THE INVOLVEMENT OF SUPRASPINAL SYSTEMS IN THE DEVELOPMENT OF PAIN-INDUCED SECONDARY INJURY AFTER SPINAL CORD INJURY

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2021

Major Subject: Neuroscience

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ABSTRACT

Pain input after spinal cord injury can be detrimental to acute and long-term processes. In a rodent model of incomplete spinal cord injury, it has been shown that nociceptive stimulation (pain input) administered below the injury can exacerbate secondary injury mechanisms, increasing tissue loss and expanding hemorrhage. Recent data suggests that supraspinal signals are involved in the development of secondary injury and long-term locomotor deficits. In this dissertation, I explored the role of brain-dependent processes in the development of hemorrhage at the spinal cord lesion site.

I first examined whether communication with the brain is required to induce hemorrhage and engage a cardiovascular response. Rats received a lower thoracic (T10-11) contusion injury followed by a rostral (T2) transection. A day after the contusion injury, nociceptive fibers were engaged by applying electrical stimulation (shock) to the tail or the irritant capsaicin to one hind paw. Noxious stimulation increased hemorrhage at the site of injury. This effect, and the rise in blood pressure/flow elicited by shock, were blocked by a rostral transection. Further, pharmacologically inducing a rise blood pressure with norepinephrine did not promote hemorrhage.

To explore whether noxious stimulation increases the permeability of the blood spinal cord barrier (BSCB), contused rats were injected with Evans blue. Exposure to shock allowed Evan's blue to enter the area of injury and this effect was blocked by a rostral transection.

The remaining experiments examined whether the adverse effects of noxious stimulation are driven by a brain-mediated pain state. Inhibiting pain with morphine did not attenuate the shock-induced hemorrhage or decline in locomotor performance. When the same noxious stimulus was applied rostral versus caudal to injury, only the latter produced evidence of increased hemorrhage. It was unclear, however, whether stimulation above the site of injury induced a comparable level of pain. To address this issue, a new procedure was developed wherein shock intensity was modified so that it elicited a comparable brain-dependent (vocalization) response. After verifying that shock at vocalization threshold induces hemorrhage when given caudal to injury, I assessed the effect of stimulation applied rostral to injury. Again, it did not induce hemorrhage.

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my advisor and committee chair, Dr. James Grau, for the continuous support of my Ph.D. research and development, for his patience, motivation, and immense knowledge. I would also like to thank my committee members, Dr. Rajesh Miranda, Dr. Michelle Hook, and Dr. Kayla Bayless, for their invaluable feedback and support throughout the course of this research. Their plentiful experience has encouraged me in all the time of my academic research.

Thanks also go to the department faculty and staff for making my time at Texas A&M University a great experience. Specifically, I would like to thank Sylvia Bernal for her support and advise throughout these past five years. Her devotion to her students exceeds all expectations. She provided me a solid foundation when I felt like I was sinking.

Thanks to all my friends, both near and far, for your support. Thank you, Katherine Rosario, for your lifelong friendship and love. Thank you to all my fellow graduate students (current and graduated). Your constant support and gave me strength during the last five years. Special thanks to Jessica Huebschman and Kushal Bakshki for helping me relieve stress and constantly reminding me of my love and passion for neuroscience. I am sincerely thankful to Jacob Davis, who, from the very beginning, endured graduate school as my closest friend and comrade. Thank you for making me laugh from deep in my belly during the toughest times of my life. Thanks to Misty Malamakal, for your valuable mentorship in my first year of graduate school. Thanks for

Kie Huang, Joshua Reynolds, Melissa Henwood, Travis Johnston, Kelsey Hudson, Rachel Baine, and Megan Tarbet for your patience and assistance in my projects. And thanks to all the undergraduate students in the Grau lab for your help and patience.

I would like to thank my family: my parents and to my brothers and sister. Without their tremendous understanding and encouragement in the past few years, it would be impossible for me to complete my thesis. Specifically, thank you to my mother for always picking up my countless phone calls and to my sister for reminding me how awesome I am, even when I didn't feel that way. And to my dad for always reminding me of the end goal. And thank you to my in-laws who always stood behind me with endless love and enthusiasm.

And finally, thank you to my best friend and husband, Jonathan Fauss, for constantly listening to me rant and talk things out, for comforting me in times of struggle, for your unconditional love, and for the sacrifices you have made for me to pursue this degree.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was informed by a dissertation committee consisting of Professor Jim Grau of the Department of Psychological and Brain Sciences, Professors Rajesh Miranda and Michelle Hook of the Department of Neuroscience and Experimental Therapeutics, and Professor Kayla Bayless of the Department of Molecular and Cellular Medicine.

The student completed all work for this dissertation independently.

Funding Sources

Graduate study was supported by Mary Tucker Currie, the Department of Psychological and Brain Sciences, and the Institute for Neuroscience.

This project was funded through the Mary Tucker Currie Professorship and grants from the Department of Defense (DoD) to J.W.G., the National Institute of Neurological Disorders and Stroke (NS104422), and the Office of the Assistant Secretary of Defense for Health Affairs, through the Spinal Cord Injury Research Program under Award No. W81XWH-18-1-0807.

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CHAPTER I

INTRODUCTION AND OVERVIEW

Overview

Spinal cord injury (SCI) is a debilitating condition that affects over 296,000 people in the United States (NSCISC, 2021). According to NSCISC, the most prevelant cause of SCI is vehicular accidents, followed by falls, violence, sports, medical/surgical and other various causes, all of which have the potential to induce further tissue damage to the body. This is important because prior work has shown that additional pain (nociceptive) input soon after SCI can increase tissue loss and impair long-term functional outcomes (Grau et al., 2004; Turtle et al., 2019; Turtle et al., 2018). More specifically, nociceptive stimulation after SCI has been shown to exacerbate secondary injury mechanisms, such as increasing lesion volume, hemorrhage, inflammation, and cell death.

