THE EFFECT OF PERIPHERAL PAIN ON SPINAL CIRCUITS FOLLOWING SPINAL CORD INJURY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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Events soon after spinal cord trauma alter spinal cord function and drastically impact functional outcomes. Guided by our work in a transection model of spinal learning, we hypothesized that uncontrollable noxious input (C fiber activation) undermines spinal function by engaging a pro-inflammatory state (central sensitization). In a clinically relevant model of spinal cord injury (SCI), C fiber input exacerbates inflammatory processes within the lesion, increases cell death, and impairs functional outcomes. However, the mechanisms that underlie increased cell death and inflammation following C fiber activation have not been fully described. Specifically, the extent to which descending, brain-mediated processes are required is unknown. Here, we examined whether local spinal circuits, or descending brain-mediated processes, contribute to the increased inflammation associated with C fiber activation. In a contusion model of SCI, a spinal transection was used to isolate the spinal cord from the brain. Electrical stimulation of the tail provided C fiber activation. Examination of hemoglobin, IL-18, and IL-1β concentrations revealed that complete spinal transection reversed the detrimental effects of C fiber input. These results suggest that brain-mediated processes are required for the development of secondary injury cascades associated with C fiber activation following SCI.

CHAPTER I

INTRODUCTION

Spinal cord injury (SCI) is a challenge that impacts the lives of over 240,000 people in the U.S. alone, with an average of 12,500 new cases per year. There are many complications associated with a spinal trauma, foremost of which is the rigorous and difficult process of recovery. Another common complication is the development of chronic neuropathic pain. Peripheral tissue damage and inflammation, known as polytrauma, is common with SCI. This secondary injury provides a source of pain input (noxious stimulation) through ascending C fibers. Understanding how the processes that accompany SCI unfold, and the impact they have on recovery, will equip us to better treat the detrimental long-term effects of spinal trauma.

Recent work suggests that nociceptive input undermines recovery in a contusion model of spinal cord injury (Grau, Washburn, & Hook, 2004). Further, it has been shown that noxious C fiber input following SCI exacerbates inflammatory processes within the lesion, increases cell death, and induces neuropathic pain (Garraway et al., 2014). It has been suggested that these detrimental effects are due to the activation of secondary injury cascades, characterized by hemorrhage and increased cell death (Gerzanich et al., 2009). Hemorrhage is thought to occur as a result of progressive hemorrhagic necrosis (PHN) (Simard et al., 2007), while the increased cell death is believed to be a result of the pro-inflammatory form of cell death known as pyroptosis (Vacarri et al., 2008).

Progressive hemorrhagic necrosis (PHN) is one of the most devastating secondary injury pathways and reflects the destruction of the neurovascular membranes resulting in massive hemorrhage (Simard et al., 2007). This pathway is characterized by the formation of SUR1-TRPM4 channels. SUR1-TRPM4 is a heteromer formed by the co-assembly of the SUR1 receptor protein with the TRPM4 calcium channel (Woo et al., 2013). The formation of this heteromer has been shown to play a destructive role following SCI, which can be prevented with the SUR1 antagonist glybenclamide (Simard et al., 2013).

Pyroptosis is an inflammatory form of cell death characterized by the activation of caspase 1 and the processing of IL-1β and IL-18 from their precursor forms into the biologically active mature forms, and is thought to play a central role in the increased cell death seen with C fiber activation following SCI. Inhibition of this pyroptotic cell death cascade has been shown to improve functional outcomes following SCI (Vacarri et al., 2008).

Prior work in our laboratory has shown that in a spinal transection model of neuronal learning, nociceptive stimulation impairs plasticity. However, while C fiber stimulation causes hemorrhage and cell death in a contusion model of spinal cord injury, there is no evidence of hemorrhage or cell death occurring in the transection model of spinal learning. This suggests that in a contusion model of injury, spared fibers may contribute to the detrimental effects of C fiber activation. It is unknown whether the pro-inflammatory state that occurs following contusion injury is localized within the spinal cord or spreads to other regions of the body. The present proposal explores these questions by comparing the impact of noxious stimulation in subjects that have undergone a contusion injury alone or a contusion injury followed by a full spinal cord

transection. If spared fibers play an important role, this may impact the development of secondary injury and lead to the creation of novel therapeutic treatments.

