C-FIBERS ARE NOT NECESSARY FOR THE DEVELOPMENT OF PAIN-INDUCED HEMORRHAGE FOLLOWING SCI

An Undergraduate Research Scholars Thesis

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ABSTRACT

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Spinal cord injuries (SCI) are commonly accompanied by additional injuries (polytrauma), such as lacerations, bone fractures, and burns. These injuries serve as origins for pain (nociceptive) input to the spinal cord. Previous work has shown that this nociceptive input undermines locomotor recovery and exacerbates the infiltration of blood into the spinal cord (hemorrhage) after injury. Hemorrhage within the spinal cord then further damages the tissue, leading to an increase in the size of injury. Prior studies have found that engaging nociceptive fibers through intermittent electrical stimulation (shock), or application of the irritant capsaicin, is sufficient to induce hemorrhage in the spinal cord. While electrical stimulation activates both myelinated A-delta and unmyelinated C-fibers, capsaicin selectively binds C-fibers through the transient receptor potential vanilloid receptor-1 (TRPV-1). Therefore, as C-fiber activation has been shown to be sufficient to induce hemorrhage, this study served to determine if it is necessary. To eliminate C-fibers while leaving A-delta fibers intact, three excitotoxic doses of capsaicin were administered 12 hours apart. Thirteen days later, rats received a moderate T12 contusion injury. The next day they were exposed to intermittent tail shock or nothing (unshocked). Locomotor scores and hemodynamic measurements were collected at 3 one-hour

time points following nociceptive stimulation. Rats were then sacrificed, and the injured region of the spinal cord was collected for tissue analysis. Drabkin's assay was used to measure hemoglobin content in each sample. Ablating C-fibers attenuated the cardiovascular response to nociceptive stimulation and its adverse effect on locomotor performance. It did not, however, attenuate pain-induced hemorrhage.

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NOMENCLATURE

SCI	Spinal Cord Injury
BSCB	Blood-Spinal Cord Barrier
TRPV-1	Transient Receptor Potential Vanilloid Receptor-1

CHAPTER I

INTRODUCTION

Spinal cord injury (SCI) is a devastating affliction that can leave individuals with long-term motor and sensory deficits. These injuries are often the result of traumatic accidents such as car wrecks or explosions in combat. As a result, those individuals who suffer a spinal cord injury also experience a multitude of other injuries, including broken bones, lacerations, and burns (polytrauma). These accompanying injuries serve as sources of pain (nociceptive) input through the spinal cord. Prior work has demonstrated that nociceptive stimulation after injury increases tissue loss at the site of injury and impairs long term recovery (Grau et al., 2004, Grau et al., 2017).

Researchers have explored the effect of pain input on recovery after SCI using rats that have received a contusion injury of the lower thoracic (T12) spinal cord. A day after injury, nociceptive fibers below the injury are engaged by applying electrical stimulation at an intensity that engages myelinated (A-delta) and unmyelinated (C) nociceptive fibers or the irritant capsaicin. Capsaicin, the active ingredient in chili peppers, engages C-fibers that express the transient receptor potential vanilloid receptor-1 (TRPV-1). Both forms of noxious stimulation expand the area of tissue loss (secondary injury) and impair long-term locomotor recovery (Grau et al., 2017, Turtle et al., 2019)

Further studies linked pain-induced tissue loss after SCI to a breakdown of the blood spinal cord barrier (BSCB) and an infiltration of blood into the spinal cord (hemorrhage) (Turtle et al., 2018, Turtle et al., 2019). As blood is neurotoxic, hemorrhage at the site of injury kills

surviving neurons, increasing the area of tissue loss. This in turn would undermine long-term recovery.

Nociceptive stimulation is transmitted through two types of nerve fibers, A-delta fibers and unmyelinated C-fibers. While electrical stimulation indiscriminately activates both A-delta and C-fibers, capsaicin selectively engages C-fibers. C-fiber release of the neuropeptide substance P has previously been shown to impact the formation of edema following traumatic brain injury (TBI) by increasing the vascular permeability of the blood-brain barrier, leading to further motor deficits (Nimmo et al., 2004, Donkin et al., 2009). Treatment with either a TRPV-1 antagonist, or inducing C-fiber neuropeptide depletion, both blocked the neurogenic inflammation following TBI and significantly decreased the permeability of the blood-brain barrier. Therefore, since the activation of C-fibers has been shown to be sufficient in producing hemorrhage following SCI and has demonstrated a connection to increased vascular permeability of the blood-brain barrier, this study sought to determine if C-fiber activation is necessary to produce the hemorrhage effect.

