

COMBINATIONAL TREATMENT APPROACH FOR TRAUMATIC
SPINAL CORD INJURY

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Medical Neuroscience Program,
Indiana University

December 2016

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate this dissertation to my parents, Gloria and Gary Walker, my sister, Shelley Walker, and to my adopted grandparents, Grandpa Pat Kuhns, and in loving memory of Grandma Alice Kuhns.

ACKNOWLEDGEMENTS

I would like to thank my dissertation advisor, Dr. Xiao-Ming Xu, for training me to become an independent scientist, to think critically, and for guiding me through my dissertation research. I would like to thank all current and former Dr. Xu lab, Dr. Xiaoming Jin lab, and Dr. Jinhui Chen lab members who have been part of my journey. I also appreciate the support and guidance of Dr. Yi Ping Zhang and Dr. Christopher Shields, from the University of Louisville. I am gracious for the generous support from the *Wings for Life*, Spinal Cord Injury Foundation, Austria.

I would like to thank the members of my dissertation committee for their invaluable guidance, suggestions, and encouragement: Dr. Ellen Chernoff, Dr. Rajesh Khanna, Dr. Theodore Cummins, and Dr. Wei-Hua Lee. I would like to thank Dr. Andy Hudmon and his lab members for their encouragement, support, and brainstorming: Aarti Chawla, Derek Kaiser, and Ross Nelson.

I would also like to extend my appreciation to wonderful friends who have encouraged me so greatly during my graduate studies: Joyce Schneider, Sandi Thomas, Nicole Ashpole and Matt McBride, Joel and Alyssa Brittain-Duncan, Sherry Pittman, Michael and Danielle Kalwat, Hoa Nguyen and Justin Babcock, Natalia Ryan, Ben and Jess Thirlby, Natalie and Josh Case, Jenny LeVora, Nipun Chopra, Sarah Wilson, Jessica Pellman, and Sreeparna Majumdar.

My greatest appreciation goes to my family, for their encouragement, love, support, and unflinching guidance, during my journey through graduate school and throughout life: Gary and Gloria Walker, Shelley Walker, and Grandpa Pat Kuhns.

COMBINATION TREATMENT APPROACH FOR TRAUMATIC SPINAL CORD INJURY

Spinal cord injury (SCI) is devastating and debilitating, and currently no effective treatments exist. Approximately, 12,000 new cases of SCI occur annually in the United States alone. The central nervous system has very low repair capability after injury, due to the toxic environment in the injured tissue. After spinal cord trauma, ruptured blood vessels cause neighboring cells and tissues to be deprived of oxygen and nutrients, and result in the accumulation of carbon dioxide and waste. New blood vessels form spontaneously after SCI, but then retract as the injured tissue forms a cavity. Thus, the newly formed vasculature likely retracts because it lacks a structural support matrix to extend across the lesion. Currently, in the field of spinal cord injury, combinational treatment approaches appear to hold the greatest therapeutic potential. Therefore, the aim of these studies was to transplant a novel, non-immunogenic, bioengineered hydrogel, into the injured spinal cord to serve as both a structural scaffold (for blood vessels, axons, and astrocytic processes), as well as a functional matrix with a time-controlled release of growth factors (Vascular endothelial growth factor, VEGF; Glial cell line-derived neurotrophic factor, GDNF). The benefit of this hydrogel is that it remains liquid at cooler temperatures, gels to conform to the space surrounding it at body temperature, and was designed to have a similar tensile strength as spinal cord tissue. This is

advantageous due to the non-uniformity of lesion cavities following contusive spinal cord injury. Hydrogel alone and combinational treatment groups significantly improved several measures of functional recovery and showed modest histological improvements, yet did not provoke any increased sensitivity to a thermal stimulus. Collectively, these findings suggest that with further investigation, hydrogel along with a combination of growth factors might be a useful therapeutic approach for repairing the injured spinal cord.

Ellen A. G. Chernoff, Ph.D. – Chair

TABLE OF CONTENTS

LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
INTRODUCTION	1
History of Spinal Cord Injury	1
Spinal Cord Injury Background	3
PART I: Vascular Endothelial Growth Factor and Spinal Cord Injury	12
VEGF Background	12
Discovery of VEGF and its Receptors	12
VEGF Isoforms and Co-receptors	14
Localization of VEGF and its Receptors	22
Synergistic Activation of VEGF Receptors	23
VEGF Levels and VEGF Receptor Expression after SCI	25
Studies Employing VEGF for SCI Repair	25
VEGF Combinational Therapies for SCI Repair	28
VEGF Neuroprotection in Neurodegenerative Disease Models	29
VEGF Signaling for Neuroprotection in SCI	30
Conclusion	31
PART II: Glial cell line-derived Neurotrophic Factor and Spinal Cord Injury	35
SCI Background and Need for Therapies	35
Discovery of GDNF Family Ligands and Receptors	36
Localization of GDNF and its Receptors	37
GDNF Promotes Cell Survival and Growth	40
GDNF Signaling for Cell Survival	41
Studies Employing GDNF for SCI Repair	42
GDNF Combinational Therapies for SCI Repair	43
PART III: Hydrogel, VEGF, and GDNF for Spinal Cord Injury Repair	48
Bioengineered Tissues Background	48
Rationale	50
Materials and Methods	55
<i>In vitro</i> Neurite Outgrowth of Spinal Cord Neurons	55
<i>In vivo</i> Hydrogel Combinational Therapy for SCI	62

Novel Bioengineered Hydrogel	62
Animal Acclimation and Gentling	62
Baseline Behavior	65
Spinal Cord Injury Surgical Procedures	65
Treatment Groups Pseudo-randomization	66
Blinded Conditions	71
Transplantation Surgeries	71
Behavioral Assessments	72
Part I: Treadscan™ Gait Analysis	72
Part II: Basso, Beattie, and Bresnahan Open-field Locomotion	78
Part III: Hargreaves' Test	78
Part IV: Gridwalk Assessment	79
Part V: Modified Grooming Test for Trunk Stability	79
Transcranial Magnetic Motor Evoked Potential	80
Histopathological Analysis	80
Statistical Analysis	82
Results	82
VEGF and GDNF Promote Neurite Outgrowth from Spinal Cord Neurons	82
Treadscan™ Gait Analysis	84
Basso, Beattie, and Bresnahan Open-field Locomotion	89
Hargreaves', Gridwalk, and Modified Grooming Tests	89
Transcranial Magnetic Motor Evoked Potential	90
Histopathological Analysis	98
Discussion	118
Conclusions	126
OVERALL DISCUSSION	128
Angiogenesis following SCI	128
Bioengineered Tissues for SCI Treatment	128
Overlapping Signals in Nervous and Vascular Systems	129
VEGF and GDNF Combinational Treatment Approach	130
GDNF Treatment for SCI Repair	131
Combinational Treatment Approaches for SCI	131
VEGF and GDNF Promote Significant Neurite Outgrowth	132
Improved Locomotion with Various Hydrogel Treatment Groups	132
Putative Limitations of the Study	134
Suggested Future Directions	135
Combinational Treatment Approaches Employed for SCI	140

REFERENCES

142

CURRICULUM VITAE

LIST OF TABLES

PART I: Vascular Endothelial Growth Factor and Spinal Cord Injury

TABLE 1	VEGF Ligand and Receptor Cytogenetic Locations	20
TABLE 2	VEGF Ligand and Receptor Expression Patterns	21

PART III: Hydrogel, VEGF, and GDNF for Spinal Cord Injury Repair

TABLE 3	<i>In Vivo</i> Experimental Design	70
TABLE 4	Treadscan™ Parameters	85

LIST OF FIGURES

INTRODUCTION

FIGURE 1	History of Spinal Cord Injury	2
----------	-------------------------------	---

PART I: Vascular Endothelial Growth Factor and Spinal Cord Injury

FIGURE 2	Intracellular Signaling of VEGF	18
FIGURE 3	Pathological Switch Promoting Angiogenesis	24
FIGURE 4	VEGF Pleiotropism	34

PART II: Glial cell line-derived Neurotrophic Factor and Spinal Cord Injury

FIGURE 5	GDNF Family of Ligands and Receptors	39
FIGURE 6	GDNF Modulation of the Inhibitory Glial Scar	47

PART III: Hydrogel, VEGF, and GDNF for Spinal Cord Injury Repair

FIGURE 7	Histology of Hydrogel Transplantation Time Course Study	53
FIGURE 8	Quantification of Transplantation Time Course Study	54
FIGURE 9	Neurite Outgrowth of E15 Spinal Cord Neurons	57
FIGURE 10	Neuroprotection by VEGF and GDNF	60
FIGURE 11	ImageXpress®	61
FIGURE 12	Hydrogel Release Profiles	63
FIGURE 13	<i>In vivo</i> Experimental Timeline	64
FIGURE 14	Louisville Impactor System Apparatus	67
FIGURE 15	Surgical Stabilization Device	68
FIGURE 16	LISA Injury Device Parameters	69
FIGURE 17	Treadscan™	74
FIGURE 18	Basso Beattie and Bresnahan Open Field Locomotion	75
FIGURE 19	Hargreaves' and Gridwalk tests	76
FIGURE 20	Modified Grooming Test	77
FIGURE 21	Treadscan™ Motor Assessments at 6 Weeks Post SCI	87
FIGURE 22	Treadscan™ Motor Assessments at 6 Weeks Post SCI	88
FIGURE 23	Basso, Beattie, and Bresnahan Motor Assessment	91
FIGURE 24	Hargreaves' Test	92
FIGURE 25	Gridwalk Test	93
FIGURE 26	Modified Grooming Test	94
FIGURE 27	tcMMEP Recording Traces	95
FIGURE 28	Verification of Injury Epicenter	96
FIGURE 29	Injury Epicenter	97
FIGURE 30	Rostral-Caudal Cavity Spanse	99
FIGURE 31	Histopathology	100
FIGURE 32	Epicenter Lesion Cavity Area	101

FIGURE 33	RECA-1 & GFAP Immunofluorescence Staining	102
FIGURE 34	RECA-1 & GFAP Immunofluorescence Staining	103
FIGURE 35	RECA-1 & Laminin Immunofluorescence Staining	104
FIGURE 36	Epicenter Lesion and Spared White Matter	105
FIGURE 37	Hydrogel Animal Immunofluorescence Staining	106
FIGURE 38	Hydrogel + VEGF + GDNF Immunofluorescence Staining	107
FIGURE 39	Immunofluorescence Staining	109
FIGURE 40	Vascular Immunofluorescence Staining	111
FIGURE 41	Vasculature and Glial Scar Immunofluorescence Staining	113
FIGURE 42	Vasculature and Glial Endfeet Immunofluorescence Staining	114
FIGURE 43	Cresyl-echt Violet Staining	115

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AAV	Adeno-Associated Virus
Akt	Protein kinase B
ALS	Amyotrophic Lateral Sclerosis
AMPA	Alpha-amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid
ANG-1	Angiopoietin-1
ANOVA	Analysis Of Variance
ARTN	Artemin
BAD	Bcl-2-Associated Death promoter
Bax	Bcl-2-associated X protein
BBB	Basso Beattie and Bresnahan Open Field Locomotion Test
B.C.E.	Before Common Era
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
<i>C. Elegans</i>	<i>Caenorhabditis elegans</i>
CNS	Central Nervous System
cPLA2	calcium-dependent Phospholipase A2
cRET	Co-rearranged during transfection receptor
CSPGs	Chondroitin Sulfate Proteoglycans
DMEM	Dulbecco's Modified Eagle's Medium
DPX	Distyrene, Plasticizer, Xylene mounting media
DRG	Dorsal Root Ganglion
ED-1	Anti-CD68 antibody
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	endothelial Nitric Oxide Synthase
ERK	Extracellular signal-regulated Kinases
FAK	Focal Adhesion Kinase
aFGF	acidic Fibroblast Growth Factor
bFGF	basic Fibroblast Growth Factor
Flk-1	VEGF Receptor-2
Flt-1	Fms-like tyrosine kinase-1; VEGF Receptor-1
GDNF	Glial cell line-derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GDNF	Glial cell line-derived Neurotrophic Factor
GFR α	GDNF Family Receptor alpha
HGF	Hepatocyte Growth Factor
HPRD	Human Protein Reference Database
HRE	Hormone Response Element
IGF-1	Insulin-like Growth Factor 1
IL-1 β	Interleukin-1 beta
LISA [®]	Louisville Injury System Apparatus

LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinases
MEK	Mitogen/Extracellular signal-regulated kinase
mTOR	mechanistic Target of Rapamycin
NMDA	N-Methyl-D-Aspartic Acid
NO	Nitric Oxide
Nogo A	Neurite outgrowth Inhibitor A
NRP	Neuropilin
NRTN	Neuroturin
NT3	Neurotrophin-3
O.C.T.	Optimal Cutting Temperature
OV	Parapoxvirus Orf virus
OX42	Anti-CD11b/c antibody
PBS	Phosphate-buffered Saline
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PGF	Placental Growth Factor
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PLC γ	Phospholipase C gamma
PLG	Poly lactide-co-Glycolic
PLGA	Poly lactide-co-Glycolic Acid
PNS	Peripheral Nervous System
PSPN	Persephin
PTEN	Phosphatase and Tensin Homolog
Rac	Ras-related C3 botulinum toxin substrate
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcomas
RECA-1	Rat Endothelial Cell Antigen-1
RET	Rearranged During Transfection Receptor
RhoA	Ras homolog gene family, member A
ROC	Ras of Complex protein
RTK	Receptor Tyrosine Kinase
SCI	Spinal Cord Injury
SCs	Schwann Cells
SEM	Standard Error of the Mean
SMI-31	Anti-Neurofilament H Antibody (Sternberger Monoclonal)
SOD1	Superoxide Dismutase 1
T10	Thoracic level 10
TAF	Tumor-Angiogenesis Factor
tcMMEP	transcranial Magnetic Motor Evoked Potential
TUNEL	Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling Assay
VEGF	Vascular Endothelial Growth Factor
VEGF-A	Vascular Endothelial Growth Factor A
VEGF-B	Vascular Endothelial Growth Factor B
VEGF-C	Vascular Endothelial Growth Factor C

VEGF-D	Vascular Endothelial Growth Factor D
VEGF-E	Vascular Endothelial Growth Factor E
VEGFRs	Vascular Endothelial Growth Factor Receptors
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
VEGFR-3	Vascular Endothelial Growth Factor Receptor-3
VPF	Vascular Permeability Factor

INTRODUCTION

History of Spinal Cord Injury

Spinal cord injury (SCI) results in a lifetime of devastating disabilities and yet, currently no effective treatments exist. The first documented cases of SCI were written in approximately 3,000 B.C.E., with details contained within the Edwin Smith papyrus. This papyrus was translated by Dr. James Henry Breasted in 1922 (partial translations) and in its entirety in 1930 (Hughes 1988). This papyrus is noteworthy on several levels, not only to be the first document to report on SCI, and to be considered as possibly the first medical papyrus in existence, but also because it is considered to be the first written piece designated as a scientific document (Hughes 1988). It is speculated that the Egyptian physician and architect, Imhotep, detailed the first cases of SCI, either from battle wounds or construction site injuries (Hughes 1988).

Approximately 5,000 years later, neurosurgeons and neurotraumatologists are still challenged to find a treatment for the debilitating, chronic neurological deficits resulting from SCI. In the nineteenth century, Santiago Ramon y Cajal encouraged future neuroscientists to try to change the 'irreversibility' of the central nervous system (CNS), which he described as forever fixed after development and differentiation (Schwab and Bartholdi, 1996). Attempting to repair the injured CNS is demanding and perplexing, yet, neuroscientists, neurosurgeons, and neurotraumatologists of today (Figure 1) have nonetheless accepted this challenge and are forging ahead with potential therapeutic approaches for all those suffering from this devastating condition.

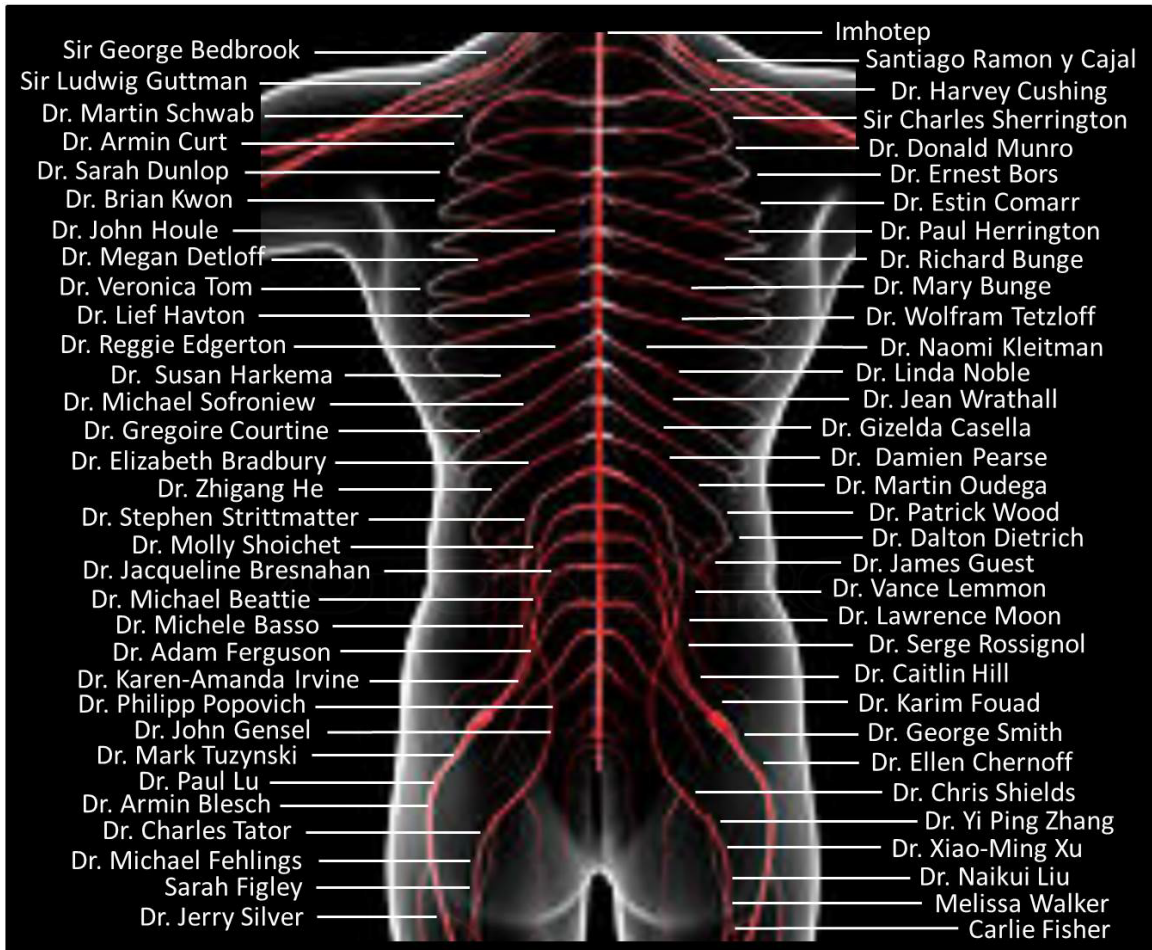


Figure 1. History of Spinal Cord Injury. Following Imhotep's initial documented cases of spinal cord injury, neuroscientists and neurotraumatologists have been working for over 5,000 years to find a cure for this devastating and debilitating condition. This is not an exhaustive list of SCI researchers. It is noteworthy that a majority of the SCI scientists (listed below Drs. Richard and Mary Bunge) are first, second, or third generation SCI scientists trained by Drs. Richard and Mary Bunge.

Spinal Cord Injury Background

This dissertation document will present an overview of spinal cord injury including the major causes; the resulting tissue destruction; inhibitory components to nervous system repair; a background of the literature, primarily focusing upon vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF), bioengineered tissues, and combinational therapeutic approaches, as rationale for our designed combinational treatment approach (including VEGF and GDNF); the endogenous repair mechanisms; data from conducted research studies; limitations of the study; VEGF and GDNF for other neurodegenerative diseases; and future directions for use of bioengineered tissues for therapeutic approaches for SCI.

Motor vehicle accidents, falls, and sports injuries are the primary causes of SCI cases worldwide (Singh et al., 2014). SCI involves an initial mechanical insult that tears, shears, punctures, or compresses the spinal cord, resulting in a breakdown of the blood-spinal cord barrier (Noble and Wrathall, 1987, 1988, 1989; Popovich et al., 1996). A secondary wave of injury ensues, comprised of vascular hemorrhage (Oudega 2012), ischemia (Tator and Fehlings, 1991), edema, excitotoxicity, and chronic inflammation (Mautes et al., 2000; Oudega 2013; Blomster et al., 2013). The resulting toxic milieu leads to neuronal, glial, and vascular cell death (all components of the neurovascular unit), axonal degeneration, loss of gray and white matter tissue (Tator and Koyanagi, 1997), and glial scar formation (Hagg and Oudega, 2006). On a systems-level this can lead to motor and sensory loss of function. On a global scale, this often leads to

paralysis, spinal shock, autonomic dysreflexia, loss of bowel and bladder function (depending upon level and severity of injury), loss of sexual function, pressure ulcers, chronic inflammation, and chronic pain.

Despite centuries of medicine and science suggesting that CNS neural networks were irrevocably 'fixed' and unable to be modulated or regenerated after degeneration or injury, the last half century has experienced tremendous strides in neural regeneration progress. Recent literature in the field of neurotrauma suggests that combinational treatment approaches appear to hold the greatest therapeutic promise (Xu et al., 1995; Guest et al., 1997; Sayer et al., 2002; Fouad et al., 2005; Bunge MB, 2008; Lutton et al., 2011; Lu et al., 2012; Deng et al., 2013; Ansorena et al., 2013). These studies will be described in more detail in later chapters.

Astonishingly, despite the toxic environment at lesion epicenter after SCI, an endogenous vascular response occurs, which peaks between 7-14 days post-injury, and regresses coincident with the onset of cystic cavitation, in both rats and higher primates (Loy et al., 2002; Casella et al., 2002; Benton et al., 2008a; Fassbender et al., 2011). We hypothesized that the angiogenic vasculature regresses due to a loss of structural support, with the onset of cystic cavitation. Therefore, the overall goal of this dissertation research was to provide a structural scaffold for vascular reorganization, axonal regrowth, and tissue repair following contusive SCI, by employing our novel bioengineered hydrogel.

In one of the earliest studies utilizing biomaterials for SCI repair (Marchand and Woerly, 1990) although an immune response was provoked,

beneficial host astrocytic and axonal regrowth into the collagen matrix occurred. A few years later, this group (Marchand et al., 1993) reported on the optimization of their biomaterial by cross-linking their collagen bioimplant with carbodiimide, which resulted in a positive modification of the glial scar, improved biomechanical properties, and enhanced axonal growth into the implant. By 2001, this group of colleagues tested their novel bioengineered hydrogel (Neurogel™) on a chronic compressive SCI model (subdural inflated balloon), transplanting their Neurogel™ at 3-months post SCI (Woerly et al., 2001a). Significant improvements were observed in BBB locomotion (enhanced by an enriched housing environment), accompanied by axonal growth, angiogenic vascular growth, and astrocytic process growth into the hydrogel implant (Neurogel™), including myelinated axons and functional (dendro-dendritic) connections (Woerly et al., 2001a). This study greatly enhanced the use of bioengineered tissues for SCI, particularly because of the beneficial motor effects and histological outcomes on a chronic injury model. Since there are over 250,000 Americans suffering from SCI, and an even greater number globally, chronic SCI treatments are even more substantial than treatments that must be administered acutely. It is difficult to administer therapeutics to injured patients within several hours after injury (acutely), partially because some patients do not make it to the medical center in under a few hours after injury. For example, people injured in the battlefield or in an earthquake may not be transported to the medical facility or even located (in the aftermath of an earthquake) for a number of hours after injury. Additionally, once suspected SCI patients arrive at the hospital, their

vertebral columns must be stabilized and then scans are performed to assess the presence of spinal cord injury, which again occupies more time post injury. Therefore, therapies that can be administered subacutely or in the chronic phases after SCI will have the widest impact for SCI patients.

In addition to promoting axonal, astrocytic, and vascular ingrowth into transplanted biomaterials for tissue repair, various groups began to realize the importance of longitudinally directed growth of regenerating axons. In 2006, Prang et al., delivered an alginate-based anisotropic capillary hydrogel into a rat model of cervical dorsal column transection, which promoted directional axonal regrowth into the transplanted hydrogel without provoking an immune response. Some SCI studies have observed axonal regrowth into the lesion area, without directed growth, thus resulting in random axonal growth within the lesion. The ultimate goal of axonal regrowth into the lesion epicenter is growth through-and-beyond the distal end of the lesion and functional reconnections of these axons with their target neurons. This is necessary in order to create a full connection of neuronal activity from the brain down through the spinal cord, to the neurons that connect to the muscles in our limbs and our internal organs, for movement and to sustain life (heartbeat, respiration, digestion, bladder function).

Aside from serving as a structural scaffold, biomaterials also serve as functional reservoirs for embedded trophic factors and transplanted cells. Through the use of a poly(lactic-co-glycolic acid) outer hydrogel membrane and a poly(ethylene glycol)/poly-L-lysine hydrobromide hydrogel inner layer, embedded with endothelial and neural progenitor cells, Rauch et al. (2009) observed two-to-

four-fold increases in the number of intralesional functional vasculature, and the formation of a blood-spinal cord barrier. Intralesional vasculature is crucial for delivering oxygen and nutrients to the tissues and for the removal of cellular waste. Moreover, an intact blood-spinal cord barrier is crucial for minimizing the inflammatory mediators that extravasate from the blood vessels and result in a wave of secondary tissue degeneration and chronic inflammation. Therefore, this study nicely demonstrates the potential of bioengineered tissues for combinational treatment approaches.

Piantino and colleagues (2006) delivered a photoactivated hydrogel in combination with neurotrophin-3, in a thoracic transection model of SCI. Significant axonal growth of the corticospinal and raphespinal tracts was observed. The authors note that the promoted outgrowth from two descending corticospinal tracts may have primarily resulted from sprouting from undamaged ventral corticospinal tract. Many studies have shown that regrowth of damaged descending tracts, such as the corticospinal and raphespinal tracts, is very challenging and greatly limited. Regrowth is much more probable with the descending propriospinal tract after SCI (Deng et al., 2013, 2014, 2015). Collectively, these studies employing biomaterials give us insight into the usefulness of bioengineered tissues for combinational treatment approaches for repairing the injured spinal cord and central nervous system.

The Belgian Anatomist, Andreas Vesalius, reported in his 1543 publication (*De humani corporis fabrica, On the fabric of the human body, 1543*) on the overlap of the nervous and vascular systems (Carmeliet and Tessier-

Lavigne, 2005). The intertwining of these two systems led to the hypothesis that a similar trophic factor might influence the development of both systems. Dr. Peter Carmeliet and colleagues have been vital in linking the nervous and vascular systems (Carmeliet and Storkebaum, 2002; Storkebaum et al., 2004a; Storkebaum and Carmeliet, 2004b; Zacchigna et al., 2008; Ruiz de Almodovar et al., 2009; Carmeliet and Ruiz de Almodovar, 2013). The neurovascular evolution of VEGF (vascular endothelial growth factor) and its influence on the nervous system was nicely summarized by Zacchigna, Carmeliet and colleagues (2008). This publication displayed the importance of the human VEGF homologue in *C. elegans* (*Caenorhabditis elegans*) and *Drosophila melanogaster*, which lack blood vessels or have very few, respectively. Additionally, Popovici et al. (2002) described receptors on neurons in *C. elegans* with structural similarity to the human VEGF receptors (VEGFRs), which can activate human VEGFRs (Zacchigna et al., 2008). This seminal paper describes the role of VEGF beyond its canonical role in the vascular system (Zacchigna et al., 2008), and details the pleiotropic influence of VEGF on the nervous, vascular, glial, and immune systems.

In addition to the publications from Carmeliet and colleagues describing the pleiotropic influence of VEGF, and the VEGF homologs in *C. elegans* (which lack blood vessels) and *Drosophila* (which have very few blood vessels), several other factors prompted us to employ VEGF embedded within bioengineered hydrogel for treatment of traumatic SCI. First, publications showing the angiogenic vasculature regresses following SCI, at the onset of

cavitation (Loy et al., 2002; Casella et al., 2002; Benton et al., 2008a; Fassbender et al., 2011). Second, deletions within the VEGF promoter region cause a neurodegenerative phenotype in mice, similar to Amyotrophic Lateral Sclerosis (ALS), showing VEGF is important for maintenance of motor function (Oosthuysen et al., 2001). Third, Lambrechts et al. (2003) showed motoneuron protection by VEGF administration in an ALS mouse model. Additionally, this study also showed that VEGF serum levels in European patients correlated with ALS susceptibility, with lower circulating VEGF levels correlating with higher risk of sporadic ALS. Next, VEGF delivered via a retroviral vector delayed disease onset, promoted neuroprotection, and prolonged survival of animals with an ALS phenotype (Azzouz et al., 2004). Similarly, VEGF delivered intracerebroventricularly prolonged the survival period, delayed the disease onset, and spared motor neurons in an ALS model (Storkebaum et al., 2005). Moreover, intact vasculature is crucial for delivering oxygen and nutrients to the tissues and for removing toxic wastes. Therefore, we hypothesized that delivering VEGF to the lesion epicenter via a bioengineered hydrogel might positively influence the endogenous angiogenic response while promoting neuron survival and growth, Schwann cell migration and survival, and influencing microglia and astrocytes (Storkebaum et al., 2004a).

In 2007, Tufro et al., showed an interaction of VEGF and GDNF (glial cell line-derived neurotrophic factor) at the RET proto-oncogene receptor, with a possible additive effect on cell outgrowth. Moreover, VEGF and GDNF robustly influenced animal survival, delayed disease onset, and maintained motor

function in an ALS neurodegenerative disease model (Krakora et al., 2013). VEGF and GDNF also had an additive effect on neuron survival and increased the axon fiber density compared to control animals or animals receiving VEGF or GDNF alone, in a Parkinson's disease model (Herran et al., 2013).

Spinal cord injury literature has shown GDNF promotion of neuroprotection (Arce et al., 1998; Soler et al., 1999; Nicole et al., 2001), axonal growth (Blesch and Tuszynski, 2001; Dolbeare and Houle, 2003), reduction of astrogliosis (Iannotti et al., 2003; Deng et al., 2011a; Ansorena et al., 2013), increased myelination (Zhang et al., 2009; Deng et al., 2013), increased intralésional vasculature (Zhang et al., 2009; Ansorena et al., 2013), and improved functional recovery (Cheng et al., 2002; Kao et al., 2008; Deng et al., 2013; Ansorena et al., 2013). Additionally, in a Parkinson's disease model, Iravani et al. (2012) observed neuroprotection following GDNF administration. In a combinational treatment approach, neural stem cells secreting GDNF conferred striatal neuroprotection in a Huntington's disease model (Pineda et al., 2007). In another Huntington's disease model study, GDNF delivered via an adeno-associated viral vector into the striatum, provided neuroprotection to both nitric oxide synthase striatal interneurons as well as parvalbumin striatal interneurons (Kells et al., 2004). Following SCI, Zhang et al. (2009) showed that GDNF administration increased the number of myelinated axons and the number of blood vessels. In an SCI combinational therapeutic strategy, GDNF released from a Schwann cell-seeded guidance channel, with Schwann cells overexpressing GDNF, positively modulated the inhibitory astrocytic glial scar,

created a more permissive environment for propriospinal axonal regrowth through-and-beyond the distal end of the lesion, conducted electrical signals through the lesion gap, and improved functional recovery (Deng et al., 2013). Collectively, these studies highlight the importance of combinational treatment approaches for traumatic spinal cord injury, and encouraged us to employ the combination of VEGF and GDNF, slowly released from a novel bioengineered hydrogel, for the treatment of traumatic spinal cord injury.

PART I: Vascular Endothelial Growth Factor and Spinal Cord Injury

VEGF Background

Vascular endothelial growth factor (VEGF) is an important signaling molecule intimately associated with angiogenesis (Folkman et al. 1971; Carmeliet et al., 1996), axonal guidance (Ruiz de Almodovar et al., 2011; Carmeliet & Ruiz de Almodovar et al., 2013; Zachary et al., 2005), neuroprotection (Storkebaum et al., 2004a; Facchiano et al., 2002; Widenfalk et al., 2003; Zacchigna et al., 2008), Schwann cell survival and migration, and proliferation of astrocytes, microglia, and neural stem cells (Storkebaum et al., 2004a). Thus, making this pro-angiogenic factor a therapeutic target for promoting spinal cord revascularization, neuroprotection, cell proliferation, tissue regeneration, and ultimately improved functional recovery. This introduction therefore, focuses on the background of VEGF as an angiogenic trophic factor and its more recently discovered pleiotropic role in the nervous systems, as well as its potential influence for tissue repair following traumatic spinal cord injury.

Discovery of VEGF and its Receptors

VEGF is well known for its influence on vasculature and has been widely characterized in cardiovascular and cancer research and medicine. In more recent decades, VEGF has also been recognized for its role in embryonic development (Carmeliet et al., 1996), its pleiotropic effects (Storkebaum et al., 2004a; Rosenstein and Krum, 2004; Carmeliet et al., 2013) on neurons and glia, and its therapeutic potential to prevent neurodegeneration (Herran et al., 2013; Azzouz et al., 2004; Emerich et al., 2010; Storkebaum and Carmeliet, 2004b).

Dr. Judah Folkman and colleagues extensively studied endothelial cell activation and angiogenesis, intricately associated with tumor growth and survival, characterizing the trophic factor as Tumor-Angiogenesis Factor (TAF; Folkman et al., 1971) with seminal papers during the 1970s. Previous studies displayed the factor's potential to act at a distance, after diffusing across a membrane (Greenblatt et al., 1992; Ehrman et al., 1992). Vascular Endothelial Growth Factor (VEGF), a gene family comprised of five major proteins along with receptors and co-receptors, was originally termed Vascular Permeability Factor (VPF) in 1983 (Senger et al., 1983) by Drs. Senger and Dvorak. In 1989, this vascular trophic factor, which resulted in extensive endothelial cell outgrowth and angiogenesis, was termed VEGF by Drs. Ferrara and Henzel at Genentech (Ferrara et al., 1989). The neurovascular evolution of VEGF and its influence on the nervous system was summarized by Zacchigna, Carmeliet and colleagues (2008), displaying the importance of the human VEGF homologue in *C. elegans* (*Caenorhabditis elegans*) and *Drosophila melanogaster*, which lack blood vessels or have very few, respectively. Additionally, Popovici et al. (2002) described receptors on *C. elegans* neurons with structural similarity to the human VEGF receptors (VEGFRs), which can activate human VEGFRs (Zacchigna et al., 2008). Dr. Peter Carmeliet has been instrumental in expanding and detailing the Belgian Anatomist, Andreas Vesalius' 1543 observations about the overlap of the nervous and vascular systems (*De humani corporis fabrica, On the fabric of the human body*, 1543), thus, proving the pleiotropic influence of VEGF on the nervous, vascular, and immune systems (Carmeliet and Tessier-Lavigne, 2005).

VEGF Isoforms and Co-receptors

The VEGF sub-family of growth factors belongs to the platelet-derived growth factor (PDGF) family, and is comprised of isoforms VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PGF (placental growth factor), summarized by Grünewald et al. (2010). Together, these trophic factors are responsible for embryonic vasculogenesis, the formation of new blood vessels, as well as the development of blood vessels from existing blood vessels (angiogenesis). Human VEGF exists as a homodimer (~45 kDa under non-reducing conditions, and ~23 kDa under reducing conditions; Ferrara et al., 1989) and is synonymous with VEGF-A. Alternative splicing of the human VEGF-A gene (cytogenetic location 6p12, 9 exons; www.ncbi.nlm.nih.gov/gene/7422) generates five distinct VEGF-A monomers including VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆, designated by the number of amino acids in the sequence. VEGF-B is located on chromosome 11 (cytogenetic location 11q13, 7 exons; www.ncbi.nlm.nih.gov/gene/7423). VEGF-C is located on chromosome 4 (cytogenetic location 4q34.3, 7 exons; www.ncbi.nlm.nih.gov/gene/7424). VEGF-D is located on the X chromosome (cytogenetic location Xp22.31, 7 exons; www.ncbi.nlm.nih.gov/gene/2277). The non-human VEGF-E, encoded by the parapoxvirus Orf virus (OV; Meyer et al., 1999) is located on chromosome 4 (cytogenetic location 4q32, 8 exons; Wise et al., 2012). PGF is located on chromosome 14 (cytogenetic location 14q24.3, 7 exons; www.ncbi.nlm.nih.gov/gene/56034). VEGF-E binds selectively to VEGFR-2, and has been shown to promote lesion angiogenesis in response to the viral infection

by the parapoxvirus Orf virus (OV; Meyer et al., 1999), and regulation of keratinocytes for wound re-epithelialization in response to the purified VEGF-E protein¹⁵ that this virus encodes, thus making it a potentially good candidate for wound healing and repair. Moreover, since VEGF-E binds only to VEGFR-2 and not to VEGFR-1, it does not result in vascular permeability or tissue inflammation, like VEGF-A (Wise et al., 2012). Thus, purified VEGF-E might have potential in tissue repair beyond just wound healing.

Two tyrosine kinase receptors, VEGF Receptor 1 (VEGFR-1, fms-like tyrosine kinase 1/Flt-1; www.ncbi.nlm.nih.gov/gene/5228) and VEGF Receptor 2 (VEGFR-2, fetal liver kinase 1/Flk-1, kinase insert domain receptor/KDR) were identified in 1992 (de Vries et al.; Terman et al.). Neuropilin (NP1 and NP-2) co-receptors bind specifically to VEGF isoform 165 (VEGF₁₆₅ in humans, VEGF₁₆₄ in rats). The human VEGFR-1 gene is located on chromosome 13 (cytogenetic location 13q12, 32 exons; www.ncbi.nlm.nih.gov/gene/2321) and VEGFR-2 gene is located on chromosome 4 (cytogenetic location 4q11-q12, 30 exons; www.ncbi.nlm.nih.gov/gene/3791). VEGF Receptor 3 (VEGFR-3, fms-like tyrosine kinase 4/Flt-4; www.ncbi.nlm.nih.gov/gene/2324) was independently characterized in 1992 by Galland et al. (1992) and Pajusola and colleagues (1992). The cytogenetic locations for all VEGF proteins and receptors are summarized in Table 1. All three VEGF Receptors are type V receptor tyrosine kinases (RTK's), consisting of an extracellular region (7 immunoglobulin-like domains), a single transmembrane domain, a juxtamembrane component, and an intracellular protein-tyrosine kinase segment with a variable (70-100 amino

acids) kinase insert and a carboxyterminal tail (Roskoski Jr. et al., 2007). The main pathway promoting angiogenesis is the interaction of VEGF-A (VEGF) and its VEGFR-2 receptor; particularly, the phosphorylation of the VEGFR-2 Tyrosine residue 1175, which binds to the SH2-domain of Phospholipase-C γ (PLC γ), upstream of the PKC mitogen-activated protein kinase/extracellular signal-related kinases (MAPK/ERK) pathway. Ji et al. (1997) showed that PLC γ knockout mice were embryonic lethal at approximately day E9. VEGFR-1 knockout mice were shown to be embryonic lethal at E8.5, resulting from disorganized vasculature and endothelial cell-overgrowth (Hiratsuka et al. 2005); this study also displays the importance of the transmembrane domain of VEGFR-1, which localizes VEGF for signaling during embryogenesis, and negatively regulates angiogenesis. Takashima et al. (2002) observed embryonic lethality (E8.5) in NRP-1 and NRP-2 knockout animals, due to lack of blood vessel formation. In 1996, both Carmeliet et al. and Ferrara et al. discovered the dose-dependent embryonic lethality of homozygous VEGF^{-/-} knockout animals (E10.5 and E11-12, respectively) and heterozygous VEGF^{+/-} animals (approximately E12.5), due to lack of formation of functional vasculature and significant cell apoptosis. Furthermore, Ferrara et al. (1996) detailed the significantly diminished capacity for tumorigenesis of VEGF^{-/-} knockout embryonic stem cells; thus, underscoring VEGF's role in tumor formation and the critical role of angiogenesis in tumor growth. While VEGF, VEGFR-1 (Flt-1), and VEGFR-2 (Flk-1) are all essential components of embryonic development, these studies (Carmeliet et al. 1996 and Ferrara et al. 1996) highlight VEGF as the most vital factor, due to VEGF^{+/-}

embryonic lethality (Ferrara et al. 1996). Collectively, these studies display the importance of VEGF, its receptors and downstream signaling pathways for angiogenesis, embryonic development, and tumorigenesis. VEGF ligand isoforms and receptor interactions are summarized in Figure 2.

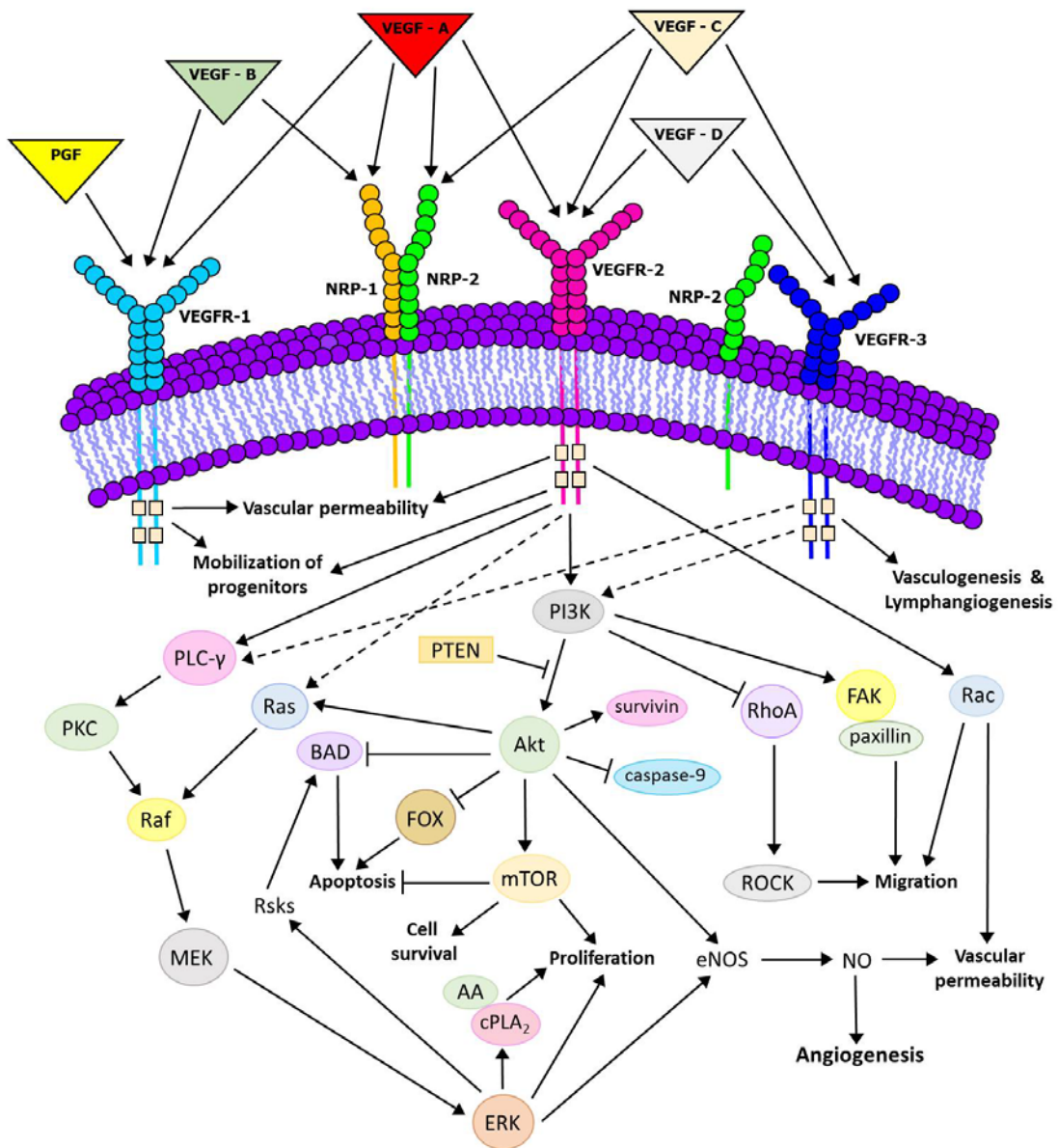


Figure 2. Intracellular Signaling of VEGF. VEGF-A binds to VEGFR-1 (VEGF Receptor 1), VEGFR-2 (VEGF Receptor 2), NRP-1 (Neuropilin-1 receptor), and NRP-2 (Neuropilin-2 receptor). VEGF-B and PGF (Placental Growth Factor) bind to VEGFR-1. VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 (VEGF Receptor 3). Downstream signaling leads to angiogenesis, vasculogenesis, lymphangiogenesis, vascular permeability, cell survival (inhibition of apoptosis), migration, proliferation, and mobilization of progenitors.

Figure 2 Abbreviations: VEGF-A (Vascular Endothelial Growth Factor A), VEGF-B (Vascular Endothelial Growth Factor B), VEGF-C (Vascular Endothelial Growth Factor C), VEGF-D (Vascular Endothelial Growth Factor D), PGF (Placental Growth Factor), VEGFR-1 (Vascular Endothelial Growth Factor Receptor 1), VEGFR-2 (Vascular Endothelial Growth Factor Receptor 2), VEGFR-3 (Vascular Endothelial Growth Factor Receptor 3), NRP-1 (Neuropilin-1 Receptor), NRP-2 (Neuropilin-2 Receptor), PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase), Rac (Ras-related C3 botulinum toxin substrate 1), Ras (Rat sarcomas, small GTPase), RhoA (Ras homolog gene family, member A), FAK (Focal Adhesion Kinase), PTEN (Phosphatase and tensin homolog), Paxillin, Survivin, Caspase-9, Akt (Protein kinase B), FOX (Forkhead box), PLC- γ (Phospholipase C, gamma), PKC (Protein kinase C), BAD (Bcl-2-associated death promoter), Raf (Rapidly Accelerated Fibrosarcoma), mTOR (mechanistic target of rapamycin), ROC (Ras of Complex protein), NO (Nitric oxide), eNOS (endothelial Nitric Oxide Synthase), AA (Arachidonic acid), cPLA2 (calcium-dependent Phospholipase A2), ERK (Extracellular signal Regulated Kinases), MEK (Mitogen-activated protein kinase kinase).

