THE ROLE OF NOXIOUS STIMULATION IN PROGRESSIVE HEMORRHAGIC NECROSIS IN THE CONTUSED SPINAL CORD

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The role of noxious stimulation in progressive hemorrhagic necrosis in the contused spinal cord.

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Traumatic spinal cord injuries are often accompanied by secondary injuries that complicate the recovery process. Our lab has shown that nociceptive stimulation engages pain fibers and negatively impacts recovery following spinal cord injury (SCI). Further, nociceptive stimulation appears to increase the amount of red blood cells at the lesion site, which may indicate compromised vasculature. Progressive hemorrhagic necrosis (PHN), a phenomenon originating from the inflammatory environment induced by the initial injury, leads to the death of endothelial cells at the lesion site and hemorrhage. The present study investigated the impact of nociceptive stimulation on the amount of lesion-site hemorrhage, a hallmark of PHN. Adult rats received a laminectomy and a moderate contusion injury at the T12 vertebra. After a 24-hour recovery period, half of the subjects received noxious input in the form of electrical shock to the tail. Three hours post-shock, subjects were sacrificed and a 1-cm section of tissue around the lesion site was collected and sectioned. Following hematoxylin and eosin staining, the amount of hemorrhage was quantified as a percentage of the total section area. Subjects that received shock had more hemorrhage at and around the lesion site, compared to the unshocked group. The amount of hemorrhage peaked at the lesion epicenter and decreased with distance in both groups. Based on these findings, PHN may play a role in the detrimental effect of noxious input on

recovery after SCI. Further work will examine of the effect of noxious input on capillary segmentation, another hallmark of PHN, and the cellular mechanisms that underlie these effects.

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CHAPTER I

INTRODUCTION

Every year, there are over 12,000 new cases of traumatic spinal cord injury (SCI) in the United States alone. The major causes of spinal cord injury, which include vehicular accidents and violence, are often accompanied by peripheral injuries that further complicate the recovery process. Our laboratory has shown that peripheral nociceptive signals, whether consciously perceived or not, can impair recovery following a contusive spinal cord injury to a significant extent (Grau et al., 2004). These nociceptive signals engage peripheral C-fibers, eventually leading to the induction of central sensitization in the spinal cord and a cascade of necrotic cell death (Ferguson et al., 2006, 2012). Understanding how noxious input contributes to cellular necrosis and undermines recovery is key for the development of novel therapeutic approaches.

In traumatic SCI, the events that unfold immediately after injury generally occur in two stages. The initial mechanical trauma to the spinal cord, characterized by the physical destruction of local cells and vasculature, constitutes the primary injury. The hypoxic, pro-inflammatory environment resulting from the necrotic cell death at this stage is thought to prime the spinal cord for the secondary stage of injury. In the 48 hours following injury, the area of necrotic tissue expands significantly as an inflammatory cascade of cell death radiates outward from the lesion. This hyper-inflammation, in combination with the loss of descending modulatory pathways, results in a state of central sensitization that characterizes the secondary stage of injury. It is during this acute post-injury period that the spinal cord is at its most vulnerable (Flanders et al, 1996).

In order to determine the effect of peripheral injury during this period, uncontrollable noxious stimulation was given to spinally-contused subjects 24 hours after injury. This was achieved with either electrical shock administered through a tail electrode or an intradermal injection of capsaicin into the hind-paw. We found that subjects who received nociceptive input following traumatic SCI demonstrated a lasting deficit in locomotor recovery, increased tissue loss, and increased indices of neuropathic pain (Garraway et al, 2011; Grau et al., 2004). Examination of lesion-site protein extracts revealed that subjects who received noxious stimulation had increased levels of pro-inflammatory cytokines as well as cellular mediators of purinergic signaling pathways. Unexpectedly, J. Turtle observed that protein extracts from shocked subjects were much darker and redder than unshocked subjects. The absorbance spectrums for the protein extracts revealed a significantly higher absorbance at 420 nm, the wavelength at which hemoglobin absorbs, in samples from shocked subjects. Further examination confirmed an increased presence of hemoglobin in shocked subjects as well as the widespread extravasation of red blood cells into the spinal parenchyma. These observations led to the hypothesis that noxious input may be exacerbating vascular damage beyond that of the primary injury, leading to extensive hemorrhage and potentially contributing to the detrimental impact of noxious input on SCI.

