or stainless steel. Finally, the presence of a permanent foreign substance leads to a specific cellular response, called a chronic foreign body response, which includes accumulation of inflammatory cells that can irritate surrounding tissues.

2.6.2 Corrosion of biodegradable metals

Corrosion is dependent on several factors including geometry and surface characteristics of the implant, rate and extent of vascular flow in and adjacent to the implant, local pH and mechanical stress on the implant, all of which can vary by tissue type and location. Timing the Mg corrosion/resorption rate to coincide with the needs for nerve regeneration will be crucial to the success of a Mg-based scaffolding approach for PN tissue regeneration.

A major mechanism for metal corrosion in an aqueous environment is electrochemical corrosion. In the presence of water, metal atoms become oxidized and water molecules are reduced into OH⁻ and H⁺ ions. The H⁺ ions accept the electrons from the metal, forming H₂ gas, while aqueous metal ions complex with OH⁻ groups forming metal hydroxide. The overall corrosion reaction for Mg is shown below:

\[
\text{Mg}(s) + 2 \text{H}_2\text{O}_{aq} \rightarrow \text{Mg(OH)}_2(s) + \text{H}_2(g) \quad (1)
\]
Magnesium is especially susceptible to this reaction as a result of its strong electrode potential. As the metal reacts with water, magnesium hydroxide accumulates on the surface of the magnesium, creating a protective film between the bulk metal and the aqueous interface. This is referred to as a passivation layer. In physiologic environments, chloride ions attack this magnesium hydroxide layer, converting it into magnesium chloride, which is highly soluble. This, in turn, causes severe pitting corrosion on the metal. Impurities in the magnesium may also facilitate galvanic corrosion (corrosion that occurs when two metals with different electrode potentials are in contact) and all of the corrosion results in subsequent hydrogen gas evolution. The rate of gas evolution is directly correlated with the rate of corrosion. In vivo, hydrogen gas evolution from rapidly corroding Mg alloys may result in the creation of gas cavities between tissue layers and the risk of a gas embolism. The constituent gases within a cavity closely resemble atmospheric concentrations as hydrogen gas evolved from the implant is rapidly exchanged with those in blood and tissues.

Corrosion is measured in mils/year and is calculated by using the following equation:

\[
\text{Mils/year (mpy)} = C \times \frac{\text{weight loss}}{\text{area} \times \text{time}} \times K
\]  

(2)

Where C is a constant (to compensate for using different units of weight loss, area and time) and K is a density factor, characteristic for each material.

The corrosion behavior of magnesium alloys in vivo is very complex and is still not well-understood. Immediately after implantation, proteins attach to the implant as part of the normal host response to a foreign body. This protein layer, and subsequent encapsulation by surrounding tissue, serves to protect the metal from aqueous exposure, slowing the corrosion rate. In addition, the diffusion of H₂ and clearance of other corrosion products is dependent on the water content and its flow in the surrounding tissues, the amount of and intactness of local blood vessels and the blood flow in these vessels. These are, in turn,
affected by the corrosion rate of the metal. Differences in water content, flow rates and vascularization between different tissues and between animal species and humans make predicting the corrosion rate of a magnesium alloy based on in vivo animal studies very difficult. Several factors have been shown to influence the rate of corrosion in vitro including pH, temperature, pressure and others (Table 2) although much work is still required to develop and standardize in vitro systems for monitoring and measuring corrosion that may accurately predict corrosion in the in vivo case. Another set of variables is the physical properties of the Mg, as mentioned above. This includes the alloy composition, trace element composition, size, shape and even surface roughness (which will affect how proteins adsorb and cells attach). Both processing and post-processing of Mg metal can dramatically alter corrosion rates.
Table 2 Variables that affect corrosion rate. Several variables have been identified that affect the rate of Mg corrosion in an aqueous environment. In the body, the rate of resorption of a metallic implant will depend on the complex interaction of all of these factors, as well as physical properties of the Mg. Table modified from 14.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Increase or decrease</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride concentration</td>
<td>Increase</td>
<td>Increased corrosion</td>
</tr>
<tr>
<td>pH</td>
<td>Increase</td>
<td>Increased passivation</td>
</tr>
<tr>
<td>pH</td>
<td>Decrease</td>
<td>Increased corrosion</td>
</tr>
<tr>
<td>Medium buffering capacity</td>
<td>Increase</td>
<td>Decreased corrosion</td>
</tr>
<tr>
<td>Temperature</td>
<td>Increase</td>
<td>Increased corrosion</td>
</tr>
<tr>
<td>CO₂</td>
<td>Increase</td>
<td>Increased corrosion (via buffering)</td>
</tr>
<tr>
<td>O₂</td>
<td>Decrease</td>
<td>Decreased corrosion</td>
</tr>
<tr>
<td>Proteins</td>
<td>Increase</td>
<td>Decreased corrosion</td>
</tr>
<tr>
<td>Mechanical compression</td>
<td>Increase</td>
<td>Decreased corrosion</td>
</tr>
<tr>
<td>Mechanical tension</td>
<td>Increase</td>
<td>Increased corrosion</td>
</tr>
</tbody>
</table>

2.6.3 Metabolism of corrosion products

Mg alloys have been shown to induce a minimal foreign body response, 29, 203 as the native buffering systems in the body are readily able to compensate for increases in the concentration of corrosion products (OH⁻ and Mg²⁺) 204. In addition to metal ion release via electrochemical corrosion, and galvanic corrosion in the case of Mg alloys, local cellular action also influences the resorption rate of Mg. In bone tissue, particles of degradable biomaterials have been observed in the cytoplasm of macrophages and giant cells in proximity to the implant 205. Also, Mg(OH)₂, the principle product of corrosion, is easily solubilized and excreted in the urine 206.

2.6.4 Strategies for controlling corrosion of biodegradable metals
One of the biggest challenges in engineering biodegradable metals is controlling the rate of corrosion. The
two principle methods of corrosion control are alloying and surface treatments.

2.6.4.1 Alloying

Alloying Mg with trace amounts of other biocompatible metals can significantly improve corrosion
resistance after implantation. Popular alloying elements in medical applications include zinc, calcium,
aluminum, yttrium, zirconium, and others. Processing of alloys i.e. rolling, extruding, temperature of
these processes etc. also has substantial effects on subsequent degradation behavior. Devices or samples
created from the same mixture of alloy will contain subtle discrepancies in microstructure that alter
corrosion behavior and these discrepancies may be exaggerated by various processing methods. The
relationship between alloy composition, processing methods and in vivo behavior is not yet well
understood and is currently the topic of much research, worldwide.

2.6.4.2 Surface treatments

Surface treatments are used to enhance the initial corrosion resistance, biocompatibility or bioactivity of
an alloy. Techniques for altering the surface of a metal include anodization (electrolytic passivation of
the surface of a metal), heat treatment (heating/cooling a metal to alter its properties e.g. annealing, etc.),
chemical vapor deposition (uniform deposition of other metals or chemicals onto the surface of an alloy)
and others. Like alloying, this is also a topic of intense global research.

2.6.5 Methods of monitoring corrosion of biodegradable metals

Monitoring the corrosion of biodegradable alloys is also challenging, even in highly controlled laboratory
environments. The International Organization for Standardization (ISO) has issued a set of standardized
tests for evaluating biomaterials (ISO 10993) although these tests were designed to assess the utility and
safety of non-resorbable biomaterials. Thus, in vivo and in vitro evaluation of biodegradable metals is a
relatively nascent field.
2.6.5.1 Methods of monitoring corrosion of biodegradable metals - In vitro

In vitro tests are generally used to evaluate biodegradable metals for corrosion behavior or toxicity. Toxicity is directly related to the concentration and composition of corrosion products in the test solution, which may be composed of buffered saline solutions with various additives to mimic physiological conditions in the body and with or without cells. Popular methods for measuring corrosion in vitro include mass loss measurement, hydrogen evolution, volumetric measurement and pH monitoring. Potentiodynamic polarization is a method that can be used to characterize instantaneous corrosion rate and other information regarding corrosion mechanisms. Electrochemical impedance spectroscopy (EIS) is another method used to characterize corrosion mechanisms of an alloy. A major challenge in this field is standardizing technical discrepancies between labs to enable the sharing of results.

2.6.5.2 Methods of monitoring corrosion of biodegradable metals - In vivo

In vivo corrosion monitoring is especially important in the evaluation of the performance of a biodegradable metal. Standard animal models for evaluating biomaterials are described in ISO 10993 and are frequently used to characterize the biocompatibility of Mg alloys. These involve surgically implanting a defined volume of material under the skin on the back of a mouse for specific periods of time, after which, the animal is sacrificed and various tissues are analyzed for markers of toxicity caused by the material. In such experiments, corrosion of the implant may be evaluated by surgical excision and mass loss measurement, histological analysis or using non-invasive imaging techniques such as micro computed tomography (micro CT) or variations of inductively coupled-plasma mass spectroscopy (ICP-MS). Chemical composition of corrosion products adjacent to or around the implant may also be evaluated.
2.7 **Summary and limitations of current and previous research**

Though much progress has been made toward understanding the mechanisms of CNS and PN injury, current standards of care are often not enough to ensure complete recovery.

2.7.1 **Summary and limitation of brain injury management strategies**

Brain damage through TBI and stroke is a major cause of death and disability worldwide. Critical advances in medicine over the last century have reduced mortality and improved functional outcome but there is still no effective ‘gold standard’ of treatment. A family of interventions referred to as *neuroprotective therapies* is currently being investigated to halt the cascade of secondary injury in the hours and days after damage by targeting the mechanisms that control the pathways of excitotoxicity, inflammation, apoptosis, and other events that cause inappropriate cell death or lead to a loss of function. A candidate neuroprotectant of interest is the essential element Mg.

Intracellular levels of Mg\(^{2+}\) decrease after brain damage and it has been shown that normalizing tissue Mg levels after injury can significantly improve neurological outcome in laboratory animals subjected to experimental damage \(^{210}\). This ameliorative effect has been attributed to a variety of mechanisms including reduced vasospasm in the injured tissue, reduced excitotoxicity \(^{22}\), reduced levels of free radicals \(^{211}\) and several others \(^{23}\). Thus, the use of Mg\(^{2+}\) solutions to treat brain tissue injury is well documented and shows tremendous promise, although there are still obstacles to translating these findings into effective treatments for humans. A prominent theory to address the discrepancies between human and animal studies of Mg intervention is that Mg\(^{2+}\) ion levels are more tightly regulated in humans than in rodents \(^{98}\).

To date, there is not a clinically significant neuroprotectant available; over 40 candidate drugs have failed to show efficacy in Phase III clinical trials \(^{63}\). The failure of so many clinical trials have led several researchers to critically analyze why neuroprotective success in animals has not translated well to humans.
Much emphasis has been placed on the narrow scope of variables often tested in animal studies and design flaws in human clinical trials.

Despite difficulties in translating laboratory results to clinical utility, many prominent researchers feel that the available animal models of brain damage are adequate and that a deeper understanding of the mechanisms is required for progress in identifying and developing successful management practices. Therefore, because more information is needed, we proposed to characterize the effects of increased levels of Mg$^{2+}$ on neural cells in culture. The main contribution of the research described in Chapter 3 is the development of a culture system capable of monitoring and characterizing neural cell responses to elevated Mg$^{2+}$ in terms of cell number and behavior.

### 2.7.2 Summary and limitations of conduits for management of PN injuries

Peripheral nerve injuries are a serious problem and are reported in over 3% of all ER visits in the United States every year. Due to the physically delicate nature of PN tissue and the complexity of the neuromuscular junction, full functional recovery is rarely achieved after severe trauma. In cases where the nerve is severed and neuroanastomosis is not possible, the current ‘gold standard’ of treatment is an autograft surgically placed at the site of injury to bridge the nerve segments. Autografts are less than satisfactory, however, because harvesting a donor nerve requires a second surgery and results in donor site morbidity. In addition, the mismatch in dimensions and functions of the donor and damaged nerves results in limited functional recovery. Although the FDA has approved 11 nerve conduits as of 2012 (Table 3), none of these devices have proved clinically useful device for long nerve gap repair. There is a great clinical need for an engineered alternative to autografts.
Table 3 FDA approved nerve conduits. Despite intense research over the past decades, relatively few conduits have been granted FDA approval. Use in the clinic has been largely relegated to bridging small gaps in non-critical sensory nerves. Table modified from 15.

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Company</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuraGen/NeuraWrap</td>
<td>Integra</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>Neurolac</td>
<td>Polyganics</td>
<td>PDLLA/CL</td>
</tr>
<tr>
<td>Neurotube</td>
<td>Synovis</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>Neuromatrix</td>
<td>Collagen matrix Inc</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>Salubridge</td>
<td>Salumedica</td>
<td>Polyvinyl alcohol hydrogel</td>
</tr>
<tr>
<td>Surgisis</td>
<td>AxoGenInc</td>
<td>Porcine Small Intestinal Submucosa</td>
</tr>
<tr>
<td>Avance</td>
<td>AxoGenInc</td>
<td>Decellularized nerve</td>
</tr>
</tbody>
</table>

One of the major challenges in PN regeneration over long gap injuries is thought to be inadequate mechanical support and contact guidance of cell growth within the lumen of the conduits. In short gaps, it is proposed that fibrin bridges form within the conduits and they physically guide support cells across the gaps by contact guidance. This initial structural support is necessary for subsequent axonal regeneration across the gap. Once this occurs, axonal regeneration all the way to the distal target can commence. Within gaps longer than 3 cm, it is proposed that one of the major issues is that the fibrin bridges don’t form, preventing Schwann cells crossing from the proximal to distal nerve stumps. The major contribution of the research described in Chapters 4 and 5 is the evaluation of a nerve conduit containing a filamentous scaffold composed of the biodegradable metal, Mg, to support regenerating nerve tissue.
3.0 Effects of Mg\(^{2+}\) supplementation of neural stem/progenitor cells in vitro

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Manuscript in preparation for submission

Abstract
After traumatic brain injury, intracellular magnesium ion levels drop drastically in injured tissue. Animal studies have shown that systemic replenishment of magnesium ion (Mg\(^{2+}\)) levels can improve functional recovery, but clinical trials in humans have had mixed success. Because recovery might benefit from raising local levels of Mg\(^{2+}\), we characterized the effects of increasing external Mg\(^{2+}\) concentrations in cell cultures of murine neural stem/progenitor cells (NSCs). Plate-reader based assays, utilizing the stains Alamar Blue and crystal violet, were used to estimate cell counts in the presence of elevated Mg\(^{2+}\). The results from these methods were compared to visual counting and to each other. Alamar Blue, a metabolic activity assay, gave low signals with NSCs and was inaccurate in the presence of elevated Mg\(^{2+}\). The crystal violet assay was unaffected by Mg\(^{2+}\) and gave similar results to visual cell counting methods. The crystal violet assay was then used to examine the effects of Mg\(^{2+}\) supplementation, time in culture, plating density and plating substrate on NSC numbers. NSC numbers increased when exposed to Mg\(^{2+}\) concentrations 3-40 mM above basal levels (0.8 mM). Above 10.8 mM, cell numbers decreased. Plating density and time in culture did not affect this biphasic response, though plating cells on laminin, an adhesive substrate that cells encounter in vivo, negated the positive influence of the Mg\(^{2+}\) on NSC number. NSCs consistently differentiated into neurons in all conditions tested and the percentage of cells that differentiated into neurons was unaffected by Mg\(^{2+}\) supplementation. These studies set the stage for further mechanistic research.
3.0.1 Chapter Aims

The purpose of the experiments outlined in this chapter was to establish a baseline for the effects of Mg\(^{2+}\) supplementation on NSC health and division (measured in terms of cell number) and behavior (measured by the percentage of NSCs differentiating into neurons, and characteristics of neurite outgrowth). We evaluated the effectiveness of two plate reader assays for measuring cell number and characterized the effects of cell-plating density, plating substrate, time in culture and level and exposure time of Mg\(^{2+}\) supplementation on NSC response. We also evaluated the effectiveness of one plate reader assay by comparing to cell counts monitored by visual methods. Then, using the best assays, we tested Hypothesis 1: Numbers of NSCs will increase in response to moderate increases in local Mg\(^{2+}\) concentration and Hypothesis 2: Normal NSC behavior, as measured by the ratio of cells that differentiate into neurons and the elaboration of neurites, will not be harmed and may be increased by moderate increases in local Mg\(^{2+}\) concentration.

3.1 Introduction

Following traumatic brain injury or hemorrhagic stroke, it was found that magnesium ions (Mg\(^{2+}\)) are significantly depleted in brain tissue and cerebrospinal fluid\(^{21, 70}\). In addition to the initial injury, ‘secondary’ cell death can occur in proximity to the injured tissues via a variety of mechanisms including oxidative stress and increased extracellular levels of potentially toxic neurotransmitters and calcium ions\(^{21}\). In animal studies, Mg\(^{2+}\) solutions, delivered either systemically or intraventricularly, improved functional recovery after traumatic brain injury\(^{23}\) and after stroke\(^{213}\). Possible mechanisms include restoring Mg\(^{2+}\) levels, which restores the action of hundreds of enzymes that depend on Mg\(^{2+}\), especially for energy usage, and Mg\(^{2+}\) blockage of N-methyl-D-aspartate (NMDA) glutamate receptors and calcium ion channels, which would reduce excessive toxic calcium influx into neurons (reducing secondary cell damage). Replenishing Mg\(^{2+}\) has also been implicated in other mechanisms, including alleviating oxidative stress and reducing vasospasm (another event that causes secondary damage)\(^{70}\).
In vivo, in humans, it is known that raising serum levels of Mg\(^{2+}\) to 2 mM is acceptable, while raising levels above that can result in undesirable clinical symptoms. However, in vitro, in cultures of rat brain neurons, raising levels of Mg\(^{2+}\) to as high as 4 mM above normal did not result in dopamine neuron degeneration and, in fact, protected those neurons from damage.\(^{214}\) In other culture studies, raising Mg\(^{2+}\) levels had mixed effects. It is known that this will stop spontaneous neuronal discharge in complex cultures of brain neurons where there are high numbers of neurons with significant synaptic connections (unlike the sparse cultures of NSCs which we are studying here) and in some studies this is damaging.\(^{215}\) In contrast, another study reported that increasing Mg\(^{2+}\) levels to 1.2 mM in culture for two weeks had no effect on cell numbers or morphology, but did increase measures of synaptic plasticity.\(^{216}\) In similar neuronal cultures, there is also an extensive body of literature studying lowered Mg\(^{2+}\) levels, which simulate epilepsy-like conditions of firing in these cultures containing mature, extensively networked neurons. These cultures are very different from the NSC cultures we are using here. We were unable to find any previous study examining the effects of elevated Mg\(^{2+}\) on neurosphere cultures or NSC differentiation in vitro.
Figure 10 Theoretical neuromodulatory pathways of Mg\(^{2+}\) action after TBI. A serious consequence of TBI may be ischemic conditions in or around damaged tissues. Ionic Mg\(^{2+}\) can interrupt the harmful biochemical cascades caused by ischemic conditions via multiple mechanisms including NMDA receptor blockade and blockade of Ca\(^{2+}\) channels \(^6,10\). Image modified from \(^{11}\).

NSCs are primary cells with neural characteristics that are relatively homogeneous compared to whole brain cell mixtures and can also be passaged several times. As primary cells, NSC cultures offer several advantages over neuronal cell lines. In addition, the relative homogeneity of NSC cultures allows us to examine the effects of Mg\(^{2+}\) directly on several types of neural cells. Finally, this culture lets us ask two types of questions: What does increased Mg\(^{2+}\) do to neural stem cell division? And: What does increased Mg\(^{2+}\) do to neural stem cell differentiation? Neither of these questions can be asked when using more traditional neuronal cell cultures. Thus, we determined the effects of increasing Mg\(^{2+}\) above normal cell
culture medium levels in cultures of murine NSCs derived from cortical tissues. We examined three assays for measuring the effect of Mg$^{2+}$ supplementation on cell number: the Alamar Blue plate-reader assay, the crystal violet plate-reader assay and visual counting of digital micrographs. It was necessary to compare the results of plate reader assays to counting data as elevated Mg$^{2+}$ concentrations have been shown to interfere with the MTT assay, a popular colorimetric assay.$^{217}$

After confirming the accuracy of the crystal violet assay, it was used to examine the effects of Mg$^{2+}$ supplementation, time in culture, plating density and plating substrate (with or without laminin pre-treatment) on NSC numbers. Immunohistochemistry was used to monitor the NSC differentiation into neurons (a measure cell behavior/health).