Protein	Cytogenetic location	Reference
VEGF-A	cytogenetic location 6p12, 9 exons	www.ncbi.nlm.nih.gov/gene/7422
VEGF-B	cytogenetic location 11q13, 7 exons	www.ncbi.nlm.nih.gov/gene/7423
VEGF-C	cytogenetic location 4q34.3, 7 exons	www.ncbi.nlm.nih.gov/gene/7424
VEGF-D	cytogenetic location Xp22.31, 7 exons	www.ncbi.nlm.nih.gov/gene/2277
VEGF-E	cytogenetic location 4q32, 8 exons, Orf virus	Wise et al., 2012
PGF	cytogenetic location 14q24.3, 7 exons	http://www.ncbi.nlm.nih.gov/gene/5228
VEGF Receptor 1	cytogenetic location 13q12, 33 exons	http://www.ncbi.nlm.nih.gov/gene/2321
VEGF Receptor 2	cytogenetic location 4q11- q12, 30 exons	www.ncbi.nlm.nih.gov/gene/3791
VEGF Receptor 3	cytogenetic location 5q35.3, 34 exons	www.ncbi.nlm.nih.gov/gene/2324

Table 1. VEGF Ligand and Receptor Cytogenetic Locations.

Isoform/ Receptor	Tissue expression	Tumor expression
VEGF-A	lungs, heart, adrenal, kidneys, liver, spleen, gastric mucosa	breast, colorectal, non-small cell lung, prostate
VEGF-B	heart, brain, kidney, testes, spleen, lung, liver	breast carcinoma, non-Hodgkins lymphoma, fibrosarcoma, benign thymoma, melanoma
VEGF-C	heart, skeletal muscle, ovaries, placenta, small intestines, lung, kidney, spleen, pancreas, prostate, testes	breast, colon, cervix, stomach, lung, prostate
VEGF-D	heart, colon, kidney, lung, liver, small intestines, ovaries, pancreas, prostate, skeletal muscles, testis, spleen	cervical intraepithelial neoplasia, colorectal, breast, glioblastoma, gastric mucosa, melanoma, thyroid carcinoma; lymph node metastasis with lung, ovarian, colorectal tumors
VEGF-E	Non-human/non-applicable	non-human protein/non-applicable
PGF	dentine matrix, endometrium, placenta, natural killer cells, serum, trophoblasts, vascular endothelium, umbilical vein endothelial cells, heart, skin, retina, skeletal muscle	non-small cell lung cancer, breast, gastric, prostate
VEGFR-1	endothelial cells, embryonic cells derived from endothelium (early yolk sac mesenchyme); soluble and membrane-bound forms	medulloblastoma, pancreas, bladder, melanoma, Kaposi sarcoma, ovarian, prostate, malignant mesothelioma, esophageal carcinoma
VEGFR-2	endothelial cells, mononuclear cells; soluble and membrane-bound forms	medulloblastoma, pancreas, bladder, melanoma, Kaposi sarcoma, ovarian, small cell lung cancer, malignant mesothelioma, myeloid leukemia, esophageal carcinoma
VEGFR-3	corneal dendritic cells, retina, microvascular endothelial cells in early development; lymphatic endothelium in later development and postnatally	small cell lung cancer
NP-1	umbilical vein endothelial cells, heart, sensory and sympathetic neurons, adipocytes, osteoblasts, bone marrow fibroblasts, microvascular endothelial cells; soluble forms - hepatocytes, renal proximal and distal tubules	prostate, colon adenocarcinoma, breast, astrocytoma, esophagus, stomach, pancreas, gall bladder, glioma, neuroblastoma, melanoma, non-small cell lung cancer, small cell lung cancer
NP-2	human umbilical vein endothelial cells, sympathetic neurons, many hindbrain nuclei, inner ear, dorsal aorta, limb buds, lung, back and tongue muscles, submandibular gland, kidney, intestinal epithelium, whisker follicles	glioblastoma, neuroblastoma, melanoma, bladder, prostate, pancreas, non-small cell lung cancer, small cell lung cancer

Table 2. VEGF Ligand and Receptor Expression Patterns.

Localization of VEGF and its Receptors

VEGF-A mRNA is widely expressed throughout the body, with the highest expression in the lungs, heart, adrenal glands, and kidneys, and lower expression in the liver, spleen, and gastric mucosa (Roskoski et al., 2007). VEGF-A is also a major target for anti-tumor therapies, as VEGF-A is expressed by the following human tumors: colorectal, breast, non-small cell lung, and prostate (Roskoski et al., 2007). VEGF-B is highly expressed in the heart, brain, testes, and kidney, with lower expression in spleen, lung, and liver (Roskoski et al., 2007). VEGF-C is expressed in the heart, intestine, ovaries, and the placenta (HPRD: 03317; ID: 01889). VEGF-D is expressed in the colon, heart, kidney, liver, lung, ovaries, pancreas, prostate, skeletal muscles, small intestine, spleen and testis (HPRD: 02102, ID: 03237). PGF is expressed in the dentine matrix, endometrium, eyes, natural killer cells, placenta, serum, trophoblasts, umbilical vein endothelial cells, and vascular endothelium (HPRD: 03076, ID: 02102). VEGFR-1 is expressed in blood vessels, bone marrow, colon, endometrium, epididymis, fetus, leydig cells, monocytes, ovaries, pancreas, placenta, prostate, seminiferous tubule, Sertoli cells, testis, and urothelium (HPRD: 01297, ID: 10529). VEGFR-2 is expressed in the bone marrow, heart, hematopoietic stem cells, mammary gland, neurons, placenta, testis, and urothelium (HPRD: 01867, ID: 03076). Neurons more widely express VEGFR-2 while VEGFR-1 is more abundant on glial cells (Zacchigna et al., 2008). As VEGF-A₁₆₅ is the most abundant and most biologically active (pro-angiogenic) isoform of VEGF molecules, the remainder of this review will primarily focus on VEGF-A₁₆₅

(VEGF₁₆₅) and its therapeutic application for spinal cord injury repair. Tissue expression of VEGF and its receptors is summarized in Table 2.

Synergistic Activation of VEGF Receptors

In 2001, Carmeliet and colleagues observed synergistic activation of the VEGFR-1 receptor by VEGF and PGF to promote angiogenesis (Carmeliet et al., 2001, adapted illustration Figure 3). During embryogenesis, VEGFR-1 is primarily a soluble receptor, which inhibits angiogenesis by binding VEGF and thus preventing VEGF from binding to the cell-surface VEGFR-2, which promotes angiogenesis (Shibuya 2006). PGF binds to both the membrane-bound VEGFR-1 and the soluble inhibitory form of VEGFR-1. Thus, during embryogenesis, PGF can bind to the soluble form of VEGFR-1 and allow VEGF to bind to the membrane-bound VEGFR-2 to promote angiogenesis. In contrast, under pathological conditions VEGFR-1 is primarily membrane-bound on endothelial cells, and PGF is upregulated. Thus, PGF can activate VEGFR-1 while VEGF binds VEGFR-2, both promoting angiogenesis. Carmeliet et al. (2001) described a synergistic effect on the promotion of angiogenesis when PGF activated the membrane-bound VEGFR-1 while VEGF activated the membrane-bound VEGFR-2. While PGF and VEGF both activate VEGFR-1, PGF results in the phosphorylation of tyrosine residue 1309 while VEGF₁₆₅ promotes the phosphorylation of tyrosine 1213 (Autiero et al., 2003).

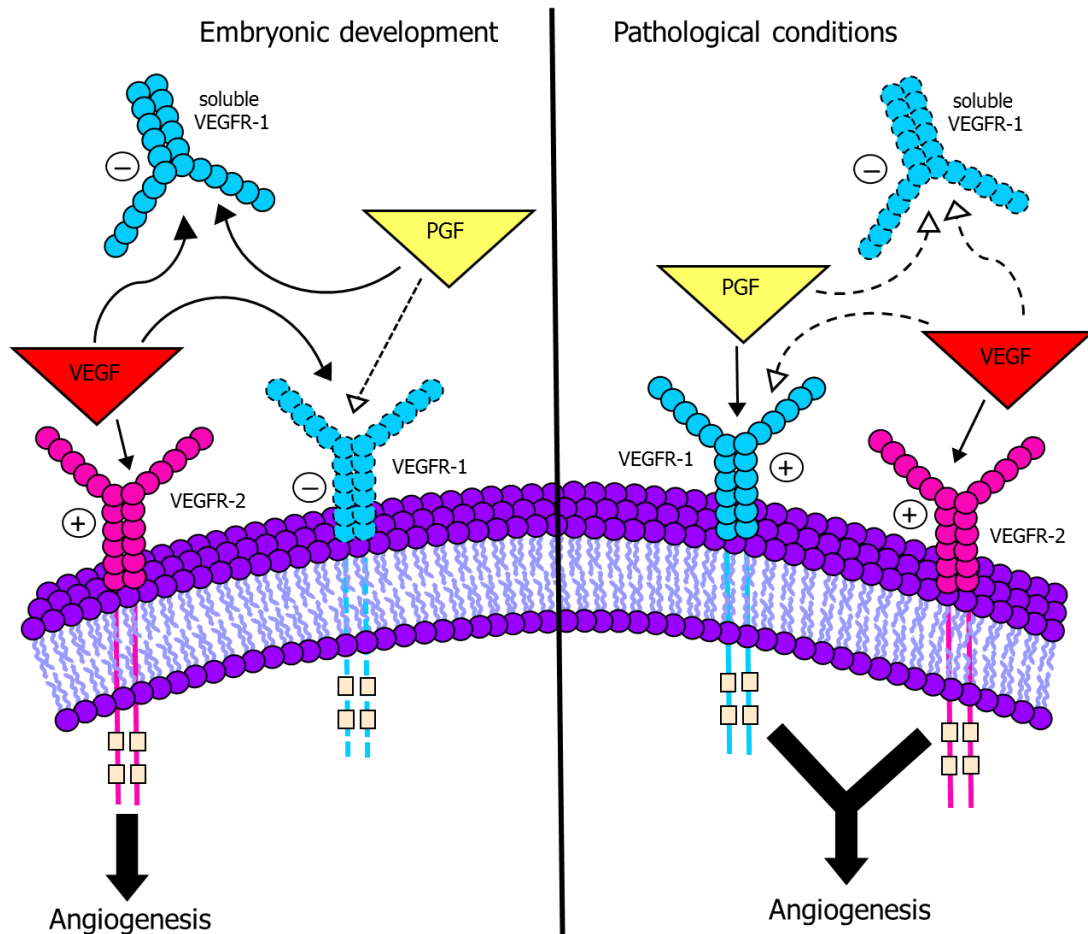


Figure 3. Pathological Switch Promoting Angiogenesis. Synergistic activation of VEGFR-1 (soluble and membrane-bound forms) under embryonic conditions, by VEGF and PGF, primarily the soluble form of VEGFR-1, which negatively regulates angiogenesis. Under pathological conditions VEGF primarily binds to VEGFR-2 while PGF primarily binds to membrane-bound VEGFR-1, and both VEGF receptors promote angiogenesis after a pathological insult.

VEGF levels and VEGF Receptor Expression after SCI

Bartholdi et al. (1997) and Herrera et al. (2009) observed reduced VEGF levels at injury epicenter at 1 day post SCI with diminished VEGF levels as far as 1 month post SCI. Additionally, Ritz et al. (2010) reported reduced levels of VEGF, Angiopoietin-1 (Ang-1), PDGF-BB, and PGF, and increased expression of the angiogenic factor (HGF, hepatocyte growth factor). VEGF receptors Flt-1 and Flk-1 have been shown to be constitutively expressed by vascular endothelial cells, neurons, and some astrocytes in the spinal cord (Choi et al., 2007). Following SCI, VEGFR1 (Flt-1), VEGFR2 (Flk-1) and neuropilin-1 receptors have been shown to be upregulated in reactive astrocytes and microglia/macrophages following contusive SCI (Choi et al., 2007; Skold et al., 2000). This receptor expression peaked between 7 and 14 days following injury and remained relatively high even at 14 days and beyond (Choi et al., 2007). Taken together, this suggests that VEGF and its two tyrosine kinase receptors play a role in inflammation and the astrocytic response following contusive spinal cord injury. However, Skold et al. (2000) *in vitro* study suggests that upregulation of VEGF, its receptors and co-receptors in astrocytes may occur in the absence of inflammatory cells, with prostaglandins being upstream of VEGF.

Studies Employing VEGF for SCI Repair

VEGF has become a therapeutic target for spinal cord injury repair primarily over the past two decades. Fassbender et al. (2011) nicely reviews the literature on microvascular dysfunction following SCI, and details the importance of putative therapeutic approaches targeting the microvasculature. VEGF routes

of administration vary from: exogenously applied (intrathecal and intraspinal injections, osmotic mini pumps; Widenfalk et al., 2003; Benton and Whittemore 2003; Herrera et al., 2009); engineered transcription factor activation of endogenous VEGF expression (Liu et al., 2010); overexpression via cells (Kim et al., 2009), viral vectors (Facchiano et al., 2002; Figley et al., 2014), or in response to other neurotrophic factor administration (GDNF; Kao et al., 2008), or as a result of shockwave therapy (Yamaya et al., 2014), amongst others. VEGF has been shown to be neuroprotective (Facchiano et al., 2002); promote angiogenesis (Facchiano et al., 2002; Figley et al., 2014; des Rieux et al., 2014) and oligodendrogenesis; improve myelin integrity (Sundberg et al., 2011); reduce tissue lesion volume (Widenfalk et al., 2003); increase white matter (Facchiano et al., 2002) and gray matter (Figley et al., 2014) sparing; promote neuritogenesis into the lesion (des Rieux et al., 2014); decrease glial scar (Widenfalk et al., 2003); and improve locomotion (Facchiano et al., 2002; Kim et al., 2009; Liu et al., 2009; Yamaya et al., 2014).

However, the time window of treatment onset, number of doses and duration of treatment, and VEGF dosage are crucial factors in employing this trophic factor following SCI, as some studies have reported exacerbation of lesion and decreased motor performance compared to controls (Benton and Whittemore, 2003), aberrant excessive sprouting of axons (Nesic et al., 2010) and increased mechanical allodynia (Nesic et al., 2010; Sundberg et al., 2011). Drs. Benton and Whittemore administered a supraphysiological dosage of VEGF (0.5 µg/µL) at 3 days post injury; considering the peak of the inflammatory phase

and the very high VEGF dosage, it is reasonable to have observed exacerbation of lesion, likely due to excessive vascular permeability and extravasation of inflammatory mediators. It is unknown whether the increased mechanical allodynia in these studies is a result specifically of VEGF₁₆₅ or perhaps VEGF₁₈₈, as suggested by Nestic et al. (2010). However, it is noteworthy that a subset of saline injected SCI control animals also developed mechanical allodynia (Sundberg et al., 2011) similar to other studies (Figley et al., 2014). Thus, VEGF may just be one of the key players involved in mechanical hypersensitivity after SCI. Interestingly, van Neerven et al. (2010) had a similar route of intrathecal VEGF administration as Sundberg et al. (2011); however, van Neerven gave daily injections for the first week post-SCI while Sundberg's group gave only one injection immediately following injury. Sundberg and colleagues observed exacerbated forepaw mechanical allodynia (Nestic et al., 2010); yet van Neerven and colleagues observed a decrease in mechanical allodynia of the hindpaw (2010). Additionally, Figley et al. (2014) reported significantly decreased mechanical allodynia in VEGF treated rats compared to saline or viral vector vehicle controls. Observed differences across these studies are likely due to the duration of VEGF administration and dosages.

In a study of cerebral ischemia, Manoonkitiwongsa et al. (2004) reported neuroprotection with low (2 μ g) and medium (8 μ g) doses of VEGF₁₆₅, subthreshold to promote angiogenesis. However, higher (60 μ g) doses of VEGF₁₆₅ resulted in angiogenesis without neuroprotection in ischemic brains and neuronal injury in VEGF₁₆₅ treated non-ischemic (uninjured/normal) brains. This

study further demonstrates the crucial aspect of VEGF dosage, in addition to timing, particularly for studies targeting angiogenesis and neuroprotection concomitantly. Shinozaki et al. (2014) investigated the contributions of VEGFR-1 and VEGFR-2 activation on neuroprotection following SCI, through neutralizing antibodies, and determined VEGFR-1 plays a major role in vascular permeability, while VEGFR-2 promotes neuron survival.

VEGF Combinational Therapies for SCI Repair

Similar to other neuroprotective and neural regeneration therapies, VEGF alone might be insufficient to produce significant axon regeneration/sparing, functional synapse formation, and improved functional recovery following SCI. Thus, further investigation of VEGF is necessary, and more studies are employing VEGF as part of combinational treatments. In 2012, Lutton et al. showed reduced lesion cavity, glial scar density, and the inflammatory response (macrophage/microglia) in response to VEGF and PDGF following SCI. This combination of trophic factors (VEGF and PDGF) was also shown to promote improved functional recovery following SCI (Chehrehasa et al. 2014). Gong et al. (2015) observed neuroprotection of spinal cord neurons through VEGFR-2 (Flk-1), after application of the endothelin-A/B dual receptor antagonist (Bosentan). In a 2011 combinational study (de LaPorte et al.), poly (lactide-co-glycolide) (PLG) bridges loaded with VEGF and FGF-2 (fibroblast growth factor 2) promoted neurite growth and angiogenesis within the lesion site, and prevented the formation of cystic cavity. Additional trophic factors might be necessary in order to promote significant axonal re-growth and functional recovery.

VEGF Neuroprotection in Neurodegenerative Disease Models

Dysfunctional vasculature or aberrant VEGF levels negatively influence a number of neurodegenerative diseases including Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS; Lou Gehrig's disease), Huntington's disease, Parkinson's disease, and stroke (Storkebaum and Carmeliet, 2004b). In a mouse model of epilepsy, VEGF administration preserved learning and memory function (Morris water maze) and reduced anxiety-like behaviors that are typically observed in status-epilepticus rodents (Nicoletti et al., 2010). Reduced VEGF levels and deletion of the HRE (hormone response element) in the VEGF promoter (Oosthuysen et al., 2001) can both lead to rodent ALS-like phenotypes: decline of motor function and decreased grooming behavior in a SOD1 (Superoxide dismutase 1) mouse model of ALS (Carmeliet & Ruiz de Almodovar, 2013). Moreover, in similar studies of human patients, Carmeliet & Ruiz de Almodovar (2013) and Lambrechts et al. (2003) determined that human ALS patients had lower VEGF levels compared to healthy population-based controls, with the lowest VEGF serum levels correlating with the greatest ALS susceptibility (Lambrechts et al., 2003). Additionally, VEGF was shown to be neuroprotective in rodent models of Parkinson's disease (Herran et al., 2013), ALS (Azzouz et al., 2004), Huntington's disease (Emerich et al., 2010), and cerebral ischemia (Sun et al., 2003; Manoonkitiwongsa et al., 2004). Interestingly, VEGF-A165 competes with Semaphorin 3A for signaling through the neuropilin-1 receptor. VEGF promotes axonal outgrowth and chemoattraction while Semaphorin 3A influences axonal guidance by

chemorepulsion, axon pruning, and growth cone collapse (Zachary et al., 2005). Thus, inhibition of Semaphorin 3A is another putative target for SCI therapies for promoting axonal outgrowth via VEGF-A165, making this a potential combinational treatment approach worth investigating.

VEGF Signaling for Neuroprotection in SCI

VEGF also influences many cell types, including neurons (Kawai et al., 2006; Jin et al. 2006; Ruiz de Almodovar et al. 2009), Schwann cells (Sondell et al., 1999), astrocytes (Krum et al., 2002; Mani et al. 2010), microglia, neuronal stem cells (Maurer et al., 2003; Meng et al., 2006), and oligodendrocyte precursors, to promote angiogenesis, neurogenesis (Sun et al. 2006), dendritogenesis, synaptic plasticity, axon growth and guidance, cell survival (Sun et al. 2003), proliferation (Zhu et al. 2003), migration, differentiation, neuromuscular junction innervation, and neuroendothelial junction maintenance. Jin et al. (2000) detailed the neuroprotective effects of hippocampal neurons by VEGF activation of VEGFR-2, and downstream signaling of PI3K, with reduced caspase-3. Hao and Rockwell (2013) showed the neuroprotection of hippocampal neurons via signaling through VEGF activation of VEGFR-2, with downstream signaling through the PI3K/Akt and MEK/ERK pathways. This study also suggests that VEGFR-1 and NP-1 likely serve as backup signaling pathways for neuroprotection with blockade of VEGFR-2. The pleiotropic mechanisms of VEGF are summarized in Figure 4, as well as Storkebaum et al. (2004a), Nowacka and Obuchowicz et al. (2012), and Carmeliet and Ruiz de Almodovar (2013).

Conclusion

Since the discovery of VEGF in early 1970's, by Dr. Judah Folkman (Folkman et al., 1971) and its official naming by Drs. Ferrara and Henzel (Ferrara et al., 1989), it has been determined that this vascular trophic factor has much broader implications than its canonical role in development of the vascular system. VEGF's pleiotropic mechanisms include: angiogenesis (Folkman et al. 1971; Carmeliet et al., 1996), axonal guidance (Ruiz de Almodovar et al., 2011; Carmeliet & Ruiz de Almodovar et al., 2013; Zachary et al., 2005), neuroprotection (Storkebaum et al., 2004a; Facchiano et al., 2002; Widenfalk et al., 2003; Zacchigna et al., 2008), Schwann cell survival and migration, and proliferation of astrocytes, microglia, and neural stem cells (Storkebaum et al., 2004a). Moreover, deletions within the VEGF promoter region cause a neurodegenerative phenotype in mice, similar to Amyotrophic Lateral Sclerosis (ALS), showing VEGF is important for maintenance of motor function (Oosthuysen et al., 2001). Additionally, Lambrechts et al. (2003) showed motoneuron protection by VEGF administration in an ALS mouse model. This study also showed that VEGF serum levels in European patients correlated with ALS susceptibility, with lower circulating VEGF levels correlating with higher risk of sporadic ALS. VEGF delivered via a retroviral vector delayed disease onset, promoted neuroprotection, and prolonged survival of animals with an ALS phenotype (Azzouz et al., 2004). Similarly, VEGF delivered intracerebroventricularly prolonged the survival period, delayed the disease onset, and spared motor neurons in an ALS model (Storkebaum et al., 2005).

After spinal cord injury, an angiogenic response occurs that peaks approximately 7-14 days post-injury, and regresses coincident with the onset of cystic cavitation, in both rats and higher primates (Loy et al., 2002; Casella et al., 2002; Benton et al., 2008a; Fassbender et al., 2011). Intact vasculature is crucial for delivering oxygen and nutrients to the tissues and for removing toxic wastes. In studies of SCI, VEGF has been shown to: 1) promote angiogenesis (Facchiano et al., 2002; Figley et al., 2014; des Rieux et al., 2014), 2) decrease the glial scar (Widenfalk et al., 2003), 3) increase white matter sparing (Facchiano et al., 2002), 4) increase gray matter sparing (Figley et al., 2014), 5) promote neuroprotection (Facchiano et al., 2002), 6) promote neuritogenesis into the lesion (des Rieux et al., 2014), 7) promote oligodendrogenesis and improved myelin integrity (Sundberg et al., 2011), 8) reduce tissue lesion volume (Widenfalk et al., 2003), and 9) promote improved locomotion (Facchiano et al., 2002; Kim et al., 2009; Liu et al., 2009; Yamaya et al., 2014). Therefore, VEGF appears to be a promising target for repair of the injured nervous system, due to trauma and degenerative diseases.

However, the main factors for consideration in applying this trophic factor in models of SCI are time point of administration and VEGF concentration, as some studies have observed exacerbation of SCI lesion (Benton and Whittemore, 2003), likely due to early time point after SCI insult and supraphysiological doses of VEGF. It is also important to consider that VEGF administered alone might be insufficient to promote neuroprotection, axon regeneration/sparing, functional synapse formation, and improved functional

recovery following SCI. Current literature suggests that VEGF in combination with other therapeutic approaches for SCI appears to hold the greatest potential for promoting angiogenesis, neuroprotection, axonal regeneration, and functional recovery (de LaPorte et al., 2011; Chehrehasa et al., 2014; Gong et al., 2015).

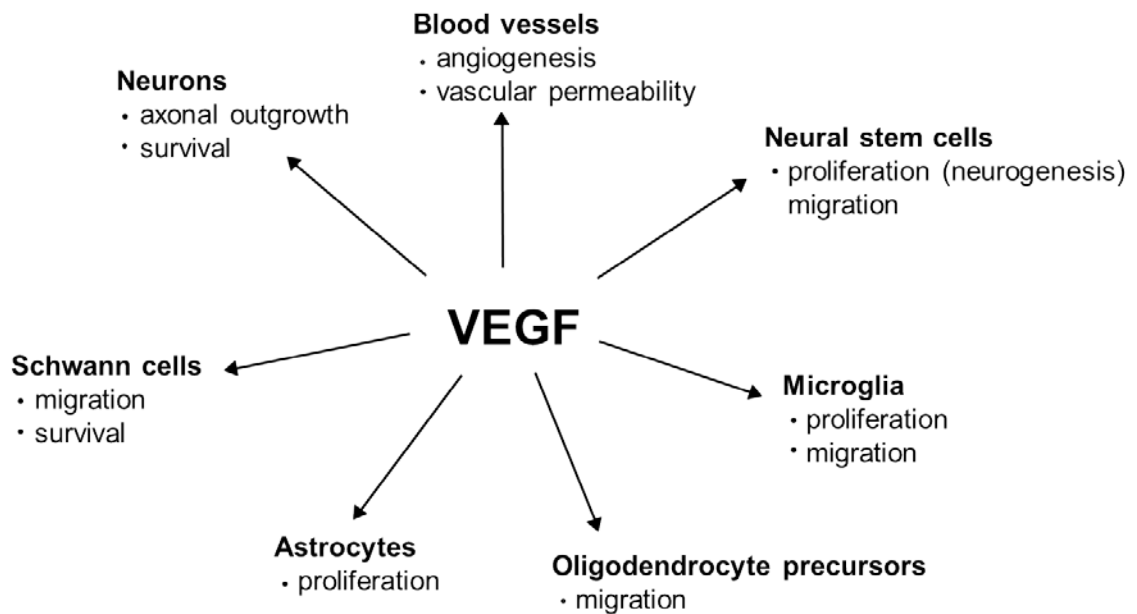


Figure 4. VEGF Pleiotropism. Influence of VEGF on vasculature, neurons, neural stem cells, and glial cells (astrocytes, Schwann cells, microglia, and oligodendrocyte precursors).

PART II: Glial cell line-derived Neurotrophic Factor and Spinal Cord Injury

SCI Background and Need for Therapies

Spinal cord injury (SCI) is a devastating chronic condition for which no effective treatments currently exist. Singh, Fehlings et al. (2014) conducted a systematic review of global statistics, beginning with 5,874 articles with a final inclusion of 48 articles, reporting worldwide SCI statistics, with the United States having the highest prevalence (906 cases per 1 million people); New Zealand having the highest reported national incidence (49.1 cases of SCI per 1 million people); and Spain (8 cases of SCI per 1 million people) and Fiji (10 cases of SCI per 1 million people) showing the lowest national incidences. The primary cause of SCI cases worldwide is motor vehicle accidents, followed by falls and sports injuries, for most countries (Singh et al., 2014). The long-term potential of chronic pain, inflammation, and devastating disabilities that SCI patients endure are compounded by the extensive lifetime costs of care. Approximately 1 - 4.5 million United States dollars is spent over the lifetime of an SCI patient, depending upon the patient's age and level of injury (Christopher Reeve Foundation website, NSCISC – National Spinal Cord Injury Statistical Center). The national cost in the United States is estimated at more than \$400 billion US dollars for current and future healthcare for patients suffering from SCI.

The initial SCI mechanical trauma disrupts local vasculature and leads to a breakdown of the blood-spinal cord barrier (Noble and Wrathall, 1987; Popovich et al. 1996; Schnell et al., 1999). This is followed by secondary wave of injury (Schwab and Bartholdi, 1996), comprised of hemorrhage, microvascular

dysfunction (Fassbender et al., 2011), ischemia (Tator and Fehlings, 1991), excitotoxicity, edema, neuronal apoptosis, loss of gray and white matter tissue (Tator and Koyanagi, 1997), axonal die-back, chronic inflammation (Mautes et al., 2000; Oudega 2013; Blomster et al., 2013), and the formation of a dense astrocytic glial scar. During the acute phase after SCI, the astrogliosis is presumed to contain the spread of excitotoxic molecules, thus limiting the lesion area. However, in chronic phases after SCI, this inhibitory scar impedes axonal regeneration and tissue repair by surrounding the lesion area and creating a structural border, separating injured from spared tissue. Literature has shown GDNF's promising influence on reducing astrogliosis (Iannotti et al., 2003; Deng et al., 2011a; Ansorena et al., 2013), in addition to its known neuroprotective effects, thus making astrocytes a target for potential therapies for SCI.

Discovery of GDNF Family Ligands and Receptors

The Glial cell line-derived neurotrophic factor (GDNF) subfamily of neurotrophic ligands consists of GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN), which bind to the glycosylphosphatidylinositol-anchored GFR α receptors 1-4, respectively (Trupp et al., 1998). The molecular structures of the GDNF family ligands and receptors are nicely detailed by Wang (2013), as well as in Figure 5. While ARTN (Widenfalk et al., 2009; Wong et al., 2015), NRTN (Buj-Bello et al., 1997; Horger et al., 1998; Golden et al., 2003), and PSPN (Tomac et al., 2002; Milbrandt et al., 1998) have all been shown to be neuroprotective, this chapter focuses specifically on GDNF and its applications for the treatment of spinal cord injury (SCI).

GDNF was first identified as a neurotrophic factor released from glial cells by Engele et al. (1991) and Lin. et al. (1993), in its promotion of the survival of dopaminergic neurons. The GFR α -1 receptor was first reported in *Cell* in 1996 (Jing et al.), following its isolation, cloning, and characterization from rat retinal cells; a study which also detailed the interaction between GDNF, GFR α -1, and the cRET receptor. Interestingly, the following week a *Nature* publication (Treanor et al., 1996) revealed concurrent work with similar findings on a cloned and characterized GFR α -1, as well as the GDNF, GFR α -1, and cRET multi-subunit receptor complex.

Localization of GDNF and its Receptors

Expression patterns of GDNF, GFR α -1, and cRET indicate that the three are not mutually exclusive for GDNF's trophic actions, as GFR α -1 is expressed in regions lacking cRET, and cRET has expression in regions lacking GFR α -1 expression, well-characterized by Trupp et al. (1997). In 1996, Trupp et al. identified GDNF's activation of the cRET proto-oncogene, resulting in neuronal survival, while Jing et al. (1996) identified GFR α -1 as mediating the interaction between GDNF and cRET. In 2001, Nicole et al. demonstrated the expression of GDNF mRNA and protein, as well as GFR α -1 and cRET on both neurons and astrocytes. Heparan sulphate, a key glycosaminoglycan, was identified as crucial for the phosphorylation of the c-Ret co-receptor, thus, also necessary for GDNF signaling through its GFR α -1 receptor (Barnett et al. 2002).

Satake et al. (2000) showed a dramatic upregulation of GDNF mRNA expression within 3 hours post SCI that was maintained for approximately 2-4

weeks following injury. Additionally, changes in GDNF's expression pattern following CNS injury are nicely illustrated by Trupp et al. (1995, 1997) and Donnelly and Popovich (2008). GDNF targets in the CNS and PNS, as well as the administration of GDNF gene therapy for motoneuron protection were nicely highlighted in a review by Bohn (2004).

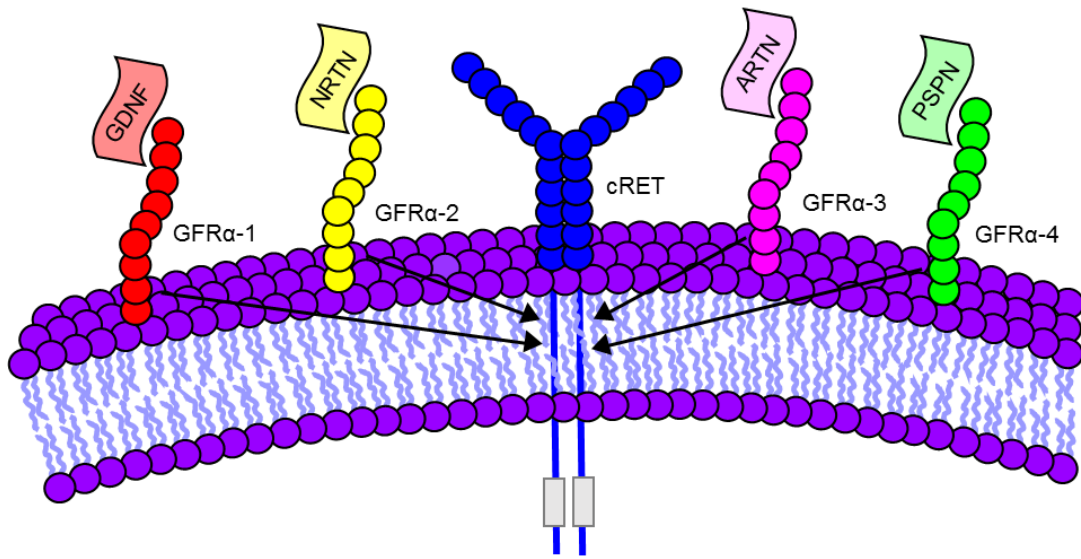


Figure 5. GDNF Family of Ligands and Receptors. GDNF binds to GFR α -1, NRTN binds to GFR α -2, ARTN binds to GFR α -3, and PSPN binds to GFR α -4. GFR α 1-4 bind to cRET co-receptors.

GDNF Promotes Cell Survival and Growth

One of the earliest studies to report GDNF induced reduction of astrogliosis was a study by Trok et al. (1996), in which spinal cord explants were allotransplanted into Sprague-Dawley anterior eye chambers. GDNF was shown to promote graft survival and growth, in addition to the reduced GFAP immunoreactivity. Klocker et al. (1997) identified a new subpopulation of neurons responsive to GDNF in a study showing significantly reduce cell death of axotomized retinal ganglion cells in response to GDNF treatment. The upregulation of GDNF in the distal portion of peripheral injured nerves was assessed and quantified, along with the localization of its cRET receptor, as reported by Bar et al. (1998). Similarly, Hoke et al. (2002) showed upregulation of GFR α 1 receptor on the distal segment of the sciatic nerve following injury; this upregulation and the upregulation of GDNF by Schwann cells was maintained for approximately six months following injury. The GFR α 1 receptor was localized to peripheral Schwann Cells in a study by Hase et al. (2005), showing another target of GDNF for the repair of injured nervous system. Arce et al. (1998) reported a 75% inhibition of neuron survival after exposure to Schwann cell cultured media containing a blocking antibody against GDNF. This study demonstrates the importance of GDNF for the Schwann cell-mediated neuroprotection. Paratcha et al. (2001) highlighted the recruitment of cRET to neuronal cell membrane lipid rafts, in response to soluble GFR α 1. Rind et al. (2002) showed anterograde transport of GDNF in dorsal root ganglion (DRG) and motor neurons, both with undetectable levels of GDNF mRNA in their current

state. The radiolabeled GDNF in this study was provided to the DRGs and motor neurons and by Schwann cells and oligodendrocytes, respectively. In 2004, a novel *in vivo* study was published showing for the first time the endogenous release of GDNF from astrocytes, which was neuroprotective to neighboring neuronal populations, in utero during development (Zhao et al.).

GDNF Signaling for Cell Survival

In addition to its neuroprotective effects (Oppenheim et al., 1995; Beck et al., 1995; Tomac et al., 1995), GDNF has also been shown to: 1) attenuate astrocyte cell death via reduced activation of caspase-3 (Yu et al., 2007) as well as through caspase-3/Akt independent mechanisms (Chu et al., 2008); 2) minimize activation of microglia and production of nitric oxide (Xing et al., 2010; Hermann et al., 2001); and 3) promote the survival (Liu et al., 2014) and proliferation (Hoke et al. 2003; Zhang et al., 2009) of Schwann cells. GDNF activates rat primary cortical microglial cells through GFR α -1 and cRET receptors, with downstream signaling through the MAPK pathway, as illustrated in a study by Honda et al. (1999). This study demonstrates microglia as another putative therapeutic target for GDNF in CNS injury and disease. However, a pro-inflammatory response, resulting in increased levels of IL-1 β likely led to the GDNF neuroprotection observed in a lipopolysaccharide (LPS)-induced nigral degeneration model of Parkinson's disease (Iravani et al., 2012).

Soler et al. (1999) characterized the downstream signaling of GDNF in motoneurons, which includes activation of both the PI3K and ERK-MAPK pathways. Further investigation revealed that the neuroprotective effects of

GDNF signaled through the PI3K pathway (Soler et al., 1999). In 2001, Nicole et al. described a novel mechanism of cortical neuroprotection from excitotoxicity-induced necrotic cell death after GDNF application; however, in this study GDNF failed to rescue cortical neurons from apoptotic cell death. Moreover, this study illustrated the indispensable nature of the MAPK (MEK) pathway, and GDNF's reduction of NMDA-triggered calcium influx, resulting in the attenuation of necrotic cell death. However, glutamatergic excitotoxicity induced by non-NMDA agonists (AMPA and kainate) was unable to be attenuated by GDNF administration (Nicole et al., 2001). Additionally, this study highlighted GDNF's neuroprotective effects were likely through diminished NMDA receptor activity and not the result of free radical scavenging. Cheng et al. (2002) investigated the downstream neuroprotection signaling of GDNF and determined that GDNF activated the MAPK signaling pathway and resulted in increased levels of Bcl-2. Liu et al. (2014) described a similar upregulation of Bcl-2 and downregulation of Bax, which provided neuroprotection *in vitro* and Schwann cell survival *in vivo*, in rats treated with Schwann cells overexpressing GDNF, as compared to SCI rats.

Studies Employing GDNF for SCI Repair

After avulsion injury, axotomized motoneuron cell death was reduced by 50% and somatic atrophy was reduced, after treatment with GDNF (Li et al., 1995). In another study of avulsion injury, GDNF administered via AAV-viral vector significantly attenuated spinal cord ventral horn motor neuron death (Watabe et al., 2000). In one of the earliest studies of GDNF administration after

SCI, Ramer et al. (2000) reported the ability of GDNF to rescue spinal cord motoneurons. In a contusive SCI model, GDNF showed significant improvement in motor function (Basso, Beattie, Bresnahan, BBB locomotor rating scale), increased cell survival and number of spared neuronal fibers compared to PBS-controls (Cheng et al., 2002).

Iannotti et al. (2004) reported significantly increased spared white matter and significantly attenuated lesion volume in response to GDNF administration via an osmotic minipump, following contusive SCI. Quite noteworthy, Mills et al. (2007) described the GDNF enhancement of axonal regeneration occurs within a narrow therapeutic dosage range. In a compressive clip model of SCI, Kao et al. (2008) demonstrated significantly improved motor functional recovery (inclined plane), significantly reduced infarct zone, a dramatic increase in the number of VEGF-positive and GDNF-positive cells (undetectable in sham and SCI-only groups), and significantly reduced TUNEL staining.

GDNF Combinational Therapies for SCI Repair

Iannotti et al. (2003) showed robust remyelination, axonal regeneration, and reduced cavitation, as well as modest yet significantly reduced astrogliosis and immune infiltration, in response to GDNF releasing matrigel guidance channels transplanted following hemisection SCI. Additionally, there was synergistic promotion of axonal regeneration and myelination in response to guidance channels containing both Schwann cells (SCs) and GDNF (Iannotti et al., 2003). Despite significant axonal regrowth into the SCI lesion site accompanied by the recruitment of myelinating Schwann cells, Blesch and

Tuszynski (2003) highlighted the difficulty of promoting axonal regrowth through and beyond the lesion site, following secretion of GDNF from genetically modified, transplanted fibroblasts. In a novel study of chronic spinal cord injury, using a peripheral nerve graft, GDNF treatment enhanced axonal regeneration by 7-fold compared to controls (Dolbeare and Houle, 2003). In a study with Schwann cell seeded-guidance channels (Zhang et al., 2009) observed significantly enhanced axonal regeneration, myelination, and number of blood vessels within the regenerated tissue. GDNF was also shown to increase the diameter of the regenerated axons in this study (Zhang et al., 2009).

The inhibitory astrogliosis was positively modulated and an intermingling of host and graft tissue was observed at the hemisection lesion interface, in a combinational study of GDNF and Schwann cells (SCs) in semi-permeable guidance channels (Deng et al., 2011b). In a moderate, contusive, midline SCI study, we observed a harsh glial scar border juxtaposed to the host spinal cord tissue after administration of saline, our novel hydrogel (non-immunogenic, *in situ* gelable, bioengineered hydrogel comprised of a multi-arm thiolated polyethylene glycol, thiolated laminin-derived loopshaped short peptide sequences (CDPVCC GTARPGYIGSRGTARCCAC), thiolated hyaluronan, and thiolated recombinant human collagen; Li et al., 2013; 2014), and hydrogel + brain-derived neurotrophic factor (BDNF). In contrast, in the presence of GDNF an intermingling of glial fibers into the host spinal cord tissue (Figure 6) occurred (Xu *unpublished*). Another notable study was performed by Zhao et al. (2004) in which GDNF reduced axotomy-induced astrogliosis of the facial nerve. In a more

recent study, a growth-promoting bridge was formed by transplantation of Schwann cell-seeded guidance channels, with Schwann cells overexpressing GDNF (Deng et al., 2013). This GDNF overexpression modulated the inhibitory astrocytic glial scar, created a more permissive environment for propriospinal axonal regrowth through and beyond the distal end of the lesion, conducted electrical signals through the lesion gap, and improved functional recovery (Deng et al., 2013). This study highlights the importance of combinational treatment approaches for traumatic spinal cord injury.

In another combinational treatment approach, GDNF was embedded into an alginate hydrogel for slow release and employed in a hemisection SCI model (Ansorena et al., 2013). In this study, GDNF promoted increased functional recovery, increased numbers of intralesional and perilesional neurites, reduced astrogliosis, and increased intralesional vasculature, as compared to controls. Using PLGA (polylactide-co-glycolic acid) microspheres for slow release, Zhang et al. (2013) administered GDNF, Chondroitinase ABC, and a Nogo A antibody following a transection SCI. Lu et al. (2012) showed remarkably robust axonal regeneration up to 12mm in length, in a severe SCI transection model (2mm of cord removed), with a combinational treatment approach including transplantation of neural stem cells in fibrin matrices containing a trophic factor cocktail (GDNF, BDNF (brain-derived neurotrophic factor), PDGF-AA (platelet-derived growth factor), NT3 (neurotrophin-3), IGF-1 (insulin-like growth factor 1), EGF (epidermal growth factor), aFGF (acidic fibroblast growth factor), bFGF (basic fibroblast growth factor), HGF (hepatocyte growth factor), and calpain

inhibitor/MDL28170). Moreover, this tissue graft resulted in: 1) significantly enhanced motor recovery, 2) significantly improved electrical signals across the lesion gap, 3) survival and differentiation of the neural stem cells, 4) an intermingling of host axons into tissue grafts, 5) increased myelination, and 6) functional synapse formation likely leading to the observed significant improvement in locomotion (Lu et al., 2012). Collectively, these studies demonstrate the high potential of GDNF, particularly in combinational treatment approaches, for use for repair of the injured spinal cord.

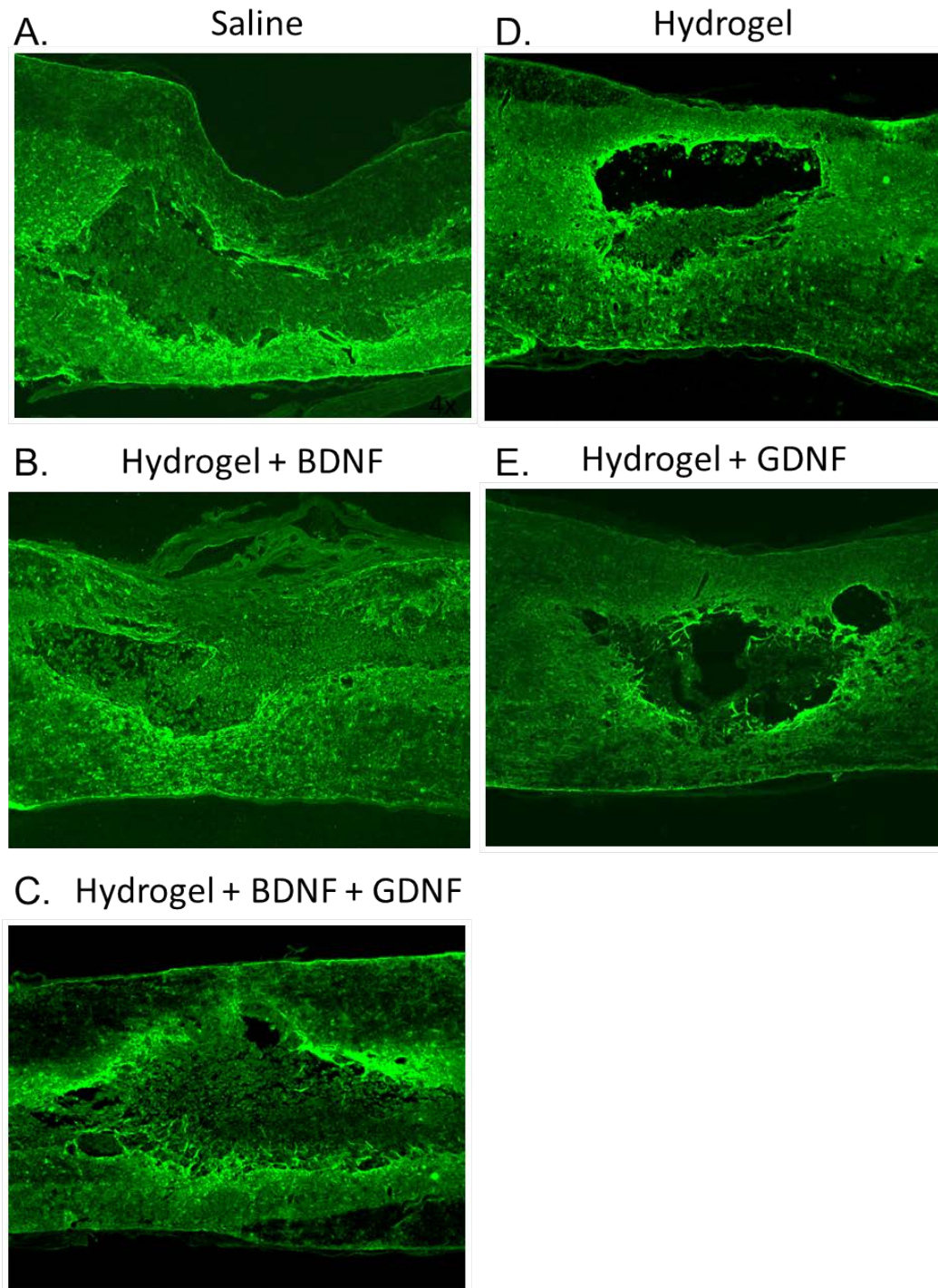


Figure 6. GDNF Modulation of the Inhibitory Glial Scar. A harsh border was observed with administration of Saline (A), Hydrogel (D), and Hydrogel + BDNF (B) after midline contusive SCI. Contrasted by a more permeable glial border with glial fibers appearing to intermingle with host lesion tissue in the presence of added GDNF, Hydrogel + GDNF (E), and Hydrogel + BDNF + GDNF (C).