Rats received a systemic injection of capsaicin at a dosage that was previously shown to ablate C-fibers (Krahl et al., 2001). Thirteen days later, C-fiber ablation was confirmed prior to spinal contusion. 24-hours after injury, animals then received 6 min of uncontrollable intermittent electrical stimulation to the tail or nothing (unshocked). Electrical stimulation was applied at an intensity that would normally engage both A-delta and C-fibers. The current study will establish whether electrical stimulation affects locomotor performance, cardiovascular function, or hemorrhage after C-fibers have been ablated.

CHAPTER II

METHODS

Subjects

Adult male Sprague-Dawley rats (100-120 days old) were purchased from Envigo (Houston, TX). Rats were then acclimated to handling and open enclosures used for behavioral assessment at least 7 days prior to experiment. Prior to contusion injury, rats we housed in pairs with water and food *ad libitum* and maintained on a 12-hour light-dark cycle. Subjects were also acclimated to blood pressure testing prior to experimentation. Surgeries and testing we carried out during the light portion of the cycle. All experiments were carried out in accordance with National Institutes of Health (NIH) standards for the care and use of laboratory animals (NIH Publication 80-23) and were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Every effort was made to minimize suffering and limit the number of animals used to what was scientifically necessary.

Capsaicin Treatment and C-fiber Assay

Subjects received capsaicin or vehicle (10% Tween 20, 10% ethanol, and 80% saline) injections administered subcutaneously while under isoflurane anesthesia in three separate injections (25, 50, and 50 mg/kg capsaicin) (Krahl et al., 2001). The three injections were spaced 12 hours apart. Experimental procedures were carried out 14 days following capsaicin treatment.

On the day prior to experimentation, a corneal chemosensitivity test was given to both capsaicin and vehicle groups to assess the effectiveness of C-fiber ablation from capsaicin treatment. A drop of 1% ammonium hydroxide (NH_4OH) was placed into both eyes and the

number of eye wipes in a 10 second interval were counted. Number of eye wipes were compared between capsaicin and vehicle-treated subjects to determine the success of treatment.

Surgery

Rats received a contusion injury at the level of the T11–12 vertebrae using the NYU MASCIS device. Animals were anesthetized with 5% isoflurane gas and a surgical level of anesthesia was maintained with 2–3% isoflurane. A longitudinal incision extending approximately 2 cm rostral and caudal to the injury site was made on both sides of the vertebral column. The T11–12 vertebrae were then palpated and exposed. A laminectomy was performed, exposing the spinal cord while keeping the dura intact. The vertebral column was held steady and a 10 g impactor was dropped onto the spinal cord from a height of 12.5 mm. Following surgery, the incision was closed with Michel clips and rats were administered 100,000 units/kg of penicillin and three ml of saline to prevent infection and replace lost fluids.

For the 24 h following surgery, animals were housed individually in a temperature-controlled room and allowed to recover overnight. After surgery, animals had free access to food and water. Bladders were checked at regular intervals (every 8–10 h after surgery and 2–4 h during testing) and expressed. While prior work has shown that nociceptive stimulation delays the recovery of bladder function (Grau et al., 2004), these effects were observed weeks after injury. Because animals in the present study were sacrificed within 48 h of injury, all required expression after surgery.

Noxious Stimulation

Prior work has established that a brief (6 min) exposure to intermittent electrical stimulation can induce a form of maladaptive plasticity and impair recovery after a contusion injury (Grau et al., 2004; Hook et al., 2008). An advantage to this procedure is that both the

duration and intensity of stimulation can be readily controlled. Further, there is no tissue damage at the intensity used. In the present study, stimulation was applied to the tail while the rat was loosely restrained in an opaque Plexiglas tube. The electrodes were coated with electrode gel and taped to the tail centered approximately 3.5 cm from the tip. Using a constant current shocker, animals (Shocked) received 180 shocks, 100-msec in duration on a variable inter-stimulus interval that ranged from 0.2–3.8 s (mean 2 s). After the last shock, the electrodes were removed, and the rat was returned to a holding bin. Unshocked controls were treated the same, except they received no electrical stimulation.

Locomotor Scores

Before surgery, animals were acclimated to a 45-inch plastic pool on three separate days for four minutes. Locomotor function was then assessed, beginning the day following contusion, using the Basso, Beattie, Bresnahan (BBB) scoring system. Animals were scored prior to shock to establish baseline scores, and then at 0, 1, 2, and 3 hours following shock.