### 3.2 Methods

#### 3.2.1 Neurosphere preparation

Cell cultures containing neurospheres (floating cell clusters composed mostly of NSCs) were prepared from newborn (P5-P8) C57/Bl-6 mice (6-8 mice per neurosphere preparation) via methods modified from published procedures.$^{218, 219}$ All animal procedures were approved by the University of Cincinnati IACUC and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH. After animal euthanasia, the cerebral cortices and subcortical tissues adjacent to the anterior lateral ventricles (including hippocampi and excluding the lower hypothalamus, cerebellum and brainstem) were minced in sterile Hank’s Balanced Salt Solution with additives (HBSS+: HBSS plus 2 mM Glutamax (Invitrogen, Carlsbad, CA) and an antibiotic/antimycotic solution (AAA, Invitrogen, Carlsbad, CA; final concentrations: 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B). Tissues were disaggregated with papain for 30 min at room temperature (RT) (16 U/mL papain, 2 mM EDTA, 1 mM cysteine in HBSS+, pre-activated at 37°C for 30 min) and enzyme action was terminated with soybean trypsin inhibitor (1.25 mg/mL; Sigma, St. Louis, MO). After mechanical disaggregation by passage through 18, 21, and 23 gauge needles and centrifugation (10 min,
377g), cells were centrifuged again in sucrose (0.9 M in HBSS, 15 min) to remove myelin and debris and plated (1.5x10⁶ cells per 20 mL) in uncoated T75 flasks in neurosphere growth medium (NSGM) - DMEM/F12 (15 mM HEPES, 1.2 g/L bicarbonate, 3.15 g/L glucose; Sigma) with 2% B27 supplements (Invitrogen), 2 mM Glutamax, 1x AAA, 27.5 µM β-mercaptoethanol (Invitrogen), 20 ng/mL epidermal growth factor (EGF) and 10 ng/mL fibroblast growth factor-1 (FGF-2) (both from Sigma). Cells were maintained in a tissue culture incubator (5% CO₂ and 100% humidity at 37ºC), fed via partial media changes every 2-3 days and passaged 1-2 times per week (15 minutes of 1x trypsin/EDTA, Fisher Scientific (Pittsburgh, PA) with centrifugation), replating at the same density in NSGM. Neurosphere preparations were used for less than 10 passages.

3.2.2 Cell plating

For experiments, cells were dissociated (as for passing), resuspended in neurosphere differentiation medium (NSDM) - NSGM minus EGF and FGF-2, with 1% fetal calf serum (Fisher-Hyclone Defined), filtered (70 µm filter) to remove clumps, and plated in 96-well (100 µL/well) or 24-well (0.5 mL/well) plates. For laminin pre-treatment, 96 well plates were filled with 50 µL/well of either NSDM or NSDM with laminin (20 µg/mL), incubated for two hours and cells were added in another 50 µL NSDM. Several plating densities were examined; cells were plated at 25K/96-well plate (78K/cm²) unless noted otherwise.

3.2.3 Mg²⁺ supplementation

After incubation for 24 hours (to allow for cellular attachment and initial growth), NSDM or 2x concentrated Mg²⁺ solutions diluted in NSDM were added to wells (200 µL/well final volume for-96 well plates, 1mL final volume for 24 well-plates). MgCl₂ solutions were added at 24 hours with media changes (half well replenishment) every other day. Materials were from Fisher if not otherwise described.

3.2.4 Crystal Violet Assay
At the appropriate times, cells were fixed (10 min at room temperature in 2% glutaraldehyde (in PBS), then 10 min in 4% glutaraldehyde), rinsed and stained with crystal violet (CV) (Allied Chem. Corp., NY, NY, 0.1% in water, 30 min), rinsed thoroughly (water), and air-dried. Color was extracted (10% acetic acid in water) and staining intensity was read (abs. 540 nm) in an EL800 (Bio-Tek Instruments, Winooski, VT) plate reader. Readings were blanked and normalized to the average normal medium control (0.8 mM Mg²⁺) per experiment.

3.2.5 **Alamar Blue Assay**

NSCs were plated in NSDM on 96-well plates (BD Falcon Microtest™ Optilux™, Belford, MA) at cells numbers of 5K to 100K per well and grown for 24 h. For the Alamar blue assay, 200 µL sterile filtered 0.5 mg/mL resazurin sodium salt (Sigma, St. Louis, MO) solution in phosphate buffered saline (PBS; pH 7.4) were added to each well. After 1 hour in the culture incubator, the fluorescence ($\lambda_{ex}$ 530 nm, $\lambda_{em}$ 590 nm) was measured with a Spectra max Gemini XPS (Molecular Devices, Sunnyvale, CA) plate reader. For tests without cells, solutions (NSDM with and without addition of MgCl₂) were equilibrated in the assay plates in the incubator for 1 h. The resazurin solution was added, incubated for 1 hour and fluorescence was recorded. The Alamar Blue assay was repeated in the same manner with SW872 cells (ATCC: HTB-92) that were maintained in DMEM/F12 with 10% FBS and Primocin (antibiotic solution, Invitrogen) in humidified 5% CO₂ in air at 37 °C.

3.2.6 **Cell Staining**

Cultured cells grown on glass coverslips were fixed with 4% paraformaldehyde (pH 7.4 in PBS, 20 minutes at room temperature), then immunostained for neuron-specific tubulin (NST), the type III β form of tubulin, monoclonal antibody (Sigma) via standard methods with donkey anti-mouse secondary antibody conjugated with Alexa 488 (Jackson ImmunoResearch Labs, West Grove, PA). Nuclei were stained by including 4',6-diamidino-2-phenylindole (DAPI) (Sigma, 1:1000 dilution) in the secondary
antibody incubation. After staining, coverslips were mounted on glass slides using Fluoromount (SouthernBiotech, Birmingham, AL).

3.2.7 Microscopy

CV-stained cells were photographed on an Olympus IMT-2 (Olympus, Japan) inverted microscope with a QICam cooled CCD camera (Q Imaging, Canada). Cells stained with NST and DAPI and mounted on slides were viewed and photographed on an upright Zeiss Axioplan Imaging 2e microscope with a Zeiss Axiocam digital camera. All photos were arranged using Photoshop (Adobe, San Jose, CA).

3.2.8 Cell counting by digital image analysis

Digital 8-bit photographs of DAPI staining were taken for eleven microscope fields per well, using a preset pattern; five adjacent, non-overlapping fields across the center, three fields above and three below, all taken at least one microscope field in from the edge of the coverslip (Figure 11). The number of DAPI+ nuclei per image (cell count) was found using the Cell Profiler (http://www.cellprofiler.org/) digital object recognition software package. The accuracy of CellProfiler was confirmed by comparison to manual counts for a subset of randomly selected images (data not shown). The eleven counts per coverslip were averaged and normalized to the mean control value and 3-4 coverslips were examined per condition, per experiment, in four experiments.

3.2.9 Measuring characteristics of neuronal differentiation

NSCs plated on untreated and laminin–pre-coated glass coverslips with 144 hour Mg$^{2+}$ exposure were fixed after 7 days in vitro and stained for NST and DAPI. A blinded observer then analyzed digital micrographs of NST stained samples. For each sample, 6 fields were systematically chosen toward the periphery of each well, where cell density was greatest, and examined. The total number of cells, indicated by DAPI+ staining, and NST+ cells (neurons) were recorded and averaged to represent each
well. For each NST+ cell, the number neurites, and length of each neurite were measured and recorded using Image J and then averaged to represent each well.  

**Figure 11 Schematic for photographing NSCs on a 12mm glass coverslip.** White = DAPI+ cell nuclei. A pre-determined template of 11 microscope fields (shown as green boxes, true to scale) was used to guide microscopy of NSCs on glass coverslips. The mean was calculated and used to represent a ‘cell number’ value for each coverslip. Ununiform cell distribution, as seen in this composite (7 days in vitro, 5 mM Mg²⁺ supplementation over 6 days), resulted in high variance between fields on a coverslip and among coverslips of like conditions. Scale bar = 1mm.
Figure 12 Cell counting using the program Cell Profiler. Cell nuclei in digital micrographs of DAPI stained slides (A) were identified and counted using Cell Profiler. Sample Cell Profiler graphical output shown in (B); unique cells shown in solid colors. Scale bar = 50 µm.

3.2.9 Statistical Analysis

For all analyses, the person analyzing data was blinded to the conditions being examined. Statistical analysis involved a one-way ANOVA with Student-Neuman-Keuls post hoc analysis. When data were not normally distributed, analysis involved a Kruskal-Wallis ANOVA with Dunn’s multiple comparison post hoc test. Significance was concluded for p ≤ 0.05. In short-term CV assays, occasional wells (~1-3/experiment) contained residual clumps of tissue that were not removed by filtration, resulting in values at least 3-4 standard deviations above sister wells, so these wells were not included in analysis. Analysis used the Sigma Plot/Stat (v. 11.0) statistics package (Systat Software Inc., San Jose, CA).

3.3 Results

3.3.1 The Alamar Blue assay provided inaccurate estimates of relative NSC cell numbers and was affected by high concentrations of Mg$^{2+}$.

Plate-reader based assays allow relatively rapid analysis of cell numbers or cell viability. However, a recent report outlined that the presence of soluble Mg$^{2+}$ gave anomalous readings in the absence of cells, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay $^{217}$. To examine Mg$^{2+}$ interference with the Alamar Blue assay of cell viability, Mg$^{2+}$ solutions were added to culture
medium without cells present. In the Alamar Blue assay, a blue resazurin dye is added to the medium over living cells and mitochondrial reductases reduce resazurin, which is blue and non-fluorescent, to resorufin, which is pink and fluorescent \(^{221}\). The amount of resorufin produced is proportional to the metabolic activity of the cells. As shown in Figure 13A, addition of MgCl\(_2\) to medium significantly increased the relative fluorescence units (RFUs) at concentrations of 40 mM or greater (ANOVA (28-108/condition) \(p<0.001\)).

To determine if readings were proportional to NSC numbers, NSCs were plated at different densities in normal differentiation medium and incubated for 48 hours. A more quantifiable determination of the readings per cell number might have been achieved if we removed the cells from the dishes and did the assay on floating cells. However, with these delicate primary cells, this would not be an accurate comparison; the process of removing the cells from the plate to make them into a floating cell suspension is damaging and the metabolism of the cells is like to be very different from that of cells attached to the substrate. So this comparison of cells plated at different densities was considered to be a relatively strong indication of the assay effectiveness. For NSCs, significantly increased numbers compared to controls were seen only with the 50K and 100K plating densities (Figure 13B, hatched dark gray bars) (ANOVA (26-32/condition) \(p<0.001\)). The highest increase, at 100,000 cells per 96 well, was only \(~20\%\) above control. This low level increase in the RFU was comparable to the increase observed with addition of 40 mM MgCl\(_2\) to medium without cells. Figure 13A, right panel, shows the data for cells plated at 0 and 100K, shown here again to allow comparison to the increase in RFU with 40 mM or more Mg\(^{2+}\) to medium alone (left panel). As an assay control, the plating density experiment was repeated with a cell line with high metabolic activity, a liposarcoma cell line: SW872 cells \(^{222,223}\). The SW872 cells (Figure 13B, plain light gray bars) showed a much more significant increase in signal over control values. Significant increases were seen for all but the 5K plating density (ANOVA (26-32/condition) \(p<0.001\)). The signals were much more robust than for NSCs (at 100K plating density, SW872 cells showed a \(~3,000\%\) increase over control). This made the Mg\(^{2+}\) interference insignificant, even at the lowest plating
density (5,000 SW872 cells/well). The low values with NSCs suggest that either low mitochondrial activity or low activity of any of the many intracellular enzymes that can convert this dye.

Figure 13 The Alamar blue assay. A) In the absence of cells (No Cells), Mg$^{2+}$ concentrations above 40 mM increased relative fluorescence units (RFU) in the Alamar Blue assay. The maximal increase in RFU at 40-160 mM was comparable to that observed with live NSCs at 100K per 96 well (N panel, on left, shows cell data from 1B). B) Alamar Blue values after culturing SW872 (S) or NSC (N) cells at the specified densities for 48 hours. Significant increases were determined by a Kruskal-Wallis ANOVA with Dunn’s post-hoc and are marked * or +, for S or N cells, respectively.
3.3.2 The Crystal Violet assay was responsive to changes in NSC number and provided accurate estimates of relative NSC cell numbers, confirmed by cell counting, in the presence of elevated Mg$^{2+}$.

To avoid the interference with Mg$^{2+}$ and to use an assay that did not depend on the metabolic activity of the NSC cells, we chose staining with crystal violet $^{224,225}$. In this assay, the medium containing Mg$^{2+}$ is removed, the cells are fixed, air dried and stained with crystal violet, which selectively binds to nuclei. The dye is extracted with acetic acid and the color in the solution is read on a plate reader. We first tested the validity of the CV assay in the same way as for Alamar Blue, NSC cells were grown in 96 well plates with the same dosing schedule, using the same plating densities. As seen in Figure 14, there was positive correlation between plating density and the signal. The signal in all densities tested was significantly different from cells plated at 5K, which was used for comparisons.

To further test the validity of this assay, we treated the cells with elevated Mg$^{2+}$ levels and determined relative cell numbers with both the CV assay and using a cell counting technique. Cells were plated in parallel in 96 well plates and in 24 wells on glass coverslips at the same plating density, with the same volume to plate area ratio. Both were exposed to elevated MgCl$_2$ beginning at 24 hours and remaining on for three days, then removed. Cells were fixed at seven days after plating. CV assays were done on cells in 96 wells and DAPI+ nuclei were counted in photographs of the cells grown in 24 wells, using a semi-automated image analysis method. As seen in Figure 15, nearly identical dose response data were obtained with both assays. The CV data in this graph are also shown in Figure 16. Healthy neurons, as observed by IHC microscopy, were present in both 24-well and 96well plates.
Figure 14 The crystal violet assay. NSCs were grown for 48 hours at the specified plating densities in control medium (0.8 mM Mg$^{2+}$) and assayed using crystal violet. Staining showed a correlary increase with plating density (data were normalized to 5K plating density per experiment) with higher precision at lower plating densities.
**Figure 15** Crystal violet versus cell counts, long-term analysis. NSCs (12k/96-well, 39k/cm²) were exposed to MgCl₂ solutions from day one to four after plating (72 hour exposure) and then grown in control medium until day seven. Fixed cells were analyzed via crystal violet (black bars) or by counting visually imaged DAPI+ nuclei (gray bars). Asterisks mark significant changes in cell number compared to control medium for each condition by one-way ANOVA with Tukey’s HSD post hoc.

3.3.3 Mg²⁺ supplementation affected NSC cell number; this effect was relatively unaltered by cell plating density, time in culture or length of Mg²⁺ exposure.

3.3.3.1 Effects of plating density/Mg²⁺ exposure on cell number measured by the crystal violet assay.

In longer-term cultures (seven days), longer exposures to Mg²⁺ (72 and 144 hour) were tested, with two cell plating densities (low and high, 39K and 156K cells/cm², respectively), all analyzed with the CV assay. For all four conditions (Figure 16), addition of 2.5 mM MgCl₂ resulted in increased cell numbers and addition of 20 mM resulted in decreased cell numbers. At 5 mM MgCl₂, three conditions showed a significant increase and at 10 mM, the condition of 144-hour exposure, high plating density gave a significant increase. Although this higher plating density appeared to give better results and therefore might be a preferred density, visual inspection suggested that the cells were not as healthy, since vacuoles were observed in the majority of the cells, in all Mg²⁺ concentrations (not shown). Healthy neurons, as observed by IHC microscopy, were present in all conditions tested.
Figure 16 Long-term growth, dose response curves for MgCl₂. NSCs were plated at two different densities (39K cells/cm² (bars with no pattern) and 156K cells/cm² (bars with hatched pattern)) in uncoated 96 wells. They were treated with MgCl₂ beginning at 24 hours after plating and continuing for either 72 hours (bars without shading) or 144 hours (gray bars). All cells were fixed at seven days and analyzed with crystal violet. Asterisks mark significant changes in cell number compared to control medium for each condition by Kruskal-Wallis one-way ANOVA with Dunn’s post hoc. Error bars = standard deviation.

3.3.3.2 Effects of plating density on cell number in short-term assays as measured by the crystal violet assay

The same biphasic dose-response curve to Mg²⁺ in cell number observed in long-term cultures was also present in short term cultures; various plating densities of cells exposed to increased [Mg²⁺] for 24 hours, 24 hours after plating, showed mild increases in cell numbers. At the lowest cell density (5K/well), addition of 20 mM Mg²⁺ resulted in significantly decreased cell numbers. Similar trends were observed at higher densities, but none were significantly different from control. Variability within density conditions did increase with the highest density tested, indicating possible overgrowth in cultures. Healthy cells, as observed by IHC microscopy, were present in all conditions tested. These were all biphasic curves in the
sense that only the middle range of Mg\(^{2+}\) concentrations resulted in increased cell numbers. Thus, time and plating density make only minor changes in the basic effect observed. 25K/well was chosen as for further experimentation that represented a good balance between cell density in the wells and responsiveness to Mg\(^{2+}\).

**Figure 17 Short-term effects of Plating Density and Mg\(^{2+}\) supplementation on NSC cell number.** NSCs were grown for 48 hours at the specified plating densities in either control differentiation medium or control medium plus MgCl\(_2\) at the specified added Mg\(^{2+}\) concentration. Cell number was assayed with CV. Asterisks mark significant changes in cell number compared to control medium by Kruskal-Wallis one-way ANOVA with Dunn’s post hoc. Error bars = standard deviation.

3.3.3.3 *The observed increase in cell number was due to Mg\(^{2+}\).*

To determine if the effects observed were due to the addition of Mg\(^{2+}\), and not due to other effects, such as osmolarity increases, solutions containing MgCl\(_2\), MgSO\(_4\) and NaCl were added to cells in 96 well plates for the last 24 hours of a 48-hour cell culture period and relative cell numbers were assayed with CV. As shown in Figure 17, a 24-hour exposure to all concentrations of MgCl\(_2\) (clear bars), except 20 mM, resulted in increased cell numbers over control medium (ANOVA (24-40 counts/condition) p<0.001). Asterisks mark conditions significantly different from control (gray bars) (ANOVA (12-40/condition) p<0.001). Addition of NaCl (black bars) resulted in a significant decrease in cell numbers only at 40 mM NaCl (ANOVA (16-40/condition) p<0.006). The 20 mM MgCl\(_2\) condition, which contains
40 mM chloride, surprisingly (compared to NaCl) did not show decreases, but there was a trend toward a
decrease and see long-term application below.

Figure 18 Short-term growth, dose response curves for MgCl₂, MgSO₄, and NaCl. NSCs (25k/well) were plated on uncoated
plastic and treated for 24 hours with the Mg²⁺ concentrations shown (added to control medium). Asterisks mark significant
changes in cell number compared to control medium by Kruskal-Wallis one-way ANOVA with Dunn’s post hoc. Error bars =
standard deviation.