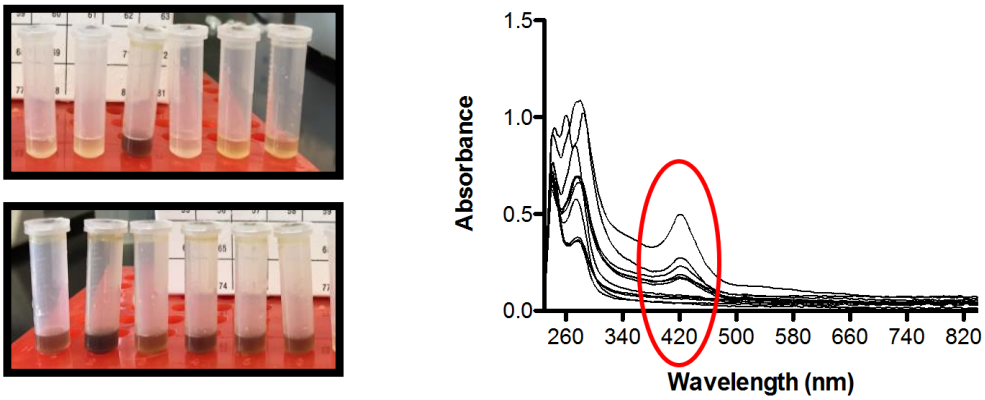

Figure 1. Lesion-site protein extracts (left) are darker and redder in subjects with SCI that receive noxious input (bottom) than those that don't (top). Full-spectral analysis reveals significantly higher absorbance at 420 nm, the wavelength at which hemoglobin absorbs light, in subjects that receive noxious input.

The separation of the systemic circulatory circuit and the central nervous system is rigorously maintained by the blood-spinal cord barrier (BSCB), and is crucial to normal functioning. It has been shown that there is a disruption in the functioning of the BSCB following SCI in a manner that correlates with severity of injury. However, the hemorrhage observed after noxious stimulation appears too extensive to be explained by a transient disruption of BSCB functionality. Increased hemorrhage could reflect a phenomenon called progressive hemorrhagic necrosis (PHN), a devastating process that begins to develop during the secondary injury phase of SCI (Simard et al., 2009). PHN is characterized by the appearance of petechial hemorrhages around the injury site that eventually coalesce into an expansive region of necrotic cell death. This condition has previously been correlated with an increase in tissue loss, edema, and the formation of glial scar tissue following central nervous system trauma (Tator et al., 1997). The destruction of spinal vasculature, and the integrity of the BSCB, is associated with an increase in tissue loss, edema, the formation of glial scar tissue and reduced neurological recovery. If noxious input is increasing vascular destruction, it would have significant functional

consequences (Mautes et al., 2000). Specifically, noxious input after SCI could enhance endothelial cell death, leading to catastrophic vascular compromise and contributing to the observed recovery deficit. The alignment of such observations with vascular damage, a primary pathognomonic feature of PHN, could potentially indicate an interaction between the two processes. Understanding the relationship between the effect of noxious input and the development of PHN may reveal common mechanisms, presenting an attractive target for therapeutic intervention.

The aim of this study is to determine the relationship between noxious input and hemorrhage, an indication of PHN, following traumatic SCI. Using histological methods, the amount of lesion-site hemorrhage will be compared between spinally-contused subjects that receive C-fiber activation soon after injury and those that do not. If noxious stimulation is facilitating additional vascular damage, we would expect to see increased hemorrhage at and around the lesion site in subjects that receive C-fiber stimulation.

CHAPTER II

METHODS

Subjects

Subjects were adult, male Sprague-Dawley rats obtained from Harlan (Houston, TX, USA) and weighed approximately 350-400 g. Free access to food and water was provided throughout the experiment. All procedures were approved by the institutional animal care committee at Texas A&M, and all efforts were made to minimize suffering and reduce the number of animals used.