SCI unfolds in two major phases, the primary injury and secondary injury. Primary injury is characterized as the initial mechnaical insult to the spinal cord. The damage to the cord is irreversible and dependent on the severity of impact to the tissue. Secondary injury, however, unfolds over the hours to days following the primary injury and is dependent on the neurobiological processes that increase the area of cell death, potentially doubling the area of injury (Beattie, Hermann, Rogers, & Bresnahan, 2002; Ducker, Kindt, & Kempe, 1971; Hausmann, 2003; McVeigh, 1923). Microglial activation, inflammation, vascular destruction, and necrotic cell death have been shown

to contribute to the deleterious consequences of secondary injury (Alizadeh, Dyck, & Karimi-Abdolrezaee, 2019; Mautes, Weinzierl, Donovan, & Noble, 2000). The expansion of secondary injury has been linked to symptoms of allodynia, hyperalgesia, and poor functional recovery (Hook et al., 2017; Turtle et al., 2017). Given the nature of secondary injury, its various mechanisms, and time course, it serves as a valuable therapeutic target to suppress the expansion of cell death and improve functional outcomes.

The current study builds upon work showing that uncontrollable stimulation administered to rats that have been spinally transected impairs spinal learning (Ferguson, Crown, & Grau, 2006). This effect has been generalized over two pain models, uncontrollable electrical stimulation, and the irritant capsaicin (Grau et al., 2017; Hook, Huie, & Grau, 2008). Peripheral noxious stimulation has also been shown to drive overexcitation within the dorsal horn and promote nociceptive sensitization and enhanced mechanical reactivity (Ferguson, Huie, Crown, & Grau, 2012; Hook et al., 2008). Importantly, both models are known to activate nociceptive (C) fibers and engage neurons within the dorsal horn of the spinal cord (Latremoliere & Woolf, 2009; Turtle et al., 2018). In an incomplete SCI model, our laboratory has shown that pain (nociceptive) stimulation administered soon (1-4 days) after SCI can expand tissue loss and fuel hemorrhage, inflammation and cell death, and impair locomotor function (Grau et al., 2004; Turtle et al., 2019; Turtle et al., 2018).

Recent work has found that supraspinal systems are involved in the development of hemorrhage. Indeed, a rostral complete transection at T2 or an anesthetic dose of

lidocane has been shown to block the adverse effects of pain on hemorrhage and secondary injury (Davis et al., 2020; Reynolds et al., 2019). The current study explores the role of the brain in the development of secondary injury.

To explore how the brain contributes to the development of pain-induced hemorrhage, I first review the mechanisms by which nociception exacerbates secondary injury in a contusion model. Then, I review the dysregulation of hemodynamics after SCI. Finally, I review the role of stress and inflammation in the context of SCI.

Nociceptive Stimulation Exacerbates Acute Secondary Injury

C-fiber activation increases cell death and inflammation

In the uninjured animal, pain, or nociception, is usually detected in the periphery, where the signal travels into the spinal cord and synapses in the dorsal horn. The signal then travels to the brain for perception and motor processing. As discussed above, nociception in the context of SCI can promote nociceptive sensitization and undermine spinal plasticity. Work within a transection model of SCI has shown that spinal neurons are capable encoding multiple forms of learning, including single stimulus habituation, sensitization) (Groves & Thompson, 1970; Joynes & Grau, 1996), Pavlovian (stimulus-stimulus) (Patterson, Cegavske, & Thompson, 1973) and instrumental learning (response-outcome) (Crown, Ferguson, Joynes, & Grau, 2002b; Grau, Barstow, & Joynes, 1998; Joynes, Ferguson, Crown, Patton, & Grau, 2003). After complete SCI, spinal neurons that encode instrumental learning are susceptible to the effects of nociceptive stimulation when given in an uncontrollable manner. Interestingly, the stimulation that is sufficient to induce these effects are brief. Just six minutes of

uncontrollable stimulation administered can prevent and disable adaptive learning (Crown, Ferguson, Joynes, & Grau, 2002a; Crown et al., 2002b; Crown & Grau, 2001; Grau et al., 1998). In an incomplete injury model, the same six minutes of electrical stimulation has been shown to impair locomotor recovery and promote chronic pain (Garraway et al., 2014).

Within the spinal cord dorsal horn, nociceptive stimulation has been shown to fuel apoptotic and pyroptotic cell death after a thoracic contusion injury (Turtle et al., 2018). Both intermittent electrical stimulation and capsaicin injection lead to enhanced expression of the proinflammatory cytokine tumor necrosis factor (TNF) and the apoptotic cell death marker, caspase-3 (Turtle et al., 2018). Only electrical stimulation however, increased signals related to pyroptotic cell death (caspase-1 and interleukin-1β). Other work has found additional markers for inflammation and cell death (Turtle et al., 2017). Additionally, histological analysis has shown that lesion volume significantly expands after shock treatment (Turtle et al., 2017).

Several studies attempted to block the adverse effects of pain on locomotor function, lesion volume, inflammation, and cell death. One way to do this is to block the pain of nociceptive stimulation with the opioid analgesic, morphine. While systemic morphine resulted in robust antinociception and blocked behavioral reactivity to nociceptive stimulation, it failed to block the adverse effects pain has on locomotor recovery, lesion volume, hemorrhage, inflammation, and cell death (Hook et al., 2007; Turtle et al., 2017). This suggests that the adverse effects of pain on secondary injury and long-term function are dependent on C-fiber activation, rather than the perception of

pain. Additional evidence for this came from a study using the anesthetic lidocaine, a sodium channel blocker that blocks neural activity. Epidural lidocaine, delivered locally to the injury site, blocked the effect of pain on hemorrhage, inflammation, and cell death (Turtle et al., 2017). Furthermore, lidocaine treatment improved long-term locomotor recovery.

Progressive hemorrhagic necrosis

Recently, our laboratory has been exploring the effects of nociceptive stimulation on hemorrhage. The infiltration of blood cells into the spinal cord would exacerbate secondary injury because they are neurotoxic (Regan & Guo, 1998). Initial hemorrhaging after SCI is due, in part, to the shearing of blood vessels in the microvasculature of the gray matter (Mautes et al., 2000). As capillaries in the vicinity fail, the microhemorrhages coalesce, expanding the hemorrhagic lesion. The catestrophic fragmentation of capillaries and the resulting expansion of the hemorrhagic lesion is known as progressive hemorrhagic necrosis (PHN) (Simard, Woo, Aarabi, & Gerzanich, 2013).