3.3.4 Effects of laminin pre-coating on NSC response to Mg²⁺ supplementation

It is known that normal cell culture plastic is often not an optimal substrate for neuronal cells and NSCs.
For example, mouse and rat NSCs differ in their ability to differentiate without an adhesive substrate.
Mouse NSCs will attach to regular cell culture plastic and will differentiate, but rat NSCs require an
adhesive protein. One of the most commonly used adhesive substrates, because it is a component of
the extracellular matrix in the brain, is the protein laminin. Therefore, we sought to determine whether the
supportive effect seen with Mg²⁺ supplementation was altered by substrate effects. Mg²⁺/Substrate
interactions with have been observed previously, with endothelial cells being differentially affected by
Mg²⁺ depending on the substrate.
For these assays we used short-term growth and the CV assay. Cells were plated on uncoated plastic, as in all previous assays and in the same plates coated with laminin (20 µg/mL) and exposed to a Mg\(^{2+}\) dose response curve. The laminin pre-coating, compared to uncoated plastic, resulted in more viable cells (Figure 19A), greater process growth and cell spreading (Figure 20 - compare A-C with D-F) and less aggregation (Figure 20). However, on laminin, there were no changes in cell number with increasing MgCl\(_2\) (Figure 19), while the cells grown on uncoated plastic showed increased cell numbers in 5 and 10 mM MgCl\(_2\) (Figure 19) (ANOVA for all conditions (10-25/condition) p<0.001), with a trend towards a decrease at 20 mM. Addition of MgCl\(_2\) did not otherwise alter cellular morphology. For comparison, views of the DAPI-stained cells from the long-term image analysis experiment are also shown, at 0, 5 and 20 mM MgCl\(_2\) (Figure 20, G-I). In the long-term experiments, neurons were found in all concentrations, as shown in Figure 20 (J-L).

![Figure 19 Effects of substrate on MgCl\(_2\) dose response curve (crystal violet).](image)

In control medium, laminin precoating resulted in increased cell numbers over cells plated on untreated plastic (unfilled bar). When NSCs were grown on untreated plastic for 48 hours (unfilled bars), significant increases were seen with added 2.5 and 5 mM MgCl\(_2\), while no significant changes were observed when grown on laminin coated plastic (filled bars). Asterisks represent cell numbers different from cell numbers on untreated plastic without Mg\(^{2+}\) supplementation.
Figure 20 Representative micrographs of cells plated on bare or laminin-coated plastic after Mg\(^{2+}\) supplementation. Photomicrographs of NSCs. Cells were grown either without laminin (NO LAM) (A-C, G-L) or on laminin (LAM) (D-F) and exposed to different MgCl\(_2\) concentrations (column headings). A-F: Short-term growth (48 h), stained with crystal violet. Addition of 5 mM MgCl\(_2\) increased cell number without laminin (B), but not on laminin (E). Addition of 20 mM showed decreased cell numbers in this experiment (C), but had no effect on cells grown on laminin (F). Cells grown on uncoated plastic grew in clumps (two multi-cell clumps are seen in the insert in A), while cells on laminin remained isolated and extended longer processes (insert in D). The crystal violet stained just nuclei. (A, D). G-I: NSCs grown for 7 days with 6 days MgCl\(_2\) exposure
were stained with DAPI to show cell nuclei. Bar in I = 100 µm for G-I. K-L: NSCs grown for 7 days with 6 days MgCl₂ exposure were stained for NST. Scale bars = 50 µm.

3.3.5 Effects of Magnesium supplementation and plating substrate on neurite characteristics

To determine if elevated Mg²⁺ affects the differentiation of NSCs into neurons, we plated NSCs for 6 days total in the differentiation medium, with or without Mg²⁺ and with or without laminin. At the end of the time period the cultures were fixed and immunostained for neurons, and counterstained with DAPI to allow analysis of all cells present. Neither Mg²⁺ supplementation nor plating on laminin affected the percentage of cells that differentiated into neurons, as determined by counting stained neurons and DAPI+ cells (Figure 21). However, when imaged cells were analyzed for neurites, there were differences observed. First, plating on laminin alone altered neurite outgrowth, as it did for general cell shape, as shown in Figure 20, comparing A-C and D-F. With specific respect to neurites, plating on laminin, with or without Mg²⁺ increased the number of neurites per neuron, the average length of neurites, the sum total length per neurons of all the neurites and the maximum length of the longest neurite per cell (Figure 22).

In terms of Mg²⁺ effects on neurites, none were observed when cells were plated on laminin. But when plated on uncoated plastic, there were some effects of Mg²⁺ supplementation. Addition of 10 mM Mg²⁺ resulted in a significant increase in sum neurite length/neuron and 5 mM and 10 mM Mg²⁺ supplementation resulted in significant increases in max neurite length/neuron on uncoated plastic. Thus, Mg²⁺ supplementation does not alter the basic differentiation process of NSCs, but it does increase certain aspects of their neurite production, if cells are plated on uncoated plastic. Plating on laminin increases neurite production in a more dramatic way, but then, there is no effect of adding Mg. In addition, laminin pre-coating does not affect basic differentiation.
Figure 21 Percent of NSCs differentiating into neurons. There were no significant changes in the percent of cells differentiating into neurons from either Mg$^{2+}$ supplementation (0, 2.5, 5, 10mM) or plating on a laminin substrate (L, NL = no laminin) compared to control medium (NL – 0) by Kruskal-Wallis one-way ANOVA with Dunn’s post hoc. Error bars = standard deviation.
3.4 Discussion

3.4.1 Principle neurotherapeutic mechanism of Mg$^{2+}$ action in brain injuries

Studies in animals suggest that the beneficial effects of systemic application of Mg$^{2+}$ solutions after nervous tissue damage are not explained by just blockage of NMDA glutamate channels and calcium ion channels. Because clinical studies are in progress to use Mg$^{2+}$ solution treatments for brain injury, and
because elevation of local tissue Mg$^{2+}$ around an injury site might be an option to explore, further study is warranted to understand the mechanisms of elevated Mg$^{2+}$ on neural cells.

3.4.2 Colorimetric assays can be influenced by the presence of Mg$^{2+}$

As mentioned in the methods and results, we observed challenges using the Alamar Blue assay with this cell type and with elevated Mg$^{2+}$. A previous report described the interference of Mg$^{2+}$ with the MTT viability assay \(^{228}\) and we report here that this ion also interferes with the Alamar Blue assay by giving false positive readings at higher ion concentrations. This is not a significant problem if the cells are metabolically active and give a large signal to noise ratio, and the analysis does not depend critically on looking at low cell numbers. However, this Mg$^{2+}$ interference with Alamar Blue is a significant problem when or if cell numbers are low or when cells have a lower Alamar Blue signal, as we saw with NSC cells. Previous reports have similarly reported that the Alamar Blue readings and utility are dependent on the cell line \(^{221,229}\).

The CV assay allowed us to circumvent Mg$^{2+}$ interference, since the Mg-containing fluids are removed before staining and the readings depend on nuclear staining, not enzymatic action or metabolic activity. Another advantage was that it was also independent of cell clumping, because the dye is extracted from stained cells and color is read in the supernatant. As shown in Figure 11, the NSC cells aggregate, and this induces variability, even when counting visually. This can be seen in Figure 15, where DAPI+ cell counting was directly compared with a CV assay, and the variability with cell counting was much higher than with CV. We tried to use a plate reader to read the DAPI staining, but the variability was even higher (data not shown), presumably because of this cell aggregation, which results, on the plate reader, in overlapping nuclei being counted as single cells. This was one of the reasons why we undertook to directly compare the CV assay with a visual counting method (Figure 15).

3.4.3 Mg$^{2+}$ supplementation increased NSC numbers
In short-term experiments, using the CV assay, a biphasic dose response curve was observed in NSC numbers with increasing Mg\(^{2+}\), when cells were plated on uncoated plastic. Relative cell numbers were significantly increased at Mg\(^{2+}\) levels of 2.5-10 mM Mg\(^{2+}\) above that in the NSDM medium (0.8 mM). Then, at supraphysiological levels of 20 mM and higher, cell numbers either returned to control values or were decreased. Similar biphasic dose response curves with Mg\(^{2+}\) have been reported for primary human articular chondrocytes in culture during the proliferation phase, where increased cell numbers occurred with addition of 10 mM MgSO\(_4\), and decreased cell numbers with 15 mM and higher \(^{24}\). In human umbilical cord endothelial cells, increased proliferation was observed with addition of 5-10 mM Mg\(^{2+}\), the highest concentration tested \(^{25}\). Note that the range of effective concentrations with NSCs was also similar to the effective doses observed with other cell types. Another example is that endothelial cells increase motility and cell spreading when it is elevated to 1 or 10 mM above basal levels \(^{27}\).

Our experiments show that the stimulatory effects observed are likely to be due to Mg\(^{2+}\), because replacing MgCl\(_2\) with MgSO\(_4\) produced indistinguishable dose response curves, and sodium chloride addition did not produce an increase. The detrimental effects of 20 mM and higher levels also appear due to Mg\(^{2+}\), because they were seen with MgSO\(_4\) in short-term experiments and with MgCl\(_2\) in long-term experiments, although there may be a contribution of chloride toxicity at chloride concentrations of 40 mM or higher. Alternatively, this may be due to increased osmolality at these higher ion concentrations, as was observed in other studies \(^{28}\).

Note that there was no decrease in cell viability at 20 mM Mg\(^{2+}\) when cells were plated on laminin, suggesting that some interaction with the substrate alleviates this toxicity. Substrate interaction with Mg\(^{2+}\) effects has been observed previously, when endothelial cells were differentially affected by Mg\(^{2+}\) depending on the substrate \(^{27}\). Differences in Mg\(^{2+}\) responsiveness also occur with differences in cell cycle or degree of confluency \(^{30,31}\) and with passage number and differentiation status \(^{24}\). Thus, many
variables influence the Mg\(^{2+}\) effects. A lack of effect when grown on laminin may also explain why this phenomenon has not been reported previously.

Multiple mechanisms may underlie Mg\(^{2+}\) actions on NSCs in our cultures because of the many intercellular and extracellular roles of this ion. A wide range of cellular processes are dependent on Mg\(^{2+}\) as a co-factor, and it has been proposed that this ion is a major regulator of cellular events including proliferation and protein production\(^{230, 231}\). In NSCs, Mg\(^{2+}\) could also influence the NMDA-type glutamate receptors directly, or other neurotransmitter receptors by acting via interference with calcium ion channels. Neurosphere cultures contain multiple types of glutamate and other neurotransmitter receptors, with variable expression patterns and variable actions of antagonists and agonists\(^{232-237}\). Because of these complex responses, it is not surprising that we also found some variation in the effects of Mg\(^{2+}\) on cells grown on untreated plastic. The variations observed were primarily in terms of which Mg\(^{2+}\) concentration gave the peak increase, not in the biphasic response. Variations in responsiveness also occurred with passage number and with days since feeding, similar to changes seen with time in culture were observed for another cell type\(^{24}\), however, we did not quantify these changes. Some of these actions of Mg\(^{2+}\) may depend on the health of the neural cells because Mg\(^{2+}\) can block neurotransmitter receptors and/or calcium ion channels. For example, some of the differences seen with and without laminin may be that cells grown without attachment to laminin are more susceptible to both the beneficial and toxic effects of Mg\(^{2+}\).

The beneficial effects for NSCs of elevated external Mg\(^{2+}\) when plated on plastic could be due to improved health of the cells, or via actions on integrins or their binding. Integrin actions are a possibility because of the very significant effects of plating NSCs on laminin. Pre-plating with both laminin and elevation of Mg on uncoated plastic both resulted in increased cell numbers and increased neurite measures, but did not alter differentiation. Laminin has previously been shown to promote NSC numbers and these effects were shown to be partially due to actions on integrins, based on the effects of blocking
agents. It is well known that both Mg$^{2+}$ and Ca$^{2+}$ are necessary for binding to integrins, acting via several binding sites on both alpha and beta chains of the integrins. In understanding the effects of cations on integrins, many researchers examine effects of manganese ion (Mn$^{2+}$) addition, because Mn$^{2+}$ binds with greater affinity to the integrin cationic sites than Mg$^{2+}$. Mn$^{2+}$ elevation induces neurite outgrowth in PC12 cells via upregulation of integrins. Thus, another possible mechanism by which Mg$^{2+}$ could altering NSC numbers and neurites is via either stabilization and/or activation of integrin receptors or via stimulating greater production of integrins by the cells. The lack of Mg effects after laminin pre-coating could suggest that either 1) Mg and laminin are acting via the same mechanisms, i.e., integrin effects or 2) that Mg might be effective only when cells are perhaps producing low levels of integrins. Once integrin production may be restored, by plating on laminin, these effects may no longer be required.

3.4.4 NSC differentiation patterns were minimally affected by Mg$^{2+}$ supplementation and plating substrate

NSCs were observed to differentiate into neurons in every combination of variables tested i.e. neurons were observed regardless of plating density, Mg$^{2+}$ supplementation, or time in culture. When neuronal numbers and characteristics were quantified after 6 days in culture, the percentage of NSCs that differentiated into neurons was so low that it was not possible to accurately quantify the effects of Mg$^{2+}$ supplementation. The changes in neurite characteristics for neurons plated on laminin were likely due to the fact that the NSCs adhere to laminin more robustly than to culture plastic.

3.4.5 Study limitations

One of the biggest technical hurdles in performing these experiments was obtaining consistent results with different batches of neurospheres. We found that our neurospheres were most consistent when used before passage 8 and responded to magnesium dosing erratically when used past that passage. There are also compromises to using dissociated neurospheres in culture rather than traditional primary cultures or
immortalized cell lines. Primary cultures and neurospheres both will contain mature cell types, but the neurosphere cultures offer an advantage over primary cultures because any kind of rapid-throughput experiments that test multiple variables require large cell numbers. For primary neurons, this would require a prohibitive number of animals because mature neurons cannot be passaged. Then, while both neurospheres and cell lines differ from primary neurons in that they can be readily passaged, can be expanded to increase cell numbers and one set of cells be used for several months of experimentation, immortalized cell lines usually suffer from some disruption of normal intracellular signaling pathways and may not be the best reflection of processes occurring in the intact animal \(^\text{101}\). For our purpose, dissociated neurosphere cultures provide the best compromise between preparation time, passagability, and cell types present with the added benefit that cell differentiation, which is one measure of the health and appropriateness of the treatments, can be readily assessed.
4.0 Digital methods for evaluating cellular response to magnesium filaments in peripheral nerve tissue in vivo

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Abstract

Magnesium metal has emerged as a leading candidate material for non-toxic, bioresorbable medical implants. Though much work has been done to examine the resorption rate and biological effects of Mg in muscle and bone, there have been few reports in healthy or injured peripheral nerve tissue. The objective of this study was to grossly assess Mg biocompatibility in nerve tissue in addition to developing digital image analysis techniques to quantifiably assess nerve tissue response.

To test basic nerve biocompatibility and develop analysis techniques, Mg metal filaments (150-200 µm diameter, 99.9% purity) were placed in the epineurial tissues of the sciatic nerve, immediately outside nerve bundles (+Mg). The nerves were crushed (+Cr) or uncrushed (-Cr), as a first test of whether Mg might be affected by an injury environment. Tissue samples were removed 7 days post-surgery from each of four conditions (-Mg/-Cr, -Mg/+Cr, +Mg/-Cr, +Mg/+Cr), processed for paraffin sectioning, and stained for macrophages, axons and Schwann cells and GLUT1, an antibody marker for injured nerve tissue. Slides from conditions without Mg (Uncrushed and Crushed) were used in the development of digital methods for IHC stain coverage assessment. Slides with Mg (Uncrushed and Crushed) were used to examine IHC gradient effects that may have arisen due to the Mg filament. All slides were examined for gross tissue response.
Our results showed that coverage of immunohistochemical (IHC) stains in histologic sections of nerve tissue could be estimated with high accuracy and precision by examining less than 10% of the total nerve area. We found no evidence of a gradient effect in the presence of a Mg filament for the stains examined for either Uncrushed or Crushed nerves. Histological analysis also showed little evidence of Mg resorption or inflammation around the Mg filament after one week in vivo; only a thin rim of macrophages encapsulated the Mg metal filaments at one week. These results set the stage for analysis of large volumes of IHC stain data in future experiments examining repairs of nerve tissue.

4.0.1 Chapter aims

The purpose of the experiment outlined in this chapter was to investigate techniques for characterizing nerve tissue by quantification of IHC staining and also to establish a gross baseline for Mg biocompatibility and resorption in peripheral nerves. We also examined the corrosion of and cellular response to a Mg filament of 150-200 um diameter placed in the epineurium of healthy and crushed sciatic nerves in a rat model after one week. Through this study, we tested Hypothesis 3: Mg filaments will be biocompatible when implanted immediately adjacent to healthy and crushed peripheral nerve tissue and started initial testing of Hypothesis 4: Untreated Mg filaments will resorb at a rate appropriate for nerve regeneration. In addition, digital analysis methods for assessing various aspects of tissue response were developed.

4.1 Introduction

The potential advantages of magnesium (Mg) metal and its alloys in in several medical applications were first described as long as a century ago 29. Their safety in human use, including minimum foreign body response, and their excellent capacity for resorption, leaving little traces behind, has also been well documented. Recent advancements in manufacturing technology and techniques for overcoming and observing the corrosion process in biological environments, as well as increased availability of Mg metal (compared to early 20th century supplies) have significantly renewed interest in developing Mg-based
bioresorbable metals. This is especially true in orthopedic and vascular applications where implanted devices have entered clinical trials in humans in recent years. After providing mechanical support to a broken bone in the form of a screw or plate, or keeping a blood vessel open as a stent, the metal then slowly resorbs into the body as native tissue remolds itself and takes over load-bearing functions.

One of the ways that the health of tissues in proximity to an implant may be assessed is by histology. Although traditional histologic methods for sectioning and staining tissue are well established, the presence of a metallic implant can make implementing these methods challenging. Large masses of metal cannot be sectioned by traditional means. If the metal in a sample is small and soft enough to section, as is true with the Mg filament we are using, there is still a difference in stiffness between the metal and surrounding tissue that can cause separation of the sample during sectioning, resulting in large artifacts that can make analysis difficult. In addition, the resorption of Mg in vivo is often non-uniform and difficult to predict and there are not established methods for quickly quantifying the cellular response to this environment using traditional histologic methods. Furthermore, there are very few ways to detect or characterize the byproducts of Mg corrosion, including both ionic Mg and Mg complexes (oxides and phosphates). This is partly why the effect of corroding Mg on peripheral nerves, which are present in close proximity to nearly any potential musculoskeletal implant location, has not been thoroughly investigated, beyond that it appears function is unaffected.

In this experiment the gross reaction of peripheral nerve tissue to an implanted Mg filament after 1 week in vivo was examined. IHC stained sections of nerve tissue were used to develop digital analysis methods for quantitatively describing IHC staining in sections of nerve tissue and for describing gradient effects within nerve tissue in the presence of a Mg filament. We also show that reliable estimates of total stain coverage in nerve tissue can be obtained by observing a relatively small portion of a nerve, a result that could prove helpful in examining tissue sections containing large artifacts due to the presence of metal.
4.2 Methods

4.2.1 Preparation and sterilization of magnesium filaments

Mg filaments ~17 mm long × 150-200 µm diameter were machined from Mg rods (12.7 mm diameter, 99.9% pure, Goodfellow, USA (PA)) using a lathe. Mild heat treatment was used to relieve stresses from machining (the filaments were heated in a tube furnace at 205°C for 1.5h in an Ar and 0.1% SF₆ atmosphere). Filaments were cleaned by sonication in 100% ethanol (three, 3 minute cycles, dried in air) and were stored in a vacuum until use. For implantation, the filaments were cut into 5 mm lengths and sterilized by UV exposure (20 minutes, turned, 20 minutes). All filaments had a gray oxidation layer that was not removed prior to implantation.