CHAPTER II

METHODS

Subjects

The study was conducted using Sprague-Dawley rats, purchased from Envigo (Houston, TX) and acclimated for at least 7 days prior to experimentation. All subjects were adult males, weighing approximately 308g to 373g. Subjects were dual housed with water and food ad libitum and maintained on a 12-hour light-dark cycle. Behavioral testing and surgeries were performed during the light portion of the cycle. All experiments and procedures followed the NIH standards for the care and use of laboratory animals (NIH publication No. 80-23) and were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. We worked to ensure that the minimal number of subjects was used, and that subjects did not experience undue suffering.

Procedures

Contusion Surgery

Subjects were anesthetized with 5% isoflurane using an induction chamber. An area approximately 6 cm wide was shaved from the base of the skull to the tail. After shaving, the surgery area was cleaned with iodine and alcohol. Anesthesia was continued with 2-4% isoflurane administered through a nose cone. An incision was made 6 cm around the twelfth thoracic vertebra (T12). Next, an incision was made on either side of the spinal column cut to the depth of the rib cage. 1cm lateral incisions were made immediately above the T10 vertebra and below the T13 vertebra. The superficial tissue within the surgery area was then removed from the

dorsal surface of the spinal column with rongeurs. After cleaning tissue from the surgery area, a laminectomy was performed on the T12 vertebra and the caudal half of the T11 vertebra. The surgery area was gently cleaned with sterile gauze. The MASCIS device was used to perform the contusion injury (Gruner, 1992; Constantini and Young, 1994). Clamps on the MASCIS device were inserted into the previously made incisions, and the subjects were suspended by their spine. The spine was aligned and pulled taut prior to set up of the impactor. The impactor was then centered on the exposed cord. The contusion injury was performed by dropping the 10-g impactor (2.5 mm head) from a height of 12.5 mm directly onto the exposed cord. Following contusion, the surgery site was closed with Michel clips. Subjects were administered 100,000 units/kg of penicillin and 3 mL of saline after surgery through intraperitoneal injection to prevent infection and account for blood loss that occurred during the procedure.

Behavioral Analysis

The locomotor activity of subjects was scored on the Basso, Beattie, and Bresnahan (BBB) scale (Basso et al., 1995) to obtain a measurement of injury severity.

Transection Surgery

Twenty-four hours following contusion, subjects received a secondary transection surgery rostral to the contusion injury (T2). Subjects were anesthetized with 5% isoflurane using an induction chamber. Once under anesthesia, subjects were transferred to the nose cone where anesthesia was decreased to 2-4% isoflurane. Following palpation of the T2 vertebra, an incision was made 6cm around of the T2 vertebra. A deep V cut was made originating just rostral to the T2 vertebra and extending caudally. Spreaders were used to open the surgery site. Rongeurs were used to

clear away tissue back to the spinous process of the T2 vertebra, where it was then followed down to the cord. Impeding tissue was removed and the cord was exposed. The exposed cord was then transected. Transections were performed with the Thermal Cautery Unit, manufactured by Geiger. Next, the wound was closed with Michel clips. Subjects were administered 3 mL of saline following surgery. Subjects who underwent a sham surgery had the procedure replicated in its entirety, barring the transection.

Electrical Stimulation

Subjects received six minutes of mild electrical stimulation applied to the tail (180 stimuli; 100 ms; 0.2 - 3.8 s ISI) at an intensity that engages C fibers (1.5 mA) (Thompson et al., 1990; Hathway et al., 2009). Because all subjects received spinal injury, the flow of pain signals to the brain was muted and little pain should have been perceived. Unshocked subjects were treated identically, but did not receive any electrical stimulation.