PHN is further characterized by the up-regulation of the sulfonylurea receptor 1/transient receptor potential melastatin 4 (Sur1-Trpm4) channel complex (Simard et al., 2013). While not expressed constitutively, it is upregulated in the event of spinal cord trauma in endothelial and other cells. This channel complex has been associated with capillary fragmentation and necrotic cell death in a contusion model of SCI (Lee, Choi, Na, Ju, & Yune, 2014, 2015; Simard et al., 2013). In our own model, we have found that pain input upregulates Sur1-Trpm4 and capillary fragmentation (Turtle et al., 2019).

Effect of blocking brain and spinal cord communication after SCI

Prior work has shown that the adverse effects of pain on adaptive learning are dependent on descending serotonergic (5-HT) fibers within the dorsolateral funiculus (DLF) (Crown & Grau, 2005). Indeed, rats that have been given selective lesions to the DLF or a 5-HT antagonist fail to perform an instrumental learning task. Similarly, administration of a 5-HT agonist in transected rats blocked the adverse effect of uncontrollable stimulation on spinal learning.

Given that descending fibers play a protective role in spinal learning, it was hypothesized that blocking brain/spinal cord communication by disrupting descending fibers would expand pain-induced hemorrhage. Reynolds and colleagues instead found that a subsequent, complete rostral transection (T2) blocked hemorrhage, suggesting that supraspinal systems are involved in the development of pain-induced hemorrhage (Reynolds et al., 2019). Similarly, a pharmacological transection with the anesthetic lidocaine, delivered at T2, blocked the adverse effects of pain on hemorrhage and long-term recovery (Davis et al., 2020). Taken together, these data suggest that the descending brain systems play an active role in the development of secondary injury at the lesion site.

Hemodynamics After SCI

Dysregulated blood pressure after SCI

Other work suggests that a rise in blood pressure (BP) after SCI can fuel hemorrhage and increase the area of tissue loss (Guha, Tator, & Rochon, 1989; Nielson et al., 2015). Indeed, blood pressure around the time of injury is a strong predictor of

locomotor recovery after thoracic SCI in rats (Nielson et al., 2015). It is also known that noxious electrical stimulation induces an elevation in BP (Canon et al., 2015; Karlsson, 1999; Lindan, Joiner, Freehafer, & Hazel, 1980; Snow et al., 1978). Other models have attributed hypertension to increased blood-brain barrier permeability and hemorrhage (Guha & Tator, 1988; Hardebo & Beley, 1984; Heistad & Marcus, 1979; Ito et al., 1980). The effect of hypertension on lesion pathology and recovery could be mediated by direct projections from the mid-thoracic (T1-T6) spinal cord through sympathetic fibers (Krassioukov, Furlan, & Fehlings, 2003; Rabchevsky, 2006).

Autonomic dysreflexia

Dysregulated blood pressure after SCI could be a result of a syndrome known as autonomic dysreflexia. Autonomic dysreflexia develops as a result of damage to the thoracic spinal cord at and above T6 that interrupts descending sympathetic fibers, causing an acute rise in systolic pressure, followed by a bout of hypotension and bradycardia (Eldahan & Rabchevsky, 2018; Marsh & Weaver, 2004). Autonomic dysreflexia is often triggered by noxious visceral or somatic stimulation below the injury, typically bladder or colongeric distension, that results in the massive sympathetic reflex. This is thought to be caused by afferent sprouting of nociceptive fibers in the dorsal horn, caudal to the injury (Hou, Duale, & Rabchevsky, 2009). This intraspinal sprouting of pain fibers leads to amplification of afferent inputs, such as from the bladder and bowel, resulting in enhanced sympathetic activity.

In rodent models, AD is often studied in animals that have received a high thoracic transection and a form of noxious stimulation (Hou et al., 2009; Hou, Lu, &

Blesch, 2013; Laird, Carrive, & Waite, 2006). In our own model of pain with a lower thoracic injury, we have found that electrical stimulation leads to an acute rise in BP that is associated with hemorrhage (Misty M. Strain et al., under review). Additionally, treatment with norepinephrine is sufficient to elevate blood pressure and induce an acute locomotor deficit however (Johnston, Lout, Baine, & Grau, 2021). It is currently unknown, however, if a higher thoracic injury within our model will induce changes in BP.

Stress Systems and SCI

Inflammation and stress after SCI

The introduction of stressors, psychological or physiological, activates the hypothalamic-pituitary-adrenal (HPA) axis, which then triggers the release of glucocorticoids (stress hormones) from the adrenals into the bloodstream. At low levels, the release of glucocorticoids can be beneficial, promoting anti-inflammatory effects (Kern, Lamb, Reed, Daniele, & Nowell, 1988; Marx, 1995) and even facilitate learning and memory (McEwen & Sapolsky, 1995). However, in higher concentrations, such as in cases of chronic stressors, stress hormones are correlated with increased pro-inflammatory cytokines (Turnbull et al., 1994; Zhou, Kusnecov, Shurin, Depaoli, & Rabin, 1993) and an increase in cell loss in the central nervous system (Armanini, Hutchins, Stein, & Sapolsky, 1990; Chou, 1998). Stressors such as uncontrollable shock have already been shown to elevate pro-inflammatory cytokines such as IL-1β, IL-18, and IL-6 (Maier & Watkins, 1998; Nguyen et al., 2000; O'Connor et al., 2003).

Other studies suggest that stress factors, such as corticosterone and immune cells, may play a role in secondary injury (Popovich, Stuckman, Gienapp, & Whitacre, 2001; Washburn, 2007). For example, uncontrollable shock drives an increase in corticosterone that persists for 72 hours in spinally injured rats (Washburn, Patton, Ferguson, Hudson, & Grau, 2007). This was associated with an increase in IL-1β and decreased spleen weights. As discussed previously, work with morphine on the effects of nociception after SCI suggests that this effect is dependent on nociception and C-fiber activation, rather than psychological pain. Given this, it is possible that the consequences of noxious input on blood pressure, hemorrhage, and tissue loss are due, in part, to a brain-dependent activation of the stress system.