4.2.2 Animals

All procedures and housing were in AAALAC approved animal facilities. All animal treatment protocols were reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

A total of 6 adult male Lewis rats weighing between 215 g and 235 g were randomly assigned to one of the 3 following groups: Mg filament without crush injury (+Mg/-Cr) (n = 2); Mg filament with crush injury (+Mg/+Cr) (n = 2); Crush injury without Mg filament (-Mg/+Cr) (n = 2). Crush injuries were performed only on the left legs, and the sciatic nerves on the contralateral (right) leg served as controls. Few animals were used as the purpose of the experiment was primarily to develop methods for quantifying IHC stain coverage and, secondarily, to grossly examine tissue response to a Mg filament.

4.2.3 Surgery

Each animal was anesthetized with isoflurane gas. The sciatic nerve was exposed by dorsolateral gluteal splitting incision according to previous methods 156. After shaving the surgical site, alternating scrubs of
Betadine and 70% ethanol were used to clean the skin. The correct incision site could be identified by palpating the femur through the skin, then using a permanent marker to draw a line parallel to the femur, 2-3 mm caudal to the long axis of the bone. The sciatic nerve was easily identified after cutting through the gluteal musculature, as shown in Figure 23.

For placement of a Mg filament implant in the epineurium, the sciatic nerve was isolated and elevated with forceps. A Mg filament was then inserted into the epineurium, proximally to distally, parallel to the nerve fibers, with the insertion site proximal to the sciatic, peroneal bifurcation. For animals receiving a crush wound, the sciatic nerve was clamped with a smooth-jawed hemostat approximately 3 mm proximal to the sciatic, peroneal bifurcation for 2 consecutive 30-second periods, separated by approximately 10 seconds. If applicable, the filament was inserted after the crush and distal to the crush site. The gluteal muscle and skin were closed using 4-0 Vicryl sutures (Ethicon Endo Surgery, NJ, USA). Lidocaine hydrochloride analgesic gel (Akorn, IL, USA) was applied to the wound after closure and bitter apple spray was applied daily to the affected foot to prevent autotomy. No signs of autotomy were observed, not even toe licking.

**Figure 23 Animal Surgery.** After shaving the hind leg and then cleaning the skin with alcohol and Betadine, the sciatic nerve was exposed in adult male Lewis rats by dorsolateral gluteal muscle splitting incision (A). The nerve was isolated and a 5mm Mg
filament was placed in the epineurium (connective tissue) adjacent to nerve bundles (C). In ‘crush’ conditions, the nerve was crushed prior to Mg filament implantation.\textsuperscript{256}

4.2.4 Functional Assessment of Crush Wound

To determine if the crush wounds were successful, rats were lifted by the tail to induce a ‘toe spreading’ reflex. In the event of a successful crush, Rats were unable to spread their toes on the crushed side. Each crush was effective by this measure. The crush wound was also confirmed by histological examination of both hematoxylin & eosin (H&E) staining and immunostaining with the GLUT1 antibody.

4.2.5 Sacrifice/Dissection

All animals were euthanized by CO\textsubscript{2} asphyxiation followed by cervical dislocation on post-surgical day 7 (1 week). The sciatic nerves were removed, with both proximal and distal nerve stumps attached. Contralateral samples were also excised and assigned to non-operated negative controls (-Mg/-Cr). All tissues were fixed in 4% paraformaldehyde for 48 hours.

4.2.6 Histology

Fixed control nerves or nerves plus conduits were embedded in paraffin and sectioned (5 μm thick) for histological examination. The Mg pieces were readily sectioned and the Mg was visible within paraffin sections. However, upon further processing, the Mg either floated away or was corroded, so in the final sections, only cavities remained. Midpoint sections were stained with H&E or immunostained for the presence of neuronal axons (rabbit antibody to the 200 molecular weight neurofilament protein (NF200), 1:500 dilution (Sigma, St. Louis, MO)), Schwann cells (rabbit antibody to the protein S100 (S100), 1:500 (Dako, Carpinterio, CA)) macrophages (mouse monoclonal ED1 antibody, specific for the cytokine CD68 (ED1), 1:500 (Abcam, Cambridge, MA)), and for an intact nerve-blood barrier (mouse monoclonal antibodies to the glucose transporter-1 protein (GLUT1), 1:500 (Thermo, Fremont, CA)). In all conditions, cell nuclei were labeled by staining with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) (Sigma-Aldrich). Combinations of two primary antibodies plus DAPI were ED-1/S100 (protocol referred
to as *ESD staining* and GLUT1/NF200 (protocol referred to as *GND staining*) and the secondary antibodies were anti-mouse Alexa 488 and anti-rabbit Alexa 594 (1:1000 dilution each) (Invitrogen, Grand Island, NY). Consecutive sections were stained with each protocol.

**Table 4 Antibody Summary.** Summary of IHC antibodies used including the target cell or structure for each antibody and a description of the physiological function of that structure.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>@S100</td>
<td>Schwann Cells</td>
<td>S100 proteins are small, acidic proteins of 10–12kDa found in many tissues, including Schwann cells and others derived from the neural crest, where they function as intracellular Ca(^{2+}) signaling or buffering proteins(^{240}).</td>
</tr>
<tr>
<td>@ED1</td>
<td>Macrophages</td>
<td>ED1 targets the rat homologue of human CD-68, a glycoprotein that binds to low-density lipoprotein, expressed by most tissue macrophages and by peripheral blood granulocytes(^ {241}).</td>
</tr>
<tr>
<td>@NF200</td>
<td>Axons</td>
<td>NF200 bind to the heaviest neurofilament, which is ~200-220 kDa in weight. The presence of this protein is characteristic of mature nerve tissue.</td>
</tr>
<tr>
<td>@GLUT1</td>
<td>Glucose transporter 1</td>
<td>Glucose transporter 1 facilitates the transport of glucose across the plasma membranes of mammalian cells(^ {242}). It is heavily expressed in red blood cells and endothelial cells in barrier tissues e.g. the blood brain barrier in the CNS and endothelial cells and perineurium in the PNS.</td>
</tr>
<tr>
<td>@DAPI</td>
<td>Cell nuclei (non-specific)</td>
<td>DAPI is a fluorescent stain with excitation potential in the ultraviolet range that will strongly bind to adenine-thymine regions in DNA. DAPI is cell membrane permeable and will bind non-specifically to cell nuclei.</td>
</tr>
</tbody>
</table>
4.2.7 Microscopy

Stained sections were viewed and photographed on an upright Zeiss Axioplan Imaging 2e fluorescence microscope with a Zeiss Axiocam digital camera.

4.2.8 Digital Image Post Processing

8-bit grayscale images of each stain were stitched together to create composites in Photoshop. After setting the Image>Mode to ‘RGB color’, grayscale images of all three channels were brought into the same Photoshop document and aligned. In order to color a layer, that later was selected and then under the ‘channel’ tab, all information in two of the three color channels was deleted e.g. to create a ‘red layer’, all of the information in the ‘blue’ and ‘green’ channels was deleted by selecting that color channel, pressing ‘control-A’ and then delete. This process was then repeated for the other two microscope channel layers. This resulted in a ‘red’, a ‘green’, and a ‘blue’ composite layer, each corresponding to one of the microscope channel composites. The final step was taking the two composite layers closest to the top of the image and choosing the effect ‘lighten’, so that all three composites could be viewed simultaneously. Documents were then cropped to remove extraneous background and saved in TIF (Tagged Image File) format. Figure 24 illustrates this process.
Figure 24 Colored composite micrograph storyboard. A composite micrograph of an IHC stained nerve bundle from an uncrushed nerve without Mg (-Mg/-Cr) is shown (A) along with each isolated IHC stain used to create the composite (B-D). Grayscale composites of individual stains (B-D) were created in Photoshop by stitching together high magnification digital photographs taken under fluorescence. Grayscale composites were then colored and combined in Photoshop (D) to create a single, colored representation of the tissue in select slides (‘B’ (S100) corresponds to the red channel, ‘C’ (ED1) corresponds to the green channel and ‘D’ (DAPI) corresponds to the blue channel).

4.2.9 Quantification of stain coverage

In slides of Crushed and Uncrushed conditions, nerve tissue was isolated from surrounding tissues, imported to Matlab and custom code was used to analyze: nerve area, stain area and percent stain
coverage (stain area/nerve area). Random sampling protocols for estimating stain coverage were then examined.

4.9.1 Quantifying true stain coverage

The stain coverage was calculated for each of the channels, in pixels, for the nerve tissue in each Uncrushed and Crushed composite without Mg (Figure 26) using custom Matlab code. The basic function of this code was to identify the total area of the nerve tissue and the total area of each stain e.g. ‘DAPI’, ‘NST’ etc. so that a proportion of ‘total stain area’ divided by ‘total nerve area’ could be determined.

4.9.1.1 Notes on area calculations

8-bit images, like the grayscale composites that we created, can be thought of as a matrix of pixels, each with a value between 0 and 255. The number ‘8’ in 8-bit refers to be amount of information stored in each pixel, with the maximum number of levels of information calculated based on the expression $2^n$, where ‘n’ is the number of bits ($2^8 = 256$, there are 256 discrete values between 0 and 255). Pixels with a value of ‘0’ are displayed as black and pixels with a value of ‘255’ are displayed as white. Pixels with a value between 0 and 255 are displayed as a shade of gray, with darker shades being closer to 0 and lighter shades being closer to 255. Colored images are simply combinations of the three primary colors of light, red, green and blue. When these colors are combined in equal amounts, the resulting color is a shade of gray. This is why we can easily convert ‘colorless’ grayscale images into color images; by removing the color information from two of the three primary color channels in each composite, we are isolating the data from each microscope channel to a single primary color. In this way, three unique datasets i.e. the histochemical stains viewed in the ‘red’, ‘green’ and ‘blue’ channels of a microscope, can be displayed simultaneously in the same image with each represented by one of the three primary colors of light.

To identify the ‘true’ staining in each channel, a threshold was identified for each stain that conveyed the true amount of stain in the thresholded picture. The threshold for a digital image is defined as a value
between the minimum and maximum pixel values (0 – 255 for an 8-bit image) after which all pixels in the image are converted to either black (0) or white (255) depending on whether or not they fall below or above the threshold value. A threshold value was defined for each channel so that structures that were truly stained were white and everything else was black. In addition, a threshold value was also defined for the composite nerve image to distinguish nerve tissue from background or artifact from sectioning. When thresholding a color image, pixels are converted to black or white depending on whether or not the average (generally) of the three, color channels is below or above the defined threshold value.

Matlab was used to automate the process of applying a threshold to each channel of each image and then counting the white pixels in that channel to quantify stain or nerve coverage.

4.9.2 Random sampling for estimating stain coverage

When dealing with these sections, we realized that there would be many instances where we would not be able to image all of the nerve staining in any one cross section of nerve. The technical issues that contribute to this include the fact that the tissue can have wrinkles, non-specific debris, artifactual holes, etc. These are unavoidable with this type of tissue and sectioning, and they were increased by the difficulties in sectioning the hard Mg pieces, as mentioned previously. Also, the labor involved in analyzing the entire image is time consuming and expensive. Therefore, with this first experiment, where the tissue was more intact than we anticipated with the future implanted and regenerating tissues, we set out to determine how the estimation would change with different sampling schemes. We wanted to determine how to make an informed choice of a minimal sampling scheme. Variables that were of importance to test were the sampling window size (we chose a square sampling window) and the numbers of samples that were analyzed per image. Within these windows, we would determine the percent of area covered by an antibody stain. Stain coverage in both the Crushed and Uncrushed conditions, without Mg, was estimated by random sampling in Matlab. Both staining protocols, ESD and GND, were assessed. Low magnification schematics of the images sampled are shown in Figures 25 and 28. For each stain in
each image, we determined the effects on estimated stain coverage of Sampling Window Size, designated as the size (in µm) of one edge of a square sampling region, and Samples per analysis, the number of Sampling Windows analyzed per image (Table 5). The size of individual samples compared to a nerve bundle is shown in Figure 26.

Table 5 Sampling Window Size and Total Number of Samples values tested. Combinations of Sampling Window Size and Total Number of Samples were exhaustively examined to determine how the estimation of each stain would change.

<table>
<thead>
<tr>
<th>Sampling Window Size (in µm)</th>
<th>Samples per analysis</th>
</tr>
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<tbody>
<tr>
<td>40, 50, 60, 70, 80, 90</td>
<td>3-10, 12, 15, 20, 30, 50</td>
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</tbody>
</table>
Figure 25 Overview of images without Mg (nerve tissue only) subjected to random sampling. Full composites of each image examined are shown to give the reader perspective on the size of the images being sampled. Both Uncrushed (A, B) and Crushed (C, D) nerves were examined. Two different staining protocols were used so that several histochemical stains could be evaluated. In the ESD protocol, slides were stained for the presence of the ED1 antibody (A, C – green), a marker for macrophages (an indicator of inflammation), S100 (A, C – red), a marker for Schwann cells, and DAPI (A, C – blue), a marker for cell nuclei. In the GND protocol, slides consecutive with the ESD slides were stained for GLUT1 (B, D – green), a marker for Glucose transporter protein (an indicator of the integrity of the nerve/blood barrier), NF200 (B, D – red), a marker for axons, and DAPI (B, D – blue). Scale bars = 250 µm.
Random coordinates within each bundle were selected by custom Matlab coding utilizing the Matlab function, \texttt{rand}. After identifying a random coordinate on a nerve bundle, a sampling window of specified size was digitally placed at that site and recorded. A threshold was then applied to each channel of the image within the sampling window; the sum of white pixels was used to estimate \textit{Stain Coverage} within the sampling window for each stain. Another threshold was applied to the composite image within the sampling window, using all background staining, to include all of the nerve tissue in the window. The sum of those white pixels was used to estimate \textit{Nerve Area} within the sampling window. The proportion of stain coverage within each sampling window was calculated by dividing the calculated \textit{Stain Coverage} by the calculated \textit{Nerve Area} for each sampling window (Figure 26). The average of all sampling windows was then calculated for each stain and used as an estimate for the stain coverage in that image. This process was repeated 10 times for each combination of Sampling Window Size and Samples per analysis. Threshold values for each stain were determined subjectively and kept constant for all analyses.

If there were multiple bundles in an image, the total number samples taken from each bundle was calculated based on the relative size of the bundles to each other e.g. in the case of an image with two bundles, where one bundle was twice as large as the other, the larger bundle would be sampled twice for every time the smaller bundle was sampled.
Figure 26 Random sampling storyboard. Matlab was used to isolate bundles of nerve tissue in each image ((A) shows an individual bundle from the Uncrushed condition, ESD staining in Figure 25). The six boxes on the nerve tissue in (A) show the 6 sampling windows examined (40 µm – 90 µm, left to right). A pre-determined number of random points, represented by asterisks, were selected on the body of each bundle (B). A sampling window was then created at each point. A 50 µm sampling window, created around the red asterisk in (B) is shown in (C). For each sampling window, a threshold was applied to each channel of the image and the white pixels were summed to estimate the stain coverage (S100 (red) stain coverage shown in (D)). Another threshold was applied to the composite image and the white pixels were summed to estimate the total nerve tissue contained in the sampling window (E). The proportion of stain coverage within the window was calculated for each stain by dividing the ‘stain coverage’, in pixels, by the ‘nerve area’, in pixels. The average stain coverage of all sampling windows (from all bundles, if applicable) was then calculated and used as an estimate for the total stain coverage of the nerve. Scale bar in (A) = 50 µm.
4.2.10 Gradient effects calculations

A major consideration when using biodegradable materials in medical devices is the local tissue response to corrosion products in vivo. It is important to understand how the local tissues respond to the degradation of the biomaterial, and, then, how far away from the implant does this reaction occur. Diffusion away from the implant will determine the total size/extent of the local response. With Mg metal, we are limited in our analyses by the fact that we cannot readily detect Mg$^{2+}$ or even Mg complexes (oxides, phosphates, etc.) in the intact tissues or in histological samples. Therefore, we have to find other methods to determine if the Mg metal is degrading and releasing by-products, or otherwise chemically affecting the local tissues. One method is to determine if there is a difference in the reaction of the tissues immediately adjacent to the Mg implant and then farther away. Another way to think of this is that if the Mg metal is resorbing, the concentration of Mg ions or other materials might be higher closer to the implant, i.e. could create a standing gradient in the local tissues. This might then produce a gradient in the response of any cell type affected by the degradation products. Likely scenarios include that the Mg$^{2+}$ or Mg complexes (or perhaps even trace elements in the Mg despite its purity) released from the implant might attract macrophages toward it (stimulate inflammation), or might repel/attract Schwann cells or axons, depending on their response to these elements. If so, we would expect to see gradients in the amounts of staining for one of these cell types relative to the implant. If we do not see any gradients, then either the Mg has not yet started to biodegrade, or the cell types are not reacting to the corrosion by-products. Either way is important to know, to extend this research further. Therefore, to assess gradient effects for each of the cell types (except for GLUT1 staining, which has a different type of distribution), we sought to develop a method for rapidly assessing gradient effects in IHC staining due to Mg corrosion products. We were primarily interested in the effects on the cells within the nerve bundles, since those are the cell types important for nerve regeneration.

A custom Matlab code was used to create graphs of stain intensity as a function of distance from the magnesium wire. A user-defined circle, calculated by the selection of 3 points on the perimeter of the Mg
cavity, was used to represent the magnesium wire. The program would assign each pixel from each channel in the image a ‘distance’ from the center of the magnesium wire. After each channel was thresholded, as in the Area Calculations, the program would then add up, or *bin*, white pixels (those that were labeled as ‘positive’ for each strain) according to their distance from the magnesium wire. A storyboard outlining this process can be seen in Figure 27.

![Figure 27 General image-processing schematic (A-C) used to assess correlation between distances from the magnesium wire and stain coverage.](image)

Composite micrographs of the staining for each section were composed in Photoshop (A – (+Mg/+Cr) stained for macrophages (ED 1 - green), Schwann cells (S100 – red) and cell nuclei (DAPI – blue). Nerve bundles were then isolated in Photoshop (B) and then the average proportion of stain coverage per nerve area was analyzed using custom Matlab code as described in Figure 26. Stain coverage per nerve area as a function of distance from the magnesium wire was then calculated. A simplified diagram of how the algorithm calculated stain coverage as a function of distance from the magnesium wire is shown (C). A user-defined circle (green, generated by the user choosing 3 points on the border of the wire) was used to represent the magnesium wire. Yellow stars mark the cavity left by the Mg filament.

The data generated from this process was then used to create graphs that allowed us to visually look for correlation between ‘distance from the magnesium wire’ and ‘stain intensity’. For each analysis, a filtered and unfiltered dataset was created. In the filtered dataset, stain coverage values were removed if the area of the nerve being examined was less than 200 pixels. This method effectively removed the misleading extremes often found around the edges of nerve bundles i.e. the stain coverage value for very small areas of nerve was more likely to be close to 0% or 100% and filtering by ‘area’ removed those extremes. A sample output comparing filtered and unfiltered data is shown in Figure 39. (This analysis was performed on both whole composites that included the epineurial connective tissue and composites where this tissue had been removed and we were focused only on the nerve fascicles proper.)
Figure 28 Overview of images with Mg (nerve tissue only) subjected to random sampling. Full composites of each image examined are shown to give the reader perspective on the size of the images being sampled. Both Uncrushed (A, B) and Crushed (C, D) nerves were examined for gradient effects due to a Mg filament. Two different staining protocols were used so that several histochemical stains could be evaluated. In the ESD protocol, slides were stained for the presence of the ED1 antibody (A, C – green), a marker for macrophages (an indicator of inflammation), S100 (A, C – red), a marker for Schwann cells, and DAPI (A, C – blue), a marker for cell nuclei. In the GND protocol, slides consecutive with the ESD slides were stained for GLUT1 (B, D –
green), a marker for Glucose1 transporter protein (an indicator of the integrity of the nerve/blood barrier), NF200 (B, D – red), a marker for axons, and DAPI (B, D – blue). Yellow stars indicate the cavity left by a Mg filament. Scale bars = 250 µm.