Blood Pressure Analysis

Before surgery, rats were acclimated on three separate days to the blood pressure assessment system. Animals were placed in clear acrylic tubes with adjustable nose cones atop a warming platform (Kent Scientific). Blood pressure measurements were obtained using the CODA High Throughput Noninvasive Blood Pressure System and data acquisition software. Measurements were taken prior to shock to establish baselines, and then at 0, 1, 2, and 3 hours following shock.

Tissue Collection

Rats were euthanized with 100 mg/kg of pentobarbital 3 hours after the application of nociceptive stimulation. One centimeter of spinal cord tissue centered at the lesion was dissected

and flash frozen in liquid nitrogen. Protein was extracted using the QIAzol lysis reagent according to the manufacturer's instructions.

Spectrophotometric Hemoglobin Analysis

Spectral analyses for free hemoglobin were conducted on protein extracts from lesion tissue. Spectrophotometric absorbance was measured from 200 to 800 nm from 1.0 µl of protein extract (NanoDrop, Thermo Scientific). Absorbance at 420 nm was used as a measure of hemoglobin content (Prahl, 1999). A second photometric assay based on cyanmethemoglobin colorimetry was conducted to verify that the absorbance reflected the in- filtration of blood. Briefly, 10 µl of protein extract was added to 40 µl of Drabkin's reagent (Ricca Chemical, #RC266032) and incubated at room temperature for 20 min. 1 µl of each sample was loaded onto a spectrophotometer and absorbance was assessed at 540 nm (as per the manufacturer's instructions), against a standard curve generated with known concentrations of native rat hemoglobin (Lifespan Biosciences, #LS-G11201-10, Seattle, WA) in Drabkin's reagent.

Statistics

All data were analyzed using analysis of variance (ANOVA) or analysis of covariance (ANCOVA). In all cases, a criterion of $p < .05$ was set as the threshold for statistical significance. For our primary measures, we also report the proportion of variance accounted for [eta squared (η^2)].

CHAPTER III

RESULTS

C-fiber ablation was verified using the corneal chemosensitivity task, which assess the eye-wipes elicited by the administration of one drop of 1% ammonium hydroxide to the right and left eyes (Krahl et al., 2001). As expected, ammonium hydroxide elicited eye-wipes in the vehicle animals (mean = 5.83 $\pm$ 0.78), but not in those that received capsaicin treatment (mean = 0.0 $\pm$ 0.0). A rank sum test confirmed that this difference was statistically significant, $Z = 3.81$ and $p < 0.0001$.

Locomotor performance was assessed prior to and after shock treatment. Prior to shock, there were no differences in locomotor performance between groups, $F(1, 20) < 1.0$, $p > 0.05$. Exposure to noxious electrical stimulation induced an acute impairment in locomotor performance and this effect was attenuated by C-fiber ablation (Figure 1). To control for variation in baseline locomotor performance, an analysis of covariance (ANCOVA) was performed with the baseline score serving as the covariate. This analysis revealed that both shock and capsaicin treatment had a significant effect, both F's > 16.55, $p < 0.001$. There was also a significant interaction between shock and capsaicin treatment, $F(1, 19) = 21.50$, $p = 0.0002$. The magnitude of these effects did not vary across time, all F's < 1.0, $p > 0.05$. *Post hoc* comparisons of the group means showed that the vehicle shocked animals differed from the other three groups ($p < 0.05$). In addition, the capsaicin shocked group differed from the capsaicin unshocked group ($p < 0.05$). No other group comparisons were significant ($p > 0.05$). The results imply that noxious stimulation disrupted locomotor performance and that this effect was attenuated, but not eliminated by, prior treatment with systemic capsaicin.