Specific Aims

In the current study, I explored the role of supraspinal systems in the exacerbation of hemorrhage and secondary injury within the spinal cord. My central hypothesis is that the brain plays a modulatory (necessary) role in the development of hemorrhage. To test this hypothesis, I examine the effects of pain on blood pressure, BSCB permeability, and the effects of stimulation at pain threshold.

Aim 1 (Chapter III) examined the effects of a rostral transection on pain-induced hemorrhage and blood pressure. I expand upon previous work by examining how a rostral transection affects blood pressure in a capsaicin model. Aim 2 (Chapter IV) determined the role of the brain on BSCB permeability using Evan's Blue dye. Aim 3 (Chapter V) examined the role of brain-dependent pain on hemorrhage through the application of morphine and rostral nociceptive stimulation. Finally, Aim 4 (Chapter VI)

examined the impact of stimulation at pain threshold on hemorrhage, blood pressure, and locomotor performance.

CHAPTER II

GENERAL METHODS

Subjects

Adult male Sprague Dawley rats were obtained from Envigo (Houston, TX) and were acclimated to their holding environment for at least 7 days prior to experimentation. Prior to surgery, rats were dual housed, with food and water *ad libitum* and maintained on a 12-hour light/dark cycle with all behavioral testing performed during the light cycle. All experiments were carried out in accordance with NIH standard for the care and use of laboratory animals (NIH publications No. 80-23) and were approved by the University Laboratory Animal Care Committee at Texas A&M University. Every effort was made to limit the number of animals and minimize unnecessary suffering.

Contusion and Transection Surgery

All subjects received a moderate contusion injury at the T10-T11 vertebral level using the MASCIS (NYU) device. Rats were anesthetized with a 5% isoflurane and maintained at 2-3% isoflurane during surgery. After they were shaved and the surgical site cleaned with iodine and alcohol, a single longitudinal incision was made, extending approximately 3 cm over to the injury site. Two longitudinal incisions were made on either side of the vertebral column cut to the depth of the rib cage. One cm lateral incisions were made immediately below the T10 vertebra and above the T13 vertebra. After clearing the tissue, a laminectomy was performed on the T12 vertebra. The New

York University (NYU) Multicenter Animal Spinal Cord Injury Study (MASCIS) device was used to perform the contusion injury (Gruner, 1992). Clamps were used to secure the spinal cord, and the 10-gram impactor was centered on the lesion site. The drop height was set at 12.5 mm. After impact, the wound was closed with Michel clips. To prevent urinary tract infection and compensate for fluid loss, animals received 100,000 units/kg of penicillin and 3 mL of saline intraperitoneal (i.p.) after surgery.

Animals were given 18 hours to recover in a temperature-controlled room (25° C). Food and water were available *ad libitum*. The rats' bladders were expressed twice daily and immediately after all BP time points. After experimentation was complete, all animals were euthanized with a lethal dose of pentobarbital [100 mg/kg; i.p.].

For transection experiments, animals received a complete spinal transection 18 hours after the contusion injury. Anesthesia was induced with 5% isoflurane gas and maintained at 2-3% isoflurane throughout the procedure. Their heads were then secured in a stereotaxic apparatus. The skin over the upper thoracic region was shaved and disinfected with iodine and alcohol. A longitudinal incision was made over the second thoracic vertebrae (T2) and the tissue just rostral to T2 was cleared away. At this point, the sham rats had their wound closed with Michel clips. The remaining rats had their spinal cord transected with a cautery device and the wound was closed using Michel clips. All animals then received 3 ml of saline (i.p.). The transections were visually confirmed post-mortem at the time of sacrifice.

Locomotor recovery assessment

Before surgery, rats were acclimated over 3 days to the open observation arena for 4 minutes per day. Twenty-four hours after contusion injury, rats' locomotor scores were analyzed using the Basso, Beattie, and Bresnahan (BBB) locomotor scale (Basso, Beattie, & Bresnahan, 1995) by placing the rats in the open arena for 4 minutes with a trained observer unaware of the animal's treatment condition. BBB scores were resampled at various time points during each experiment to examine any changes in locomotor performance. In all cases, there were no group differences prior to treatment (all Fs < 2.73, $p > 0.05$).

In experiments where the spinal cord was transected 18 hr after contusion injury, BBB was assessed prior to transection surgery to balance groups based on BBB score. In all cases, there were no group differences prior to spinal transection (all Fs < 2.73, $p > 0.05$). BBB was not assessed after spinal transection due to the complete nature of the injury.

Blood Pressure

Before surgery, rats are acclimated to the non-invasive blood pressure apparatus in a warm room (27°C) with dim lighting for three sessions that are identical to experimental testing in procedure. For blood pressure assessment, rats were placed in clear acrylic tubes with black, matte nose cones. The tubes were placed on top a Far-infrared warming platform (Kent Scientific) and temperature was monitored with an infrared thermometer aimed at the tail to ensure that temperatures are maintained between 32-35°C. Prior to the session's start, the rats were acclimated to the tubes for 5

minutes. Then, an occlusion cuff and Volume Pressure Recording (VPR) cuff was secured at the base of the tail. After five additional minutes of acclimation to the apparatus and cuffs, the subjects underwent 15 cycles of BP measurement (Kent Scientific). Body temperature was monitored and maintained at approximately 32° to 35° Celsius. Blood pressure was assessed in the minutes before treatment and 0, 1, 2, and 3 hours after. This non-invasive BP method has been validated for accuracy of measurement in rodents (Feng et al., 2008).

Six measures of cardiovascular function were obtained: systolic BP, diastolic BP, mean arterial BP, heart rate, blood flow, and blood volume. Preliminary analyses of the baseline values, and the change observed after treatment, showed that the three measures of BP were highly correlated (all r’s > 0.905, $p < 0.0001$). Due to this redundancy, only one measure (systolic BP) is presented. Likewise, blood flow and volume were highly correlated (r’s > 0.954, $p < 0.0001$). For this reason, and because blood flow has proven to be more reliably related to our experimental effects, we present blood flow. Finally, because a pain-induced rise in heart rate was not strongly correlated with the changes observed in systolic BP and blood flow (all r’s < 0.404), it too was analyzed.