Tissue Preparation

Tissue Collection

Subjects were sacrificed by intraperitoneal administration of pentobarbital (100 mg/kg). The tissues of interest were harvested and then flash frozen in liquid nitrogen. We collected spinal tissue from the lesion site, rostral to the injury, and caudal to the injury. Additionally, we collected the brain, spleen, adrenal glands, kidneys, liver, tibialis anterior muscle, cervical spinal cord and blood from the subjects.

Protein Extraction

Protein was isolated from the collected tissue using Trizol RNA extraction followed by a protein extraction using the Qiagen kit.

Assays

Bradford Assay

The concentration of the extracted protein was determined using the Bradford assay. Protein concentrations were diluted to a final concentration of 3 μg/μl in 4X Lamelli buffer.

Western Blot

Immunoblotting was performed using pre-cast 26-well 15% Tris-HCl Criterion gel according to the manufacturer's instructions. Protein samples were heated for 10 minutes at 96°C prior to being loaded into the gels. Proteins were pulled down at a constant voltage of 180V for 75 minutes in chilled running buffer. Gels were then removed and washed in transfer buffer. Transfer to an activated nitrocellulose membrane was achieved at a constant voltage of 100V over the course of an hour. The transfer solution was kept chilled with an ice pack, which was changed after 30 minutes. Following transfer, extraneous areas of the membranes were removed and the biotinylated ladder was separated. The membranes were submerged in blocking solution (5% milk in TBST) for an hour. The primary antibody was then added, with the targets of interest for this study being IL-18, IL-1β, and Hgb. Membranes were incubated in primary antibody overnight. The following day, membranes were washed 3X for ten minutes in TBST. Secondary antibody solutions were then added and allowed to incubate for an hour. Another

series of TBST washes were performed prior to visualization with Enhanced Chemiluminescence (ECL).

CHAPTER III

RESULTS

Current research suggests that secondary injury cascades that occur in SCI, such as pyroptosis and PHN, are exacerbated by noxious input associated with secondary injuries. This experiment examines whether the activation of these processes is brain-dependent or occurs independent of central processing.

Procedure

Twenty-four rats received a spinal contusion at T12. Eighteen hours following contusion, locomotor function was assessed with the BBB scoring system and used as an indicator of injury severity. Subjects were randomly assigned to one of four groups (transection/shock, transection/unshock, sham/shock, sham/unshock; n=6) such that BBB scores were balanced (randomized block design). Subjects then underwent either a transection surgery or a sham procedure. Six hours following the second surgery, subjects received either electrical stimulation or no stimulation. Three hours following electrical stimulation, subjects were sacrificed and the tissues of interest were collected.

Injury Site Tissue

Images of protein extracts revealed a visual difference between groups (Figure 1). Replicating prior work, the extracts of subjects receiving electrical stimulation without spinal transection were notably dark red. However, spinal transection seems to have prevented this effect.