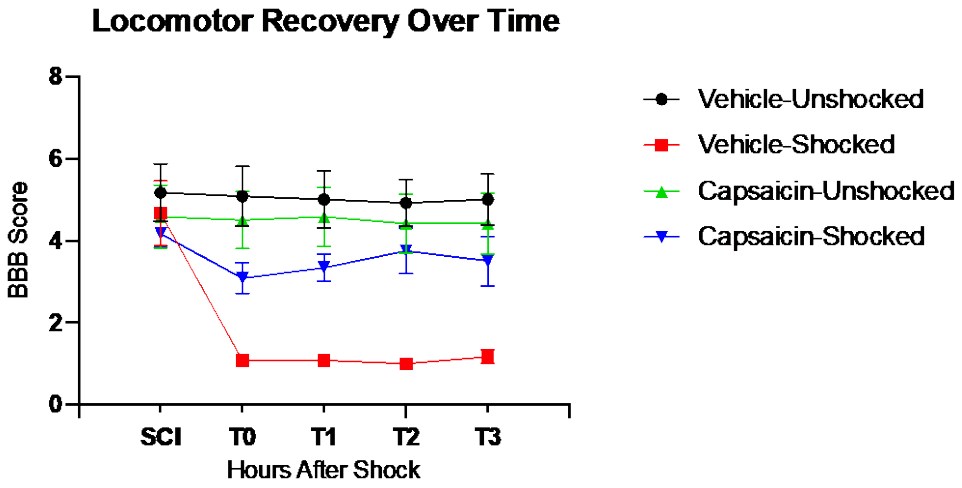

Figure 1. Ablating C-fibers attenuates the effect of nociceptive stimulation (shock) on locomotor performance. Exposure to shock induced an acute disruption in locomotor performance. This effect was attenuated, but not eliminated, by earlier treatment with systemic capsaicin.

Exposure to noxious stimulation is known to increase heart rate and blood pressure. The latter effect could contribute to nociception-induced hemorrhage. The study found that capsaicin treatment reduces baseline heart rate, $F(1, 20) = 6.63$, $p = 0.0181$. Ablating C-fibers also altered how shock affects heart rate, with shocked rats exhibiting a lower rate (Figure 2A). An ANCOVA using heart rate as a covariate showed that the interaction between shock and capsaicin treatment approached significance $F(1, 19) = 4.10$, $p = 0.052$. Furthermore, there was a significant change in heart rate over time, $F(3, 57) = 3.65$, $p = 0.0178$, and the nature of this effect varied with capsaicin treatment, $F(3, 57) = 5.17$, $p = 0.032$. *Post hoc* comparisons of the group means showed that the capsaicin and vehicle treated shocked groups differed ($p < 0.05$). No other group difference was significant ($p > 0.05$).

Treatment with capsaicin did not affect baseline systolic blood pressure, all F's < 2.00, $p > 0.05$. Nor did this variable reveal a significant effect of shock treatment (Figure 2B). An ANCOVA, with baseline systolic blood pressure serving as the covariate, showed that neither

shock nor capsaicin had a significant effect F's < 2.90, $p > 0.05$. In addition, neither effect varied over time, all F's < 2.18, $p > 0.05$. Systolic blood pressure over time is shown in Figure 2 (B).

Blood flow (Figure 2C) and blood volume (Figure 2D) were also assessed. Capsaicin treatment did not affect baseline blood flow, all F's < 2.30, $p > 0.05$. There was an effect of shock treatment on blood flow and the magnitude of this effect varied with time, $F(3, 57) = 3.70$, $p = 0.0167$. No other term approached statistical significance, all F's < 2.51, p > 0.05.

The results for blood volume paralleled what was found for blood flow. The volume of blood flow did not differ prior to shock treatment, all F's < 1.56, $p > 0.05$. Here too, shock had a significant effect and the magnitude of this effect varied with time, $F(3, 57) = 2.88$, $p = 0.0436$. No other term approached statistical significance, all F's < 2.65, $p > 0.05$.