Uncontrollable electrical stimulation

Rats were loosely restrained in opaque Plexiglas tubes housed in an acoustic isolation chamber. Electrical stimulation to the tail was applied through tail electrodes formed from a modified fuse clip. Electrodes were coated with electrode gel (Harvard Apparatus, Holliston, MA, USA) and attached 2 cm from the tip of the tail with Orthaletic tape. The electrodes were then connected to a BRS/LVE shock generator

(Model SG-903) and a constant current 1.5 mA, AC (60 Hz) electrical stimuli (100 ms in duration) was applied on a variable intermittent schedule (0.2-3.8 s) for 6 minutes. In experiments where shock was applied at vocalization threshold, the intensity required to elicit a vocalization was determined (see below), and intermittent electrical stimulation was given at that intensity. Unshocked controls were treated the same except with the absence of shock treatment. To evaluate whet the vocalization threshold test affected hemorrhage at the site of injury, we included a group that underwent threshold assessment but no subsequent electrical stimulation.

Electrical stimulation to the hind limbs was applied through thin (26-gauge, 0.4039 mm AWG diameter) wire electrodes. One wire was inserted through the skin at 1.5 cm from the end of the paw and another was inserted in the skin 1.7 cm above the first electrode. After insertion, the wires ends were twisted to assure they did not move and the leads from a BRS/LVE shock generator were connected to the wires with alligator clips. Stimulation to the forelimbs was conducted in the same manner, except the electrodes were placed in equivalent spots on the forelimb. The 6 minutes of stimulation was delivered exactly as described above with a constant current (1.5mA or vocalization threshold), AC (60 Hz) electrical stimuli (100 ms in duration) on a variable intermittent schedule (0.2-3.8 s). Unshocked control groups either received the wire insertion treatment with no shock delivered, or no wire insertion with no shock delivered. After set-up and baseline testing, unshocked animals remained in the Plexiglas tubes for an additional 6 min, to equate the period of restraint across groups.

Vocalization thresholds were determined as described previously (King, Joynes, Meagher, & Grau, 1996). Briefly, after rats were prepared for electrical stimulation, they were given 3 minutes to acclimate to the test environment. A continuous constant current shock was then applied, with the intensity incremented in intervals of 0.05 mA once every 3 seconds, beginning at 0 mA. For tail shock, the electric current was terminated when the rat exhibited a vocalization response to the shock, or when the current reached 1.2 mA. For hindlimb shock, the electric current was terminated when the rat exhibited a vocalization response to the shock. Vocalization thresholds did not differ between treatment groups of the same electrode type and location, Fs < 1.0, $p > 0.05$.

Drug preparation and administration

Capsaicin

Capsaicin (3%) dissolved in Tween-20 (5%) and EtOH (5%) was injected intradermally into the dorsal surface of the hind paw with a 27-gauge needle while rats were restrained in Plexiglas tubes. Controls were injected with an equal volume of vehicle solution. Animals were randomly injected on the left or right paw. Animals were left in the Plexiglass tubes for 6 minutes to equate treatment time across experiments.

Norepinephrine

After shock treatment, animals were transferred to BP tubes and given a 2 mL subcutaneous injection of norepinephrine (0.1 mg/kg) or vehicle (saline) to the trunk. The first assessment of BP was conducted immediately after administration of drug. The dosage used was based on pilot work demonstrating that it produced an increase in

systolic BP comparable to that induced by noxious electrical stimulation (Johnston et al., 2021).

Evans Blue Dye

Immediately after shock treatment, 2.5 mL of 2% Evans blue Dye (dissolved in saline) was injected intraperitoneally. The rats were observed for 15 minutes after injection to determine if the injection was successful, causing the solution to disperse through the body which turned a blue color. If a rat did not turn blue (due to a mis-placed injection), an additional injection was given.

Morphine

Morphine was administered 15 minutes prior to shock treatment using a dose and injection protocol that has been previously shown to induce a robust anti-nociception (Hook et al., 2007). Morphine sulfate was dissolved in saline and injected intraperitoneally (i.p.) at a dose of 20 mg/kg.

Tissue collection and protein extraction for analysis

For hemorrhage analysis, rats were sacrificed with a lethal injection of pentobarbital (100 mg/kg; i.p.). One cm of spinal cord tissue centered over the injury site was collected. The collected tissue was then flash frozen in liquid nitrogen and stored in -80°C. The protein was isolated from the collected spinal tissue using Trizol RNA extraction followed by a protein extraction procedure using the Qiagen kit.

For Evans blue analysis, the rats were given a lethal dose of pentobarbital and perfused with cold saline three hours after electrical stimulation. The spinal cord was extracted after perfusion and a one-centimeter section encompassing the lesion site was

collected and flash frozen. Spinal cords were stored in -80°C until analysis. For preparation, the spinal cords were homogenized in 50% trichloroacetic acid solution. Samples were then centrifuged at 10,000 x g for 10 minutes and the supernatant was preserved and diluted at a 4:1 ratio in 100% EtOH.

Immunoblotting

The concentration of the extracted protein was measured using the Bradford assay. The samples were diluted to a final concentration of 3 µg/µL in 4X Lamelli buffer. Western blotting was performed using a 26-well 12% Tris-HCL Criterion precast gels (BioRad, Hercules, CA) according to manufacturer's instructions. The diluted samples were heated to 95°C for 10 minutes and centrifuged for a quick spin cycle (3-5 sec). Then, equal amounts of the protein (30 µg) were loaded into each well. After the addition of SDS-PAGE running buffer, electrophoresis was performed at 180 V for approximately one hour. Proteins were then transferred onto a polyvinylidene difluoride (PVDF; Millipore, Bedford, MA) membrane for one hour in an ice bucket at 100V in cold transfer buffer. The membrane was then blocked in 5% blotting-grade milk (BioRad, Hercules, CA) for one hour prior to overnight incubation in primary antibodies hemoglobin α [1:1000; Abcam (Cambridge, MA) ab92492; RRID: AB10561594], Lamin B1 (1:1000; Abcam ab16048; RRID: AB443298), and Beta Tubulin [1:5000; Upstate (Lake Placid, NY) RRID: AB309885] at 4°C. After three washes in Tris-buffered saline and Tween-20 (TBST) at 10 minutes each, the blots were incubated for one hour in HRP-conjugated goat anti-rabbit secondary antibodies [1:5000; Thermo Scientific (Rockford, IL); RRID: AB228341] or HRP-conjugated goat anti-mouse