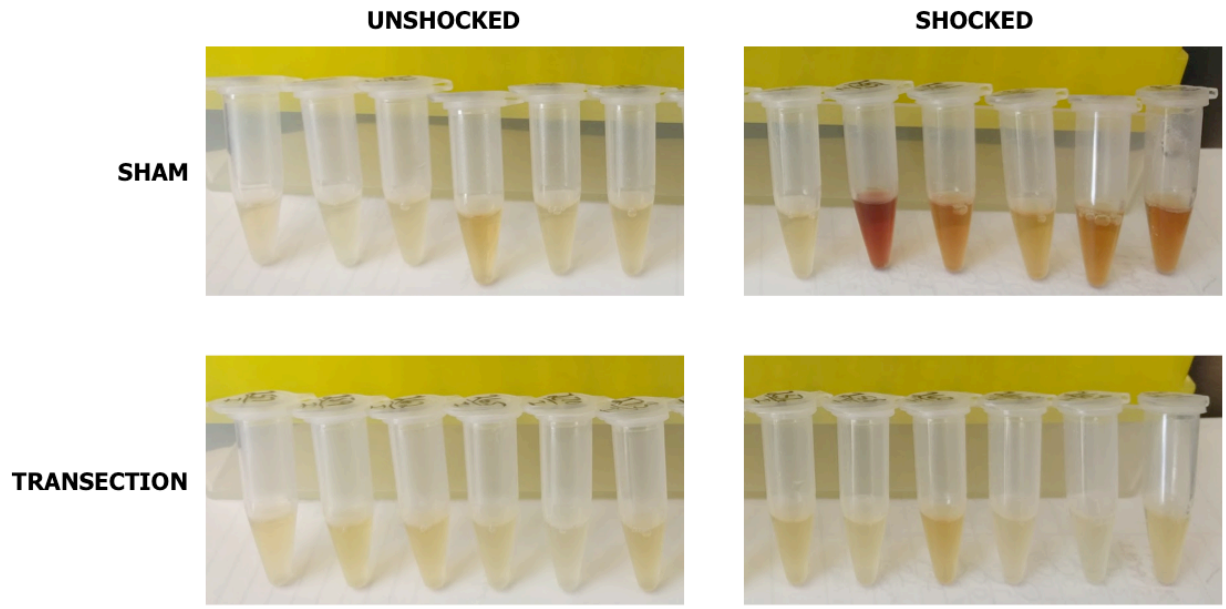

Figure 1. Color difference seen in protein extracts. *Top Left*- Sham/Unshocked subjects, whose extracts are notably clear. *Bottom Left*- Transection/Unshocked subjects, displaying normal clear extracts. *Top Right*- Sham/Shocked subjects, who have distinguishably redder extracts. *Bottom Right*- Transection/Shocked subjects, exhibiting a complete reversal of the blood infiltration seen in the sham/shocked subjects.

To quantify this color difference, full spectral analysis (FSA) was performed using the NanoDrop system (Figure 2). Absorbance values were recorded for 420nm, the wavelength that correlates with hemoglobin absorbance (Figure 3). These values were then subjected to statistical analysis. An analysis of variance (ANOVA) revealed a significant interaction between stimulation and surgery condition, $F(1,20) = 5.37$, $p < .05$.

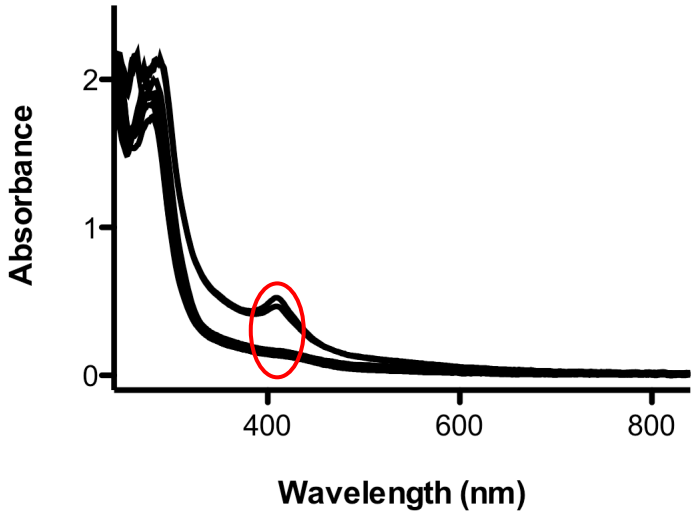

Figure 2. FSA absorbance of injury site protein for all subjects. An absorbance peak around 420nm was seen in the subjects that underwent a sham surgery and received electrical stimulation (circled).

