Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2003	**N/A**	**-**

4. TITLE AND SUBTITLE
810 NM LIGHT TREATMENT OF ACUTE SPINAL CORD INJURY ALTERS THE IMMUNE RESPONSE AND IMPROVES AXONAL REGENERATION AND FUNCTIONAL RECOVERY

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Byrnes /Kimberly R.

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Uniformed Service University of the Health Sciences

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Spinal cord injury (SCI) results in substantial and often permanent impairment of function due to the lack of regeneration of damaged axons. Despite vigorous research, no cure for SCI has been found. Light therapy (LT), through the absorption of light by target tissue, improves healing in a number of injury models. However, no study to date has assessed the ability of LT to facilitate the regeneration of specific spinal cord tracts. Our hypothesis was that transcutaneous application of 810 nm light promotes axonal regeneration and functional reinnervation following transection of the corticospinal tract (CST) by changing the extracellular milieu of the spinal cord. Three studies were implemented to investigate this hypothesis. First, anterograde and retrograde tract tracing techniques were used to investigate axonal regrowth after SCI and LT. LT (810 nm) was applied at the site of acute injury to the CST of adult rats. Anterograde tract tracing demonstrated that LT improved axonal regrowth after injury, with significant increases in axon number (199 +/- 12) and distance of regrowth (8.7 +/- 0.8 mm) as compared to controls ($p<0.01$). Double label retrograde tract tracing revealed that transected axons regrew and reinnervated motor neurons in the lumbar spinal cord in the light treated group only ($p<0.05$). Functional analyses revealed that this regeneration was coupled with significant improvement in 2 tests of CST performance, angle of rotation and ladder beam cross time ($p<0.05$). Second, to explore the effect of LT on the spinal cord cellular environment, we investigated the inflammatory response after SCI, using quantitative immunohistochemistry techniques. This study revealed that LT suppressed the invasion/activation of macrophages, microglia and T lymphocytes after SCI ($p<0.001$) and delayed the activation of astrocytes. The third study explored gene expression after SCI and LT. A number of cytokines and chemokines were assessed using reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of interleukin 6, monocyte chemoattractant protein 1 (MCP-1) and inducible nitric oxide synthase (iNOS) was suppressed at 6 hours post-injury by LT ($p<0.01$). These results demonstrate that LT has an anti-inflammatory effect on the spinal cord after injury and significantly improves axonal regeneration and functional recovery.

15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**153**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

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ABSTRACT

810 NM LIGHT TREATMENT OF ACUTE SPINAL CORD INJURY ALTERS THE IMMUNE RESPONSE AND IMPROVES AXONAL REGENERATION AND FUNCTIONAL RECOVERY

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Directed by Juanita J. Anders, Ph.D., Associate Professor of Anatomy, Physiology, and Genetics, and Neuroscience

Spinal cord injury (SCI) results in substantial and often permanent impairment of function due to the lack of regeneration of damaged axons. Despite vigorous research, no cure for SCI has been found. Light therapy (LT), through the absorption of light by target tissue, improves healing in a number of injury models. However, no study to date has assessed the ability of LT to facilitate the regeneration of specific spinal cord tracts. Our hypothesis was that transcutaneous application of 810 nm light promotes axonal regeneration and functional reinnervation following transection of the corticospinal tract (CST) by changing the extracellular milieu of the spinal cord. Three studies were implemented to investigate this hypothesis. First, anterograde and retrograde tract tracing techniques were used to investigate axonal regrowth after SCI and LT. LT (810 nm) was applied at the site of acute injury to the CST of adult rats. Anterograde tract tracing demonstrated that LT improved axonal regrowth after injury, with significant increases in axon number (199 +/- 12) and distance of regrowth (8.7 +/- 0.8 mm) as compared to

controls ($p<0.01$). Double label retrograde tract tracing revealed that transected axons regrew and reinnervated motor neurons in the lumbar spinal cord in the light treated group only ($p<0.05$). Functional analyses revealed that this regeneration was coupled with significant improvement in 2 tests of CST performance, angle of rotation and ladder beam cross time ($p<0.05$). Second, to explore the effect of LT on the spinal cord cellular environment, we investigated the inflammatory response after SCI, using quantitative immunohistochemistry techniques. This study revealed that LT suppressed the invasion/activation of macrophages, microglia and T lymphocytes after SCI ($p<0.001$) and delayed the activation of astrocytes. The third study explored gene expression after SCI and LT. A number of cytokines and chemokines were assessed using reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of interleukin 6, monocyte chemoattractant protein 1 (MCP-1) and inducible nitric oxide synthase (iNOS) was suppressed at 6 hours post-injury by LT ($p<0.01$). These results demonstrate that LT has an anti-inflammatory effect on the spinal cord after injury and significantly improves axonal regeneration and functional recovery.

810 NM LIGHT TREATMENT OF ACUTE SPINAL CORD INJURY ALTERS THE IMMUNE RESPONSE AND IMPROVES AXONAL REGENERATION AND FUNCTIONAL RECOVERY

By

Kimberly R. Byrnes

Dissertation submitted to the faculty of the

Program in Neuroscience of the

Uniformed Service University of the Health Sciences

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy 2003

ACKNOWLEDGEMENTS

I would first and foremost like to thank Dr. Juanita J. Anders, my advisor and mentor over these past 6 years. Without her help, guidance, and friendship, this thesis would not have been possible. I've learned an immeasurable amount about science, academics and research. Thank you.

I would also like to thank all of the members of my committee, Drs. Rosemary Borke, Sonia Doi, Howard Bryant, Leslie McKinney and Ronald Waynant. Without their advice, time and commitment, I would not have been able to see this project through to its conclusion. Their effort in helping me to complete this journey has been inspiring.

I owe an enormous thank you to the many people over the years that helped me with the actual workload of this project. For his patience, countless answers to my countless questions, and the amount of laser information that I never knew I'd need to learn, I thank Dr. Ilko Ilev. To Xingjia Wu, Kimberly Smith, Lauren Barna, Reed Heckert, Amy Van Horn, Heather Gerst, and Dr. Holly Nash, thank you for your hours upon hours of hard work for caring about my project.

I would like to acknowledge the faculty and staff of the Neuroscience Program and the Graduate Education Office, specifically Drs. Cinda Helke, Michael Sheridan and Regina Armstrong and Ms. Janet Anastasi, for encouraging me to become involved in the

graduate education process on more than one level and helping me to learn more than I ever thought I would in graduate school.

I would also like to acknowledge the advice and support of several faculty and staff of USUHS, including Drs. Diane Borst, Michelle Chenault, Cathy Joswick, Joe McCabe, Harvey Pollard and Linda Porter. And I'd like to thank those outside of USUHS who also supported my journey, Drs. David Baxter, Laure Haak, Leonardo Longo and Jan Tunér.

I am extremely grateful to the friends that I have made along the way. Without their support, guidance and advice, I would not be where I am today. I would be remiss if I did not specifically acknowledge Ashley Poindexter, Tara Romanczyk, Alisa Schaefer and Sharon Dominick for their love and encouragement.

Finally, I thank my parents, Rose and Randolph Byrnes, for their never ending support and love. Thank you for giving me a chance to make you proud. I still owe you a beach house, but hopefully this will hold you till then.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BBB	Beattie-Bresnahan-Basso
BDNF	Brain derived neurotrophic factor
cAMP	Cyclic adenosine – 3',5' monophosphate
C-C	Cysteine-cysteine
cDNA	Complementary Deoxyribonucleic Acid
cm	Centimeter
CNS	Central nervous system
CREB	cAMP response element binding protein
CST	Corticospinal tract
CX3C	Cysteine – 3 amino acids – cysteine
CXC	Cysteine – any amino acid – cysteine
Da	Dalton
DNA	Deoxyribonucleic acid
DPI	Days post-injury
eNOS	endothelial nitric oxide synthase
ETC	Electron transport chain
FADH	Flavin-adenine-dinucleotide
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Hematoxylin and Eosin
ICAM	Intracellular cell adhesion molecule
IL1β	Interleukin 1 β
IL6	Interleukin 6
iNOS	Inducible nitric oxide synthase
I.P.	Intra-peritoneal
J	Joules
K	Potassium
Kg	Kilogram
L3	Lumbar level 3
LASER	Light amplification by stimulated emission of radiation
LT	Light therapy
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MIP1α	Macrophage inflammatory protein 1α
ml	Milliliter
mm	Millimeter
MP	Methylprednisolone
mRNA	Messenger Ribonucleic acid
mW	Milliwatt
μm	Micrometer

Na	Sodium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFκB	Nuclear factor κ B
NGF	Nerve growth factor
nm	Nanometer
nNOS	Neuronal nitric oxide synthase
NT3	Neurotrophin 3
PNS	Peripheral nervous system
ROS	Reactive oxygen species
RST	Rubrospinal tract
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCI	Spinal cord injury
T9	Thoracic level 9
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
UV	Ultraviolet
W	Watts

CHAPTER 1

Introduction and Background

Spinal Cord Injury

Spinal cord injury (SCI) is a significant clinical problem with an incidence of 11,000 new cases per year, primarily young adults (Center, 2001). Fifty-five percent of injuries occur between the ages of 16 and 30. Most injuries occur due to motor vehicle accidents and gunshot wounds, and 46% occur in the thoracic or lumbar region of the spinal cord. Injury in this region of the spinal cord typically results in paralysis of the lower limbs, with a loss of sensation below the level of the injury.

Damage to the spinal cord is generally permanent, due to the inability of axons in the central nervous system (CNS) to regenerate and reinnervate the appropriate targets after injury. The average cost to spinal cord injured patients to cope with this lifelong impairment is $209,074 for the first year after injury, followed by a yearly cost of $21,274. However, SCI leads to a drain not only on financial resources, but also on emotional and physical resources, as the life expectancy of SCI victims is reduced by over 10 years for those sustaining a thoracic or lumbar injury and over 20 years for those with a cervical injury. Despite vigorous research in the field, there currently is no cure for SCI.

Organization of the Spinal Cord

The brain and spinal cord comprise the CNS. The spinal cord is composed of four segments in both humans and rats, and these segments are divided based on the origin of their associated spinal nerves and vertebrae. In humans, there are 8 cervical vertebrae, 12 thoracic, 5 lumbar and 5 sacral. Rats have one more thoracic and lumbar vertebrae and 1 less sacral than humans. In both humans and rats, the innervation of the upper limbs originates from the lower cervical and upper thoracic segments (C5 – T1) and lower limbs are innervated by the lower thoracic, lumbar and upper sacral segments (T12 – S2; (Greene, 1935; Spence, 1982). This innervation originates from and travels to a variety of tracts within the spinal cord, including the major supraspinal motor tract, the corticospinal tract (CST).

The CST originates from pyramidal neurons in layer V of the motor cortex, as well as sensory cortices, in humans and the sensorimotor cortex in rats (Hicks and D'Amato, 1977; Zilles et al., 1980; Ullan and Artieda, 1981; Donoghue and Wise, 1982; Leong, 1983). These neurons receive inputs from sensory, premotor and supplementary areas of the cortex, as well as the thalamus and basal ganglia (Kennedy, 1990). The CST descends through the internal capsule, the cerebral peduncle, and most of the fibers decussate in the lower medulla (Vahlsing and Feringa, 1980; Whishaw and Metz, 2002). Once crossed, those fibers course in the lateral funiculus in humans or the ventral portion of the dorsal funiculus in rats and are termed the lateral or dorsal CST, respectively (Molander et al., 1984; Brosamle and Schwab, 1997). Approximately 5 – 10% of the axons in this tract remain uncrossed and course in the anterior funiculus of humans or

ventral funiculus of rats and are termed the anterior or ventral CST, respectively (Vahlsing and Feringa, 1980; Brosamle and Schwab, 1997; Whishaw and Metz, 2002). Within the spinal cord, both the Lateral/Dorsal and Anterior/Ventral portions of the CST terminate on alpha motor neurons in layer IX of the ventral horn, as well as on interneurons in the intermediate gray matter (Molander et al., 1984; Kennedy, 1990).

Damage to the Spinal Cord

Damage to the spinal cord typically involves an initial injury to the cord, usually the result of transection or crush, followed by a secondary injury. The secondary injury results from the imbalance of intra and extracellular ionic concentrations, excitatory amino acid build up, protease activity and lipid peroxidation and breakdown (Bao and Liu, 2002; Eng and Lee, 2003). These events lead to demyelination, axonal degeneration, neuronal death, cavitation and glial scarring surrounding the area of the initial injury (Dusart and Schwab, 1994; Koshinaga and Whittemore, 1995; Fitch et al., 1999). Secondary injury often causes greater impairment of axonal regeneration and functional recovery than the initial injury would have caused alone (Popovich et al., 2002). While the causes of secondary injury are not fully understood, invasion/activation of cells such as neutrophils, macrophages, microglia, and T and B lymphocytes has been under investigation as potential mediators of secondary injury (Popovich et al., 2002).

Cell Invasion and Activation

Neutrophils are the first cells to respond to an insult. Neutrophils secrete several cytokines and proteinases and are responsible for much of the early tissue degradation

following injury (Fujita et al., 1994; Anderson, 1995). Activated neutrophils also release reactive oxygen species that damage endothelial cells (Tator and Fehlings, 1991; Taoka and Okajima, 1998), further contributing to secondary injury. These cells infiltrate the wound area between 3 and 6 hours following CNS injury, first adhering to blood vessel walls before invading the tissue and peaking at approximately 24 hours post-injury (Perry and Gordon, 1991; Dusart and Schwab, 1994; Bartholdi and Schwab, 1997; Carlson et al., 1998; Streit et al., 1998).

Macrophages and activated microglia, which are immunologically identical, appear in the spinal cord between 12 and 24 hours post-injury, with a maximal infiltration at 4 – 8 days post-injury (Popovich et al., 1997; Carlson et al., 1998). Microglia are flattened cells with antler-like processes when resting; upon activation, these cells retract their processes and become round and phagocytic-like (Popovich et al., 1997; Watanabe et al., 1999). The activated microglia expresses a variety of cell surface molecules and cytokines, including MHC Class II antigens and complement receptors, that, in combination with their round morphology, makes them indistinguishable from blood-borne macrophages (Popovich et al., 1997; Carlson et al., 1998). Both of these cell types possess receptors for carbohydrates, antibodies and complement that are expressed on the cell surface after injury (Delves and Roitt, 2000). Once they have found such a marker for a 'foreign' cell, it is phagocytosed. The phagocytosed cell is disposed of using superoxide anions, hydroxyl radicals, hypochlorous acid, nitric oxide and lysozymes (Delves and Roitt, 2000), the release of which may damage non-injured tissue in the area, leading to secondary injury. Popovich et al. (Popovich et al., 2002) and Fitch et al. (Fitch et al., 1999) have shown that activated macrophages alone were sufficient to cause

significant cavitation and glial activation *in vitro* and *in vivo*. Macrophage invasion has also been closely correlated with the production of neurite growth inhibitory molecules by microglia and astrocytes, such as chondroitin sulfate proteoglycans (Fitch and Silver, 1997; Fitch et al., 1999) and keratan sulfate proteoglycan (Jones and Tuszynski, 2002). Inhibition of macrophage activity or invasion at the site of injury spares white matter, preserves myelinated axons and reduces cavitation (Popovich et al., 1999).

At approximately this same time, astrocytes are activated in the spinal cord. They migrate to the site of the injury and surround the injured portion of the spinal cord, walling off the injury site (Lagord et al., 2002). GFAP immunoreactivity, a marker of astrocyte activation, appears around the lesion site at 1 day post-injury, and outlines the lesion margins over the first week after SCI (Schnell et al., 1999; Lagord et al., 2002). Over this time period, astrocyte activation is apparent several millimeters from the injury site, extending beyond the macrophage and microglial invasion (Schnell et al., 1999). Astrocytic activation is restricted to the lesion site at time periods longer than 2 weeks (Popovich et al., 1997). Astrocytes may contribute to the lack of regeneration through the production of a number of proteins, such as proteoglycans, that inhibit neurite outgrowth (McKeon et al., 1991; Fitch and Silver, 1997). However, they also produce several extracellular matrix proteins and growth factors that may promote neuronal growth (Liesi and Kauppila, 2002).

T and B lymphocytes have an infiltration pattern similar to macrophages/activated microglia, entering the spinal cord at 1 – 2 days post-injury, peaking at 7 days, and then dropping in number and activation thereafter (Popovich et al., 1997; Schnell et al., 1999). T and B lymphocytes may also contribute to demyelination of the spinal cord after injury

(Popovich et al., 1997), but have a significantly lower presence in the injured spinal cord than macrophages/activated microglia.

Gene Expression

Damage to the spinal cord results in significant changes in gene expression. Increases in intracellular calcium levels and alterations in cellular oxidative states play a role in gene expression after injury. SCI increases the intracellular calcium level, which then results in activation of several kinases and downstream transcription factors, including protein kinase C, calcium calmodulin kinase II, transcription factor serum response factor and the cAMP response element binding transcription factor (CREB) (Marciano et al., 2002). These factors activate the transcription factors c-fos and c-jun by 3 hours post-SCI, and result in the transcription of a number of genes, such as those involved in apoptosis (Hayashi et al., 2000). Redox sensitive NFκB is also upregulated by changes in cellular oxidation state early after SCI; activation of this transcription factor results in upregulation of gene expression of a variety of cytokines, chemokines, cell adhesion molecules, iNOS, growth factors and receptors (Chikawa et al., 2001).

The degree of recovery after SCI is dependent upon the pattern of cytokine and chemokine gene expression as well as other genes that result in a pattern of cell invasion/activation. mRNA of pro-inflammatory cytokines, such as interleukin 1 (IL1β), tumor necrosis factor α (TNFα), IL6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), are produced quickly after SCI. Expression occurs within 1hr for IL1β and TNFα and within 6hr for IL6 and GM-CSF (Benveniste, 1992; Bartholdi and Schwab, 1997; Klusman and Schwab, 1997; Hayashi et al., 2000; Pan et al., 2002). The

early expression of these cytokines after injury suggests that they are produced primarily by resident cells of the spinal cord following stimulation, such as endothelial cells, astrocytes, microglia and neurons.

TNFα is a 17,000 Da transmembrane protein produced by astrocytes and microglia (Benveniste, 1992; Aggarwal et al., 2001). In spinal cord injuries, TNFα is first detectable between 45 and 60 minutes post-injury, and disappears by 24 hours (Bartholdi and Schwab, 1997; Streit et al., 1998; Hayashi et al., 2000). This cytokine modulates the expression of surface antigens and adhesion molecules, changing the permeability of blood vessels to circulating immune cells (Aggarwal et al., 2001). It may play a role in secondary injury and impaired regeneration by inducing the production of inducible nitric oxide synthase (iNOS), IL1β, GM-CSF and promoting the activity of neutrophils.

IL-1β is expressed in a similar pattern, appearing within the first hour after CNS damage (Bartholdi and Schwab, 1997; Streit et al., 1998; Hayashi et al., 2000). This 17,000 Da cytokine, produced by macrophages, endothelial cells, microglia and astrocytes (Benveniste, 1992), reaches peak levels by 1 hour post-injury and decreases by 12 hours (Bartholdi and Schwab, 1997; Hayashi et al., 2000). IL1β has a complex role in the injured spinal cord, inducing remyelination after spinal cord damage (Mason et al., 2001). However, it also increases astrocyte and macrophage activation and expression of other pro-inflammatory cytokines, proteoglycans, intracellular cell adhesion molecule (ICAM), and iNOS, which may contribute to secondary injury and impair functional recovery (Benveniste, 1992; Lotan and Schwartz, 1994; Klusman and Schwab, 1997; Dinarello, 2001).

IL6, a 26,000 Da polypeptide, is secreted by microglia and astrocytes after stimulation by IL1β or TNFα. This pro-inflammatory cytokine stimulates astrocyte and macrophage activation (Benveniste, 1992; Lotan and Schwartz, 1994). Additionally, IL6 has been found to increase the production of neurite growth inhibitory molecules in astrocytes (Fitch and Silver, 1997), supporting its role in secondary injury and prevention of axonal regeneration. The 14,000 Da protein GM-CSF is also produced by fibroblasts and macrophages (Nicola, 2001). This cytokine is pro-inflammatory, increasing activity of both neutrophils and macrophages (Nicola, 2001).

Pro-inflammatory cytokines are considerably damaging to the spinal cord. These cytokines are responsible for inducing and/or altering the activity of various cells within the spinal cord, including macrophages, microglia and astrocytes (Benveniste, 1992; Bartholdi and Schwab, 1997; Klusman and Schwab, 1997), and can induce cell death and demyelination (Selmaj and Raine, 1988; Simmons and Willenborg, 1990; Jenkins and Ikeda, 1992), suggesting that they may contribute to the secondary damage observed after SCI. Direct injection of pro-inflammatory cytokines induces demyelination in the spinal cord (Simmons and Willenborg, 1990), while blockage of their activity results in decreases in macrophage, T lymphocyte and neutrophil recruitment, as well as a decrease in demyelination (Ousman and David, 2001). Pro-inflammatory cytokine receptor knockout aids in recovery after injury, as demonstrated by Raivich and colleagues (Raivich et al., 2002).

Anti-inflammatory cytokines, on the other hand, may promote recovery. TGFβ, whose expression is upregulated by 1 day post-injury, has both pro- and anti-inflammatory properties, making its role in SCI complex and currently debated. This

cytokine is secreted by macrophages and astrocytes (Flanders and Roberts, 2001). *In vitro*, TGFβ reduces microglial adhesion to astrocytes and/or laminin (Lagord et al., 2002; Milner and Campbell, 2002). It also inhibits macrophage activity and decreases expression of pro-inflammatory cytokines, such as TNFα (Aggarwal et al., 2001; Flanders and Roberts, 2001). However, TGFβ has been shown to increase astrocyte proliferation and provide chemoattraction for macrophages (Frisen et al., 1994; Lotan and Schwartz, 1994; Flanders and Roberts, 2001).

Chemokines also play a role in SCI. The C-C chemokines, macrophage inflammatory protein 1 (MIP1α) and monocyte chemoattractant protein 1 (MCP-1), are responsible for the recruitment of inflammatory cells, such as macrophages, T lymphocytes and, in the case of MIP1α, neutrophils, into the injured spinal cord (Oppenheim et al., 1991; Glabinski et al., 1996; Rollins, 2001; Sherry and Franchin, 2001). There are four classes of chemokines based on their activities and the position of cysteine residues in their amino acid structure (Eng and Lee, 2003). The cysteines of the C-C family are adjacent to each other, the other family types are the C (single cysteine residue), CXC (cysteines separated by another amino acid) and CX3C (cysteines separated by 3 other amino acids). These chemokines are normally upregulated within the first few hours after SCI. MIP1α transcription increases between 45 minutes and 6 hours post-injury and MCP-1 expression is upregulated by 6 hours post-injury and peaks at approximately 12 hours post-injury (Bartholdi and Schwab, 1997; Glabinski et al., 1998; McTigue et al., 1998; Ma et al., 2002). These chemokines are produced by fibroblasts, endothelial cells, macrophages, neutrophils and astrocytes in response to cytokine or toxin stimulation (Rollins, 2001; Sherry and Franchin, 2001). Once

produced, as well as performing chemotactant duties, they are responsible for stimulation of macrophages and increasing cellular adhesion to cell adhesion molecules, such as ICAM (Rollins, 2001; Sherry and Franchin, 2001).

Ultimately, the interaction between these chemokines and cytokines results in the inflammatory response in the spinal cord, characterized by invasion/activation of macrophages, microglia, astrocytes, and T and B lymphocytes. Genes expressed within these immune cells that play a fundamental role in the inflammatory response, such as iNOS, are also altered by SCI. Three isoforms of NOS have been identified within the CNS, neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. iNOS is the only member of the NOS family of enzymes that is calcium independent and responds to stress signals. It is produced by neurons, astrocytes, macrophages and microglia after exposure to endotoxins or cytokines (Chatzipanteli et al., 2002). After SCI, iNOS peaks at approximately 24 hours post-SCI, and plays a neurodestructive role through the production of toxic free radicals that cause lipid peroxidation (Hall and Braughler, 1986; Hamada et al., 1996b; Hu et al., 2000).