4.2.11 Mg corrosion calculations

The location of each Mg filament placed within the epineurium was clearly detectable as an empty cavity in the nerves of each animal that had received a filament. The diameter of this cavity was measured in pixels using the ‘ruler’ tool in Photoshop and recorded. The units of these measurements were then converted to ‘µm’ from pixels. The mean diameter of the Mg cavity was compared to the original diameter of the Mg filament.

4.3 Results

4.3.1 Qualitative observations

4.3.1.1 There was minimal Mg degradation after 1 week in vivo

The metal filaments were readily cut using a regular microtome knife and the Mg metal pieces were readily observed in almost all paraffin sections. However the shapes of the Mg filament pieces were often irregular and were almost always separated from the surrounding tissues. With rare exceptions, the pieces of Mg metal were no longer present after slides were processed for immunostaining, presumably because they either dissolved in the aqueous solutions or physically lifted off the slides. The location of each Mg filament placed within the epineurium was clearly detectable as an empty cavity in the nerves of each animal that had received a filament. The average measured internal diameter of the cavities that had contained the filaments (see cavity surrounded by macrophages (stained green) in Figures 29, 31) was 160 µm, with no difference between crushed and uncrushed nerves, indicating that very little total corrosion had taken place. The original needles implanted were ~150 to 200 um, with some irregularity in dimensions because these were handcrafted on a lathe. Furthermore, the tissue shrinks during preparation. So given these variables, we can’t be completely accurate in saying there was no corrosion, but we can confirm that any corrosion that did occur was very slight at this. This is consistent with the one study in
the literature that showed that Mg wires of 500 um diameter, implanted subcutaneously, were still detectable at 10 weeks after implantation.

4.3.1.1 There was minimal inflammation in response to an implanted Mg filament

The resorption of Mg has not been described when placed specifically in neural tissue. One previous study, performed over 30 years ago, described the placement of Mg wire into a rabbit peripheral nerve. However, all that was reported was that it did not alter relatively simple measures of nerve conduction velocity and no histological analyses were shown. Other reports have anecdotally mentioned that nerves in the vicinity of Mg implants were normal and no studies, to our knowledge, have demonstrated harmful effects of Mg corrosion products on nerve tissue in proximity to the Mg implant. We had hypothesized that the physically delicate nature of neural tissue compared to that of bone or skin would result in more pronounced fluid flow at the site of implantation, due to the local edema and inflammation caused by implantation of the Mg filament. We further hypothesized that this might be exacerbated by a crush injury. This enhanced fluid flow around the implant would result in faster corrosion rates that those previously described. However, we see no evidence that neural tissue supports rapid resorption of the Mg filaments, and there were no suggestions that the crush injury affected the resorption rate of the filaments in neural tissue. The caveat is that the number of animals used in this study was small, so these represent preliminary results.
**Figure 29 Representative IHC staining and epineurial implantation of a Mg filament.** IHC staining in control and crushed tissue with Mg filament implant in connective tissue at 1 week. Yellow stars mark the cavity left by the Mg filament (which was displaced in sectioning). There is a concentrated population of macrophages encapsulating the space occupied by the magnesium needle as seen in Mg/crush condition (A). Nerves that had been crushed (A,B) were clearly distinguishable from those that had not (C,D) due to the lack of GLUT1 staining around the nerve bundles in the crush conditions (B) as opposed to the control (D). GLUT1 also positively stained blood vessels, visible as tiny green circles within the nerve fascicle (B,D). Scale bar = 250 µm.
Figure 30 Representative IHC staining in Crushed and Uncrushed nerves without Mg (higher magnification). Differences in IHC staining were readily identified between Crushed and Uncrushed nerves. In slides stained with the ESD protocol (S100 - red, ED1 - green, DAPI - blue), Crushed nerves showed less S100, more ED1, and more cells overall than Uncrushed nerves. In slides stained with the GND protocol (NF200 - red, GLUT1 - green, DAPI - blue), Crushed nerves showed less NF200, and more cells overall than Uncrushed nerves. The absence of GLUT1 around the perimeter of a nerve was used as a marker for a successful crush injury; the presence of GLUT1 within nerve bundles was not examined. Scale bars = 50 µm.
Figure 31 Mg implantation resulted in minimal inflammation in surrounding tissues. ED1 (macrophages, green), S100 (Schwann cells, red) and DAPI (cell nuclei, blue) composite staining of control (-Mg/-Cr) nerve tissue (A) and implanted nerve tissue with an implanted Mg filament (+Mg/-Cr) (B). ED1 staining was primarily limited to only 1 or 2 cell layers immediately adjacent to the cavity left by the Mg filament (indicated by yellow arrowheads), indicating very mild irritation.

4.3.2 Random sampling

4.3.2.1 Random sampling provided a reliable estimate for stain coverage in nerve tissue.

Qualitative differences between Crushed and Uncrushed nerve sections were reflected in the quantitative stain coverage results e.g. there were marked decreases in NF200 and S100 staining between Uncrushed and Crushed nerves; the amount of ED1 stain coverage, and total number of cells, as indicated by DAPI staining, was higher in Crushed sections than in Uncrushed sections and also reflected by quantitative random sampling.

4.3.2.2 Accurate results could be obtained with relatively few sampling windows.

In order to determine the accuracy of sampling a subset of the images, we systematically determined how subset sampling compared to the percent of stain coverage done on the entire image. These analyses were
done for each stain type in Figures 33-37. Our conclusions are that, in general, across each stain, taking only 4 samples per image was comparable to taking 50 samples per image. The variance of the mean of stain coverage was generally very small if 4 or more samples were taken from an image and increasing the Samples per analysis did not greatly increase the precision or accuracy of the stain coverage estimate. Using the area chart in Figure 32 as a guide along with the ‘t-value’ and ‘% difference’ tables in Figures 33-37, reasonable estimates of stain coverage (within 10% of the actual value) could be obtained by sampling <10% of the total nerve area for both Crushed and Uncrushed sections using one of several combinations of ‘Sampling Window Size’ and ‘Samples per analysis’. The estimates that were farthest from the true stain coverage (ED1 – Uncrushed, DAPI_GND – Crushed) were still relatively accurate and displayed high precision across sampling protocols. The error in the estimate in each case was the result of inconsistencies in the image being examined e.g. most of the ‘ED1+’ staining was due to ‘noise’ (random staining of debris on the coverslip) in the Uncrushed ED1 image and discrepancies in ‘DAPI’ stain estimates was due to the slightly greater cell density at the edge of the perimeter of the bundles in the DAPI_GND image.

Figure 32 Maximum nerve area sampled for images of Uncrushed (A) and Crushed (B) nerves. Since samples were taken around random points on the nerve bundle, there was always a possibility that samples could overlap. The values shown in this
Figure 33 Summary of random sampling analysis for S100 staining in Uncrushed and Crushed nerves. Representative samples of the S100 channel (displayed in grayscale) are shown for Uncrushed (A) and Crushed (B) nerve tissue; scale bar = 50 µm. The graphs in (C) and (D) show the results of stain coverage estimation by random sampling in Uncrushed (C – the image used is shown in Figure 25A) and Crushed (D – the image used is shown in Figure 25C) tissue stained for S100. Error bars = standard deviation. Stain coverage estimates for each combination of ‘Samples per analysis’ and ‘Sampling Window’ by random sampling were calculated 10 times, each with a different set of random samples. The data shown in (C) and (D) includes the estimates from each of these trials. The solid black horizontal line on each graph represents the true stain coverage for the composite nerve image. ‘T-value’ charts show whether the stain coverage estimate for each combination of ‘Samples per analysis’/’Sampling window’ differed from the true mean of the image by a Student’s T-test (9 degrees of freedom, t-values > 2.262 represent mean values significantly different from the true stain coverage value); Green = t-value <2.262, Red = t-value
‘% difference’ charts show how widely estimates of the stain coverage mean differed from the true mean in terms of percent; Dark green = <3%, Light green = <5%, Yellow = <10%, Orange = <15%, Red = >15%.

Sampling provided a good estimate of S100 coverage in that the estimated values were consistently close to true mean of the image, especially in the Crushed condition. In the Uncrushed condition, mean stain coverage values tended to increase with larger sampling windows because smaller bundles, which, in the specific image tested, had lower stain coverage were excluded, as they were smaller than the sampling window. The number of samples per analysis had little impact on the mean. The variance of the estimated stain coverage mean was minimal in all cases tested as long as more than 3 samples per analysis were taken. A decrease in S100 between the Uncrushed and Crushed conditions was clearly evident.

Figure 34 Summary of random sampling analysis for ED1 staining in Uncrushed and Crushed nerves. Representative samples of the ED1 channel (displayed in grayscale) are shown for Uncrushed (A) and Crushed (B) nerve tissue; scale bar = 50 µm. The graphs in (C) and (D) show the results of stain coverage estimation by random sampling in Uncrushed (C – the image used is shown in Figure 25A) and Crushed (D – the image used is shown in Figure 25C) tissue stained for ED1. Error bars =
standard deviation. Stain coverage estimates for each combination of ‘Samples per analysis’ and ‘Sampling Window’ by random sampling were calculated 10 times, each with a different set of random samples. The data shown in (C) and (D) includes the estimates from each of these trials. The solid black horizontal line on each graph represents the true stain coverage for the composite nerve image. ‘T-value’ charts show whether the stain coverage estimate for each combination of ‘Samples per analysis’/‘Sampling window’ differed from the true mean of the image by a Student’s T-test (9 degrees of freedom, t-values > 2.262 represent mean values significantly different from the true stain coverage value); Green = t-value <2.262, Red = t-value >2.262. ‘% difference’ charts show how widely estimates of the stain coverage mean differed from the true mean in terms of percent; Dark green = <3%, Light green = <5%, Yellow = <10%, Orange = <15%, Red = >15%.

Sampling provided an acceptable estimate of ED1 coverage in the Crushed condition though estimates were consistently high in the Uncrushed condition. In the Uncrushed condition, mean stain coverage values tended to increase with larger sampling windows, as in Figure 33. The number of samples per analysis had little impact on the mean. The variance of the estimated stain coverage mean was minimal in all cases tested as long as more than 3 samples per analysis were taken. An increase in ED1 staining between the Uncrushed and Crushed conditions was clearly evident.
Figure 35 Summary of random sampling analysis for NF200 staining in Uncrushed and Crushed nerves. Representative samples of the NF200 channel (displayed in grayscale) are shown for Uncrushed (A) and Crushed (B) nerve tissue; scale bar = 50 µm. The graphs in (C) and (D) show the results of stain coverage estimation by random sampling in Uncrushed (C – the image used is shown in Figure 29A) and Crushed (D – the image used is shown in Figure 29C) tissue stained for NF200. Error bars = standard deviation. Stain coverage estimates for each combination of ‘Samples per analysis’ and ‘Sampling Window’ by random sampling were calculated 10 times, each with a different set of random samples. The data shown in (C) and (D) includes the estimates from each of these trials. The solid black horizontal line on each graph represents the true stain coverage for the composite nerve image. ‘T-value’ charts show whether the stain coverage estimate for each combination of ‘Samples per analysis’/‘Sampling window’ differed from the true mean of the image by a Student’s T-test (9 degrees of freedom, t-values >2.262 represent mean values significantly different from the true stain coverage value); Green = t-value <2.262, Red = t-value >2.262. ‘% difference’ charts show how widely estimates of the stain coverage mean differed from the true mean in terms of percent; Dark green = <3%, Light green = <5%, Yellow = <10%, Orange = <15%, Red = >15%.
Sampling provided a good estimate of NF200 coverage in both Uncrushed and Crushed conditions. Higher ‘% difference’ in the Crushed condition, was likely due to the relatively low stain coverage value i.e. similar absolute variation from the true stain coverage value would register as a larger ‘% difference’ in the Crushed condition than the Uncrushed condition. The number of samples per analysis had little impact on the mean. The variance of the estimated stain coverage mean was minimal in all cases tested as long as more than 3 samples per analysis were taken. A decrease in NF200 staining between the Uncrushed and Crushed conditions was clearly evident.

Figure 36 Summary of random sampling analysis for DAPI staining in Uncrushed and Crushed nerves (ESD staining).

Representative samples of the DAPI channel (displayed in grayscale) are shown for Uncrushed (A) and Crushed (B) nerve tissue; scale bar = 50 µm. The graphs in (C) and (D) show the results of stain coverage estimation by random sampling in Uncrushed (C – the image used is shown in Figure 29B) and Crushed (D – the image used is shown in Figure 29D) tissue stained for DAPI. Error bars = standard deviation. Stain coverage estimates for each combination of ‘Samples per analysis’ and ‘Sampling

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Window’ by random sampling were calculated 10 times, each with a different set of random samples. The data shown in (C) and (D) includes the estimates from each of these trials. The solid black horizontal line on each graph represents the true stain coverage for the composite nerve image. ‘T-value’ charts show whether the stain coverage estimate for each combination of ‘Samples per analysis’/’Sampling window’ differed from the true mean of the image by a Student’s T-test (9 degrees of freedom, t-values > 2.262 represent mean values significantly different from the true stain coverage value); Green = t-value <2.262, Red = t-value >2.262. ‘% difference’ charts show how widely estimates of the stain coverage mean differed from the true mean in terms of percent; Dark green = <3%, Light green = <5%, Yellow = <10%, Orange = <15%, Red = >15%.

Sampling provided a good estimate of DAPI coverage in both Uncrushed and Crushed conditions. The slightly low stain coverage estimate in the Crushed condition was likely due a slightly higher density of DAPI+ cells on the perimeter of the nerve, a region that was not sampled as heavily as the interior; in order to avoid samples with only a small amount of nerve tissue, random sampling coordinates were selected at least 1 sampling window from the edge of each bundle. The number of samples per analysis had little impact on the mean. The variance of the estimated stain coverage mean was minimal in all cases tested as long as more than 3 samples per analysis were taken. An increase in DAPI staining between the Uncrushed and Crushed conditions was clearly evident.
Figure 37 Summary of random sampling analysis for DAPI staining in Uncrushed and Crushed nerves (GND staining).

Representative samples of the DAPI channel (displayed in grayscale) are shown for Uncrushed (A) and Crushed (B) nerve tissue; scale bar = 50 µm. The graphs in (C) and (D) show the results of stain coverage estimation by random sampling in Uncrushed (C – the image used is shown in Figure 29B) and Crushed (D – the image used is shown in Figure 29D) tissue stained for DAPI. Error bars = standard deviation. Stain coverage estimates for each combination of ‘Samples per analysis’ and ‘Sampling Window’ by random sampling were calculated 10 times, each with a different set of random samples. The data shown in (C) and (D) includes the estimates from each of these trials. The solid black horizontal line on each graph represents the true stain coverage for the composite nerve image. ‘T-value’ charts show whether the stain coverage estimate for each combination of ‘Samples per analysis’/‘Sampling window’ differed from the true mean of the image by a Student’s T-test (9 degrees of freedom, t-values > 2.262 represent mean values significantly different from the true stain coverage value); Green = t-value <2.262, Red = t-value >2.262. ‘% difference’ charts show how widely estimates of the stain coverage mean differed from the true mean in terms of percent; Dark green = <3%, Light green = <5%, Yellow = <10%, Orange = <15%, Red = >15%.
Sampling provided a good estimate of DAPI coverage in both Uncrushed and Crushed conditions. The slightly low stain coverage estimate in the Crushed condition was likely due to a slightly higher density of DAPI+ cells on the perimeter of the nerve, a region that was not sampled as heavily as the interior; in order to avoid samples with only a small amount of nerve tissue, random sampling coordinates were selected at least 1 sampling window from the edge of each bundle. The number of samples per analysis had little impact on the mean. The variance of the estimated stain coverage mean was minimal in all cases tested as long as more than 3 samples per analysis were taken. An increase in DAPI staining between the Uncrushed and Crushed conditions was clearly evident.

4.3.2.3  The size of the sampling window had little impact on the stain coverage estimate.
Choosing a sampling window of adequate size is crucial for achieving an accurate estimate. Using a sampling window that is too small will increase the risk of large estimations errors i.e. extreme value are more common, and using a sampling window that is too large will increase the risk of oversampling the specimen i.e. sampling the same area multiple times. Square Sampling Windows with edges between 40 and 90 µm were tested. Each sampling window tested yielded similar results, though using the 40 µm sampling window generally resulted in greater variability of the stain coverage estimate, even with high numbers of samples taken, than the other Sampling Window sizes tested. The larger sampling windows tended to yield higher estimates of stain coverage for S100 and ED1 in the ESD control slide because, as the size of the Sampling Window increased, nerve bundles that were smaller than the Sampling Window, and had a lower stain coverage than the larger bundles, were excluded from the analysis. As shown in Figure 32, oversampling was likely to occur at relatively low numbers of samples taken when using larger sampling windows for the examined specimens.

4.3.2.4  IHC stains with low coverage were more susceptible to greater estimate deviation from the true stain coverage value.
As shown in the Figures 33-37, the stain coverage estimates for IHC stain with low coverage e.g. ED1 in the Uncrushed condition and S100 and NF200 in the Crushed condition were not as accurate in terms of
‘% difference’ as those with higher coverage. However, estimates for all stains were still relatively accurate as the stain coverage estimate was generally less than two points from the actual mean.

4.3.3 Gradient effects

The coverage of each stain as a function of distance from the Mg filament was calculated for each slide containing a Mg filament. There was a large increase in total cells and macrophages in the epineurial connective tissue immediately adjacent to the Mg filament, but there were no consistent stain density gradients within the adjacent nerve bundles. Visual inspection of all graphs did not detect consistent gradient effect differences that would have warranted a more intensive mathematical analysis of the graph data. A characteristic graph can be seen in Figure 39.
Figure 38 Representative IHC staining in Crushed and Uncrushed nerves with Mg (higher magnification). Differences in IHC staining were readily identified between Crushed and Uncrushed nerves. In slides stained with the ESD protocol (S100 - red, ED1 - green, DAPI - blue), Crushed nerves showed less S100, more ED1, and more cells overall than Uncrushed nerves. In slides stained with the GND protocol (NF200 - red, GLUT1 - green, DAPI - blue), Crushed nerves showed less NF200, and more cells overall than Uncrushed nerves. The absence of GLUT1 around the perimeter of a nerve was used as a marker for a successful crush injury; the presence of GLUT1 within nerve bundles was not examined. There appeared to be less S100 and NF200 staining in nerve tissue containing a Mg filament than nerve tissue that did not, possibly due to unintentional injury induced during placement of the Mg filament. Scale bars = 50 µm.
Figure 39 Graphs of correlation between distances from the magnesium wire and stain coverage. The proportion of stained pixels as a function of the distance from the center of the magnesium wire was calculated for each applicable slide (the graphs in this figure came from the analysis of the image in Figure 27). There was generally no signal for the first 100 to 150 pixels from the center of the magnesium wire (as that area was occupied by the wire) followed by a strong signal from the cells encapsulating the wire and then by signal generated by the stain within the nerve tissue and each image. The amount of measurable area could be very small when examining the aspect of nerve bundles most proximal or distal to the Mg wire cavity, leading to extremes in calculated stain coverage in unfiltered data (A-B). This was corrected for by filtering out any data points generated by nerve areas less than 200 pixels (C-D, black arrows show where the total nerve area was less than 200 pixels and the data was filtered).

4.4 Discussion

4.4.1 Qualitative observations – Implanted Mg filaments showed minimal signs of corrosion or inflammation in local tissues after 1 week in vivo.

The Mg filaments in this experiment were thoroughly encapsulated at 1 week and showed very little sign of corrosion, based on the size of the cavity left behind in histologic sections and the lack of any deleterious gradient effects on neighboring cells. In vitro, when we placed similar size Mg filaments to those used in this experiment in a typical cell culture medium in a cell culture incubator, they were completely corroded within 24 hours. When CNS-derived primary cells were plated in proximity to the filament, there was a measurable gradient of cell death immediately adjacent to it though cell numbers were normal a few millimeters away (data not shown). This highlights a major difficulty that currently challenges the field of degradable biomaterials: Mg corrosion in vivo can be orders of magnitude slower than corrosion in vitro and current in vitro models of corrosion do not correlate well with in vivo observations.