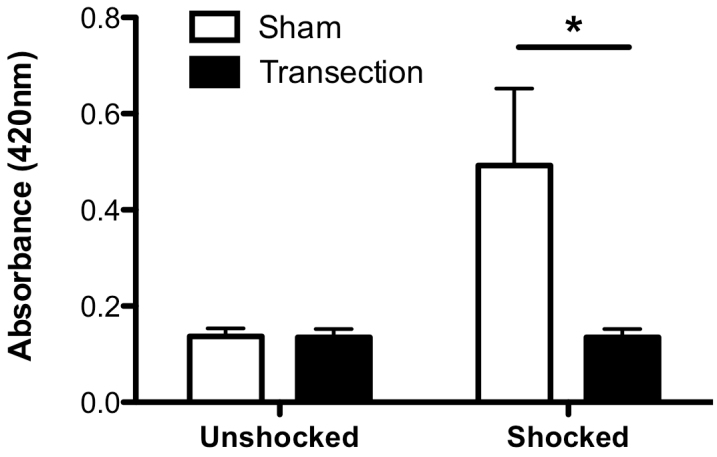

Figure 3. Graphical representation of injury site protein extract absorbance at 420nm. Transection/shock subjects, and unshocked groups, displayed significantly less absorbance at 420nm than sham/shocked subjects. * indicates a significant difference with a p-value < .05. Error bars represent SEM (n=6).

Hemoglobin concentration was examined by immunoblotting in order to verify that 420 nm absorbance correlates with blood infiltration (Figure 4). An ANOVA yielded a significant main effects of surgery condition and stimulation, and a significant interaction between stimulation and surgery condition, all Fs > 9.94, p < .05. This confirms that blood is infiltrating the spinal

cord in sham subjects that received electrical stimulation, while transected subjects were protected from this effect.

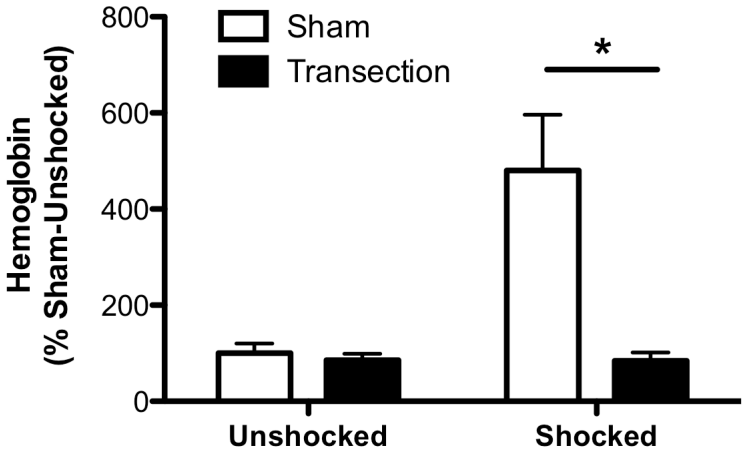

Figure 4. Concentration of hemoglobin in the injured cord. Sham/shocked subjects showed significantly higher levels of hemoglobin, with transection reversing this infiltration. * indicates a significant difference with a p-value < .05. Error bars represent SEM (n=6).

The mature forms of IL-18 and IL-1β were used as markers of inflammation and cell death. The concentrations of IL-18 and IL-1β were obtained using immunoblotting and subjected to statistical analysis (Figure 5). An ANOVA examining the percent mature IL-18 concentration revealed significant main effects of surgery condition and stimulation, and a significant interaction between surgery condition and stimulation, all $Fs > 5.98$, $p < .05$. Analyses of the percent mature IL-1β concentration displayed significant main effects of surgery condition and stimulation as well as a significant interaction between surgery condition and stimulation, all $Fs > 12.63$, $p < .05$. This indicates that spinal transection blocked the pyroptotic effects of nociceptive input on the injured cord, in addition to blocking hemorrhage.