Lack of Regeneration

It is well known that adult neurons are capable of regrowth after injury (Zhou et al., 2003). However, following SCI there is limited spontaneous sprouting that reaches only 3 – 4 mm caudal to the lesion and fails to reinnervate target neurons (Li et al., 1994). Several studies have suggested that scar formation and cavity development after injury impair axonal growth (Fawcett and Asher, 1999; Fitch et al., 1999). Inhibitory molecules, such as myelin associated glycoprotein and Nogo-A, that block regeneration,

are present within the CNS (Niederost et al., 2002; Fournier et al., 2003). Astrocytes also produce growth-inhibitory molecules, such as chondroitin sulfate proteoglycan, which is significantly upregulated after SCI (Lemons et al., 1999). There is also a lack of growth promoting factors in the spinal cord after injury, such as neurotrophin 3 (NT3) and brain derived neurotrophic factor (BDNF; Hayashi et al., 2000).

Functional Impact

Several spinal cord tracts are involved in locomotion. The reticulospinal tract has been implicated in the initiation of movement, while vestibulospinal and rubrospinal tracts are responsible for posture maintenance (Cheng et al., 1997). Voluntary gait modification and response to changes in the environment are controlled by the CST (Cheng et al., 1997). Armstrong (Armstrong, 1986) demonstrated that lesion of the CST in rats impairs limb adduction and flexion and prolongs the activity of extensor muscles, thus affecting locomotion. However, the CST shares functions with the rubrospinal tract (RST), so specific, sensitive functional assays are necessary to detect and identify CST impairments (Li et al., 2003). As humans, cats and monkeys have an overlapping CST and RST in the spinal cord (Kennedy, 1990), correlation of specific impairments to each tract is difficult. Rats, however, have a separate RST, located in the lateral funiculus of the spinal cord (Kennedy, 1990; Liu et al., 2002), allowing for investigation of this tract alone.

Lesion of the CST impairs the ability to perform a number of quantifiable tasks. The ability of rats to reflexively place their hindlimbs onto a surface for support after stimulation, also called contact placing, or to climb onto that surface with their forelimbs

supported is often lost after SCI (Kunkel-Bagden et al., 1993). Ladder crossing, in which the ability of animals to grasp a ladder rung and maneuver across the ladder is tested, is significantly affected by CST lesion (Merkler et al., 2001; Metz and Whishaw, 2002). Footprint analysis assesses stride length, base of support and angle of rotation measurements of normal locomotion in rats; these measurements are also significantly altered by SCI (Kunkel-Bagden et al., 1993).

Treatment Modalities

Current Research

Current research into the amelioration of the effects of SCI includes studies on decreasing secondary injury, removal of inhibitory factors, re-introduction of lost growth factors and bridging of the injury site. Anti-inflammatory drug treatment, application of growth factors and transplantation of different cell types are all under vigorous investigation. The combination of several treatment modalities is also being investigated as therapy for SCI.

Delaying or impairing the inflammatory response, such as by methylprednisolone (MP) treatment, improves function and decreases traumatic edema after SCI (Xu et al., 1992). MP significantly reduces vascular permeability after injury (Xu et al., 1992) and reduces secondary injury (Hall et al., 1978; Green et al., 1980; Braughler and Hall, 1982). Clinical studies of MP have shown that administration within hours after SCI has a beneficial effect, although there is an increased incidence of infection, most likely due to systemic immune suppression (Bracken et al., 1984; Bracken et al., 1997). Minocycline,

an antimicrobial drug with anti-inflammatory properties has also been shown to improve function after SCI (Lee et al., 2002; Wells and Yong, 2002).

Removal of the potentially inhibitory cells or the inhibitory proteins they produce also results in an improvement in axonal growth and sprouting. Kalderon et al. (Kalderon and Fuks, 1996a) applied x-irradiation at day 17/18 after spinal cord injury and demonstrated an increase in axonal regeneration, as defined by double labeling of cortical neurons. X-irradiation treatment at earlier time points also improves performance on the 'Beattie Bresnahan Basso' functional test (BBB score) after injury and decreases cellular proliferation (Zeman et al., 2001). This improvement is proposed to be due to x-irradiation induced elimination of proliferating cells, such as astrocytes, which form the glial scar and produce inhibitory proteoglycans. Elimination of the effects of Nogo by IN-1 antibody treatment improves CST function in rats (Schwab, 1990a, b; Bregman et al., 1995; Merkler et al., 2001). Cleavage of chondroitin sulfate proteoglycan also removes inhibitory factors from the injured CNS and improves axonal sprouting (Zuo et al., 1998; Lemons et al., 1999).

Providing neurotrophic factors to the injured spinal cord is another method for improving recovery. Nerve growth factor (NGF) and NT3 were also found to reduce axonal degeneration after lesion of the CST (Sayer et al., 2002). Implantation of collagen matrices containing NT3 promotes axonal sprouting into grafts and functional improvement (Houweling et al., 1998a). Application of BDNF promotes RST neuronal survival and recovery of function (Giehl et al., 1998; Houweling et al., 1998b; Jakeman et al., 1998; Liu et al., 2002).

Transplantation of cells and tissues has been studied as a treatment for SCI.

Several types of tissues and cells have been used as bridging material or to provide a growth-promotive environment. Implantation of peripheral nerves or Schwann cells, the glial support for peripheral nerves, into the injured area improves axonal sprouting and function (David and Aguayo, 1981; Li and Raisman, 1994; Cheng et al., 1996). Schwann cells synthesize and secrete neurotrophic and ECM molecules, including NGF, BDNF, ciliary neurotrophic factor, laminin and collagen, as well as express cell adhesion molecules such as N-cadherin and β1 integrin, which all play a role in increasing axonal regrowth following damage (Paino et al., 1994). Transplants of fetal cortical or spinal cord tissue supports axonal growth and gross locomotor function after SCI (Diener and Bregman, 1998; McDonald et al., 1999). Olfactory ensheathing cell transplantation also improves axonal growth and functional recovery (Ramon-Cueto, 2000; Nash et al., 2002; Li et al., 2003). However, a common problem with transplantation therapies is the lack of growth beyond the grafted tissue (Xu et al., 1997), necessitating further research into combining grafting therapies with other treatments. A recent combination therapy for SCI that has resulted in axonal growth beyond the graft is MP and transplants (Chen et al., 1996; Guest et al., 1997; Nash et al., 2002).

Recently, macrophages have been considered as a treatment for SCI. Despite macrophage involvement in secondary injury, several researchers have found that implantation or stimulation of macrophages improves recovery after SCI. Implantation of activated macrophages, preincubated with sciatic nerve sections or a macrophage stimulant, improved axonal sprouting and locomotor function in rats after SCI (Prewitt et al., 1997; Rabchevsky and Streit, 1997; Rapalino et al., 1998; Popovich et al., 1999; Schwartz et al., 1999).

However, the majority of these treatment methodologies are invasive and carry the risk of additional damage to the spinal cord. Additionally, many carry unwelcome side effects, such as global immune suppression, as is the case with MP treatment, or transplantation rejection. Therefore, investigation into alternative therapies is warranted. Particularly, a therapy that is non-invasive and lacks notable side effects is needed.

Light Therapy

Light is an important factor in both the normal function of organisms as well as in the correction of dysfunction. Low intensity red light was used in ancient times as a treatment for illness and early researchers have found that rearing animals with constant irradiation of different colored (or different wavelengths) of light led to different weights among the animals (Karu, 1989).

The LASER, an acronym for 'light amplification by stimulated emission of radiation', provided researchers and clinicians with an easy to use, collimated, monochromatic, coherent light source for experimental and clinical situations (Shaffer, 2002). Although whether the specific features of laser light is necessary for light therapy is under investigation, the advent of lasers advanced research in the light therapy field. Light therapy (LT), also referred to as low level intensity laser, low power laser irradiation, biostimulation, or photo-biomodulation, traditionally uses light sources with powers of 200 mW or less, significantly lower than the power of lasers used for tissue ablation or cutting, or even less than your average light bulb (60 – 100 W). Clinically, the wavelengths used range from the visible spectrum to the infrared: green (540), red (633) and infrared (780 and above; Karu, 1998). Currently, light therapy research is in a

relatively young state, and standardized treatment procedures are not available. However, researchers are attempting to establish a consensus on light therapy terminology and dosimetry. The state of the art in the field is presentation of light treatment parameters in terms of wavelength and power (W), both of which were discussed previously, as well as size of the spot being treated, pulsing rate, treatment time and power density (W/cm^2). The most common treatment parameter statement is a demonstration of the energy density, or dosage, determined using the following calculation:

$$\frac{\text{Power (W) x treatment time (seconds)}}{\text{Spot size } (cm^2)} = J/cm^2.$$

Currently, the exact mechanism underlying the effects of LT is unknown. Several theories have been postulated. A variety of photoreceptors or chromophores have been theorized to absorb photons and produce the effects seen after LT. Components of the electron transport chain (ETC) of mitochondria and a variety of enzymes are possible photon acceptors. The presence of several maxima in the action spectra of cells suggests that more than one of these mechanisms may play a role in LT (Karu et al., 1994; Karu et al., 1997).

Several researchers have suggested that components of the ETC of mitochondria are the primary photon acceptors (Passarella et al., 1984; Enwemeka, 1988). The integral role of the ETC in cellular energy makes it a tempting LT photon acceptor. Normally, the ETC absorbs electrons from the electron carriers NADH and FADH. The electrons are passed along the various ETC components and the energy released from this passage

is used to emit protons from the mitochondria, creating an electrochemical gradient across the organelle membrane. This gradient drives the production of ATP.

Chromophores within the ETC, such as the cytochrome a/a_3 complex, also known as cytochrome c oxidase, have been shown to absorb light of specific wavelengths and increase their activity following light absorption (Yu et al., 1997c). It is hypothesized that these chromophores, once excited by the absorption of a photon, increase their ability to absorb electrons, thereby increasing the general activity of the ETC (Karu, 1989). This increase in ETC activity has been measured as an increase in ATP production, a finding observed following light treatment *in vitro* (Passarella et al., 1984; Karu et al., 1995; Mochizuki-Oda et al., 2002).

Interestingly, comparison of the wavelengths absorbed by components of the ETC (absorption spectrum) and the wavelength dependence of light-induced alterations in DNA, RNA and reactive oxygen species production (action spectra) reveals a large amount of similarity in the two spectra, suggesting that these alterations are due to the absorption of light by the ETC (Karu, 1989, 1998). Additionally, pharmacological blockage of ETC activity has been shown to decrease LT's effect *in vitro* (Enwemeka, 1988; Karu, 1989), further supporting the ETC light absorption theory.

It has also been theorized that LT induces a change in ROS production, which has a number of biological effects within the cell. Karu et al. (Karu, 1998) found that light irradiation of macrophages led to an increase in chemiluminescence, a measurement of the presence of ROS within the cell. This increase was found to be due to an increase in non-mitochondrial respiration, where light absorption by NADH-dehydrogenase in the plasma membrane affects ROS production. Increases in ROS production have been

detected in several cell and tissue types after LT, including skin, sperm and lymphocytes (Chi et al., 1995; Lubart et al., 2001; Oren et al., 2001; Lubart et al., 2002), further supporting ROS production as a mechanism of LT's effects.

It is important to note that, while the mechanism of LT is not fully understood and experiments to elucidate this are still inconclusive, it has been shown that the effects of LT are not thermal. Several researchers, including ourselves, have determined that there is a negligible 0.5 – 1°C amount of heat production following LT (Anders et al., 1993; Castro et al., 2003). Other researchers have found that application of the same amount of heat produced during LT did not result in the effects observed after LT. The lack of heat-induced effects suggests that, while LT may cause some minimal heating, this temperature change does not induce the biological effects observed. (Maegawa et al., 2000; Mochizuki-Oda et al., 2002).

While the mechanism of LT remains under investigation, wavelength dependent action spectra and dose response curves have been determined in several experimental models. Karu studied the action spectra for DNA and RNA synthesis in HeLa cells and showed that LT produced peaks in synthesis at 400, 630 – 680, and 780 – 810nm (Karu, 1998). Wavelength dependent effects have also been identified. Mochizuki-Oda et al. (Mochizuki-Oda et al., 2002) demonstrated an increase in ATP production after 830 nm treatment, but 652 nm light irradiation using the same parameters and identical increases in local tissue temperature had no effect. A co-culture system, in which macrophage stimulation was found to induce proliferation of fibroblasts, was used to test the effects of different wavelengths of light on macrophage stimulation (Young et al., 1989). This study demonstrated that 660 nm light induced greater stimulation of fibroblast

proliferation than 820 and 870 nm light, and 880 nm of the same dosage inhibited proliferation.

The outcome of LT has been shown to be dose dependent, with dosages of 0.001 – 10 J/cm^2 stimulating cellular activity and dosages greater than 10 J/cm^2 inhibiting activity (Tuner and Hode, 2002). Dosages in the 10,000 J/cm^2 range have been shown to have lethal effects on *E. coli* (Karu, 1998).

In vitro, dosages of LT below 10 J/cm^2 increase DNA, RNA and protein synthesis, mitochondrial membrane potential and fibroblast proliferation (Kana et al., 1981; Yew et al., 1982; Greco et al., 1989; Karu, 1989; Vacca et al., 1993; Conlan et al., 1996; Greco et al., 2001). Yu et al. (Yu et al., 1997b) found that a 5 J/cm^2 dosage of LT applied to mitogen stimulated lymphocytes *in vitro* resulted in increased proliferation and increased ATP production. *In vivo*, LT with dosages below 10 J/cm^2 enhances wound healing, both in terms of wound closure rates (Kana et al., 1981; Enwemeka, 1988; Schindl et al., 1999) and histological assessment (Yu et al., 1997a). Low dose LT (10 J/cm^2) was also found to preserve the normal electrophysiology of crushed nerves (Rochkind et al., 1987a; Rochkind et al., 1987b).

Cellular activities such as proliferation (Bolton et al., 1995), enzyme activity (Bolton et al., 1995), DNA synthesis (Tsai and Kao, 1991), RNA synthesis (Funk et al., 1993), and protein synthesis (Funk et al., 1992) are decreased after high doses (22 – 37.8 J/cm^2) of LT are applied. LT has also been shown to have an inhibitory effect on the immune system, suppressing pro-inflammatory cytokine production (Funk et al., 1992; Funk et al., 1993; Shimizu et al., 1995), decreasing macrophage activity (Young et al., 1989; Funk et al., 1993) and decreasing ROS production by neutrophils (Karu et al.,

1997). The potential of high dosages of light (> 10 J/cm^2) as a therapeutic tool for immune suppression has only recently become a target of investigation.

High dosage LT of peripheral nerves has been shown to improve axonal regeneration. Anders et al. (Anders et al., 1993) determined that 632.8 nm light with a dosage of 162.4 J/cm^2 significantly increased facial nerve reinnervation of target tissue after crush injury. The same LT treatment parameters also resulted in significant decreases in neuronal death and increases in calcitonin gene related peptide mRNA production after facial nerve transection (Snyder et al., 2002).

Within the CNS, LT research is not extensive. *In vitro* experiments on CNS tissue have shown that low dose (3.6 J/cm^2) LT stimulates neurite outgrowth (Wollman et al., 1996). This neuronal outgrowth can be directed by light as well, possibly due to electromagnetic gradients influencing the arrangement of actin monomers (Ehrlicher et al., 2002).

In vivo, low doses of LT (under 10 J/cm^2) restored normal electrophysiology (Erlich et al., 1988) and delayed axonal degeneration (Assia et al., 1989) after crush injury of the optic nerve. Rochkind et al. (Rochkind et al., 1988; Rochkind and Ouaknine, 1992) found that homologous sciatic nerve transplants in combination with high dosages of LT (70 J/cm^2) were able to repair histological and functional deficits following spinal cord transection or crush (T12 - L2) in dogs. Analysis of the light treated spinal cord histology revealed a decreased scar surrounding the peripheral nerve transplant and an increase in the amount of axonal sprouting into the graft in comparison to the non-irradiated spinal cords. Spinal cord injured dogs also demonstrated an increased ability to support their weight and walk several steps following laser treatment,

as opposed to those who remained paralyzed following transplantation alone. Transplantation of embryonic spinal cord tissues into transected adult rat spinal cord in combination with LT (approximately 1500 J/cm^2, 780 nm) resulted in improved gait performance and re-establishment of limb function, as well as electrophysiological and histological indications of axonal regeneration (Rochkind et al., 1997, 1998).

Hypothesis and Aims

Despite vigorous research in the field, no cure has been found for the motor or sensory deficits seen after SCI and no study to date has assessed the ability of light to regenerate specific tracts within the spinal cord or determined the recovery of specific locomotor functions. The aim of this research was to determine the effectiveness of LT to promote CST axonal regeneration and recovery of specific CST functions after SCI. The hypothesis was that transcutaneous application of 810 nm light promotes axonal regeneration and functional reinnervation of spinal cord neurons following transection of the CST by changing the extracellular milieu of the spinal cord in adult rats. The experimental model that was used to test this hypothesis was an *in vivo* model of spinal cord injury in the adult rat, which involved the complete transection of the CST in the dorsal funiculus. The methods used to test this hypothesis included anterograde and retrograde tract tracing techniques, functional analyses, reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. The methods were performed in order to: a) determine the origin of regenerating axons; b) quantitate the axonal regrowth caudal to a lesion of the CST; c) determine restoration of CST functions after injury and; d) identify changes in gene and protein expression within the lesion site. The long term

goal of this research is to determine if transcutaneous application of LT is effective as a treatment for acute and chronic spinal cord injury and other types of CNS injury in humans.

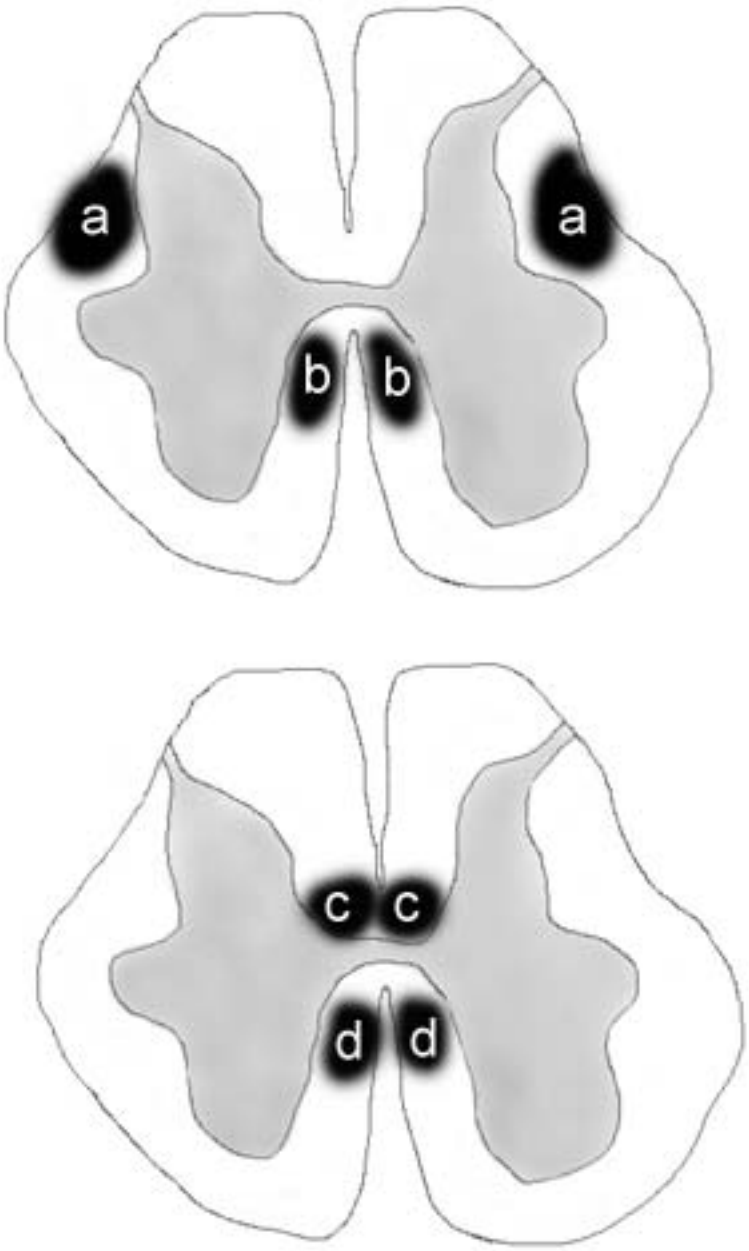

Figure 1: Human and Rat Corticospinal Tract Locations. Diagrams of the CST location in humans (a, b) and rats (c, d). a) The lateral, primarly crossed CST in humans. b) The anterior, uncrossed CST in humans. c) The dorsal, primarily crossed CST in rats. d) The ventral, uncrossed CST in rats.

CHAPTER 2

LIGHT PROMOTES REGENERATION AND FUNCTIONAL RECOVERY AFTER SPINAL CORD INJURY

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Keywords: axonal regeneration, corticospinal tract, dorsal hemisection, fluorogold, footprint analysis, ladder test, laser, LPLI, mini-ruby, photobiomodulation, rat, Sprague Dawley

Spinal cord injury (SCI) in the adult mammalian central nervous system (CNS) leads to permanent paralysis due to the failure of damaged CNS nerves to regenerate. Light therapy (LT), or photobiomodulation, is an effective treatment in healing cutaneous wounds and promoting regeneration of peripheral nerves (Rochkind et al., 1989; Anders et al., 1993; Whelan et al., 2001; Snyder et al., 2002). The biological changes and tissue regeneration seen in these injury paradigms are attributed to a light absorption mechanism (Karu, 1998). LT research is not extensive within the area of CNS injury and no study to date has assessed the ability of light to regenerate specific tracts within the spinal cord or determined the

recovery of specific locomotor functions. To test the efficacy of light in supporting axonal regeneration and functional recovery after SCI, we applied 810 nm light transcutaneously at the site of acute injury to the corticospinal tract (CST) of adult rats. The effectiveness of LT was determined using anterograde and retrograde tract tracing techniques and functional analyses. Here we show that the application of LT at the site of injury significantly improves axonal regeneration and restores specific CST functions. Our results demonstrate that light can be delivered to the CNS transcutaneously and suggest that light will be a useful treatment for human spinal cord injuries.

A series of experiments involving *in vivo* spectrophotometric analysis were performed to assess whether transcutaneous application of 810 nm laser diode emission with an output power of 150 mW was able to penetrate to the depth of the spinal cord (Figure 1a). Peak penetration through all tissue layers overlying the spinal cord was found between the 770 nm and 810 nm wavelengths (Figure 1b). Six percent of the initial power output penetrates to the spinal cord. These data show that 810 nm light, with 9 mW of energy, reaches the spinal cord.

To determine if application of 810 nm light to the spinal cord increased axonal growth, an anterograde tracer, mini-ruby (Molecular Probes, Eugene, OR), was injected 5 weeks after CST lesion. Analysis revealed that all mini-ruby labeled axons were found in the white matter, in the region of the spinal cord normally occupied by the CST (Figure 2a, b). There were few (30.7 +/- 17 axons per animal) mini-ruby labeled axons caudal to the lesion in the control group (Figure 2c). These labeled axons extended an average distance of 2.9 +/- 0.8 mm caudal to the lesion (Figure 2d), with a maximal distance of 7 mm reached by 17% of counted axons (Figure 2e), which is comparable to spontaneous

post-lesional sprouting previously reported (Li et al., 1994). The average number of mini-ruby labeled axons in the light treated group was significantly greater than that of the control group ($p<0.0001$, one way ANOVA with Tukey post-test; Figure 2a), with an average of 199 +/- 13 labeled axons caudal to the lesion. The mini-ruby labeled axons in the light treated group extended an average of 8.7 +/- 0.8 mm caudal to the lesion, a significantly increased length over the control group ($p<0.01$, one way ANOVA with Tukey post-test; Figure 2b). The maximum distance traversed over 5 weeks in the light treated group was 14 mm caudal to the lesion, a distance that was reached by 8% of the counted axons (Figure 2e).