In vivo, the rate of degradation is constantly changing due to a number of factors. Immediately after implantation, serum proteins rapidly adsorb to the surface of the metal and cells attach as part of the foreign body response. This decreases the surface area exposed to aqueous solution. In addition, the intensity of the foreign body response can depend on the local concentrations of the byproducts of metal
corrosion, which are, in turn, determined by the rate of corrosion and the fluid access to the metal surface and the fluid composition, whose ionic composition can vary, i.e., because of different extents of cell injury and release of cellular contents. The complexity of this relationship is still largely unknown for biological systems and depends on several factors including alloy composition, geometry, surface treatment of the metal, physiologic flow, cellular environment, tissue type and others 195, 243.

4.4.2 Random sampling analysis revealed that accurate estimates of IHC coverage could be obtained by observation of a small proportion of total nerve area.

The random sampling method used to quantify the intensity of each stain delivered results consistent with visual examination of stained tissue sections. Assessment of stain coverage (Figures 33-37) and stain coverage as a function of distance from the Mg wire (Figure 39) were readily acquired and provided an objective and repeatable method for rapidly evaluating nerve tissue response. Visual examination of stained slides revealed an increase in the relative presence of macrophages and decrease in viable neurons in the crushed nerve relative to undamaged nerve tissue. These observations were reflected in the results generated by our algorithm. The mild foreign body response observed next to the filaments, characterized by a small capsule of macrophages with very few blood elements or excess tissue growth, was also clearly distinguishable in the graphs of stain coverage as a function of distance from the Mg wire.

4.4.3 Gradient effects due to Mg corrosion products were not observed.

Implantation of a Mg filament in the absence of a crush resulted in a significant increase in ED-1+ macrophages, and total cell number, but no other significant changes. There was no distance effect in either total cell count or ED-1+ staining, relative to the Mg filament (Figure 39). This suggests that the Mg metal is not corroding/releasing corrosion products that are influencing the macrophage population or total cell population. Therefore, we speculate that this cell increase is due to irritation caused by manipulation of the nerves during placement of the Mg filament. Though we did not detect any gradient effects by IHC, sophisticated spectroscopic methods (LA-ICP-MS) have been used to detect
concentration gradients of Mg alloying materials in bone tissue. While precise, this type of complex analysis is technically challenging and can only provide gradient information from the Mg in one, small linear portion of tissue at a time.

4.4.4 Study limitations
The small number of animals in each experimental condition excluded us from the comparing conditions using traditional statistical methods, thus, the qualitative observations described can only serve as estimates in changes in IHC stain coverage.

Other technical challenges included that it was difficult to accurately place the Mg filament at the site of crushed nerve tissue. The nerve was crushed prior to implantation of the Mg filament and the location of the crush was not clearly marked, making it difficult to precisely identify during fixation and sectioning. Another issue was that the crush was not uniformly effective at damaging all the nerve fascicles within the nerve. We could detect nerves that were crushed by either examining the H&E staining or by examining the staining with the GLUT1 antibody although there were several samples in the Crushed condition with intact GLUT1 membrane around some or all nerve bundles. Thus, the degree of crush injury was variable throughout the sectioned nerves, making it challenging to more definitively describe tissue/Mg interactions.

4.5 Conclusions
The findings from this experiment offer an interesting first glimpse at the effects of Mg metal implanted in nerve tissue. In this study, we demonstrated, by random sampling, that IHC stain coverage in nerve tissue could be reliably estimated by examining relatively small areas of a sample. This was confirmed by qualitative observations of differences between Uncrushed and Crushed nerve in macrophage and NF200 staining. We also introduced a method for measuring stain intensity as a function of distance from a Mg filament and used it to show that, in this case, no gradients were detected. This observation, combined
with the measurements of the Mg implant cavities, suggest no degradation of the Mg filament after 1 week. Collectively, these qualitative observations indicate that Mg metal is biocompatible in nerve tissue. Bioresorbable metal materials show great promise for applications in medicine although the complex interactions that govern the rate of implant corrosion, bioresorption of corrosion products and host tissue response still need to be understood in greater detail to advance this nascent field. The work presented here represents the first steps in the development of a system to quantitatively study this intricate environment at the cellular level in neural tissues.
5.0 Magnesium metal as a scaffolding material for peripheral nerve regeneration

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Abstract

Magnesium metal (Mg) is very promising as a non-toxic, bioresorbable medical implant. The objective of this work was to evaluate the feasibility of using Mg metal as a tissue scaffold for peripheral nerve (PN) regeneration. Mg metal was placed inside the lumen of a biodegradable poly(caprolactone) (PCL) nerve conduit to promote repair across a 6 mm nerve defect in the rat model. Some conduits were filled with a growth promoting keratin hydrogel as a positive control for nerve tissue growth. We investigated Mg resorption, cellular responses to Mg and nerve repair parameters.

After a nerve gap repair, Mg microfilaments placed inside nerve conduits were surrounded by healthy, organized regenerating nerve tissue. There was a minimal inflammatory response to the Mg filament as measured by immunohistochemical (IHC) staining of macrophages in the surrounding tissues. A measure of functional recovery, the relative size of the nerve’s target muscle, the gastrocnemius, was improved in animals treated with conduits containing Mg, or keratin hydrogel without Mg, versus empty conduits. The rate of Mg resorption within the conduit, monitored by micro computed tomography (microCT), was appropriate to match the time course of PN regeneration and was accelerated by the presence of keratin hydrogel. These promising results warrant further exploration of Mg as a scaffolding material for regenerating PN tissue.
5.0.1 Chapter Aims

The purpose of the experiment outlined in this chapter was to characterize the use of a novel nerve conduit containing a scaffold composed of a centrally located filament of Mg metal in a rat sciatic nerve defect model. Regenerating nerve tissue was assessed by histology, the resorption of Mg metal was assessed by microCT and several behavioral methods for monitoring functional recovery were attempted. Through this study, we tested Hypothesis 4: Untreated Mg filaments will resorb at a rate appropriate for nerve regeneration; Hypothesis 5: Because the Mg filaments will remain intact and will provide the appropriate physical support (a scaffold), a Mg-containing nerve conduit will support regeneration of PN tissues across a short nerve injury gap, and this repair will be as good as or better than the regeneration through an empty conduit; Hypothesis 6: When used inside a nerve conduit, a Mg filament will be biocompatible with regenerating nerve tissue and; Hypothesis 7: Keratin hydrogel filler in a nerve conduit will enhance nerve regeneration above that seen with a saline-filled conduit, and when combined with a centrally located Mg filament, would synergistically improve nerve regeneration.

5.1 Introduction

Peripheral nerve (PN) injuries are a serious problem and are reported in over 3% of all emergency room visits in the United States every year, comprising over 300,000 injuries \(^{16, 244}\). Due to the physically delicate nature of PN tissue and the complexity of the neuromuscular junction, full functional recovery is rarely achieved after severe trauma. In cases where the nerve is severed and neuroanastomosis is not possible, the current ‘gold standard’ of treatment is an autograft surgically placed at the site of injury to bridge the nerve segments. Autografts, however, are less than satisfactory, because harvesting a donor nerve typically requires a second surgical site and results in donor site morbidity. In addition, the mismatch in dimensions and functions of the donor and damaged nerves results in limited functional recovery \(^{19}\). Thus, novel solutions are much needed.
To replace autografts, researchers have actively pursued hollow nerve conduits made of synthetic biomaterials. Since 1995, the FDA has approved 11 different biodegradable nerve conduits composed of various natural and synthetic materials. However, all of these have only been shown to be effective for nerve gaps of up to 20-25mm. With longer gaps, tissue regrowth from proximal to distal nerve stumps does not occur. One of the hypothesized reasons for this failure is that proteinaceous fibrin bridges cannot physically form. In short gaps, these protein bridges form strong ‘contact guidance’ filaments that enable fibroblasts and Schwann cells to cross the gap. One current strategy being explored to correct this situation is to place linearly aligned biomaterial substrates in the lumen of nerve conduits to provide this contact guidance. It has been shown by several investigators that peripheral nerve regeneration can also be improved by addition of biomaterial fillers to the lumen of the conduit. Fillers such as extracellular matrix proteins, various polysaccharides and synthetic polymers provide regenerating tissue with a three dimensional scaffold conducive to cellular migration across the gap and (potentially) enhanced recovery.

We proposed that microfilaments of the biodegradable metal Mg would be an effective material to provide contact guidance for a regenerating nerve when placed within an experimentally established nerve conduit. The physical characteristics of Mg and its alloys, including stiffness close to that of native bone and ready degradation in a physiologic environment, have made Mg appealing for a variety of biomedical applications including bioresorbable orthopedic screws and plates, vascular stents and bone tissue scaffolding material. As such, extensive studies have been performed to test the biocompatibility of Mg subcutaneously and bone tissue in vivo. Although there are still technical limitations that must be overcome to prevent problems such as severe local corrosion, gas cavity formation from corrosion byproducts or unpredictable resorption rates, the metal itself has been shown to be non-toxic and biocompatible.
We hypothesized that a Mg filament placed axially in the lumen of a nerve conduit would significantly improve regeneration of peripheral nerves by providing the strength and linear nature required for initial cell growth across a nerve gap, followed by resorption into the body, which allows the nerve to continue growing without interference. As outlined previously in this dissertation, we implanted Mg microfilaments alone into the epineurial connective tissue (epineurium) around nerve bundles in normal and injured large PNs in the leg of rats (sciatic nerves) and left them in the animal for one week. This tested the basic biocompatibility and bioresorption of magnesium in nerve tissue and provided an initial examination of resorption rate. In the experiment described in this Chapter, we tested our Mg scaffold idea by repairing a short (6mm) injury gap in rat sciatic nerves with a well-studied poly(caprolactone) (PCL) conduit containing Mg metal and filled with keratin hydrogel, a hydrogel that has shown promising nerve regeneration potential in recent animal studies or saline as a control. These implants were left in the animal for 6 weeks.

5.2 Methods

5.2.1 Preparation and Sterilization of magnesium filaments

0.250 µm diameter Mg wire (99.9% pure) (Goodfellow, PA, USA) was cut into 10 mm lengths then cleaned by sonication (10 minutes in 100% ethanol) and sterilized by UV light as in 2.2.1.

5.2.2 Preparation and sterilization of PCL nerve conduit

PCL nerve conduits were constructed according to previous methods by Danielle Minteer, a graduate student in the lab of Kacey Marra, PhD. Conduits were stored under vacuum at the University of Cincinnati for up to 6 weeks until needed. Prior to surgery, conduits were cut to length under a cell culture hood using a razor blade and a ruler. Conduits were then sterilized by exposure to UV light for 20 minutes; 10 minutes, turned, 10 minutes.
5.2.3 Animals

A sciatic nerve defect was created in 24 adult male Lewis rats weighing between 215 g and 250 g. The defect was surgically repaired in one of 4 ways: PCL conduits flushed with saline (-Mg/Sa) \(n = 4\) or filled with keratin hydrogel (-Mg/Kr) \(n=9\) via syringe after implantation; PCL conduit with added Mg filament flushed with saline (+Mg/Sa) \(n = 4\) or filled with keratin hydrogel (+Mg/Kr) \(n=9\) after implantation.

5.2.4 Surgery

The sciatic nerve was exposed by dorsolateral gluteal incision as in Chapter 4, after which the nerve was sharply transected with scissors 5 mm proximal to the sciatic peroneal bifurcation and allowed to retract for 30 seconds. A 6mm defect was created by repairing the gap with an 8mm PCL conduit, overlapping the proximal and distal stumps by 1 mm, and held in place by two 8-0 polypropylene stitches (Ethicon Endo Surgery, NJ, USA) suturing the conduit to the epineurium on each end. The lumen of the conduit was then filled with either keratin hydrogel, or flushed with saline as a control. Keratin hydrogel was prepared with keratose, a preparation of keratin derived from human hair using a chemical oxidant. Conduits containing a Mg filament were constructed by inserting a 10 mm Mg filament 2 mm deep into the perineurium of the proximal nerve stump, threading the conduit over the filament, stitching the proximal end of the conduit in place, threading the distal stump onto the Mg needle, then suturing the distal end of the conduit to the distal nerve stump. This effectively positioned the Mg filament coaxially with conduit. A storyboard outlining this process can be seen in Figure 41. The muscle was closed with 2-3 buried stitches using 4-0 Vicryl suture (Ethicon Endo Surgery, NJ, USA). The skin was closed with a running stitch using 4-0 Proline (polypropylene) suture (Ethicon Endo Surgery, NJ, USA). External stitches were removed two weeks after surgery. Meir (Matt) Hershcovitch, MD, Department of Otolaryngology-Head and Neck Surgery, University of Cincinnati, Cincinnati, OH and Kevin Little, MD, Department of Pediatrics, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH performed the surgeries, each on an equal number of animals.
Figure 40 Mg-containing nerve conduit for peripheral nerve repair. A bioresorbable magnesium filament (represented by the light blue rod) is embedded into the stumps created by a nerve defect (represented by the gray cylinders on either side of the image). The defect is bridged by a biodegradable, PCL conduit (represented by the transparent cylinder) and held in place by very fine sutures (represented by the curved arrows). We hypothesized that the magnesium filament would act as a physical, contact-guidance support for regenerating nerve tissue, improving the functional outcome of the repair, before resorbing into the body.\textsuperscript{256}

Figure 41 Placement of a Mg-containing neural conduit. After the sciatic nerve was exposed by dorsolateral gluteal muscle splitting incision (A), it was sharply transected approximately half way between its proximal origin and the sciatic/tibial bifurcation. The teal ‘P’ and ‘D’ in panel (A) indicate proximal and distal aspects of the nerve. Next, a cylindrical 0.25mm x 10mm Mg filament was inserted 2 mm into the internal epineurium of the proximal stump (B). An 8mm PCL conduit was then
threaded over the Mg filament and sutured to the external epineurium using two opposing 8-0 horizontal mattress sutures with a 1mm overlap between the nerve and the conduit (C). Finally, the distal nerve stump was threaded 2mm onto the distal end of the Mg filament before being sutured to the distal conduit, with a 1mm gap between the nerve and the conduit, effectively creating a 6mm gap in the nerve. Images in this figure were obtained during a dissection that took place in the week before the surgery, hence, the lack of skin cleaning agents around the incision.

5.2.5 Behavioral testing

Three behavioral tests were performed weekly in an effort to identify differences in functional recovery between groups. Rats were exposed to each test twice in the week before experimental measurements were made in order to condition them to each process.

5.2.5.1 Grid walk

Rats were placed at the edge of a metal sheet with diamond-shaped cutout cutouts opposite to their home cage. A video camera was positioned level to the metal sheet such that both the rat and the home cage were within the recording frame. The rat was then allowed to make its way across the metal sheet to its home cage. This was recorded on video and repeated five times consecutively for each animal. Recordings were taken weekly. Afterwards, a student in the lab of Sheila Fleming, PhD (Neurology, University of Cincinnati) watched each video trial and recorded how many times each animal's limbs fell through the diamond cutouts in the metal sheet. Four measurements were recorded: forelimb slips (defined as either forelimb falling below the wire mesh), Hindlimb slips (defined as either hindlimb, including the whole foot, falling beneath the wire mesh), Heel slips (defined as the heel, but not the toes falling through the mesh) and total number of steps taken. It was hypothesized that the degree of nerve recovery would be correlated to the number of mistakes made by each animal. A diagram of this testing set up is shown in Figure 42.
A grid composed of commercial heavy wire mesh was placed on top of two empty rat cages, raising the middle portion of wire grid above the ground. The experimental rat was placed on one side of the grid and made to walk to his home cage, which was placed on the other side of the grid. A static video camera was set up, level with the wire grid, to record the number of steps taken and the number of times the rat misstepped and allowed his foot to fall through the mesh.

5.2.5.2 Cylinder test

Animals were placed at the bottom of a translucent plastic cylinder on a piece of clear glass. A camera was then set up to record the movement of each animal from underneath the glass by placing the camera on the lab bench end arranging a mirror so that the recording frame included the entirety of the plastic cylinder. Three-minute videos capturing this movement were recorded weekly. Afterwards, a student in the lab of Sheila Fleming watched each video trial and recorded the number of steps each animal took with their right (surgery side) or left foot. It was hypothesized that animals with the best nerve recovery would use their right foot more often than those that had not recovered as well. A diagram of this testing set up is shown in Figure 43.
Figure 43 Cylinder test experimental setup. Rats were placed in a clear cylinder on a piece of transparent glass for 3 minutes once a week. A camera and mirror were positioned so that the activity of the rat could be monitored from beneath the glass (A). A representative frame from the video feed is shown (B). The number of left (surgery) and right (control) hindlimb steps taken by the rat during the 3-minute session in the cylinder was recorded. The ratio of left and right steps taken was then calculated. A ratio near 1 was considered normal. A ratio <1 indicated the animal was favoring the surgery leg and not using it as often as the non-surgery leg. The lack of toe spread, as seen on the left hind foot in B, is a signal of denervation.

5.2.5.3 Footprint analyses

Two legal-sized sheets of paper (8.5” x 14”) were taped together long ways, overlapping by approximately 1”, to create a single sheet approximately 8.5” x 27” in size. This sheet of paper was placed on the floor of a corridor, constructed using empty plastic rat housing cages. After the hind feet of each animal were painted using non-toxic, washable paint (Crayola, Easton, PA) (Figure 44), the animal was placed at one end of the corridor and allowed to run to its home cage, which was placed at the other end of the corridor. Two consecutive trials were conducted weekly for each animal. A diagram of this testing set up is shown in Figure 45. The size and toe-spread of each footprint (an indicator of functional recovery) were measured.
Figure 44 Painting feet for footprint analysis. The feet of rats were painted with non-toxic, washable paint using a cotton applicator before walking down a paper-floored corridor. The rats were held as shown in the picture above; thumb and forefinger were used to lightly hold the knees of the rat together to prevent kicking during paint application. The body and head of the rat were held against the body to reduce squirming.
Figure 45 Footprint analysis experimental setup. A corridor leading to the home cage was constructed by placing empty cages next to each other on top of blank paper, which was used to record footprints (A). Animals were positioned at the end of the corridor, opposite their home cage, allowing their front paws down on the paper before releasing their painted hind paws. Animals then ran down the corridor, toward their home cage leaving footprints on the paper (B). Animals were encouraged in the right direction with light touches on their hindquarters if they began to veer off course e.g. between the empty cages, turning around.

5.2.6 Sacrifice/Dissection

All animals were euthanized by CO₂ asphyxiation followed by cervical dislocation on post-surgical day 42 (6 weeks) with the exception that 2 animals were sacrificed at day 7 (1 each for +Mg/Sa, +Mg/Kr) to
grossly assess Mg resorption. All tissues were fixed in 4% paraformaldehyde for 48 hours. A simple interrupted stitch was sutured the proximal end of each nerve for identification of tissue orientation.

5.2.7 Micro Computed Tomography

Before further tissue processing, the position and resorption state of Mg filaments in the sciatic nerves were assessed by micro computed tomography (microCT) (Siemens - Inveon Multimodality System, CA, USA) in the UC Vontz Imaging Core Facility. Samples were submerged in PBS after fixation and kept immersed throughout scanning. Samples were scanned at half-degree increments with 384 steps (step and shoot) for 192 degrees. Images were acquired with high magnification and a pixel matrix binning of 2, resulting in an effective voxel size of 17.27 µm. The voltage used was 80kVp, current was 300uA, and the exposure time was 2100 ms with 25 ms settle time.