PART III: Hydrogel, VEGF, and GDNF for Spinal Cord Injury Repair

Bioengineered Tissues and SCI

Bioengineered tissues serve as both structural and functional scaffolding matrices, through their capacity to serve as reservoirs for various pharmacological reagents, growth factors, and enzymes that would otherwise be broken down within minutes-to-hours within the lesion environment. Bioengineered tissues can be designed and synthesized to have various viscosities, rheological properties, and electrospun parameters for fiber alignment. The hydrogel used in this study was designed to match the tensile strength of the spinal cord (Li et al., 2013; 2014), and to have a release profile of approximately 3-4 weeks of embedded trophic factors (Figure 12; Xuejun Wen, unpublished).

Bioengineered tissues have been utilized for models of SCI primarily over the past two decades. Woerly and colleagues synthesize and transplanted a novel biocompatible hydrogel (Neurogel™) comprised of poly(N-[2-hydroxypropyl]methacrylamide) into a 3mm transection gap at the 5th thoracic cord, and observed angiogenesis and axonal regrowth into the bioimplant (2001b). Similarly, a study employing a bioengineered poly(D,L-lactic acid) guidance channel observed increased angiogenesis and neuron survival, following a 4mm thoracic complete transection SCI (Patist et al., 2004). Hurtado et al. (2011) observed robust axonal alignment and regeneration (up to 2mm) from a poly-L-lactic acid microfiber-based conduit, inserted into a 3mm gap in the thoracic spinal cord, after complete transection. Through the use of a

poly(ethylene glycol)-poly(serinol hexamethylene urethane) bioengineered tissue, Riffeld et al. (2014) demonstrated improved functional recovery, 66% increase in spared spinal cord tissue, and enhanced survival of the transplanted bone marrow stromal cells. This study displays the use of bioengineered tissues as matrices for transplanted cells, and promotes the combinational use of bioengineered tissues, beyond just structural scaffolds or reservoirs for trophic factor release. Other recent studies utilizing bioengineered tissues also demonstrate the importance of combinational therapies for SCI repair. For example, Rauck and colleagues (2015) observed that their novel poly(ethylene argininy laspartate diglyceride) and heparin coacervate did not provoke an immune response and nor did it affect the glial scar, axon density, or neuron sparing. Another beneficial aspect of this bioengineered tissue was that in combination with Sonic Hedgehog it decreased the intensity of the glial scar (Rauck et al., 2015), thus, creating a more permissive environment for tissue repair.

Dr. Peter Carmeliet has been vital in expanding and detailing the Belgian Anatomist, Andreas Vesalius' 1543 observations (*De humani corporis fabrica, On the fabric of the human body, 1543*) about the overlap of the nervous and vascular systems, thus, displaying the pleiotropic influence of VEGF on the nervous, vascular, and immune systems (Carmeliet and Tessier-Lavigne, 2005). The neurovascular evolution of VEGF and its influence on the nervous system was summarized by Zacchigna, Carmeliet and colleagues (2008), displaying the importance of the human VEGF homologue in *C. elegans* (*Caenorhabditis*

elegans) and *Drosophila melanogaster*, which lack blood vessels or have very few, respectively. Additionally, Popovici et al. (2002) described receptors on *C. elegans* neurons with structural similarity to the human VEGF receptors (VEGFR's), which can activate human VEGFR's (Zacchigna et al., 2008).

Rationale

We employed a novel, non-immunogenic, *in-situ* gelable bioengineered hydrogel to serve as a structural support matrix for growing axons, blood vessels, and astrocytic processes. This hydrogel then served as a functional reservoir with a time-controlled release of both VEGF and GDNF, and was designed to have an approximate release profile of 3-4 weeks (Figure 12; Xuejun Wen, unpublished). The biodegradable nature of our hydrogel along with the breakdown of VEGF and GDNF (by endogenous enzymes) give the treatment profile a terminal point, thus relieving any concerns of unregulated trophic factor administration, unregulated cell growth or tumor formation, which exist when using viral vector administration or cells genetically modified to overexpress trophic factors. The overall aim of this study was to create a permissive environment for lesion revascularization, neuronal and axonal sparing, tissue regrowth, and ultimately for functional recovery, after a moderate thoracic contusive SCI.

Preliminary data from pilot studies, with hydrogel transplanted at 2 days (acute phase), 7 days (subacute phase), and 4 weeks (chronic phase) post SCI, reflected that 7 days post SCI is a more optimal transplantation time point than either 2 days or 4 weeks (Figures 7 and 8). These time points were chosen in

order to determine the optimal time window of transplantation post SCI. Due to the inflammatory wave of secondary tissue damage after SCI, and the fact that the onset of cystic cavitation occurs at approximately 7 days post SCI. Anywhere from 7-14 days post SCI is on the tail-end of the inflammatory phase, and the beginning stages of cavitation and glial scar formation.

After performing a moderate, midline, thoracic contusive SCI with various transplantation time points for saline and hydrogel, we concluded that the hydrogel might be exacerbating the lesion area with transplantation at 2 days post SCI due to the fact that the onset of cavitation typically occurs at approximately 7 days post SCI (Figures 7 and 8). Thus, at 2 days post SCI, the cavity may not be developed to hold the hydrogel. Therefore, injection of 3-4 μ L of fluid that will then become semi-solid as it reaches body temperature, will compress the adjacent spinal cord tissues, and cause more injury than tissue protection or repair. Since the hydrogel is proposed to serve primarily as a structural support network for the injured spinal cord, 4 weeks post SCI is in the chronic injury stage, and the transplanted hydrogel may not confer any reduction in lesion area or cavity size. Seven days post SCI is the onset of cavitation, the onset of angiogenic vascular regression, and is at the tail-end of the inflammatory response as well as the early stages of the astrocytic (glial wall) response following injury (Donnelly and Popovich 2008).

Based on the pilot studies discussed above (Figures 7 and 8), we chose a transplantation time point of 7-8 days post SCI. However, in an attempt to minimize inflammation following SCI, 12 days post SCI was chosen for the

transplantation time point of the last study (Table 3). Most of the axonal regrowth observed will therefore be a result of axonal sparing or sprouting as opposed to neuroprotection, due to the 12 day transplantation time point which is late enough in the secondary phase of injury that not much neuroprotection is occurring.

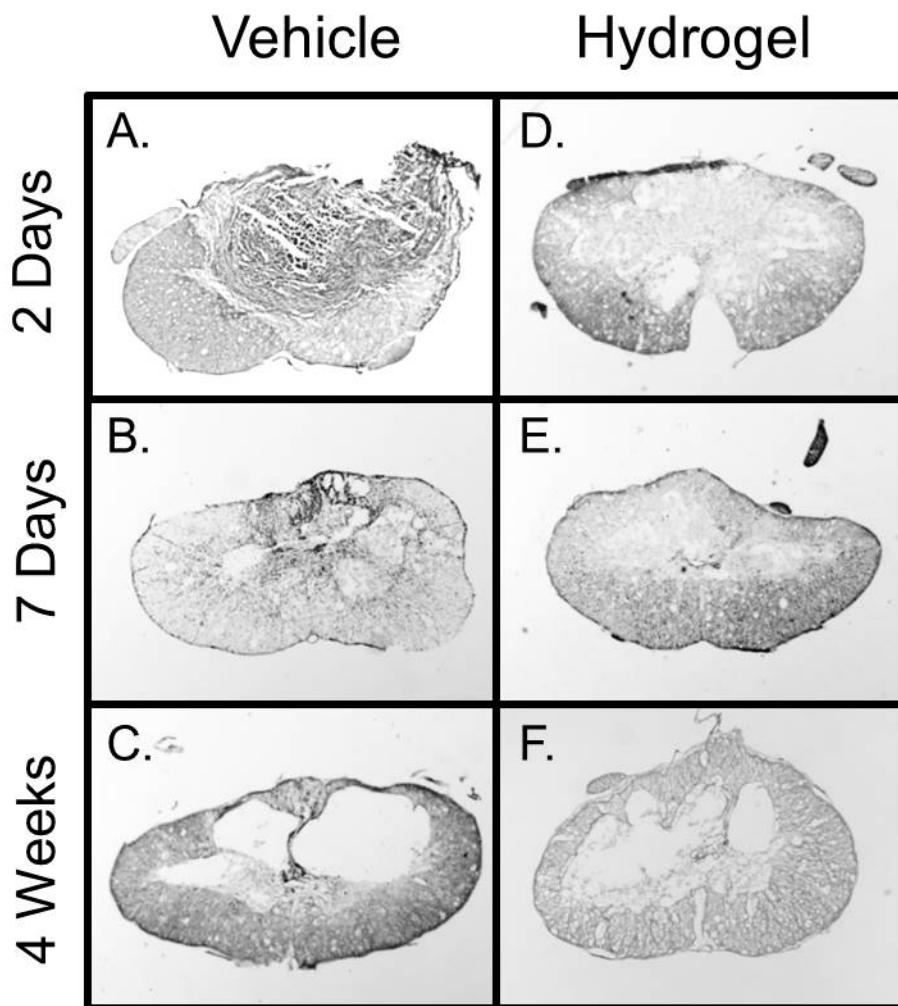


Figure 7. Histology of Hydrogel Transplantation Time Course Study. Saline (Vehicle) transplanted at 2 days (A), 7 days (B), and 4 weeks (C) post moderate, midline, contusive SCI. Hydrogel transplanted at 2 days (D), 7 days (E), and 4 weeks (F) post moderate, midline, contusive SCI.

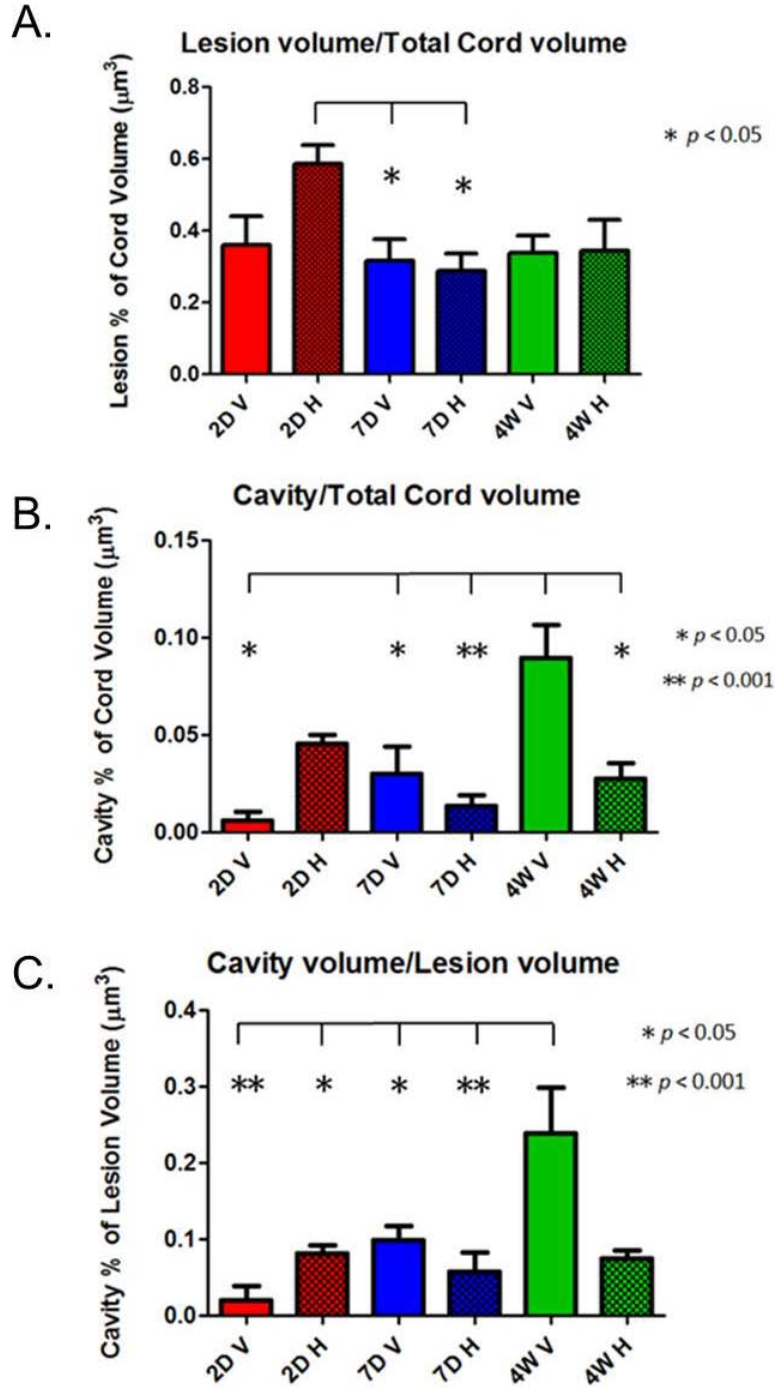


Figure 8. Quantification of Hydrogel Transplantation Time Course Study. (A) Lesion volume as percent of total cord volume. (B) Cavity volume as percent of total cord volume. (C) Cavity volume as percent of total lesion volume. V = Saline (Vehicle), H = Hydrogel, 2D = 2 days, 7D = 7 days, and 4W = 4 weeks.

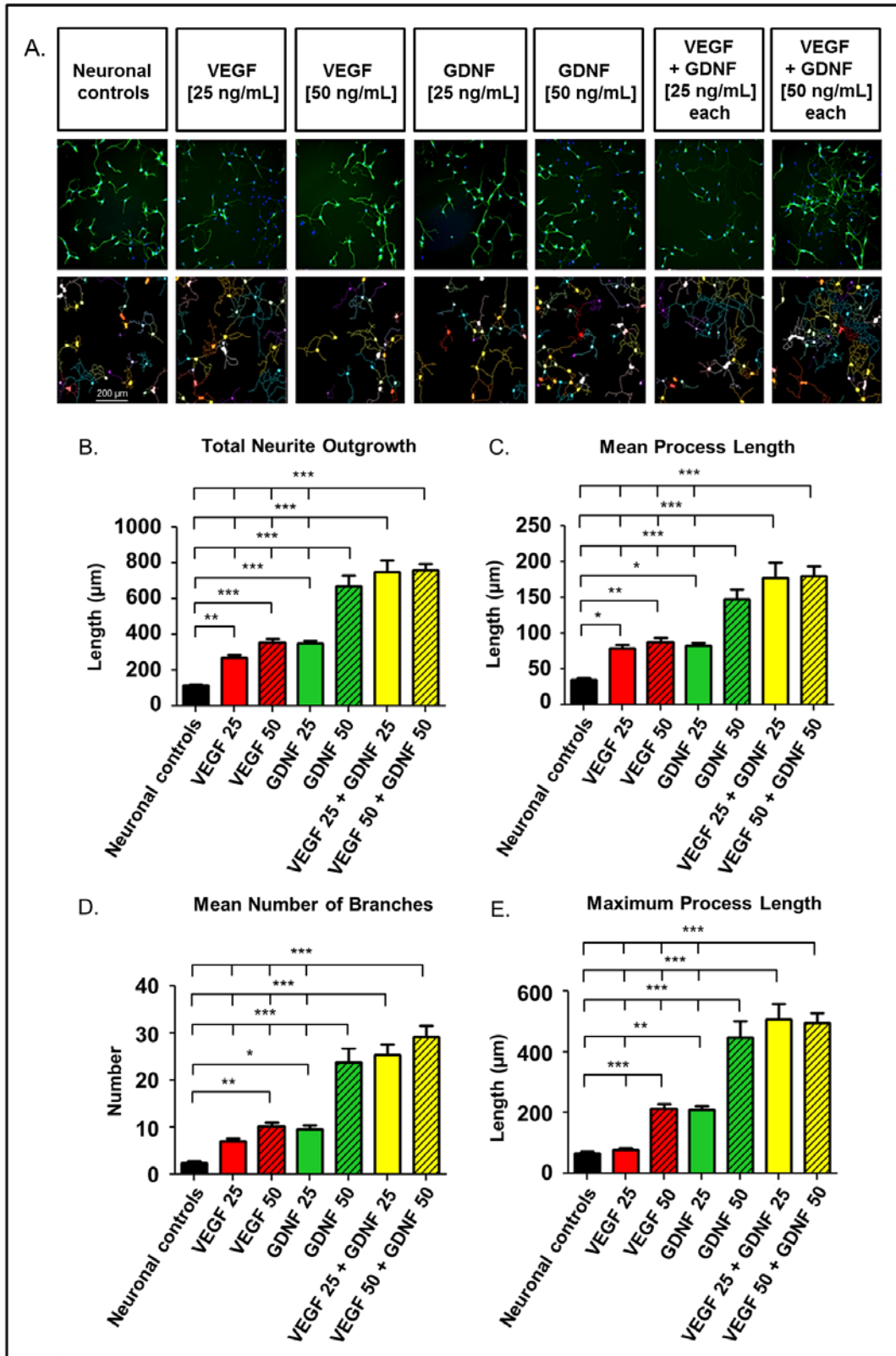
Materials and Methods

All animal procedures were performed in accordance with the approved Institutional Animal Care and Use Committee of the Indiana University School of Medicine guidelines (Protocol #10406).

***In vitro* Neurite Outgrowth of Spinal Cord Neurons**

Embryonic day 15 (E15) spinal cord neurons were isolated from Sprague-Dawley embryonic rats (Harlan, Indianapolis, IN) from a previously established protocol (Jiang et al., 2006). Briefly, E15 rat spinal cords were isolated and placed in Leibovitz's L-15 medium (Gibco). Meninges were carefully removed. Spinal cords were cut into small pieces, dissociated with 0.05% trypsin/EDTA for 15-20 min at 37°C, and then gently triturated. Neurons were placed in 37°C for 30 minutes, to allow for adherence and to eliminate glial cells and fibroblasts. Neurons were then plated on poly-L-lysine pre-coated 48-well plates (Corning® CellBind® Surface) at a density of 280,000 cells/well, and incubated in a humidified atmosphere containing 5% CO₂ (37°C), DMEM + 10% heat-inactivated fetal calf serum + 5% heat-inactivated horse serum + 2 mM glutamine. After 16 hours, medium was replaced with Neurobasal medium with 2% B27, 1% N2 and 2 mM glutamine (all from Life Technologies, Inc.). The purity of the cell culture was evaluated and estimated to be approximately 94% neurons. Forty-eight hours after plating, the medium (Neurobasal 2% B27 and 1% N2) was refreshed (half-on), and trophic factors were added (VEGF and GDNF) for treatment group wells. Neuronal control wells received half-on refreshed media only at this time point. Groups consisted of the following: 1)

neuronal (medium) controls, 2) VEGF 25 ng/mL, 3) VEGF 50 ng/mL, 4) GDNF 25 ng/mL, 5) GDNF 50 ng/mL, 6) VEGF 25 ng/mL + GDNF 25 ng/mL, and 7) VEGF 50 ng/mL + GDNF 50 ng/mL. 72 hours later, cells were fixed with 4% PFA. Cells were then stained for β -tubulin (neurites) and Hoechst 33342 (1:100; Invitrogen; nuclear marker), with FITC (Fluorescein isothiocyanate) secondary antibody used for detection of β -tubulin. Cells were imaged using the ImageXpress® Micro XL (Figure 11), and 90 cells per treatment group were analyzed using the MetaXpress® 5.1 software (Molecular Devices).



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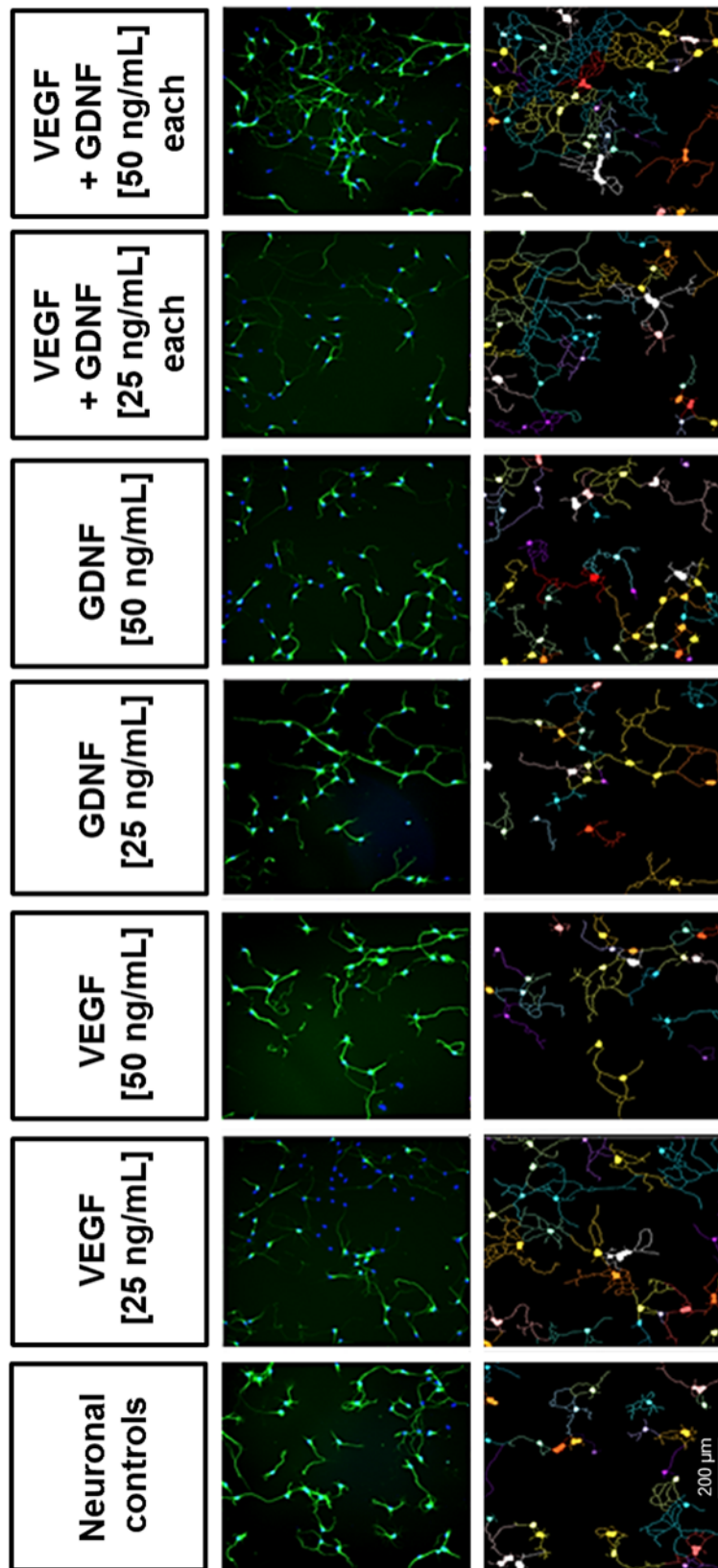


Figure 9. Neurite Outgrowth of E15 Spinal Cord Neurons. (A) Neurite outgrowth stained with β -tubulin (green) and Hoechst (blue), top row; computer software (MetaXpress®) generated pseudo-color schematic of outgrowth from individual neurons, bottom row). Neuronal controls are neurons with media that does not contain added VEGF or GDNF. (B) Total Neurite Outgrowth. (C) Mean Process Length. (D) Mean Number of Branches. (E) Maximum Process Length. (F) Larger image of (A). (n = 90 neurons per treatment group, all from one plating. All images are 20x magnification. All data include SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

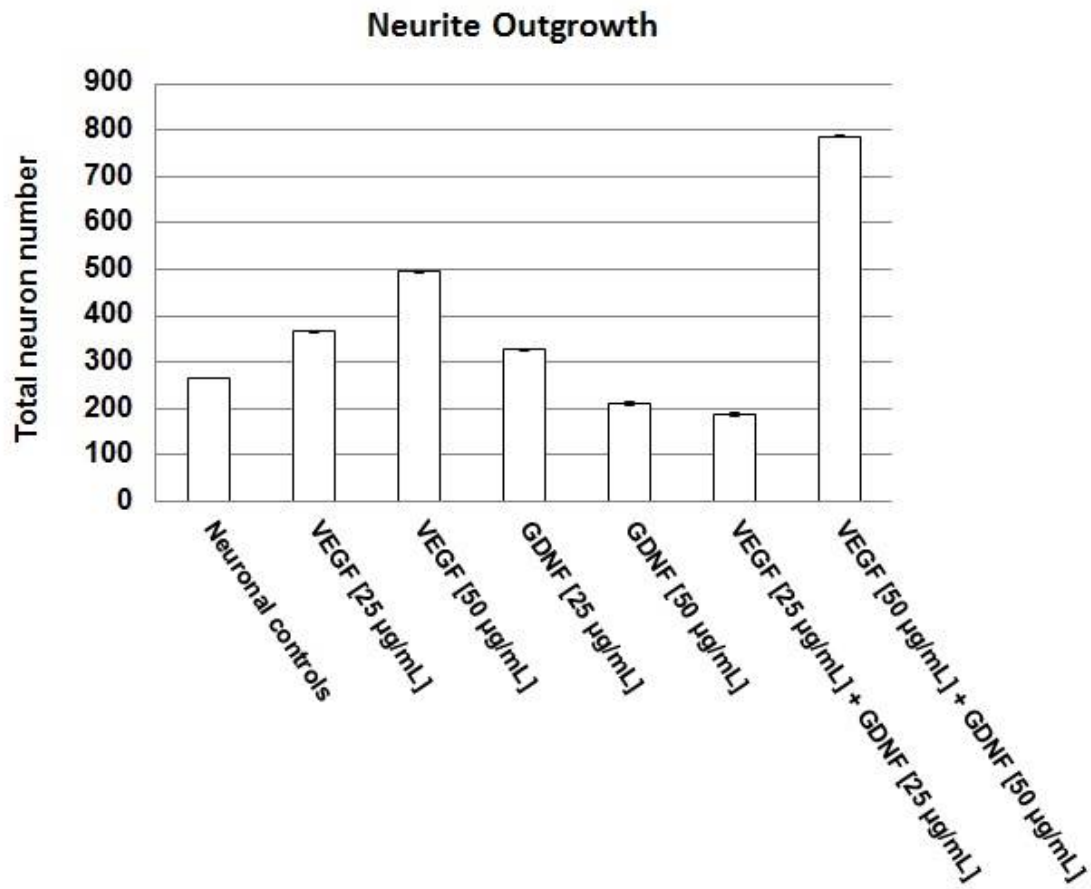


Figure 10. Neuroprotection by VEGF and GDNF. VEGF and GDNF resulted in significantly more E15 neurons, which appears to be largely an influence of VEGF, with a dramatic increase in neuron number for the combined VEGF + GDNF group at higher concentrations.

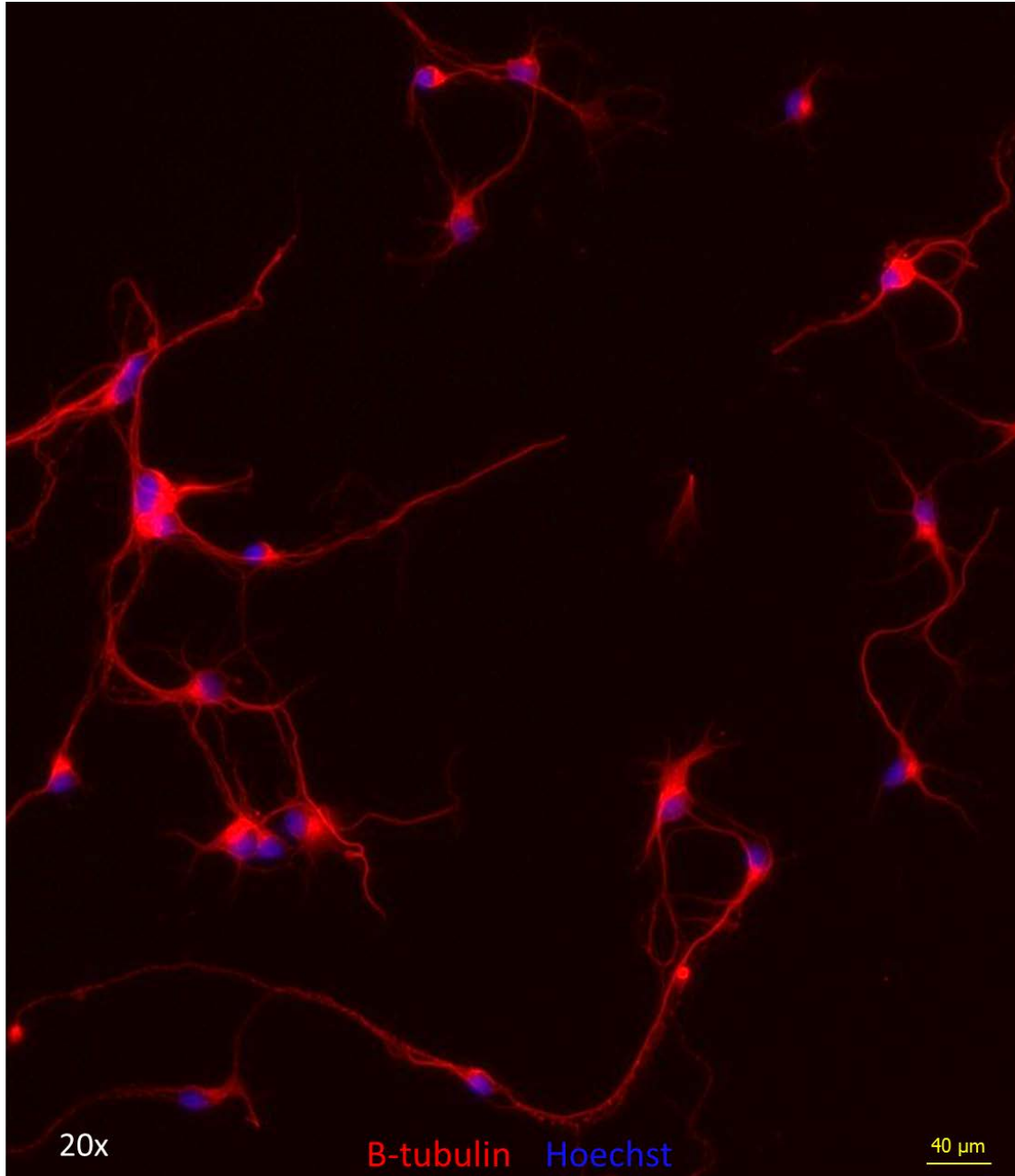


Figure 11. ImageXpress[®]. Neurons stained for β -tubulin and Hoechst for visualization of neurite outgrowth via ImageXpress[®] device as well as MetaXpress[®] software.

***In vivo* Hydrogel Combinational Therapy for SCI**

Novel Bioengineered Hydrogel

A novel, non-immunogenic, *in situ* gelable, bioengineered hydrogel comprised of a multi-arm thiolated polyethylene glycol, thiolated laminin-derived loopshaped short peptide sequences (CDPVCC GTARPGYIGSRGTARCCAC), thiolated hyaluronan, and thiolated recombinant human collagen was obtained from our collaborator, Dr. Xuejun Wen and Dr. Ning Zhang (Li et al., 2013; 2014). This non-immunogenic hydrogel provides a structural support scaffold to the surrounding tissues by conforming to the irregularly-shaped lesion cavities, as it solidifies into a semi-solid gel with similar mechanical properties to endogenous CNS tissue. At cooler temperatures, the gel remains in a liquid state, whereas, once it reaches body temperature it becomes semi-solid, conforming to the surrounding shape, thus, creating a physical continuity with the host spinal cord.

Animal Acclimation and Gentling

Forty adult female Sprague-Dawley rats (195-230g, Harlan, Indianapolis, IN) were acclimated to the housing facility, testing environments, and investigators (animal gentling) prior to evaluation. However, animals were not placed in the Treadscan™ for familiarity, prior to baseline assessments, as animals perform better when untrained on the Treadscan™ (personal communication with Johnny Morehouse, University of Louisville). Original *n* number (40) for statistical power analysis. Animal housing included a 12-hour light and 12-hour dark cycle, air filtration system, and food and water *ad libitum*.

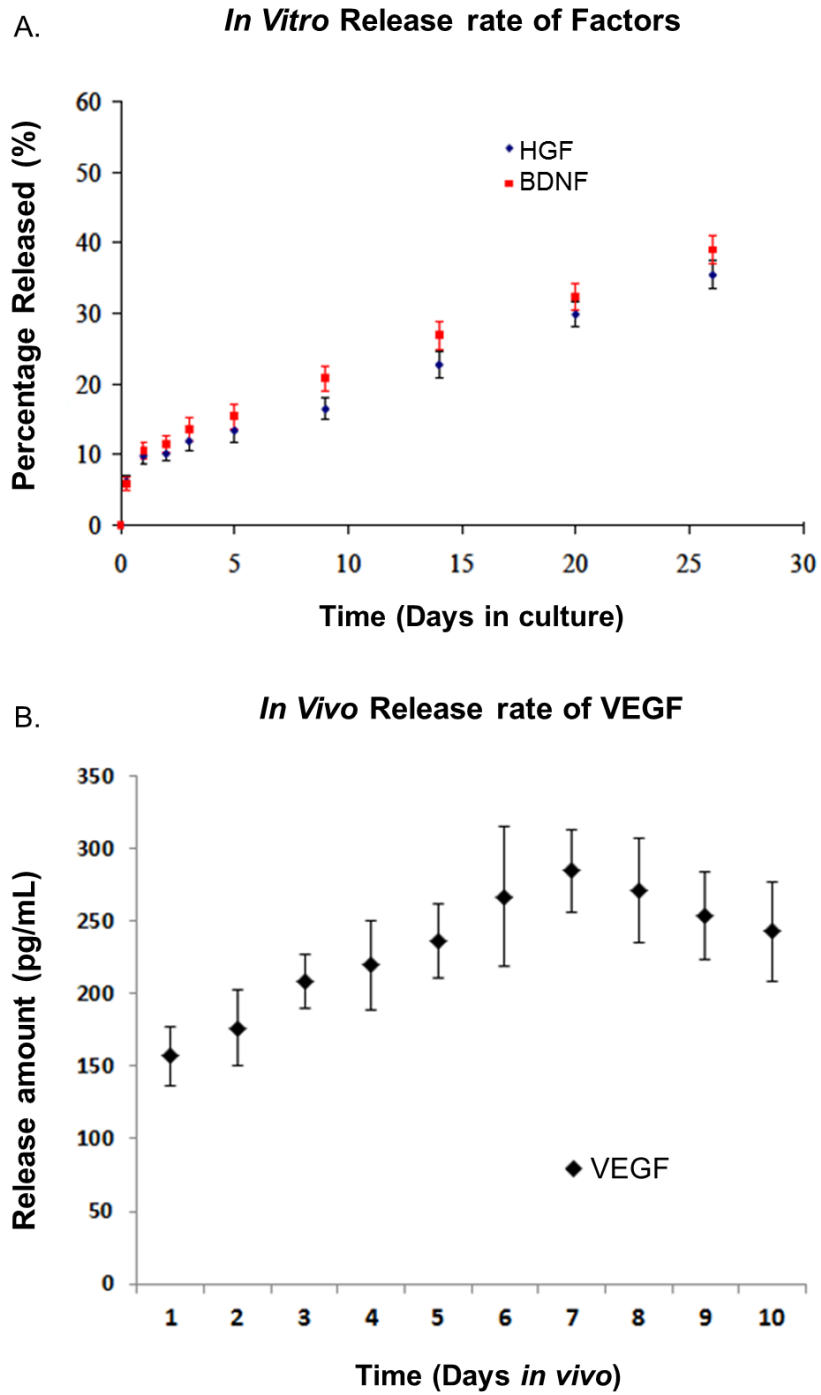


Figure 12. Hydrogel Release Profiles – *in vitro* and *in vivo*. (A) *In vitro* release profile of HGF and BDNF. (B) *In vivo* release profile of VEGF. Dr. Xuejun Wen (unpublished).

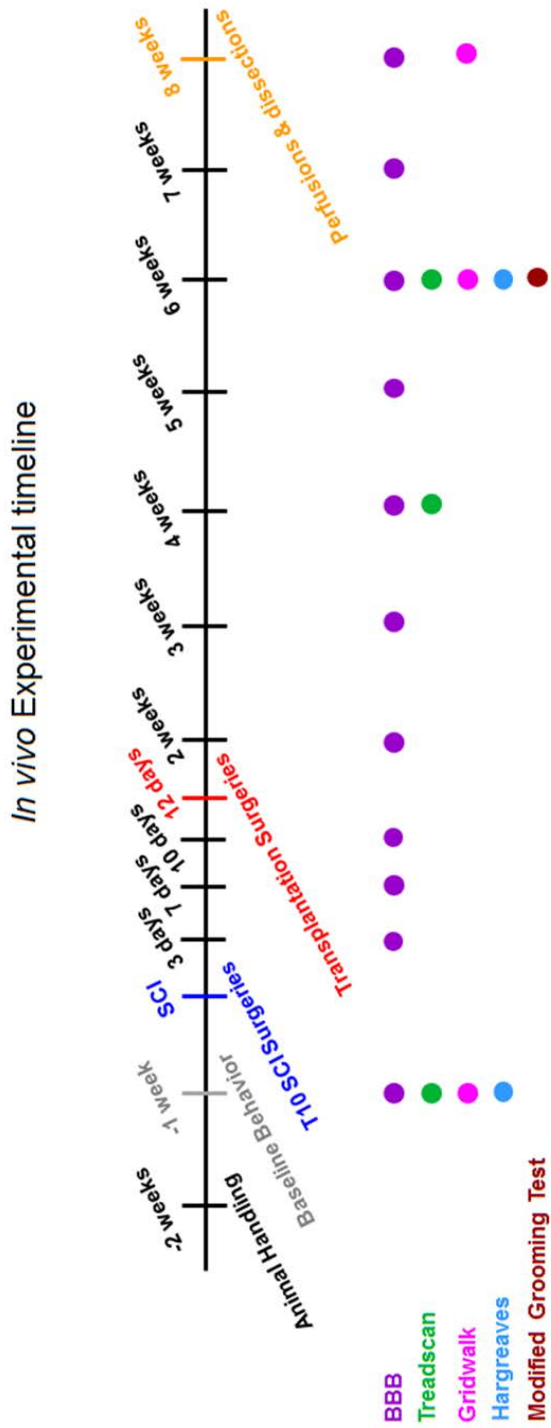


Figure 13. In Vivo Experimental Timeline. Moderate, contusive T10 SCI *in vivo* experimental design with transplantation of hydrogel, VEGF, and GDNF, and behavioral assessments.

Baseline Behavior

Forty animals were assessed for baseline motor and sensory function one week prior to SCI surgeries (Figure 13). Original *n* number of animals per group was calculated in order to have a high enough statistical power analysis for quantification of behavior and histological measurements. Assessments included the Basso Beattie and Bresnahan (BBB) open field locomotion, Treadscan™ (Clever Sys, Inc., Reston, VA), Gridwalk, and Hargreaves' thermal sensitivity measurement (Plantar Test Analgesia Meters, Harvard Apparatus). Each assessment will be explained in detail below.

Spinal Cord Injury Surgical Procedures

Rats were randomly assigned to receive Sham surgery (laminectomy only, *n* = 5) or T10 midline contusive SCI (*n* = 35). All animals were anaesthetized with an intraperitoneal (IP) injection of a Ketamine (40 mg/kg)/Xylazine (5 mg/kg IP) mixture. Animals were then placed on pre-warmed heating pads to maintain body temperature. A midline incision was made on the dorsal surface of the skin, and tissue and muscle retracted until the vertebral column was reached. Animals were placed in a custom vertebral stabilizer (Walker et al., 2015; Figure 15), with stabilization arms (Figure 15.B) affixed to the thoracic T10 vertebrae, and a laminectomy was performed on the T9 and T10 vertebrae, without durotomy. Midline thoracic contusive SCI was performed employing the Louisville Injury System Apparatus (LISA® device, *n* = 35; Figure 14), with a preset depth of 0.85 mm. Sham animals received laminectomy only. Injury time, velocity, and injury depth were recorded from the LISA® device and analyzed to ensure there were

no significant differences across animals or treatment groups (Figure 16). All animals received 5 mL subcutaneous injections of 0.9% saline for hydration following surgery. Animals were placed in temperature controlled housing with moist food and water, with monitoring for recovery. Once alert from anesthesia, animals were administered 0.2 mL [0.03 mg/mL] of Buprenorphine and 0.2 mL of [10 mg/mL] Baytril every 12 hours for the first 4 days. Post-SCI, animals' bladders were expressed manually 3 times daily, until bladder reflexes recovered.

Treatment Groups Pseudo-randomization

LISA® device SCI parameters (time, seconds; velocity, meters/second; and depth, millimeters) were assessed to ensure no significant differences existed across treatment groups. Animals were tested behaviorally (BBB) at 3 and 7 days post SCI, prior to transplantation surgery. SCI rats were then pseudo-randomized into treatment groups based on body weight and BBB scores, so that all five treatment groups had a mean BBB score of 10. Sham animals had a mean BBB of 21 points. Two animals died during SCI surgeries and one animal died during the transplantation surgeries due to anesthetic effects. Thus, after pseudo-randomization and transplantation surgeries our groups included: Sham (n = 5), Saline (n = 5), Hydrogel (n = 7), Hydrogel + VEGF (n = 6), Hydrogel + GDNF (n = 7), Hydrogel + VEGF + GDNF (n = 7).

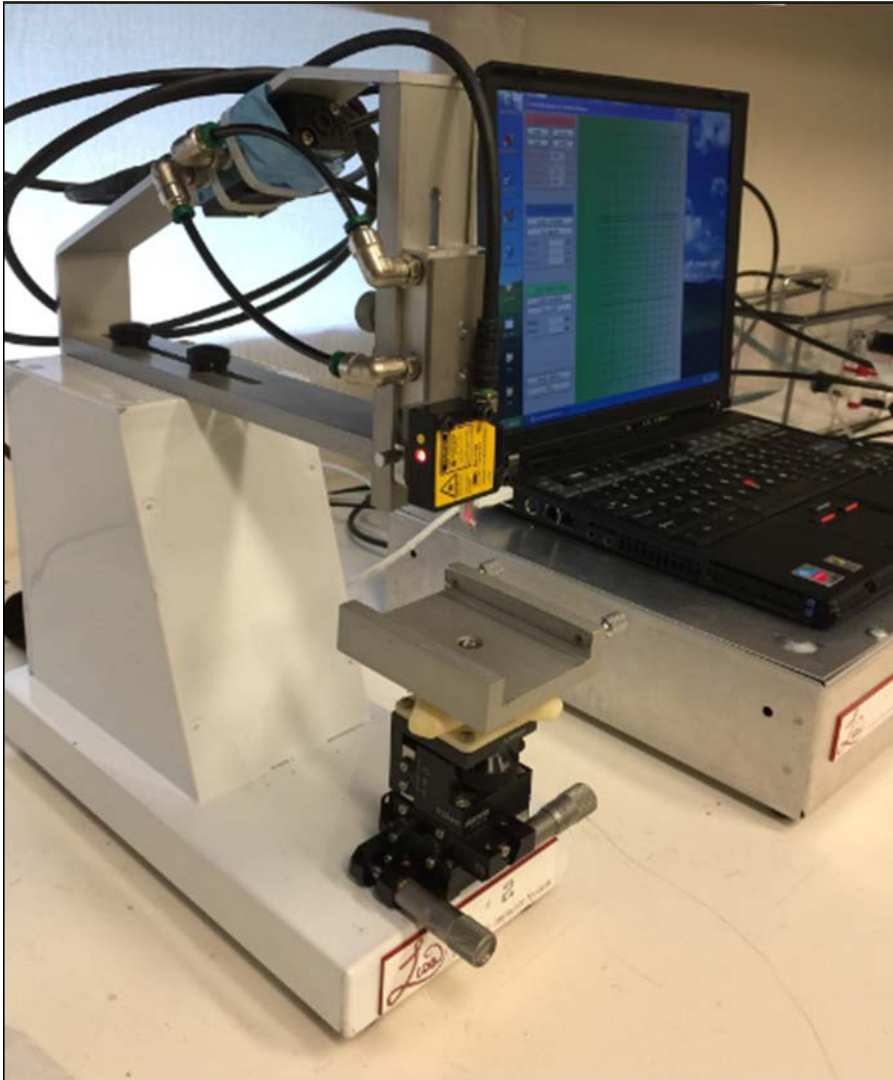
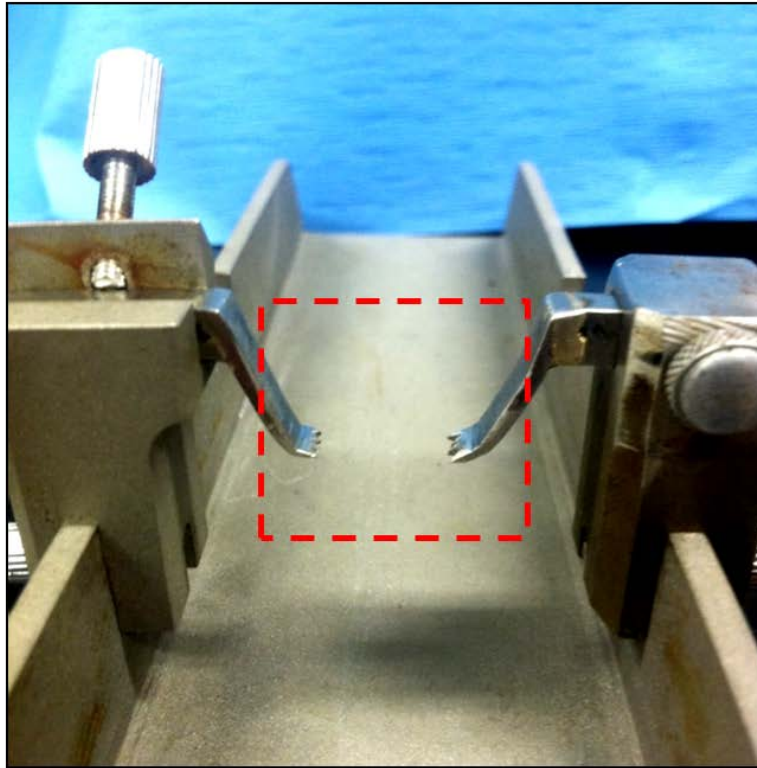


Figure 14. Louisville Impactor System Apparatus. LISA® injury device and associated laptop with software.