A similar amount of regrowth was found following methylprednisolone treatment of a vertebral level C3 lesion at 6 weeks post-injury (Nash et al., 2002), suggesting that light treatment and methylprednisolone may have similar beneficial effects after SCI. This theory is supported by previous findings (Byrnes et al., 2003b) that LT significantly reduces macrophage but not neutrophil invasion, similar to methylprednisolone treatment (Xu et al., 1992; Mabon et al., 2000).

Anterograde analysis does not definitively determine whether transected axons have regrown past the lesion, as spared axons may also be counted caudal to the lesion. Therefore, to determine if LT promotes regeneration of transected axons, a double label, retrograde tracing analysis was performed. Based on the mini-ruby data, axons in the light treated group grew at a rate of 0.25 – 0.4 mm per day. Using these data, we calculated that axons would require at least 10 weeks to reach the mid-lumbar region and innervate motor neurons responsible for lower limb function. At the time of CST lesion, transected neurons were labeled by a retrograde tracer, hydroxystilbamidine

methanesulfonate (HM), inserted into the lesion. Ten weeks after CST lesion, axons terminating at vertebral level L3, approximately 24 mm caudal to the initial lesion, were labeled by injection of fast blue into the ventral horn. Numbers of single (HM or fast blue) and double (neurons with axons that were transected and regrew to L3) labeled neurons in the motor cortex were assessed using unbiased stereology.

Analysis of single labeled neuron number revealed no significant difference ($p>0.05$) between control and light treated groups, suggesting no difference in labeling efficacy between groups (Figure 3a, b, c). The average number of HM labeled neurons is 8,860 +/- 3408 in the control group and 13,270 +/- 3236 in the light treated group, which is comparable to the number of CST axons reported in the lower thoracic region of the spinal cord (Hicks and D'Amato, 1977; Brosamle and Schwab, 1997). The average number of fast blue labeled neurons is 129 +/- 109 in the control group and 131 +/- 120 in the light treated group, which is comparable to the number of neurons found in the motor cortex after injection of a retrograde tracer into the ventral, uncrossed portion of the CST at vertebral level L4 (Brosamle and Schwab, 1997). Fast blue has been shown to spread approximately 2 mm from its injection site (Kalderon and Fuks, 1996a), therefore neurons in laminae III – VI, where ventral CST axons terminate, may have taken up the tracer. Since fibers of the dorsal and ventral CST originate from the same area of the motor cortex (Brosamle and Schwab, 1997), it is likely that these fast blue labeled neurons are from the ventral CST that was not lesioned in the dorsal hemisection surgical procedure. The ventral CST axons, however, do not play a significant role in motor function in the rat (Whishaw and Metz, 2002).

Double labeled neurons, with both HM and fast blue labeling, were found only in the light treated group (Figure 3d, e, f) and the percentage of these neurons represented a statistically significant increase in comparison to the control group (Figure 3d, $p<0.05$, Mann-Whitney U Test). This increase in double labeling indicates that only CST axons in the light treated group regrew and terminated in the gray matter of vertebral level L3 after transection. The percentage of double labeled neurons found in this study is comparable to that seen in a previous double labeling study by Kalderon et al. (Kalderon and Fuks, 1996a).

This study revealed that double labeled neurons accounted for approximately 30% of the number of mini-ruby labeled axons observed at 5 weeks post-lesion in the light treated group. As double labeling represents neurons with axons that terminated in the L3 area, it is understandable that the total number of regenerating axons found at the T12 level at 5 weeks post-injury would be greater.

Complete lesion of the dorsal portion of the adult rat CST at vertebral level T9 results in loss of several CST controlled functions (Kunkel-Bagden et al., 1993; Whishaw and Metz, 2002). To determine if the axonal regeneration and reinnervation resulted in functional improvement, performance of rats in two functional tests, the ladder/grid walking test and footprint analysis, was assessed prior to and after CST lesion. Five measurements were taken, including footfalls (failure of hindpaws to grasp ladder rungs and falling below the plane of the ladder), time to cross the ladder, base of support, stride length, and angle of rotation. Data are presented as mean percentage of pre-surgical measurement, to control for variations among animals.

One week after CST lesion, rats had significant impairments in angle of rotation ($p<0.05$, Figure 4a, repeated measures ANOVA with Newman-Keuls post-test) and footfalls ($p<0.05$, Figure 4b) in comparison to pre-surgical measurements in both control and light treated groups. An increase in ladder cross time was also observed in both groups at this time point (Figure 4c).

At 9 weeks post-injury, rats underwent these functional tests again. At this time point, animals in the light treated group had no significant difference ($p>0.05$; Figure 4a, c) in angle of rotation (Figure 4d) and ladder beam cross time in comparison to the pre-surgical measurements, demonstrating a recovery of these functions. Comparison of ladder beam cross time and angle of rotation measurements in light treated and control groups also revealed a significant improvement in the light treated group ($p<0.05$, one way ANOVA with Tukey post-test; Figure 4a, c). Measurements for rats that received CST lesions but were not light treated remained at the 1 week post-surgery levels at this time point, significantly greater than pre-surgical measurements ($p<0.05$).

Angle of rotation and ladder cross time are both associated with CST function and are significantly lengthened by CST lesion (Kunkel-Bagden et al., 1993; Metz and Whishaw, 2002). Ladder crossing time is positively correlated with hindlimb errors in step placement (Metz and Whishaw, 2002). We found a significant increase in footfalls in both control and light treated animals post-surgery ($p<0.05$, Figure 4b), but there was no significant difference between these two groups. However, analysis of errors in ladder crossing, including correct placement of hindpaws on ladder rungs and grasping of ladder rungs, was not assessed in this study and may have been modified by light treatment, leading to the observed improvement in crossing time.

No significant change was found in stride length or in base of support in either group at any time point after CST lesion ($p>0.05$, data not shown). Previous studies have shown that CST lesion in adult rats does not necessarily impair these functions, as this lesion does not affect the rubrospinal or propriospinal tracts, which play a greater role in these functions than the CST (Kunkel-Bagden et al., 1992; Hamers et al., 2001).

These data suggest that LT promotes significant improvement in specific CST controlled functions after lesioning. This study demonstrated particular improvement in functions mediated by innervation from the L1-L3 vertebral level. Similar results have been found with other treatment modalities, such as transplantation of fetal tissue (Kunkel-Bagden and Bregman, 1990; Kunkel-Bagden et al., 1992).

The mechanism of how LT causes change in the spinal cord is unknown, however, several theories have been postulated (Lubart et al., 1992; Karu, 1998; Lubart and Breitbart, 2000). It is important to note that the effects of LT are not due to heat (Maegawa et al., 2000; Mochizuki-Oda et al., 2002; Castro et al., 2003). Several significant changes have been found after LT of the injured spinal cord. Rochkind et al. (Rochkind et al., 1998) found that LT at similar treatment parameters in combination with embryonic cortical tissue transplants after SCI resulted in improved weight bearing abilities of hindlimbs in rats and decreased degradation of transplanted tissue. Research in our laboratory has demonstrated that LT significantly decreased inflammatory cell invasion into the spinal cord after injury, shifted the activation peak time of astrocytes, and suppressed pro-inflammatory cytokine and chemokine mRNA production (Byrnes et al., 2003a; Byrnes et al., 2003b), suggesting that LT alters the spinal cord environment after injury. Recently, 800 nm light was also found to direct axonal guidance *in vitro*

(Ehrlicher et al., 2002), suggesting that light may have also played a role in directly promoting axon growth through the lesion site.

In conclusion, we found that transcutaneous application of 810 nm light significantly improved CST axonal regeneration, reinnervation at vertebral level L3 and specific locomotor functions. These results demonstrate that LT is a novel and non-invasive treatment for acute spinal cord injury.

Methods

Spectrophotometric measurement. An incoherent broadband white light was directed at the surface of the skin in the lower thoracic region of adult Sprague Dawley rat. Rats were anesthetized with sodium pentobarbital (50 mg/Kg, I.P.) prior to all measurements. A smart, tissue-activated optical fiber probe (Ilev et al., 2002) was inserted sequentially into the skin, sub-cutaneous connective tissue layer, deep connective tissue layer, muscle and the spinal cord within the vertebral column. At each of these layers, a transmission spectrum in the range of 500 – 1200 nm was collected while white light was applied to the skin surface.

Corticospinal tract lesion. Thirty adult female Sprague Dawley rats (150 – 200 g) were used in this study. For all surgical techniques, rats were anesthetized with sodium pentobarbital (50mg/Kg, I.P.). Dorsal hemisection was performed by an investigator blinded to group assignment. The ninth thoracic vertebra was identified and a laminectomy was performed to expose the spinal cord. A suture was passed beneath the dorsal funiculus. Iridectomy scissors were used to carefully incise this isolated portion of the spinal cord, thereby transecting the CST. Inspection of the lesion and visualization of

the central gray commissure verified that the CST had been completely transected. After the hemisection was completed, the exposed spinal cord was covered with gelfoam (Pharmacia, Upjohn; Kalamazoo, MI), and the overlying muscles and skin were sutured. During the recovery period, urinary bladders were manually expressed until spontaneous voiding returned approximately 1 – 2 days post-injury.

Retrograde labeling. At the time of CST lesion, gelfoam soaked in hydroxystilbamidine methanesulfonate (HM; 3% in 0.9% saline; Molecular Probes, Eugene, OR) was inserted into the lesion site of 20 rats. Ten weeks after the surgery, a laminectomy was performed at vertebral level L3, approximately 24 mm caudal to the original lesion site, and 1 μl of a 2% fast blue solution (in PBS, Sigma, St. Louis, MO) was bilaterally injected (0.5 μl into each side) into the spinal cord at a depth of 1.3 mm.

Anterograde labeling. Five weeks after CST lesion, 10% tetramethylrhodamine biotinylated dextran (mini-ruby, Molecular Probes) was injected into the motor cortex of one group of 10 rats using stereotaxic coordinates (from bregma, -0.11 AP and ±1.60 ML; -1.33 AP and ±1.50 ML; -2.85 AP and ±1.40 ML; depth = 1.0 – 1.2 mm). 2 μl of mini-ruby was injected into each of the 6 sites, for a total injection volume of 12 μl.

Tissue analysis for labeling detection. Eight days after the injections of mini-ruby or fast blue, rats were anesthetized and transcardially perfused with 4% paraformaldehyde. Brains and spinal cords were carefully dissected, post-fixed for 24 hours and cryoprotected in 30% sucrose for 24 hours prior to sectioning of the tissue at a thickness of 20 μm. Sections utilized for counting mini-ruby labeled axons from the lesion site to 16 mm caudal to the lesion were collected and mounted at a ratio of 1/6. For neuronal counting, cortical sections were collected and mounted at a ratio of 1/8. The fractionator

method of unbiased stereology was used to count HM and/or fast blue labeled neurons in the motor cortex (2.6 mm from midline to lateral edge of brain per hemisphere). The percentage of neurons that regenerated an axon was calculated according to:

$$\frac{\text{Double labeled neurons}}{\text{Fast Blue + HM + Double Labeled neurons}} \text{ X } 100$$

Cortical and spinal cord injection sites were studied prior to counting to ensure labeling efficacy; only those with adequate injections, without leakage of the tracer significant distances away from the injection site, and with adequate uptake into the intended neurons, were included in the final analysis.

Light treatment. Beginning immediately after surgery, half of the rats (randomly assigned; n=15/group) were transcutaneously irradiated at the lesion site for a total of 14 consecutive days with an 810 nm diode laser (Thor International, UK; 150 mW output through a delivery fiber optic, 2,997 seconds treatment time/day). Dosage was 1589 J/cm^2 per day (irradiance = 0.53 W/cm^2, 450 J).

Functional testing. One week prior, and 1 and 9 weeks after dorsal hemisection surgery, the same rats undergoing retrograde labeling were trained and then tested on two functional tests. One test required rats to walk across a ladder beam (Columbus Instruments, Columbus, OH) that recorded the length of time required to cross the beam as well as the number of footfalls. This test was videotaped for confirmation. Rats also underwent footprint analysis: hindpaws were dipped in ink and the rats walked across sheets of white paper. Base of support, stride length and angle of rotation were analyzed as described previously (Kunkel-Bagden et al., 1993; Hamada et al., 1996a).

Statistical analysis. Functional test data are presented as mean percentage of baseline scores recorded one week prior to surgery +/- SEM. Neuronal counts are presented as mean percentage of total neuronal number counted +/- SEM. Axonal counts are presented as mean +/- SEM. Functional data were analyzed using Repeated Measures ANOVA with Newman-Keuls post-test to assess changes over time or one-way ANOVA with Tukey post-test to assess differences between groups at individual time points. Axonal count data were analyzed using One Way ANOVA, with Tukey post-test. Neuronal count data were analyzed using Mann-Whitney U analysis.

We thank Tara Romanczyk for technical assistance and Dr. Albert McManus for helpful advice on presentation of quantitative data. We also thank Drs. Rosemary Borke, Howard Bryant, Sonia Doi and Leslie McKinney for thorough reading and editorial comments regarding this work. This work was funded by a Defense Veterans Head Injury Program Grant and a pilot grant from the Defense Advanced Research Projects Agency. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

Competing Interests statement.

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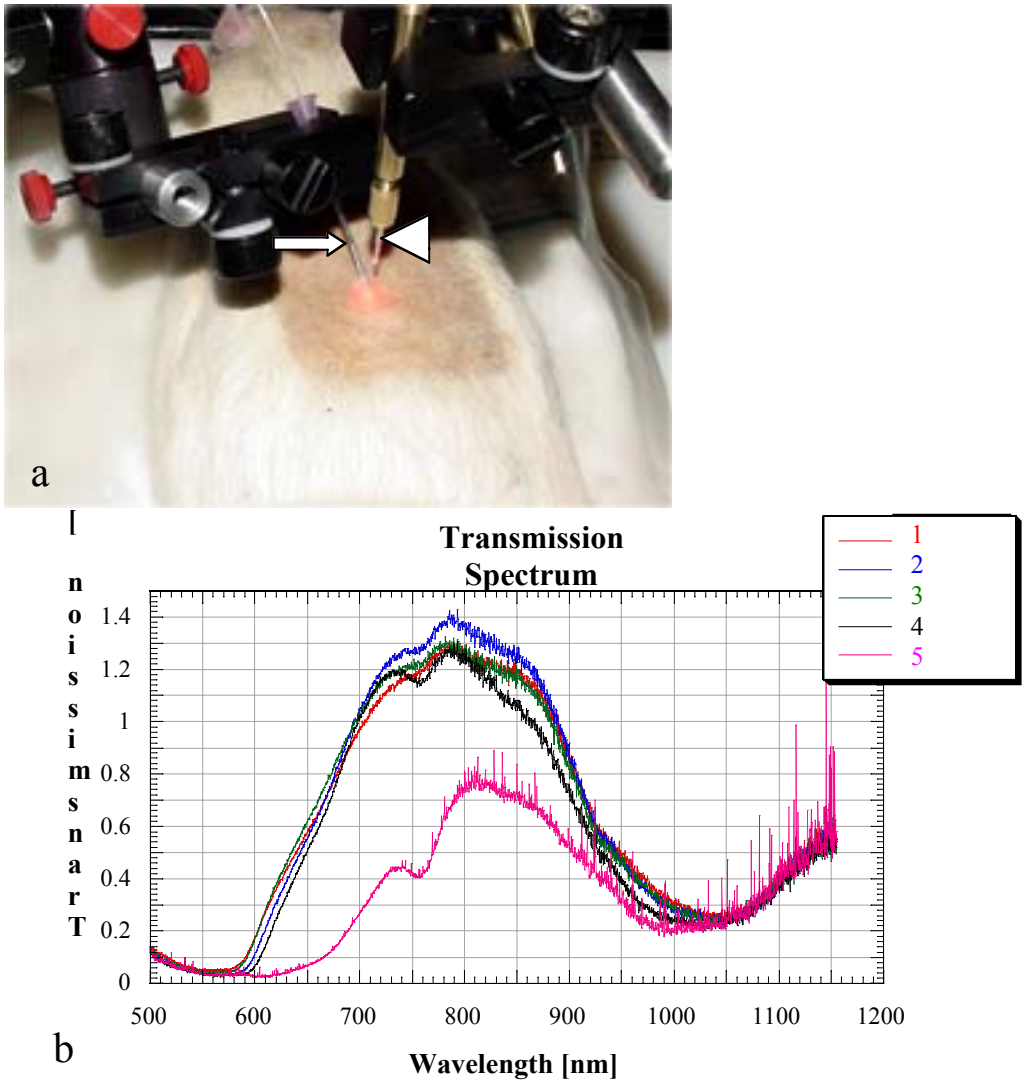


Figure 1. a) Photograph of spectrophotometric analysis experimental set-up. The smart fiber (arrow) is inserted below the skin of the rat, the light source (arrowhead) is positioned above the skin for transcutaneous application of light. b) Graphical representation of transmission (in arbitrary units) through each layer of tissue, depending on wavelength (nm). Layer 1 = skin, 2 = loose connective tissue, 3 = dense connective tissue, 4 = muscle, 5 = vertebral column and spinal cord. The graph demonstrates that wavelengths in the 770 – 810 nm range had the greatest transmission, or penetration, through all levels.

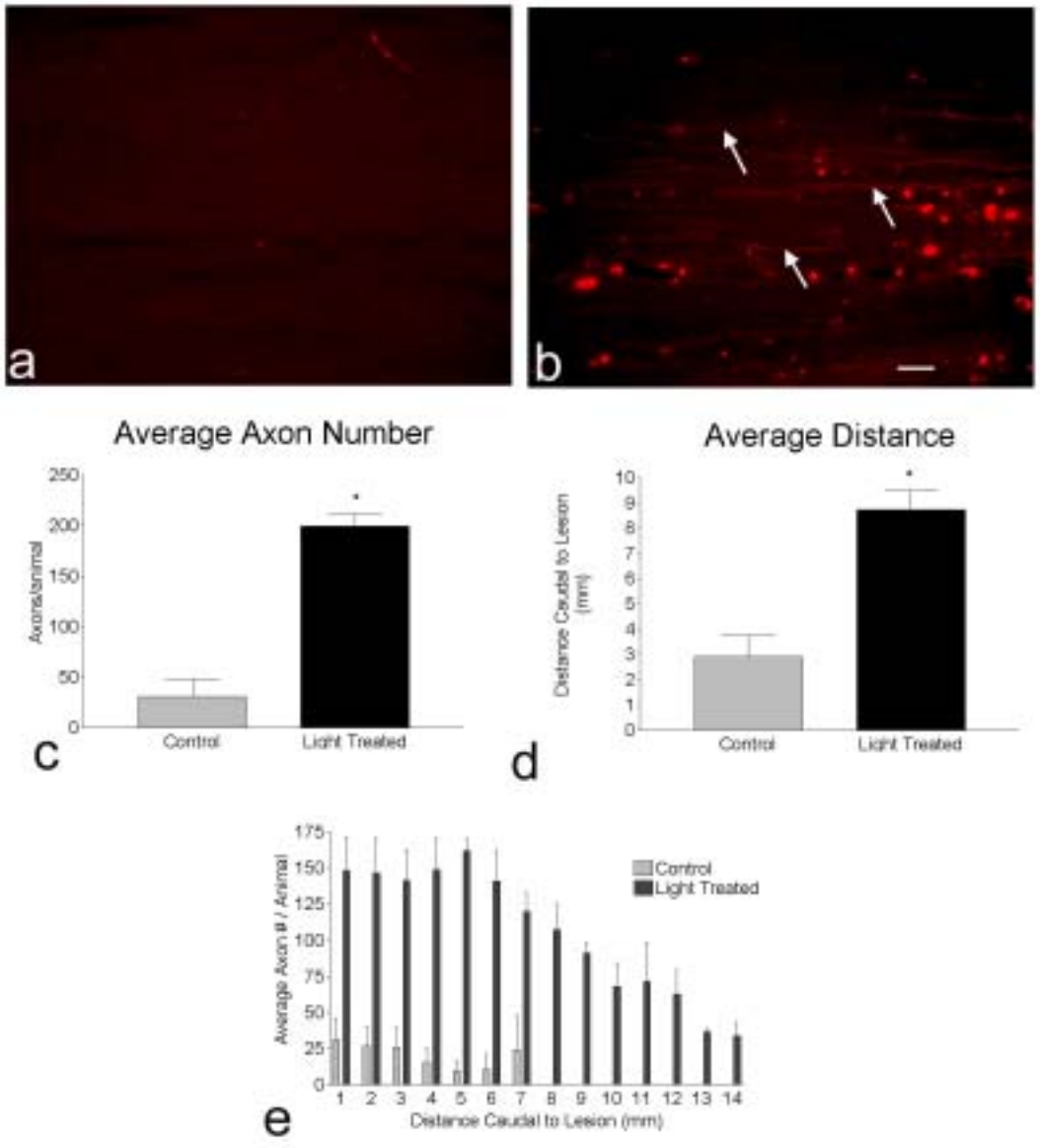
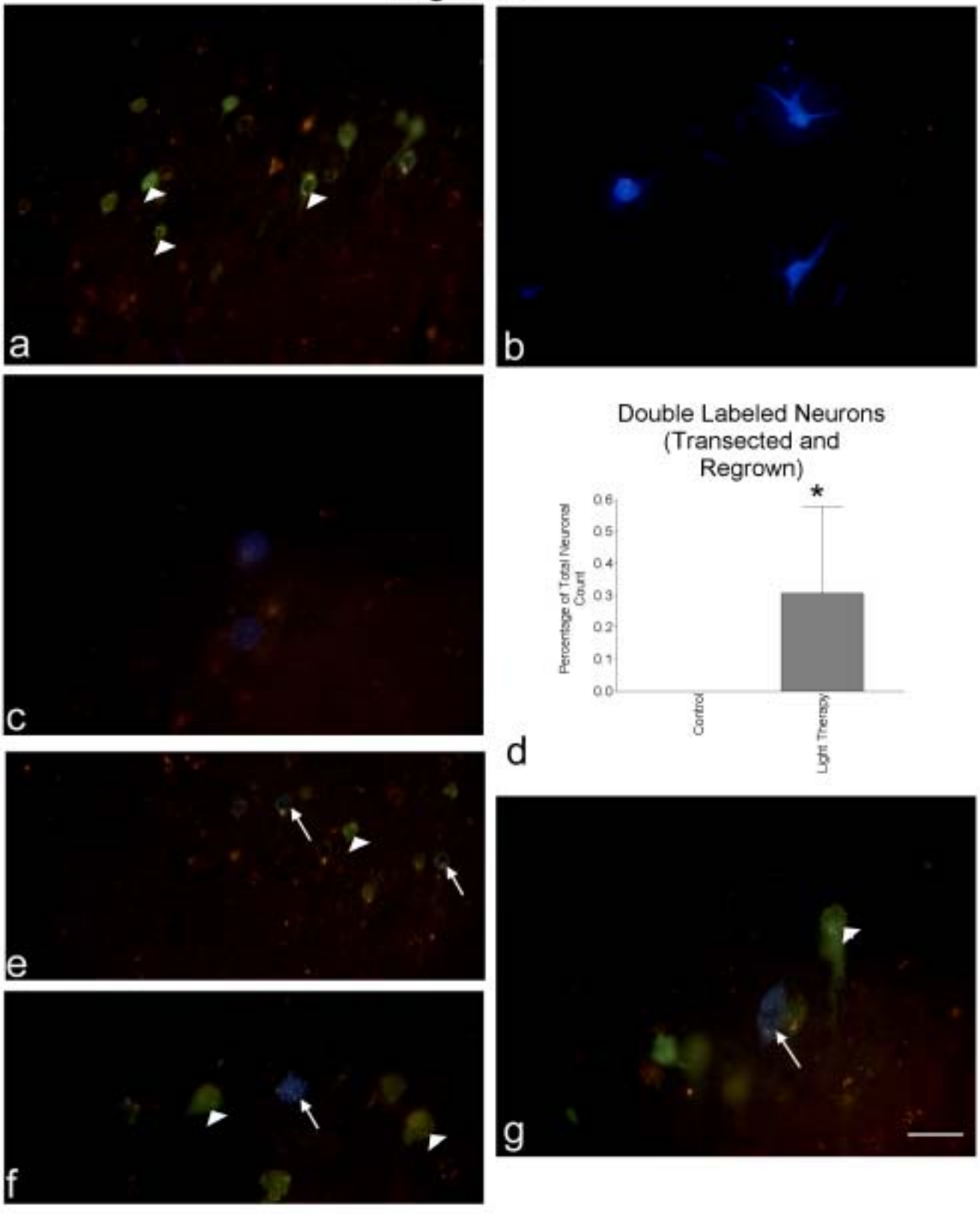


Figure 2. Photomicrographs of mini-ruby labeled axons and related quantitation. a) Photomicrograph of white matter 4 mm caudal to the lesion site in control rat. b) Photomicrograph of white matter 4 mm caudal to lesion in light treated rat. Note that mini-ruby labeled axons, indicated with arrows, are found at this distance only in the light treated group. Bar = 43um. Comparisons of average axon number (c) and average distance caudal to the lesion (d) are shown. *p<0.01; **p<0.001 using one way ANOVA. N = 5/group. e) Number of axons counted in control and light treated group per mm caudal to the lesion. Bars represent mean +/- SEM.