5.2.7.1 Apparatus for holding tissue samples during CT scanning

The CT scans took approximately one hour from start to finish. Tissue had to be hydrated for that time so that it could be preserved for histological examination. In addition, the vessel holding the tissue had to be transparent to the x-rays from the CT scan so that the signal from the sample would not be distorted. We accomplished this by building a ‘boat’ from a Styrofoam plate, blocking the end with tape and creating watertight seals using a silicone epoxy (Kwik-Sil, World Precision Instruments, Inc., Sarasota, FL.). The tissue was placed in the ‘boat’ and held in place with Kwik-Sil (Figure 46). Next, the boat was flooded with PBS, submerging the tissue samples. The entire boat was then scanned. Up to 6 samples were scanned simultaneously; 3 on the bottom of the boat, then a layer of Styrofoam with 3 samples on top.
Figure 46 Apparatus for holding tissue samples during CT scanning. Tissue samples were held in place using a waterproof silicone epoxy (Kwik-Sil) at the bottom of a watertight boat constructed out of Styrofoam plates, tape and Kwik-Sil that was flooded with PBS prior to being scanned. The Kwik-Sil successfully held the tissue samples in place and did not damage them during the removal process. Blue sutures were used to mark the proximal aspect of each sample during excision.

5.2.8 Histology

Fixed nerves or nerves plus conduits were embedded in paraffin and sectioned (10 µm thick) for histological examination. The Mg pieces were readily sectioned and the Mg was visible within paraffin sections. However, upon further processing, the Mg either floated away or was corroded, so in the final sections, only cavities remained. Midpoint sections were stained with hematoxylin & eosin (H&E) or immunostained for the presence of neuronal axons (rabbit antibody to neurofilament protein, NF200, 1:500 dilution (Sigma, St. Louis, MO)), Schwann cells (rabbit antibody to the protein S100, 1:500 (Dako, Carpinterio, CA)) macrophages (mouse monoclonal ED1 antibody, specific for the cytokine CD68, 1:500 (Abcam, Cambridge, MA)), and for an intact nerve-blood barrier (mouse monoclonal antibodies to the glucose transporter-1 protein, GLUT1, 1:500 (Thermo, Fremont, CA)). In all conditions, cell nuclei were labeled by staining with 4’,6-diamidino-2-phenylindole (DAPI) (1:1000) (Sigma-Aldrich). Combinations of two primary antibodies plus DAPI were: ED-1/NF200 and GLUT1/S100 the secondary antibodies were anti-mouse Alexa 488 and anti-rabbit Alexa 594 (1:1000 dilution each) (Invitrogen, Grand Island, NY). Table 6 (reprinted from Chapter 4) outlines the target of each stain.
Table 6 Antibody Summary. Summary of history of chemical antibodies used including the target cell or structure for each antibody and a description of the physiological function of that structure (Reprint of Table 4).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>@S100</td>
<td>Schwann Cells</td>
<td>S100 proteins are small, acidic proteins of 10–12kDa found in many tissues, including Schwann cells and others derived from the neural crest, where they function as intracellular Ca$^{2+}$ signaling or buffering proteins$^{240}$.</td>
</tr>
<tr>
<td>@ED1</td>
<td>Macrophages</td>
<td>ED1 targets the rat homologue of human CD-68, a glycoprotein that binds to low-density lipoprotein, expressed by most tissue macrophages and by peripheral blood granulocytes$^{241}$.</td>
</tr>
<tr>
<td>@NF200</td>
<td>Axons</td>
<td>NF200 bind to the heaviest neurofilament, which is ~200-220 kDa in weight. The presence of this protein is a signal of mature nerve tissue.</td>
</tr>
<tr>
<td>@GLUT1</td>
<td>Glucose transporter 1</td>
<td>Glucose transporter 1 facilitates the transport of glucose across the plasma membranes of mammalian cells$^{242}$. It is heavily expressed in red blood cells and endothelial cells in barrier tissues e.g. the blood brain barrier in the CNS and endothelial cells and perineurium in the PNS.</td>
</tr>
<tr>
<td>@DAPI</td>
<td>Cell nuclei (non-specific)</td>
<td>DAPI is a fluorescent stain with excitation potential in the ultraviolet range that will strongly bind to adenine-thymine regions in DNA. DAPI is cell membrane permeable and will bind non-specifically to cell nuclei.</td>
</tr>
</tbody>
</table>

5.2.8.1 Paraffin embedding and sectioning

After fixation, nerve tissue and conduits were cut approximately in half with a scalpel before both halves were embedded in a single paraffin block (Figure 47). Histological sections, 10 $\mu$m thick, were then prepared. In order to obtain representative sections from all levels of the conduit and nerve, 15 sections were collected at a time (three per slide, for a total of 5 slides containing adjacent sections) (Figure 48)
and then 35 sections consecutive sections were discarded. Using this method, approximately 50 slides, each with three sections, were created for each nerve/conduit sample, with higher numbered sections containing samples both more proximal and more distal to the midpoint.

Figure 47 Nerve tissue/conduit embedding. Conduits were cut approximately in half before being embedded in paraffin. There were sections that were both proximal and distal to the midpoint of the conduit in each histological section.

Figure 48 Histologic sections on slides. Sections contained samples both proximal (‘P’) and distal (‘D’) to the cutting plane of the conduit (Left image). Three sections were systematically placed on each microscope slide (Right image) so that proximal and distal sections were readily identifiable.

5.2.8.2 Determining the midpoint of each conduit

We were interested in comparing histological sections of the midpoint of each conduit, which we defined as halfway between the proximal and distal stumps within the conduit. The midpoint was not necessarily at the plane at which the conduits were transected prior to being embedded in paraffin as a result of:
- **Accuracy of cutting the conduit in half after fixation.** One half of the conduit could be longer than the other as a result of imprecise manual transection.

- **Variations in paraffin embedding.** One conduit half could be embedded in the paraffin more deeply to the cutting plane than the other half i.e. the first several histologic sections may only contain tissue from the proximal or distal stump.

To account for these variables, we recorded 1) The slide at which each half of the conduit became visible in sectioning and 2) The slide at which each conduit ended during the sectioning process. This information, coupled with knowledge of the thickness of each section, the number of sections skipped in between sample collection and the number of sections per/coverslip, allowed us to quickly and dependably calculate the true midpoint of each conduit using custom coding in Excel.

5.2.9 **Microscopy**

Stained sections were viewed and photographed on an upright Zeiss Axioplan Imaging 2e fluorescence microscope with a Zeiss Axiocam digital camera.

5.2.10 **Digital Image Post Processing**

Grayscale images across the complete cross sections of the nerves were taken at 100x magnification, in each of three channels. These were combined into a complete composite and colored in Photoshop using the same methods outlined in Chapter 4.

5.2.10.1 **Evaluation of IHC staining**

Tissue organization and quality of histologic sections led to difficulties quantifying stain coverage, as described in Chapter 4. This led us to attempt assessing the degree of stain coverage through qualitative assessment. Composite micrographs of midpoint sections from each sample were examined for the presence of NF200+ and S100+ nerve fascicles and a GLUT1+ sheath around fascicles to measure the health of regenerated tissue.
5.2.11 Gastrocnemius cross-sectional area calculation

Immediately after sacrifice in the sciatic nerve defect experiments, the gastrocnemius muscles were excised from the surgical and contralateral sides and placed in 4% paraformaldehyde in PBS for 24 hours. Muscles were then removed from solution and transected through the thickest point. Digital photographs of the cross-section of each muscle were then used to calculate the cross-sectional area of each muscle. The ratio of the cross-sectional area of the surgical side divided by the contralateral side was calculated for each animal.

(See 12 for a depiction of the musculature of the rat leg)

Figure 49 Leg anatomy of a rat. The sciatic nerve enervates the gastrocnemius and the soleus. As such, those muscle groups atrophy significantly when the sciatic nerve is cut. We hypothesized that functional recovery could be assessed by comparing the relative size of the muscles enervated by the sciatic nerve on the surgery side to those on the contralateral side i.e. that ratio would be larger in groups with better functional recovery. Image from 12.
Figure 50 Muscle size calculation storyboard. The gastrocnemius and soleus on both the surgery side (A – right) and contralateral (A – left) sides were excised after immediately after sacrifice. After fixation in paraformaldehyde, the muscles were transected at their thickest point and photographed (B). Digital photographs were imported into Matlab to calculate the area of each cross section (C). The area of the ‘surgical side’ (C – top) was divided by that of the ‘contralateral side’ (C – bottom) to get a ratio. These ratios were then used to compare the functional recovery of different groups.

5.2.12 Statistical Analysis

Gastrocnemius cross-sectional area and behavioral data were compared for each of the four conditions tested using 1-way ANOVA with a Student Newman-Keuls post hoc test. Alpha = 0.05 was used to denote significance.

5.3 Results

5.3.1 Tissue reactions to the Mg metal are minimal

An advantage of using microfilaments made out of Mg, a relatively soft metal, was that they were readily sectioned and therefore allowed visualization of virtually all cells surrounding the implant. The immunostaining for ED-1 suggests that the cells immediately adjacent to the Mg were macrophages, as is expected of a foreign body response (Figure 51). These were, at most, only a few layers thick, and appeared to completely surround the Mg as a capsule. At six weeks, inside the regenerating nerve, there appeared to be thinner layers of ED-1+ cells than at 1 week when the Mg microfilament was placed in the
epineurium as described in the previous chapter. This suggests that, as might be expected, either time or placement (regenerating nerve vs connective tissue) had some impact on the macrophage reaction. Giant cells were not observed in the cells adjacent to the Mg, although they were abundantly visible in the degrading conduit layer, which exhibited a more significant foreign body response (Figure 52).

**Figure 51 Cells surrounding Mg filament are macrophages.** IHC staining in proximity to a Mg filament surrounded by regenerating nerve tissue in the center of a PCL conduit 6 weeks after placement in a sciatic nerve defect. The presence of ED1+ cells (green) is concentrated immediately adjacent to the Mg filament. The size of the cavity indicates minimal Mg resorption at 6 weeks. A yellow star marks the cavity left by the Mg filament (which was displaced in sectioning). Red = NF200, green = ED1, blue = DAPI. Scale bar = 250 µm.

**Figure 52 Inflammation at the tissue/conduit interface.** Yellow and black arrowheads indicate the border between regenerating nerve tissue (left side of each image) and conduit material (right side of each image) at the midpoint of a (Mg/Kr)
conduit after 6 weeks in vivo. ED1+ cells (A, green) and other cell types densely infiltrated the PCL conduit at 6 weeks (B) shows H&E staining in an adjacent section. Arrows point to possible giant cells, indicated by clusters of nuclei. Red = NF200, green = ED1, blue = DAPI. Scale bar = 50 µm.

5.3.2 Gradient effects due to Mg corrosion products were not detected

The area coverage of each stain as a function of distance from the Mg filament was calculated at the midpoint of the sciatic nerve or conduit for each animal with a Mg filament (Figure 53). Histology of the regenerated tissue at the midpoint of the conduit revealed a densely packed core or cells, often consisting of S100+ bundles, in the center of the regenerated tissue surrounded by looser connective tissue. Analysis of DAPI coverage as a function of distance from the Mg filament clearly identified the dense core of regenerated tissue. Analysis of ED1 coverage (an indicator of inflammation) did not reveal any gradient effects. The presence of keratin hydrogel did not seem to have any effect on relative ED1 staining or any gradient effects compared to conduits flushed with saline.
Figure 53 Representative gradient effects due to Mg filament corrosion. Red circles in ((A), +Mg/Sa condition shown) are 500 and 1000 µm away from the center of the cavity left by the Mg filament (yellow star). Blue = DAPI+, green = ED1+, red = NF200+, scale bar = 250 µm. Regenerating tissue tended to nucleate around the Mg filament within the lumen of the conduit at
the midpoint of the nerve gap, presenting with a zone of dense cellular tissue around the Mg and fewer cells in the periphery. This gradient is clearly depicted in the graphs of ‘Proportion of nerve area stained’ vs. ‘Distance from the center of the Mg filament’ for the DAPI (B – blue, E) and NF200 (B – red, C) stains. The presence of ED1 cells was consistent throughout the regenerated nerve tissue with exception of a very thin layer surrounding the Mg filament. i.e. no gradient effects were observed. This trend held true for conduits filled with either saline or keratin. S100+ staining tended to nucleate around Mg filaments as well, similar to DAPI staining. Tissue morphology was similar in conduits without Mg as well, with a central core of dense cellular tissue and looser tissue toward the periphery. Conduit material and defects in the histological section (A) were removed in Photoshop prior to analysis.

5.3.3 Rate of Mg resorption was dependent on conduit filler

MicroCT provided excellent visualization of Mg mass loss at both 1 and 6 weeks in vivo (Figure 54). Mg filaments were intact after 1 week in conduits filled with saline or keratin (one shown in Fig 54C). At 6 weeks, Mg filaments in the saline group (n=3, although only 2 were images, as shown in Figure 54A) were largely intact with either very small or no gaps, although they appeared to have lost some volume. All of the Mg filaments in conduits filled with keratin hydrogel (n=8, Figure 54B) showed significantly more resorption, with at least one significant defect in the middle section of each filament. Despite these defects, the remaining pieces of the filament remained linearly aligned in all samples.
Figure 54 MicroCT. MicroCT visualization of an Mg filament and PCL conduit, in situ, after 6 weeks in vivo. Filaments in conduits filled with saline (A – only 2 of 3 conduits were scanned) resorbed more slowly than those in conduits filled with keratin (B). Imaging at 1 week after surgery revealed negligible corrosion in one conduit filled with saline (not shown) and a second filled with keratin (C). Micro CT was also used to confirm the central location of the filament within select conduits at 1 week (Mg filament in a conduit with keratin filler shown, (C)). Scale bars = 1 mm.

5.3.4 Regenerating nerve tissue shows normal organization in the presence of whole and resorbed Mg metal

As stated above, regenerating nerve tissue crossed the 6mm defect in all conditions tested and thoroughly surrounded the Mg filament when present (Figure 55). When the organization of the regenerating nerve
was examined at a higher magnification, it could be seen that normal regenerating nerve tissue mini-fascicles were present. These were organized like normal tissue in that they contained a core of S100+ bundles and were encapsulated by a GLUT1+ sheath of perineurial fibroblasts and these were surrounded by layers of connective tissue equivalent to the epineurium, including adjacent to the Mg filament (Figure 57).

Figure 55 Regenerating tissues-Low power views. H&E midpoint composites of the four conditions tested in the sciatic nerve defect model at 6 weeks. Black stars mark the cavity left by the Mg (B,D). All conditions showed normal tissue organization at the midpoint. The Mg filament was centrally located within the conduit and thoroughly surrounded by regenerating tissue in every animal that received a conduit with Mg (B,D). Scale bar = 250 µm.
Figure 56 IHC composite images. IHC composites of S100 (red), GLUT1 (green) and DAPI (blue) at the midpoint of (-Mg/Sa) (A) and (+Mg/Sa) (B) conditions. A yellow star marks the cavity left by the Mg filament in (B). S100 positive regenerating tissue was centrally located in both empty conduit controls (A) and Mg-containing conduits (B). GLUT1+ sheaths (shown in higher magnification in Figure 57) were present in both conditions, including adjacent to the Mg filament. Conduit material appeared yellow as a result of non-specific fluorescence in both the red (S100) and green (GLUT1) channels being present in approximately equal magnitudes. Scale bars = 1mm.
Figure 57 Regenerating tissues – IHC. H&E and IHC staining of midpoint composites of the central regenerating nerve bundle in each of the four conditions tested in the sciatic nerve defect model at 6 weeks. Arrows point to regenerated nerve fascicles. Arrowheads point to the outline of the Mg cavity in (D-F) and (J-L). S100 positive bundles of regenerating nerve tissue surrounded by a Glut1 positive sheath were present in all conditions (indicated by yellow arrows) though were not as prevalent in conduits containing keratin. Bundles were observed adjacent to Mg wire in the center of regenerating tissue. There were comparable numbers of ED1+ macrophages in all conditions and they formed only a thin ring, 2-3 cell layers thick surrounding the Mg filament. NF200 positive staining was also seen in regenerating nerve bundles in each condition tested though was not as prevalent in conduits containing keratin. Scale bar = 100 µm.
5.3.5 S100+/NF200+/GLUT1+ fascicles were less prevalent in conduits filled with keratin hydrogel

Although mini-fascicles could be identified by the presence of S100/NF200+ fibers surrounded by a GLUT1+ stained perineurium in all conditions, the occurrence of these mini-fascicles was not observed in all animals at the midpoint sections. Table 7 shows the percentage of the animals in each condition that had mini-fascicles. In addition, another observation (not quantified) was that the fascicles or bundles of Schwann cells without GLUT1 surrounds were generally smaller and more diffuse in keratin-filled compared to saline-filled conduits (Figure 57). As can be seen in Table 7, the presence of Mg did not appear to affect the formation of bundles in either condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>NF200+ present in bundles (% of animals)</th>
<th>S100+/Glut1+ bundles present (% of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-Mg/Sa)</td>
<td>100% (3 of 3)</td>
<td>100% (3 of 3)</td>
</tr>
<tr>
<td>(+Mg/Sa)</td>
<td>100% (3 of 3)</td>
<td>100% (3 of 3)</td>
</tr>
<tr>
<td>(-Mg/Kr)</td>
<td>25% (2 of 8)</td>
<td>50% (4 of 8)</td>
</tr>
<tr>
<td>(+Mg/Kr)</td>
<td>38% (3 of 8)</td>
<td>38% (3 of 8)</td>
</tr>
</tbody>
</table>

5.3.6 Regenerating tissue was present adjacent to actively resorbing, or recently resorbed Mg

In several of the animals with conduits containing Mg and keratin, there were gaps at the midline in the Mg filaments (representative sample shown in Figure 58). Histologic sections were chosen from the regions proximal to the gaps, in the gaps and distal to the gaps and stained. Nerve fascicles were present in the distal regions; the process of Mg resorption did not destroy the newly regenerating nerve fascicles. The midpoint of the gaps was identified by the lack of a Mg implant cavity, confirming that the Mg was
gone. Of significant interest was that the tissue in the central position where the Mg had been (based on the other sections) (Figure 58, E-G). Here, in the center where the Mg filament had been originally, the space was filled with connective tissue without nerve bundles. This tissue did not contain S100+ Schwann cells. In fact, the nerve bundles remained in roughly the same positions that they had been proximal to this tissue. Examination of overall macrophage density showed no change in comparison to sections both proximal and distal to the gap in Mg.

In the proximal and distal areas, it was obvious that the Mg was resorbing, because the cavity was smaller and the edges were often more irregular. In these regions, there was evidence of a mild inflammation, according to our neuropathologist (Dr. Matthew Hagen, UC Pathology). The blood vessels were enlarged in these regions, one sign of an inflammation, and there was some evidence of greater ED1+ macrophages, although this was not quantified, and some evidence of other extravascular blood elements (white blood cells, plasma cells) being more common in these regions. This inflammation phenomenon was reduced in the center of gaps, where there was no evidence of a Mg capsule. In particular, blood vessels were of more normal diameter. This suggests that once resorption of the Mg is complete, the inflammatory status of the tissue resolves back to a more normal appearance.
Figure 58 Tissue filled the gap left by resorbing Mg. Representative H&E and IHC staining of regenerating nerve tissue in a gap left by resorption of the Mg filament in the Mg/Keratin condition at 6 weeks. The yellow dashed lines in (A), shown on a microCT image of a Mg/Keratin filament, represent where ‘Proximal’, ‘Gap’ and ‘Distal’ sections were taken. Arrows point to regenerated nerve minifascicles. Arrowheads point to the outline of the Mg cavity in (B-D) and (H-J). Fibrous tissue readily infiltrated the void left by the resorption of the Mg filament (E-G). Yellow areas in F and G are the result of blood products in the tissue. Red-orange areas surrounding (or in) the Mg cavity in C are likely the result of non-specific staining of Mg corrosion byproducts. There was no evidence of hydrogen gas evolution or toxicity from the build-up of corrosion byproducts. Scale bar = 100 µm.
5.3.7 *Animals receiving (\(+Mg/Sa\)) and (\(-Mg/Kr\)) conduits showed improved functional recovery compared to controls (\(-Mg/Sa\)) when compared by gastrocnemius cross-sectional area.*

Measuring the size of the gastrocnemius muscles, which are innervated by the sciatic nerve, provided insight into functional recovery after nerve repair. All injured muscles showed significant atrophy relative to the muscles from the intact, contralateral leg. As shown in Figure 50, addition of Mg microfilaments to saline-filled conduits resulted in increased size of recovering muscles when compared to the contralateral side. A comparable increase in muscle size was also seen with the keratin filler alone, which was expected as keratin-filled conduits have been shown to increase nerve regeneration\(^{33, 34}\). However, addition of Mg to the keratin filled conduits did not significantly alter muscle size compared to keratin-filled alone, which suggests no synergism of these two treatments, perhaps due to the increased Mg resorption. The increased muscle size suggests that the presence of the Mg microfilaments improved growth of nerves sufficiently to allow them to reach muscles and start stimulating re-growth.