Surgery

Subjects received a contusive spinal injury with the use of a MASCIS device. Subjects were anesthetized with isoflurane gas (5%) and the area surrounding the injury site was shaved and disinfected with iodine. A stable level of anesthesia was maintained with 2-3% isoflurane gas. A 7.0 cm midline incision was made followed by an incision on either side of the vertebral column. A laminectomy was performed, exposing the lower thoracic spinal cord while leaving the dura intact. The spinal column was then immobilized using a MASCIS device, and a moderate contusion injury was produced with a 10 g weight dropped from 12.5cm. The lesion site was closed with Michel clips and subjects were administered intraperitoneal injections of 3mL of 0.9% filtered saline and 100kUnits/Kg of penicillin. For the 24 hours following surgery, subjects were housed individually in a heated room with free access to food and water. Subjects had their bladders and colons manually expressed as needed.

Procedure

Twenty-four hours following injury, half of the subjects received nociceptive stimulation in the form of electrical stimulation applied to the tail. Subjects were restrained in dark plastic tubes with their tail and hindlimbs hanging freely. An electrode made from a modified fuse-clip was coated in electrode gel and secured to the tail. Next, subjects received 6 minutes of intermittent, uncontrollable shock at an intensity that engages pain fibers (80ms duration, 1.5mA), as described by Baumbauer et al (2008). Due to the spinal injury, however, pain transmission in the brain was muted. Control animals were restrained in the plastic tubes for the shock period. At three hours post-shock, all subjects were euthanized with an intraperitoneal injection of 0.2 mL of pentobarbital. Immediately following euthanasia, subjects were perfused intracardially with saline, followed by 4% paraformaldehyde. The spinal cord was dissected out and submerged in paraformaldehyde, followed by cryoprotection with a 30% sucrose solution for 48 hours.

Tissue Analysis

In order to investigate the effect of acute nociceptive stimulation on the development of PHN, the amount of hemorrhage present at the lesion site was quantified. A 1-cm-long segment of the spinal cord surrounding the lesion site was collected and prepared for cryostat sectioning. After suspending the tissue segment in Tissue-Tek, the sample was put in the freezer (-80 °C) for 24 hours. The tissue was then sectioned into 20 μm-thick sections and mounted on a charged microslide. The tissue underwent H&E staining using a protocol adapted from *Histopathologic Technique and Practical Histochemistry* (Lillie, 1965). Images of the sections were obtained using light microscopy at 40x magnification. For each subject, ten sections were collected at 800 nm intervals across the lesion site for hemorrhage quantification. The sections were analyzed by

blinded observers using ImgaeJ software. Hemorrhage was traced and quantified as a percentage of the total section area. The lesion epicenter for each subject was aligned using the section with the highest percent hemorrhage. Three sections both rostral and caudal to the lesion were also analyzed.

CHAPTER III

RESULTS

The amount of lesion site hemorrhage was quantified for both shocked and unshocked subjects that received a midline contusion injury. Sections stained with H&E demonstrate characteristic differences between the treatment groups, most notably in the distribution of hemorrhage (Figure 2). Hemorrhage in unshocked subjects is primarily found in the white matter, while hemorrhage in shocked subjects was evenly distributed between white and gray matter. In addition, the sections of shocked subjects typically exhibited gross morphological disfigurements.