Figure 3. Photomicrographs of single and double labelled neurons at 10 weeks post-injury. a) Numbers of HM labelled neurons (arrowheads) in the motor cortex, b) fast blue labelled neurons in the injection site at L3, and c) fast blue labelled neurons in the motor cortex were similar in both groups. d) Graphical representation of comparison of double labelled neurons between light treated and control groups. *$p<0.05$ using Mann Whitney U. Bars represent mean percentage of counted neurons +/- SEM. e – g) Double labelled neurons were found only in motor cortex of light treated rats. Arrows indicate double labelled neurons, identifiable by green punctate label in blue cytoplasm, which is consistent with labelling pattern previously described (Pyner and Coote, 2000). Bar = 67μm (a – e); 34μm (f – g).

Figure 3


a
b
c
Double Labeled Neurons
(Transected and
Regrown)
*
Percentage of Total Neuronal
Count
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Light Therapy
d
e
f
g

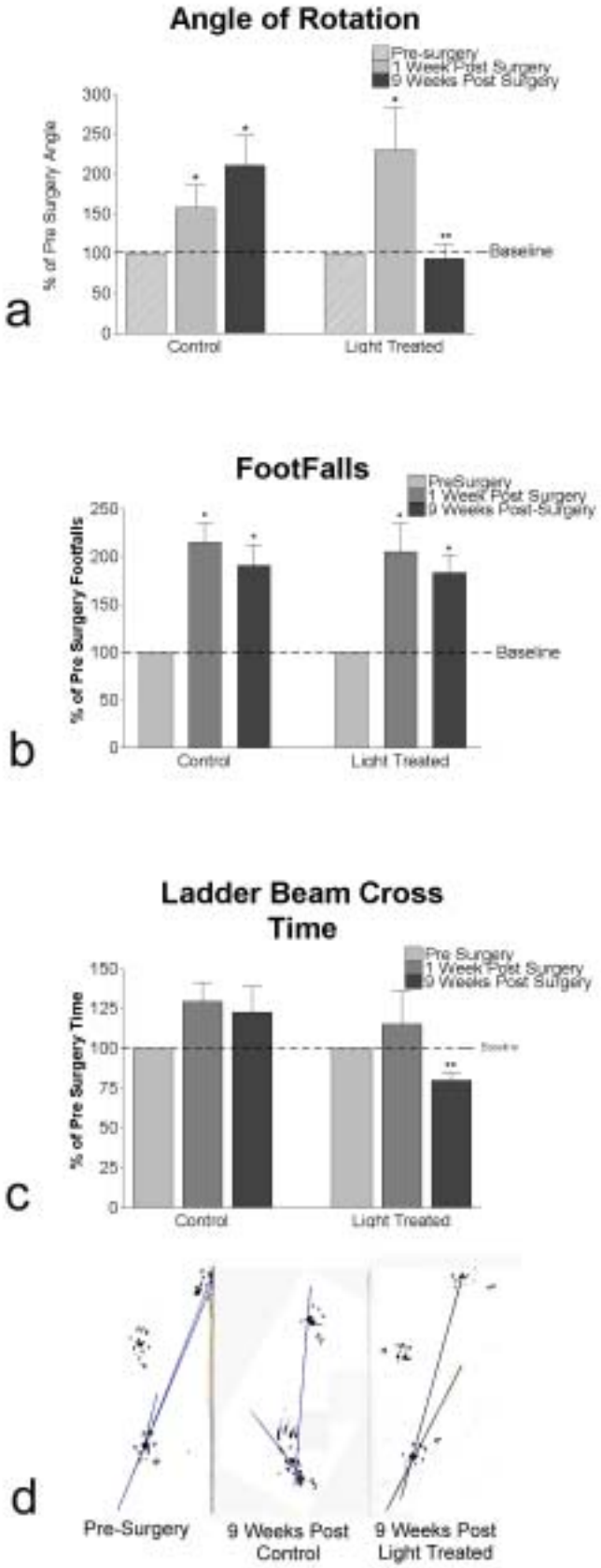


Figure 4. Angle of rotation (a), footfalls (b) and ladder beam crossing time (c) measurements are presented for pre-surgical, 1 week and 9 weeks post-surgical time points. significant improvement was found only in the light treated group. Graph bars are mean percentage of pre-surgical measurements +/- SEM. *$p<0.05$ using repeated measures ANOVA with Newman Keuls post-test between time points. **$p<0.05$ using one way ANOVA with Tukey post-test between control and light treated groups at 9 week time point. N = 10/group. d) Footprints from pre-surgical and 9 weeks post-surgical time points. Notice the increased angle of rotation at 9 weeks in the control group. In the light treated group the angle has returned to pre-surgical values.

CHAPTER 3

LIGHT ALTERS CELLULAR INVASION AND ACTIVATION FOLLOWING SPINAL CORD INJURY

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Abbreviated Title: LIGHT ALTERS CELL INVASION/ACTIVATION AFTER INJURY

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Acknowledgements: This work was funded by a Summer Student Research Grant from the American Society for Lasers in Surgery and Medicine and a Defense Veterans Head Injury Program Grant. The authors would like to thank Ms. Tara Romanczyk and Ms. Amy van Horn for their technical assistance. We would also like to thank Drs. Rosemary Borke, Howard Bryant, Sonia Doi, and Leslie McKinney for their thorough reading and editorial comments. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

Keywords: 810nm, astrocytes, B lymphocytes, immunohistochemisry, Low Power Laser Irradiation, macrophages, microglia, photo-biomodulation, rats, Schwann cells, Sprague-Dawley, T lymphocytes

Abstract

Cell invasion and activation are currently prime suspects as causes of secondary damage after spinal cord injury (SCI). A variety of anti-inflammatory drugs have been studied as a means to alleviate the immune response following SCI, with varying degrees of success. Non-invasive light therapy (LT) improves axon growth and functional recovery in SCI (Rochkind et al., 1988; Rochkind and Ouaknine, 1992; Rochkind et al., 1997; Byrnes et al., 2000). The impact of LT on cell invasion/activation after SCI was investigated in the current study. The dorsal halves of spinal cords were lesioned in adult rats and transcutaneously irradiated for a maximum of 14 days, beginning immediately after surgery, using a 150 mW 810 nm laser (1589 J/cm^2). The response of various cell types to SCI and LT was quantified at 2, 4, 14 and 16 days and 5 weeks post-injury. Immunohistochemistry for macrophages/activated microglia, neutrophils, T and B lymphocytes, astrocytes and Schwann cells revealed that LT suppressed invasion/activation of macrophages and microglia as well as T lymphocytes. The activation of astrocytes was delayed two days by LT, with a peak in activation occurring at 4 days post-injury. A similar trend was found in the migration and activation of B lymphocytes and neutrophils, in which decreases were found in the light treated group, although these were not statistically significant. LT had no effect on Schwann cell migration into the spinal cord. These results indicate that light alters the spinal cord environment and the immune response following SCI. LT, therefore, is a promising, non-invasive therapy for SCI.

After spinal cord injury (SCI), demyelination, axonal degeneration, neuronal death, cavitation and glial scarring surrounding the area of the initial injury occur, comprising a 'secondary injury' that often causes greater impairment of recovery than the initial injury would have caused alone (Dusart and Schwab, 1994; Koshinaga and Whittemore, 1995; Fitch et al., 1999; Popovich et al., 2002). Invasion/activation of immune cells has been under investigation as a potential mediator of secondary injury (Popovich et al., 2002).

Neutrophils invade the spinal cord by 6 hours post-injury, with a maximal response 2 days post-injury (dpi; Perry and Gordon, 1987; Dusart and Schwab, 1994; Bartholdi and Schwab, 1997; Carlson et al., 1998; Isaksson et al., 1999). Activated neutrophils produce free radicals (Fujita et al., 1994; Anderson, 1995; Wakabayashi et al., 1995) and obstruct capillaries (Hallenbeck et al., 1986), contributing to the secondary damage. Macrophages and activated microglia appear in the spinal cord at approximately 12 to 24 hours post-injury and peak at 4 – 8 dpi (Perry and Gordon, 1987; Dusart and Schwab, 1994; Frisen et al., 1994; Popovich et al., 1997; Carlson et al., 1998; Dai et al., 2000). These cells can produce several harmful substances, such as hydroxyl radicals and nitric oxide (Delves and Roitt, 2000), and studies have demonstrated that macrophages/activated microglia contribute to axonal damage and demyelination (Fitch et al., 1999; Popovich et al., 2002). At approximately 12 – 24 hours post-injury, astrocytes are activated in the spinal cord. They migrate to and surround the injured portion of the spinal cord, walling off the injury site (Lagord et al., 2002) and producing a number of growth inhibitory proteins (McKeon et al., 1991; Fitch and Silver, 1997).

Recent evidence suggests that alteration of cell invasion/activation after SCI improves functional recovery. Research demonstrated that depletion of macrophages improved locomotion, spared white matter, preserved myelinated axons, supported axonal sprouting and reduced cavitation (Popovich et al., 1999). Anti-inflammatory drugs also increase tissue sparing (Hirschberg et al., 1994) and promote functional recovery (Chikawa et al., 2001; Nash et al., 2002).

Light therapy (LT), or photo-biomodulation, has been under investigation as a treatment for a number of tissue injury models, including cutaneous wounds (Rochkind et al., 1989; Yu et al., 1997a; Whelan et al., 2001) and peripheral nerve damage (Anders et al., 1993; Snyder et al., 2002). While the mechanism of LT is unknown, it has been established that the effects of low power LT are due to the absorption of light rather than through the production of heat (Anders et al., 1993; Mochizuki-Oda et al., 2002; Castro et al., 2003). This therapy has been tested *in vitro* and *in vivo*, and it has become clear that dosages of 0.001 – 10 J/cm^2 stimulate cellular activity and dosages greater than 10 J/cm^2 inhibit activity (see Tuner and Hode, 2002 for review).

The potential of high dosages of light as a therapeutic tool for immune suppression has only recently become a target of investigation, and has been shown to suppress pro-inflammatory cytokine RNA and protein production (Funk et al., 1992; Funk et al., 1993; Shimizu et al., 1995), macrophage activity (Young et al., 1989; Funk et al., 1993) and reactive oxygen species production by neutrophils (Karu et al., 1997). Following SCI, high dosage LT has been found to have a number of therapeutic effects. Research showed that sciatic nerve or embryonic cortical tissue transplants in combination with LT improved axonal sprouting, decreased scar formation, and

improved weight bearing and step taking by hind limbs in dogs and rats (Rochkind et al., 1988; Rochkind and Ouaknine, 1992; Rochkind et al., 1997, 1998).

The current study aims to determine the effect of LT on an *in vivo* model of acute SCI. Our previous work demonstrated that 810 nm LT with a dosage of 1589 J/cm^2 resulted in increased axonal regrowth following SCI (Byrnes et al., 2000, K. R. Byrnes, R. W. Waynant, I. K. Ilev, J. J. Anders, unpublished observations). Using these parameters, cellular invasion/activation following SCI and LT was determined.

Materials and Methods

Subjects. Fifty adult female Sprague-Dawley rats (200 – 300g, Taconic Farms, Germantown, NY) were used in this study under an approved Uniformed Services University IACUC protocol. Food and water were provided ad libitum and the rats were exposed to 12-hour cycles of light and dark periods.

Dorsal hemisection of spinal cord. Rats were randomly assigned to light treated or control groups (25 rats/group) and an investigator, blinded to group assignment, performed the dorsal hemisection surgery. Each animal was anesthetized with sodium pentobarbital (50 mg/Kg, I.P.) and placed on an isothermal heating pad warmed to 37^{o}C. The ninth thoracic vertebra was identified and a laminectomy was performed, exposing the spinal cord between vertebral levels T8 and T10. A suture was passed beneath the dorsal funiculus of the spinal cord. This thread was lifted and iridectomy scissors were used to carefully incise the dorsal funiculus, thereby completely transecting the corticospinal tract (CST). The suture thread was removed and confirmation that the CST had been completely lesioned was verified by inspection of the lesion and visualization of the central gray commissure.

After the dorsal hemisection was completed, the exposed spinal cord was covered with gelfoam (Pharmacia, Upjohn; Kalamazoo, MI), and the overlying muscles and skin were sutured. During the recovery period, urinary bladders of the rats were manually expressed until spontaneous voiding returned approximately 1-2 dpi.

Light treatment. Treatment began immediately after SCI and continued daily for a total of 14 days. Prior to treatment, all animals were lightly anesthetized with sodium

pentobarbital (20 mg/Kg, I.P.) and placed on isothermal heating pads. All treatments were done in the dark.

Light was applied to the rats in the treatment group (n = 25) with a 200 mW 810nm diode laser (Thor DDII, Thor International LTD., Basildon, Essex, UK). The laser was modified with a delivery fiber optic to produce a homogenous 6 mm diameter spot with a power of 150 mW. During treatment, this spot was centered on the skin directly above the location of the spinal cord hemisection. Treatment was applied for 2,997 seconds per day, for a final energy density, or dosage, of 1589 J/cm^2 per day (irradiance = 0.53W/cm^2, 450J). Approximately 6% of the power of the 810nm laser reached the spinal cord level, as measured by an *ex vivo* analysis of light penetration through the layers of tissue between the skin and the spinal cord (K. R. Byrnes, R. W. Waynant, I. K. Ilev, J. J. Anders, unpublished observations). These parameters have been found successful in promoting axonal regrowth in our previous studies (Byrnes et al., 2000, K. R. Byrnes, R. W. Waynant, I. K. Ilev, J. J. Anders, unpublished observations).

The control group (n= 25) received no laser irradiation, but was handled identically to the light treatment group.

Analysis of cell invasion and activation. Spinal cord tissue from rats was collected at 48 hours, 4, 14, and 16 days and 5 weeks post-injury. At each time point, 5 rats per treatment group were deeply anesthetized with 10% chloral hydrate (1ml/100g, I.P.) and euthanized via intracardiac perfusion with 4% paraformaldehyde. The thoracic spinal cord at the lesion site and 3 mm rostral and 5 mm caudal to the lesion site was dissected, post-fixed for 24 hours in 4% paraformaldehyde, and cryoprotected for 24 hours in 30% sucrose. The 10 mm spinal cord segments were sectioned longitudinally on

a freezing microtome at 20 μm, from the dorsal aspect of the spinal cord through the level of the gray commissure. Sections were serially mounted onto 10 slides, with 3 sections per slide. One slide from each rat was processed for histological analysis using an H&E stain and one slide/rat was processed for each cell type under investigation. Immunolabeling was repeated for each animal to ensure labeling efficacy. Negative controls, in which primary antibody was not added during immunohistochemistry, were run for each cell type (Fig. 2i).

The tissue was rehydrated and blocked with an appropriate blocking solution. Tissue was incubated overnight with primary antibodies (Table 1) followed by incubation with an appropriate fluorescently labeled secondary antibody (Jackson Immunochemicals, West Grove, PA) at room temperature for 30 minutes.

The lesion site and the surrounding tissue of at least 6 sections per animal per antibody were digitally photographed using a Leica/Spot system (Version 2.2 for Windows, Diagnostic Instruments, Inc. Sterling Heights, MI). The proportional area of tissue occupied by immunohistochemically stained cellular profiles within a defined target area (the lesion site and surrounding tissue) was measured using the Scion Image Analysis system (www.rsb.info.nih.gov/nih-image/) as described previously (Popovich et al., 1997). All tissue sections were coded prior to measurement to prevent bias and all image backgrounds were normalized prior to quantitation.

Statistical analysis. Area of spinal cord occupied by cell type is expressed as mean +/- SEM. Kruskal-Wallis statistical analysis with Dunn's post-test was used to compare means (due to large mean number of pixels and large standard errors leading to the necessity of using a non-parametric test). Tests were performed using the GraphPad

Prism Program, Version 3.02 for Windows (GraphPad Software, Inc. San Diego, CA) and SPSS 11.0 for Windows (SPSS, Inc., Chicago, Illinois).

Results

Assessment of temporal invasion/activation of the various cell types of interest was investigated within the lesion site and the surrounding tissue. Gross observation of longitudinal H&E stained sections of the thoracic spinal cord revealed a cavity at the location of the initial injury (Fig. 1). Between 48 hours and 4 dpi the size of this cavity increased (Fig. 1a, b) and expanded longitudinally from 4 to 16 dpi (Fig. 1a – d). This expansion extended a greater distance (1 – 2 mm) in the rostral direction than the caudal direction (0 – 0.5 mm). The cavitation spread was found primarily in the white matter of the spinal cord. The cavitation, both in the initial injury site and rostral/caudal to it, appeared to be decreased at the 5 weeks post-injury time point (data not shown). No observable difference in cavity size was seen between the control and light treated groups, however there appeared to be a decrease in longitudinal spread of the rostral cavitation in the 14 dpi light treated tissue (Fig. 1d).

Due to the clustering behavior of cells within and surrounding the lesion following SCI and the inability to discern individual cell nuclei, assessment of numbers of individual cells was not possible. Therefore, measurement of tissue area occupied by immuno-positive label within a defined target space was used to assess cell invasion/activation. As an increase in immunolabeling does not necessarily reflect an increase in cell number, this measurement is a method of quantifying the magnitude of a cellular response, both in terms of cell invasion and activation. The current work does not attempt to distinguish between these two cellular response parameters.

Neutrophils, macrophages/activated microglia and astrocytes were the primary cells found in the lesioned spinal cord. T lymphocytes, B lymphocytes and Schwann cells

were also identified. However, based on our measurement of the number of immuno-positive pixels in the area surrounding the lesion, there was approximately 80% less ($p<0.0001$) immunolabeling of T and B lymphocytes and Schwann cells than macrophages/activated microglia and astrocytes.

Neutrophils

Immunohistochemical labeling with the antibody against the RP3 clone revealed small, round, cellular profiles that were detected at all time points investigated in both control and light treated groups (Fig. 2a, b). These cells chiefly bordered the lesion site, but some cellular profiles were perivascular or adjacent to the meninges. The largest amount of positive labeling occurred at 4 dpi. This labeling was significantly increased at this time point for both light treated and control groups when compared to all other time points ($p<0.05$; Fig. 2c). However, when the amount of positive immunolabeling for neutrophils in the light treated and control tissues was analyzed, there was no significant difference at any time point (Fig. 2c). Immunolabeling for neutrophils decreased after 4 days. At 14 dpi, the remaining neutrophils in the control tissue were found not only along the edges of the lesion but also 1 mm caudal to the lesion edge. Neutrophil migration was not found in any of the light treated tissue.

Macrophages/Activated Microglia

Macrophages and activated microglia are not distinguishable from each other in the mammalian CNS since activated microglia express the same cell surface molecules and cytokines and have the same round morphology as blood borne macrophages

(Popovich et al., 1997; Carlson et al., 1998). Immunolabeling for ED1, an antibody against a macrophage/microglia lysosomal glycoprotein revealed many of these large, amoeboid cells in the injured spinal cord (Fig. 2d – h). At 48 hours post-injury, immuno-positive macrophages/activated microglia were located in and around blood vessels, in the dorsal roots and along the edges of the lesion site, with no infiltration into the surrounding tissue. At this time point, there were observably fewer labeled macrophages/activated microglia in the light treated group than in the control group. By 4 dpi, a large cavity had formed and macrophages/activated microglia were localized to the lesion edges. Similar to the 48 hour situation, there were significantly fewer immunolabeled cells at 4 dpi in the light treated tissue compared to the control tissue. By 14 dpi, the immuno-positive cells were found along the edges of the lesion and within the lesion cavity (Fig. 2d), and had also invaded the tissue rostral and caudal to the lesion site, reaching up to 4 mm rostral to the lesion and 2 – 3 mm caudal. This cellular migration was predominantly in the white matter of the dorsal funiculus around small cavities in the rostral/caudal tissue. The light treatment group had less migration of macrophage/activated microglia at this time point (Fig. 2e), with migration reaching approximately 1 mm in the rostral direction and absent in the caudal direction. At 16 dpi, there appeared to be fewer ED1 labeled cells in the control tissue, so that control and light treated tissue looked similar (Fig. 2g, h).

In both control and light treated groups, ED1 expression showed an initial peak at 48 hours post-injury and a subsequent peak at 14 dpi, with a decline at 4 dpi. Both peaks were reduced in the light treated group, with significant reductions in ED1 expression at 4 and 14 dpi in the light treated group ($p<0.001$, Fig. 2f). Moreover, ED1 expression was

further reduced at 5 weeks post-injury in the light treated group compared to controls ($p<0.001$, Fig. 2f). While there wasn't a significant decrease ($p=0.156$) in ED1 expression in the light treated group at 48 hours post-injury, a trend toward suppression of ED1 expression in the light treated group in comparison to the control group was found.

Astrocytes

Astrocytes were detected using an antibody against GFAP, an intermediate filament primarily expressed in astrocytes. Immunolabeling with this antibody revealed long thin processes that were heavily labeled near the lesion site in both the light treated and control groups. GFAP positive processes were also found throughout the entire length (10 mm) of the sections studied, extending 3 mm rostral and 5 mm caudal to the lesion. At 48 hours post-injury, heavy GFAP positive labeling was found to outline the lesion in all rats of the control group and GFAP positive processes were found throughout the 10 mm section in 3 of the 5 rats of the control group (Fig. 3a). Light treated tissue, however, had only a light band of GFAP positive label near the lesion edge and along the meninges/blood vessels in all 5 rats (Fig. 3b). By 4 dpi, however, GFAP labeling in the light treated tissue had increased to the degree observed in the 48 hour control tissue in 3 of the 5 rats. In both groups, immunolabeling for GFAP decreased over the remaining time periods (Fig. 3c – f), eventually becoming restricted to the lesion site by 5 weeks post-injury. Interestingly, at 16 dpi, 2 days after light treatment ended, there was a slight increase in rostral/caudal extension of GFAP labeling in 3 of the 5 rats in the light treated group (Fig. 3f).