A.



B.



Figure 15. Surgical Stabilization Device. (A) U-shaped holder for surgical stabilization. (B) Serrated arms for lateral vertebral stabilization.

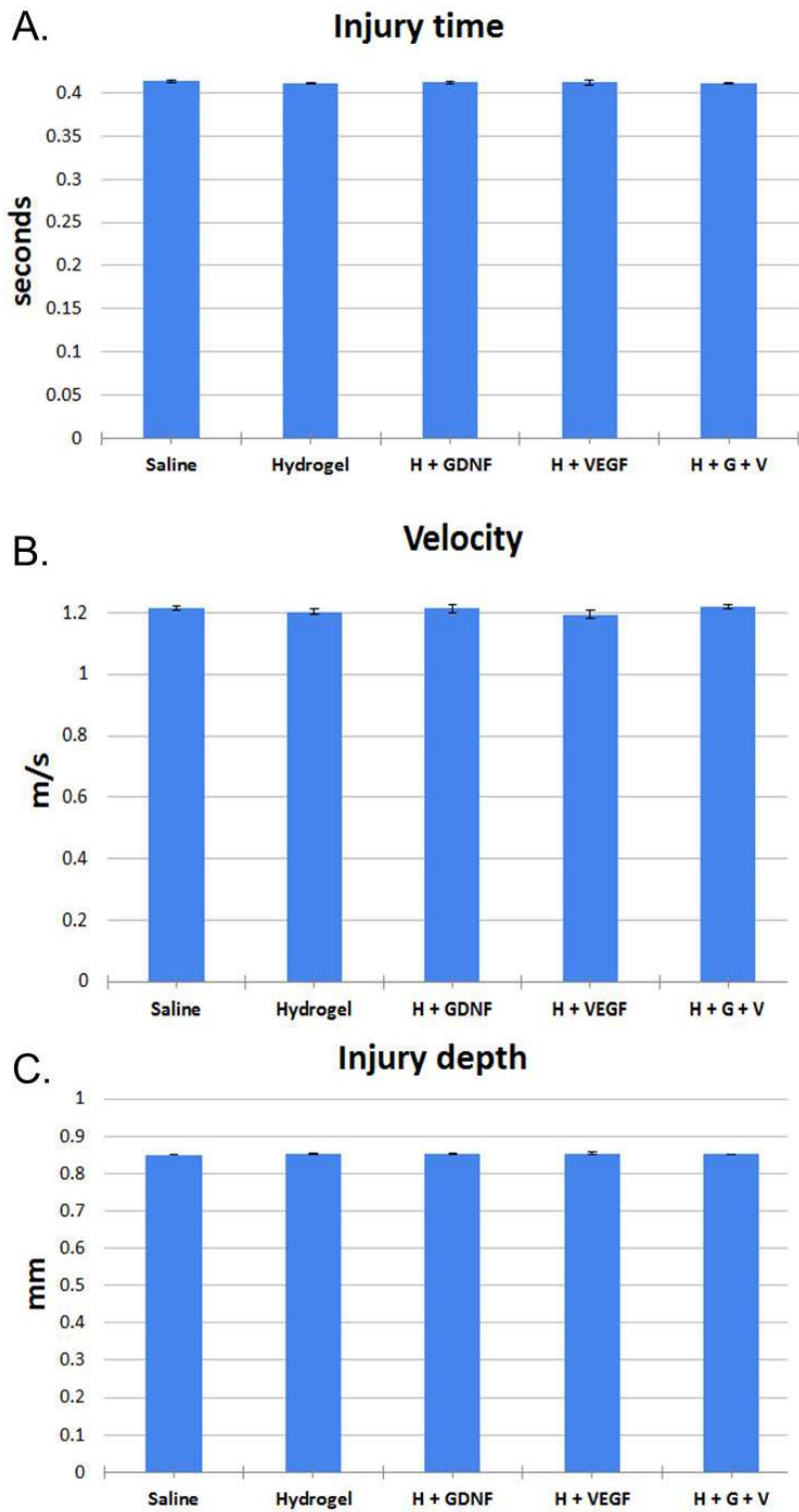


Figure 16. LISA® Injury Device Parameters.
 (A) Injury time, (B) Velocity, and (C) Injury depth.

Treatment Groups	Transplantation 12 days post SCI	Initial <i>n</i>	Cryosectioning 25 µm cross-sections	Behavioral Assessments	Sprague Dawley (female)	T10 contusion LISA	BBB 10 days post SCI	Final <i>n</i>
Sham	✓	<i>n</i> = 5	Cresyl-violet	BBB, Gridwalk, Hargreaves ¹ , Treadscan, Modified Grooming, tcMMEP	✓	✓	21	<i>n</i> = 5
SCI + Saline	✓	<i>n</i> = 5	RECA-1		✓	✓	10.6	<i>n</i> = 5
SCI + Hydrogel	✓	<i>n</i> = 6	GFAP		✓	✓	10.7	<i>n</i> = 5
SCI + Hydrogel + VEGF	✓	<i>n</i> = 8	SMI-31		✓	✓	10.6	<i>n</i> = 5
SCI + Hydrogel + GDNF	✓	<i>n</i> = 8	ED-1		✓	✓	10.5	<i>n</i> = 5
SCI + Hydrogel + VEGF + GDNF	✓	<i>n</i> = 8			✓	✓	10.8	<i>n</i> = 5

Table 3. *In Vivo* Experimental Design

Blinded Conditions

All rats, including sham, were coded with numbers and treatment group information remained separated from the coded numbers throughout the study. Thus, all behavioral testing, SCI and transplantation surgeries, histological analysis, and quantification were performed under blinded conditions with animal treatment group information separated from the investigators.

Transplantation Surgeries

At twelve days following SCI, animals received transplantation of Saline (n = 5), Hydrogel (n = 7), Hydrogel + VEGF (n = 6), Hydrogel + GDNF (n = 7), or Hydrogel + VEGF + GDNF (n = 7). Hydrogel components were mixed together approximately 12 hours prior to transplantation surgeries, with VEGF [15 µg/mL] and GDNF [5 µg/µL], or both factors added to the hydrogel within 6 hours prior to transplantation surgeries. Animals were anesthetized with Isoflurane, USP (Piramel Healthcare, NDC 66794-013-10) administered up to effect (for each animal). A midline incision was made on the dorsal surface, and tissue and muscle were retracted until the vertebral column was reached. Tissue was cleared until the T10 a vertebra was reached, and the dural suture located (directly above injury epicenter). Hydrogel with and without trophic factors was kept on ice prior to and during the transplantation surgeries, otherwise the hydrogel would become a semi-solid gel in the tube or glass pipette. The glass pipettes were then loaded with saline, hydrogel, or hydrogel plus corresponding trophic factor. Surgeon was kept blinded as to treatment group. A stereotaxic frame was used to lower the glass pipettes to reach a depth of 0.8 mm into the

spinal cord. Injections (Saline or Hydrogel) were made at injury epicenter via pressure injection through hand-pulled beveled glass pipettes (outer diameter 50-70 μm). The total volume of injection was 3 μL per rat, at approximately 1 $\mu\text{L}/\text{min}$, 0.8 mm in depth, and occurred over duration of approximately 3 minutes, with a needle dwell time of approximately 5 minutes to prevent hydrogel or saline leakage. Following surgery, all animals received 5 mL subcutaneous injections of 0.9% saline for hydration and were placed in temperature controlled housing with moist food and water, with monitoring for recovery. Once alert from anesthesia, animals were administered 0.2 mL [0.03 mg/mL] of Buprenorphine and 0.2 mL of [10 mg/mL] Baytril every 12 hours for the first 4 days. Animals' bladders were expressed 2-3 times daily, until reflexes returned.

Behavioral Assessments

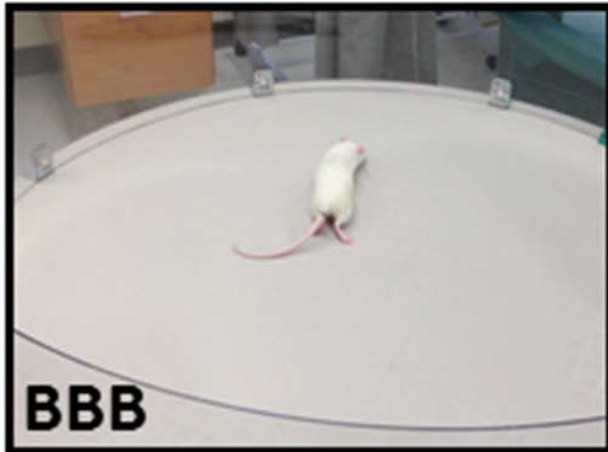
Part I: Treadscan™ Gait Analysis

Quantitative measures of locomotor stability and coordination were measured using the TreadScan™ system (Beare et al., 2009). The Treadscan™ apparatus consists of a motorized treadmill with translucent belt, beneath which angled mirrors allow a digital camera to capture the locomotion of the animals. A plexiglass chamber sits above the treadmill to ensure a consistent frame of the animal walking for digital capture. The accompanying Treadscan™ software (Clever Sys, Inc., Reston, VA) allowed us to identify animal paws, and set criteria for the software to recognize individual paws using captured animal footage. Baseline data for the Treadscan™ system was collected for each animal before the beginning of the experiment, along with other behavioral baseline tests.

Each animal was placed in the Treadscan™ chamber. The treadmill was then started and the speed was gradually increased until a comfortable gait was reached for each animal. Investigators then prompted the software to capture the animal's gait for 2,000 consecutive frames, at 60 frames per second. Once the blinded investigator was satisfied with the quality of captured locomotion, each video file was saved for further analysis. Once the tester was satisfied with the lighting and other parameters of the setup, a background image was captured and calibration video recorded for the session. A sampling of 10-15 paw traces (per paw) were required for an acceptable 'foot model', which was built for each animal using the Treadscan™ software (Figure 17). Upon preview, a video clip containing 10-15 consecutive steps was selected to be analyzed for each animal. Once an acceptable foot model was built, the analysis was run using the input of calibration, background, animal video, and foot model. Appropriate gait analysis parameters were selected from previously published literature (Beare et al., 2009; Tom et al., 2009; Myers et al., 2012; Figley et al., 2014; Chehrehasa et al., 2014), in addition to several other parameters only available in the newest versions of the software, and therefore not commonly used in the literature. Testing was repeated at weeks 4 and 6 post SCI for animals with BBB scores above 13 (consistent plantar stepping). Results from 6 weeks post SCI are presented (Figures 21-22). Statistical outliers beyond two standard deviations away from the mean were excluded; thus, the *n* number (*n* = 5) is the same for all behavioral assessments.

Figure 17. Treadscan™. (A) Rat on translucent belt with each foot highlighted for individual foot models. (B) Outline of rat with foot models, and horizontal and perpendicular body axes labeled. (C) Individual foot models with body axes labeled. (D) Overview of Treadscan™ setup.

A.



B.



C.



Figure 18. Basso Beattie and Bresnahan (BBB) Open Field Locomotion. Three examples of rats moving in the open field for BBB assessment.

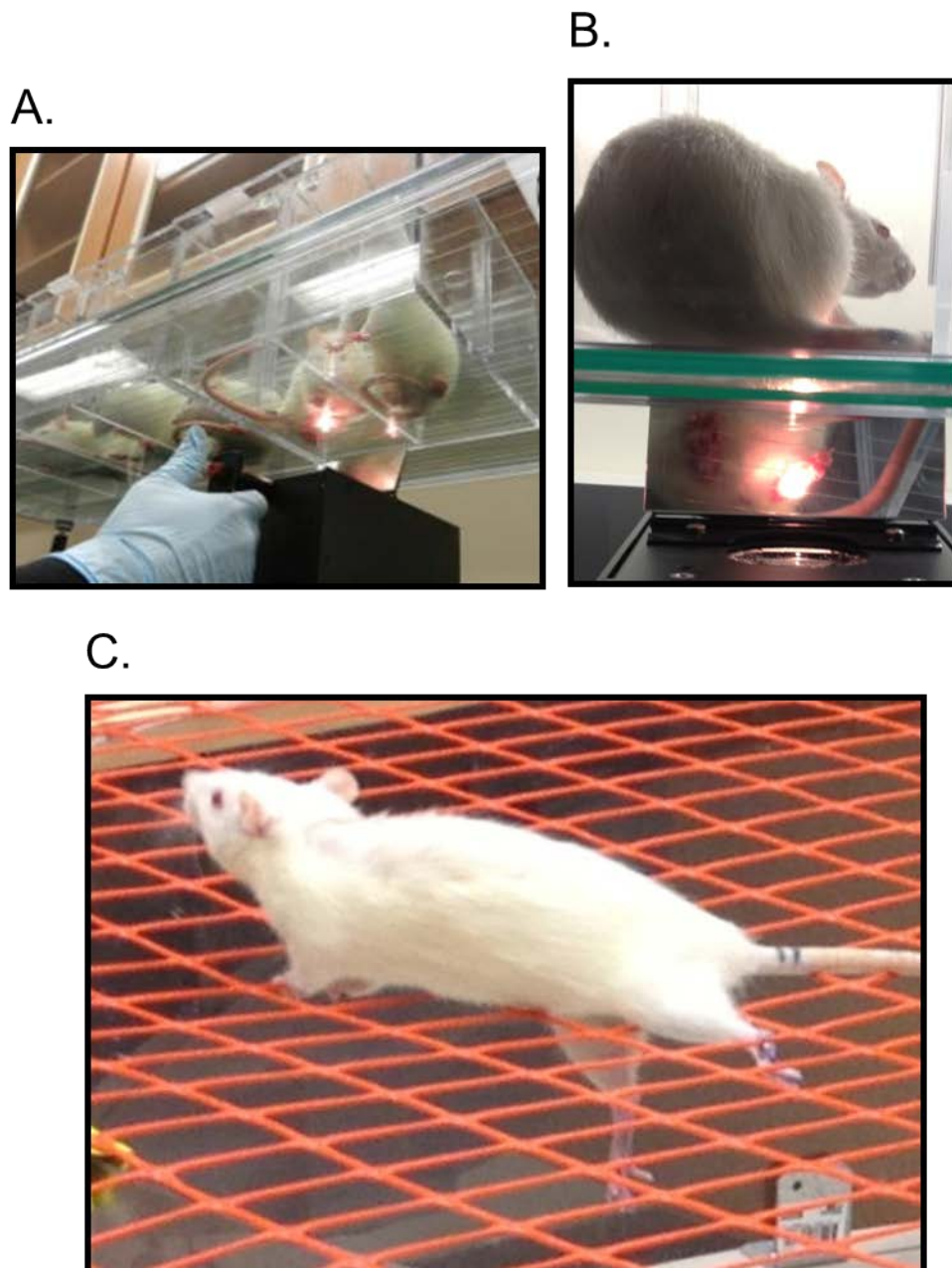


Figure 19. Hargreaves' and Gridwalk tests. (A) and (B) Hargreaves' test of thermal sensitivity. (C) Rat traversing grid during Gridwalk test.

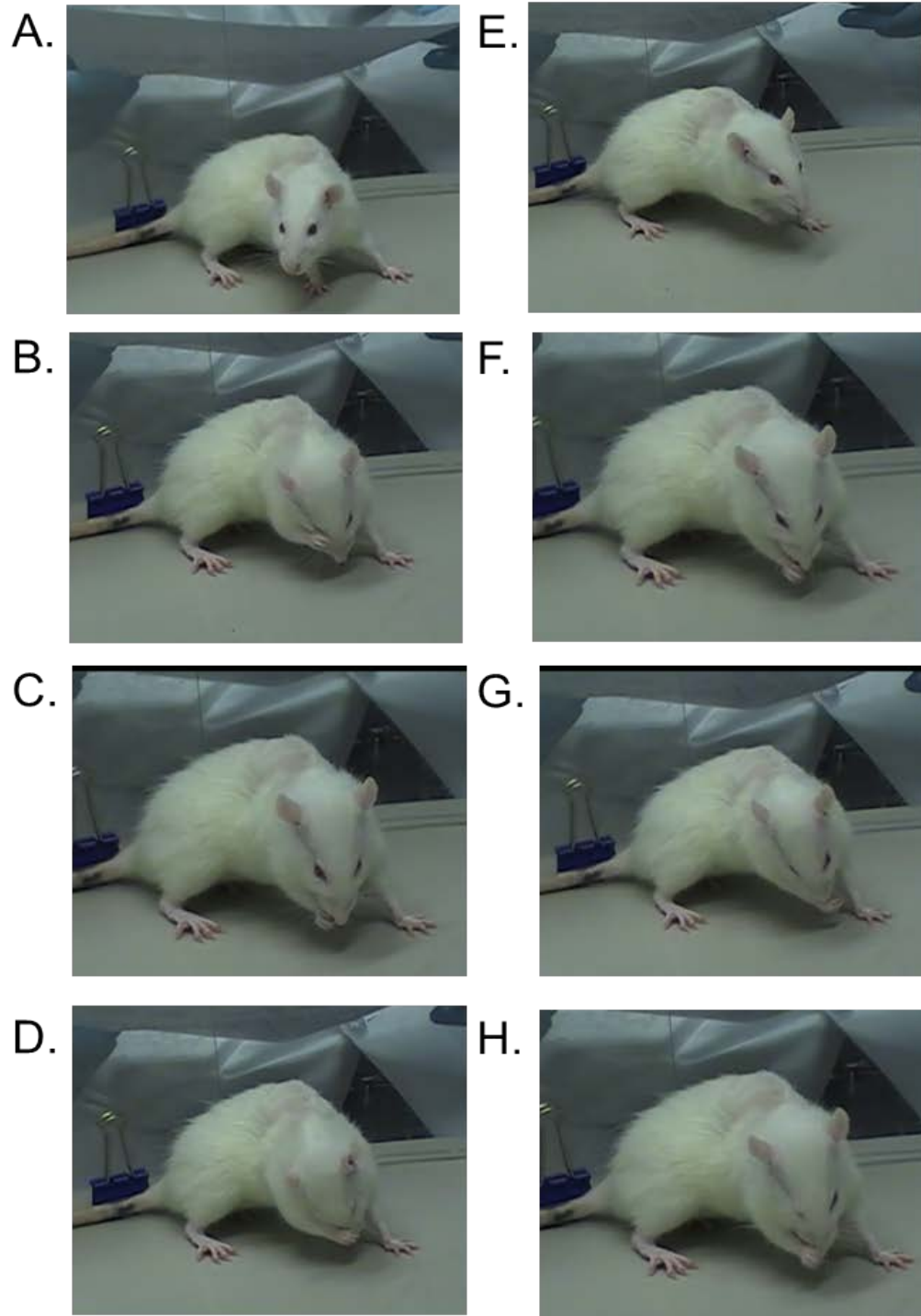


Figure 20. Modified Grooming Test. (A) Rat with weight on both forepaws. (B) - (H) Rat grooming with only one forepaw, with body weight propped on the other forepaw.

Part II: Basso Beattie and Bresnahan Open-field Locomotion

Animals were tested for recovery of locomotion in an open field environment using the Basso Beattie and Bresnahan (BBB) Locomotor Rating Scale (Basso, Beattie, and Bresnahan 1995, 1996). Animals were tested at baseline, 3 and 7 days post SCI, and weekly for 8 weeks. Animals' scores from 3 and 7 days post SCI were evaluated in order to pseudo-randomize the rodents into treatment groups so that all treatment groups would have a mean BBB of 10. Animals were tested for 4 minutes in the open field, with two blinded investigators scoring, and a third blinded investigator video recording each animal in each session (Figure 18). Locomotion was averaged then across both rear hindlimbs, due to the nature of a midline contusive injury.

Part III: Hargreaves' Test

Alterations in thermal sensitivity were assessed using the Hargreaves' test (Plantar Test Analgesia Meters, Harvard Apparatus; Hargreaves et al., 1988; Hill et al., 2009). Animals were placed in individual plexiglass boxes on top of a pre-warmed glass surface (34°C), for 10 minutes in order to acclimate to the testing environment. A thermal stimulus was then presented beneath the paw until the animal moved the paw in response to the stimulus, or until 20 seconds passed, whichever occurred first (Figure 19). The second paw was tested, and then each successive animal (across 6 animals) was tested before the first paw was repeated. A minimum of 3-4 minutes was maintained between trials of any paw, in order to allow the stimulated paw to return to a pre-stimulated level. Animals were tested at baseline and week 6 post SCI. Five trials were acquired per

paw/time point per animal, and three trials/paw were eventually averaged – the three closest paw withdrawal responses per paw.

Part IV: Gridwalk Assessment

Functional recovery was also evaluated using the Gridwalk test (Metz et al., 2000). Animals were placed on a preconstructed grid with gaps of ~3.25cm x 4.25cm between rungs (Figure 19). Animals were tested for 3 minutes, by two investigators. Animals were tested at baseline and weeks 6 and 8 post SCI (Figure 23). Only animals with consistent plantar stepping (BBB score of 13 or above) were tested on the Gridwalk test.

Part V: Modified Grooming Test for Trunk Stability

The Grooming test was adapted for cervical spinal cord injuries (Gensel et al., 2006; Inoue et al., 2013), yet it was originally developed for assessing brachial plexus injury and recovery (Bertelli and Mira, 1993). Once rodents feel comfortable with their surrounding environment, they often halt their locomotion or exploration and begin grooming. Thus, it came to our attention during the open-field BBB locomotion and Hargreaves' test that some rodents were able to support their body weight on their hindlimbs and partially rear, using both forepaws for grooming simultaneously, similar to Sham animals. However, a portion of rats could only groom with one forepaw at one time. These rodents supported their body weight on both hindlimbs and one forepaw, while grooming with the other forepaw (Figure 20). The animals then shifted their body weight to the opposite forepaw and groomed with the second forepaw. Animals were assigned a score for the ability to use both forepaws simultaneously for

grooming, similar to Sham, and a lessor score to grooming with only one forepaw at-a-time. The observation of the rodents' differing ability to groom was made at 6 weeks post SCI. Thus, the Modified Grooming test was established as a measure of trunk stability, and the animals were tested at week 6 post SCI (Figure 6).

Transcranial Magnetic Motor Evoked Potential

At 7 weeks post SCI, animals were restrained beneath a cloth, and electrodes were placed in the tibialis anterior muscle, as well as a grounding electrode placed in the tendon. A magnetic wand was placed over the motor cortex to evoke transcranial magnetic motor evoked potentials (tcMMEPs). Muscle evoked potentials were recorded and traced for motor activity (Figure 27). All traces were assessed and background threshold 'noise' level (inherent within the signal) was defined. Three traces per leg were averaged, and both legs were assessed for all rats. Statistical outliers beyond two standard deviations away from the mean were excluded.

Histopathological Analysis

Eight weeks post SCI, tissue was collected and processed, methods previously described (Walker et al., 2012). Briefly, after transcardial perfusion with 0.01 M Saline and 4% paraformaldehyde, the brain and spinal cords were dissected, and dehydrated in 30% sucrose. Spinal cord segments 1.5 cm in length, surrounding the injury epicenter, were identified (Figure 28), isolated (Figure 29), and frozen in Tissue-Tek® O.C.T. compound (Fisher Scientific) with 3 spinal cords per block. Tissue was sectioned cross-sectionally at 25 µm

thickness on Leica CM (1860, 1950) cryostats and mounted onto Superfrost® glass plus slides (Fisher Scientific) in sets. Therefore, approximately 600-800 tissue slices were cut and mounted per animal. Tissue was stained with cresyl violet acetate (Nissl) stain with eosin counterstaining and coverslip mounted with DPX (Fisher Scientific), for lesion area and cavity measurements. Tissue was also stained using immunofluorescence for the following primary antibodies: mouse anti-rat endothelial cell antigen-1 (1:100; ABd Serotec MCA970F), rabbit anti-glial acidic fibrillary protein (GFAP, 1:200; Chemicon, AB5804), mouse SMI-31 (1:1000; Sigma), and mouse anti-rat ED-1 (1:200; Sigma). The following secondary antibodies were used for detection: goat anti-mouse TRITC (1:100; Jackson Immunoresearch, 115-025-003), goat anti-rabbit FITC (1:100; Jackson Immunoresearch, 115-095-003), and goat anti-mouse TRITC (1:100; Jackson Immunoresearch, 115-025-003). Tissue sections were coverslip mounted post staining using Fluoromount-G (Southern Biotech) and Hoechst 33342 (1:100; Invitrogen) was used for fluorescence staining of nuclei.

Brightfield images were obtained using an Olympus BX60 microscope in combination with Neurolucida 9 software (MicroBrightfield, Inc.). Based upon morphological appearance from the cresyl-echt violet stained tissue sections, lesion and spared white matter tissue was identified. Cavity was then identified. Lesion, cavity, and spared white matter areas were subsequently traced from 4x images using ImageJ software. Approximately 600 cord sections traced for brightfield quantification. Immunofluorescent slides were imaged using a Zeiss Axio Imager M2 and Neurolucida 9 software (MicroBrightfield, Inc.). RECA-1

positive staining was assessed morphologically for blood vessels. Similarly, SMI-31 positive staining was assessed for axons and GFAP positive staining was assessed for astrocytes. Fluorescence integrated density (intensity/total cord area) was traced for regions of interest in the penumbra region of the lesion, from 10x images and quantified using ImageJ software. Approximately 100 cord sections traced for IF quantification.

Statistical Analysis

All quantified data are presented as the mean +/- SEM (standard error of the mean). A one-way analysis of variance (ANOVA) was used to analyze: 1) the *in vitro* results of neurite outgrowth, and the *in vivo* portion of the study, including 2) BBB, 3) Treadscan™, 4) Gridwalk, 5) Hargreaves' test, and the 6) Modified Grooming test, as well as the histological quantification of 7) lesion, 8) cavity, and 9) spared white matter area, as well as integrated fluorescence density for the 10) vasculature (RECA-1), 11) axons (SMI-31), and 12) astrocytes (GFAP). Statistical outliers beyond two standard deviations away from the mean were excluded. Only $p < 0.05$, data was considered significant, and the null hypothesis was rejected. Tukey's post hoc analysis was used following the one-way ANOVA for all quantification, for pair-wise comparisons (Kao and Green, 2008). GraphPad Prism® was used for all statistical analyses.

Results

VEGF and GDNF Promote Neurite Outgrowth from Spinal Cord Neurons

To determine whether VEGF and GDNF have any effect on neurite outgrowth, spinal cord neurons were treated with the two factors, alone or in

combination. Using the ImageXpress® Micro XL, we observed and quantified (MetaXpress 5.1 software) significant neurite outgrowth from spinal cord neurons (Figure 11) in response to both VEGF and GDNF, and an additive effect when combined, as compared to neuronal controls (not containing any factors). Ninety neurons were assessed per treatment group (Figure 9). Treatment groups included: 1) neuronal controls (media and neurons), 2) VEGF 25 ng/mL, 3) VEGF 50 ng/mL, 4) GDNF 25 ng/mL, 5) GDNF 50 ng/mL, 6) VEGF + GDNF 25ng/mL each, and 7) VEGF + GDNF 50 ng/mL each. VEGF at 25ng/mL significantly increased total neurite outgrowth, mean process length, and mean number of branches, compared to neuronal (media) controls (Figure 9). VEGF at 50 ng/mL significantly increased total neurite outgrowth, mean process length, maximum process length, and mean number of branches, compared to neuronal (media) controls. While GDNF at 25 ng/mL significantly increased all four measures of neurite outgrowth over neuronal controls, it was not significant compared to VEGF at 25 ng/mL or VEGF at 50 ng/mL. However, GDNF at 50 ng/mL significantly increased all measures of neurite outgrowth compared to neuronal controls, VEGF at 25 and 50 ng/mL and GDNF at 25 ng/mL. VEGF 25 ng/mL + GDNF 25 ng/mL combined, and VEGF 50 ng/mL + GDNF 50 ng/mL combined, significantly increased total neurite outgrowth, mean process length, maximum process length, and mean number of branches compared to neuronal controls, VEGF at 25 and 50 ng/mL, and GDNF at 25 ng/mL. Both VEGF + GDNF combination groups (25 and 50 ng/mL) appear to have additive effects on all measures of neurite outgrowth, compared to VEGF or GDNF alone. However,

neither combination group (VEGF + GDNF) promoted statistically significant outgrowth more than GDNF at 50 ng/mL.

VEGF and GDNF also resulted in a significant increase in the total number of neurons (Figure 10). Thus it appears that VEGF and GDNF had neuroprotective effects on the neurons in culture as well as neurotrophic influence on neurite outgrowth.

Treadscan™ Gait Analysis

Hydrogel alone, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF significantly improved ($p < 0.05$, ANOVA) a number of parameters of locomotion and coordination, as detected by the Treadscan™ (Figures 21-22). All three of these treatment groups improved the following parameters: 1) Stance maximum area, 2) Print angle, 3) Base of support (rear track width), 4) Homologous gait coupling, 5) Homolateral gait coupling, 6) Diagonal coupling, 7) Brake time, 8) Minimum Longitudinal deviation, and 9) Maximum Longitudinal deviation, close to Sham levels (dotted lines in Figures 21-22). Tukey's post hoc analysis was run on statistically significant groups from the ANOVA analysis. Thus, it appears that the hydrogel alone is having a structural support of the endogenous repair mechanism that occurs in rodents after SCI. Similar to the *in vitro* results, GDNF appears to be the greatest contributor to functional recovery. Additionally, none of these parameters are measured by the BBB, aside from print angle, which is qualitatively measured by BBB and quantified by the Treadscan™.

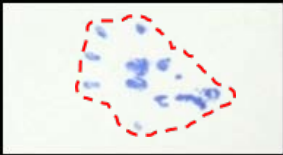
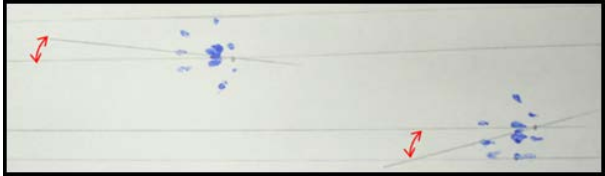
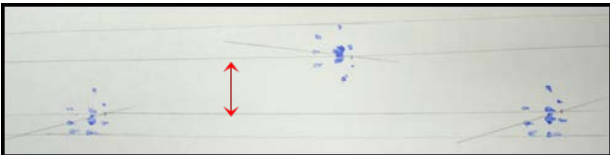
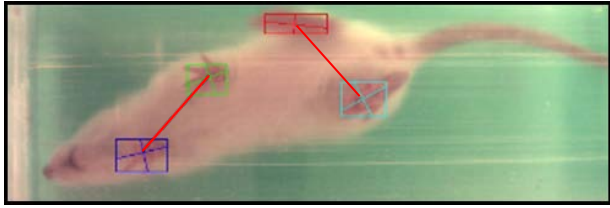
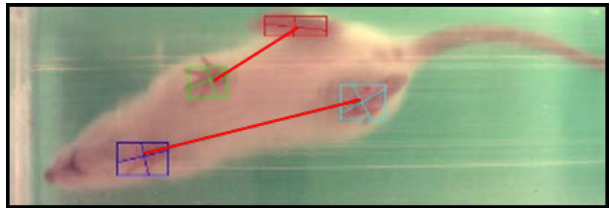
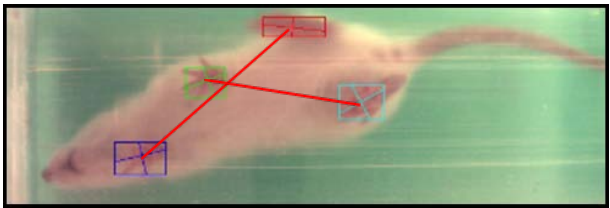
Parameter	Description	Visual description
Stance Maximum Area	The surface area occupied by the paw	
Print Angle	The angle of rotation of the paw from the central body axis	
Base of Support	The distance between the two rear hind-paws	
Homologous Gait Coupling	The coupling that occurs between the two front paws or the two rear paws	
Homolateral Gait Coupling	The coupling that occurs between both paws on the same side of the body	
Diagonal Gait Coupling	The coupling between the fore-paw and rear-paw on the opposite sides of the body	

Table 4. Treadscan™ Parameters



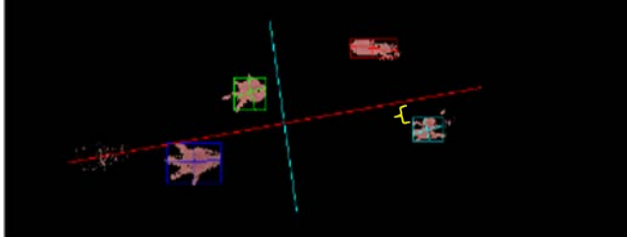
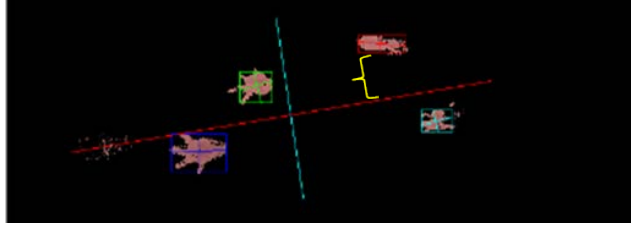
Parameter	Description	Visual description
Brake Time	The time it takes the paw to come to a halt once reaching the ground from the swing phase.	<p style="text-align: center;">Gait Cycle (Right Hind Limb)</p> <p style="text-align: center;">← Stance phase (60%) →</p> <p style="text-align: center;">← Braking phase → ← Propulsive phase →</p>  <p style="text-align: center;">← Swing phase (40%) →</p> 
Longitudinal Minimum Deviation	The smallest deviation of the limbs from the central axis of the body.	
Longitudinal Maximum Deviation	The largest deviation of the limbs from the central axis of the body.	

Table 4. Treadscan™ Parameters (continued)

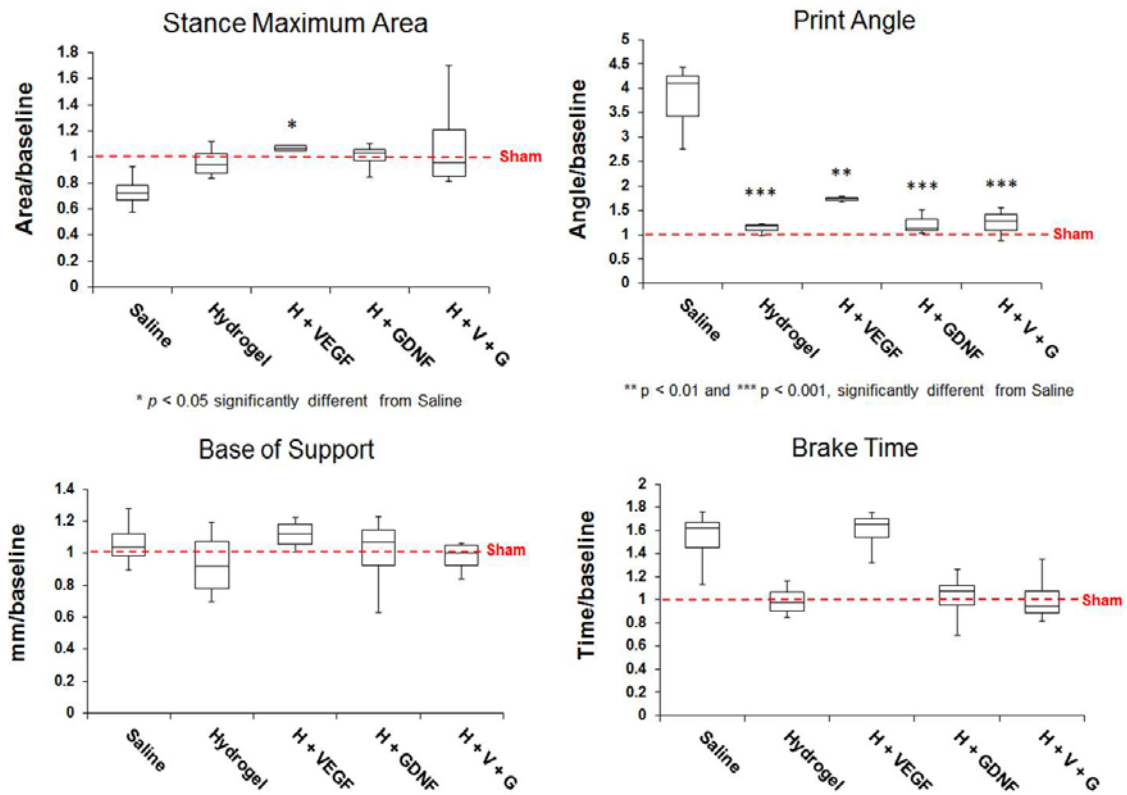


Figure 21. Treadscan™ Motor Assessments at 6 Weeks Post SCI. Hydrogel alone, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF treatment significantly improved Maximum Stance Area (A), Print Angle (B), Base of Support, and (D) Brake time. All data was normalized to baseline, and the Sham animal data is represented by the horizontal dashed lines. $n = 5$ per treatment group. SEM, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

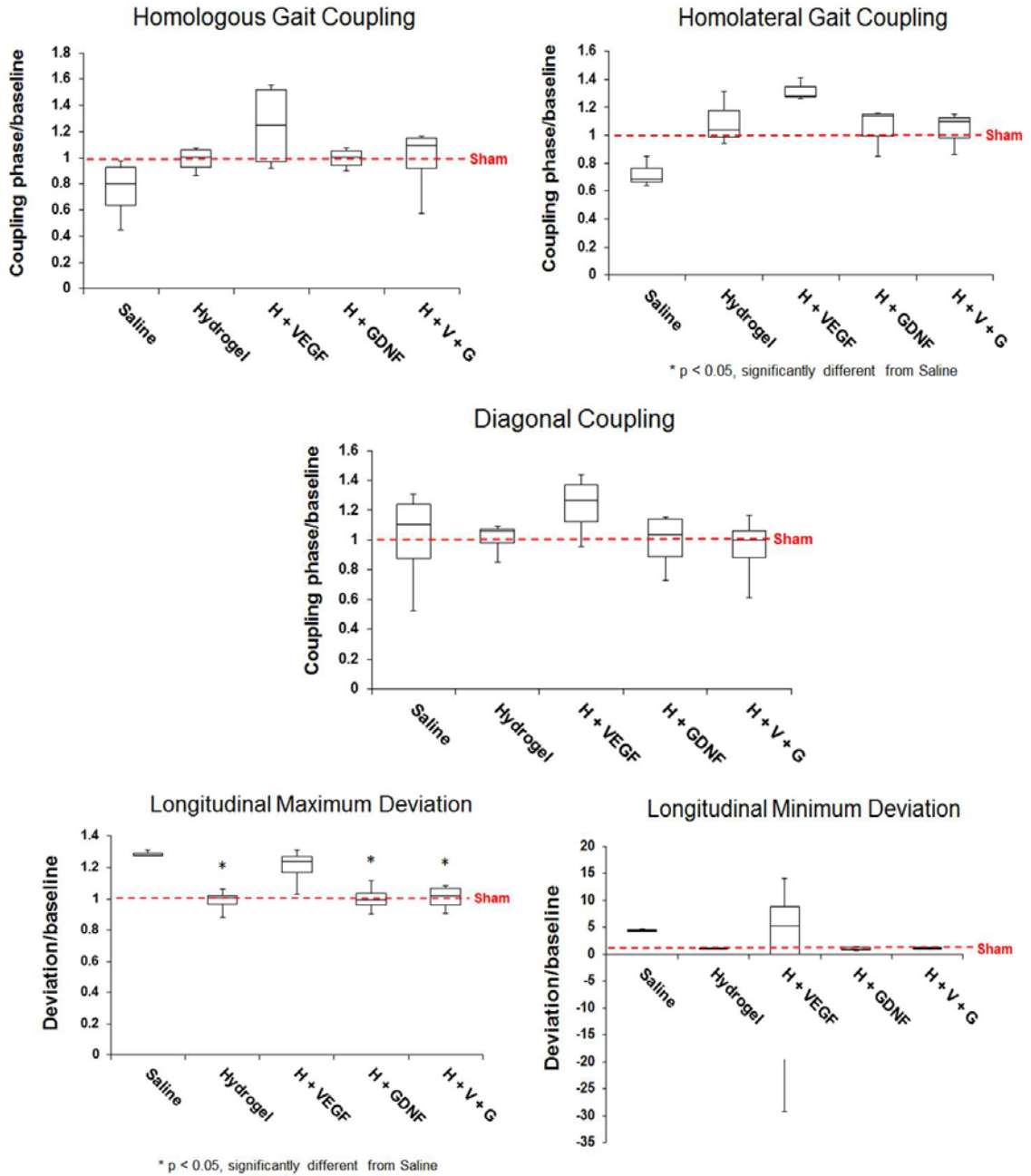


Figure 22. Treadscan™ Motor Assessments at 6 Weeks Post SCI. Hydrogel alone, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF treatment significantly improved Homologous Gait Coupling (A), Homolateral Gait Coupling (B), Diagonal Gait Coupling (C), Minimum Longitudinal Deviation (D), and Maximum Longitudinal Deviation (E) compared to Saline controls and Hydrogel + VEGF. All data was normalized to baseline, and the Sham animal data is represented by the horizontal dashed lines. $n = 5$ per treatment group. SEM, * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.001$.

Basso, Beattie, and Bresnahan Open-field Locomotion

All animals (except for Sham) displayed functional deficits following injury, evident at 3 and 7 days post SCI (Figure 23). Rodents have an endogenous spontaneous recovery following SCI, which is apparent in the Saline-treated animals. Thus, all treatment groups significantly improved over the course of 8 weeks following injury, compared to 3 days post injury. However, no statistical differences were detected across treatment groups at any particular time point, $p > 0.05$ (ANOVA).

Hargreaves', Gridwalk, and Modified Grooming Tests

The Hargreaves' test did not show any significant differences across treatment groups at baseline or 6 weeks post SCI (Figure 24). The paw withdrawal time (seconds) also did not differ significantly between baseline and 6 weeks post SCI, $p > 0.05$ (ANOVA). Thus, none of the treatments have caused an increased sensitivity to a thermal stimulus (hyperalgesia) in Sprague-Dawley rats, which is always a concern with SCI treatments and pre-clinical trials. We used the Hargreaves' test as a measure of thermal sensitivity to ensure that our treatments were not causing allodynia or hyperalgesia. Allodynia is the perception of a noxious stimulus in response to a non-noxious stimulation. Hyperalgesia is the perception of a highly noxious stimulus in response to a mildly noxious stimulation. It is important for preclinical studies of spinal cord injury to determine whether the putative treatments are causing allodynia or hyperalgesia, as these would be undesirable side-effects for spinal cord injured patients. We could have also utilized the von Frey hair test of mechanical

sensitivity to measure allodynia or hyperalgesia. However, we feel that the Hargreaves' test provides us with consistent, reliable, and reproducible data to measure treatment-induced allodynia or hyperalgesia.

The Gridwalk and Modified Grooming tests did not show any significant differences across treatments at any time points, $p > 0.05$ (ANOVA, Figures 25 and 26, respectively). The Grooming test is typically employed for cervical models of SCI. However, in noticing animals pausing to groom during the Hargreaves' test and BBB assessment, we modified the standard Grooming test and utilized it as a measure of trunk stability in our thoracic SCI model. If further developed and explored as a putative measure of trunk stability, the Grooming test may have broader implications for detecting SCI deficit and recovery. With higher n numbers, thus more statistical power for analysis, assessments such as Gridwalk, Modified Grooming test, and BBB might presumably show more significant differences across treatment groups. However, the final n number for this study was $n = 5$ per treatment group.

Transcranial Magnetic Motor Evoked Potential

No significant differences were detected in tcMMEP tracings across treatment groups. As locomotor activity significantly differed across treatment groups, there is a possibility that the tcMMEP signal was lost within the background 'noise' of the assessment. Higher n numbers should reduce the variability observed across animals within and across treatment groups.