[1:5000; Pierce Biotech (Rockford, IL); RRID: AB258492] at room temperature. Finally, the blots were washed for another 3 x 10 min series in TBST and developed using electrochemiluminescence (ECL; Pierce, Rockford, IL). The blots were imaged with Fluorchem HD2 (ProteinSimple, Santa Clara, CA) and were analyzed by calculating the ratios of the integrated densitometry of each protein of interest to the loading control (lamin B1 or beta tubulin), then normalizing this ratio to a control group (run on the same blot) that did not receive nociceptive treatment or drug.

Spectrophotometry

Hemorrhage Analysis

Spectral analysis for hemoglobin was conducted with protein extracted from spinal cord tissue. Approximately 1.5 μL of protein extract was loaded onto the spectrophotometer and absorbance at 420 nm was used to measure the hemoglobin content of the samples.

To verify the absorbance reflected from our samples, we conducted another photometric assay based on the breakdown of hemoglobin to cyanmethemoglobin using Drabkin's reagent to determine hemoglobin concentration in our samples. Briefly, 160 μL of Drabkin's reagent was incubated in 10 μL of protein for one hour. Then, 1.5 μL was loaded onto the spectrophotometer and absorbance was measured at 540 nm (as per manufacturer's instructions).

Evans Blue Analysis

Spectral analysis for Evans blue was conducted with the diluted samples extracted from spinal cord tissue. Approximately 1.5 μL of protein extract was loaded

onto the spectrophotometer and fluorescence intensity of Evans blue was quantified at an excitation wavelength of 620 nm (Kumar et al., 2018). Concentration was determined using a standardization curve made from known concentrations of Evans blue diluted in saline.

Statistics

All data were analyzed using student's t-test, analysis of variance (ANOVA) or analysis of covariance (ANCOVA). Differences between group means were assessed using Duncan's New Multiple Range *post hoc* tests when necessary. Statistical significance was set at a p of 0.05.

CHAPTER III

DOES THE BRAIN REGULATE PAIN-INDUCED HEMORRHAGE THROUGH BLOOD PRESSURE?

Previous work has shown that cutting brain and spinal cord communication can block the development of pain-induced hemorrhage at the spinal cord contusion site (Reynolds et al., 2019). What is currently unknown is the brain-dependent process that underlies this effect. Prior data suggests that BP increases as a response to pain input in a contusion model and this is associated with an increase in hemorrhage (Misty M. Strain et al., under review). Here, I explore if a pain-dependent rise in BP is necessary and sufficient to induce hemorrhage and if this effect is dependent on the brain.

Experiment 1: Spinal transection blocked shock-induced increase in blood pressure and hemorrhage

Past work has shown that a rise in BP can increase tissue loss and impair long-term recovery after SCI (Guha et al., 1989; Nielson et al., 2015). Noxious stimulation leads to a rise in BP that could fuel hemorrhage (Canon et al., 2015; Snow et al., 1978). We hypothesized that cutting communication with the brain blocks the nociception-induced rise in BP, protecting against the consequences of prolonged elevated BP. Alternatively, work on AD suggests that nociceptive input can induce a rise in BP, through the disruption of descending modulating pathways and the resulting unregulated control of sympathetic reflexes. To study this phenomenon, researchers typically cut communication with the brain by means of a high thoracic (T1-T6) transection, an

experimental treatment that can enhance the effect of noxious stimulation on cardiovascular function (Laird et al., 2006; Rabchevsky et al., 2012; West, Popok, Crawford, & Krassioukov, 2015; West et al., 2016). If this process contributes to the nociception-induced rise in BP in our paradigm, cutting the spinal cord should promote rather than block the effect.

Procedure

Thirty-two rats received a moderate T12 contusion injury and eighteen hours later, half of the subjects were randomly assigned to undergo a T2 transection surgery. Twenty-four hours after the original contusion injury, half of the animals in each group received six minutes of intermittent shock to the tail (1.5 mA, 0.2-3.8 second ISI) or an equal period of restraint. Systolic blood pressure, heart rate, and blood flow were measured immediately before spinal transection and at hourly intervals for 3 hours after shock treatment (experimental design and timeline in Figure 1A).

After obtaining the last cardiovascular measurement, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized, and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin’s analysis) and with gel electrophoresis and immunoblotting for hemoglobin. The experimental design involved a full 2 (Sham vs. Transection) x 2 (Shock vs. Unshock) factorial (n = 8).

Results

Prior to transection surgery (Baseline), systolic BP ranged from 104.78 ± 9.68 to 107.52 ± 7.21 (mean ± SE) across groups. These differences were not statistically significant, all Fs < 1.0, $p > 0.05$. Analysis of systolic BP across the three hours showed that contused rats that were not transected (Sham) exhibited higher systolic BP after shock (Shk) treatment (Figure 1B). This phenomenon was blocked by a rostral transection. An analysis of covariance (ANCOVA), with baseline systolic BP serving as the covariate, confirmed that the effect of shock treatment depended upon whether animals had received a spinal transection, $F_{(1, 27)} = 6.66$, $p = 0.0156$ ($\eta^2 = 0.148$). *Post hoc* comparisons of the group means showed that the sham-operated group that received shock (Sham Shk) differed from the other three ($p < 0.05$). No other group comparisons were significant ($p > 0.05$).