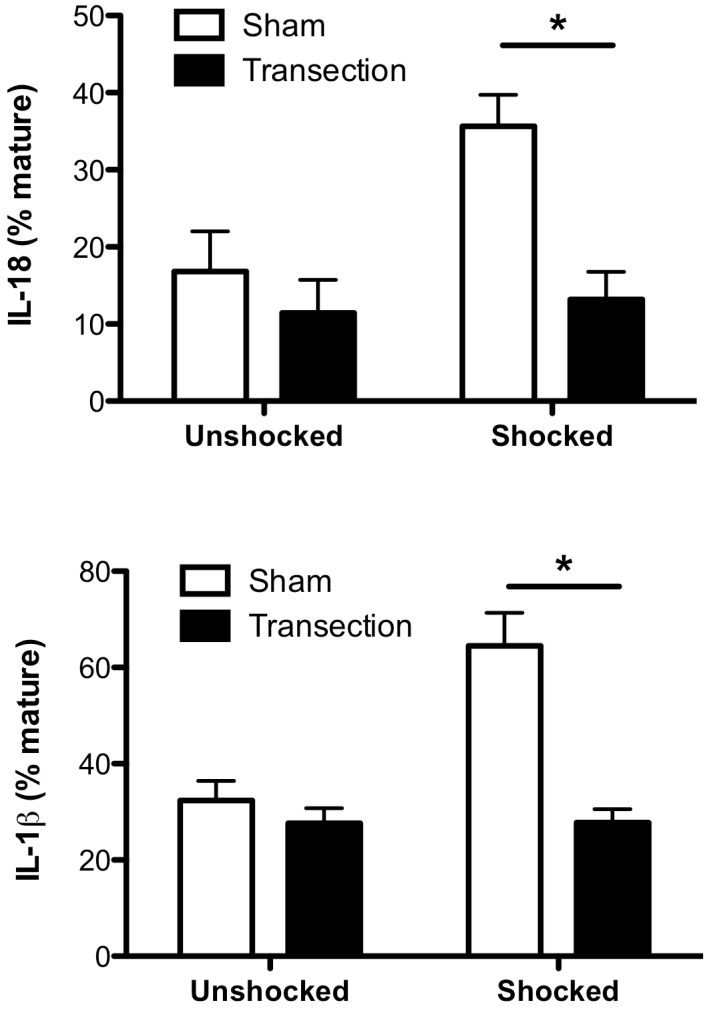

Figure 5. Concentration of percent mature IL-18 and IL-1β in the injured cord. *Top*- Sham/shocked subjects exhibited an increase in relative mature IL-18 concentration, while transection/shocked subjects were protected from this effect. *Bottom*- This protective effect is seen again in IL-1β, with sham/shocked subjects having a significantly higher concentration of mature protein when compared to transection/sham subjects. * indicates a significant difference with a p-value < .05. Error bars represent SEM (n=6).

Cervical Tissue

Absorbance of cervical protein extract was measured using full spectral analysis (Figure 6). Values for absorbance at 420 nm was recorded and subjected to statistical analysis. An ANOVA showed that neither the main effects of treatment condition, nor their interaction, approached significance, all Fs < 1.00, p > 0.05.