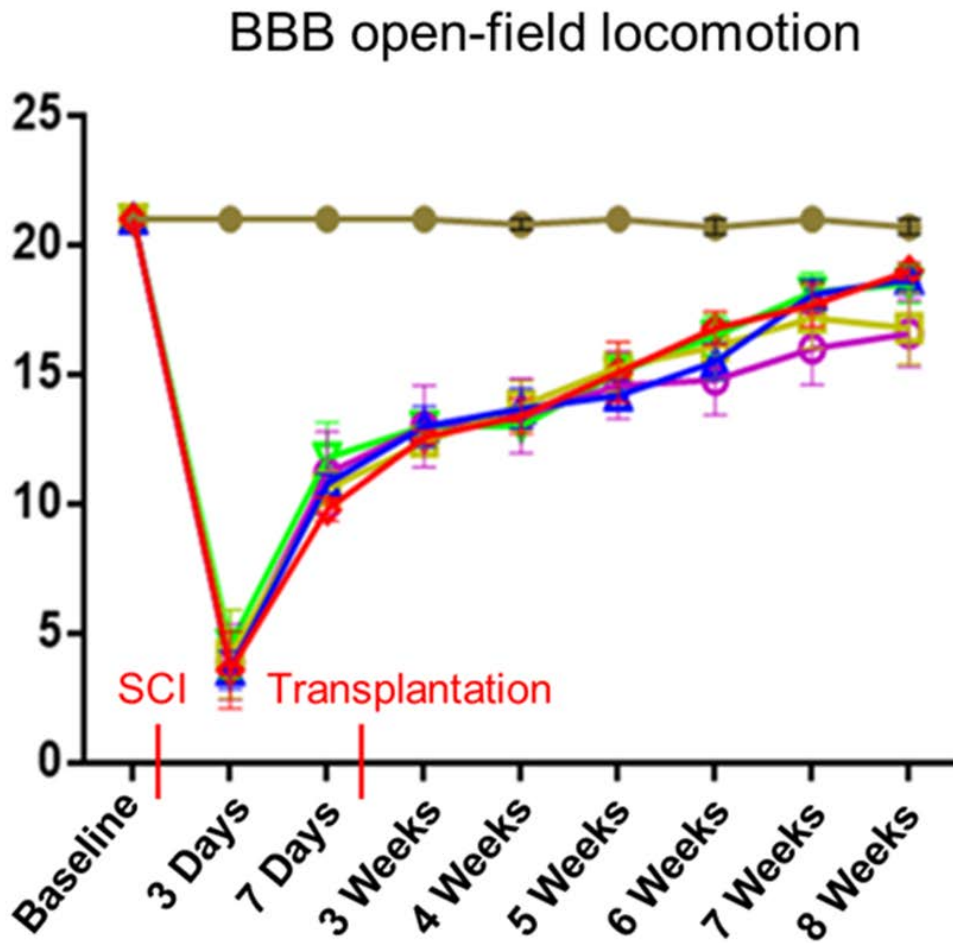


Figure 23. Basso, Beattie, and Bresnahan (BBB) Motor Assessment. No significant differences were observed across treatment groups over 8 weeks post SCI. $n = 5$ per treatment group. SEM, $p < 0.05$.

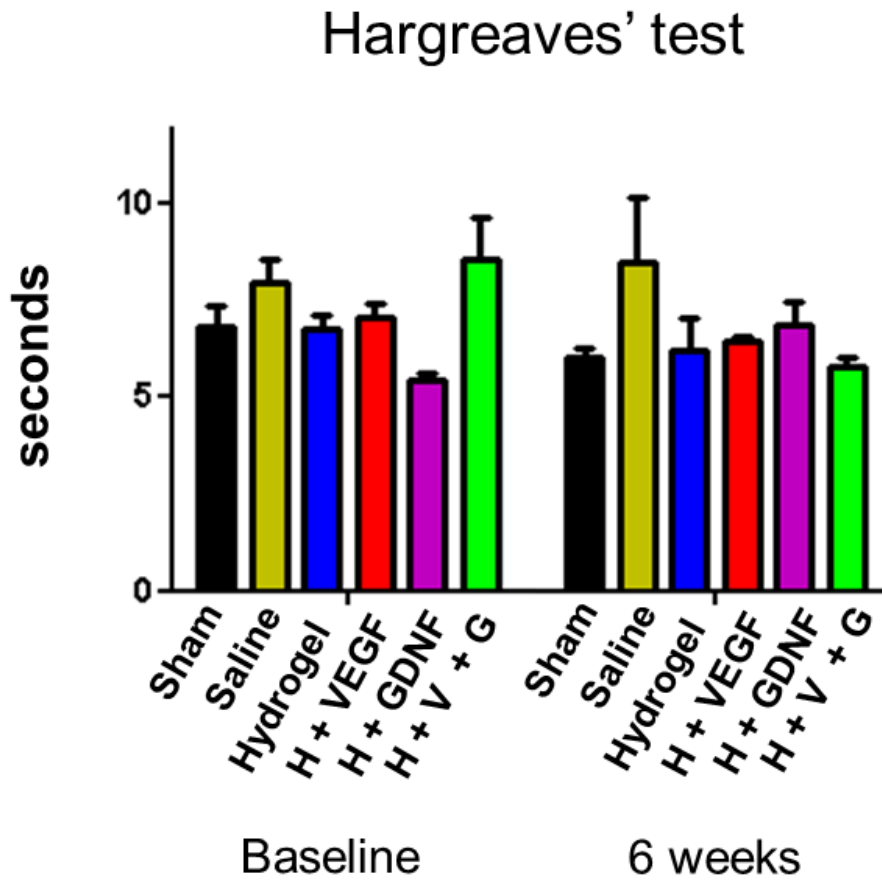
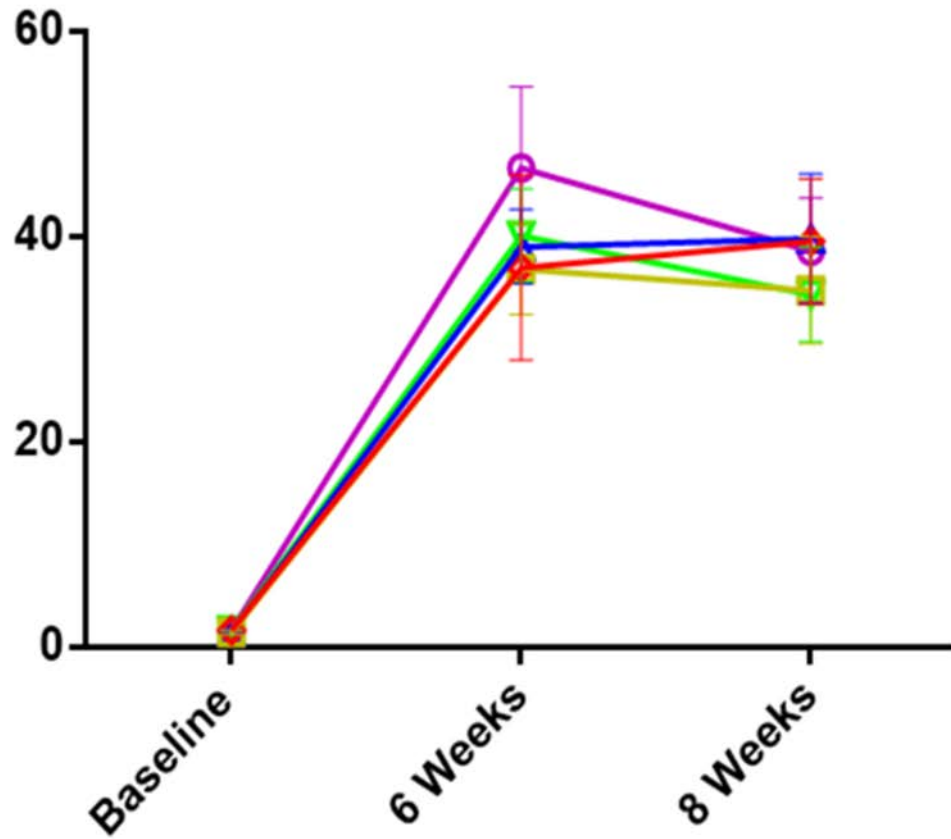


Figure 24. Hargreaves' Test. No significant differences were observed across treatment groups at either time point, and there were no significant differences between baseline and 6 weeks post SCI for any of the groups. $n = 5$ per treatment group. $p > 0.05$, ANOVA.

Gridwalk



- Saline
- Hydrogel
- Hydrogel + GDNF
- Hydrogel + VEGF
- Hydrogel + GDNF + VEGF

Figure 25. Gridwalk Test. No significant differences were observed across treatment groups at 6 weeks or 8 weeks post SCI. $n = 5$ per treatment group. $p > 0.05$, ANOVA.

Modified Grooming Test - Trunk Stability

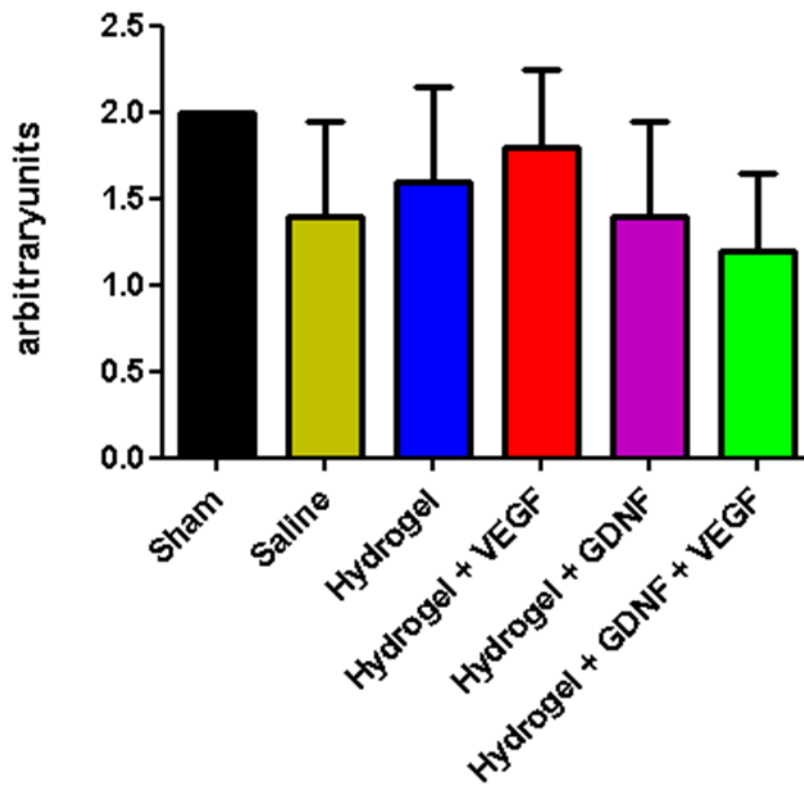


Figure 26. Modified Grooming Test. The grooming test was modified from cervical SCI models as a measure of trunk stability with thoracic SCI injury model. No significant differences were observed across treatment groups. However, this test may have broader applications for thoracic SCI models in the future, if further developed. $n = 5$ per treatment group. $p > 0.05$, ANOVA.

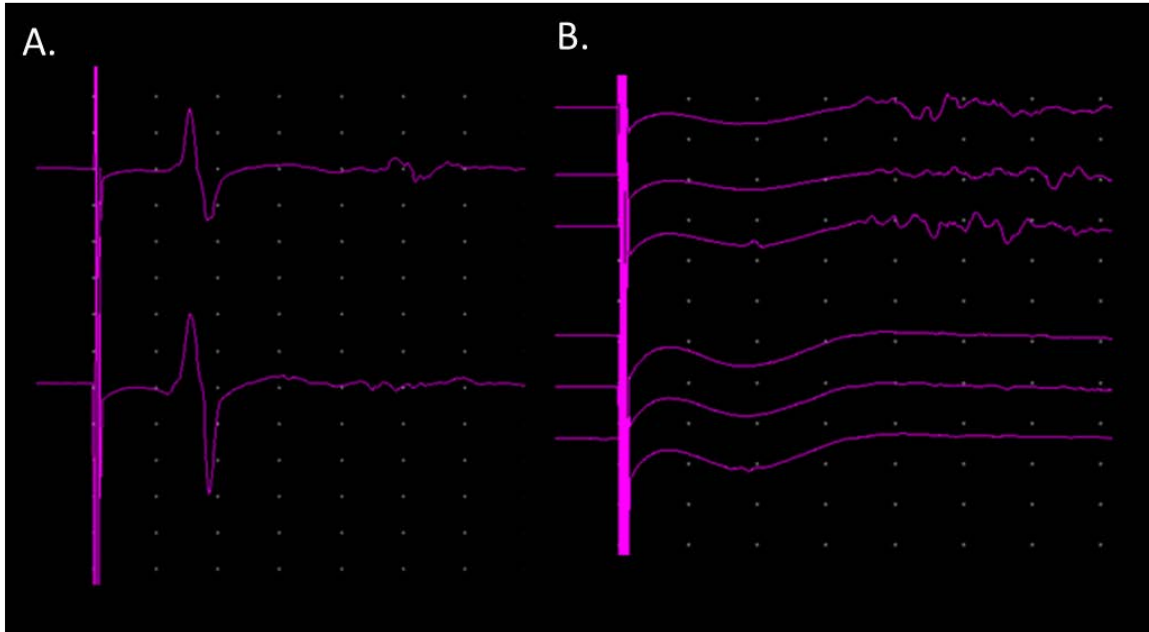


Figure 27. tcMMEP Recording Traces. (A) Baseline (Sham) left and right hind limbs. (B) Left and right hind limbs, respectively, at 7 weeks following injury. $n = 5$ per treatment group.

Verification of Injury Epicenter



Figure 28. Verification of Injury Epicenter. Brains and spinal cords were dissected following perfusion, and injury epicenter was verified (yellow dotted line).



Figure 29. Injury Epicenter. Case sample of injury epicenter at T10 (thoracic 10), 8 weeks following a moderate, midline, contusive SCI with the LISA device (depth of 0.85mm). Higher magnification of inset, below.

Histopathological Analysis

No significant differences were detectable across treatment groups for lesion area, cavity area, spared white matter area, or total cord area, measured from Cresyl-echt violet stained and imaged cross sections (Figure 31; $p > 0.05$, ANOVA). Similarly, no significant differences were observed in astrocyte reactivity (GFAP), vasculature (RECA-1), or axons (SMI-31), measured by integrated fluorescence density across total cord area (for each parameter), $p > 0.05$ (ANOVA). However, non-significant trends were observed in reduced lesion cavity area at injury epicenter for Hydrogel alone, Hydrogel + VEGF, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF (Figures 32 and 36). Hydrogel + VEGF showed a non-significant trend of increased vasculature density at lesion epicenter, compared to Saline animals and other treatment groups (data not shown), and also a non-significant trend of decreased rostral-caudal cavity span (Figure 30). Hydrogel + GDNF showed a non-significant trend of lower GFAP integrated density as compared to Saline and other treatment groups (Figure 31). These trends are noteworthy, particularly because they might be the histological differences underlying the functional locomotor differences detected with the Treadscan™. Higher n numbers should correspond to lower variability across animals in each treatment group. Thus, with higher n numbers we would expect to see more parameters with statistical significance in histological outcomes and behavioral assessments (BBB, Gridwalk, Modified Grooming test), similar to the differences detected with the Treadscan™ in this study.

The SCI resulted in an immune response (Figures 37 and 39), an attempt by microglial cells to clear tissue debris. However, no significant increase in immune response (ED-1) was observed across treatment groups (quantified data not shown). Thus, hydrogel did not provoke a significant immune response. This was expected as the hydrogel is designed to be non-immunogenic. Individual microglia (red) and reactive astrocytes (green) can be visualized by immunofluorescence histopathology in Figure 39. Immunofluorescence integrated densities of RECA-1 (vasculature), GFAP (astrocytes), and SMI-31 (axons) were compared across treatment groups (Figure 31). Higher power immunofluorescence images reveal the overlap of the vasculature (RECA-1) and glial endfeet (GFAP) that are part of the neurovascular unit (Figures 33, 34, 38, and 42). The vascular basement membrane (laminin) is visualized in Figure 35 along with RECA-1. Higher power images of the vasculature (RECA-1) reveal individual red blood cells (Figures 40 and 41).

Rostral-Caudal Cavity Spanse

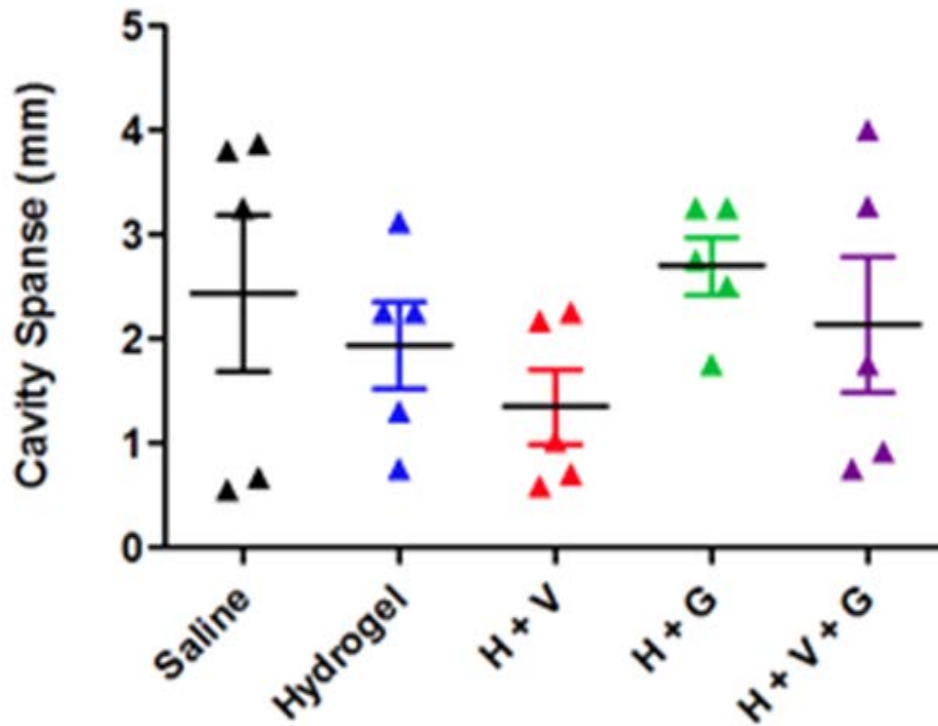


Figure 30. Rostral-Caudal Cavity Spanse. The spanse of cavity extending rostrally and caudally was measured. No significant difference exists across treatments groups, although Hydrogel, Hydrogel + VEGF, and the combined group of Hydrogel + VEGF + GDNF showed trends of less cavity compared to Saline and Hydrogel + GDNF groups. $n = 5$ per treatment group.

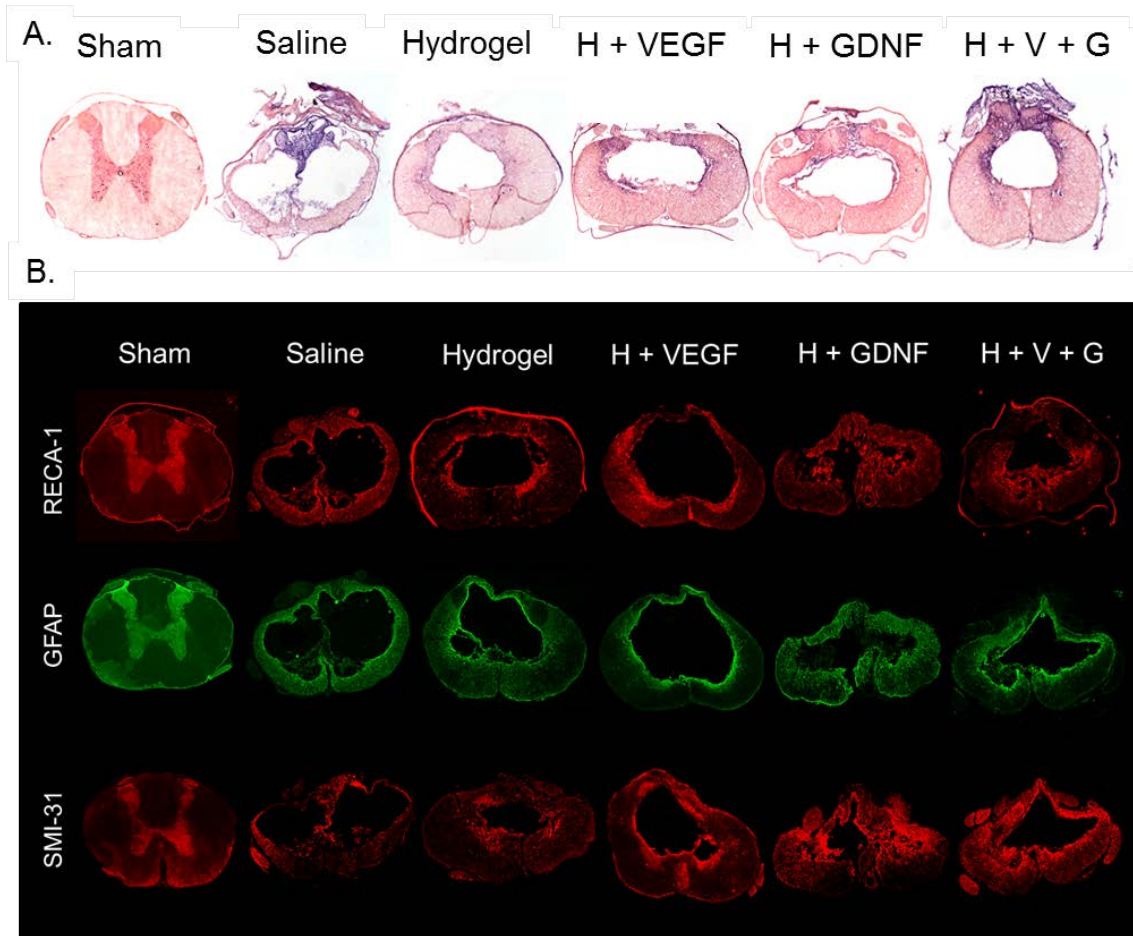


Figure 31. Histopathology. (A) Cresyl-echt violet staining of cross-sections. (B) RECA-1 staining for the vasculature (top row). GFAP staining for astrocytes (middle row). SMI-31 staining for axons (bottom row). No statistical significance was observed from histopathological results (data not shown). Image magnification for brightfield images is 4x, and for immunofluorescence is 10x magnification was used.

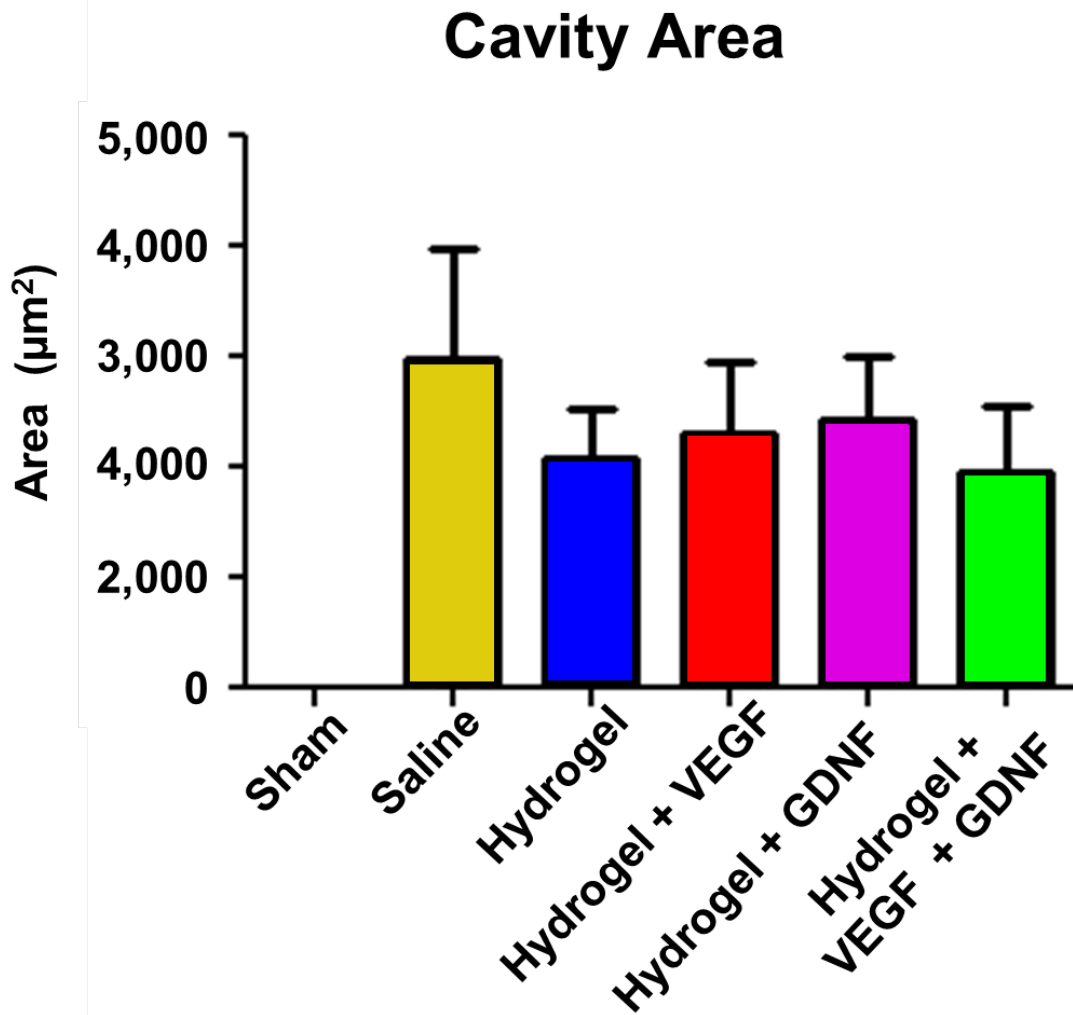


Figure 32. Epicenter Lesion Cavity Area. A non-significant trend of reduced lesion cavity area (μm^2) was observed for all treatment groups containing Hydrogel. Sham tissue was uninjured and displayed no cavity. $n = 5$ per treatment group. $p > 0.05$.

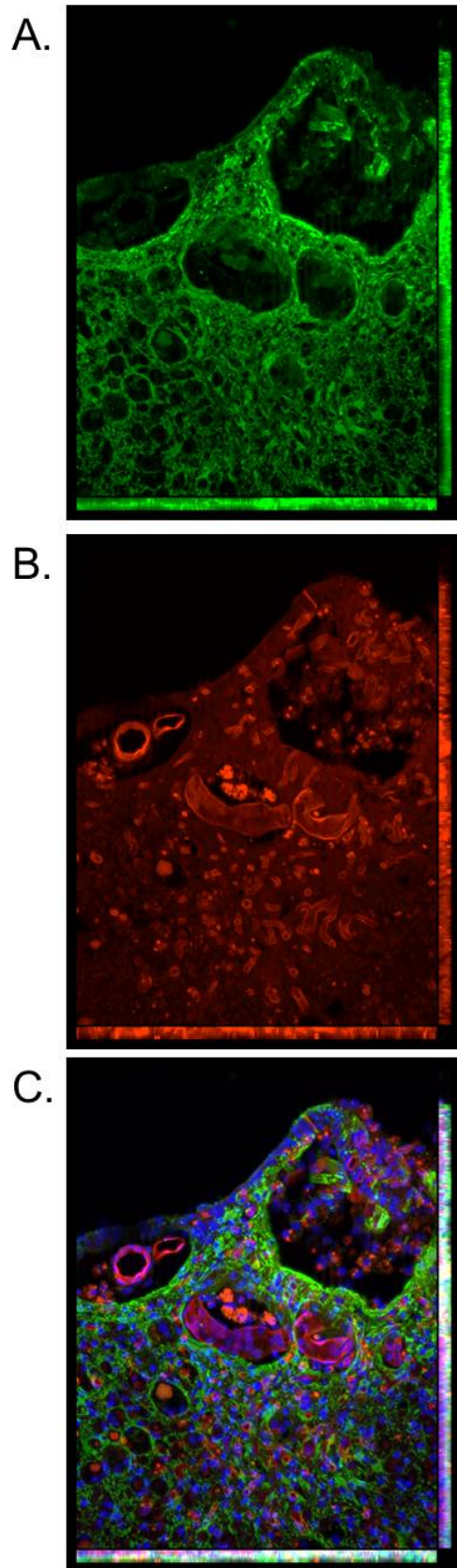


Figure 33. RECA-1 & GFAP Immunofluorescence Staining. (A) GFAP (green) for astrocytes, (B) RECA-1 (red) for vasculature, and (C) merged image with Hoechst included for cell nuclei (blue).

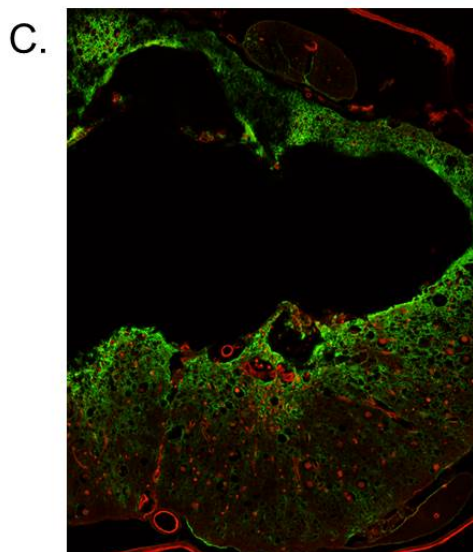
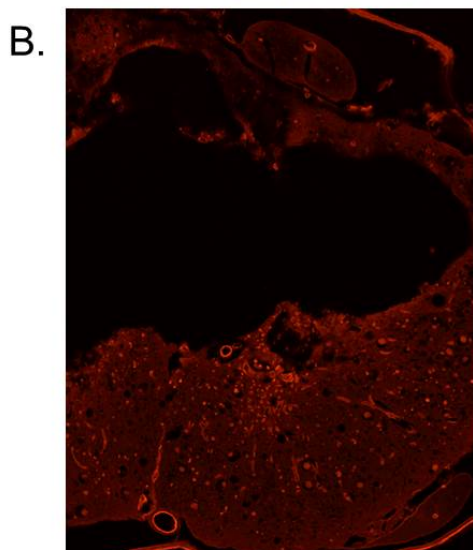
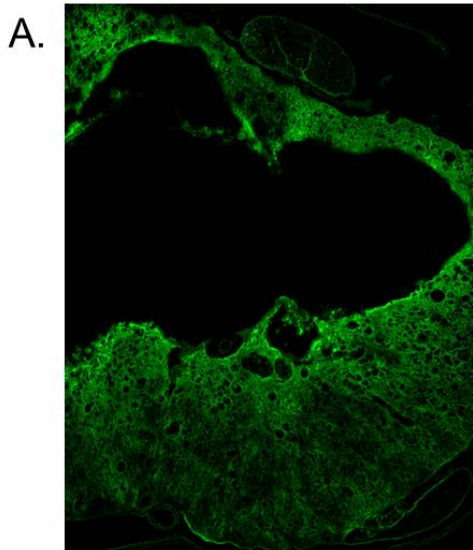


Figure 34. RECA-1 & GFAP Immunofluorescence Staining.
(A) GFAP (green) for astrocytes,
(B) RECA-1 (red) for vasculature,
and (C) merged image.

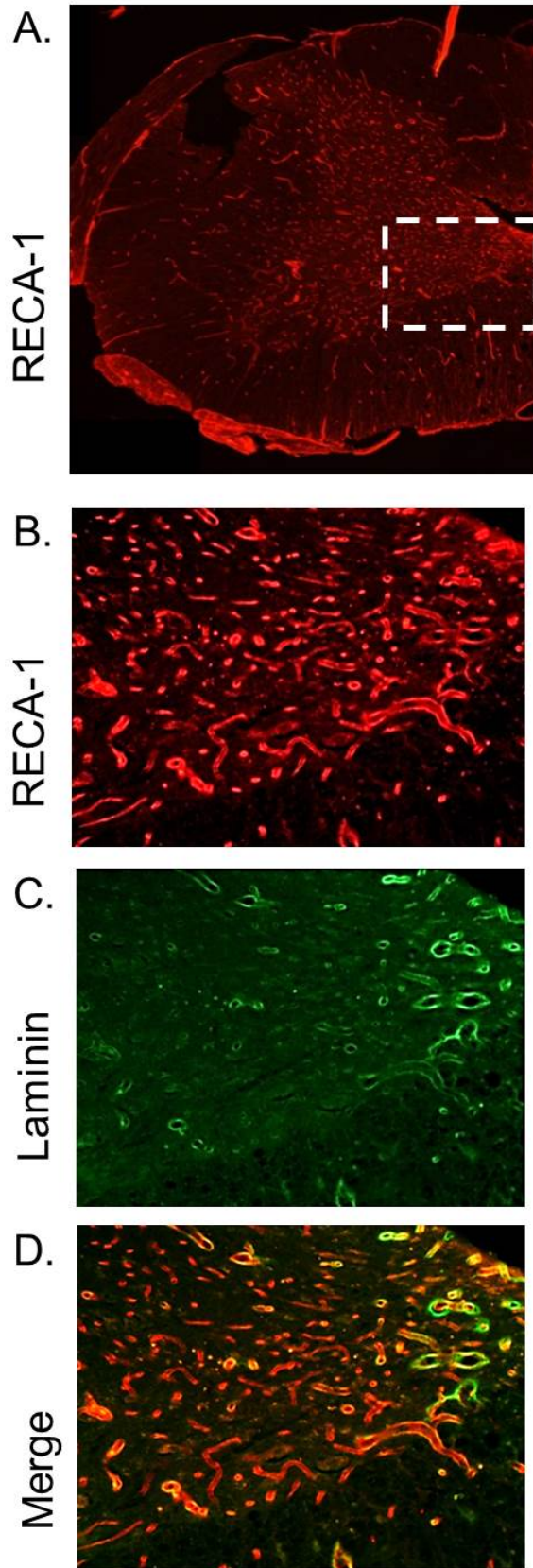


Figure 35. RECA-1 & Laminin Immunofluorescence Staining. RECA-1 (red) for vasculature, (A) and (B). Laminin (green,C). Merged image (D).

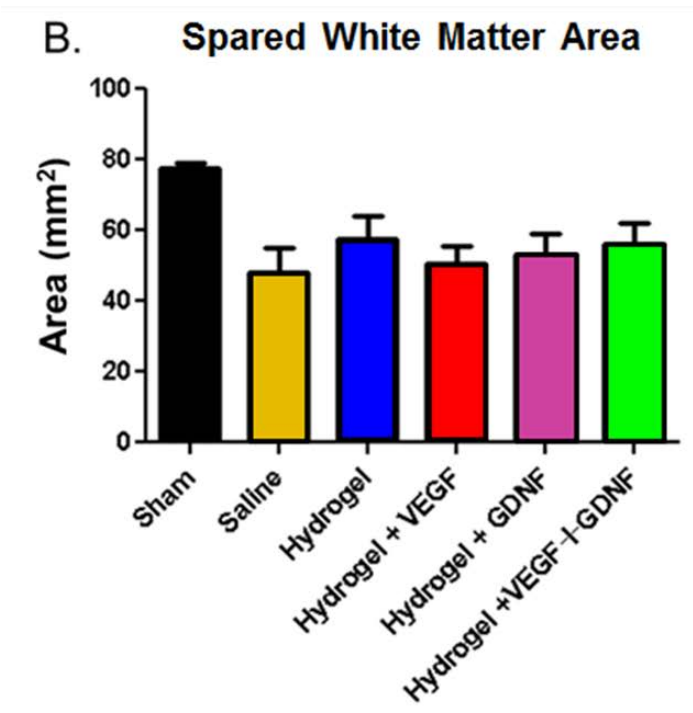
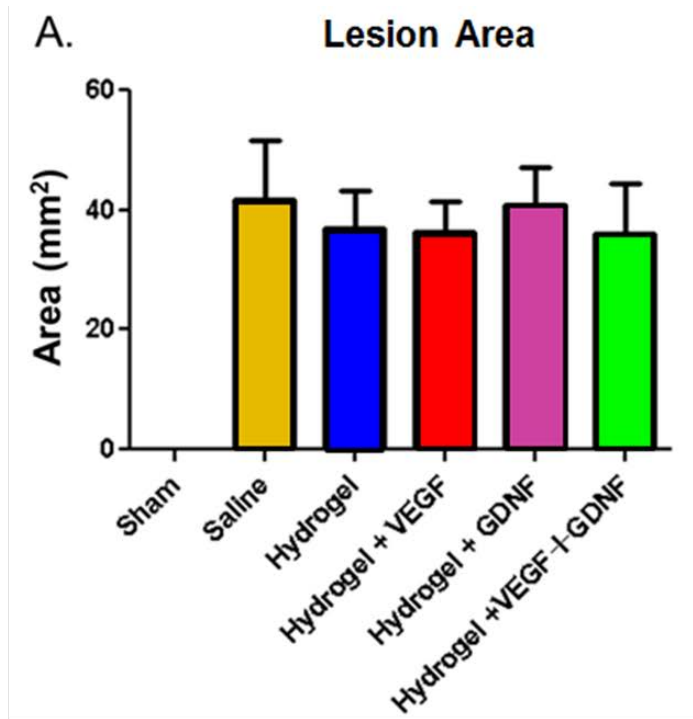


Figure 36. Epicenter Lesion and Spared White Matter. (A) Lesion Area at epicenter. (B) Spared white matter area at epicenter. Neither parameter is statistically significant. $n = 5$ per treatment group. $p > 0.05$.

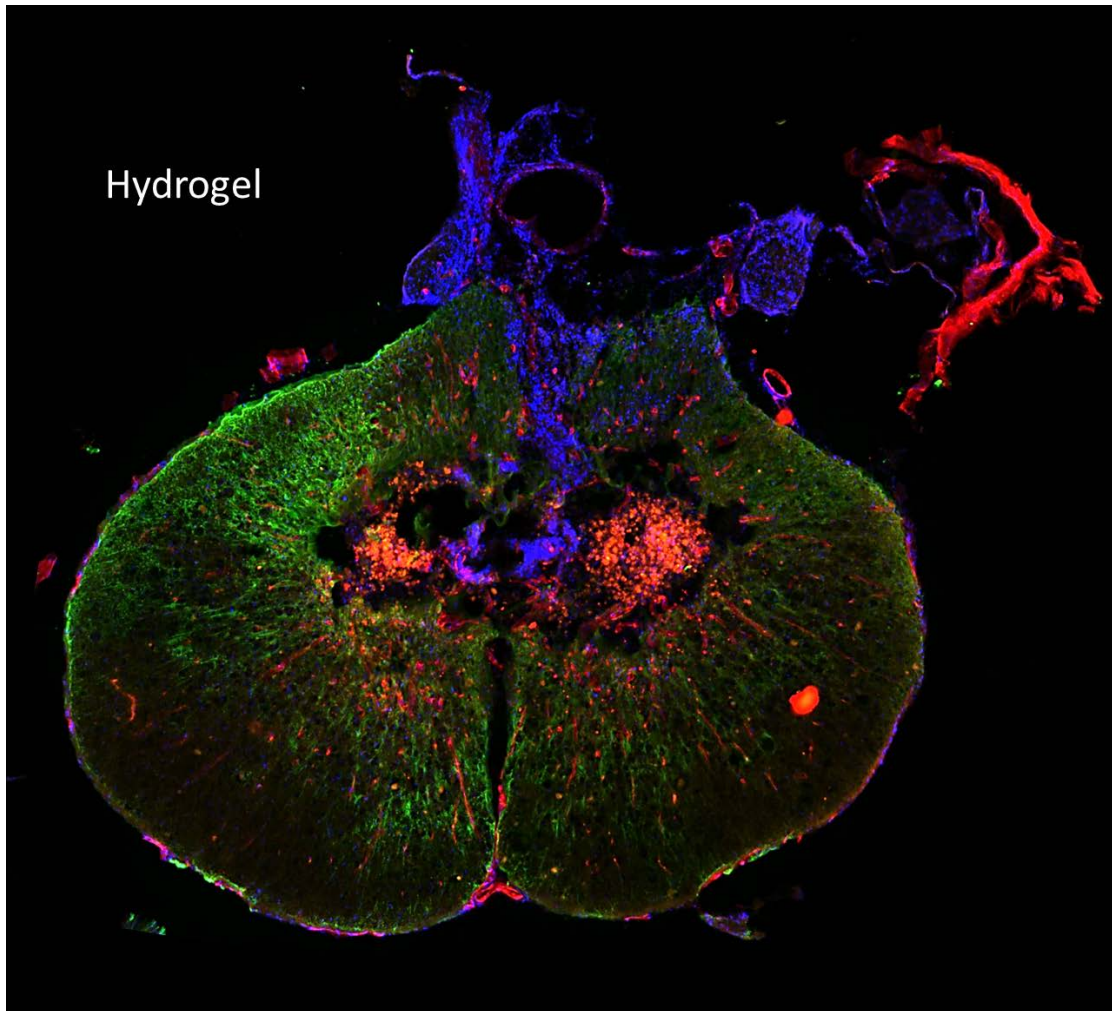


Figure 37. Hydrogel Animal Immunofluorescence Staining. GFAP (green) highlights the reactive astrocytes. RECA-1 (red) displays the vasculature. Hoechst (blue) marks the cell nuclei. Some blood vessel fragments have likely been phagocytized by microglial cells, with a more globular shape appearance and highlighted in red, within the lesion epicenter. Tissue taken several mm rostral from injury epicenter.

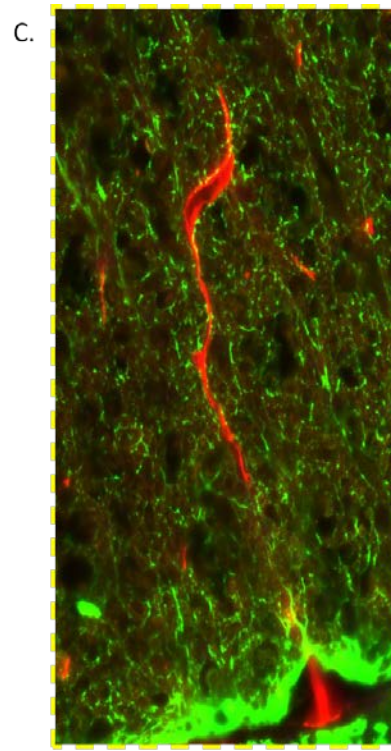
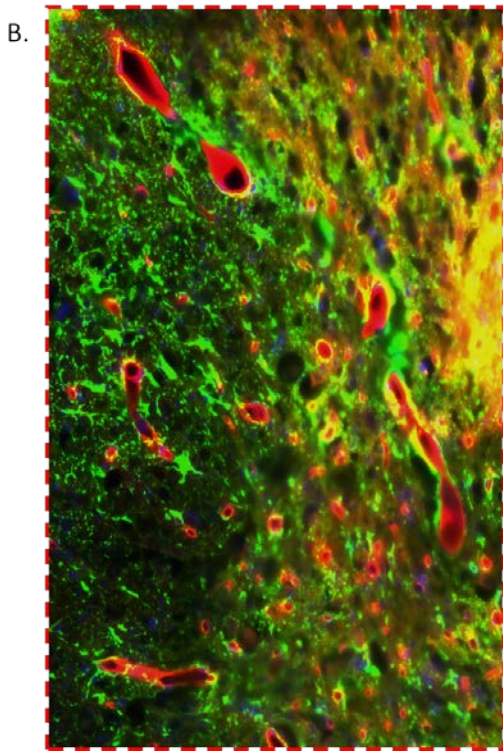
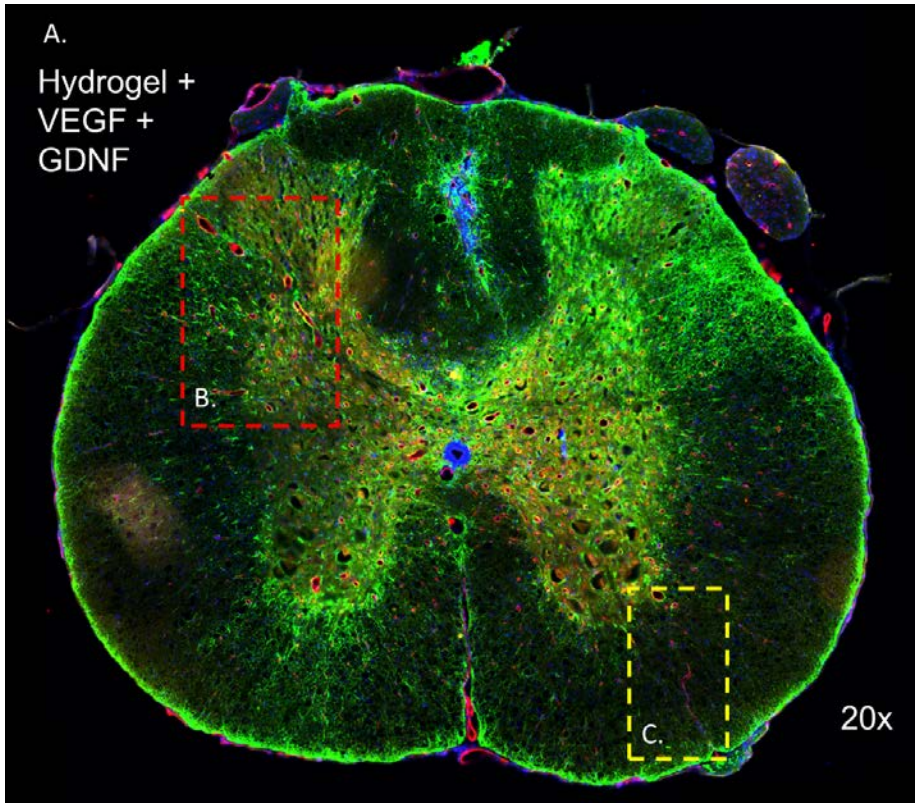
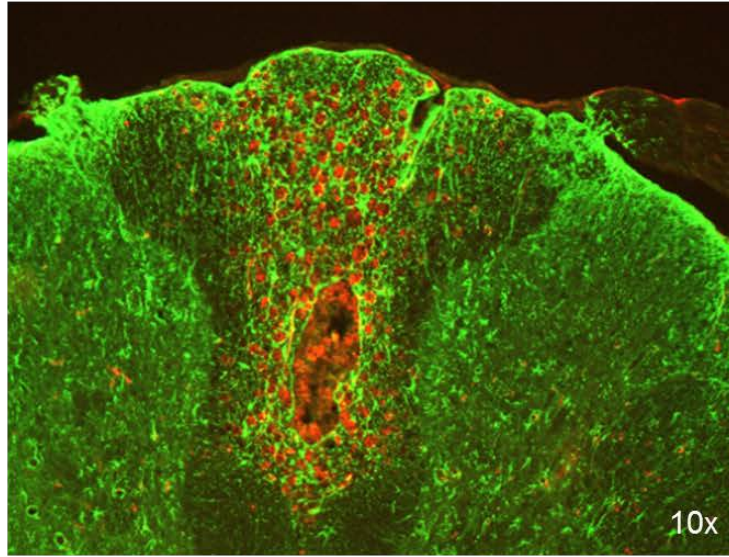
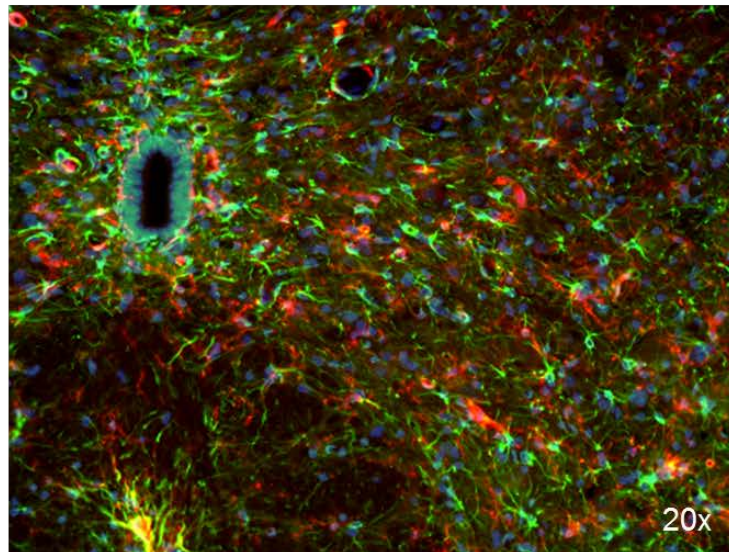


Figure 38. Hydrogel + VEGF + GDNF Immunofluorescence Staining. GFAP (green) highlights the reactive astrocytes. RECA-1 (red) displays the vasculature. Hoechst (blue) marks the cell nuclei. Tissue taken ~3mm rostral to injury epicenter. (A) A small amount of lesion can be seen in the dorsal white matter of the cord. Several areas of vasculature are highlighted below with corresponding boxes (B and C) above. (B) Astrocytic endfeet are visible on several blood vessels.