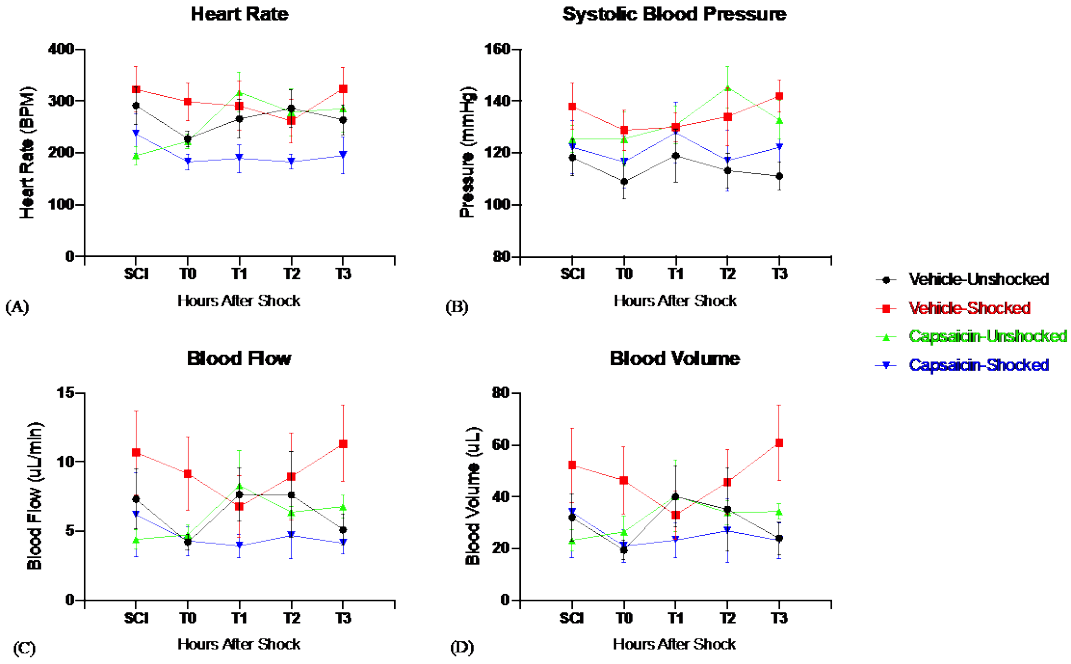

Figure 2. Effect of capsaicin and shock treatment on cardiovascular function. **(A)** The baseline heart rate of capsaicin treated animals was significantly reduced and capsaicin-shocked animals showed a significant reduction in heart rate over time. **(B)** No significant differences in systolic blood pressure were observed between treatment and shock groups at baseline and over time. Blood flow **(C)** and blood volume **(D)** measurements demonstrated nearly identical results. On both measures, there was a significant effect of shock treatment and the magnitude of this effect varied over time.

Three hours after noxious stimulation, the injured region of the spinal cord was removed and assessed for hemoglobin content. This was accomplished by assessing absorbance at the wavelength (420 nm) associated with hemoglobin and the Drabkin's assay. Results demonstrated that administration of shock induced a statistically significant increase in hemorrhage at the site of injury, independent of treatment group (Figure 3). Both capsaicin and vehicle treated groups showed an increase in hemorrhage compared to unshocked groups. Shock treatment significantly increased absorbance at 420 nm, $F(1, 20) = 5.69$, $p = 0.0271$. Likewise, a Drabkin's assay revealed a significant effect of shock, $F(1, 20) = 5.03$, $p = 0.0363$. No other term was statistically significant, all F's < 1.0, $p > 0.05$.