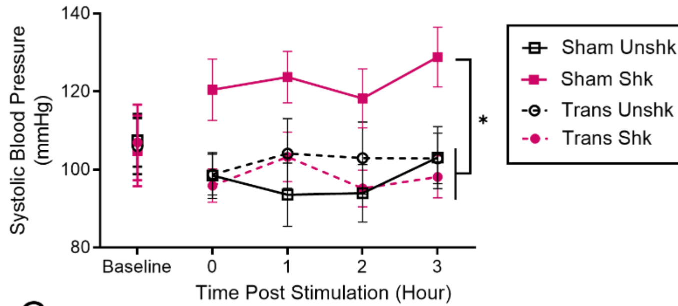
Baseline heart rate ranged from 233 ± 24.9 to 282 ± 20.4 (mean ± SE) across groups. These differences were not statistically significant, all Fs < 1.0, $p > 0.05$. The sham-operated shocked rats (Sham Shk) exhibited greater heart rate throughout the three hours (Figure 1C). An ANCOVA with baseline heart rate serving as a covariate revealed a main effect of transection, $F_{(1, 27)} = 6.336$, $p = 0.0181$ ($\eta^2 > 0.177$). *Post hoc* comparisons revealed a significant difference between the sham-operated shocked rats and the transected unshocked rats. There was also a main effect of time and an interaction between time and baseline heart rate, Fs > 2.79, $p < 0.0458$. No other comparisons were significant ($p > 0.05$).

Before spinal transection (Baseline), blood flow ranged from 3.64 ± 1.04 to 4.98 ± 4.58 (mean ± SE) and did not differ between groups, all Fs < 1.0, $p > 0.05$. Analysis of flow over the 3 hours of testing showed that the sham-operated shocked rats (Sham Shk) and the transected rats (Trans Unshk and Trans Shk) exhibited an increase in blood flow after treatment (Figure 1D). An ANCOVA, with baseline flow serving as the covariate, revealed a between subjects main effect of transection surgery, and an interaction between shock treatment and transection, both Fs > 9.142, $p < 0.0054$ ($\eta^2 > 0.153$). *Post hoc* comparisons of the group means revealed a significant difference between the sham-operated unshocked group (Sham Unshk) from the other three, and a difference between the sham-operated shocked group (Sham Shk) and transected unshocked group (Trans Unshk) ($p < 0.05$). There was also a within subjects main effect of time, $F_{(3, 81)} = 5.639$, $p = 0.0015$, and a trend for significance between time and group ($p = 0.0604$). No other comparisons were significant ($p > 0.05$).

A

T12 Contusion	18 Hours	Baseline BBB & BP	Sham	6 Hours	Unshock	BP 0-3hr	Collect Tissue
					Shock		
			T2 Transection		Unshock		
					Shock		

B



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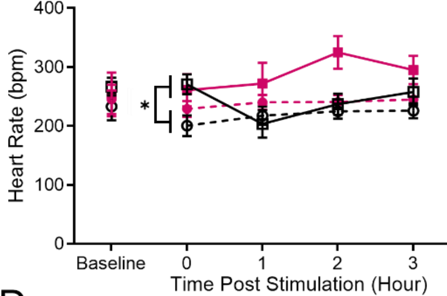

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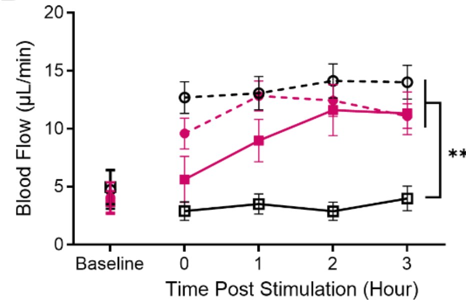


Figure 1. A rostral transection blocks the rise in blood pressure induced by noxious electrical stimulation. (A) Experimental design and timeline of Experiment 1. (B) Sham-operated rats that received electrical stimulation (Shk) exhibited higher systolic blood pressure over the next 3 hrs (T0-T3). Transection surgery blocked this effect. (C) Rats treated with electrical stimulation exhibited higher heart rate over the 3 hrs. (D) Both transected groups and the sham-operated rats that received shock displayed a significant increase in blood flow. Sham-operated unshocked animals remained unchanged. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Blood content from tissue at the site of injury was first assessed by measuring absorbance at 420 nm [the absorbance peak for hemoglobin; (Choudhri, Hoh, Solomon, Connolly, & Pinsky, 1997; Sadie, 1920; Turtle et al., 2019; Vankampen & Zijlstra, 1961)]. Contused rats that were not transected exhibited greater absorbance relative to the sham unshocked group and both transected groups. Shock treatment had no effect on absorbance in transected rats. An ANOVA showed that the main effects of transection and shock treatment were statistically significant both Fs > 5.060, $p < 0.0325$ ($\eta^2 > 0.094$). The interaction between these variables approached significance, $F_{(1, 28)} = 3.77$, $p = 0.0623$. *Post hoc* comparisons confirmed that the non-transected (Sham) group that received shock differed from the other three groups ($p < 0.05$) (Figure 2A).

A similar pattern was obtained with the Drabkin's assay, with transected animals exhibiting lower hemoglobin content at the site of injury, $F_{(1, 28)} = 9.046$, $p = 0.0055$ ($\eta^2 > 0.067$). *Post hoc* comparisons found that the two non-transected groups differed from the transected groups ($p < 0.05$) (Figure 2B).

Alpha hemoglobin was also assessed using western blotting. Again, contused rats that were not transected (Sham) and received shock exhibited a higher concentration of hemoglobin relative to both the unshocked groups and transected rats that received shock (Figure 2C). An ANCOVA, with blot serving as a covariate, confirmed that shock and transection had a significant effect, both Fs > 7.64, $p < 0.01$ ($\eta^2 > 0.082$). *Post hoc* comparisons showed that the non-transected group given shock differed from the other three groups ($p < 0.05$). There were no other significant group comparisons ($p > 0.05$).