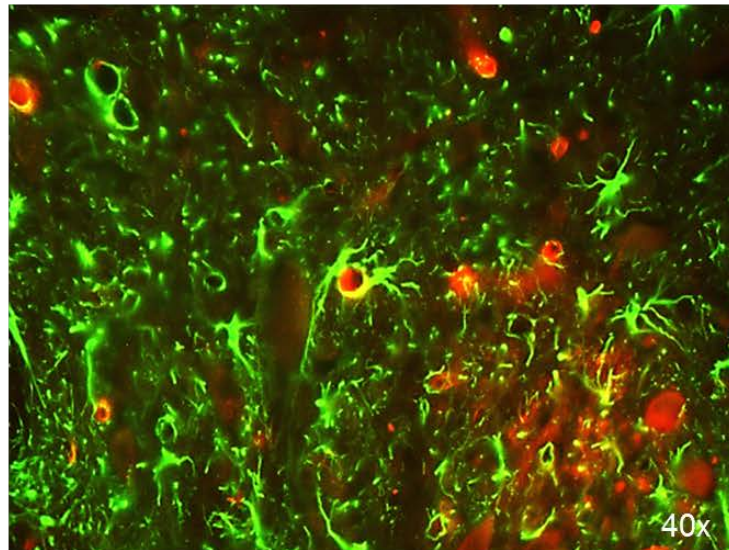
A.



B.



C.



4
0
x

Figure 39. Immunofluorescence Staining. GFAP (green) highlights the reactive astrocytes. OX42 (red) labels microglia. Hoechst (blue) marks the cell nuclei. (A) Dorsal white matter injury visible with microglia (red) and reactive astrocytes (green). (B) Central canal region with many individual astrocytes visible (green). (C) Reactive astrocytes (green) and microglia (red).

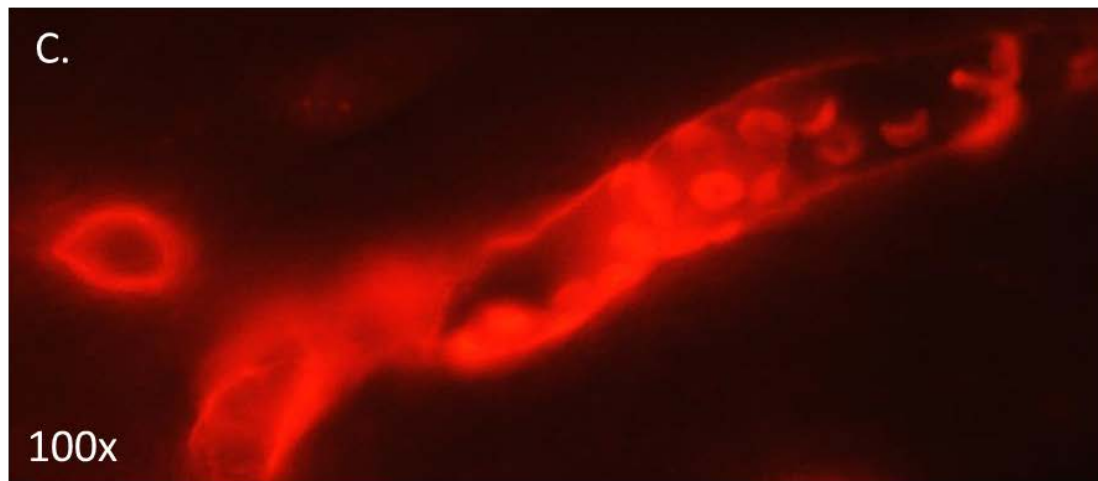
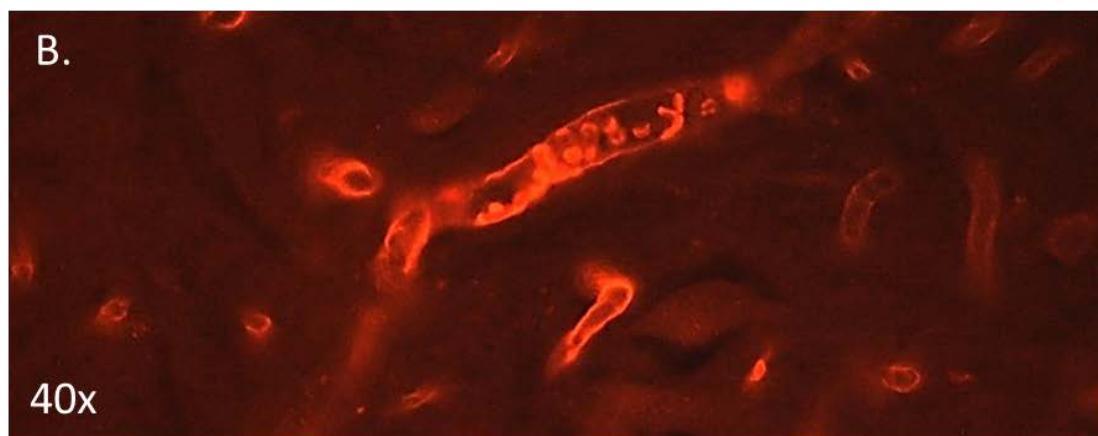
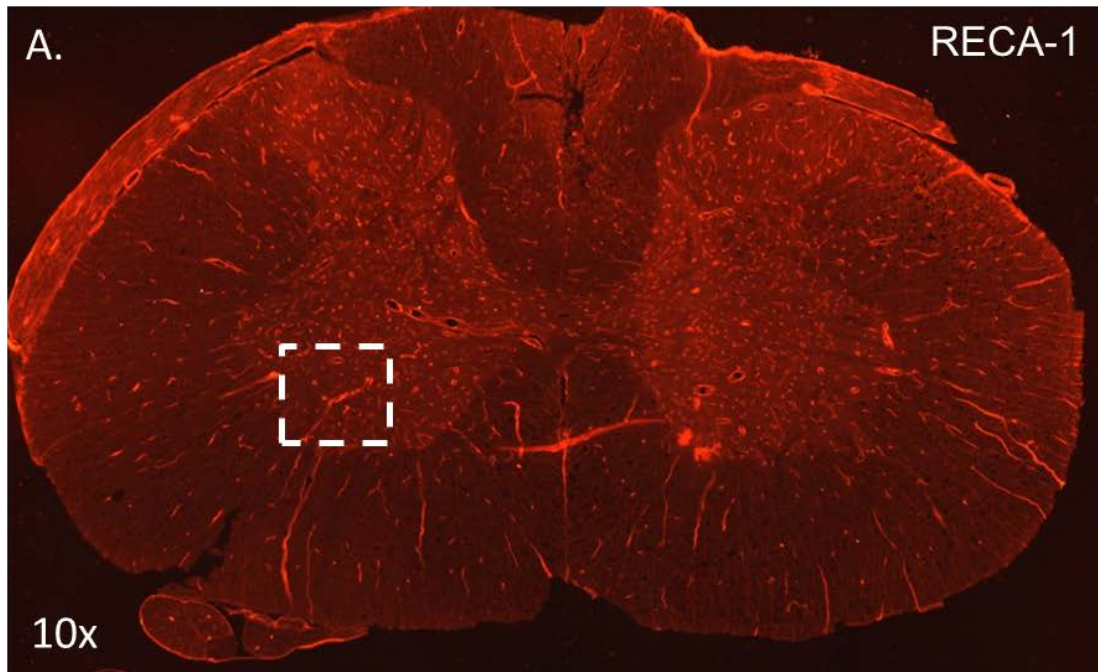


Figure 40. Vascular Immunofluorescence Staining. (A) 10x image of entire spinal cord several mm from injury epicenter. At 40x (B) and 100x (C) individual red blood cells were visualized within spinal cord grey matter vasculature.

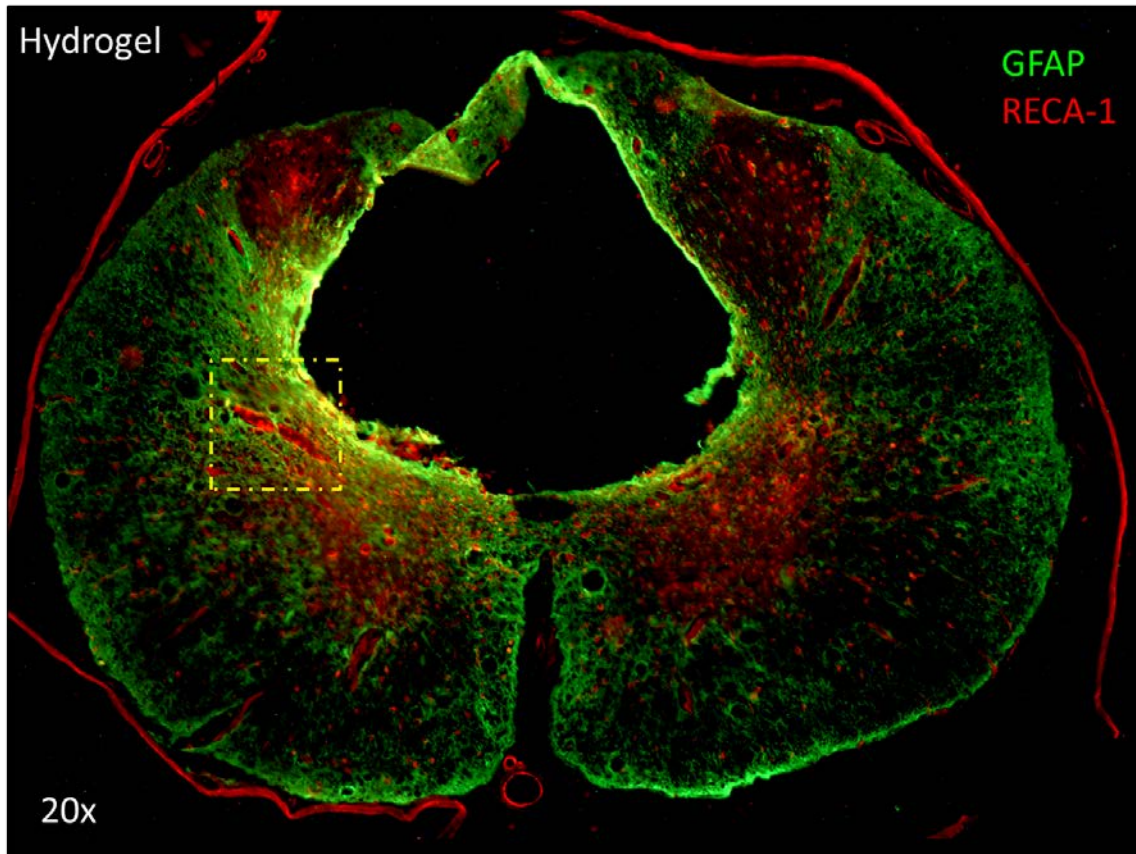


Figure 41. Vasculature and Glial Scar Immunofluorescence Staining. Hydrogel cord at injury epicenter. Red blood cells can be visualized in the blood vessel highlighted with the yellow (dotted) box.

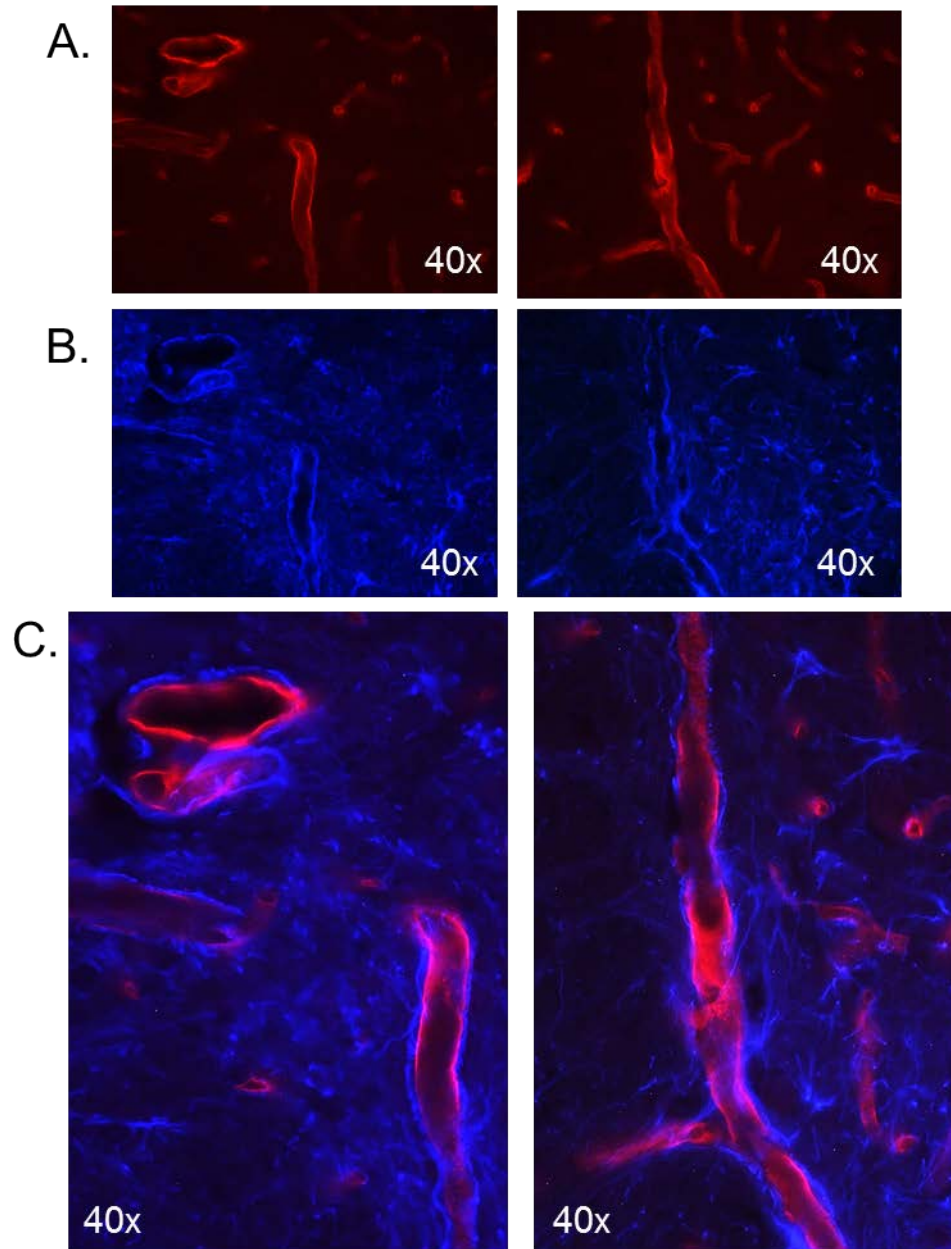


Figure 42. Vasculature and Glial Endfeet Immunofluorescence Staining. (A) RECA-1 (red) staining for blood vessels. (B) GFAP (blue) staining for reactive astrocytes. (C) Merged image of RECA-1 and GFAP, showing astrocytic endfeet as well as intertwining of astrocytes with the vasculature.

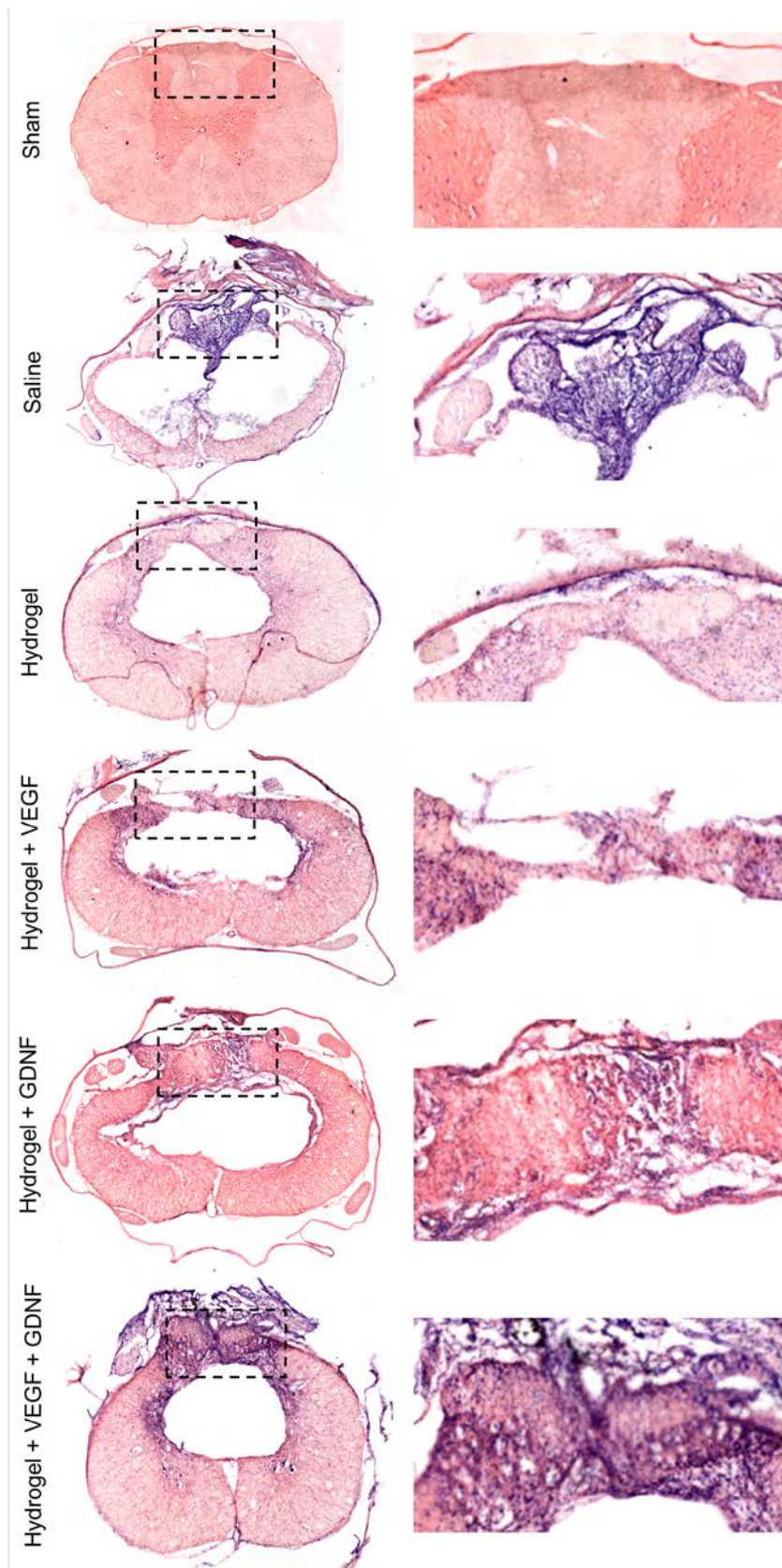


Figure 43. Cresyl-echt Violet Staining. Representative images of epicenter tissue show grey and white matter sparing. Higher magnification shows more white matter sparing in the dorsal columns of the Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF treatment groups as compared to the Saline and Hydrogel + VEGF groups. This may account for the significant locomotor recovery of these three groups, as observed by the Treadscan™ assessment.

Discussion

While GDNF is a well characterized neurotrophic factor, VEGF's influence on the nervous system has been primarily recognized over the past two decades. VEGF and GDNF have been shown to synergize at the cRET receptor (Tufro et al., 2007) *in vitro*, and to have synergistic neuroprotective benefits in other *in vivo* studies, such as ALS (Krakora et al., 2013) and Parkinson's disease (Herran et al., 2013). Moreover, the combination of VEGF and GDNF has also been shown to delay disease onset, increase survival period, significantly maintain motor function (BBB), and provide motor neuron protection (Lambrechts et al., 2003; Azzouz et al., 2004; Storkebaum et al., 2005; Krakora et al., 2013). A thorough review of the literature provided us with background for *in vitro* and *in vivo* concentrations of VEGF and GDNF.

In the present study, the first objective was to assess the neurite outgrowth in response to VEGF and GDNF, specifically of spinal cord neurons. The findings suggest that VEGF and GDNF both promote neurite outgrowth and have an additive effect when combined. This effect appears to be largely driven by GDNF for all measures of neurite outgrowth, as GDNF [50 ng/mL] showed significantly greater total neurite outgrowth, mean process length, maximum process length, and mean number of branches than GDNF [25 ng/mL], VEGF [25 ng/mL], VEGF [50 ng/mL], and neuronal controls. Whereas, the outgrowth and branching promoted by GDNF [50 ng/mL] did not significantly differ from either of the combined VEGF + GDNF groups, indicating that GDNF likely contributed the most to the effects in the combination group.

In SCI animal models, GDNF has been shown to promote significant neurite and axonal growth (Iannotti et al., 2003; Blesch and Tuszynski, 2003; Dolbeare and Houle, 2003), and increased neurite branching (Deng et al., 2016). Additionally, *in vivo* GDNF has been shown to reduce astrogliosis (Deng et al., 2013; Ansorena et al., 2013), increase myelination (Zhang et al., 2009; Deng et al., 2013), increase intralésional vasculature (Zhang et al., 2009; Ansorena et al., 2013), and improve functional recovery (Cheng et al., 2002; Kao et al., 2008; Deng et al., 2013; Ansorena et al., 2013). Thus, it was expected that the combination of VEGF and GDNF (slowly released from hydrogel) would create a favorable environment, *in vivo*, for tissue repair and improved functional recovery. We anticipated that the hydrogel would serve as both a structural matrix to fill the contusion injury-induced cavitation and a functional reservoir to protect the VEGF and GDNF proteins from degradation by the surrounding toxic lesion milieu, and slowly release those over the course of approximately four weeks post transplantation.

Significant functional recovery was observed with Hydrogel alone, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF groups using the Treadscan™ system, whereas the BBB, Gridwalk, and Modified Grooming test did not detect significant differences across treatment groups. First, we note that Hydrogel alone significantly improved locomotor outcomes as measured by the Treadscan™. Thus, the Hydrogel itself had a positive effect on locomotion. Similarly, a hyaluronan and methylcellulose based-hydrogel delivered intrathecally resulted in reduced inflammatory cytokines, a reduction in the glial

scar deposition (CSPGs), reduced lesion volume, improved axonal conduction, and improved motor function (Austin et al., 2012). Other studies utilizing a bioengineered tissue as the sole treatment after SCI have revealed beneficial axonal and astrocytic growth into the lesion (Marchand and Woerly, 1990; Marchand et al., 1993). Similarly, in a T9 transection injury model, significant axonal regrowth was observed in response to transplanted thiolated hyaluronic acid hydrogels (Horn et al., 2007). A study employing a Neurogel™ treatment after SCI resulted in significant axonal growth, angiogenic vascular growth, and astrocyte process growth into the hydrogel implant, along with significant improvements in motor function (Woerly et al., 2001a), which were enhanced by enriched housing environments. The non-significant trend of increased spared white matter in the Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF groups (Figure 36) is evident in examination of the dorsal columns of the spinal cord (Figure 43), as well as greater spared grey matter for these three groups.

Second, in considering the significant recovery observed using the Treadscan™ system, one possible explanation for no significant differences in other behavioral measurements is that the final *n* number in each group might not be high enough to tease apart minor differences in the other behavioral measures. Another possible explanation is that the Treadscan™ has the capability to measure some parameters of locomotion that BBB assessment does not. For example, Treadscan™ and Catwalk™ can measure footprint area, pressure maps, stride length, stride width (base of support), gait parameters (swing time and break time), and more detailed measures of coordination. Figley

et al. (2014) observed significant improvement after treatment with a bioengineered zinc-finger VEGF-A using the Catwalk™, although no differences were observed through BBB assessments. Additionally, the Treadscan™ reduces inter-rater variability and investigator bias, even for blinded studies, particularly on the most subjective measures (toe clearance and coordination). Taken together, these indicate the usefulness in supplementing the BBB assessment with other motor assessments following SCI, such as the Treadscan™, Catwalk™, or Footprint. A combination of behavioral assessments provides the most thorough and in-depth knowledge of functional deficits and recovery (BBB, Treadscan™, Catwalk™, Footprint, Gridwalk, Grooming).

For example, in this study we adapted the grooming test and used it as a measure of trunk stability. We observed the animals stopping to groom during the BBB and Hargreaves' tests (Harvard Apparatus), whenever they felt comfortable in their surroundings. It was obvious that some rodents could groom using both forepaws simultaneously, like Sham animals, while others could only groom with one forepaw at-a-time and required the second forepaw to be on the ground supporting the rodent's body weight. We observed an interesting non-significant trend, which supports the idea that this modified grooming test might be a useful measurement for trunk stability after SCI, when used in conjunction with the BBB, Treadscan™ or Catwalk™, and Gridwalk.

Some SCI treatments have been shown to provoke increased sensitivity to thermal or mechanical stimuli. We feel that the Hargreaves' test provides us with consistent, reliable, and reproducible data to measure treatment-induced

hyperalgesia. Thus, we employed the Hargreaves' test in this study, and no significant differences were observed across time or across treatment groups. This has important clinical implications because putative therapeutic approaches which do increase sensitivity to thermal stimuli, thus corresponding to hyperalgesia, do not progress on to clinical trials.

VEGF significantly promoted neurite outgrowth *in vitro*, but failed to promote significant improvements *in vivo*. Here we discuss some important considerations of factors that might contribute to these observed differences. First, *in vitro* and *in vivo* conditions require different concentrations of factors, as the surrounding cells, tissue, and connected organs of the body influence the exposure of the host tissue to the transplanted trophic factors. Whereas, in the *in vitro* experiment, the neurons were directly exposed to the trophic factors with less than 5% other cells types in the culture competing for trophic factor access.

Second, the *in vitro* study did not include hydrogel, thus the neurons had immediate, direct exposure to VEGF and GDNF concentrations, whereas, *in vivo* the hydrogel protected the proteins from degradation and had a time-controlled release profile of approximately four weeks. The *in vitro* study could be conducted again in the future, with hydrogel (mixed with VEGF and GDNF) added to the neuronal cultures. However, this experiment was not performed in this study. If hydrogel embedded with VEGF and GDNF were added to the previously plated neuron cultures, we might expect to see a slower neurite outgrowth compared to the current study which did not include the hydrogel. We would anticipate possibly seeing greater outgrowth from the hydrogel + GDNF +

VEGF or hydrogel containing either factor alone, as compared to media controls, but likely only if neurite outgrowth were observed for longer periods of time than the current study, due to the fact that the hydrogel withholds the trophic factors, thus it takes longer time before the neurons are exposed to all of the factors.

In contrast, if the neurons had been mixed with hydrogel before plating, and then the trophic factors were added directly to the hydrogel neuronal co-culture, then we would expect to see greater outgrowth of the neurites compared to our current study, without hydrogel, before any factors were added. However, hydrogel and neuronal cultures with subsequent addition of trophic factors should yield greater outgrowth than hydrogel and neuronal cultures with only media added. Similar to the previously described future study, in order to see this outgrowth from the hydrogel and neuronal cultures, it will likely take longer for the trophic factors to reach all of the neurons, as the factors must diffuse across the hydrogel. Thus, to observe the increased outgrowth, the plates might need to be assessed at time points longer than 5 days, perhaps on the order of 10-14 days in culture. This proposed study of mixing hydrogel with neurons, plating them, and then adding the trophic factors would be a good simulation model for an *in vivo* study in which cells (Schwann cells, mesenchymal stromal cells, oligodendrocyte precursors, neural progenitors, endothelial cells) are embedded within the hydrogel, trophic factors are mixed with the hydrogel, and this combination is transplanted into the injured spinal cord.

Third, the *in vitro* study was performed on embryonic day 15 (E15) isolated spinal cord neurons and the *in vivo* study was performed on adult

Sprague Dawley rats. Thus, the axonal growth response on adult neurons *in vivo* might not be as robust as the outgrowth observed from younger neurons *in vitro*. Fourth, it is important to recall that VEGF's neurite outgrowth effects (*in vitro*) were modest, thus, possibly not sufficient enough *in vivo* to promote significant improvements in functional recovery or histopathology. Fifth, the *in vivo* study included a transplantation time point of 12 days post SCI. Therefore, this leads us to another important consideration – the optimal *in vivo* transplantation time point might differ between VEGF and GDNF. Perhaps the transplantation time point needs to be further optimized, and determined if the optimal time point for VEGF and GDNF differ. Twelve days post SCI is a subacute treatment as it is just at the end of the acute phase following injury. VEGF given too early in the acute phase, following SCI has been shown to be detrimental (Benton and Whittemore, 2003). If the optimal time point for transplantation does differ between VEGF and GDNF, then two different time points can be achieved through one transplantation of hydrogel containing both factors by embedding one factor (most likely VEGF) into nanoparticles and then embed those nanoparticles into hydrogel containing GDNF. Thus, the GDNF will have a release profile that begins sooner than the VEGF. Therefore, the hydrogel can actually be transplanted as early as 7-8 days post SCI, when a cavity has formed. Thus, there is a space to hold the hydrogel.

Another consideration is that VEGF might need to be in combination with other factors in order to have *in vivo* efficacy at promoting significant histopathological or functional outcomes, as indicated by two combinational SCI

papers administering VEGF and PDGF (Lutton et al., 2011, Chehrehasa et al., 2014), and possibly by the *in vivo* Hydrogel + VEGF data from this study. In the first of these two cited studies (hemisection SCI model), VEGF and PDGF were only beneficial when administered in combination (Lutton et al., 2011). Similarly, in the second study (contusion SCI model), either factor alone resulted in behavioral results similar to control animals (BBB of 10), whereas VEGF and PDGF in combination resulted in significantly improved locomotion (BBB of 18) and significantly reduced lesion cavity (Chehrehasa et al., 2014). One argument is that VEGF causes permeability of the vasculature and too much permeability can lead to extravasation of inflammatory mediators, likely exacerbating the lesion environment (Benton and Whittemore, 2003). However, it is noteworthy that in this study there was no evidence of an exacerbated inflammatory response (as assessed with ED-1) with any treatment groups, as compared to Saline treated animals or Sham controls. Thus, we do not believe any of our treatments caused a more permeable blood-spinal cord barrier, above and beyond the initial mechanical injury and the endogenous secondary wave of injury effects on the intactness of this barrier.

Lutton and colleagues (2011) suggest that the addition of PDGF and VEGF as a combinational therapy stabilizes angiogenic vasculature and forms an intact blood-spinal cord barrier, necessary for correct maintenance of fluids and molecules inside the blood circulation and inside the surrounding tissues. Thus another potential combinational treatment approach would be to include VEGF, GDNF, and PDGF inside the hydrogel. However, the caveat is that the optimal

transplantation time point for each of the factors might not be the same, which necessitates a time course study to identify the optimal transplantation time points. Once identified, the time course for each factor could then be achieved by embedding the factor to be released last (presumably PDGF) in nanoparticles designed with layers and concentrations that allow it to have the slowest release. These nanoparticles could then be embedded into hydrogel containing other nanospheres which have a different biodegradable profile thus that their contents (presumably VEGF) would be released slightly before the PDGF nanoparticles. Then, contained within the hydrogel itself, could be the third factor (likely GDNF) that would be released at the earliest time point. Thus, instead of transplanting at 12 days post SCI, this combinational hydrogel could be transplanted as early as 7-8 days post SCI. This would afford an earliest release of GDNF, followed by VEGF with a final release of PDGF. Thus, GDNF's neuroprotective benefits could begin during the acute phase of SCI, with VEGF's influence on the neurons and vasculature would not begin too early during the peak of the inflammatory phase (leaky/disrupted blood vessels), and the final release would be the PDGF, to stabilize the angiogenic vasculature and stem cell proliferation.

Conclusions

In summary, VEGF and GDNF promoted significant neurite outgrowth and branching from embryonic spinal cord neurons *in vitro*. Furthermore, the combination of VEGF and GDNF provoked an additive response of neurite outgrowth and branching, as anticipated based on *in vivo* literature of ALS and Parkinson's disease models (Krakora et al., 2013; Herran et al., 2013).

Additionally, *in vitro* results matched *in vivo* results – treatment groups containing GDNF showed the most neurite outgrowth from spinal cord neurons while animal treatment groups containing GDNF resulted in the greatest improvement in functional recovery.

The *in vivo* Treadscan™ parameters revealed that hydrogel alone improved functional recovery as much as hydrogel + GDNF, and hydrogel + VEGF + GDNF. Therefore, the *in vivo* data supports the idea that hydrogel's role as a structural scaffold, for growing vasculature, axons, and astrocytic processes, is just as important as its functional purpose of slowly releasing trophic factors while protecting them from degradation. Moreover, due to its non-immunogenic and its *in-situ* gelable properties, the hydrogel has the capability of conforming to the non-uniform lesion cavity of each individual host without provoking an immune response. This, along with the fact that no increased sensitivity to a thermal stimulus was observed, supports the further exploration of this hydrogel for future therapeutic use for repairing the injured spinal cord. Thus, future studies employing hydrogel will likely include various trophic factor combinations, some possibly embedded within nanoparticles prior to embedding within the hydrogel itself, to allow for various time-controlled release profiles of the different trophic factors. Designing bioengineered matrices to promote growth guidance and directionality is also an important consideration for future studies. Combinations of transplanted cells, trophic factors, and enzymes – all embedded within bioengineered matrices to protect and slowly release the factors, cells, and enzymes – also appear to be promising therapeutic approaches.

OVERALL DISCUSSION

SCI is a devastating and disabling condition and yet, currently no effective treatments exist. Neurosurgeons, neuroscientists, and neurotraumatologists have been working for over 5,000 years to find an effective treatment. However, it was not until the twentieth century that neuroscientists first started seeing breakthroughs in reaching the goals set by Santiago Ramon y Cajal when he encouraged future neuroscientists to try to change the ‘irreversibility’ of the central nervous system (Schwab and Bartholdi, 1996).

Angiogenesis following SCI

Despite the toxic lesion environment at injury epicenter after SCI, an endogenous angiogenic response occurs, peaking between 7-14 days post-injury, but regressing coincident with the onset of cavitation, in both rats and higher primates (Loy et al., 2002; Casella et al., 2002; Benton et al., 2008a; Fassbender et al., 2011). We therefore, hypothesized that the angiogenic vasculature regresses due to a loss of structural support, with the onset of cystic cavitation. Therefore, the overall goal of this dissertation research was to provide a structural scaffold for vascular reorganization, axonal regrowth, and tissue repair following contusive SCI, by employing a novel bioengineered hydrogel.

Bioengineered Tissues for SCI Treatment

Bioengineered matrices have been employed for treatment for SCI since the early 1990’s (Marchand and Woerly, 1990). The next few years saw major advancements in optimizing the bioengineered tissues for the structural and

functional properties. The ultimate goal is axonal regrowth through-and-beyond the distal end of the lesion, for functional reconnections of axons with their target neurons, and ultimately for improved functional recovery. Thus, providing directionality to the bioengineered tissue is worthwhile, and might be useful for future approaches with our bioengineered hydrogel. This is necessary in order to create a full connection of neuronal activity from the brain down through the spinal cord, to the neurons that connect to the muscles in our limbs and our internal organs, for movement and to sustain life (heartbeat, respiration, digestion, bladder function).

Overlapping Signals in Nervous and Vascular Systems

Andreas Vesalius gave us clues to the overlapping development of the vascular and nervous systems in his 1543 publication (*De humani corporis fabrica, On the fabric of the human body*, 1543) as noted by Carmeliet and Tessier-Lavigne (2005). Dr. Peter Carmeliet and colleagues have been instrumental in linking the vascular and nervous systems and the overlapping signaling between the two, as well as overlapping signaling to other various cell types (Carmeliet and Storkebaum, 2002; Storkebaum et al., 2004a; Storkebaum and Carmeliet, 2004b; Zacchigna et al., 2008; Ruiz de Almodovar et al., 2009; Carmeliet and Ruiz de Almodovar, 2013). Additionally, other publications also encouraged us to employ VEGF and GDNF embedded within bioengineered hydrogel for treatment of traumatic SCI. Publications showing the angiogenic vasculature regresses with the onset of cystic cavitation (Loy et al., 2002; Casella et al., 2002; Benton et al., 2008a; Fassbender et al., 2011) prompted us

to utilize our hydrogel to rescue this endogenous angiogenic response by providing a structural support matrix for the angiogenic vasculature. Studies showing deletions in the VEGF promoter region result in neurodegenerative phenotypes in mice, similar to ALS, and showing VEGF is important for maintenance of motor function (Oosthuysen et al., 2001) also prompted us to hypothesize that the VEGF will positively influence the CNS tissue repair, provide neuroprotection to neurons, if the treatment is applied at an early enough time point post-injury. Furthermore, intact vasculature is crucial for delivering oxygen and nutrients and for removing toxic wastes from the tissues. Therefore, we hypothesized that delivering VEGF to the lesion epicenter via a bioengineered hydrogel might positively influence the endogenous angiogenic response while promoting neuron survival and growth, Schwann cell migration and survival, and influencing microglia and astrocytes due to the pleiotropic mechanisms of VEGF (Storkebaum et al., 2004a).

VEGF and GDNF Combinational Treatment Approach

Tufro et al. (2007) encouraged us to use GDNF in combination with VEGF, as both have been shown to synergize at the cRET receptor. Additionally, VEGF and GDNF significantly promoted animal survival, delayed disease onset, and maintained motor function in an ALS neurodegenerative disease model (Krakora et al., 2013). Likewise, in a 2013 Parkinson's disease model (Herran et al.) VEGF and GDNF had an additive effect on neuron survival and increased axon fiber density compared to control animals or animals receiving VEGF or GDNF alone.

GDNF Treatment for SCI Repair

Additionally, SCI literature has shown GDNF promotion of neuroprotection (Arce et al., 1998; Soler et al., 1999; Nicole et al., 2001), increased intralesional vasculature (Zhang et al., 2009; Ansorena et al., 2013), enhanced axonal growth (Blesch and Tuszynski, 2001; Dolbeare and Houle, 2003), reduced astrogliosis (Iannotti et al., 2003; Deng et al., 2011a; Ansorena et al., 2013), increased myelination (Zhang et al., 2009; Deng et al., 2013), and improved functional recovery (Cheng et al., 2002; Kao et al., 2008; Deng et al., 2013; Ansorena et al., 2013). In a Parkinson's disease model, Iravani et al. (2012) observed neuroprotection following GDNF administration. Furthermore, in a Huntington's disease model, neural stem cells secreting GDNF conferred striatal neuroprotection, showing the advantage of a combinational treatment approach (Pineda et al., 2007). Moreover, in another Huntington's disease model GDNF delivered via an adeno-associated viral vector into the striatum, provided neuroprotection to both nitric oxide synthase striatal interneurons as well as parvalbumin striatal interneurons (Kells et al., 2004). Following SCI, Zhang et al. (2009) showed that GDNF administration increased the number of myelinated axons and the number of blood vessels.

Combinational Treatment Approaches for SCI

Based on current literature within the SCI field, combinational treatment approaches appear to hold the greatest therapeutic potential (Xu et al., 1995; Guest et al., 1997; Sayer et al., 2002; Fouad et al., 2005; Lutton et al., 2011; Lu et al., 2012; Deng et al., 2013; Ansorena et al., 2013; Zhang et al., 2013).

Collectively, these studies highlight the importance of combinational treatment approaches and the beneficial effects of VEGF and GDNF for repairing the injured spinal cord. Therefore, we utilized a combinational treatment approach employing a novel, non-immunogenic bioengineered hydrogel embedded with VEGF and GDNF following a moderate midline T10 contusion SCI.

VEGF and GDNF Promote Significant Neurite Outgrowth

In vitro, we observed significant neurite outgrowth in response to VEGF and GDNF, with a possible additive effect from the combination of both factors. VEGF and GDNF also resulted in a significant increase in the mean number of neurite branches, the maximum neurite length, the mean neurite length, and the total number of neurons. Thus it appears that VEGF and GDNF had neuroprotective effects on the neurons in culture as well as neurotrophic influence on neurite outgrowth. These results correspond well to findings from the literature on both VEGF and GDNF for neuroprotection and neurotrophic support. The data supports the idea that GDNF is having a stronger influence on the neurons than the VEGF. It is important to keep in mind that hydrogel was not mixed with the neurons and trophic factors *in vitro*. However, adding the hydrogel in with the cultured neurons and trophic factors is a good future direction.

Improved Locomotion with Various Hydrogel Treatment Groups

Similar results were observed *in vivo* as *in vitro*, with groups containing GDNF having greater influence on restoring locomotion than VEGF. Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF groups all showed Sham-like

locomotion in the following Treadscan™ parameters: 1) Stance maximum area, 2) Print angle, 3) Base of support, 4) Brake time, 5) Homologous gait coupling, 6) Homolateral Gait coupling, 7) Diagonal gait coupling, 8) Longitudinal maximum deviation, and 9) Longitudinal minimum deviation. In other words, the area on the ground for which the paw occupied (stance maximum area) was significantly improved with Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF treatment. Oftentimes, after SCI, the paws can have an altered footprint, typically a smaller footprint due to injury and the angle in which the paw makes contact with the ground due to the injured limb. The angle of the paw from the central body axis (print angle) was significantly improved with the same three treatment groups as well. Internal and external paw rotation is quite common after SCI, and is a major component of the BBB assessment. The distance between the rear hind limbs (base of support) was also similar to sham animals, for the same three treatment groups (Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF). The base of support can be thought of similar to a person learning to roller blade – the wider the stance the less stable, the narrower the stance the more stable. Thus, after SCI, rodents typically have a wider base of support which begins to narrow and return closer to baseline (sham) levels with treatment or recovery. The amount of time it takes the animal to stop the paw from motion once it touches the surface of the ground (brake time) was also similar to sham levels for Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF groups. This again shows more stability in open field locomotion. The coupling between the front set of paws or rear set of paws (homologous gait

coupling), the coupling between the right front and rear paw as well as the coupling between the left front and rear paw (homolateral gait coupling), and the coupling between the opposite limbs – front right and rear left as well as front left and rear right (diagonal gait coupling) were returned to sham levels for the Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF treatment groups. The minimum and maximum distance of the paws from the central body axes (longitudinal minimum deviation and longitudinal maximum deviation, respectively), were also similar to sham animals for the same three treatment groups. Again, this shows that Hydrogel, Hydrogel + GDNF, and Hydrogel + VEGF + GDNF treatment groups significantly improved locomotor function, as detected by the Treadscan™ system. These *in vivo* results are similar to the *in vitro* findings from the study, in which GDNF played a more significant role in neurite outgrowth than VEGF, particularly at higher doses.

Putative Limitations of the Study

We can only speculate that the small *n* number of the study, variability across animals, and the subjective nature of some of the BBB assessments resulted in no significant findings from the BBB assessments. Additionally, it is notable that the significant parameters in this study, as measured by the Treadscan™, aside from print angle (paw rotation), are not measurements in the BBB. Paw rotation, as measured by the BBB is a qualitative assessment – internal or external – while the Treadscan™ provides a quantitative measurement of paw rotation (in degrees) from the central body axes. Thus, this study emphasizes the importance of multiple motor and sensory assessments

following SCI. Batteries of tests provide the most comprehensive picture of functional deficits as well as recovery and treatment effects. Therefore, we feel it is quite useful to employ the Treadscan™, Catwalk, or Footprint measures in addition to the BBB, along with other motor and sensory assessments, such as the Hargreaves' test, von Frey test, Gridwalk, sticker removal test, and Grooming test.