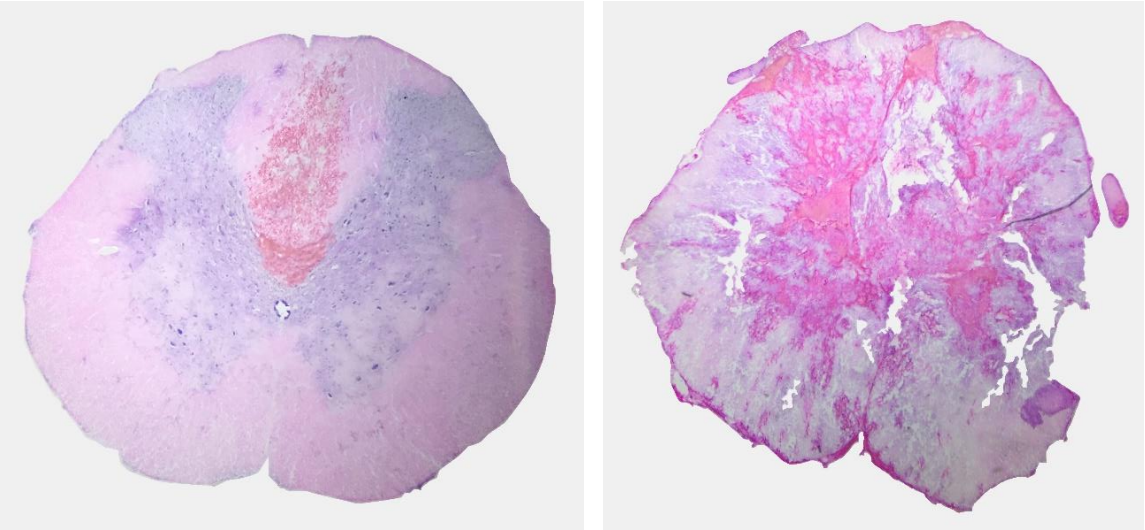

Figure 2. Representative H&E-stained sections of spinal cord tissue collected at the lesion site for unshocked (left) and shocked (right) subjects. Unshocked subjects typically exhibit a concentration of hemorrhage in the dorsal white matter. Shocked subjects generally show increased hemorrhage dorsally, but there is no discernable difference in white and gray matter distribution. Tissue was collected three hours after electrical shock or control, which was administered 24 hours after SCI.

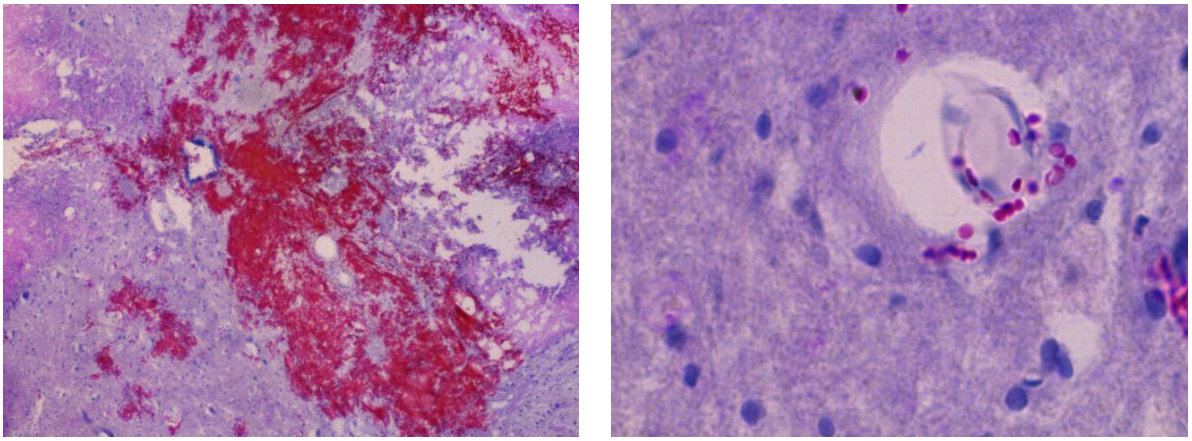

Figure 3. H&E stained tissue from the lesion site of a shocked subject show the presence of extravascular erythrocytes. Low-power magnification show wide-spread hemorrhage in the spinal parenchyma (left). Erythrocytes, determined by their archetypal biconcave shape and red color, were identified in areas of hemorrhage upon closer examination (right).