![Figure 59 Muscle Analysis](image)

**Figure 59 Muscle Analysis.** Analysis of muscle atrophy after nerve repair showed that repair with conduits containing Mg and saline (\(+Mg/Sa\)) and without Mg but with keratin (\(-Mg/Kr\)) showed significantly larger recovering muscle area compared to empty conduits containing saline (negative control) (\(-Mg/Sa\)). Cross sectional areas through the bellies of the injured
gastrocnemius muscles were expressed as a percent of the contralateral, uninjured muscle area using 1-way ANOVA with Student-Newman-Keuls post hoc test, p<0.05 (*), SigmaStat program.

5.3.8 Behavioral analyses were inconclusive at 6 weeks.

None of the behavioral analyses undertaken were able to effectively resolve differences between groups. Differences in missteps between conditions in the grid walk test (Figures 60-61) were inconsistent at best and there was nearly always no difference between the trials before and immediately after surgery. Two-way ANOVA was used in an attempt to assign variability within experimental groups to the presence of ‘Mg’ or ‘Keratin’ (Table 8). These results were inconsistent as well with neither variable, nor their interaction, being consistently significant. Three-way ANOVA was used to examine the significance of ‘time after surgery’ in addition to the presence of ‘Mg’ and ‘Keratin’ (Table 9). By this analysis, ‘time after surgery’ was the only significant factor. Data from the Cylinder test and Footprint analyses revealed no difference between experimental conditions (data not shown).
Figure 60 Comparing ‘slips/step’ by condition at each time point. Rats were made to walk across a wire grid with spacing such that their limbs could slip through if they misstepped. Five trials were recorded weekly for each animal. The average number of slips/step for the forelimbs, hindlimbs and heels were compared by condition at each time point using ANOVA. A lower number of slips/step was taken to represent better function of the leg musculature. Although there were occasional differences between experimental conditions, differences were inconsistent when compared by consecutive timepoint e.g. The number of Forelimb slips/step (A) at 28 days is highest in the (Mg/Sa) and then, at 35 days, the (Mg/Sa) condition shows the lowest number of slips/step.
Figure 61 Comparing ‘slips/step’ by timepoint for each condition. This figure contains the same data as in Figure 60. No conditions showed improvement (defined as a trend of fewer slips/step over time). When comparing data by condition, the only detectable differences were from the pre-surgery control. Many conditions showed no difference from the control at any timepoint, including 1 week after surgery. Asterisks mark differences by 1-way ANOVA with Student-Newman-Keuls post hoc test, p<0.05. Error bars = standard deviation.
Table 8 P-values of two-way ANOVA with interactions with ‘slips/step’ as the response variable and factors ‘Mg’ and ‘Filler’. The presence of ‘Mg’ (-Mg or +Mg), ‘filler’ (saline or keratin) or their interaction could not consistently predict differences in missteps from the pre-surgery control for the grid walk test.

<table>
<thead>
<tr>
<th></th>
<th>Forelimb/step</th>
<th>Hindlimb/step</th>
<th>Heel slip/step</th>
<th>(H+2+H L)/step</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-pretest</td>
<td>7</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0076</td>
</tr>
<tr>
<td>Filler</td>
<td>-</td>
<td>0.0359</td>
<td>-</td>
<td>0.0001</td>
</tr>
<tr>
<td>Mg x Filler</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 9 P-values of three-way ANOVA with interactions with ‘slips/step’ as the response variable and factors ‘Mg’, ‘Filler’ and ‘timepoint’. The only significant factor in predicting foot faults by three-way ANOVA was timepoint for the measures of ‘Hindlimb Slips/Step’ and ‘Heel Slips/Step’ (highlighted).
5.4  Discussion

5.4.1  There was a minimal inflammatory response to Mg implantation.

Consistent with current literature examining other tissues \(^{195}\), there was a minimal inflammatory response to Mg metal in PN tissue. There were relatively few ED1+ macrophages in surrounding regenerating tissues and a general lack of giant cells present in H&E sections (Figures 55B,D and 57D,J) when compared to no-Mg controls. Within the regenerating PN tissue, regenerating nerve fascicles were present, consistent with normal regeneration within a conduit at this time point \(^{245,248}\). These showed a re-organizing perineurial layer (GLUT1+), suggesting establishment of normal nerve organization. The fascicles were found immediately outside the thin layer of macrophages covering the Mg microfilament, which suggests no interference of the Mg with formation of nerve fascicles.

5.4.2  Mg resorption occurred on a time scale that is appropriate for nerve regeneration.

One of the principle challenges in designing a metallic bioresorbable device is controlling the rate of corrosion. This is especially true for magnesium, which is highly reactive within an aqueous environment. Corrosion is dependent on several factors including geometry of the implant rate of vascular flow, local pH and mechanical stress on the implant, all of which can vary by tissue type and location \(^{195}\). Timing the Mg corrosion/resorption rate to coincide with nerve regeneration will be crucial to the success of a Mg-based scaffolding approach.

In normal regenerating peripheral nerves, a protein-rich fluid containing neurotrophic factors pools in the gap between the proximal and distal stumps in the day following injury. Over the next week, a fibrin scaffold begins to form, followed by cell migration between one and two weeks after injury and formation of axonal cables during the second and third week after injury \(^{245}\). It takes regenerating axons approximately 3-4 weeks to cross a 10mm gap \(^{137}\), therefore, the Mg filaments in this experiment would have remained intact long enough to provide a physical support for these processes. But as resorption was beginning even in conduits flushed with saline, these specific filaments might not be adequate for a much
longer gap. Evidence that supports the idea that the Mg filaments may have assisted in supporting regeneration is given by the fact that all Mg filaments were completely surrounded by regenerating tissue.

5.4.3 Mg resorption rate was dependent on the selection of conduit filler.

Keratin hydrogel has been shown to enhance regeneration across long nerve gaps in rabbits. A major advantage of using keratin hydrogel as a conduit filler is that there are no known native keratin specific proteases in mammals. This ensures that after the hydrogel sets within the conduit, it is passively resorbed into the body over weeks as the nerve tissue regenerates, and does not elicit an immune response. Our microCT data show that Mg resorption was more rapid with keratin than with saline as a filler. A hypothesis for this enhanced resorption is that the keratin rendered the local environment within the conduit more acidic than with saline, due to an abundance of acidic residues in the hydrogel. This acidic microenvironment could have accelerated the electrochemical corrosion reaction of the Mg while insulated in the conduit from physiological buffers.

In terms of resorption, unlike our previous study at 1-week post-implantation, at 6 weeks, we saw significant resorption. Some suggestion of needle thinning was observed at 6 weeks, even when just using saline flushed conduits, which remained intact for the full 6 week time period, in contrast to the greater resorption when using the keratin hydrogel filler. Therefore, to address the issue of longer gaps, of a more clinically relevant gap size (1.5 cm in the rat, comparable to 2.5 cm in the human), corrosion control techniques such as Mg alloy selection, surface treatment or choice of conduit filler will need to be considered to ensure that the Mg filament remains intact long enough to effectively enhance regeneration by contact guidance.
5.4.4  **S100+/NF200+/GLUT1+ fascicles were less prevalent at the midpoint in conduits filled with keratin hydrogel**

We were surprised that there were less S100+/NF200+/GLUT1+ fascicles in conduits with keratin than those with Mg as keratin hydrogel has been shown to improve the quality of axonal regeneration in multiple animal models\(^{33,34}\). Conduits without these markers still contained a robust mass of regenerating tissue. However, morphologically, they generally did not contain a distinct, dense central core and less dense periphery. Some of the conduits in the keratin conditions appeared ‘crushed’ to variable degrees, as if the conduit had mechanically failed and had been compressed and therefore collapsed against the animals’ leg (between muscle groups) while in vivo. This might have impacted the quality of tissue able to regenerate. Despite these issues, the size and quantity of S100+/NF200+/GLUT1+ tissue was also less in uncrushed keratin-filled conduits, compared to control and Mg/saline conduits.

5.4.5  **Mg-containing conduits improved gastrocnemius size at 6 weeks**

The gastrocnemius cross-sectional data show preliminary evidence that a Mg-containing conduit may perform better than a conduit without Mg. We hypothesize that the increase in muscle size (Figure 59) was due to greater stimulation of the regeneration of nerves in the conditions (+Mg/Sa) and (-Mg/Sa), and that this resulted in faster reinnervation of the gastrocnemius. In the (+Mg/Sa) group, we hope that this was due to the immediate access of regenerating tissue to a scaffold, causing the muscle to begin to recover more quickly than in the (-Mg/Sa) control group. This would be in concordance with the fact that we see excellent incorporation of the Mg in the tissue and because of the presence of nerve fascicles in all tissues. However, an alternative hypothesis is that there was decreased initial denervation atrophy as a result of the Mg filament forming a conductive bridge between the proximal and distal stumps, perhaps serving to stimulate the denervated muscle in the absence of nerves. This is less likely, but it is known that electrically conductive nerve scaffolds and/or internal components are advantageous to nerve repair\(^{249,250}\). Future experiments will be needed to differentiate between these alternatives.
5.4.6 Behavioral analyses were not effective at 6 weeks

A number of modalities exist for characterizing the tissue inside a conduit e.g. morphometric analyses such as the number of axons, density of axons in regenerating tissue (N-ratio) or axon diameter compared to myelin thickness (G-ratio), though there is not currently a ‘gold standard’ for assessing functional recovery $^{194}$. However, histological examination (binary presence of regenerated axons in distal segments of regenerating nerves) and muscle size comparisons have been shown to reliably resolve differences between groups between empty conduits and autografts $^{194}$. Other behavioral analysis, such as footprint analysis are not always as effective $^{194}$. Another issue is that the rat has muscles in the leg that support locomotion and are not affected by our lesion, making gait-type analyses less informative. It is also not surprising that our behavioral analyses did not show clear differences between our experimental conditions, as it may take longer than 6 weeks for functional regenerating nerve tissue to cross a gap and innervate the appropriate distal targets $^{119}$.

5.4.7 Mg is a viable scaffolding biomaterial

Conduits containing Mg filaments filled with saline or keratin hydrogel performed as well as the same conduits without Mg wire in terms of the appearance and organization of regenerating nerve tissues at the midpoint of the regenerating nerve bundle. Well-defined minifascicles of S100+ axons were observed in every condition, including adjacent to the Mg filament, in the center of the regenerating tissue as have been reported in the literature $^{248}$. The presence of Mg did not have any histologically measurable negative effects, such as a decrease in the presence of S100+ or NF200+ bundles (although this was not quantified). Regenerating connective tissue also migrated to fill the gaps where the Mg filament had resorbed. No evidence was detected, in any sections, of gas cavities caused by hydrogen evolution during the resorption process, as has been seen in other tissues $^{29}$. This suggests that the resorption rate is slow enough, or minor enough due to the size of the Mg, that the local tissue can accommodate any hydrogen gas produced $^{251}$. These properties justify further investigation for using Mg filaments in conduits as a
very promising and novel method to enhance nerve regeneration by providing supportive contact
guidance for regenerating nerves.
6.0 Discussion and Conclusions

The long-term goal of our research is to better understand how ionic and metallic Mg can be used to therapeutically interact with CNS and PNS tissues. The studies outlined in this document represent the initial steps toward this goal. The central hypotheses of this dissertation were 1) neural cells (NSCs) would show altered biological responses, including beneficial effects, to moderately elevated Mg\(^{2+}\) concentrations in vitro and 2) metallic Mg, placed in the lumen of a nerve conduit, would improve nerve regeneration by serving as a scaffold that would safely resorb into the body over time, after mechanical support is no longer required by regenerating tissue. The studies discussed in Chapter 3 validate this first hypothesis through the investigation of Aim 1, described in Chapter 1. The studies discussed in Chapters 4 and 5 support this second hypothesis through the investigation of Aim 2, also described in Chapter 1. In this Chapter, the results of these studies are summarized and discussed in addition to suggested future strategies for using Mg-based therapeutics for nervous system repair.

**Aim 1: Test the hypothesis that neural cells will show altered biological responses, including beneficial effects, to moderately elevated Mg\(^{2+}\) concentrations in vitro.**

The goal of Aim 1 was to characterize an in vitro system to study the response of NSCs to Mg\(^{2+}\) supplementation. This Aim tested Hypothesis 1) Numbers of NSCs will increase in response to moderate increases in local Mg\(^{2+}\) concentration and Hypothesis 2) Normal NSC behavior, as measured by the ratio of cells that differentiate into neurons and the elaboration of neurites, will not be harmed and may be increased by moderate increases in local Mg\(^{2+}\) concentration.

Our results suggest that Mg\(^{2+}\) plays a role in the proliferation of NSCs and that elevating external Mg\(^{2+}\) to levels that are possible to achieve by external application of Mg\(^{2+}\) in vivo, can stimulate an increase in proliferation of NSCs. However, the exact conditions under which this finding might be translated to an in vivo situation remain to be determined. In the process, we also showed that a popular colorimetric plate
reader assay, the Alamar Blue assay, was inaccurate in the presence of elevated Mg\(^{2+}\). In addition, acute exposure to higher levels of Mg\(^{2+}\) may have a toxic affect, again dependent on the cell density and substrate. In other words, our results contribute to the larger body of works that show that cellular response to external Mg\(^{2+}\) is highly sensitive to the microenvironment surrounding the affected cells. While the responses to Mg were slightly variable depending on substrate and cell plating density, we still saw some positive responses in a wide variety of situations, i.e. different times in culture, cell plating densities or length of Mg\(^{2+}\) exposure beyond 24 hours. Thus, there is support for a consistent positive effect of Mg\(^{2+}\) supplementation at ranges of ~3-10 mM and evidence for some toxicity under certain conditions at 20 mM or higher. In addition, the processes that drive NSCs to differentiate into neurons appeared unaffected by Mg\(^{2+}\) increase.

The ultimate goal of the research described in Chapter 3 is to elucidate the mechanisms behind these potentially beneficial effects of Mg\(^{2+}\) supplementation so that more effective strategies for management of CNS injury can be developed. With regard to an in vivo application, these results suggest that that increased external Mg\(^{2+}\) in CNS tissue could stimulate expansion of the NSC population or even other CNS cell types and/or affect the neurite outgrowth of these cells. Given these baseline data, future developments could explore aspects such as the Mg\(^{2+}\) receptor molecules that might be involved, since there are over 10 possible molecules that interact with Mg\(^{2+}\), and then the signal transduction pathways and the subsequent processes that result in increased cell numbers.

The results of the studies described in Aim 1 are significant because they provide valuable information regarding the baseline behavior of CNS neural cell type in culture exposed to elevated levels of Mg\(^{2+}\) and also establish a culture system for further analysis.
Aim 2: Design and begin characterization of a novel use of Mg metal for repair of traumatized peripheral nerves.

The goal of Aim 2 was to characterize a novel nerve conduit system in order to evaluate Mg metal as a candidate scaffold for peripheral nerve tissue regeneration. In the study discussed in Chapter 4, we sought to test Hypothesis 3) Mg filaments will be biocompatible when implanted immediately adjacent to healthy and crushed peripheral nerve tissue and we started initial testing of Hypothesis 4) Untreated Mg filaments will resorb at a rate appropriate for nerve regeneration. The results from this study confirmed Hypothesis 3 and showed initial confirmation of Hypothesis 4. Inflammation, assessed by histology, was almost non-existent in nerve tissue with an implanted Mg filament outside of a 2 or 3 cell-layer thick ED1+ capsule surrounding the filament. After 1 week in vivo, the implanted Mg filament showed minimal signs of corrosion, determined by measuring the diameter of the capsule left by the Mg in histologic sections, and by showing that there were no obvious or quantifiable gradients of cell reactions occurring in adjacent tissues. Given the fact that Mg filaments of the same diameter (~150 µm), corroded completely in culture media within 24 hours, our data are consistent with observations in the literature that the rate of Mg corrosion in vivo can be orders of magnitude slower than that observed in vitro. We also determined, through a random sampling study, that IHC stain coverage in histologic samples of nerve tissue can be reliably estimated by examining a relatively small proportion of the total nerve area. This finding will be useful in resolving differences in nerve tissue between experimental conditions with a larger n in future experiments.

The results from Chapter 4, that a filament of Mg appeared biocompatible with nerve tissue and showed little signs of resorption after 1 week in vivo, encouraged us to attempt using a Mg filament as a novel tissue scaffold for regenerating peripheral nerve tissue within a nerve conduit. In the study discussed in Chapter 5, we further tested Hypothesis 4) Untreated Mg filaments will resorb at a rate appropriate for nerve regeneration, and also tested Hypothesis 5) Because the Mg filaments will remain intact and will provide the appropriate physical support (a scaffold), a Mg-containing nerve conduit will support
regeneration of PN tissues across a short nerve injury gap, and this repair will be as good as or better than
the regeneration through an empty conduit, Hypothesis 6) When used inside a nerve conduit scaffold, a
Mg filament will be biocompatible with regenerating nerve tissue, and Hypothesis 7) Keratin hydrogel
filler in a nerve conduit will enhance nerve regeneration above that seen with a saline-filled conduit, and
when combined with a centrally located Mg filament, would synergistically improve nerve regeneration.

The results from this study confirmed Hypotheses 4, 5 and 6 and repudiated Hypothesis 7. Mg filaments
placed in nerve conduits were still largely intact after 6 weeks in vivo, confirming Hypothesis 4. After 6
weeks, filaments were surrounded by healthy regenerating nerve tissue and were comparable by
histologic and muscle weight analyses to an empty conduit, confirming Hypothesis 5. The regenerated
tissue surrounding each Mg filament showed minimal signs of inflammation as assessed by histology,
confirming Hypothesis 6. Conduits filled with keratin hydrogel did not perform as well in some measures
of nerve fascicle development as those filled with saline, although with keratin hydrogel alone, functional
recovery was better, which is an oddly contradictory finding. We repudiated Hypothesis 7, that there
would be synergy between the keratin hydrogel and Mg filaments. Instead, possibly due to the increased
corrosion in the presence of the hydrogel, the performance of the combined treatment was less on
functional tests.

To investigate whether a Mg filament will improve nerve regeneration by enhanced contact guidance, it
will be necessary to test this conduit in a longer gap size (1.5 cm in the rat) and over a longer time frame
with analysis that includes functional testing of the regenerated tissue e.g. muscle weights,
electrophysiology. It will also be necessary to monitor the corrosion of the Mg filament over longer time
periods. We predict that strategies for controlling the rate of corrosion will need to be implemented as it
will be important for the Mg filament to stay intact long enough for regenerating tissue to bridge a longer
defect. Current experiments are underway in the Pixley lab to address these issues.
The results of the studies describe in Aim 2 are significant because the resorption rate of Mg metal in nervous tissue and the effects of Mg metal/resorption on PN tissue have not been previously described in detail. These results also suggest that Mg may be useful in supporting the regeneration of nerve tissues when used as a scaffold in combination with previously established nerve conduits. In broader terms, this research provides further evidence that Mg is a safe biomaterial, expanding the scope of safe Mg/tissue interactions to include healthy, injured and regenerating PN tissue.
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