Suggested Future Directions

Further study is needed in order to tease apart why the Hydrogel + VEGF did not confer as much functional recovery as the Hydrogel only group. It is possible that the optimal transplantation time point for VEGF is not at 2 weeks post injury. The GDNF might be having such a large effect on functional improvement that it had a greater influence on the Hydrogel + VEGF + GDNF group, in order for functional improvement to be significant in this group as well. This hypothesis matches the *in vitro* results, in which GDNF played a greater role than the VEGF. However, our *in vivo* study contained an additional variable (hydrogel), thus a direct comparison cannot be made.

Therefore, in order to further evaluate the factors influencing the *in vivo* study, a larger *n* number is necessary. A larger *n* number would provide higher statistical power for analysis, and might tease apart minor differences in the BBB assessment.

Further development of the modified Grooming test might also show promise for this assessment for future SCI studies using thoracic contusion injury models. The Grooming test is already well-established for cervical SCI models,

but appears to hold great promise for use with thoracic SCI models as well, if further developed and explored.

One method to strengthen the current study would be to verify the biological activity of the VEGF and GDNF at various time points from *in vivo* tissue. Doing an enzyme-linked immunosorbent assay (ELISA) would detect the presence of two trophic factors at various time points post-injection. However, it does not reveal the biological activity of the trophic factors. Thus, to attempt to determine the biological activity of the transplanted trophic factors, a section of spinal cord tissue (including injury epicenter) would be extracted at various time points post-hydrogel transplantation. The tissue segment would be sonicated and ground up, and exposed to cultured embryonic spinal cord neurons, similar to our *in vitro* study (previously described). If greater neurite outgrowth is observed in the VEGF, GDNF, and VEGF + GDNF treated animals compared to Saline controls then it is likely that the VEGF and GDNF are still biologically active at that time point. A caveat is that the neurite outgrowth might be a direct outcome of VEGF and GDNF on neurons or an indirect outcome through VEGF and GDNF influence on other cell types. For instance, VEGF promotion of angiogenic vasculature – creating a more favorable environment for neuron survival; VEGF and GDNF recruitment of glial cells (Schwann cells) to the lesion area – to aid in tissue repair and remyelination; VEGF promotion of migration and proliferation of microglia – to clean up the dead cellular debris; oligodendrocytes, VEGF promotion of astrocyte proliferation – to contain the toxic lesion environment; GDNF positive modulation of the astrocytic glial scar – to

encourage intermingling between neuron and astrocytic processes. Additionally, the cellular debris and toxic lesion environment from the ground tissue graft might cause neuron apoptosis within the neuronal culture.

Another way to approach this question is to incubate hydrogel with embedded VEGF, GDNF, or both at 37°C (body temperature) for varying time points, for instance 1-4 weeks. Then, at 5 days, 7 days, 2 weeks, 3 weeks, and 4 weeks take the hydrogel out of the incubator and add to the cultured spinal cord neurons. Next, assess neurite outgrowth, to determine if the VEGF and GDNF have an even greater neurite outgrowth than the hydrogel only control group or the media only neuronal control group. This does not exactly simulate *in vivo* conditions, but would be a way to assess biological activity of the VEGF and GDNF at various time points post-incubation in hydrogel, at simulated body temperatures and carbon-dioxide conditions. This would also reduce some of the confounding variables of taking tissue plugs from the *in vivo* study and exposing to the neuronal cultures, as described above, such as apoptosis (cellular debris), toxic molecules such as nitric oxide and other reactive oxygen species, endogenous VEGF and GDNF, the activation of other cell types (microglia, oligodendrocytes, astrocytes, Schwann cells) by VEGF and GDNF.

A more complex, and possibly more effective combinational treatment approach would be to embed cells (Schwann cells, neural progenitor cells, oligodendrocyte progenitors, endothelial cells) into the hydrogel matrix and then mix trophic factors (encapsulated within nanospheres or without encapsulation) into the hydrogel. Dr. Mary Bunge has been investigating the beneficial effects of

Schwann cells (SCs) for the repair of tissue following SCI, and in combinational treatment approaches for the past ~40 years, work started by Dr. Richard Bunge in 1975 (Bunge MB, 2016). In a more recent study, Williams and Bunge (2015) detail the positive effects of SCs on promoting axonal regeneration, myelination of regenerated axons, reducing cystic cavitation and secondary injury, as well as some functional improvement. Combining SC therapy with other neurotrophic factors, enzymes, cells, and other treatments, for a combinational approach enhances the functional recovery and results in greater axonal regrowth (Williams and Bunge, 2015). This work, among Dr. Bunge's enormous expanse of SCI and SC literature, once again nicely highlights the importance of combinational approaches for the treatment of SCI. Another study conducted by this group used a bioengineered bridge to support and encourage the extension of axons, astrocytes, and Schwann cells across the matrigel bridge and beyond the distal end of the lesion site. Brainstem axons regenerated across the matrigel bridge and formed functional connections onto dendrites in the caudal host tissue following a T8 complete transection injury. Improvement in hindlimb motor function directly correlated with the number of brainstem regenerated axons and GFAP positive astrocytic fibers which entered the matrigel bridge along with the transplanted SCs (Williams et al., 2015). This study also demonstrates the importance of combinational treatment approaches.

Schwann cells are currently the only FDA (Food and Drug Administration) approved cell type for human clinical spinal cord injury studies (Xu XM, 2012; Guest et al., 2013). While transplanted cells can be genetically engineered to

overexpress VEGF or GDNF, studies in which cells are embedded within the hydrogel containing trophic factors have a much greater propensity for safety as far as clinical trials, due to the fact that the trophic factors will ultimately be biodegraded over time, as will the hydrogel. Transplanting hydrogel containing cells that are genetically modified to overexpress certain trophic factors holds the concern of long-term cell survival, even after hydrogel biodegradation, and thus long-term synthesis and release of growth factors which could result in unregulated cell growth or tumor formation; which is always a major concern with administering trophic factors with no terminal time point of synthesis or release.

Additionally, enzymes could also be mixed within the hydrogel in addition to cells and trophic factors, such as Chondroitinase ABC (ChABC; Houle et al., 2006), which helps to degrade the inhibitory components of the glial scar, namely the chondroitin sulfate proteoglycans (Nogo-A, aggrecan, keratin, brevican, neurocan, phosphocan). Due to ChABC's enzymatic nature, it can be degraded quickly by the surrounding tissues. Thus, embedding ChABC into hydrogel or into nanospheres within the hydrogel will not only afford tighter control over its release profile at the site of injury, but will also protect the enzyme from being quickly degraded. Hydrogel containing ChABC (embedded in nanospheres; Houle et al., 2006), VEGF (in nanoparticles), GDNF (in different nanoparticles; Dolbeare and Houle, 2003; Deng et al., 2013), and endothelial cells and neural progenitor cells (Rauch et al. 2009), for example, might be a powerful combinational treatment approach, based on previous SCI literature with each of these individual treatment approaches, and some combinational therapies.

Combinational Treatment Approaches Employed for SCI

In a combinational treatment approach, Rauch et al. (2009) transplanted a co-culture of endothelial cells and neural progenitor cells embedded within a PLGA biodegradable polymer following a hemisection SCI. Compared to SCI lesion or PLGA alone, the PLGA plus endothelial cells as well as the PLGA plus neural stem cells both promoted twice the number of angiogenic vessels. Furthermore, the combination of PLGA with endothelial cells and neural progenitor cells resulted in twice as much vasculature as PLGA with either cell type alone. Only the treatment group containing both endothelial cells and neural progenitor cells showed the formation of a blood-spinal cord barrier, highlighting the beneficial effects of the multiple cell type combinational treatment approach.

In 2006, significant axonal growth of the corticospinal and raphespinal tracts was observed following thoracic SCI, in response to a combinational therapy comprised of a photoactivated hydrogel embedded with NT3 (Piantino et al.). This study is significant considering the difficulty of promoting outgrowth from descending corticospinal tracts. Additionally, this study also observed significant improvement in functional recovery, which is also not trivial to achieve with a complete transection injury model (T8).

In addition to combinational treatment approaches appearing to hold the greatest therapeutic potential for SCI, the current study emphasized the importance of combining multiple locomotor assessments and measures of functional recovery. When observing grooming behavior during the BBB and Hargreaves' tests, it was apparent that some of the rats could support their entire

body weight on their hindlimbs and groom with both forepaws simultaneously, similar to sham animals. However, some rats could not fully support their body weight on their hindlimbs, thus it was necessary for them to prop themselves up on one forepaw while grooming with the other forepaw, and then switch their body weight to the other forepaw to be able to groom with the second forepaw. Therefore, the grooming test was modified for use as a functional measure of trunk stability. The trends observed were not statistically significant, although it appears that this might be a useful measurement for thoracic SCI, in conjunction with the BBB, Treadscan™ or Catwalk™, and Gridwalk. The Grooming test requires no animal training and can be observed within a number of environments, including the animal's home cage, the BBB open field, and the Hargreaves' testing environment. Rodents are compulsive groomers and stop to groom frequently once they are comfortable in their surrounding environment.

Therapies targeting the vasculature appear to be promising and necessary for minimizing tissue ischemia, reducing tissue toxicity, and promoting tissue repair. Intact vasculature is crucial for delivering oxygen and nutrients to the tissue, and for removing cellular wastes. In a combinational therapy approach, Han et al. (2010) administered angiopoietin-1 and alpha v beta 3 integrin (factors known to promote endothelial cell survival), after contusive, thoracic SCI. This treatment strategy resulted in spared lesion vasculature, increased white matter sparing, decreased inflammatory response, and improved locomotion; thus, emphasizing the importance of the vasculature in the secondary wave of injury caused by the inflammatory response (Han et al., 2010).

Popovich and colleagues illustrated the high potential of vascular plasticity, up to 28 days following SCI (1996). Benton et al. (2008a) used a novel technique for identifying vasculature, and determined that a specific subclass of spinal microvasculature (within the lesion core), demonstrates tight junction dysfunction. Later in 2008, Benton and colleagues published another report detailing the microvascular dysfunction and identification of a number of upregulated mRNAs as early as 24 hours post SCI (Benton et al., 2008b).

Collectively, the studies highlighted in this dissertation demonstrate the importance of combinational treatment approaches (Bunge MB, 2008), combinations of functional assessments to measure deficits and recovery following SCI, the usefulness of bioengineered tissues as therapies, and the positive impact on tissue repair with VEGF and GDNF for SCI repair. Furthermore, we and others have highlighted the fact that VEGF might have a positive effect in vivo, primarily when combined with other factors, such as GDNF or PDGF. Additionally, the background literature demonstrates that the combination of VEGF and GDNF has broader implications for targeting the vasculature and for neuroprotection beyond SCI, for other neurodegenerative diseases (Alzheimer's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, and stroke).

REFERENCES

- Airaksinen, M. S., & Saarma, M. (2002). The GDNF family: signalling, biological functions and therapeutic value. *Nature Reviews. Neuroscience*, 3(5), 383–394. <http://doi.org/10.1038/nrn812>
- Ansorena, E., De Berdt, P., Ucakar, B., Simón-Yarza, T., Jacobs, D., Schakman, O., des Rieux, A. (2013). Injectable alginate hydrogel loaded with GDNF promotes functional recovery in a hemisection model of spinal cord injury. *International Journal of Pharmaceutics*, 455(1-2), 148–158. <http://doi.org/10.1016/j.ijpharm.2013.07.045>
- Arce, V., Pollock, R. A., Philippe, J. M., Pennica, D., Henderson, C. E., & deLapeyrière, O. (1998). Synergistic effects of schwann- and muscle-derived factors on motoneuron survival involve GDNF and cardiotrophin-1 (CT-1). *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 18(4), 1440–1448.
- Austin, J. W., Kang, C. E., Baumann, M. D., DiDiodato, L., Satkunendrarajah, K., Wilson, J. R., ... Fehlings, M. G. (2012). The effects of intrathecal injection of a hyaluronan-based hydrogel on inflammation, scarring and neurobehavioural outcomes in a rat model of severe spinal cord injury associated with arachnoiditis. *Biomaterials*, 33(18), 4555–4564. <http://doi.org/10.1016/j.biomaterials.2012.03.022>
- Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., ... Carmeliet, P. (2003). Role of PIGF in the intra- and intermolecular

- cross talk between the VEGF receptors Flt1 and Flk1. *Nature Medicine*, 9(7), 936–943. <http://doi.org/10.1038/nm884>
- Azzouz, M., Ralph, G. S., Storkebaum, E., Walmsley, L. E., Mitrophanous, K. A., Kingsman, S. M., ... Mazarakis, N. D. (2004). VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. *Nature*, 429(6990), 413–417. <http://doi.org/10.1038/nature02544>
- Bär, K. J., Saldanha, G. J., Kennedy, A. J., Facer, P., Birch, R., Carlstedt, T., & Anand, P. (1998). GDNF and its receptor component Ret in injured human nerves and dorsal root ganglia. *Neuroreport*, 9(1), 43–47.
- Barnett, M. W., Fisher, C. E., Perona-Wright, G., & Davies, J. A. (2002). Signalling by glial cell line-derived neurotrophic factor (GDNF) requires heparan sulphate glycosaminoglycan. *Journal of Cell Science*, 115(Pt 23), 4495–4503.
- Bartholdi, D., Rubin, B. P., & Schwab, M. E. (1997). VEGF mRNA induction correlates with changes in the vascular architecture upon spinal cord damage in the rat. *The European Journal of Neuroscience*, 9(12), 2549–2560.
- Basso, D. M., Beattie, M. S., & Bresnahan, J. C. (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. *Journal of Neurotrauma*, 12(1), 1–21.
- Basso, D. M., Beattie, M. S., & Bresnahan, J. C. (1996). Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop

- device versus transection. *Experimental Neurology*, 139(2), 244–256.
<http://doi.org/10.1006/exnr.1996.0098>
- Beare, J. E., Morehouse, J. R., DeVries, W. H., Enzmann, G. U., Burke, D. A., Magnuson, D. S. K., & Whittemore, S. R. (2009). Gait analysis in normal and spinal contused mice using the TreadScan system. *Journal of Neurotrauma*, 26(11), 2045–56. <http://doi.org/10.1089/neu.2009.0914>.
- Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., ... Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature*, 373(6512), 339–341. <http://doi.org/10.1038/373339a0>
- Benton, R. L., & Hagg, T. (2011). Vascular Pathology as a Potential Therapeutic Target in SCI. *Translational Stroke Research*, 2(4), 556–574. <http://doi.org/10.1007/s12975-011-0128-7>
- Benton, R. L., Maddie, M. A., Minnillo, D. R., Hagg, T., & Whittemore, S. R. (2008). Griffonia simplicifolia isolectin B4 identifies a specific subpopulation of angiogenic blood vessels following contusive spinal cord injury in the adult mouse. *The Journal of Comparative Neurology*, 507(1), 1031–1052. <http://doi.org/10.1002/cne.21570>.
- Benton, R. L., Maddie, M. A., Worth, C. A., Mahoney, E. T., Hagg, T., & Whittemore, S. R. (2008). Transcriptomic screening of microvascular endothelial cells implicates novel molecular regulators of vascular dysfunction after spinal cord injury. *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood*

Flow and Metabolism, 28(11), 1771–1785.

<http://doi.org/10.1038/jcbfm.2008.76>

Benton, R. L., & Whittemore, S. R. (2003). VEGF165 therapy exacerbates secondary damage following spinal cord injury. *Neurochemical Research*, 28(11), 1693–1703.

Bertelli, J. A., & Mira, J. C. (1993). Behavioral evaluating methods in the objective clinical assessment of motor function after experimental brachial plexus reconstruction in the rat. *Journal of Neuroscience Methods*, 46(3), 203–208.

Blesch, A., & Tuszynski, M. H. (2001). GDNF gene delivery to injured adult CNS motor neurons promotes axonal growth, expression of the trophic neuropeptide CGRP, and cellular protection. *The Journal of Comparative Neurology*, 436(4), 399–410. <http://doi.org/10.1002/cne.1076>

Blesch, A., & Tuszynski, M. H. (2003). Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination. *The Journal of Comparative Neurology*, 467(3), 403–417. <http://doi.org/10.1002/cne.10934>

Bohn, M. C. (2004). Motoneurons crave glial cell line-derived neurotrophic factor. *Experimental Neurology*, 190(2), 263–275. <http://doi.org/10.1016/j.expneurol.2004.08.012>

Buj-Bello, A., Adu, J., Piñón, L. G., Horton, A., Thompson, J., Rosenthal, A., ... Davies, A. M. (1997). Neurturin responsiveness requires a GPI-linked

receptor and the Ret receptor tyrosine kinase. *Nature*, 387(6634), 721–724.
<http://doi.org/10.1038/42729>

Bunge, M. B. (2008). Novel combination strategies to repair the injured mammalian spinal cord. *The Journal of Spinal Cord Medicine*, 31(3), 262–269.

Bunge, M. B. (2016). Efficacy of Schwann Cell (SC) transplantation for spinal cord repair is improved with combinatorial strategies. *The Journal of Physiology*. <http://doi.org/10.1113/JP271531>

Carmeliet, P., & Collen, D. (1997). Molecular analysis of blood vessel formation and disease. *The American Journal of Physiology*, 273(5 Pt 2), H2091–2104.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., ... Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, 380(6573), 435–439.
<http://doi.org/10.1038/380435a0>

Carmeliet, P., Moons, L., Lutun, A., Vincenti, V., Compernelle, V., De Mol, M., ... Persico, M. G. (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nature Medicine*, 7(5), 575–583.
<http://doi.org/10.1038/87904>

Carmeliet, P., Ruiz de Almodovar, C., & Carmen, R. de A. (2013). VEGF ligands and receptors: implications in neurodevelopment and neurodegeneration.

Cellular and Molecular Life Sciences: CMLS, 70(10), 1763–1778.
<http://doi.org/10.1007/s00018-013-1283-7>

Carmeliet, P., & Storkebaum, E. (2002). Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. *Seminars in Cell & Developmental Biology*, 13(1), 39–53.
<http://doi.org/10.1006/scdb.2001.0290>

Casella, G. T. B., Marcillo, A., Bunge, M. B., & Wood, P. M. (2002). New vascular tissue rapidly replaces neural parenchyma and vessels destroyed by a contusion injury to the rat spinal cord. *Experimental Neurology*, 173(1), 63–76. <http://doi.org/10.1006/exnr.2001.7827>

Chehrehasa, F., Cobcroft, M., Young, Y. W., Mackay-Sim, A., & Goss, B. (2014). An acute growth factor treatment that preserves function after spinal cord contusion injury. *Journal of Neurotrauma*, 31(21), 1807–1813.
<http://doi.org/10.1089/neu.2013.3294>

Cheng, H., Wu, J.-P., & Tzeng, S.-F. (2002). Neuroprotection of glial cell line-derived neurotrophic factor in damaged spinal cords following contusive injury. *Journal of Neuroscience Research*, 69(3), 397–405.
<http://doi.org/10.1002/jnr.10303>

Choi, J.-S., Kim, H.-Y., Cha, J.-H., Choi, J.-Y., Park, S. I., Jeong, C. H., ... Lee, M.-Y. (2007). Upregulation of vascular endothelial growth factor receptors Flt-1 and Flk-1 following acute spinal cord contusion in rats. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, 55(8), 821–830. <http://doi.org/10.1369/jhc.6A7139.2007>

- Chu, L.-F., Wang, W.-T., Ghanta, V. K., Lin, C.-H., Chiang, Y.-Y., & Hsueh, C.-M. (2008). Ischemic brain cell-derived conditioned medium protects astrocytes against ischemia through GDNF/ERK/NF-kB signaling pathway. *Brain Research*, 1239, 24–35. <http://doi.org/10.1016/j.brainres.2008.08.087>
- De Laporte, L., des Rieux, A., Tuinstra, H. M., Zelivyanskaya, M. L., De Clerck, N. M., Postnov, A. A., ... Shea, L. D. (2011). Vascular endothelial growth factor and fibroblast growth factor 2 delivery from spinal cord bridges to enhance angiogenesis following injury. *Journal of Biomedical Materials Research. Part A*, 98(3), 372–382. <http://doi.org/10.1002/jbm.a.33112>
- Deng, L., Ruan, Y., Chen, C., Frye, C. C., Xiong, W., Jin, X., ... Xu, X.-M. (2015). Characterization of dendritic morphology and neurotransmitter phenotype of thoracic descending propriospinal neurons after complete spinal cord transection and GDNF treatment. *Experimental Neurology*, 277, 103–114. <http://doi.org/10.1016/j.expneurol.2015.12.018>
- Deng, L.-X., Deng, P., Ruan, Y., Xu, Z. C., Liu, N.-K., Wen, X., ... Xu, X.-M. (2013). A novel growth-promoting pathway formed by GDNF-overexpressing Schwann cells promotes propriospinal axonal regeneration, synapse formation, and partial recovery of function after spinal cord injury. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 33(13), 5655–5667. <http://doi.org/10.1523/JNEUROSCI.2973-12.2013>
- Deng, L.-X., Hu, J., Liu, N., Wang, X., Smith, G. M., Wen, X., & Xu, X.-M. (2011a). GDNF modifies reactive astrogliosis allowing robust axonal

- regeneration through Schwann cell-seeded guidance channels after spinal cord injury. *Experimental Neurology*, 229(2), 238–250.
<http://doi.org/10.1016/j.expneurol.2011.02.001>
- Deng, L.-X., Hu, J., Liu, N., Wang, X., Smith, G. M., Wen, X., & Xu, X.-M. (2011b). GDNF reverses the inhibitory properties of reactive astrocytes allowing robust axonal regeneration through Schwann cell-seeded guidance channels after spinal cord injury. *Experimental Neurology*, 229(2), 238–250.
<http://doi.org/10.1016/j.expneurol.2011.02.001>
- des Rieux, A., De Berdt, P., Ansorena, E., Ucakar, B., Damien, J., Schakman, O., ... Pr eat, V. (2014). Vascular endothelial growth factor-loaded injectable hydrogel enhances plasticity in the injured spinal cord. *Journal of Biomedical Materials Research. Part A*, 102(7), 2345–2355.
<http://doi.org/10.1002/jbm.a.34915>
- de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., & Williams, L. T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science (New York, N.Y.)*, 255(5047), 989–991.
- Dolbeare, D., & Houle, J. D. (2003). Restriction of axonal retraction and promotion of axonal regeneration by chronically injured neurons after intraspinal treatment with glial cell line-derived neurotrophic factor (GDNF). *Journal of Neurotrauma*, 20(11), 1251–1261.
<http://doi.org/10.1089/089771503770802916>
- Donnelly, D. J., & Popovich, P. G. (2008). Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal

cord injury. *Experimental Neurology*, 209(2), 378–388.
<http://doi.org/10.1016/j.expneurol.2007.06.009>

Ehrmann, R. L., & Knoth, M. (1968). Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. *Journal of the National Cancer Institute*, 41(6), 1329–1341.

Emerich, D. F., Mooney, D. J., Storrie, H., Babu, R. S., & Kordower, J. H. (2010). Injectable hydrogels providing sustained delivery of vascular endothelial growth factor are neuroprotective in a rat model of Huntington's disease. *Neurotoxicity Research*, 17(1), 66–74. <http://doi.org/10.1007/s12640-009-9079-0>

Engel, J., Schubert, D., & Bohn, M. C. (1991). Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: role of mesencephalic glia. *Journal of Neuroscience Research*, 30(2), 359–371. <http://doi.org/10.1002/jnr.490300212>

Facchiano, F., Fernandez, E., Mancarella, S., Maira, G., Miscusi, M., D'Arcangelo, D., ... Pallini, R. (2002). Promotion of regeneration of corticospinal tract axons in rats with recombinant vascular endothelial growth factor alone and combined with adenovirus coding for this factor. *Journal of Neurosurgery*, 97(1), 161–168.
<http://doi.org/10.3171/jns.2002.97.1.0161>

Fassbender, J. M., Whittemore, S. R., & Hagg, T. (2011). Targeting microvasculature for neuroprotection after SCI. *Neurotherapeutics: The*

- Journal of the American Society for Experimental NeuroTherapeutics*, 8(2), 240–251. <http://doi.org/10.1007/s13311-011-0029-1>
- Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., ... Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, 380(6573), 439–442. <http://doi.org/10.1038/380439a0>
- Ferrara, N., & Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochemical and Biophysical Research Communications*, 161(2), 851–858.
- Figley, S. A., Liu, Y., Karadimas, S. K., Satkunendrarajah, K., Fettes, P., Spratt, S. K., ... Fehlings, M. G. (2014). Delayed administration of a bio-engineered zinc-finger VEGF-A gene therapy is neuroprotective and attenuates allodynia following traumatic spinal cord injury. *PloS One*, 9(5), e96137. <http://doi.org/10.1371/journal.pone.0096137>
- Folkman, J., Merler, E., Abernathy, C., & Williams, G. (1971). Isolation of a tumor factor responsible for angiogenesis. *The Journal of Experimental Medicine*, 133(2), 275–288.
- Fouad, K., Schnell, L., Bunge, M. B., Schwab, M. E., Liebscher, T., & Pearse, D. D. (2005). Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 25(5), 1169–1178. <http://doi.org/10.1523/JNEUROSCI.3562-04.2005>

- Galland, F., Karamysheva, A., Mattei, M. G., Rosnet, O., Marchetto, S., & Birnbaum, D. (1992). Chromosomal localization of FLT4, a novel receptor-type tyrosine kinase gene. *Genomics*, *13*(2), 475–478.
- Gensel, J. C., Tovar, C. A., Hamers, F. P. T., Deibert, R. J., Beattie, M. S., & Bresnahan, J. C. (2006). Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. *Journal of Neurotrauma*, *23*(1), 36–54. <http://doi.org/10.1089/neu.2006.23.36>
- Gluzman-Poltorak, Z., Cohen, T., Herzog, Y., & Neufeld, G. (2000). Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. *The Journal of Biological Chemistry*, *275*(24), 18040–18045. <http://doi.org/10.1074/jbc.M909259199>
- Golden, J. P., Milbrandt, J., & Johnson, E. M. (2003). Neurturin and persephin promote the survival of embryonic basal forebrain cholinergic neurons in vitro. *Experimental Neurology*, *184*(1), 447–455.
- Gong, S., Seng, Z., Wang, W., Lv, J., Dong, Q., Yan, B., ... He, X. (2015). Bosentan protects the spinal cord from ischemia reperfusion injury in rats through vascular endothelial growth factor receptors. *Spinal Cord*, *53*(1), 19–23. <http://doi.org/10.1038/sc.2014.147>
- Greenblatt, M., & Shubi, P. (1968). Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. *Journal of the National Cancer Institute*, *41*(1), 111–124.
- Grünewald, F. S., Prota, A. E., Giese, A., & Ballmer-Hofer, K. (2010). Structure-function analysis of VEGF receptor activation and the role of coreceptors in

- angiogenic signaling. *Biochimica Et Biophysica Acta*, 1804(3), 567–580.
<http://doi.org/10.1016/j.bbapap.2009.09.002>
- Guest, J., Santamaria, A. J., & Benavides, F. D. (2013). Clinical translation of autologous Schwann cell transplantation for the treatment of spinal cord injury. *Current Opinion in Organ Transplantation*, 18(6), 682–689.
<http://doi.org/10.1097/MOT.0000000000000026>
- Hagg, T., & Oudega, M. (2006). Degenerative and spontaneous regenerative processes after spinal cord injury. *Journal of Neurotrauma*, 23(3-4), 264–280. <http://doi.org/10.1089/neu.2006.23.263>
- Han, S., Arnold, S. A., Sithu, S. D., Mahoney, E. T., Gerald, J. T., Tran, P., ... Hagg, T. (2010). Rescuing vasculature with intravenous angiopoietin-1 and alpha v beta 3 integrin peptide is protective after spinal cord injury. *Brain: A Journal of Neurology*, 133(Pt 4), 1026–1042.
<http://doi.org/10.1093/brain/awq034>
- Hao, T., & Rockwell, P. (2013). Signaling through the vascular endothelial growth factor receptor VEGFR-2 protects hippocampal neurons from mitochondrial dysfunction and oxidative stress. *Free Radical Biology & Medicine*, 63, 421–431. <http://doi.org/10.1016/j.freeradbiomed.2013.05.036>
- Hargreaves, K., Dubner, R., Brown, F., Flores, C., & Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*, 32(1), 77–88.
- Hase, A., Saito, F., Yamada, H., Arai, K., Shimizu, T., & Matsumura, K. (2005). Characterization of glial cell line-derived neurotrophic factor family receptor

- alpha-1 in peripheral nerve Schwann cells. *Journal of Neurochemistry*, 95(2), 537–543. <http://doi.org/10.1111/j.1471-4159.2005.03391.x>
- Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., ... Rosenthal, A. (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science (New York, N.Y.)*, 266(5187), 1062–1064.
- Hermann, D. M., Kilic, E., Kügler, S., Isenmann, S., & Bähr, M. (2001). Adenovirus-mediated glial cell line-derived neurotrophic factor (GDNF) expression protects against subsequent cortical cold injury in rats. *Neurobiology of Disease*, 8(6), 964–973. <http://doi.org/10.1006/nbdi.2001.0448>
- Herrán, E., Ruiz-Ortega, J. Á., Aristieta, A., Igartua, M., Requejo, C., Lafuente, J. V., ... Hernández, R. M. (2013). In vivo administration of VEGF- and GDNF-releasing biodegradable polymeric microspheres in a severe lesion model of Parkinson's disease. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Für Pharmazeutische Verfahrenstechnik e.V*, 85(3 Pt B), 1183–1190. <http://doi.org/10.1016/j.ejpb.2013.03.034>
- Herrera, J. J., Nestic, O., & Narayana, P. A. (2009). Reduced vascular endothelial growth factor expression in contusive spinal cord injury. *Journal of Neurotrauma*, 26(7), 995–1003. <http://doi.org/10.1089/neu.2008-0779>
- Hill, R. L., Zhang, Y. P., Burke, D. A., Devries, W. H., Zhang, Y., Magnuson, D. S. K., ... Shields, C. B. (2009). Anatomical and functional outcomes

- following a precise, graded, dorsal laceration spinal cord injury in C57BL/6 mice. *Journal of Neurotrauma*, 26(1), 1–15.
<http://doi.org/10.1089/neu.2008.0543>
- Hiratsuka, S., Nakao, K., Nakamura, K., Katsuki, M., Maru, Y., & Shibuya, M. (2005). Membrane fixation of vascular endothelial growth factor receptor 1 ligand-binding domain is important for vasculogenesis and angiogenesis in mice. *Molecular and Cellular Biology*, 25(1), 346–354.
<http://doi.org/10.1128/MCB.25.1.346-354.2005>
- Höke, A., Gordon, T., Zochodne, D. W., & Sulaiman, O. A. R. (2002). A Decline in Glial Cell-Line-Derived Neurotrophic Factor Expression Is Associated with Impaired Regeneration after Long-Term Schwann Cell Denervation. *Experimental Neurology*, 173(1), 77–85.
<http://doi.org/10.1006/exnr.2001.7826>
- Höke, A., Ho, T., Crawford, T. O., LeBel, C., Hilt, D., & Griffin, J. W. (2003). Glial cell line-derived neurotrophic factor alters axon schwann cell units and promotes myelination in unmyelinated nerve fibers. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 23(2), 561–567.
- Honda, S., Nakajima, K., Nakamura, Y., Imai, Y., & Kohsaka, S. (1999). Rat primary cultured microglia express glial cell line-derived neurotrophic factor receptors. *Neuroscience Letters*, 275(3), 203–206.
- Horger, B. A., Nishimura, M. C., Armanini, M. P., Wang, L. C., Poulsen, K. T., Rosenblad, C., ... Phillips, H. S. (1998). Neurturin exerts potent actions on

- survival and function of midbrain dopaminergic neurons. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 18(13), 4929–4937.
- Horn, E. M., Beaumont, M., Shu, X. Z., Harvey, A., Prestwich, G. D., Horn, K. M., ... Panitch, A. (2007). Influence of cross-linked hyaluronic acid hydrogels on neurite outgrowth and recovery from spinal cord injury. *Journal of Neurosurgery. Spine*, 6(2), 133–140. <http://doi.org/10.3171/spi.2007.6.2.133>
- Houle, J. D., Tom, V. J., Mayes, D., Wagoner, G., Phillips, N., & Silver, J. (2006). Combining an autologous peripheral nervous system “bridge” and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 26(28), 7405–7415. <http://doi.org/10.1523/JNEUROSCI.1166-06.2006>
- Huang, S.-M., Chen, T.-S., Chiu, C.-M., Chang, L.-K., Liao, K.-F., Tan, H.-M., ... Lu, D.-Y. (2014). GDNF increases cell motility in human colon cancer through VEGF-VEGFR1 interaction. *Endocrine-Related Cancer*, 21(1), 73–84. <http://doi.org/10.1530/ERC-13-0351>
- Hughes, J. T. (1988). The Edwin Smith Surgical Papyrus: an analysis of the first case reports of spinal cord injuries. *Paraplegia*, 26(2), 71–82. <http://doi.org/10.1038/sc.1988.15>
- Hurtado, A., Cregg, J. M., Wang, H. B., Wendell, D. F., Oudega, M., Gilbert, R. J., & McDonald, J. W. (2011). Robust CNS regeneration after complete spinal cord transection using aligned poly-L-lactic acid microfibers.

<http://doi.org/10.1016/j.biomaterials.2011.05.006>

Iannotti, C., Li, H., Yan, P., Lu, X., Wirthlin, L., & Xu, X. M. (2003). Glial cell line-derived neurotrophic factor-enriched bridging transplants promote propriospinal axonal regeneration and enhance myelination after spinal cord injury. *Experimental Neurology*, 183(2), 379–393.

Iannotti, C., Ping Zhang, Y., Shields, C. B., Han, Y., Burke, D. A., & Xu, X.-M. (2004). A neuroprotective role of glial cell line-derived neurotrophic factor following moderate spinal cord contusion injury. *Experimental Neurology*, 189(2), 317–332. <http://doi.org/10.1016/j.expneurol.2004.05.033>

Inoue, T., Lin, A., Ma, X., McKenna, S. L., Creasey, G. H., Manley, G. T., ... Beattie, M. S. (2013). Combined SCI and TBI: recovery of forelimb function after unilateral cervical spinal cord injury (SCI) is retarded by contralateral traumatic brain injury (TBI), and ipsilateral TBI balances the effects of SCI on paw placement. *Experimental Neurology*, 248, 136–147. <http://doi.org/10.1016/j.expneurol.2013.06.006>

Iravani, M. M., Sadeghian, M., Leung, C. C. M., Jenner, P., & Rose, S. (2012). Lipopolysaccharide-induced nigral inflammation leads to increased IL-1 β tissue content and expression of astrocytic glial cell line-derived neurotrophic factor. *Neuroscience Letters*, 510(2), 138–142. <http://doi.org/10.1016/j.neulet.2012.01.022>

Jiang, X.-Y., Fu, S.-L., Nie, B.-M., Li, Y., Lin, L., Yin, L., ... Xu, X.-M. (2006). Methods for isolating highly-enriched embryonic spinal cord neurons: a

- comparison between enzymatic and mechanical dissociations. *Journal of Neuroscience Methods*, 158(1), 13–18.
<http://doi.org/10.1016/j.jneumeth.2006.05.014>
- Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., ... Fox, G. M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell*, 85(7), 1113–1124.
- Jin, K. L., Mao, X. O., & Greenberg, D. A. (2000). Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proceedings of the National Academy of Sciences of the United States of America*, 97(18), 10242–10247.
- Jin, K., Mao, X. O., & Greenberg, D. A. (2006). Vascular endothelial growth factor stimulates neurite outgrowth from cerebral cortical neurons via Rho kinase signaling. *Journal of Neurobiology*, 66(3), 236–242.
<http://doi.org/10.1002/neu.20215>
- Ji, Q. S., Winnier, G. E., Niswender, K. D., Horstman, D., Wisdom, R., Magnuson, M. A., & Carpenter, G. (1997). Essential role of the tyrosine kinase substrate phospholipase C-gamma1 in mammalian growth and development. *Proceedings of the National Academy of Sciences of the United States of America*, 94(7), 2999–3003.
- Kao, C.-H., Chen, S.-H., Chio, C.-C., Chang, C.-K., & Lin, M.-T. (2008). Exogenous administration of glial cell line-derived neurotrophic factor improves recovery after spinal cord injury. *Resuscitation*, 77(3), 395–400.
<http://doi.org/10.1016/j.resuscitation.2008.01.023>

- Kao, L. S., & Green, C. E. (2008). Analysis of Variance: Is There a Difference in Means and What Does It Mean? *The Journal of Surgical Research*, 144(1), 158–170. <http://doi.org/10.1016/j.jss.2007.02.053>
- Kawai, T., Takagi, N., Mochizuki, N., Besshoh, S., Sakanishi, K., Nakahara, M., & Takeo, S. (2006). Inhibitor of vascular endothelial growth factor receptor tyrosine kinase attenuates cellular proliferation and differentiation to mature neurons in the hippocampal dentate gyrus after transient forebrain ischemia in the adult rat. *Neuroscience*, 141(3), 1209–1216. <http://doi.org/10.1016/j.neuroscience.2006.04.064>
- Kells, A. P., Fong, D. M., Dragunow, M., During, M. J., Young, D., & Connor, B. (2004). AAV-mediated gene delivery of BDNF or GDNF is neuroprotective in a model of Huntington disease. *Molecular Therapy: The Journal of the American Society of Gene Therapy*, 9(5), 682–688. <http://doi.org/10.1016/j.ymthe.2004.02.016>
- Kim, H. M., Hwang, D. H., Lee, J. E., Kim, S. U., & Kim, B. G. (2009). Ex vivo VEGF delivery by neural stem cells enhances proliferation of glial progenitors, angiogenesis, and tissue sparing after spinal cord injury. *PLoS One*, 4(3), e4987. <http://doi.org/10.1371/journal.pone.0004987>
- Klöcker, N., Bräunling, F., Isenmann, S., & Bähr, M. (1997). In vivo neurotrophic effects of GDNF on axotomized retinal ganglion cells. *Neuroreport*, 8(16), 3439–3442.

- Krum, J. M., Mani, N., & Rosenstein, J. M. (2002). Angiogenic and astroglial responses to vascular endothelial growth factor administration in adult rat brain. *Neuroscience*, *110*(4), 589–604.
- Lambrechts, D., Storkebaum, E., & Carmeliet, P. (2004). VEGF: necessary to prevent motoneuron degeneration, sufficient to treat ALS? *Trends in Molecular Medicine*, *10*(6), 275–282.
<http://doi.org/10.1016/j.molmed.2004.04.004>
- Lambrechts, D., Storkebaum, E., Morimoto, M., Del-Favero, J., Desmet, F., Marklund, S. L., ... Carmeliet, P. (2003). VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nature Genetics*, *34*(4), 383–394.
<http://doi.org/10.1038/ng1211>
- Lee, G. A., Lin, C.-H., Jiang, H.-H., Chao, H.-J., Wu, C.-L., & Hsueh, C.-M. (2004). Microglia-derived glial cell line-derived neurotrophic factor could protect Sprague-Dawley rat astrocyte from in vitro ischemia-induced damage. *Neuroscience Letters*, *356*(2), 111–114.
- Li, L., Wu, W., Lin, L. F., Lei, M., Oppenheim, R. W., & Houenou, L. J. (1995). Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(21), 9771–9775.
- Lin, C.-H., Cheng, F.-C., Lu, Y.-Z., Chu, L.-F., Wang, C.-H., & Hsueh, C.-M. (2006). Protection of ischemic brain cells is dependent on astrocyte-derived

- growth factors and their receptors. *Experimental Neurology*, 201(1), 225–233. <http://doi.org/10.1016/j.expneurol.2006.04.014>
- Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S., & Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science (New York, N.Y.)*, 260(5111), 1130–1132.
- Liu, G., Wang, X., Shao, G., & Liu, Q. (2014). Genetically modified Schwann cells producing glial cell line-derived neurotrophic factor inhibit neuronal apoptosis in rat spinal cord injury. *Molecular Medicine Reports*, 9(4), 1305–1312. <http://doi.org/10.3892/mmr.2014.1963>
- Liu, Y., Figley, S., Spratt, S. K., Lee, G., Ando, D., Surosky, R., & Fehlings, M. G. (2010). An engineered transcription factor which activates VEGF-A enhances recovery after spinal cord injury. *Neurobiology of Disease*, 37(2), 384–393. <http://doi.org/10.1016/j.nbd.2009.10.018>
- Li, X., Liu, X., Cui, L., Brunson, C., Zhao, W., Bhat, N. R., ... Wen, X. (2013). Engineering an in situ crosslinkable hydrogel for enhanced remyelination. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 27(3), 1127–1136. <http://doi.org/10.1096/fj.12-211151>
- Li, X., Liu, X., Zhang, N., & Wen, X. (2014). Engineering In Situ Cross-Linkable and Neurocompatible Hydrogels. *Journal of Neurotrauma*, 31(16), 1431–1438. <http://doi.org/10.1089/neu.2013.3215>
- Loy, D. N., Crawford, C. H., Darnall, J. B., Burke, D. A., Onifer, S. M., & Whittemore, S. R. (2002). Temporal progression of angiogenesis and basal

- lamina deposition after contusive spinal cord injury in the adult rat. *The Journal of Comparative Neurology*, 445(4), 308–324.
- Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., ... Tuszynski, M. H. (2012). Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. *Cell*, 150(6), 1264–1273. <http://doi.org/10.1016/j.cell.2012.08.020>
- Lutton, C., Young, Y. W., Williams, R., Meedeniya, A. C. B., Mackay-Sim, A., & Goss, B. (2012). Combined VEGF and PDGF treatment reduces secondary degeneration after spinal cord injury. *Journal of Neurotrauma*, 29(5), 957–970. <http://doi.org/10.1089/neu.2010.1423>
- Mani, N., Khaibullina, A., Krum, J. M., & Rosenstein, J. M. (2010). Vascular endothelial growth factor enhances migration of astroglial cells in subventricular zone neurosphere cultures. *Journal of Neuroscience Research*, 88(2), 248–257. <http://doi.org/10.1002/jnr.22197>
- Manoonkitiwongsa, P. S., Schultz, R. L., McCreery, D. B., Whitter, E. F., & Lyden, P. D. (2004). Neuroprotection of ischemic brain by vascular endothelial growth factor is critically dependent on proper dosage and may be compromised by angiogenesis. *Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, 24(6), 693–702. <http://doi.org/10.1097/01.WCB.0000126236.54306.21>
- Marchand, R., & Woerly, S. (1990). Transected spinal cords grafted with in situ self-assembled collagen matrices. *Neuroscience*, 36(1), 45–60.

- Marchand, R., Woerly, S., Bertrand, L., & Valdes, N. (1993). Evaluation of two cross-linked collagen gels implanted in the transected spinal cord. *Brain Research Bulletin*, 30(3-4), 415–422.
- Maurer, M. H., Tripps, W. K. C., Feldmann, R. E., & Kuschinsky, W. (2003). Expression of vascular endothelial growth factor and its receptors in rat neural stem cells. *Neuroscience Letters*, 344(3), 165–168.
- Mauters, A. E., Weinzierl, M. R., Donovan, F., & Noble, L. J. (2000). Vascular events after spinal cord injury: contribution to secondary pathogenesis. *Physical Therapy*, 80(7), 673–687.
- Meng, H., Zhang, Z., Zhang, R., Liu, X., Wang, L., Robin, A. M., & Chopp, M. (2006). Biphasic effects of exogenous VEGF on VEGF expression of adult neural progenitors. *Neuroscience Letters*, 393(2-3), 97–101. <http://doi.org/10.1016/j.neulet.2005.09.044>
- Metz, G. A. S., Merkler, D., Dietz, V., Schwab, M. E., & Fouad, K. (2000). Efficient testing of motor function in spinal cord injured rats. *Brain Research*, 883(2), 165–177. [http://doi.org/10.1016/S0006-8993\(00\)02778-5](http://doi.org/10.1016/S0006-8993(00)02778-5)
- Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H. G., Ziche, M., ... Dehio, C. (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *The EMBO Journal*, 18(2), 363–374. <http://doi.org/10.1093/emboj/18.2.363>

- Milbrandt, J., de Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., ... Johnson, E. M. (1998). Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron*, *20*(2), 245–253.
- Mills, C. D., Allchorne, A. J., Griffin, R. S., Woolf, C. J., & Costigan, M. (2007). GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord. *Molecular and Cellular Neurosciences*, *36*(2), 185–194. <http://doi.org/10.1016/j.mcn.2007.06.011>
- Myers, S. A., DeVries, W. H., Gruenthal, M. J., Andres, K. R., Hagg, T., & Whittemore, S. R. (2012). Sildenafil improves epicenter vascular perfusion but not hindlimb functional recovery after contusive spinal cord injury in mice. *Journal of Neurotrauma*, *29*(3), 528–538. <http://doi.org/10.1089/neu.2011.2036>
- Nesic, O., Sundberg, L. M., Herrera, J. J., Mokkalapati, V. U. L., Lee, J., & Narayana, P. A. (2010). Vascular endothelial growth factor and spinal cord injury pain. *Journal of Neurotrauma*, *27*(10), 1793–1803. <http://doi.org/10.1089/neu.2010.1351>
- Nicole, O., Ali, C., Docagne, F., Plawinski, L., MacKenzie, E. T., Vivien, D., & Buisson, A. (2001). Neuroprotection mediated by glial cell line-derived neurotrophic factor: involvement of a reduction of NMDA-induced calcium influx by the mitogen-activated protein kinase pathway. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *21*(9), 3024–3033.