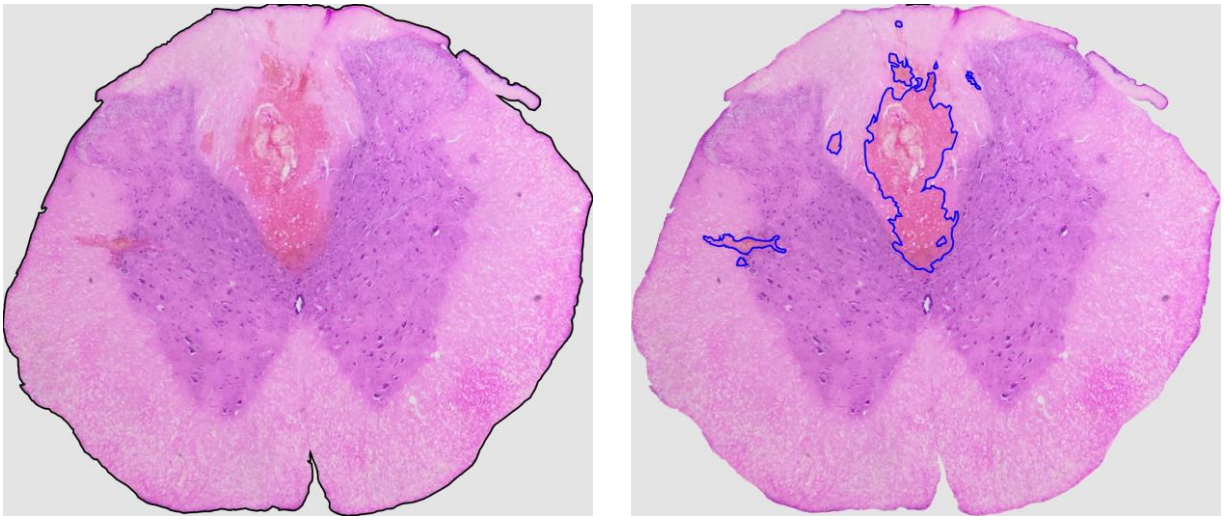

Figure 4. Demonstration of hemorrhage tracing technique for an unshocked subject using ImageJ software. The total area of the section (left) and the area of hemorrhage (right) was traced and the percent hemorrhage was calculated. Hemorrhage was defined as areas of red-tinted tissue which, upon closer magnification, contained extravascular erythrocytes.

The amount of hemorrhage was measured as a percentage of total section area (Figure 4), with hemorrhage defined as areas of red-tinted tissue which, upon further examination, were shown to contain red blood cells (Figure 3). In addition to the lesion epicenter, hemorrhage was analyzed in tissue immediately rostral and caudal to the injury site.

A mixed design two-way analysis of variance (ANOVA) yielded significant main effects of stimulation and section, $Fs > 4.47$, $p < 0.05$. Subjects who received noxious input demonstrate significantly increased hemorrhage at all measured points around the lesion site (Figure 5). There was no statistically significant interaction between section and condition, indicating a uniform increase in hemorrhage across the all analyzed sections.

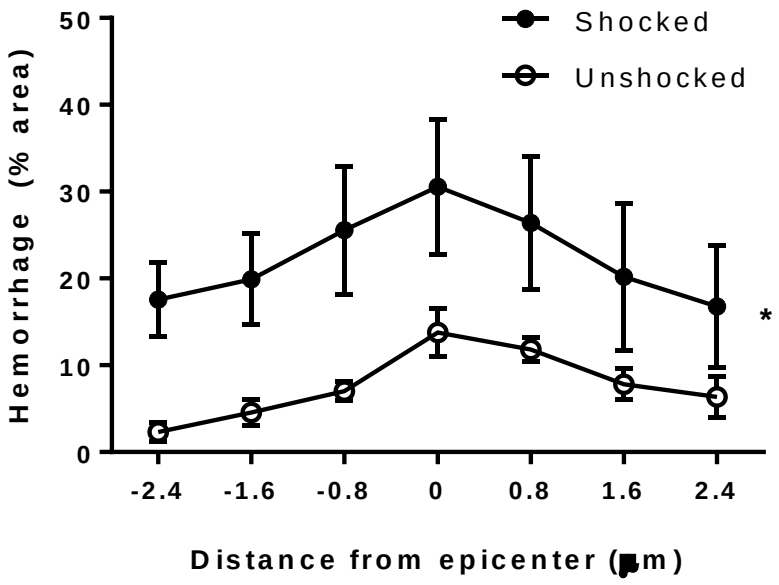

Figure 5. Noxious input increases hemorrhage across the lesion site. Negative numbers represent distance from the epicenter rostrally, and positive numbers represent distance from the epicenter caudally. Noxious input significantly increases hemorrhage in subjects with SCI ($p < 0.05$). Error bars represent SEM ($n = 6$).