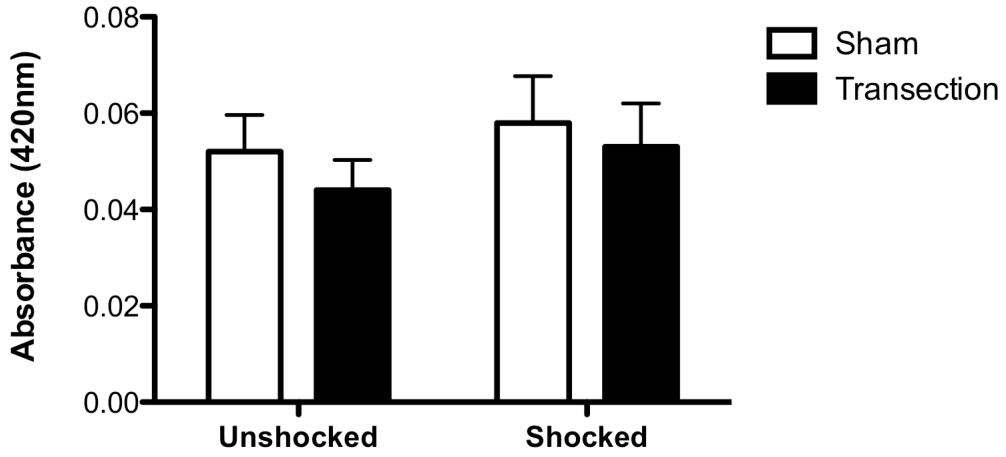

Figure 6. Graphical representation of cervical protein extract absorbance at 420nm. Protein extracts of sham/shocked subjects displayed the largest amount of absorbance at 420nm, but this effect was minimal in the cervical cord. Error bars represent SEM (n=6).

Hemoglobin concentration was measured by immunoblotting, and the relative values analyzed by ANOVA (Figure 7). Here too, neither the main effect of shock treatment, nor a main effect of surgery condition, was statistically significant, both $Fs < 1.00$, $p > .05$. However, there was a trend towards an interaction between shock treatment and surgery condition, $F(1,20) = 2.63$, $p < .12$. This indicates that hemorrhage is occurring in the cervical region of the spinal cord, although not at the levels seen at the injury site.

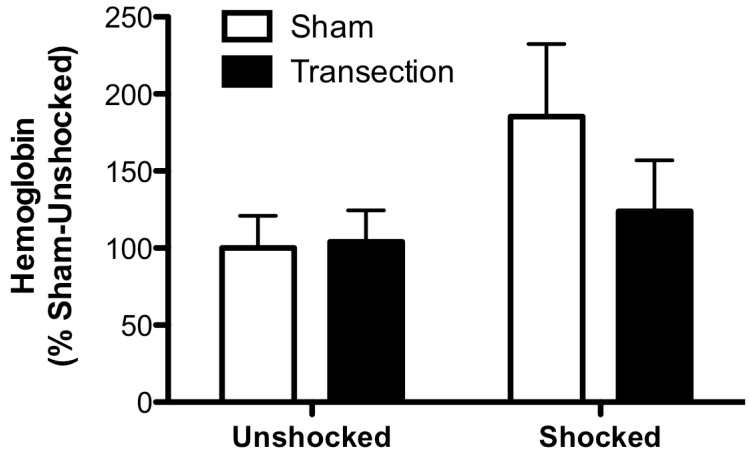

Figure 7. Concentration of hemoglobin in the cervical cord. Sham/shocked subjects show a trend towards increased levels of hemoglobin. Transection appears to reverses this effect. Error bars represent SEM (n=6).

Concentration of mature IL-18 and IL-1β was measured using immunoblotting, and percent mature values were recorded (Figure 8). Here, too, an ANOVA did not reveal any statistically significant effects, all Fs < 1.00, p > .05. The same held true for IL-1β, all Fs < 1.00, p > .05.