- Nicoletti, J. N., Lenzer, J., Salerni, E. A., Shah, S. K., Elkady, A., Khalid, S., ... Croll, S. D. (2010). Vascular endothelial growth factor attenuates status epilepticus-induced behavioral impairments in rats. *Epilepsy & Behavior: E&B*, 19(3), 272–277. <http://doi.org/10.1016/j.yebeh.2010.07.011>
- Noble, L. J., & Wrathall, J. R. (1987). The blood-spinal cord barrier after injury: pattern of vascular events proximal and distal to a transection in the rat. *Brain Research*, 424(1), 177–188.
- Noble, L. J., & Wrathall, J. R. (1988). Blood-spinal cord barrier disruption proximal to a spinal cord transection in the rat: time course and pathways associated with protein leakage. *Experimental Neurology*, 99(3), 567–578.
- Noble, L. J., & Wrathall, J. R. (1989). Distribution and time course of protein extravasation in the rat spinal cord after contusive injury. *Brain Research*, 482(1), 57–66.
- Nowacka, M. M., & Obuchowicz, E. (2012). Vascular endothelial growth factor (VEGF) and its role in the central nervous system: a new element in the neurotrophic hypothesis of antidepressant drug action. *Neuropeptides*, 46(1), 1–10. <http://doi.org/10.1016/j.npep.2011.05.005>
- Oosthuyse, B., Moons, L., Storkebaum, E., Beck, H., Nuyens, D., Brusselmans, K., ... Carmeliet, P. (2001). Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nature Genetics*, 28(2), 131–138. <http://doi.org/10.1038/88842>

- Oppenheim, R. W., Houenou, L. J., Johnson, J. E., Lin, L. F., Li, L., Lo, A. C., ... Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature*, 373(6512), 344–346. <http://doi.org/10.1038/373344a0>
- Oudega, M. (2012). Molecular and cellular mechanisms underlying the role of blood vessels in spinal cord injury and repair. *Cell and Tissue Research*, 349(1), 269–288. <http://doi.org/10.1007/s00441-012-1440-6>
- Oudega, M. (2013). Inflammatory response after spinal cord injury. *Experimental Neurology*, 250, 151–155. <http://doi.org/10.1016/j.expneurol.2013.09.013>
- Pajusola, K., Aprelikova, O., Korhonen, J., Kaipainen, A., Pertovaara, L., Alitalo, R., & Alitalo, K. (1992). FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. *Cancer Research*, 52(20), 5738–5743.
- Paratcha, G., Ledda, F., Baars, L., Couplier, M., Besset, V., Anders, J., ... Ibáñez, C. F. (2001). Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. *Neuron*, 29(1), 171–184.
- Patist, C. M., Mulder, M. B., Gautier, S. E., Maquet, V., Jérôme, R., & Oudega, M. (2004). Freeze-dried poly(D,L-lactic acid) macroporous guidance scaffolds impregnated with brain-derived neurotrophic factor in the transected adult rat thoracic spinal cord. *Biomaterials*, 25(9), 1569–1582.
- Piantino, J., Burdick, J. A., Goldberg, D., Langer, R., & Benowitz, L. I. (2006). An injectable, biodegradable hydrogel for trophic factor delivery enhances

- axonal rewiring and improves performance after spinal cord injury. *Experimental Neurology*, 201(2), 359–367. <http://doi.org/10.1016/j.expneurol.2006.04.020>
- Pineda, J. R., Rubio, N., Akerud, P., Urbán, N., Badimon, L., Arenas, E., ... Canals, J. M. (2007). Neuroprotection by GDNF-secreting stem cells in a Huntington's disease model: optical neuroimage tracking of brain-grafted cells. *Gene Therapy*, 14(2), 118–128. <http://doi.org/10.1038/sj.gt.3302847>
- Popovich, P. G., Horner, P. J., Mullin, B. B., & Stokes, B. T. (1996). A quantitative spatial analysis of the blood-spinal cord barrier. I. Permeability changes after experimental spinal contusion injury. *Experimental Neurology*, 142(2), 258–275. <http://doi.org/10.1006/exnr.1996.0196>
- Popovici, C., Isnardon, D., Birnbaum, D., & Roubin, R. (2002). Caenorhabditis elegans receptors related to mammalian vascular endothelial growth factor receptors are expressed in neural cells. *Neuroscience Letters*, 329(1), 116–120.
- Prang, P., Müller, R., Eljaouhari, A., Heckmann, K., Kunz, W., Weber, T., ... Weidner, N. (2006). The promotion of oriented axonal regrowth in the injured spinal cord by alginate-based anisotropic capillary hydrogels. *Biomaterials*, 27(19), 3560–3569. <http://doi.org/10.1016/j.biomaterials.2006.01.053>
- Rauch, M. F., Hynes, S. R., Bertram, J., Redmond, A., Robinson, R., Williams, C., ... Lavik, E. B. (2009). Engineering angiogenesis following spinal cord injury: a coculture of neural progenitor and endothelial cells in a degradable

- polymer implant leads to an increase in vessel density and formation of the blood-spinal cord barrier. *The European Journal of Neuroscience*, 29(1), 132–145. <http://doi.org/10.1111/j.1460-9568.2008.06567.x>
- Rauck, B. M., Novosat, T. L., Oudega, M., & Wang, Y. (2015). Biocompatibility of a coacervate-based controlled release system for protein delivery to the injured spinal cord. *Acta Biomaterialia*, 11, 204–211. <http://doi.org/10.1016/j.actbio.2014.09.037>
- Rind, H. B., & von Bartheld, C. S. (2002). Anterograde axonal transport of internalized GDNF in sensory and motor neurons. *Neuroreport*, 13(5), 659–664.
- Ritfeld, G. J., Rauck, B. M., Novosat, T. L., Park, D., Patel, P., Roos, R. A. C., ... Oudega, M. (2014). The effect of a polyurethane-based reverse thermal gel on bone marrow stromal cell transplant survival and spinal cord repair. *Biomaterials*, 35(6), 1924–1931. <http://doi.org/10.1016/j.biomaterials.2013.11.062>
- Ritz, M.-F., Graumann, U., Gutierrez, B., & Hausmann, O. (2010). Traumatic spinal cord injury alters angiogenic factors and TGF-beta1 that may affect vascular recovery. *Current Neurovascular Research*, 7(4), 301–310.
- Rosenstein, J. M., & Krum, J. M. (2004). New roles for VEGF in nervous tissue--beyond blood vessels. *Experimental Neurology*, 187(2), 246–253. <http://doi.org/10.1016/j.expneurol.2004.01.022>
- Rosenstein, J. M., Krum, J. M., & Ruhrberg, C. (2010). VEGF in the nervous system. *Organogenesis*, 6(2), 107–114.

- Roskoski, R. (2007). Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Critical Reviews in Oncology/Hematology*, 62(3), 179–213. <http://doi.org/10.1016/j.critrevonc.2007.01.006>
- Ruiz de Almodovar, C., Fabre, P. J., Knevels, E., Coulon, C., Segura, I., Haddick, P. C. G., ... Carmeliet, P. (2011). VEGF mediates commissural axon chemoattraction through its receptor Flk1. *Neuron*, 70(5), 966–978. <http://doi.org/10.1016/j.neuron.2011.04.014>
- Ruiz de Almodovar, C., Lambrechts, D., Mazzone, M., & Carmeliet, P. (2009). Role and therapeutic potential of VEGF in the nervous system. *Physiological Reviews*, 89(2), 607–648. <http://doi.org/10.1152/physrev.00031.2008>
- Sariola, H., & Saarma, M. (2003). Novel functions and signalling pathways for GDNF. *Journal of Cell Science*, 116(Pt 19), 3855–3862. <http://doi.org/10.1242/jcs.00786>
- Satake, K., Matsuyama, Y., Kamiya, M., Kawakami, H., Iwata, H., Adachi, K., & Kiuchi, K. (2000). Up-regulation of glial cell line-derived neurotrophic factor (GDNF) following traumatic spinal cord injury. *Neuroreport*, 11(17), 3877–3881.
- Sayer, F. T., Oudega, M., & Hagg, T. (2002). Neurotrophins reduce degeneration of injured ascending sensory and corticospinal motor axons in adult rat spinal cord. *Experimental Neurology*, 175(1), 282–296. <http://doi.org/10.1006/exnr.2002.7901>

- Schnell, L., Fearn, S., Klassen, H., Schwab, M. E., & Perry, V. H. (1999). Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. *The European Journal of Neuroscience*, *11*(10), 3648–3658.
- Schwab, M. E., & Bartholdi, D. (1996). Degeneration and regeneration of axons in the lesioned spinal cord. *Physiological Reviews*, *76*(2), 319–370.
- Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., & Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science (New York, N.Y.)*, *219*(4587), 983–985.
- Shibuya, M. (2006). Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *Journal of Biochemistry and Molecular Biology*, *39*(5), 469–478.
- Shinozaki, M., Nakamura, M., Konomi, T., Kobayashi, Y., Takano, M., Saito, N., ... Okano, H. (2014). Distinct roles of endogenous vascular endothelial factor receptor 1 and 2 in neural protection after spinal cord injury. *Neuroscience Research*, *78*, 55–64.
<http://doi.org/10.1016/j.neures.2013.09.011>
- Singh, A., Tetreault, L., Kalsi-Ryan, S., Nouri, A., & Fehlings, M. G. (2014). Global prevalence and incidence of traumatic spinal cord injury. *Clinical Epidemiology*, *6*, 309–331. <http://doi.org/10.2147/CLEP.S68889>
- Sköld, M., Cullheim, S., Hammarberg, H., Piehl, F., Suneson, A., Lake, S., ... Risling, M. (2000). Induction of VEGF and VEGF receptors in the spinal

- cord after mechanical spinal injury and prostaglandin administration. *The European Journal of Neuroscience*, 12(10), 3675–3686.
- Soler, R. M., Dolcet, X., Encinas, M., Egea, J., Bayascas, J. R., & Comella, J. X. (1999). Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 19(21), 9160–9169.
- Sondell, M., Lundborg, G., & Kanje, M. (1999a). Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 19(14), 5731–5740.
- Sondell, M., Lundborg, G., & Kanje, M. (1999b). Vascular endothelial growth factor stimulates Schwann cell invasion and neovascularization of acellular nerve grafts. *Brain Research*, 846(2), 219–228.
- Storkebaum, E., & Carmeliet, P. (2004). VEGF: a critical player in neurodegeneration. *The Journal of Clinical Investigation*, 113(1), 14–18. <http://doi.org/10.1172/JCI20682>
- Storkebaum, E., Lambrechts, D., & Carmeliet, P. (2004). VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, 26(9), 943–954. <http://doi.org/10.1002/bies.20092>

- Storkebaum, E., Lambrechts, D., Dewerchin, M., Moreno-Murciano, M.-P., Appelmans, S., Oh, H., ... Carmeliet, P. (2005). Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS. *Nature Neuroscience*, 8(1), 85–92. <http://doi.org/10.1038/nn1360>
- Sundberg, L. M., Herrera, J. J., & Narayana, P. A. (2011). Effect of vascular endothelial growth factor treatment in experimental traumatic spinal cord injury: in vivo longitudinal assessment. *Journal of Neurotrauma*, 28(4), 565–578. <http://doi.org/10.1089/neu.2010.1533>
- Sun, Y., Jin, K., Childs, J. T., Xie, L., Mao, X. O., & Greenberg, D. A. (2006). Vascular endothelial growth factor-B (VEGFB) stimulates neurogenesis: evidence from knockout mice and growth factor administration. *Developmental Biology*, 289(2), 329–335. <http://doi.org/10.1016/j.ydbio.2005.10.016>
- Sun, Y., Jin, K., Xie, L., Childs, J., Mao, X. O., Logvinova, A., & Greenberg, D. A. (2003). VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *The Journal of Clinical Investigation*, 111(12), 1843–1851. <http://doi.org/10.1172/JCI17977>
- Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., ... Hori, M. (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 99(6), 3657–3662. <http://doi.org/10.1073/pnas.022017899>

- Tator, C. H., & Fehlings, M. G. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *Journal of Neurosurgery*, 75(1), 15–26. <http://doi.org/10.3171/jns.1991.75.1.0015>
- Tator, C. H., & Koyanagi, I. (1997). Vascular mechanisms in the pathophysiology of human spinal cord injury. *Journal of Neurosurgery*, 86(3), 483–492. <http://doi.org/10.3171/jns.1997.86.3.0483>
- Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., & Böhlen, P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochemical and Biophysical Research Communications*, 187(3), 1579–1586.
- Tomac, A. C., Agulnick, A. D., Haughey, N., Chang, C.-F., Zhang, Y., Bäckman, C., ... Hoffer, B. J. (2002). Effects of cerebral ischemia in mice deficient in Persephin. *Proceedings of the National Academy of Sciences of the United States of America*, 99(14), 9521–9526. <http://doi.org/10.1073/pnas.152535899>
- Tomac, A., Lindqvist, E., Lin, L. F., Ogren, S. O., Young, D., Hoffer, B. J., & Olson, L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature*, 373(6512), 335–339. <http://doi.org/10.1038/373335a0>
- Tom, V. J., Sandrow-Feinberg, H. R., Miller, K., Santi, L., Connors, T., Lemay, M. A., & Houlié, J. D. (2009). Combining Peripheral Nerve Grafts and Chondroitinase Promotes Functional Axonal Regeneration in the Chronically

- Injured Spinal Cord. *The Journal of Neuroscience*, 29(47), 14881–14890.
<http://doi.org/10.1523/JNEUROSCI.3641-09.2009>
- Treanor, J. J., Goodman, L., de Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., ... Rosenthal, A. (1996). Characterization of a multicomponent receptor for GDNF. *Nature*, 382(6586), 80–83.
<http://doi.org/10.1038/382080a0>
- Trok, K., Hoffer, B., & Olson, L. (1996). Glial cell line-derived neurotrophic factor enhances survival and growth of prenatal and postnatal spinal cord transplants. *Neuroscience*, 71(1), 231–241. [http://doi.org/10.1016/0306-4522\(95\)00412-2](http://doi.org/10.1016/0306-4522(95)00412-2)
- Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A. S., Sieber, B. A., Grigoriou, M., ... Arumäe, U. (1996). Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature*, 381(6585), 785–789.
<http://doi.org/10.1038/381785a0>
- Trupp, M., Belluardo, N., Funakoshi, H., & Ibáñez, C. F. (1997). Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 17(10), 3554–3567.
- Trupp, M., Raynoschek, C., Belluardo, N., & Ibáñez, C. F. (1998). Multiple GPI-anchored receptors control GDNF-dependent and independent activation of

- the c-Ret receptor tyrosine kinase. *Molecular and Cellular Neurosciences*, 11(1-2), 47–63. <http://doi.org/10.1006/mcne.1998.0667>
- Trupp, M., Rydén, M., Jörnvall, H., Funakoshi, H., Timmusk, T., Arenas, E., & Ibáñez, C. F. (1995). Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. *The Journal of Cell Biology*, 130(1), 137–148.
- van Neerven, S., Joosten, E. A. J., Brook, G. A., Lambert, C. A., Mey, J., Weis, J., ... Deumens, R. (2010). Repetitive intrathecal VEGF(165) treatment has limited therapeutic effects after spinal cord injury in the rat. *Journal of Neurotrauma*, 27(10), 1781–1791. <http://doi.org/10.1089/neu.2010.1484>
- Walker, C. L., Walker, M. J., Liu, N.-K., Risberg, E. C., Gao, X., Chen, J., & Xu, X.-M. (2012). Systemic bisperoxovanadium activates Akt/mTOR, reduces autophagy, and enhances recovery following cervical spinal cord injury. *PloS One*, 7(1), e30012. <http://doi.org/10.1371/journal.pone.0030012>
- Walker, M. J., Walker, C. L., Zhang, Y. P., Shields, L. B. E., Shields, C. B., & Xu, X.-M. (2015). A novel vertebral stabilization method for producing contusive spinal cord injury. *Journal of Visualized Experiments: JoVE*, (95), e50149. <http://doi.org/10.3791/50149>
- Wang, X. (2013). Structural studies of GDNF family ligands with their receptors- Insights into ligand recognition and activation of receptor tyrosine kinase RET. *Biochimica Et Biophysica Acta*, 1834(10), 2205–2212. <http://doi.org/10.1016/j.bbapap.2012.10.008>

- Watabe, K., Ohashi, T., Sakamoto, T., Kawazoe, Y., Takeshima, T., Oyanagi, K., ... Kim, S. U. (2000). Rescue of lesioned adult rat spinal motoneurons by adenoviral gene transfer of glial cell line-derived neurotrophic factor. *Journal of Neuroscience Research*, 60(4), 511–519.
- Widenfalk, J., Lipson, A., Jubran, M., Hofstetter, C., Ebendal, T., Cao, Y., & Olson, L. (2003). Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. *Neuroscience*, 120(4), 951–960.
- Widenfalk, J., Wu, W., Hao, J., Person, J. K. E., Wiesenfeldt-Hallin, Z., & Risling, M. (2009). Treatment of transected peripheral nerves with artemin improved motor neuron regeneration, but did not reduce nerve injury-induced pain behaviour. *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery / Nordisk Plastikkirurgisk Forening [and] Nordisk Klubb for Handkirurgi*, 43(5), 245–250. <http://doi.org/10.3109/02844310903259082>
- Williams, R. R., & Bunge, M. B. (2012). Schwann cell transplantation: a repair strategy for spinal cord injury? *Progress in Brain Research*, 201, 295–312. <http://doi.org/10.1016/B978-0-444-59544-7.00014-7>
- Williams, R. R., Henao, M., Pearse, D. D., & Bunge, M. B. (2015). Permissive Schwann cell graft/spinal cord interfaces for axon regeneration. *Cell Transplantation*, 24(1), 115–131. <http://doi.org/10.3727/096368913X674657>
- Wise, L. M., Inder, M. K., Real, N. C., Stuart, G. S., Fleming, S. B., & Mercer, A. A. (2012). The vascular endothelial growth factor (VEGF)-E encoded by orf virus regulates keratinocyte proliferation and migration and promotes

- epidermal regeneration. *Cellular Microbiology*, 14(9), 1376–1390.
<http://doi.org/10.1111/j.1462-5822.2012.01802.x>
- Woerly, S., Doan, V. D., Evans-Martin, F., Paramore, C. G., & Peduzzi, J. D. (2001). Spinal cord reconstruction using NeuroGel implants and functional recovery after chronic injury. *Journal of Neuroscience Research*, 66(6), 1187–1197.
- Woerly, S., Pinet, E., de Robertis, L., Van Diep, D., & Bousmina, M. (2001). Spinal cord repair with PHPMA hydrogel containing RGD peptides (NeuroGel). *Biomaterials*, 22(10), 1095–1111.
- Wong, L. E., Gibson, M. E., Arnold, H. M., Pepinsky, B., & Frank, E. (2015). Artemin promotes functional long-distance axonal regeneration to the brainstem after dorsal root crush. *Proceedings of the National Academy of Sciences of the United States of America*, 112(19), 6170–6175.
<http://doi.org/10.1073/pnas.1502057112>
- Xing, B., Xin, T., Zhao, L., Hunter, R. L., Chen, Y., & Bing, G. (2010). Glial cell line-derived neurotrophic factor protects midbrain dopaminergic neurons against lipopolysaccharide neurotoxicity. *Journal of Neuroimmunology*, 225(1-2), 43–51. <http://doi.org/10.1016/j.jneuroim.2010.04.010>
- Xu, X.-M. (2012). Breaking News in Spinal Cord Injury Research. *Neural Regeneration Research*, 7(22), 1685–1687.
<http://doi.org/10.3969/j.issn.1673-5374.2012.22.001>
- Xu, X. M., Guénard, V., Kleitman, N., Aebischer, P., & Bunge, M. B. (1995). A combination of BDNF and NT-3 promotes supraspinal axonal regeneration

- into Schwann cell grafts in adult rat thoracic spinal cord. *Experimental Neurology*, 134(2), 261–272. <http://doi.org/10.1006/exnr.1995.1056>
- Yamaya, S., Ozawa, H., Kanno, H., Kishimoto, K. N., Sekiguchi, A., Tateda, S., ... Itoi, E. (2014). Low-energy extracorporeal shock wave therapy promotes vascular endothelial growth factor expression and improves locomotor recovery after spinal cord injury. *Journal of Neurosurgery*, 121(6), 1514–1525. <http://doi.org/10.3171/2014.8.JNS132562>
- Yu, A. C. H., Liu, R. Y., Zhang, Y., Sun, H. R., Qin, L. Y., Lau, L. T., ... Han, J. S. (2007). Glial cell line-derived neurotrophic factor protects astrocytes from staurosporine- and ischemia- induced apoptosis. *Journal of Neuroscience Research*, 85(15), 3457–3464. <http://doi.org/10.1002/jnr.21345>
- Zacchigna, S., Ruiz de Almodovar, C., & Carmeliet, P. (2008). Similarities between angiogenesis and neural development: what small animal models can tell us. *Current Topics in Developmental Biology*, 80, 1–55. [http://doi.org/10.1016/S0070-2153\(07\)80001-9](http://doi.org/10.1016/S0070-2153(07)80001-9)
- Zachary, I. (2005). Neuroprotective role of vascular endothelial growth factor: signalling mechanisms, biological function, and therapeutic potential. *Neuro-Signals*, 14(5), 207–221. <http://doi.org/10.1159/000088637>
- Zhang, L., Ma, Z., Smith, G. M., Wen, X., Pressman, Y., Wood, P. M., & Xu, X.-M. (2009). GDNF-enhanced axonal regeneration and myelination following spinal cord injury is mediated by primary effects on neurons. *Glia*, 57(11), 1178–1191. <http://doi.org/10.1002/glia.20840>

Zhao, Z., Alam, S., Oppenheim, R. W., Prevette, D. M., Evenson, A., & Parsadanian, A. (2004). Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. *Experimental Neurology*, 190(2), 356–372.
<http://doi.org/10.1016/j.expneurol.2004.06.015>

Zhu, Y., Jin, K., Mao, X. O., & Greenberg, D. A. (2003). Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 17(2), 186–193.
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California

Professional Societies & Affiliations

2015-present Journal of Spine, Neurology & Neurophysiology, Editorial
Board
2008-present IndyHub Young Professionals' Organization
2002-present International Society for the History of Neurosciences
2012-present International Society for Neuroscience
2011-2012 National Neurotrauma Society
2011-2012 Women in Neurotrauma
2011-2012 North American Vascular Biology Organization
2011-2012 Sigma Xi, The Scientific Research Society
2011-2012 American Association for the Advancement of Science

Honors & Awards

2015 Graduate Student Travel Fellowship Award • Indiana University
Purdue University Indianapolis
2014 Science Photo Contest • International Society for Neuroscience
2012 Outstanding Oral Presentation • International Neural
Regeneration Symposium, Shenyang, China
2012 IBMG Graduate Student Ambassador Volunteer Appreciation •
Indiana University School of Medicine
2011 First Place in Poster Competition • Indianapolis Society for
Neuroscience
2011 Honorary Mention • Sigma Xi Research Competition • Indiana
University School of Medicine
2010 Educational Enhancement Grant • Indiana University Graduate
Student Organization
2000 Undergraduate Teaching Assistantship • Life Science 4 •
Genetics • UCLA
1999 Honor's Content • Life Science 4 • Genetics • UCLA
1996-1998 California Dean's Honor List • 3 Semesters • Moorpark
Community College
1996 Middleton Scholarship • Royal High School • Simi Valley •
California
1995 California Girls State • Royal High School Representative •
American Legion Auxiliary

Mentoring Experience

Student Mentor

2012-2013 IBMG Graduate Student • IUSM • Indianapolis
2012-2013 IBMG Graduate Student • IUSM • Indianapolis
2013-2013 IBMG Graduate Student • IUSM • Indianapolis
2011-2012 IBMG Graduate Student • IUSM • Indianapolis
2011 Post baccalaureate Student • IUSM • Indianapolis
2011 IBMG Graduate Student • IUSM • Indianapolis
2010-2011 IBMG Graduate Student • IUSM • Indianapolis
2010-2011 IBMG Graduate Student • IUSM • Indianapolis
2009-2010 IBMG Graduate Student • IUSM • Indianapolis
2008-2009 IBMG Incoming Student • IUSM • Indianapolis
2008-2009 IBMG Graduate Student • IUSM • Indianapolis

Research Mentor

2015 Undergraduate Intern • Earlham College • Richmond Indiana •
Xu Laboratory
2015 Post baccalaureate Student Rotation • IUSM • Indianapolis •
Xu Laboratory
2011-2015 Post baccalaureate Student • IUSM • Indianapolis •
Xu Laboratory
2015 Post baccalaureate Student Rotation • IUSM • Indianapolis •
Xu Laboratory
2013 Undergraduate Student • IUPUI • Indianapolis • Xu Laboratory
2013 Master's Student • IUPUI • Indianapolis • Xu Laboratory
2012 Medical Student • IUSM • Indianapolis • Xu Laboratory
2011 Undergraduate Student • Mount Holyoke College •
Massachusetts • Xu Laboratory

Student Ambassador

2009-2012 Annual IUSM IBMG Student Ambassador
2008-2010 Annual Tokyo Student Ambassador
2008-2009 Student Ambassador, International English Language School
Students

Skills & Training Experience

- 2010-2016 **Spinal cord injury (SCI) and repair dissertation research utilizing novel Bioengineered Hydrogel**
- Earned international research grant: *Wings for Life* (2013 and 2014) • only 10 grants awarded globally in 2013
 - Eleven formal seminar presentations
- 2007-2010 **Clinical Alcohol Addiction Research (fMRI and PET Neuroimaging)**
- Proposed/designed a macro with a programmer and reduced 6 weeks of analysis down to 4 minutes.
 - Set up software/device for misting alcohol • established the idea of using Gatorade® (control drink) for fMRI
- 2004 **Oncology Research/Cancer therapeutics (lymphoma and leukemia, monoclonal antibodies)**
- Biomedical research training: Immunohistochemistry • Cell/Bacterial Cultures • Mice Tail-vein injections • Flow cytometry • Protein purification • cryopreservation • brain slicing/dissection • human autopsy
- 2000-2003 **Clinical and Rodent Functional Neuroimaging**
- Discovered/resolved vascular dilation problems resulting from inhalational anesthetics.
 - Clinical imaging: Intraoperative optical intrinsic signal imaging (iOIS) and near-infrared spectroscopy (NIRS) during human neurological surgeries (identification of eloquent language, sensory, and motor cortices)
 - Biomedical training: Neuroimaging, Anatomy, Anesthesiology, Surgical Techniques, Physiology
- 2000-2001 **Molecular Cell & Developmental Biology**
- Designed and created unique study section on Bacteriophage viruses for university students nationwide
 - Learned Authorware computer program
- 1990-2000 **Part-time Clerical Staff**
- Learned Microsoft Word and Excel programs
 - Established electronic bank and credit card statement reconciliation system; figured bi-weekly payroll and taxes
 - Coordinated: meetings, job-site walk-throughs, trips
 - Typed letters/meeting minutes
 - Filed job reports and assisted with Job-site walk-throughs

Publications

Book

2008 Gloria Walker, **Melissa Walker**, Ryan Lanigan (Illustrator). "Stop & Go Safety." • Children's book • Publisher: *The Clark Group*

Articles

1. Lin X.J., Zhao T., **Walker M.J.**, Ding A. Jiang M., Cao J. Lin S., Xu X.M., Liu S. "Pro-oligodendroblasts, activated by M1 microglia, promote functional recovery after acute contusive spinal cord injury." *Cell Transplantation (Accepted)*.
2. Walker CL, Zhang YP, Liu Y, Li Y, **Walker MJ**, Liu NK, Shields CB, Xu XM. "Anatomical and Functional Effects of Lateral Cervical Hemiconfusion in Adult Rats". *Restorative Neurology and Neuroscience (Accepted)*.
3. Wang H, Liu NK, Zhang YP, Deng L, Lu QB, Shields CB, **Walker MJ**, Li J, Xu XM. "Treadmill training induced lumbar motoneuron dendritic plasticity and behavior recovery in adult rats after a thoracic contusive spinal cord injury." *Experimental Neurology*, 2015 September (doi: 10.1016/j.expneurol.2015.07.004).
4. Ma Z, Zhang YP, Liu W, Yan G, Li Y, Shields LBE, **Walker MJ**, Chen K, Huang W, Kong M, Lu Y, Brommer B, Chen X, Xu XM, Shields CB. "A controlled spinal cord contusion for the rhesus macaque monkey." *Experimental Neurology*, 2016 February 11 (doi: 10.1016/j.expneurol.2016.02.008).
5. **Melissa J. Walker**[‡], Chandler L. Walker[‡], Y. Ping Zhang, Lisa B. E. Shields, Christopher B. Shields, and Xiao-Ming Xu. "A Novel Vertebral Stabilization Method for Producing Contusive Spinal Cord Injury." *Journal of Visualized Experiments (JoVE)*, 2015 Jan 5;(95):e50149. doi: 10.3791/50149. [‡]Co-first authors.
6. Yi Ping Zhang, **Melissa J. Walker**, Lisa B. E. Shields, Xiaofei Wang, Chandler L. Walker, Xiao-Ming Xu, Christopher B. Shields. "Controlled Cervical Laceration Injury in Mice." *Journal of Visualized Experiments (JoVE)*. 2013 May 9;(75):e50030. doi: 10.3791/50030.
7. Walker CL, **Walker MJ**, Liu N-K, Risberg EC, Gao X, Chen J, Xu X-M. "Systemic bisperoxovanadium activates Akt/mTOR, reduces autophagy, and enhances recovery following cervical spinal cord injury." *PLoS One*. 2012 7(1):e30012.

8. Kareken DA, Grahame N, Dziedzic M, **Walker MJ**, Lehigh CA, O'Connor SJ. "fMRI of the Brain's Response to Stimuli Experimentally Paired with Alcohol Intoxication." *Psychopharmacology*. 2012 Apr; 22(4): 787-97.
9. Sheth S, Nemoto M, Guiou M, **Walker M** and Toga AW. "Spatiotemporal evolution of functional hemodynamic changes and their relationship to neuronal activity." *J Cereb Blood Flow Metab*. 2005 Jul; 25(7): 830-41.
10. Guiou M, Sheth S, Nemoto M, **Walker M**, Pouratian N, Ba A and Toga AW. "Cortical spreading depression produces long- term depression of activity-related changes in cerebral blood volume and neurovascular coupling." *J Biomed Opt*. 2005 Jan-Feb; 10(1): 11004.
11. Sheth SA, Nemoto M, Guiou M, **Walker M**, Pouratian N, and Toga AW. "Linear and nonlinear relationships between neuronal activity, oxygen metabolism, and hemodynamic responses." *Neuron* 2004 Apr 22; 42(2): 347-55. **Cover article.**
12. Sheth SA, Nemoto M, Guiou M, **Walker M**, Pouratian N, Hageman N, and Toga AW. "Columnar specificity of microvascular oxygenation and volume responses: implications for functional brain mapping." *J Neurosci*. 2004 Jan 21; 24(3): 634-41.
13. Sheth S, Nemoto M, Guiou M, **Walker M**, Pouratian N and Toga AW. "Evaluation of coupling between optical intrinsic signals and neuronal activity in rat somatosensory cortex." *NeuroImage* 2003 Jul; 19(3): 884-94.

Selected Abstracts

1. Lin X., Zhao T.B., **Walker M.**, Wu W., Xu X.M. Assessing motor deficits in a rodent model of spinal cord hemisection that mimics the human Brown-Sequard syndrome. *46th International Meeting of the Society for Neuroscience*, San Diego, CA, to be held November 2016.
2. Lin X., Xiong W., Wu W., **Walker MJ.**, Jin X., Xu X.M. Imaging neural activity in the primary somatosensory cortex using GCaMP transgenic mice. *46th International Meeting of the Society for Neuroscience*, San Diego, CA, to be held November 2016. ***Dynamic poster.**
3. Lin X., Zhao T., Xiang G., Xiong W., Zhao S., Wu W., **Walker M.**, Ping X., Lin S., Jin X., Chen J., Gan W., Xu X.M. Dendritic plasticity of layer V pyramidal neurons in the primary motor cortex after lesions to the pyramid and thoracic spinal cord. *45th International Meeting of the Society for Neuroscience*, Chicago, IL, October 19, 2015.

4. Lin X., Zhao T., **Walker M.**, Ding A., Jiang M., Cao J., Lin S., Xu X.M., Liu S. Transplantation of pro-oligodendroblasts, preconditioned by LPS-stimulated microglia, promotes recovery after acute contusive spinal cord injury. *45th International Meeting of the Society for Neuroscience*, Chicago, IL, October 17, 2015.
5. Deng L., Sun Y., Ruan Y., **Walker M.**, Hamilton M., Qu W., Wang Y., Smith G.M., Xu X.M. Functional regeneration of descending propriospinal axons through and beyond a growth-promoting pathway constructed by transplanted Schwann cells overexpressing GDNF after a thoracic spinal cord transection in adult rats. *45th International Meeting of the Society for Neuroscience*, Chicago, IL, October 18, 2015.
6. Lin X., Zhao T., Xiong W., **Walker M.**, Lin S., Walker C., Jin X., Xu X.M., Liu S. Intercostal nerve-lumbar dorsal root anastomosis promotes axonal regeneration beyond a spinal cord injury. *21st Annual Kentucky Spinal Cord Head Injury Research Trust Symposium*, Louisville, KY, May 20-21, 2015. (Presented).
7. Colin M.E. Fry, Chandler L. Walker, Nai-Kui Liu, Alex Giannaris, Mathew Hamilton, Melissa J. Walker, Qingbo Lu, Xiao-Ming Xu. A gender comparison for moderate thoracic midline spinal cord contusion injury in adult rats. *21st Annual Kentucky Spinal Cord Head Injury Research Trust Symposium*, Louisville, KY, May 20-21, 2015.
8. Lin X., Zhao T., Xiong W., **Walker M.**, Lin S., Walker C., Jin X., Xu X.M., Liu S. Intercostal nerve-lumbar dorsal root anastomosis promotes axonal regeneration beyond a spinal cord injury. *44th International Meeting of the Society for Neuroscience*, Washington, DC, November 18, 2014. **Hot Topics.**
9. **Melissa J. Walker**, Lingxiao Deng, Chandler L. Walker, Wenjie Wu, Xiangbing Wu, Qingbo Lu, Sarah M. Wilson, Rajesh Khanna, Naikui Liu, Xuejun Wen, Ning Zhang, and Xiao-Ming Xu. Novel Bioengineered Hydrogel Combinational Therapy for Traumatic Spinal Cord Injury. *6th Annual Wings for Life Annual Meeting*, Salzburg, Austria, May 21-22, 2014.
10. Chandler L. Walker, Xiaofei Wang, Carli L. Bullis, Colin Frye, **Melissa J. Walker**, Lingxiao Deng, Qingbo Lu, Xiao-Ming Xu. Benefits of a novel combination acute bisperoxovanadium and subacute Schwann cell transplantation therapy following spinal cord injury. *43rd International Meeting of the Society for Neuroscience*, San Diego, CA, November 10, 2013.

11. **Melissa J. Walker**, Wenjie Wu, Lingxiao Deng, Jian Zou, Chandler L. Walker, Qingbo Lu, Naikui Liu, Xuejun Wen, Ning Zhang, and Xiao-Ming Xu. Improved Functional Recovery after Contusive Spinal Cord Injury following Transplantation of a Novel Bioengineered Hydrogel containing GDNF and BDNF. *19th Annual Kentucky Spinal Cord Head Injury Research Trust Symposium*, Louisville, KY, May 6-7, 2013.
12. **Melissa J. Walker**[‡], Wenjie Wu[‡], Lingxiao Deng, Jian Zou, Chandler L. Walker, Qingbo Lu, Naikui Liu, Xuejun Wen, Ning Zhang, and Xiao-Ming Xu. Novel Bioengineered Hydrogel Combined with BDNF and GDNF Improves Functional Recovery following Traumatic Contusive Spinal Cord Injury. *2nd International Neural Regeneration Symposium*, Shenyang, China, September 21-23, 2012. [‡]Co-first authors.
13. **Melissa J. Walker**, Wenjie Wu, Lingxiao Deng, Jian Zou, Chandler L. Walker, Naikui Liu, Xuejun Wen, Ning Zhang, Xiao-Ming Xu. Combinational treatment of transplanted bioengineered hydrogel containing GDNF and BDNF promotes improved functional recovery following contusive spinal cord injury. *42nd International Meeting of the Society for Neuroscience*, New Orleans, LA, October 13-17, 2012.
14. **Walker Melissa J**, Walker Chandler L, Xu Xiao-Ming. Development of a Novel Forelimb Assessment of Skilled Coordinated Object Manipulation following Cervical Spinal Cord Injury. *Indianapolis Society for Neuroscience Symposium*, Indianapolis, IN, October 7, 2011. **(First Place in Poster Competition)**
15. Chandler L. Walker, **Melissa J. Walker**, Nai-Kui Liu, and Xiao-Ming Xu. Bisperoxovanadium-mediated neuroprotection, functional recovery, and PI3K-Akt-mTOR activity following cervical contusive spinal cord injury. *41st International Meeting of the Society for Neuroscience*, Washington, DC, November 12-16, 2011.
16. Walker Chandler L, **Walker Melissa J**, Xu Xiao-Ming. Acute Bisperoxovanadium Therapy Stimulates Akt and mTOR Activity, Reduces Autophagy, and Promotes Neuroprotection and Functional Recovery following Cervical Contusive Spinal Cord Injury. *National Neurotrauma Symposium*, Fort Lauderdale, Florida, July 10-13, 2011. **(Poster Finalist)**
17. Kareken DA, **Walker MJ**, Cox CA, Dzemic M, Windisch, Grahame NJ, O'Connor SJ. Regional Brain Responses to Experimentally Classically Conditioned Novel Cues of Alcohol Intoxication, *International Society for Biomedical Research on Alcoholism*, Paris, France, September 13, 2010.

18. Kareken DA, **Walker MJ**, Dzemidzic M, Bragulat V, Oberlin BG, Albrecht DS, Yoder KK. Human ventral striatal dopamine receptor availability as a function of alcoholic drink tastes, *International Society for Biomedical Research on Alcoholism*, Paris, France, September 13, 2010.
19. **Walker M**, Cox C, Bragulat V, Dzemidzic M, Windisch K, O'Connor SJ, Grahame NJ, Kareken DA. Negative Prediction Error to Classically Conditioned Novel Cues of Alcohol Intoxication, *33rd Annual Research Society on Alcoholism*, San Antonio, TX, June 26, 2010.
20. **Walker M**, Bragulat V, Dzemidzic M, Cox C, Talavage T, Davidson D, O'Connor SJ, Kareken DA. Family History of Alcoholism and Smoking as Mediators of the Brain's Response to Alcohol's Olfactory Cues, *3rd Annual Integrated Biomedical Gateway Recruitment Poster Session, Indiana University School of Medicine*, Indianapolis, IN, February 19, 2009.
21. Sheth S, Guiou M, Nemoto M, **Walker M** & Toga AW. Linear and nonlinear relationships between neuronal activity, oxygen metabolism, and hemodynamic responses, *34th International Meeting of the Society for Neuroscience*, San Diego, CA, October 23-27, 2004.
22. Sheth S, Hageman N, Guiou M, **Walker M**, Nemoto M, Pouratian N & Toga AW 2003 Advantages of Functional Brain Mapping Using Cerebral Blood Volume Contrast, *9th Annual Meeting of the Organization for Human Brain Mapping*, New York City, NY, June 18-22, 2003.
23. Sheth S, Guiou M, Nemoto M, Hageman N, **Walker M** & Toga AW 2003 Coupling Between Cerebral Blood Volume and Cerebral Blood Flow in Rat Somatosensory Cortex, *9th Annual Meeting of the Organization for Human Brain Mapping*, New York City, June 18-22, 2003.
24. Hageman N, Sheth S, Guiou M, Nemoto M, **Walker M** & Toga AW 2003 Basal Conditions Affect Cerebral Perfusion in the Rat Somatosensory Cortex, *9th Annual Meeting of the Organization for Human Brain Mapping*, New York City, June 18-22, 2003.
25. Sheth S, Hageman N, Guiou M, **Walker M**, Nemoto M, Pouratian N & Toga AW 2003 Advantages of Functional Brain Mapping Using Cerebral Blood Volume Contrast, *21st International Symposium on Cerebral Blood Flow, Metabolism, and Function*, Calgary, Canada, June 29-July 3, 2003.

26. Hageman N, Sheth S, Guiou M, Nemoto M, **Walker M** & Toga AW. 2003 Coupling between cerebral blood volume and cerebral blood flow in rat somatosensory cortex, *21st International Symposium on Cerebral Blood Flow, Metabolism, and Function*, Calgary, Canada, June 29-July 3, 2003.
27. Guiou M, Sheth S, Nemoto M, **Walker M**, Pouratian N & Toga AW. Neurovascular uncoupling and suppression of intrinsic cerebrovascular motion by cortical spreading depression, *33rd International Meeting of the Society for Neuroscience*, New Orleans, LA, November 8-12, 2003.
28. Sheth S, Nemoto M, Guiou M, **Walker M**, Pouratian N and Toga AW. Spatial specificity of functional brain mapping signals is a function of timing more than etiology, *33rd International Meeting of the Society for Neuroscience*, New Orleans, LA, November 8-13, 2003.
29. Sheth S, Guiou M, Nemoto M, **Walker M**, Pouratian N and Toga AW. Optical intrinsic signal and laser Doppler imaging in rat somatosensory cortex, *32nd International Meeting of the Society for Neuroscience*, Orlando, FL, November 2-7, 2002.
30. Guiou M, Sheth S, Nemoto M, **Walker M**, Pouratian N, Ba A, Chen J and Toga AW. Cortical spreading depression produces long-term disruption of activity-related changes in cerebral blood flow and volume, *32nd International Meeting of the Society for Neuroscience*, Orlando, FL, November 2-7, 2002.
31. Sheth S, Nemoto M, **Walker M**, Guiou M and Toga AW. Hemoglobin concentration mapping using two-dimensional spectrophotometry, *8th Annual Meeting of the Organization for Human Brain Mapping*, Sendai, Japan, June 2-6, 2002.
32. Sheth S, Nemoto M, Guiou M, **Walker M**, Lee S and Toga AW. Combined optical intrinsic signal and laser Doppler imaging in rodent somatosensory cortex, *8th Annual Meeting of the Organization for Human Brain Mapping*, Sendai, Japan, June 2-6, 2002.
33. Guiou M, Sheth S, Nemoto M, **Walker M**, Ba A, Pouratian N, Chen J and Toga AW. Cortical spreading depression produces long-term attenuation and uncoupling of activity-related optical intrinsic signals to neuronal activity, *8th Annual Meeting of the Organization for Human Brain Mapping*, Sendai, Japan, June 2-6, 2002.

Conferences

- 2015 **Kentucky Spinal Cord Head Injury Research Trust Symposium**
(Poster Presentation), 21st Annual Meeting, Louisville, Kentucky
- 2015 **Wings for Life Annual Meeting** (Oral Presentation)
7th Annual Meeting, Salzburg, Austria
- 2015 **Neuroscience Innovation Day**, (Poster Presentation)
1st Annual, Indianapolis, Indiana, **IUSM, IU Health, Purdue Engineering**
- 2014 **International Meeting of the Society for Neuroscience** (Attended)
44th International Meeting, Washington, DC
- 2014 **Indianapolis Society for Neuroscience** (Attended)
Annual Meeting, Indianapolis, Indiana
- 2014 **Wings for Life Annual Meeting** (Poster and Oral Presentation)
6th Annual Meeting, Salzburg, Austria
- 2013 **Indianapolis Society for Neuroscience** (Attended)
Annual Meeting, Indianapolis, Indiana
- 2013 **Kentucky Spinal Cord Head Injury Research Trust Symposium**
(Poster Presentation), 19th Annual Meeting, Louisville, Kentucky
- 2012 **International Meeting of the Society for Neuroscience** (Poster Presentation), 42nd International Meeting, New Orleans, Louisiana
- 2012 **Linda and Jack Gill Symposium** (Poster Presentations, 2 posters)
7th Annual Symposium, Bloomington, Indiana
- 2012 **International Neural Regeneration Symposium** (2 Posters and Oral Presentation), 2nd Annual Meeting, Shenyang, China **(Outstanding Oral Presentation)**
- 2012 **Indiana Spinal Cord and Brain Injury Research Conference**
(Attended)
2nd Annual Meeting, Indianapolis, Indiana

- 2012 **Kentucky Spinal Cord Head Injury Research Trust Symposium**
(Attended), 18th Annual Meeting, Lexington, Kentucky
- 2011 **North American Vascular Biology Organization** (Attended)
Vascular Matrix Biology and Bioengineering Annual Meeting, Hyannis,
Massachusetts
- 2011 **National Neurotrauma Society** (Poster Presentation)
Annual Meeting, Fort Lauderdale, Florida
- 2011 **Indianapolis Society for Neuroscience (1st Place – Poster
Competition)**, Annual Meeting, Indianapolis, Indiana
- 2011 **Kentucky Spinal Cord Head Injury Research Trust Symposium**
(Poster Presentation), 17th Annual Meeting, Louisville, Kentucky
- 2010 **Indianapolis Society for Neuroscience** (Attended)
Annual Meeting, Indianapolis, Indiana
- 2010 **Linda and Jack Gill Symposium** (Poster Presentation)
5th Annual Symposium, Bloomington, Indiana
- 2010 **Research Society on Alcoholism** (Poster Presentation)
33rd Annual National Meeting, San Antonio, Texas
- 2009 **Indianapolis Society for Neuroscience** (Poster Presentation)
Annual Meeting, Indianapolis, Indiana
- 2008 **Indianapolis Society for Neuroscience** (Attended)
Annual Meeting, Indianapolis, Indiana
- 2008 **Research Society on Alcoholism** (Attended)
31st Annual National Meeting, Washington DC
- 2008 **Indiana Neuroimaging Symposium** (Attended)
2nd Annual Meeting, Indianapolis, Indiana
- 2008 **Indiana Symposia on Mild Cognitive Impairment** (Attended)
2nd Annual Meeting, Indianapolis, Indiana

- 2007 **Indianapolis Society for Neuroscience** (Attended)
Annual Meeting, Indianapolis, Indiana
- 2007 **Adult Skeletal Muscle Symposium: Growth, Function and Mobility, Eli Lilly** (Attended), Indianapolis, Indiana
- 2002 **International Meeting of the Society for Neuroscience** (Co-presented two posters), 32nd International Meeting, Orlando, Florida
- 2002 **International Society for the History of Neurosciences** (Attended)
7th Annual Meeting, Los Angeles, California