Quantitative analysis revealed that there was a significant decrease in GFAP expression in the light treated group at 48 hours post-injury compared to the control group ($p<0.05$), and a significant increase at 4 dpi compared to the control group (Fig. 3g, $p<0.01$). Expression peaked in the control group at 48 hours post-injury, and declined significantly ($p<0.05$) thereafter.

T Lymphocytes

T lymphocytes were detected in spinal cord tissue using UCHL1, an antibody against the surface glycoprotein CD45. Cells that were immuno-positive for UCHL1, were small, round cells and were found in very low numbers. T lymphocytes were restricted to the lesion edge and in the acellular matrix within the lesion cavity (Fig. 4a – b).

Statistical analysis of UCHL1 expression revealed that there was a peak in both the control and light treated groups at 48 hours post-injury, with a decline in expression through 16 dpi (Fig. 4c). UCHL1 expression in the light treated group was lower than the control group at 48 hours, 14 and 16 dpi, with a significant decrease found at 14 dpi ($p<0.001$).

B Lymphocytes

B lymphocytes, identified using the L26 antibody against CD20, a membrane spanning protein in B lymphocytes, were also found in very low numbers from 4 to 16 dpi. At the time points studied, L26 expression was found to be in small, round cells near the edges of the spinal cord lesion (Fig. 4d) or within the cavity, with 1 – 2 mm migration

caudal to the lesion in the white matter tract at 16 dpi in the control group only. There was no migration observed in the light treated group. Quantitative analysis of L26 expression found no significant differences between the light treated and control groups, although a non-significant trend towards a suppression of B lymphocyte activation was observed in the light treated group at 16 dpi (Fig. 4e).

Schwann Cells

Also present in very low numbers were Schwann cells, identified by antibody labeling of S100, a neural specific Ca^{2+} binding protein. These small, circular cells were found at all time points investigated, primarily along the edges of the lesion (Fig. 4f – g), without any migration rostral or caudal to the lesion. There was no significant difference in expression between LT treated and control tissue at any time point (Fig. 4h).

Discussion

This study demonstrates that LT significantly alters the normal invasion and activation of a number of cell types following SCI. Included in our study were neutrophils, normally the first to respond to an insult (peaking at 2 dpi), followed by macrophages and microglia, astrocytes, T and B lymphocytes, and Schwann cells which all peak and/or appear around 1 week post-injury (Perry and Gordon, 1987; Dusart and Schwab, 1994; Popovich et al., 1997; Carlson et al., 1998; Schnell et al., 1999; Lagord et al., 2002).

Light therapy does not deter neutrophil invasion/activation

Neutrophils secrete several cytokines and proteinases and produce reactive oxygen species that are responsible for much of the early tissue degradation following SCI (Fujita et al., 1994; Anderson, 1995; Wakabayashi et al., 1995). Neutrophils infiltrate the wound area between 3 and 6 hours following SCI, first adhering to blood vessel walls before invading the tissue and peaking at approximately 24 hours post-injury (Perry and Gordon, 1987; Dusart and Schwab, 1994; Bartholdi and Schwab, 1997; Carlson et al., 1998; Streit et al., 1998; Schnell et al., 1999; Ghirnikar et al., 2000). Our research revealed a later peak in neutrophil invasion (4 dpi) in both the light treated and control groups (Fig. 2c). LT had no significant effect on neutrophil invasion at 2, 4, 14 and 16 days and 5 weeks following SCI, as revealed by the quantitative immunohistochemistry (Fig. 2c), although we did observe a suppression in migration of neutrophils to the tissue surrounding the lesion site at 14 dpi. These data correlate well with a previous study of corticosteroid treatment of SCI, in which macrophage activity

was blocked, but neutrophil invasion (Mabon et al., 2000) and activity (Xu et al., 1992) were not.

Invasion/activation of macrophages/activated microglia is suppressed by LT

Neutrophils in the spinal cord are replaced over the first few days following SCI by activated microglia and macrophages (Dusart and Schwab, 1994; Schnell et al., 1999). These cell types are found to appear in injured tissue between 12 and 24 hours post-injury, reach large concentrations at 4 to 8 dpi, and then decrease in activity and number over the next several weeks (Perry and Gordon, 1987; Dusart and Schwab, 1994; Frisen et al., 1994; Koshinaga and Whittemore, 1995; Popovich et al., 1997; Carlson et al., 1998; Schnell et al., 1999; Dai et al., 2000). Macrophages/activated microglia, responsible for clearing debris from the damaged area (Delves and Roitt, 2000), contribute to axonal damage and demyelination, leaving behind large cavities surrounded by a glial scar (Dusart and Schwab, 1994; Koshinaga and Whittemore, 1995; Fitch et al., 1999; Popovich et al., 2002). Popovich et al. (Popovich et al., 2002) and Fitch et al. (Fitch et al., 1999) demonstrated that activated macrophages alone were sufficient to cause significant cavitation and glial activation *in vitro* and *in vivo*. Macrophage invasion has also been closely correlated with the production of neurite growth inhibitory molecules, such as keratan and chondroitin sulfate proteoglycans (Fitch and Silver, 1997; Fitch et al., 1999; Jones and Tuszynski, 2002).

The control data found in this study correlate well with the published data, with a peak in macrophage invasion occurring between 2 and 14 dpi (Fig. 2f). 810 nm light suppressed the macrophage and microglia response to acute SCI, with a significant suppression found at 4 days, 14 days and 5 weeks post-injury (Fig. 2f). This result

expands upon Rochkind et al.'s (Rochkind et al., 1988; Rochkind and Ouaknine, 1992; Rochkind et al., 1997, 1998) findings of decreased degeneration of peripheral and embryonic grafts and decreased scar formation, proposed to be due to suppression of the immune response after SCI.

Although quantification revealed a trend towards a decrease in ED1 expression, a marker for macrophage and activated microglia, at 2 and 16 dpi in light treated compared to controls, these data did not reach statistical significance. As it has been shown that macrophages/activated microglia normally reduce their presence after SCI by 2 weeks post-injury, the lack of significance observed at 16 dpi may be due to this reduction in the control group (Fig. 2g). Unexpectedly, there was also a decrease in ED1 immunolabeling at 4 dpi in the light treated and control groups. We believe that the decrease in immunolabeling at this time point was artifactual and due to the loss of the tissue that filled the lesion cavity during tissue sectioning and immunohistochemical processing.

Reduction of the macrophage response in the spinal cord leads to functional and regenerative improvement (Fitch et al., 1999; Popovich et al., 2002). Our current study demonstrates suppression of macrophage/activated microglia infiltration at various time points after SCI. Using the same LT parameters, we have demonstrated a significant improvement in axonal regeneration and functional recovery (Byrnes et al., 2000), K. R. Byrnes, R. W. Waynant, I. K. Ilev, J. J. Anders, unpublished observations), supporting the claim that macrophage reduction is beneficial to spinal cord recovery. The results of our current study are comparable to previous findings following anti-inflammatory treatment in the injured spinal cord, wherein decreasing macrophage invasion/activity after SCI preserves normal cord morphology, decreases demyelination, increases axonal

sprouting and improves motor function (Giulian and Robertson, 1990; Popovich et al., 1999; Ghirnikar et al., 2000; Chikawa et al., 2001; Nash et al., 2002).

The inhibition of macrophage/activated microglia activity/invasion and lack of a significant alteration of neutrophil response seen in this study after LT resemble the effect of methylprednisolone (Xu et al., 1992; Mabon et al., 2000). Methylprednisolone is currently the only clinically available therapy for acute SCI (Bracken et al., 1984; Bracken et al., 1997). However, methylprednisolone is administered systemically, resulting in global immune-suppression. LT may offer an alternative therapy that is effective without the overall immune-suppression.

While there is a body of evidence suggesting that depletion of macrophages leads to improved axonal growth and motor function (Giulian and Robertson, 1990; Popovich et al., 1999; Chikawa et al., 2001), other investigators have shown that increasing macrophage infiltration may promote regeneration and functional recovery following SCI (Prewitt et al., 1997; Rabchevsky and Streit, 1997; Rapalino et al., 1998; Schwartz et al., 1999). These results are in conflict with those of the current study, but as the role of macrophages in SCI is complex, it is likely that multiple approaches to modifying the presence of these cells after SCI will be effective in ameliorating damage after SCI.

The effects of LT on macrophage activity are complex as well. Using high dosages of LT, Shimizu et al. (Shimizu et al., 1995) and Funk et al. (Funk et al., 1992; Funk et al., 1993) found that LT significantly reduced production and secretion of pro-inflammatory cytokines, including prostaglandin E_2, IL1β, TNFα, and interferon γ, after periodontal ligament cell, T lymphocyte or macrophage stress. 780 nm LT inhibited vascular permeability following carrageenin induced inflammation (Honmura et al.,

1992). As increases in pro-inflammatory cytokines and vascular permeability are required for macrophage invasion/activation, these studies provide a potential mechanism by which LT can act as an anti-inflammatory agent after SCI. However, conflicting studies have demonstrated increased pro-inflammatory cytokine expression, as well as increased macrophage activity after LT treatment (Meyers et al., 1987; Funk et al., 1993). A lack of consistency between treatment parameters, including wavelength, power, and treatment time may account for these conflicting results and make it difficult to critically compare these studies.

LT delays the peak in astrocyte activation after injury

LT administered immediately after SCI and continuing for 14 dpi resulted in delayed astrocyte activation, with a peak in activation in the light treated group occurring two days after the peak in the control group. GFAP immunoreactivity, a marker of astrocyte activation, appears around the lesion site at 1 dpi and outlines the lesion margins over the first week after SCI (Schnell et al., 1999; Lagord et al., 2002). Over this time period, astrocyte activation is apparent several millimeters from the injury site, but is restricted to the lesion site at time periods longer than 2 weeks (Popovich et al., 1997), which correlates well with our data in both the light treated and control groups.

Astrocytes play a conflicting role in the spinal cord after injury. *In vitro*, astrocytes respond to macrophage stimulation by migrating and forming a cell free cavity around the macrophage injection site (Fitch et al., 1999). Studies have determined that pro-inflammatory cytokines produced by macrophages, such as IL6, IL1β and TNFα, activate astrocytes (Klusman and Schwab, 1997) and induce them to produce growth inhibitory molecules, including tenascin and chondroitin sulfate proteoglycans (McKeon

et al., 1991). The LT induced delay in astrocyte activation observed in the present study is likely due to the concurrent LT induced decrease in macrophage activation/invasion. Most notably, the decreases in ED1 and GFAP immunolabeling were both first observed at 48 hours post-injury (Fig. 2f, 3g), delaying the peak in GFAP expression to 4 dpi.

Additionally, Joosten et al. (Joosten, 1997) found that vimentin-positive astrocytes served as guides for directing axonal growth during development. Collagen transplants into the injured spinal cord were found to require astrocyte scaffolding to promote axonal growth (Joosten et al., 1995). The increase in astrocytes in this study at a later time period after SCI (Fig. 3g) suggests that these cells, particularly those distal to the lesion, are still available to provide the scaffolding necessary to direct axonal growth leading to successful regeneration.

LT reduced T and B lymphocyte invasion and/or spread in the injured spinal cord

Data from the current work on T and B lymphocyte infiltration into the injured spinal cord agree with previously published reports. These cell types have a similar infiltration pattern as macrophages/activated microglia, entering the spinal cord at 1 – 2 dpi, peaking at 7 dpi, and then dropping in number and activation thereafter (Popovich et al., 1997; Schnell et al., 1999). T and B lymphocytes may also contribute to demyelination of the spinal cord after injury (Popovich et al., 1997), but have a significantly lower presence in the injured spinal cord than macrophages/activated microglia. Although there was a decrease in infiltration of B lymphocytes into the tissue surrounding the lesion, LT had no significant effect on this cell type. T lymphocyte immunolabeling density at 14 dpi was significantly suppressed ($p<0.001$, Fig. 4c), and immunolabeling was decreased at 48 hours and 16 dpi, although this did not reach

statistical significance. As macrophages produce chemokines that attract these cell types into the injured spinal cord (Sun et al., 1997), we hypothesize that the early decrease in macrophage invasion/activation after SCI and LT (Fig. 2f) resulted in the observed effect on T and B lymphocyte invasion.

LT had no effect on Schwann cells

Schwann cells have been detected in the spinal cord following injury, appearing near spinal roots at 7 dpi (Blight, 1985). We found very few immunolabeled Schwann cells from 48 hours to 14 dpi, all in close proximity to the lesion site, with no significant difference between LT and control groups. As these cells entered the spinal cord through the spinal nerve roots rather than through the blood vessels, it is likely that LT was unable to inhibit invasion due to this alternative route of Schwann cell entry.

Conclusion

While the mechanism of LT is still under debate, several theories have been proposed and are under investigation. Light induced changes in membrane permeability to calcium (Lubart et al., 1992), mitochondrial respiration alteration via absorption of light by components of the electron transport chain (Enwemeka, 1988; Passarella et al., 1988; Karu, 1998; Pastore et al., 2000; Castro et al., 2003) or changes in cellular oxidation state through light absorption by NADPH (Lubart and Breitbart, 2000) are all currently being investigated by a number of laboratories. These LT induced alterations all lead to changes in cellular activity levels, which, in turn, leads to alterations in cellular processes including protein production, proliferation and phagocytosis.

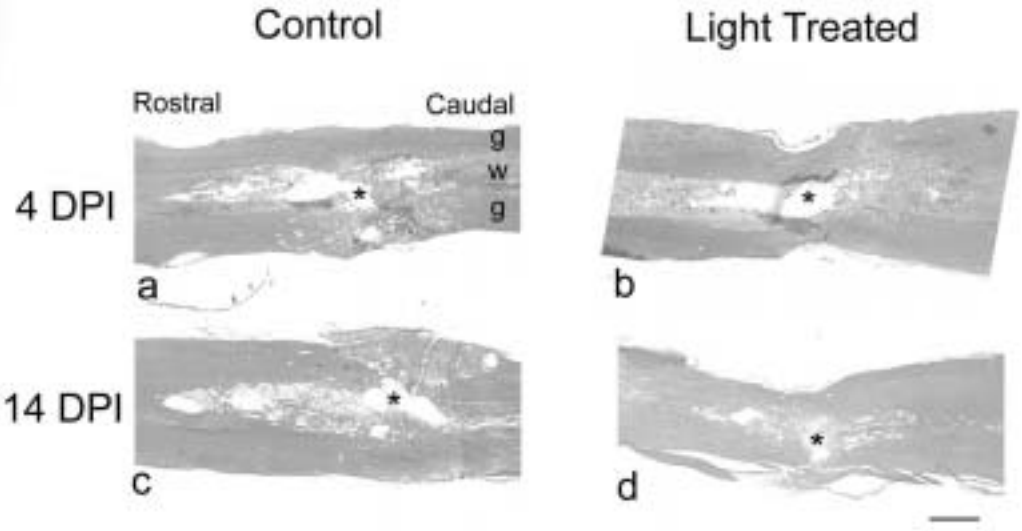
Axons have the inherent ability to regrow following injury. Altering the spinal cord environment may support this regeneration. Transcutaneous application of light

significantly affected invasion and activation of a number of cells that have a profound influence on recovery after SCI, including macrophages, microglia, astrocytes and T lymphocytes. The results of this study show that LT not only caused a significant inhibition of activation and invasion of several cell types, but also shifted the peak activation time in other cell types after SCI. This alteration in the temporal course of cellular reactions results in a change in the spinal cord environment at a time when axons are sprouting and entering the lesion zone (Fishman and Mattu, 1993). These results serve as a foundation for the novel concept of using transcutaneous application of light to promote axonal regeneration and functional recovery after SCI.

Table 1: Primary Antibodies

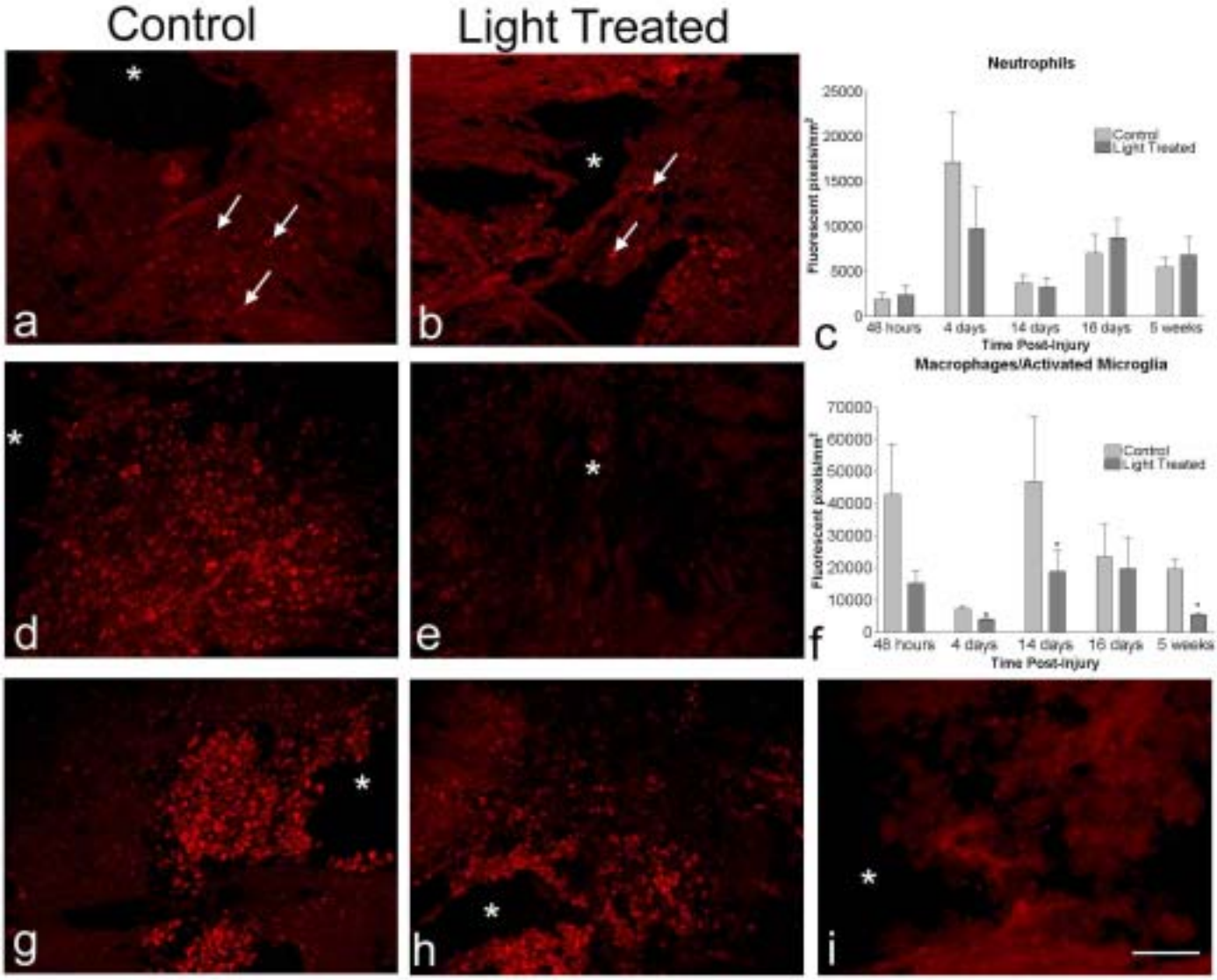
Primary Antibody	Labels	Dilution	Supplier
ED1	Macrophages Activated Microglia	1:175	Serotec, Inc. Raleigh, NC
RP3	Neutrophils	1:30	BD Pharmingen, San Diego, CA
UCHL1	T cells (resting and activated)	1:25	Dako Corp. Carpinteria, CA
L26	B cells (resting and activated)	1:75	Dako Corp. Carpinteria, CA
S100	Schwann Cells	1:100	Santa Cruz Santa Cruz, CA
GFAP	Astrocytes	1:100	Dako Corp. Carpinteria, CA

Figure 1: H&E stained spinal cord sections.



H&E representative sections for control (a, c) and light treated (b, d) groups. Increases in cavity size were observed by 4 dpi in both groups (a, b) compared to 48 hours post-injury. By 14 dpi, spread of cavitation away from the center of the lesion (*) was greater in the control group (c) than the light treated group (d), particularly in the rostral direction. Cavitation was more prominent in the white matter (w) than in the gray matter (g). All tissue is oriented in the same rostral/caudal direction. Bar = 250 mm.

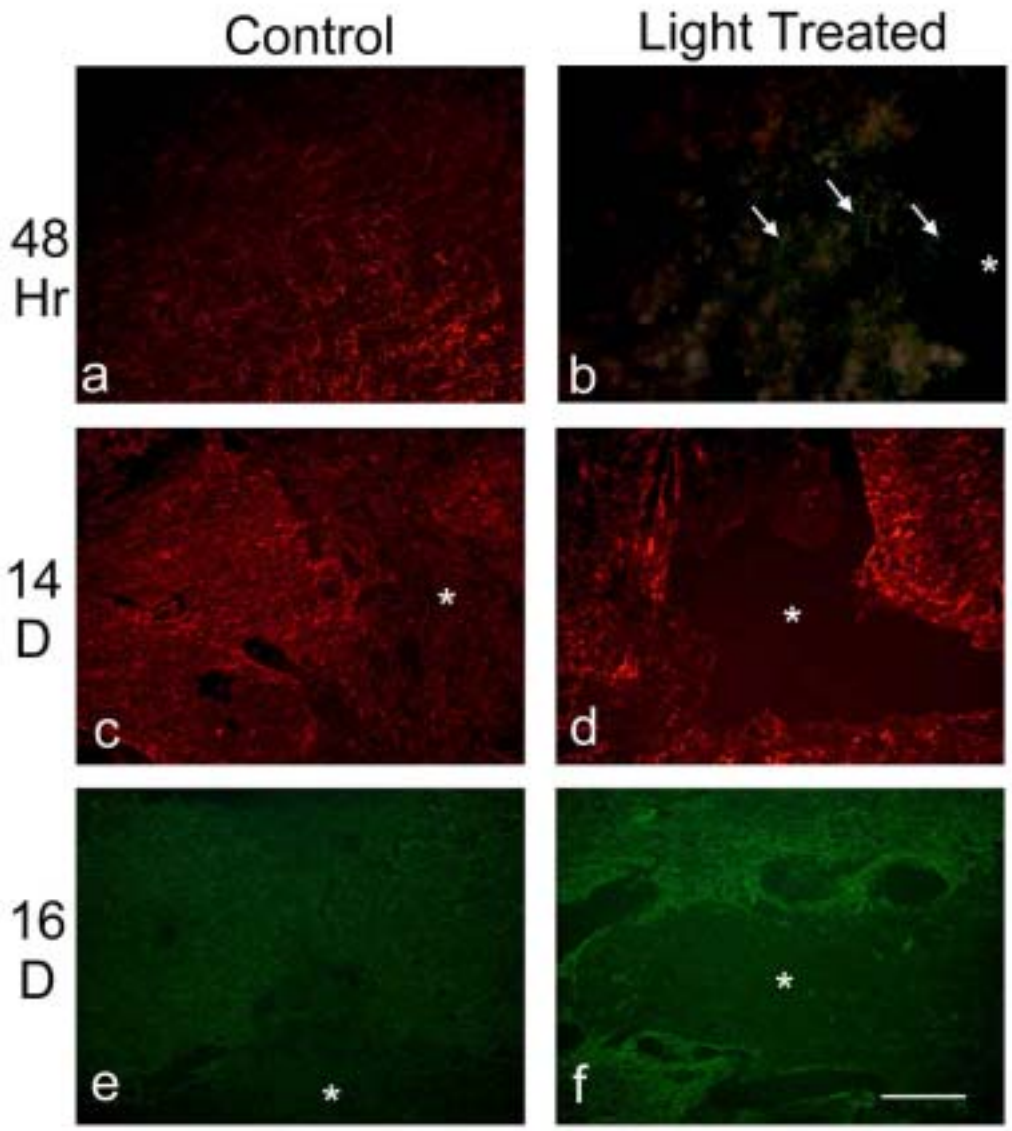
Figure 2: Neutrophils, macrophages and activated microglia.