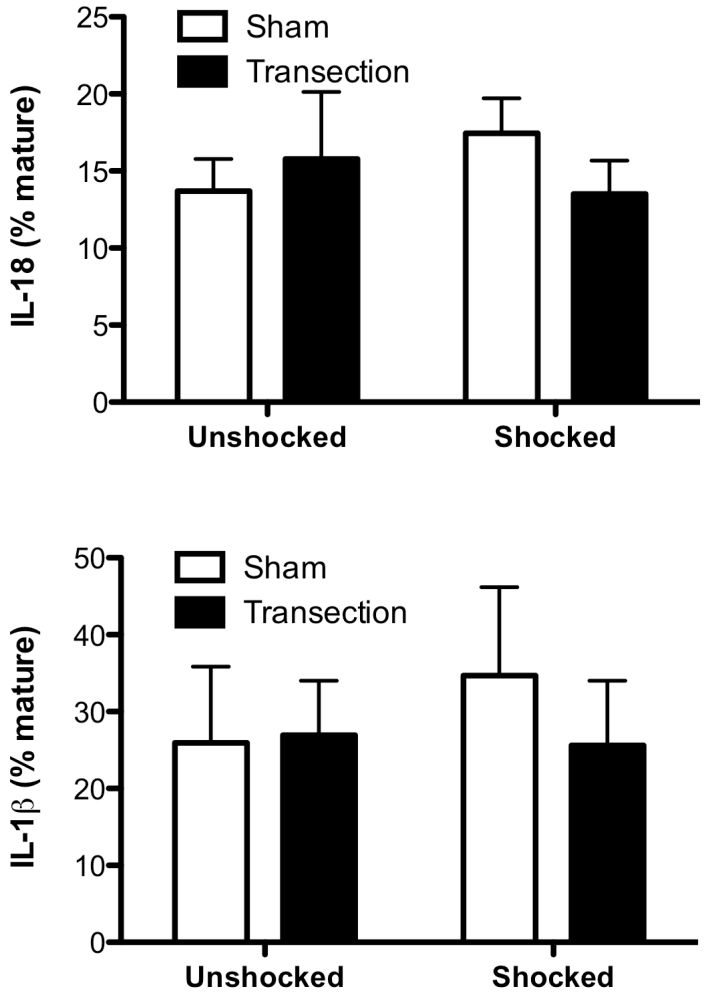

Figure 8. Concentration of IL-18 and IL-1β in the cervical cord. *Top*- Sham/shocked subjects trended towards an increase in relative IL-18 concentration. *Bottom*- Sham/shocked subjects had a higher concentration of IL-1β when compared to transection/sham subjects However, this effect was not significant. Error bars represent SEM (n=6).

CHAPTER IV

CONCLUSION

Prior research has shown that noxious stimulation following SCI negatively impacts long-term prognosis (Grau et al., 2004). Ongoing research is exploring the molecular pathways that mediate these effects. From prior work, it was not known whether brain-dependent processes play a critical role through spared fibers. It was also not known whether noxious input after injury affects tissue regions outside of the injury site.

Injury site protein extracts from subjects receiving electrical stimulation after spinal cord injury appeared visually darker, and full spectral analysis revealed that noxious stimulation in sham subjects resulted in prominent absorbance at 420nm. This reinforces our previous findings that C fiber activation following SCI increases hemorrhage and blood infiltration within the spinal cord. Interestingly, complete spinal transection fully reversed this observed peak, indicating that hemoglobin infiltration only occurred in the subjects who had access to central processing and underwent C-fiber activation. This is further supported by the increased levels of hemoglobin observed in sham/shocked subjects compared to transected/shocked subjects. Increased hemoglobin levels within the injury site indicate that hemorrhagic processes occurred, potentially through progressive hemorrhagic necrosis. Cervical protein extracts did not exhibit a notable difference in color, nor a significant difference in absorbance at 420nm. However, the trend towards increased levels of hemoglobin suggest that some amount of hemorrhage may still be occurring in the cervical cord. Spinal transection at T2 blocked the upward trend of hemoglobin infiltration that was seen in sham subjects who received C fiber stimulation.

The idea that brain-dependent processes influence the development of secondary injury was also supported by the effect of spinal transection on pro-inflammatory signal pathways. In the contused spinal cord, C fiber stimulation increased IL-18 and IL-1β processing within the lesion site. IL-18 and IL-1β processing appears to have been increased by noxious input in the cervical cord as well, although to a lesser degree. Rostral transection at T2 blocked the C fiber stimulation-induced increase in IL-18 processing as well as IL-1β processing

Based on these findings, we conclude that spared fibers contribute to the detrimental effects of C fiber activation following spinal cord injury. While my assays did not reveal any significant differences when a remote (cervical) region of the spinal cord was assessed, this could reflect a lack of statistical power. To address this issue, further work is needed to bolster the sample size. Finally, additional studies are needed to identify effective treatments based on my findings. The current work suggests the intriguing possibility that temporarily blocking afferent/efferent transmission after SCI (emulating the effect of a spinal transaction) could reduce tissue loss at the site of injury.

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