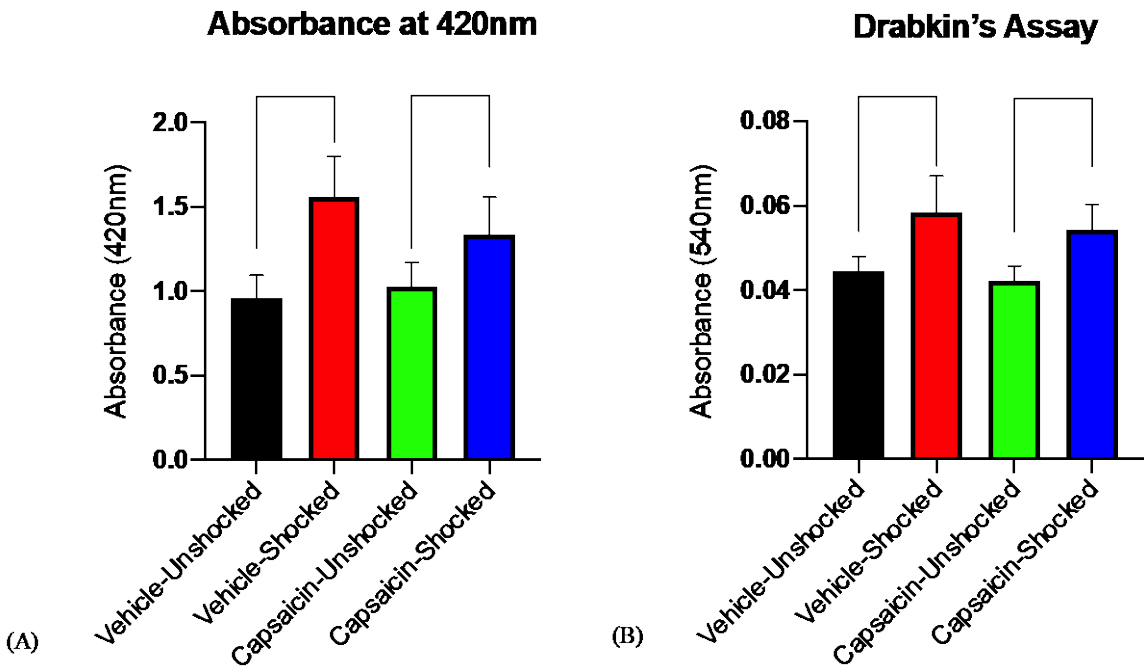

Figure 3. Effect of capsaicin and shock treatment on hemorrhage at the site of injury. **(A)** Absorbance at 420 nm demonstrated a significant increase in hemorrhage in vehicle-shocked animals, and a marked increase in capsaicin-shocked animals. **(B)** An identical pattern was observed with the Drabkin's Assay.

CHAPTER IV

DISCUSSION

Prior work has shown that pain input following SCI leads to an increase in hemorrhage at the injury site and undermines long-term recovery (Turtle et al., 2018). Furthermore, engaging nociceptive fibers using the TRPV-1 agonist capsaicin to selectively activate C-fibers was sufficient to induce hemorrhage and impair locomotor recovery. Therefore, the present study sought to evaluate the necessity of C-fibers in the development of pain-induced hemorrhage and locomotor deficits following SCI. This was explored using a noxious stimulus (intermittent electrical stimulation) that engages both myelinated (A-delta) and unmyelinated (C-fibers) nociceptive fibers. Results showed that ablating C-fibers using a neurotoxic dosage of capsaicin attenuates, but does not eliminate, the acute effect that noxious electrical stimulation has on locomotor performance. Pretreatment with capsaicin also affected the cardiovascular response but did not impact nociception-induced hemorrhage.

Myelinated A-delta fibers are involved in the body's quick response to pain. In addition, these fibers, when activated, initiate a stress and sympathetic nervous system response. This response then initiates a rise in blood pressure and heart rate. In the present study, capsaicin treatment lowered baseline heart rate and, paradoxically, induced bradycardia (lowered heart rate) in response to noxious stimulation. However, both blood flow and volume increased in shock groups, potentially indicating a compensatory mechanism to counteract lowered heart rate. Further studies into the effects of blood pressure on hemorrhage need to be performed to evaluate this potential effect. In addition, further investigation involving the production of similar hemodynamic effects, such as through sympathetic nervous system activation, while

excluding pain input, could shed light on the role of blood pressure and heart rate in pain-induced hemorrhage after SCI.

Interestingly, although both treatment groups experienced a significant increase in hemorrhage following shock, capsaicin treated animals did not demonstrate the same locomotor deficits over time as the vehicle treated animals. Vehicle treated animals exhibited a sharp decline in locomotor performance after shock treatment. While nociceptive stimulation adversely affected capsaicin treated animals, the magnitude of this effect was significantly less. The results suggest that the acute impairment in locomotor function is, to a large extent, tied to C-fiber input. An incomplete effect may have been observed either because hemorrhage was not attenuated, or some C-fibers were spared. Further work is needed to address these issues. The results also call into question the putative relation between nociception-induced hemorrhage and impairment of locomotor function.

As both shocked groups exhibited similar levels of hemorrhage, but did not demonstrate identical locomotor effects, additional work is needed to determine the processes that give rise to the locomotor deficit. Prior work has found a significant increase in proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) in response to pain input after SCI (Garraway et al., 2011, Garraway et al., 2014). TNFα activates the pro-apoptotic Caspase-8 and NFκB pathways, which could lead to further tissue loss in the spinal cord. Further protein analysis needs to be conducted to evaluate the concentrations of proteins related to cell death (caspase 1, 3 and 8) and TNFα. Additionally, experiments involving only the release of these proteins and cytokines, without prior pain-input, could help determine if their release is to blame for pain-induced locomotor deficits after SCI.

In conclusion, shock after SCI induced a significant increase of hemorrhage at the injury site in both capsaicin and vehicle treated groups, demonstrating that A-delta fibers are sufficient to produce this effect. However, effects on locomotor function did not present identically between the two groups. Although both experienced an overall locomotor detriment following the administration of shock, capsaicin treated animals experienced a milder reduction in locomotor function. C-fiber ablation therefore potentially attenuated this effect. Additionally, blood pressure and heart rate measurements produced unexpected results and need to be investigated further to determine their role in pain-induced hemorrhage and reduction in locomotor function following SCI.

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