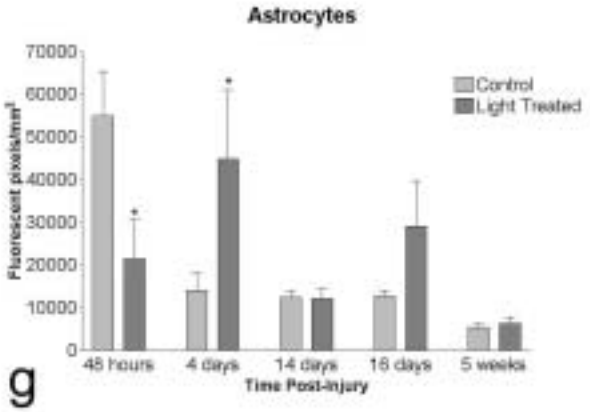


Immunohistochemistry for neutrophils (arrows; a, b) was found in 4 dpi tissue from both control (a) and light treated (b) groups. Quantitation of immunolabeling for nuetrophils is shown in (c). Immunolabeling for macrophages/activated microglia is demonstrated in (d - h). Panel (d) is a control section from 14 dpi, demonstrating cells accumulated in and around the lesion site (*). e) Light treated tissue at 14 dpi. f) Quantitation of immunolabeling for macrophage/activated microglia. g) 16 dpi control tissue. h) 16 dpi light treated tissue. i) Negative control tissue. *$p<0.001$ between the control and light treated groups; n = 5/group; ANOVA followed by Tukey test. Graph bars represent mean +/- SEM. Bar = 95 mm.

Figure 3: **Astrocytes.** GFAP immunolabeling, indicating astrocyte activation, is shown. 48 hr light treated and 16 dpi control and light treated tissue are labeled with an FITC secondary antibody (b, e, f). All others are labeled with Cy3 secondary antidoby. a) Thick astrocytic processes with heavy GFAP labeling 5 mm caudal to the lesion site in control tissue at 48 hours (48 Hr) post-injury. (b) GFAP labeling (arrows) adjacent to the lesion site (*) in light treated tissue 48 hrs post-injury. (c) GFAP labeling outlining the lesion (*) in control tissue at 14 dpi. (d) Labeling at 14 dpi in the light treated tissue. (e) 16 dpi in the control tissue. (f) Light treated tissue at 16 dpi. (g) Quantitation of GFAP labeling at all time points. *$p<0.05$ between groups ($n = 5$/group; ANOVA followed by Tukey test). Graph bars represent mean +/- SEM. Bar = 96μm.

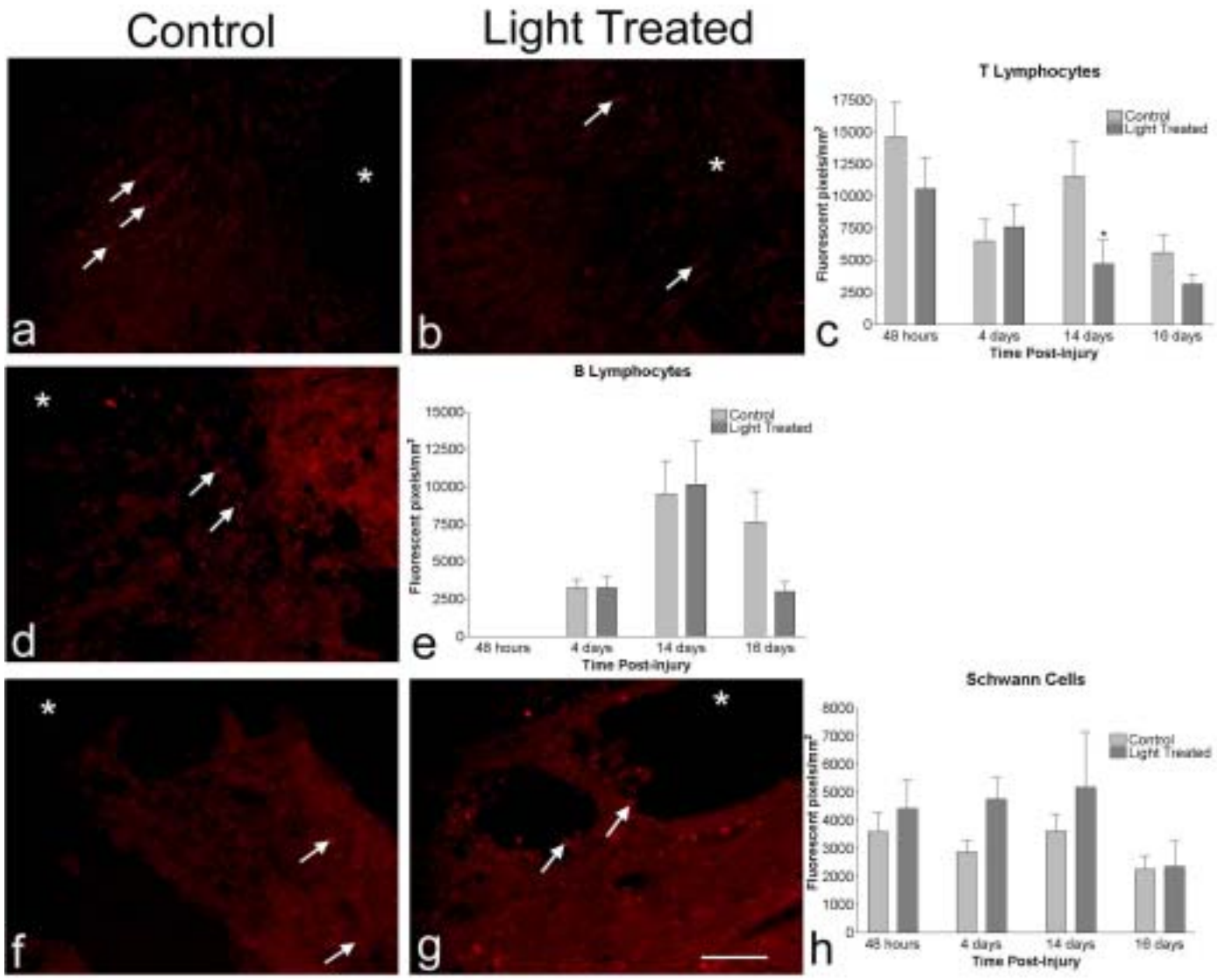
Figure 3: Astrocytes.





g

Figure 4: T and B lymphocytes and Schwann cells.



Images of 14 dpi tissue from control and light treated tissue are demonstrated in this figure. Immunolabeling for T lymphocytes (arrows, a, b) was restricted to the lesion site (*). a) Control tissue and b) light treated tissue at 14 dpi. c) Quantitation of T lymphocyte immunolabeling. *$p<0.05$ between control and light treated tissue. d) B lymphocyte immunolabeling (arrows) in control tissue. h) Quantitation of B lymphocytes immunolabeling. Schwann cell immunolabeling (arrows) near the lesion site (*) in control (f) and light treated (g) tissue. h) Quantitation of Schwann cell immunolabeling. Graph bars represent mean +/- SEM (n = 5/group; ANOVA followed by Tukey test). Bar = 96mm.

CHAPTER 4

LIGHT SUPPRESSES CYTOKINE AND CHEMOKINE GENE EXPRESSION AFTER SPINAL CORD INJURY

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Running Title: LIGHT SUPPRESSES IL6, MCP-1, INOS AFTER INJURY

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Abstract

Cytokines and chemokines are integral in the inflammatory response of tissue to injury. Following spinal cord injury (SCI), cytokines and chemokines are upregulated and play an important role in cellular invasion/activation and secondary damage. Light therapy (LT) has significant effects on the inflammatory response of cells *in vitro* and in various *in vivo* injury models. LT has also been found to improve axonal regrowth and function after injury. This study aimed to determine the impact of LT on cytokine and chemokine gene expression after SCI to further elucidate the effect of LT on the injured spinal cord. Adult rats were treated daily with LT (810 nm, 1589 J/cm^2) beginning immediately after dorsal hemisection surgery. At 6 hours and 4 days after injury, the lesioned spinal cord was removed and reverse transcriptase – polymerase chain reaction was used to detect the expression of several genes, including the pro-inflammatory cytokines interleukin (IL) 1β, tumor necrosis factor α, IL6 and granulocyte-macrophage colony-stimulating factor, the chemokines macrophage inflammatory protein 1 and monocyte chemoattractant protein (MCP-1), as well as inducible nitric oxide synthase (iNOS), intercellular adhesion molecule and transforming growth factor β. All genes studied were expressed after SCI in both the LT and control groups, however, expression of IL6, MCP-1 and iNOS was significantly suppressed in the LT group. IL6 expression was 171 fold greater in the control group than the LT group at 6 hours post-injury ($p<0.001$). MCP-1 and iNOS were also suppressed at 6 hours post-injury by LT, with 3 and 5 fold decreases ($p<0.01$), respectively. These results demonstrate that LT has an anti-inflammatory effect on the spinal cord after injury, which may contribute to improving axonal regrowth and function.

Keywords: 810nm, IL6, iNOS, LPLI, MCP-1, photo-biomodulation, rats

Introduction

Injury to the spinal cord results in the modification of expression of a number of genes, including cytokines, chemokines, and cell adhesion molecules. Transcription and translation of these groups of genes are responsible for controlling the invasion and activation of the cells that respond to injury. The degree of recovery after spinal cord injury (SCI) is dependent upon this pattern of gene expression and cell invasion/activation. mRNA of pro-inflammatory cytokines, such as interleukin 1 β (IL1β), tumor necrosis factor α (TNFα), IL6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), is produced quickly after SCI, within 1 hour for IL1β and TNFα, and within 6 hours for IL6 and GM-CSF (Benveniste, 1992; Bartholdi and Schwab, 1997; Klusman and Schwab, 1997; Pan et al., 2002). These cytokines are responsible for inducing and/or altering the activity of various cells within the spinal cord, including macrophages, microglia and astrocytes (Benveniste, 1992; Bartholdi and Schwab, 1997; Klusman and Schwab, 1997). These cytokines have also been found to induce cell death and demyelination (Selmaj and Raine, 1988; Simmons and Willenborg, 1990; Jenkins and Ikeda, 1992), suggesting that they may contribute to the secondary damage observed after SCI.

Chemokines also play a role in SCI. The C-C chemokines, macrophage inflammatory protein 1 (MIP1α) and monocyte chemoattractant protein (MCP-1), are responsible for the recruitment of inflammatory cells, such as macrophages and T cells, into the injured spinal cord (Oppenheim et al., 1991; Glabinski et al., 1996). These chemokines are normally upregulated within the first few hours after SCI. MIP1α transcription increases between 45 minutes and 6 hours post-injury and MCP-1

expression is upregulated by 6 hours post-injury and peaks at approximately 12 hours post-injury (Bartholdi and Schwab, 1997; McTigue et al., 1998; Ma et al., 2002).

Ultimately, the interaction between these chemokines and cytokines results in a vigorous inflammatory response in the spinal cord, characterized by invasion/activation of macrophages, microglia, astrocytes, and T and B lymphocytes (Dusart and Schwab, 1994; Carlson et al., 1998; Isaksson et al., 1999; Ma et al., 2002; Popovich et al., 2002). Anti-inflammatory treatments and other methods to block the action of the cytokines and/or chemokines have resulted in improvements in axonal regrowth and function (Giulian and Robertson, 1990; Ghirnikar et al., 2000; Chikawa et al., 2001; Nash et al., 2002).

Light therapy (LT), also known as photo-biomodulation or low power laser irradiation, has been shown to increase neuronal survival and regeneration in the peripheral nervous system (Anders et al., 1993; Snyder et al., 2002) and improve axonal regrowth and restore function after SCI (Rochkind et al., 1988; Rochkind and Ouaknine, 1992; Rochkind et al., 1997, 1998; Byrnes et al., 2000). This treatment modality has been under investigation for over 20 years as a therapy for wound healing and pain management (Mester et al., 1971; Rochkind et al., 1987b; Rochkind et al., 1989; Yu et al., 1997a; Jimbo et al., 1998; Schindl et al., 1999). Investigation has shown that LT, through the absorption of light by a cellular photoreceptor, rather than heating of the cell (Anders et al., 1993; Mochizuki-Oda et al., 2002; Castro et al., 2003), can increase or decrease ATP, DNA, RNA and protein synthesis, depending on the treatment parameters applied (Lam et al., 1986; Saperia et al., 1986; Greco et al., 1989; Funk et al., 1992; Mochizuki-Oda et al., 2002). Alteration of gene expression by LT has been demonstrated

in a variety of cells and injury models (Funk et al., 1993; Shimizu et al., 1995; Khanna et al., 1999; Greco et al., 2001; Snyder et al., 2002; Wong-Riley et al., 2002). LT also significantly alters inflammatory cell invasion and activity, including decreasing cytokine production when applied at high dosages (Funk et al., 1992; Funk et al., 1993; Shimizu et al., 1995).

The purpose of this study was to test the hypothesis that LT reduces the expression of pro-inflammatory cytokines and chemokines after SCI. Expression of pro-inflammatory cytokines (IL1β, TNFα, IL6 and GM-CSF), the anti-inflammatory cytokine TGFβ, chemokines (MCP-1 and MIP1α), intracellular cell adhesion molecule (ICAM), and inducible nitric oxide synthase (iNOS) were examined at 6 hours and 4 days after a dorsal hemisection in adult rats. These time points were chosen to investigate the response of injured spinal cord to LT when expression of these genes normally peaks (6 hours), as well as when our previous experiments have demonstrated significant and selective alterations in cellular infiltration/activation (4 days; Byrnes, submitted for publication). The results of this research revealed that light treatment of SCI caused a significant suppression of gene expression, particularly of genes normally upregulated soon (6 hours) after injury.

Methods

Subjects:

Twenty adult female Sprague-Dawley rats (200 – 300g, Taconic Farms, Germantown, NY) were used in this study under an approved Uniformed Services University IACUC protocol. Food and water were provided ad libitum and the rats were exposed to 12 hour cycles of light and dark periods.

Dorsal Hemisection of Spinal Cord:

Rats were randomly assigned to two groups (LT group, n= 10; control group, n = 10). Investigators were blinded to the group assignment prior to dorsal hemisection surgery. Animals were anesthetized with sodium pentobarbital (50mg/Kg, I.P.) and placed on an isothermal heating pad warmed to 37^{o}C. The ninth thoracic vertebra was identified and a laminectomy was performed to expose the spinal cord between T8 and T10. The dorsal funiculus was isolated by passing a suture thread through the spinal cord. Iridectomy scissors were used to carefully incise this isolated portion of the spinal cord, thereby transecting the corticospinal tract. Inspection of the lesion and visualization of the central gray commissure verified that the corticospinal tract had been completely transected.

After the dorsal hemisection was completed, the exposed spinal cord was covered with gelfoam (Pharmacia, Upjohn; Kalamazoo, MI), and the overlying muscles and skin were sutured. During the recovery period, bladders were manually expressed until spontaneous voiding returned at approximately 1-2 days post-injury.

Light Treatment:

Treatment began immediately after injury and continued on a daily basis. Prior to treatment, all animals were lightly anesthetized with sodium pentobarbital (20 mg/Kg, I.P.) and placed on isothermal heating pads. All treatments were done in the dark.

Light was applied daily, for the extent of the experiment, to rats in the LT group (n = 10) with a 200 mW 810 nm diode laser (Thor DDII, Thor International LTD., Basildon, Essex, UK). The laser was modified with a delivery fiber optic to produce a homogenous 6 mm diameter spot with a power of 150 mW. During treatment, this spot was centered on the skin directly above the location of the spinal cord hemisection. Treatment was applied for 2,997 seconds per day, for a final energy density, or dosage, of 1589 J/cm^2 per day (irradiance = 0.53 W/cm^2, 450 J). These parameters, which result in 6% penetration of power to the spinal cord level (Byrnes, submitted for publication), have been found to successfully promote axonal regrowth in our previous studies (Byrnes et al., 2000, Byrnes, submitted for publication).

The control group (n= 10) received no irradiation, but was handled identically to the LT group.

RT-PCR

At 6 hours or 4 days post-injury, rats (n=5/group/time point) were deeply anesthetized with chloral hydrate (1 ml/100 g, I.P., 10% solution) and euthanized by decapitation. The 5 mm of the spinal cord encompassing the lesion site and the area immediately rostral and caudal to the lesion site were dissected rapidly and placed in 500 µl of RNAlator solution (Ambion, Austin, TX). Total cellular RNA was extracted using

the Trizol (Invitrogen, Carlsbad, CA)/phenol (Sigma, St. Louis, MO)/chloroform (Sigma) technique and reverse transcribed using First-Strand Synthesis beads (Amersham Pharmacia, Piscataway, NJ) as per the protocol of the manufacturers (Invitrogen and Amersham Pharmacia). Resultant cDNA was amplified using the CytoXpress Multiplex Inflammatory Set 1 (Biosource, Camarillo, CA) or primers specific for genes of interest (Table 1). Unless otherwise noted, primer sequences were obtained with the use of the Primer3 program (Rozen and Skaletsky, 2000), with complete cDNA sequences obtained from the NIH GeneBank Entrez program. Negative (no sample added to PCR mix) and positive (provided with kit) controls were included in each PCR assay to ensure that contamination was avoided.

PCR products were assessed by electrophoresis on a 2% agarose gel containing ethidium bromide (Sigma). PCR bands were visualized using UV light and photographed. Scion Image (www.rsb.info.nih.gov/nih-image/) was used to measure band pixel density, reflecting relative gene expression. Adjustment was performed to normalize pixel intensity for samples run on different gels in order to compare the data. Pixel density for each band was obtained and normalized against the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data is presented as the ratio of the gene of interest to GAPDH.

Statistical Analysis

Resultant relative gene expression is presented as mean ratio +/- SEM. One-way ANOVA was used to compare groups. Tukey's Multiple Comparison test served as a post-test to the ANOVA for comparison of individual groups. All statistical analyses

were performed using the GraphPad Prism Program, Version 3.02 for Windows (GraphPad Software, Inc. Sand Diego, CA).

Results

Comparison of resultant bands to the molecular weight marker confirmed that each investigated gene appeared at the expected molecular weight (data not shown). Analysis of gene expression at 6 hours and 4 days post-injury revealed that all genes were detected at all time points investigated, and no significant difference ($p=0.6740$) was found in expression of GAPDH between the control and LT groups (Fig. 1). Expression profiles from each sample were only included if expression for the internal control, GAPDH, could be detected. Gene expression of GAPDH for one sample of the LT-4 day group was insufficient for measurement, so this sample was not included in any data analysis. Therefore, data for this group are from four animals; all other groups were comprised of five rats.

Pro-inflammatory Cytokines

The expression of four pro-inflammatory cytokines, IL1β, TNFα, IL6 and GM-CSF, was assessed at 6 hours and 4 days post-injury. LT immediately following injury resulted in a significant suppression ($p<0.001$; Fig. 2a, b) of IL6 expression at 6 hours post-injury. A 171 fold decrease in expression of IL6 in the LT group as compared to the control group was detected at this time point. By 4 days, transcription of IL6 had significantly decreased by 58% in the control group ($p<0.001$, Fig. 2a). The expression in the LT group remained depressed from 6 hours to 4 days, but there was no significant difference between IL6 levels at 4 days post-injury between the control and LT groups. There was no significant difference between control and LT groups in expression of TNFα, IL1β and GM-CSF at 6 hours post-injury or 4 days post-injury (Fig. 2c – e).

However, a trend was found in expression of GM-CSF at both 6 hours and 4 days post-injury, with a 10 and 3 fold decrease in expression found between the LT and control groups, respectively (Fig. 2e). A trend toward increase in transcription of TNFα in the LT group at 4 days post-injury was shown, although this increase was not significantly different from the control group (Fig. 2d).

Chemokines

Two chemokine genes were examined in this study. Analysis of mRNA quantities for MIP1α and MCP-1 were performed at 6 hours and 4 days post-injury. Quantitation of transcription revealed that LT resulted in a significant decrease in MCP-1 at 6 hours post-injury ($p<0.01$, Fig. 3a, b). The control group at this time point was found to have 66% greater expression of MCP than the LT group. This reduction in expression continued through 4 days post-injury, with a two-fold decrease in MCP-1 expression in the LT group, although this difference between the two groups was not significant. No significant differences between the LT or control group were found at 6 hours or 4 days post-injury for MIP1α (Fig. 3c); however there was a five-fold increase in MIP1α expression at 4 days post-injury in the LT group.

ICAM, iNOS, TGFβ

Gene expression was also evaluated for iNOS, ICAM and TGFβ. Analysis of gene expression revealed that LT resulted in a five-fold suppression of iNOS transcription at 6 hours post-injury ($p<0.01$; Fig. 4a), and a four-fold decrease in iNOS at 4 days post-injury that did not reach statistical signficance. Again, similar to the situation

with IL6 and MCP-1, iNOS expression was significantly decreased ($p<0.01$, Fig. 4a) in the control group from 6 hours to 4 days post-injury, but the expression levels were relatively constant over this time period in the LT group. TGFβ and ICAM, however, demonstrated a trend towards increase at both 6 hours and 4 days after injury in the LT group, although this increase did not reach statistical significance (Fig. 4b, c).

Conclusion

Following SCI there is rapid upregulation of a number of genes. The current study found that LT had a significant effect on genes that normally peak between 6 and 24 hours post-injury (Bartholdi and Schwab, 1997; McTigue et al., 1998; Streit et al., 1998; Hayashi et al., 2000; Satake et al., 2000; Pan et al., 2002), significantly decreasing the transcription of IL6, MCP-1 and iNOS at 6 hours post-injury. These three genes are all integrally involved in the immune response, and are all suggested to play an important role in secondary injury and/or the lack of regeneration after SCI (Ghirnikar et al., 2000; Satake et al., 2000; Ghirnikar et al., 2001; Bao and Liu, 2002; Ma et al., 2002; Eng and Lee, 2003).

Decreases in the expression of IL6 and MCP-1 by LT are expected to suppress macrophage invasion/activation and decrease the production of inhibitory molecules in the injured spinal cord. IL6, produced by astrocytes, fibroblasts, macrophages and microglia, plays a role in the stimulation of astrocytes and activation of macrophages (Benveniste, 1992; Lotan and Schwartz, 1994; Klusman and Schwab, 1997). Additionally, IL6 has also been found to increase the production of neurite growth inhibitory molecules in astrocytes (Fitch and Silver, 1997), supporting its role in secondary injury and prevention of axonal regeneration. MCP-1, a chemoattractant for macrophages, is produced by astrocytes and macrophages between 6 and 12 hours after injury (Sun et al., 1997; McTigue et al., 1998). When an antagonist for MCP-1 is injected after spinal cord injury, peripheral white matter is spared and macrophage numbers in the spinal cord decrease (Ghirnikar et al., 2000). MCP-1 receptor knockout has shown decreased macrophage invasion and subsequent myelin degradation (Ma et al.,

2002). This correlates well with the findings of our previous work and other studies that demonstrate LT significantly reduced the macrophage/activated microglia response after SCI and significantly improved axonal growth and functional ability (Byrnes, submitted for publication, Rochkind and Ouaknine, 1992; Rochkind et al., 1997, 1998).