CHAPTER IV

DISCUSSION

The aim of this study was to elucidate the role of noxious stimulation in the development of hemorrhage following SCI. Given the negative correlation between lesion-site hemorrhage and neurological recovery, understanding the processes that lead to vascular destruction is imperative for improving recovery from SCI. Additionally, if the negative impact of noxious stimulation is mediated by increased damage to local vasculature, understanding these processes could also improve prognosis for polytraumatic SCI victims.

Here we showed that peripheral noxious input soon after SCI significantly increases the development of hemorrhage at and around the injury site. The amount of hemorrhage decreased with distance rostral and caudal from the lesion in both groups. Further, the increase in hemorrhage in shocked subjects occurs uniformly across the lesion site. These observations align with the radial progression of tissue damage that typically develops during the first few days following SCI. Tissue collected from unshocked subjects, have hemorrhage largely restricted to the dorsal white matter and fewer observable indices of tissue destruction. This can be compared to tissue from shocked subjects, which show petechial hemorrhaging, gross structural deformities, and cavitation. The development of multiple sites of hemorrhage, which indicates extensive vascular damage, is generally considered the primary factor in the pathogenesis of PHN. The loss of vascular integrity has been shown to lead to ischemia, edema, inflammation, and increased oxidative stress, all of which contribute to the severity of the

secondary injury (Tator, et al., 1991). The significantly increased hemorrhage in shocked subjects suggest that noxious input may be exacerbating PHN.

Increased hemorrhage is not sufficient evidence to claim PHN as a mediator for the observed vascular damage. However, we have previously shown that noxious input increases the expression of a variety of pro-inflammatory cytokines that contribute to inflammation and cell death around the lesion. The increased hemorrhage at the lesion site with noxious input could suggest that additional vascular damage may be in part mediated by this inflammatory environment. These results may indicate a cellular mechanism for the progression of PHN that is related to the processes that unfold following C-fiber activation. If PHN progresses through a cascade of cellular signals, as opposed to a physical mechanism such as vessel obstruction, blockage or reversal of this pathway could alleviate some of the negative consequences.

One such pathway that has been proposed to mediate the progression of PHN during the secondary injury phase is the formation of the SUR1-Trpm4 channel/receptor complex (Simard et al., 2007). This pathway is activated in hypoxic environments and leads to apoptotic cell death. Further, it is expressed in spinal endothelial cells in rats and upregulated in these cells following exposure to a hypoxic environment *in vitro* (Simard, et al., 2009). Targeted blockage of the SUR1 channel also appears to alleviate some consequences of PHN, including tissue necrosis and hemorrhage. We have found that the SUR1-Trpm4 complex is upregulated at the lesion site in subjects that receive noxious input after SCI. This upregulation, which could result from increased levels of inflammatory cytokines, may be facilitating additional cell death and leading to the observed recovery deficit. Future studies will include examination of the impact of

noxious input on capillary segmentation, a hallmark of PHN. This would provide more evidence for the role of PHN in the detrimental effect of peripheral noxious input, which may help uncover the mechanism behind the recovery deficit. Hindering the development of PHN would promote improved recovery for victims of SCI and, based on this study, may also improve recovery for SCI victims with peripheral injuries.

Traumatic spinal cord injury impacts an estimated 275,000 people in the US, with an average age of injury of 42. Over 80% of SCI cases involve polytraumatic events, such as accidents and falls, and the majority of these victims will retain some sensation and mobility below the site of injury ("Spinal Cord," 2015). We have previously shown that these additional injuries increase tissue loss, reduce locomotor recovery, and increase indices of neuropathic pain. Understanding how nociceptive stimulation impacts recovery on a cellular level could provide direction for the development of novel therapeutic approaches. Reducing the negative impact of noxious signals on neurological recovery could result in the sparing of additional tissue and improved prognosis for the many victims of SCI.