It is unclear as to whether LT inhibits the expression of these genes within resident cells of the spinal cord, or inhibits the infiltration of the cells that express these genes into the spinal cord. However, the finding that expression was significantly altered at 6 hours post-injury, and was not significantly different at 4 days post-injury, suggests that the expression is altered within the spinal cord prior to invasion of non-resident cells. Macrophages and microglia are typically identified within the spinal cord between 12 and 24 hours after spinal cord injury, peaking between 4 – 8 days post-injury. Collaborating this theory is the finding that astrocyte activation is significantly depressed at 48 hours after spinal cord injury (Byrnes, submitted for publication), thus providing a mechanism by which the observed decreases in IL6 and MCP-1 occur.

It is important to note that IL6 and MCP-1 are also downstream genes, with production initiated by TNFα and IL1β (Sun et al., 1997; Streit et al., 1998; Sar et al., 2000). Protein production was not investigated in this study, but previous work by Funk et al. (Funk et al., 1992; Funk et al., 1993) demonstrated that LT with high energy densities (80 J/cm^2) decreased IL1, TNFα and IFNγ production and/or secretion. Therefore, the possibility remains that LT may interfere with the protein production of IL1β and TNFα, which peak approximately 7 hours after the mRNA peak (Wang et al., 1997), thereby decreasing IL6 and MCP-1 gene transcription.

LT was also found to decrease iNOS expression (Fig. 4a). This gene is expressed within 24 hours after SCI in a variety of cells, including macrophages and microglia following stimulation by a number of factors, such as TNFα or IL1β (Hu et al., 2000; Satake et al., 2000; Chatzipanteli et al., 2002). Similar results have been found by Leung et al. (Leung et al., 2002), in which iNOS protein levels decreased 38% after LT of transient cerebral ischemia. This decrease may be due to a variety of actions of LT within the injured spinal cord. LT may directly inhibit iNOS transcription, decrease iNOS producing cell types activity or invasion, or decrease production of factors that stimulate iNOS expression. Immunosuppressive agents that block inflammatory cell invasion and activation in the spinal cord after injury, such as methylprednisolone, have also been shown to inhibit the increase in iNOS expression normally seen after SCI (Hayashi et al., 2000; Chikawa et al., 2001; Diaz-Ruiz et al., 2002) and promote functional recovery (Nash et al., 2002). Blockage of iNOS activity after SCI promoted locomotor recovery and reduced contusion volume (Chikawa et al., 2001; Bethea and Dietrich, 2002)

It is interesting to note that LT had no significant effect on genes whose expression is normally upregulated within the first 1 – 3 hours after injury. Expression of IL1β, TNFα and MIP1α is upregulated within the first hour after SCI (Bartholdi and Schwab, 1997; Streit et al., 1998; Hayashi et al., 2000; Bethea and Dietrich, 2002; Pan et al., 2002) and ICAM is upregulated by 3 hours post-injury (Hamada et al., 1996a). It is likely that the rapid transcription of these genes after SCI occurs before LT is able to inhibit activity within the spinal cord. It is also noteworthy that MIP1α has been found to promote neutrophil invasion after injury (Bartholdi and Schwab, 1997; Ousman and

David, 2001; Sherry and Franchin, 2001). Previous work in the laboratory has shown that LT is unable to inhibit neutrophil invasion following SCI (Byrnes, submitted for publication), a finding that is consistent with the data from the current study, in which MIP1α mRNA production is not significantly affected by LT.

TGFβ, produced in macrophages, microglia, astrocytes and endothelial cells by 1 day post-injury and peaking between 7 and 14 days post-injury, is the only gene expressed late after injury that was not significantly affected by LT. These data conflict with the recent study by Khanna et al. (Khanna et al., 1999), in which a significant increase in TGFβ was found in cultured cells after LT. Our study did find a trend towards an increase in TGFβ, although this increase was not statistically significant. It is possible that the time points chosen were not optimal to determine the effects of LT on this cytokine *in vivo*, and further study of its response is warranted as TGFβ has a multitude of effects after SCI (Semple-Rowland et al., 1995; Streit et al., 1998; Lagord et al., 2002). Within the spinal cord, this cytokine is responsible for activating astrocytes to produce extracellular matrix proteins and neurotrophic factors (Frisen et al., 1994; Lotan and Schwartz, 1994). In addition, it can serve as a chemoattractant for macrophages (Prewitt et al., 1997). Other studies have demonstrated that TGFβ is anti-inflammatory, decreasing microglia activation *in vivo* and adhesion abilities *in vitro* and increasing neuronal survival (McNeill et al., 1994; Lagord et al., 2002; Milner and Campbell, 2002).

In the injured spinal cord, IL1β, TNFα, IL6, GM-CSF, and MCP-1 are normally downregulated by 24 hours post-injury (Bartholdi and Schwab, 1997; Wang et al., 1997; Streit et al., 1998; Bethea and Dietrich, 2002; Pan et al., 2002), which is comparable to the results found in our control group with slight decreases seen between 6 hours and 4

days post-injury (Fig. 2a, c, d, e, 3a). Expression of IL6, GM-CSF, MCP-1 and iNOS in the LT group, however, did not decrease between the 6 hours and 4 days, and the expression over these two time points did not change (Fig. 2a, d, 3a, 4a). This is possibly because these genes did not undergo the initial upregulation in gene expression early after injury, and maintained a low level of expression throughout the assessed experimental period. The LT group also demonstrated a trend ($p>0.05$, Fig. 2d) towards an increase in TNFα transcription at 4 days post-injury. Although these data were not expected, they do correlate with the significant increase in astrocyte activity observed at 4 days post spinal cord injury in LT treated animals (Byrnes, submitted for publication).

The mechanism of how LT affects gene transcription is currently unknown, although alterations in mRNA production have been found in several cell types, including T cells (Funk et al., 1993), monocytes (Funk et al., 1993), hepatocytes (Greco et al., 2001), cardiomyocytes (Khanna et al., 1999), and periodontal ligament cells (Shimizu et al., 1995), as well as in several *in vivo* injury models, including peripheral nerve injury (Snyder et al., 2002), monocular enucleation (Wong-Riley et al., 2002), and cutaneous wounds (Saperia et al., 1986). Several theories have been postulated as to the mechanism of LT, including alterations in calcium concentrations within the cell, alterations in ATP production and general cellular activity, and alterations of transcription factors themselves (Lam et al., 1986; Saperia et al., 1986; Greco et al., 1989; Funk et al., 1992; Mochizuki-Oda et al., 2002). Although the current work did not attempt to explore the mechanism of LT's effects, it is clear that the process involved in altering gene transcription is a delayed one, as changes in gene expression were only observed in genes normally expressed 6 – 12 hours after injury.

This study clearly demonstrates that transcutaneous light application has a significant effect on gene expression in the spinal cord. LT was found to suppress the production of iNOS, IL6 and MCP-1, which are integrally involved in the immune response after SCI. Our results provide support to the theory that LT has a significant anti-inflammatory effect on the injured spinal cord (Rochkind et al., 1988; Rochkind and Ouaknine, 1992; Rochkind et al., 1997, 1998). Moreover, previous work in our laboratory (Byrnes, submitted for publication) has demonstrated significant alterations in cellular invasion and activation after SCI, further supporting the theory that light has a strong potential as a non-invasive therapy for spinal cord injury.

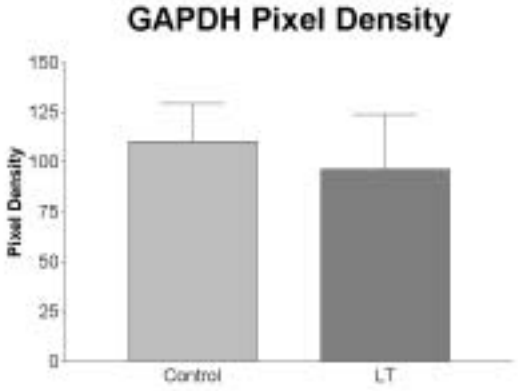
Acknowledgements

This work was funded by a Summer Student Research Grant from the American Society for Lasers in Surgery and Medicine and a Defense Veterans Head Injury Program Grant. The authors would like to thank Dr. Diane Borst and Tara Romanczyk for their advice and support. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

Table 1: Primer Sequences

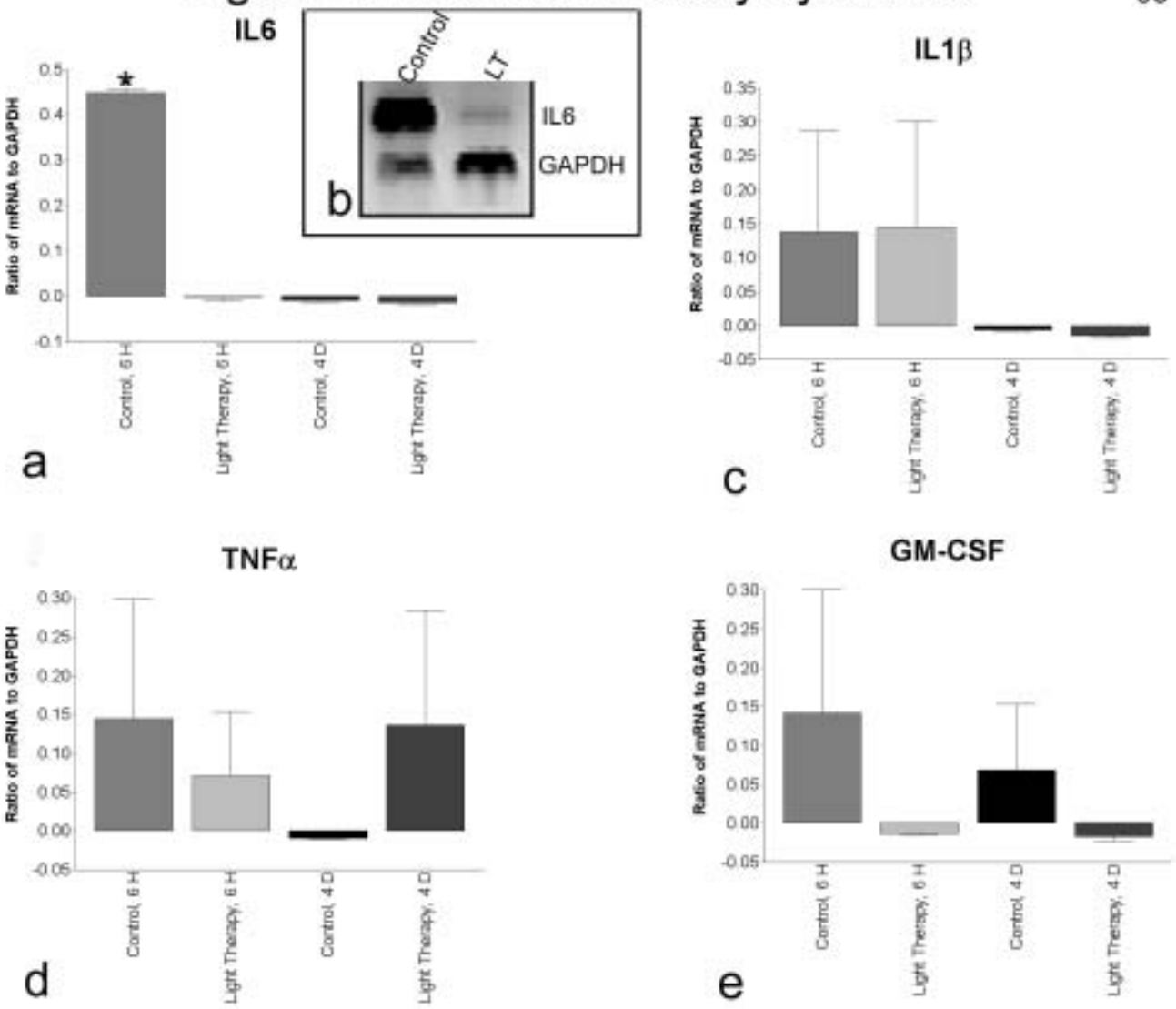
Primer	Sequence	Predicted Size	Reference
GAPDH	5' CCATGGAGAAGGCTGGG 3' 5' CAAAGTTGTCATGGATGACC 3'	195bp	(Power et al., 1995)
ICAM	5' AGGTATCCATCCATCCCACA 3' 5' GCCACAGTTCTCAAAGCACA 3'	209bp	
iNOS	5' CCCTTCCGAAGTTTCTGGCAGCAGC 3' 5' GGGTGTCAGAGTCTTGTGCCTTTGG 3'	497bp	(Satake et al., 2000)
MCP-1	5' CTTCTGGGCCTGTTGTTCAC 3' 5' GGGACGCCTGCTGCTGGTGATTC 3'	162bp	(Lee et al., 2000)
MIP-1alpha	5' TTTTGAGACCAGCAGCCTTT 3' 5' CTCAAGCCCCTGCTCTACAC 3'	191bp	

Figure 1: GAPDH Comparison



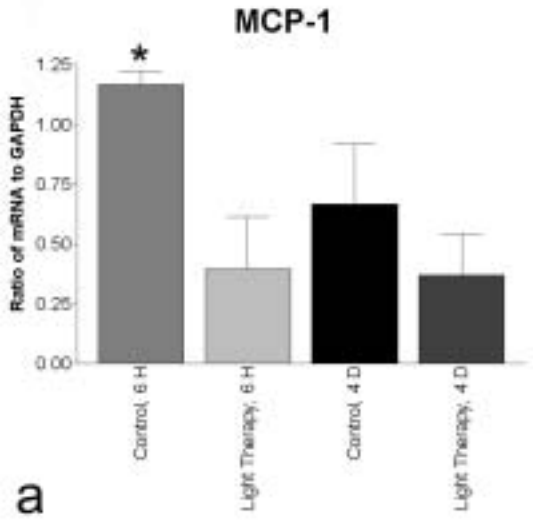
Internal control (GAPDH) expression revealed no significant difference between the control and LT groups (data from 6 hours and 4 days post-injury collapsed into one group; no significant difference seen for individual time points: data not shown). $p>0.05$ (Students t-test); bars represent mean +/- SEM.

Figure 2: Pro-inflammatory cytokines

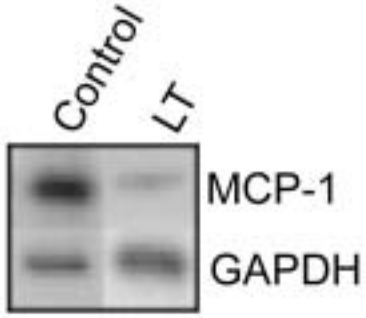


Four pro-inflammatory cytokines were semi-quantitated in this study. All samples were from the site of spinal cord injury at 6 hours (H) and 4 days (D) post-injury. A) IL6 expression analysis revealed significant inhibition of IL6 mRNA production by LT at 6 hours post-injury, but no significant difference between groups at 4 days post-injury. B) Ethidium bromide-DNA complex fluorescence for IL6 from the control and LT groups, as well as their corresponding GAPDH band, at 6 hours post-injury was digitally photographed. C) IL1b expression. D) TNFa expression. E) GM-CSF expression. *$p<0.001$ for comparison between control and LT group at individual time point (ANOVA followed by Tukey post-test), bars represent ratio of gene of interest to internal control mean +/- SEM.

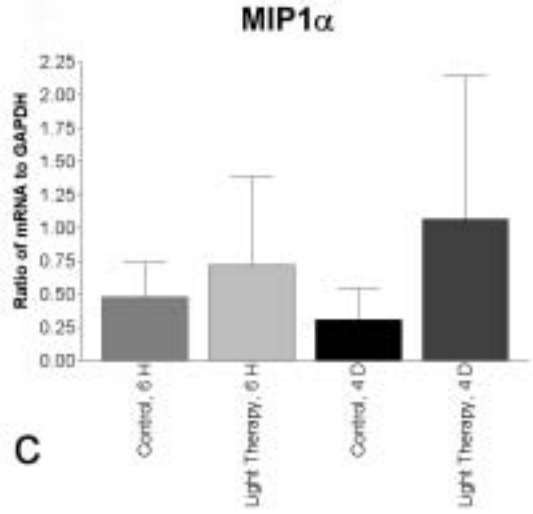
Figure 3: Chemokines



a



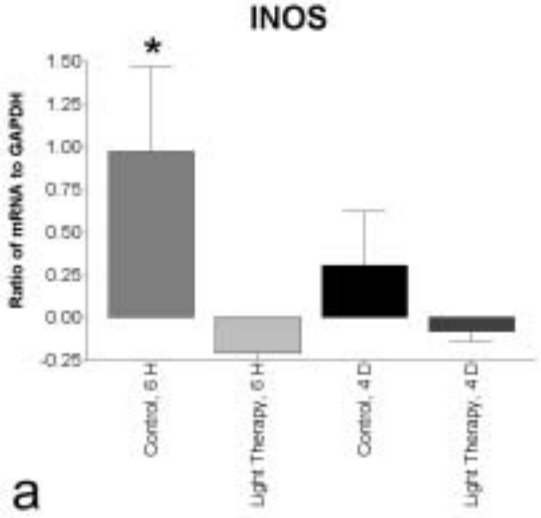
b



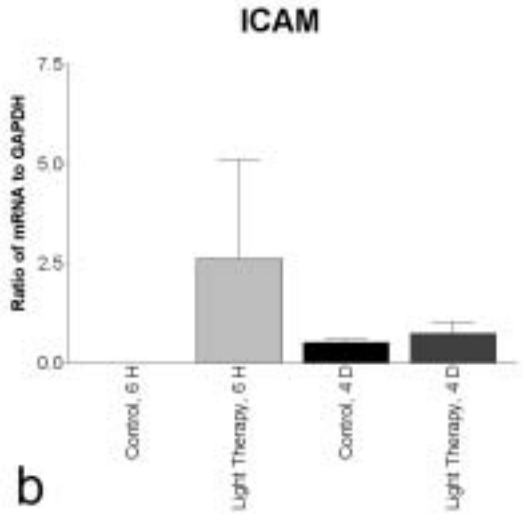
c

Two chemokines were assessed in this study. All samples were from the site of spinal cord injury at 6 hours (H) and 4 days (D) post-injury. A) MCP-1 expression at 6 hours and 4 days after SCI. B) Representative gel bands for MCP-1 from the control and LT groups, as well as their corresponding GAPDH band. C) MIP1a expression at 6 hours and 4 days after SCI. *$p<0.01$ for comparison between control and LT group at individual time point (ANOVA followed by Tukey post-test), bars represent ratio of gene of interest to internal control mean +/- SEM.

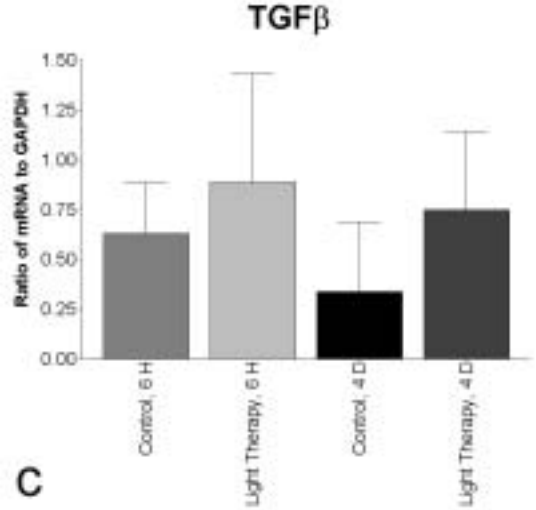
Figure 4: iNOS, ICAM, TGFbeta



a



b



c

Expression of iNOS, ICAM and TGFb were assessed at 6 hours (H) and 4 days (D) post-injury. A) iNOS expression at 6 hours and 4 days post-injury. B) ICAM expression. C) TGFb expression. *$p<0.01$ for comparison between control and LT group at individual time point (ANOVA followed by Tukey post-test), bars represent ratio of gene of interest to internal control mean +/- SEM.

Chapter 5

Discussion

The purpose of this research was to determine if transcutaneous application of 810 nm light promoted axonal regeneration and functional reinnervation of spinal cord neurons by altering the extracellular milieu of the spinal cord following transection of the CST in adult rats. A series of experiments were performed to study this hypothesis, including anterograde and retrograde tract tracing experiments, functional analyses, and cell invasion and activation assessment and gene expression measurements.

The first experiment involved assessment of neuronal regeneration after CST lesion. Unbiased stereology was used to count the number of single and double labeled neurons in layer V of the motor cortex 10 weeks after CST lesion. While there was no difference in single labeled neurons between the control and light treated groups, a significant number of double labeled neurons were found in the light treated group ($p<0.05$). Single labeled neurons were labeled with either HM, signifying the neuron had been transected at vertebral level T9, or fast blue, signifying the neuron had not been injured but did terminate in the L3 region of the spinal cord. Double labeled neurons, therefore, are those that were transected and then were able to innervate the spinal cord at vertebral level L3. Consequently, we conclude that LT promoted regeneration of transected neurons to the L3 region of the spinal cord.

Double labeling of neurons in the motor cortex not only implies that axons reached the L3 region, but that these axons terminated in the gray matter of the L3 region, as fast blue must be taken up from nerve terminals for transport. There is typically 2 – 3

mm of fast blue leakage around the injection site, which was seen in this study. Fast blue labeling was not found near the lesion site, other than fast blue labeled axons coursing in the white matter, confirming that fast blue labeled neurons in the motor cortex were only those that reached the L3 level.

Fast blue single labeled neurons in the motor cortex could be explained by one of two situations. During transection of the spinal cord, there may have been axonal sparing. Since there was no significant difference in fast blue labeling between the control and light treated groups, the axonal sparing would have to be equal. This situation is not supported by the results of the mini-ruby labeling study, however, in which there were fewer mini-ruby labeled axons that reached a shorter distance in the control group than in the light treated group ($p<0.01$). The more likely explanation is that the axons of the uncrossed, ventral CST were spared by our lesion procedure and these axon terminals pinocytosed the fast blue tracer. It has been reported that leaving this tract intact does not interfere with neuronal regeneration or functional recovery assessment (Whishaw and Metz, 2002).

It is important to note that there were considerably more HM labeled neurons than either fast blue or double labeled neurons in the motor cortex 10 weeks after injury. This fact suggests that most neurons did not regenerate to the L3 level. The number of HM labeled neurons was similar to that found in previous studies (Hicks and D'Amato, 1977; Brosamle and Schwab, 1997). A significant number of double labeled neurons were found in the light treated group, accounting for approximately 0.3% of all counted neurons, which is significantly greater than the control group ($p<0.05$). Comparison of the anterograde and retrograde studies revealed that the number of double labeled

neurons represents approximately 30% of mini-ruby labeled axons, suggesting that 30% of the regrowing axons terminate in the L3 region.

The mini-ruby labeled axons quantitated in this study were found only in the area of the dorsal funiculus normally occupied by the CST, suggesting axonal regeneration of the appropriate tract. Nash et al. (Nash et al., 2002) reported similar findings. Pettigrew et al. (Pettigrew and Crutcher, 1999) demonstrated that despite the inhibitory molecules present in white matter, neurite outgrowth is supported in directions parallel to white matter tracts, further supporting our findings. Therefore, this study found that 14 day treatment of acute SCI with 810 nm light (150 mW) resulted in a significant increase in the number of regenerating axons and in the total distance those axons regrew caudal to the lesion level.

To date, no study has evaluated axonal regrowth of specific tracts using retrograde or anterograde tracing after LT of SCI. A number of studies by Rochkind et al. (Rochkind and Ouaknine, 1992; Rochkind et al., 1997, 1998) found that LT in combination with transplantation resulted in an increase in axonal sprouting and axonal myelination within the graft, in comparison to transplantation alone. However, the source of these axons was not determined, nor were they found to extend beyond the graft. Therefore, the findings of this study are novel in demonstrating that LT, without transplantation, increased growth of axons through the lesion site and up to 24 mm caudal to the lesion.

Despite the small percentage of regeneration found in this study, studies have shown that functional improvement can be found with very small amounts of axonal regrowth (less than 10%; Bregman, 1994; Kalderon and Fuks, 1996a; Kalderon and Fuks,

1996b; Nash et al., 2002). This is supported by the current study, in which functional recovery measurements, including angle of hindlimb rotation during locomotion and duration of time necessary to cross a ladder beam, were found to return to pre-injury values at 10 weeks post-injury following LT. These measurements are specific, sensitive analyses for CST functions, and are normally impaired by CST lesion (Kunkel-Bagden et al., 1993; Merkler et al., 2001; Metz and Whishaw, 2002).

Previous studies have also shown improvement in motor function after SCI and LT (Rochkind and Ouaknine, 1992; Rochkind et al., 1998). These studies investigated non-specific recovery of function, such as weight bearance, step taking and electrophysiological measurement in the musculature of the hindlimbs, and may be influenced by reflexes below the lesion site or non-injured tracts. The current study, on the other hand, measured specific CST functions and found significant improvement after injury. It is interesting to note that the improved functions were controlled by musculature innervated by level L3 and rostral spinal cord levels. Function controlled by innervation below L3, such as footfall behavior on the ladder test, was not significantly improved, suggesting that a) regenerating axons had not extended beyond L3 within the 10 week post-injury period or b) this functional test was not sufficiently sensitive to discern an improvement in the LT group. Metz et al. (Metz and Whishaw, 2002) found that altering the distance between bars on the ladder test and assessing actual grasping behavior was necessary to determine CST function. Bar distance and specific hindlimb grasping movements were not assessed in the current research but should be addressed in future studies to precisely determine CST recovery following SCI and LT.

Stride length and base of support were not affected by CST lesion or LT. These functions have been found to be under the control of tracts other than CST, and are not normally affected by CST lesion alone. For example, tracts such as the propriospinal tract and rubrospinal tract play significant roles in base of support and stride length, respectively; these tracts are not affected by our lesioning methodology (Liu et al., 2002).

Alteration of spinal cord environment changes the ability of axons to regrow after injury. Changes in inhibitory molecule production or activity and/or decreasing secondary injury, including cavitation and demyelination, have been found to improve regeneration (Green et al., 1980; Braughler and Hall, 1982; Hall and Braughler, 1986; Schwab, 1990a; Xu et al., 1992; Bregman et al., 1995; Kalderon and Fuks, 1996a; Merkler et al., 2001). The current study determined that LT significantly altered the invasion of a number of cell types, which have substantial consequences within the injured spinal cord. LT had an immuno-suppressive effect after SCI, with the most significant impact on macrophages/activated microglia. Immunolabeling for these cells was significantly decreased at 4 days, 14 days and 5 weeks post-injury. Suppression also was found in T lymphocytes and astrocytes. T lymphocyte immunolabeling was significantly decreased at 14 days post-injury and GFAP immunolabeling was decreased at 48 hours post-injury, shifting its peak activation to 4 days post-injury. Infiltration of neutrophils and B lymphocytes into non-injured tissue around the lesion site was decreased after injury and LT. LT had no significant effect on invasion/activation of neutrophils, B lymphocyte or Schwann cells.

Decreases in these cell types would be expected to beneficially alter the environment of the injured spinal cord. Macrophages/activated microglia secrete

proteolytic enzymes and free radicals that can damage the tissue surrounding the initial injury (Delves and Roitt, 2000). Macrophages also induce astrocytes to produce proteoglycans, which inhibit neurite growth (Fitch and Silver, 1997; Fitch et al., 1999). Therefore, LT suppression of macrophages would decrease both the secondary injury and inhibitory molecule production after SCI, thereby making the spinal cord environment more supportive for axonal regrowth.

This study did not differentiate between cell number and activation state. Difficulty in discerning individual cell nuclei, particularly in cells such as astrocytes and macrophages/activated microglia, created a problem in assessing actual cell number. Immuno-positive label in a defined area was used to assess cell invasion/activation. Therefore, this study can not definitively state whether LT suppressed invasion or activation, rather that the effects of LT were a suppression of immunolabeling at the defined time points.

A possible alternative to the methodology used in this study is quantification based on cellular morphology. Prior to activation, microglia have a ramified morphology. Once activated by cytokine or endotoxin stimulation, microglia assume the round, phagocytic morphology of macrophages. Investigation of cellular morphology of macrophages and microglia may elucidate if LT prevents activation of microglia into a phagocytic state.

The finding that LT had no effect on neutrophil invasion is important to note. Several studies have found that the corticosteroid, methylprednisolone (MP), currently the only treatment available for acute SCI, fails to block neutrophil infiltration and activity after injury, while maintaining macrophage invasion inhibition (Xu et al., 1992;

Taoka and Okajima, 1998; Mabon et al., 2000). The mechanism of MP's actions is still under investigation, although several studies have found that this drug has numerous effects within the injured spinal cord. Administration of MP decreases the activation of the transcription factor NF-κB and the resultant expression of TNFα, which in turn diminishes the intensity and duration of the inflammatory response (Xu et al., 1998). While LT had no significant effect on TNFα mRNA production, a significant suppression of other down stream NF-κB genes, such as IL6 and MCP-1 (Chikawa et al., 2001), was found. This suggests that LT may have similar actions on this transcription factor.

Additionally, MP reduces lipid peroxidation after SCI through its antioxidant abilities (Anderson and Means, 1985). While lipid peroxidation and free radical production were not investigated in the current study, previous research has demonstrated that LT reduces ROS production (Karu et al., 1997). Therefore, a future study investigating the effect of LT on lipid peroxidation after SCI is warranted.

LT offers an advantage over MP. Clinically, SCI patients treated with MP exhibit a higher rate of severe sepsis and pneumonia, putting their recovery at risk (Bracken et al., 1997). This increased rate is most likely due to the global immune suppression caused by systemic MP administration. LT, on the other hand, has not been associated with any systemic effects nor have any side effects of LT been reported in the literature.

As there are several factors that can induce the invasion/activation of macrophages, microglia, T lymphocytes and astrocytes within the injured spinal cord, this study was not able to clearly identify how LT caused the immune suppression observed. To begin to explore how this immune suppression may occur, investigation of gene expression following SCI and LT was performed. The findings from this initial

investigation showed that LT depressed gene expression of the pro-inflammatory cytokine IL6, macrophage chemokine MCP-1 and iNOS at 6 hours post-injury. These data suggest that LT may affect gene expression early after injury. IL6 and MCP-1 influence the inflammatory response after injury; suppression of expression of these genes is expected to decrease inflammation intensity.

One of the most interesting results of this RT-PCR study is the finding that only the genes that are normally found to peak between 6 and 24 hours post-injury were significantly affected by LT. IL1β, TNFα, ICAM and MIP1α all experience a maximum expression at 3 hours post-injury or earlier. IL6, MCP-1 and iNOS, on the other hand, have peak expression between 6 and 12 hours post-injury. No effects were observed on expression of IL1β, TNFα, ICAM, MIP1α, or TGFβ. TGFβ was the only pro-inflammatory cytokine tested in this study, and, although changes did not reach statistical significance, increases in expression of this cytokine were found at 6 hours and 4 days post-injury. No significant change in expression of any of the investigated genes was found at 4 days post-injury, which was expected, as many of these genes are normally down-regulated by this time. The early decrease in gene expression found in this study occurs prior to invasion/activation of most immune cells, suggesting that a) the expression is altered in resident cells of the spinal cord, such as neurons, astrocytes and endothelial cells and b) this alteration may influence the change in cellular activation and invasion seen by 48 hours post-injury.

Further investigation into LT's effects on gene expression is warranted. As this study focused on gene transcription, investigation of gene translation is necessary to fully elucidate LT's effects within the spinal cord. Interestingly, similar results of 2 – 5 fold

decreases in mRNA production of pro-inflammatory cytokines and iNOS have been reported after MP treatment of acute SCI (Chikawa et al., 2001). MP has been found to significantly alter the immune response after SCI (Mabon et al., 2000); therefore, it is a logical conclusion that the LT induced changes in mRNA production would have similar effects on immune response. This conclusion is supported by our finding of decreased cell invasion/activation after SCI.

The current study details the results of LT at one dosage and one wavelength. This wavelength was found to be within the optimal range of tissue penetration, with approximately 6% of the incident power reaching the level of the spinal cord. However, there are a number of parameters that can be changed in order to more fully determine the optimal treatment parameters to improve SCI recovery. In a series of preliminary studies, we have begun to determine the optimal LT treatment parameters for acute CST lesion. Following CST lesion in adult rats, different wavelengths, dosage schedules and treatment locations were tested. The data from these experiments demonstrated that 632.8 nm light resulted in significantly less axonal growth than 810 nm light (data not shown, $p<0.01$), which agrees with the finding that 810 nm, but not 632.8 nm, is within the optimal range for light applied transcutaneously to penetrate to the spinal cord. Extending the treatment time with 810 nm light from 14 days to 21 days resulted in no significant improvement in axonal growth. However, addition of a second 810 nm light treatment with a low dosage of 1.73 J/cm^2 at the rats' motor cortex to the original 810 nm 14 day treatment significantly increased distance of regrowth (data not shown, $p<0.01$). These data suggest that the LT treatment parameters have yet to be optimized, and greater

recovery after injury can be obtained. Further experimentation is essential to determine the best parameters for axonal regeneration and recovery of function after acute SCI.

This study determined that 810 nm LT, at a dosage of 1589 J/cm^2, resulted in significant improvements in axonal regrowth and functional improvement. Although not necessarily causative, LT also induced a statistically significant suppression of immune cell invasion and pro-inflammatory cytokine and chemokine gene expression. As previous studies employing anti-inflammatory treatments have successfully improved axonal growth and return of function, it is possible that the decrease in the inflammatory response is one reason for the axonal regrowth observed after SCI and LT.

Currently, there are no national guidelines for treating acute spinal cord injuries, although administration of MP and spine stabilization are two of the most common immediate treatments. These studies demonstrate that transcutaneous application of 810 nm light is able to penetrate through multiple tissue layers to the spinal cord and have significant effects on the spinal cord environment. Light therapy, therefore, has a strong potential as a non-invasive alternative treatment for acute spinal cord injury in humans.

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APPENDIX

Additonal Methodology

Light Therapy

While optimal treatment parameters have not yet been established for treatment of spinal cord injury, a series of *ex vivo* and *in vivo* experiments were initiated to investigate wavelength and power penetration to the spinal cord level.

Ex Vivo Experiment:

A section of tissue including skin, connective tissue, muscle, vertebral bone and spinal cord was removed from the lower thoracic area of adult rats, euthanized with an overdose of 10% chloral hydrate (1ml/100g, I.P.). A 200 mW 810 nm diode laser (Thor DDII, Thor International LTD., Basildon, Essex, UK), modified with a delivery fiber optic to produce a homogenous 6 mm diameter spot with a power of 150 mW, was directed at the skin surface of the tissue section (Figure 1a). A power meter was placed below each tissue level, sequentially, or beneath the entire tissue section, to detect the amount of power transmitted through each layer. A cuvette of freshly aquired blood was also placed between the laser and the power meter to further determine transmission.

Light with a wavelength of 810 nm was found to have almost 100% penetration through blood. Forty-five mW was transmitted through the skin (70% transmission). Thirty mW was transmitted through the muscles overlying the vertebral bone, and 34 mW was able to penetrate the bone itself. Spinal cord tissue demonstrated 8%, or 12 mW, transmission through the spinal cord to the power meter, suggesting that the spinal cord absorbed 92% of the power of this wavelength of light (Figure 1b). Analysis of the

penetration through the entire tissue section revealed that 6% of the power of a 150 mW laser, or 9 mW, was transmitted to the depth of the spinal cord.

In Vivo Experiment:

An incoherent broadband white light was directed at the surface of the skin in the lower thoracic region of adult Sprague Dawley rat. Rats were anesthetized with sodium pentobarbital (50 mg/Kg, I.P.) prior to all measurements. A smart, tissue-activated optical fiber probe (Ilev et al., 2002) was inserted sequentially into the skin, sub-cutaneous connective tissue layer, deep connective tissue layer, muscle and the spinal cord within the vertebral column (Figure 1c, d). At each of these layers, a transmission spectrum in the range of 500 – 1200 nm was collected while white light was applied to the skin surface.

Analysis of the transmission spectra revealed the range of transmission was highest in the 750 to 850nm spectral interval (Figure 1e). The *in vivo* transmission spectrum obtained from the first tissue layer (skin) shows the smallest relative variations (or flat transmission) of the total transmission, suggesting that the influence of the blood transmission spectrum is minimal. The influence of blood is stronger in the rest of the spectra corresponding to the connective tissue, muscle, bone and spinal cord, and is very similar to oxygen/hemoglobin spectra measured by conventional multiwavelength pulse oximeters. Based on these results and taking into account the fact that the blood absorption is one of the major factors in the absorption of light by tissue, it is concluded that a laser source emitting at a wavelength around 810 nm would be efficient for LT.

Modification of Laser:

Analysis of the laser beam power profile revealed that the beam was non-homogeneous (Figure 1f). A fiber optic was therefore coupled to the laser diode output, effectively homogenizing the beam (Figure 1g). The homogenized beam was decreased in power at the output of the fiber optic, however, resulting in a 25% reduction in power from 200 mW to 150 mW.

Treatment Parameters

Treatment began immediately after injury and continued on a daily basis. Prior to treatment, all animals were lightly anesthetized with sodium pentobarbital (20 mg/Kg, I.P.) and placed on isothermal heating pads. All treatments were done in the dark.

Light was applied daily for 14 days to rats in the light treatment group with a 200 mW 810 nm diode laser (Thor DDII, Thor International LTD., Basildon, Essex, UK). The laser was modified with a delivery fiber optic to produce a homogenous 6 mm diameter spot with a power of 150 mW. During treatment, this spot was centered on the skin directly above the location of the spinal cord hemisection. Treatment was applied for 2,997 seconds per day, for a final energy density, or dosage, of 1589 J/cm^2 per day (irradiance = 0.53 W/cm^2, 450 J).

The control group (n= 10) received no irradiation, but was handled identically to the LT group. These animals were placed in the same area of the laser, but the laser was not turned on during their treatment.

Tract Tracing

Retrograde labeling

At the time of CST lesion, gelfoam soaked in hydroxystilbamidine methanesulfonate (HM; 3% in 0.9% saline; Molecular Probes, Eugene, OR) was inserted into the lesion site of 20 rats. HM, the active component of the retrograde tracer Fluorogold, was used as it does not have the neurotoxic properties of Fluorogold. HM has an excitation peak at 386 nm and an emission peak at 546 nm. HM is not taken up by fibers of passage and requires axonal transection for efficient labeling of the nucleus cytoplasm. Therefore, this tracer was applied to the transected CST fibers at the time of lesion.

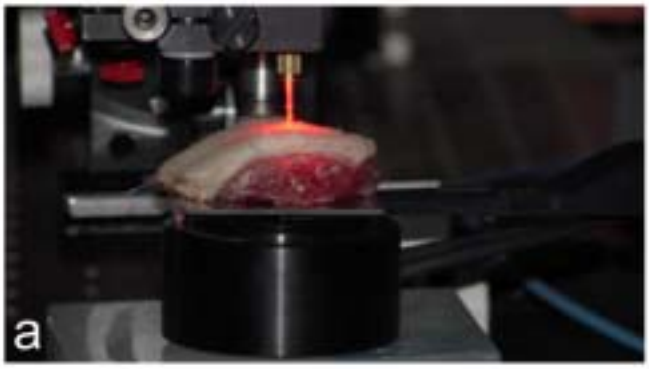
Ten weeks after the surgery, a laminectomy was performed at vertebral level L3, approximately 24 mm caudal to the original lesion site, and 1 μl of a 2% fast blue solution (in PBS, Sigma, St. Louis, MO) was bilaterally injected (0.5 μl into each side) into the spinal cord at a depth of 1.3 mm. Fast blue is excited by 405 nm light and emits at 485 nm light only. This retrograde tracer is rarely taken up by fibers of passage, and efficient labeling is obtained by either application at the site of nerve transection or nerve termination (pinocytosis).

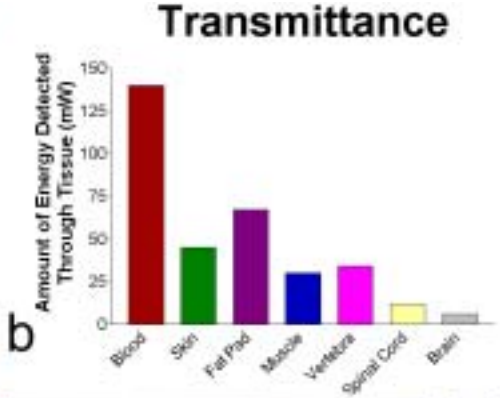
Anterograde labeling

Five weeks after CST lesion, 10% tetramethylrhodamine biotinylated dextran (mini-ruby, Molecular Probes) was injected into the motor cortex of one group of 10 rats using stereotaxic coordinates (from bregma, -0.11 AP and ±1.60 ML; -1.33 AP and ±1.50 ML; -2.85 AP and ±1.40 ML; depth = 1.0 – 1.2 mm). 2 μl of mini-ruby was injected into

each of the 6 sites, for a total injection volume of 12 µl. Mini-ruby is an anterograde tracer that is excited at 555 nm and emits at 580 nm and is taken up by neuronal cells by endocytosis.

Figure 1. *Ex vivo* and *in vivo* experimentation. a) 810 nm light was applied to a tissue section containing skin, connective tissue, muscle, bone and spinal cord that had been removed from the lower thoracic region of an adult rat. b) The power transmittance of the 150 mW 810 nm laser was measured through each type of tissue between skin and spinal cord. c) and d) *In vivo* measurement was performed by applying white light source through a fiber optic (arrow) to the surface of the skin in the lower thoracic region. A fiber optic attached to a spectrophotometer (arrowhead) was inserted sequentially beneath skin (1ln), connective tissue (2ln), muscle (3ln), vertebral bone (4ln) and spinal cord (5ln) and the spectral transmission was obtained (e). The output laser profiles of the 810 nm diode laser before (f) and after (g) fiber optic attachment.


a


Transmittance
Amount of Energy Detected Through Tissue (mW)
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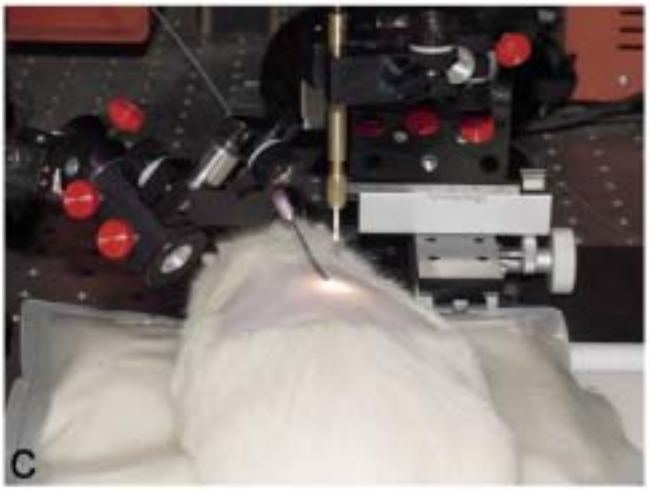
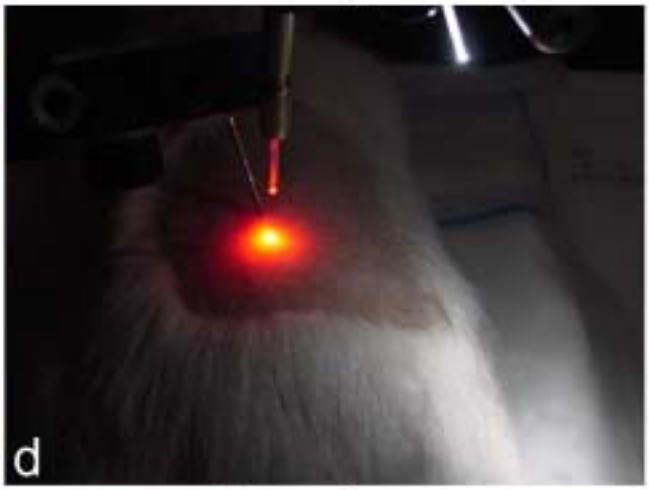
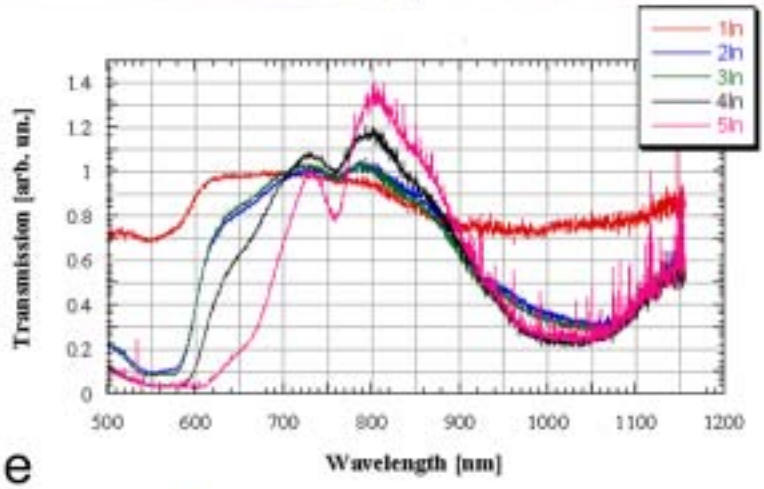
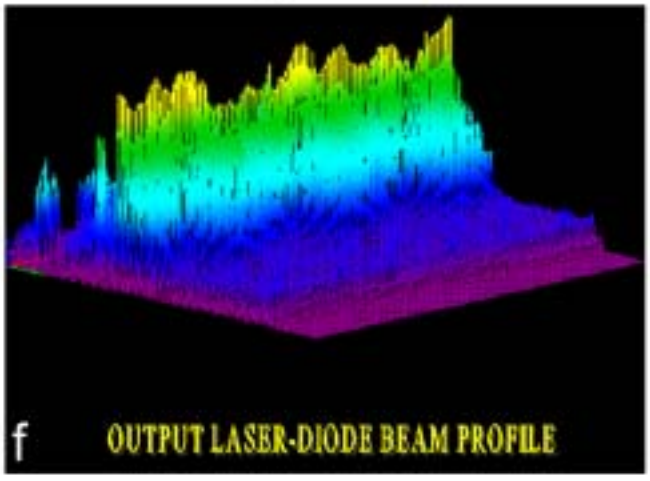
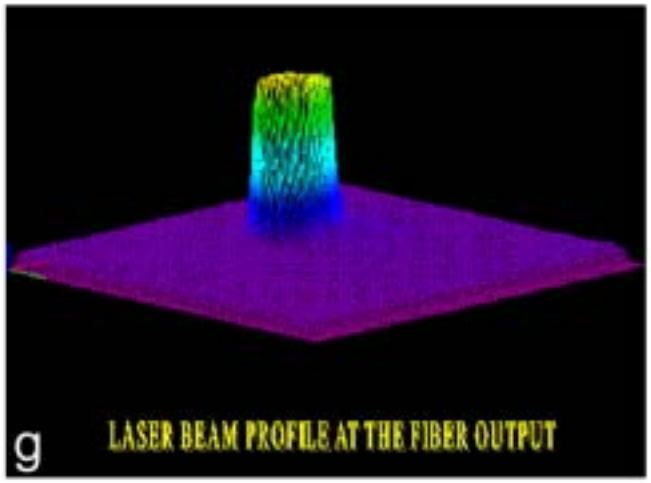
125
100
75
50
25
0
Blood
Skin
Fat Pad
Muscle
Vertebra
Spinal Cord
Brain
b


c


d


1In
2In
3In
4In
5In
Transmission [arb. un.]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
500
600
700
800
900
1000
1100
1200
Wavelength [nm]
e


f
OUTPUT LASER-DIODE BEAM PROFILE


g
LASER BEAM PROFILE AT THE FIBER OUTPUT