REFERENCES

Baumbauer, K. M., Hoy, K. C., Huie, J. R., Hughes, A. J., Woller, S. A., Puga, D. A., Grau, J. W. (2008). Timing in the absence of supraspinal input I: variable, but not fixed, spaced stimulation of the sciatic nerve undermines spinally-mediated instrumental learning. *Neuroscience, 155,* 1030-1047.

Bell, R. D., Winkler, E. A., Sagare, A. P., Singh, I., LaRue, B., Deane, R., & Zlokovic, B. V. (2010). Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron*, 68, 409-427.

Ferguson, A. R., Crown, E.D. & Grau, J. W. (2006). Nociceptive plasticity inhibits adaptive learning in the spinal cord. *Neuroscience*, 141, 421-431.

Ferguson, A. R., Huie, J. R., Crown, E. D., Baumbauer, K. M., Hook, M. A., Garraway, S. M., Lee, K. H., Hoy, K. C., & Grau, J. W. (2012). Maladaptive spinal plasticity opposes adaptive spinal learning after spinal cord injury. *Frontiers Integrative Physiology*, 3, 399.

Flanders AE, Spettell CM, Tartaglino LM, Friedman DP, Herbison GJ. (1996). Forecasting motor recovery after cervical spinal cord injury: value of MR imaging. *Radiology*, 201, 649–655.

Garraway, Sandra M., Turtle, Joel D., Huie, J. Russell, Lee, Kuan H., Hook, Michelle A., Woller, Sarah A., Grau, James W. (2011). Intermittent noxious stimulation following spinal cord contusion injury impairs locomotor recovery and reduces spinal brain-derived neurotrophic factor–tropomyosin-receptor kinase signaling in adult rats. *Neuroscience*, 199, 86-102.

Grau, J.W., Washburn, S. N., Hook, M.A., Ferguson, A. R., Crown, E. D., Garcia, G., Bolding, K.A., & Miranda, R. C. (2004). Uncontrollable nociceptive stimulation undermines recovery after spinal cord injury. *Neurotrauma*, 21, 1795-1817.

Lotocki, G., de Rivero Vaccari, J. P., Perez, E. R., Sanchez-Molano, J., Furones-Alonso, O., Bramlett, H. M., & Dietrich, W. D. (2009). Alterations in blood-brain barrier permeability to large and small molecules and leukocyte accumulation after traumatic brain injury: effects of post-traumatic hypothermia. *Journal of Neurotrauma, 26*, 1123-1134.

Kawata, K., et al. (1993). Experimental study of acute spinal cord injury: a histopathological study. *No Shinkei Geka*, 21, 45-51.

Mautes, A.E., Weinzierl, M.R., Donovan, F., and No-ble, L.J. (2000). Vascular events after spinal cord injury: con- tribution to secondary pathogenesis. *Physical Therapy*, 80, 673–687.

Nelson E., Gertz S.D., Rennels M.L., Ducker T.B., Blaumanis O.R. (1977). Spinal cord injury. The role of vascular damage in the pathogenesis of central hemorrhagic necrosis. *Archives of Neurology*, 34, 332–333.

Simard, J. M., Kilbourne, M., Tsymbalyuk, O., Tosun, C., Caridi, J., et al (2009). Key role of sulfonylurea receptor 1 in progressive secondary hemorrhage after brain contusion. *Journal of Neurotrauma*, 26, 2257-2267. 1.

Spinal Cord Injury (SCI) Facts and Figures at a Glance. *National Spinal Cord Injury Statistical Center.* August 2014. Accessed February 2016. http://www.nscisc.uab.edu.

Tator, C. H., & Fehlings, M. G. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. *Journal of Neurosurgery*, 75, 15-26.

Tator, C.H., Koyanagi, I. (1997). Vascular mechanisms in the pathophysiology of human spinal cord injury. *Journal of Neurosurgery*. 86, 483-492.

Turtle, T., Strain, M., Reynolds, J., Huang, Y., Garraway, S., Grau, J. (2015). Nociceptive stimulation induces caspase 1 activation following spinal cord injury. Abstract retrieved from *National Neurotrauma Society* online abstract archive. (Accession No. 10260)