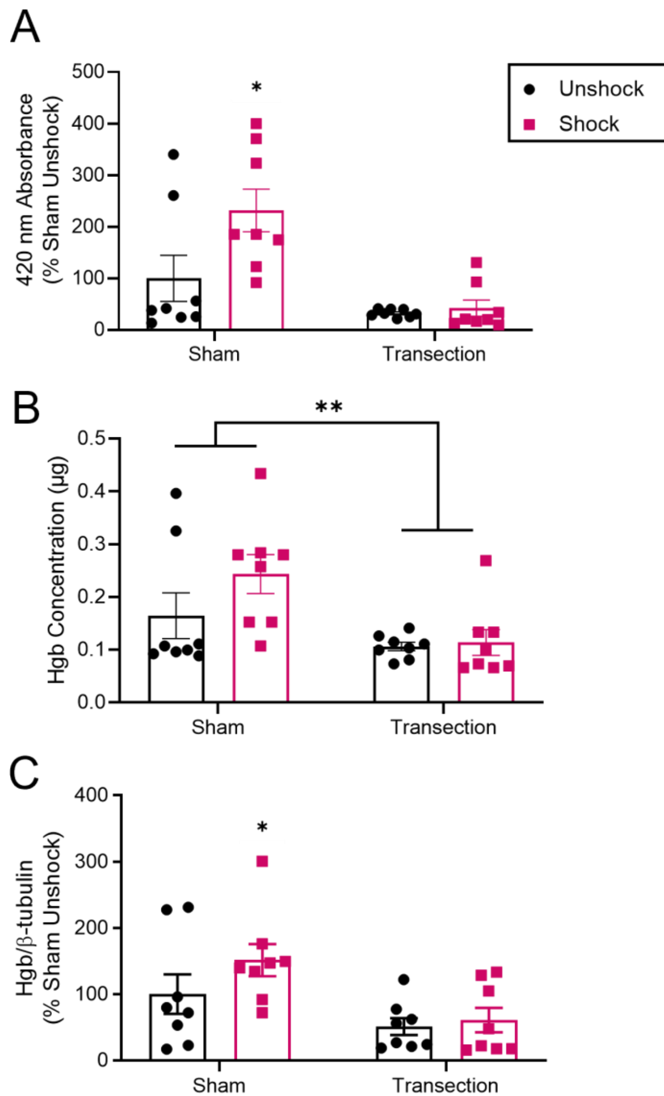


Figure 2. A rostral transection blocks nociception induced hemorrhage. (A) Quantification of peak absorbance at 420nm (the wavelength associated with hemoglobin). Sham shocked rats showed a higher peak absorbance than unshocked rats. Transection surgery blocked this effect. (B) Quantification of hemoglobin content based on formation of cyanomethemoglobin (Drabkin's assay). Tissue from sham shocked rats contained a higher concentration of hemoglobin relative to animals that had undergone a spinal transection. (C) Immunoblot quantification for hemoglobin showed that tissue samples from sham-operated rats that received shock had higher levels of hemoglobin relative to both the sham-operated unshocked group and both groups that received a transection. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 2: Spinal transection blocked capsaicin-induced increase in hemorrhage

Experiment 1 showed that nociceptive input caudal to a contusion injury produces an increase in BP and hemorrhage. Both effects were blocked by a rostral transection. To evaluate the generality of these findings, we tested another clinically relevant pain model, the irritant capsaicin. This irritant was chosen due to its common use in the pain literature (Hook et al., 2008; Huang, Lee, Murphy, Garraway, & Grau, 2016; Lamotte, Shain, Simone, & Tsai, 1991; Simone, Baumann, & LaMotte, 1989) and its specific engagement of C-fibers that express the TRPV1 receptor. In previous work, we have shown that capsaicin treatment induces enhanced mechanical reactivity (EMR) and engages cellular indices of nociceptive sensitization (Grau et al., 2012; Turtle et al., 2018). The present experiment examined whether capsaicin induces an increase in BP and hemorrhage, and whether these effects are blocked by spinal transection.

Procedure

Thirty-two rats received a contusion injury and 18 hours later half the subjects (randomly assigned) had the spinal cord transected at T2. The remaining contused rats underwent a sham surgery. Six hours later, half of the animals in each condition were given a single injection of capsaicin into the hind paw while the remaining rats received vehicle. Systolic blood pressure, heart rate, and blood flow were measured immediately before spinal transection and at hourly intervals for 3 hours after shock treatment (experimental design and timeline in Figure 3A).

After the final cardiovascular assessment, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis) and with gel electrophoresis and immunoblotting for hemoglobin. The experiment involved a 2 (Sham vs. Transection) x 2 (Capsaicin vs. Vehicle) factorial design (n = 8).

Results

Prior to transection surgery (Baseline), systolic BP ranged from 99.27 ± 7.09 to 101.88 ± 10.31 (mean ± SE) across groups. These differences were not statistically significant, all Fs < 1.0, $p > 0.05$. Contused animals that were treated with capsaicin showed no change in BP independent of whether or not they received a spinal cord transection (Figure 3B). An ANCOVA confirmed that neither capsaicin nor spinal transection had a significant effect, all Fs < 1.0, $p > 0.05$.

Baseline heart rate ranged from 213 ± 17.8 to 243 ± 26.8 (mean ± SE) across groups. These differences were not statistically significant, all Fs < 2.3, $p > 0.05$. Rats that were treated with capsaicin displayed a greater heart rate throughout the three hours (Figure 3C). An ANCOVA with baseline heart rate serving as the covariate revealed a main effect of capsaicin treatment, $F_{(1, 27)} = 18.312$, $p = 0.0002$ ($\eta^2 > 0.377$). *Post hoc* comparisons of the group means showed that the two capsaicin-treated groups differed

from those that received vehicle ($p < 0.05$). No other comparisons were significant ($p >$ 0.05).

Before transection, blood flow ranged from 2.68 ± 0.417 to 4.54 ± 2.17 (mean ± SE) and did not differ between groups, all Fs < 1.0, $p > 0.05$. Analysis of blood flow over time showed that the transected rats had significantly higher flow than sham-operated rats (Figure 3D). An ANCOVA, with baseline flow serving as the covariate, revealed a main effect of transection surgery, $F_{(1, 27)} = 120.922$, $p = 0.0001$ ($\eta^2 > 0.796$). *Post hoc* comparisons of the group means confirmed that the two transected groups differed from the non-transected (sham) animals ($p < 0.05$). No other comparisons were significant ($p > 0.05$).

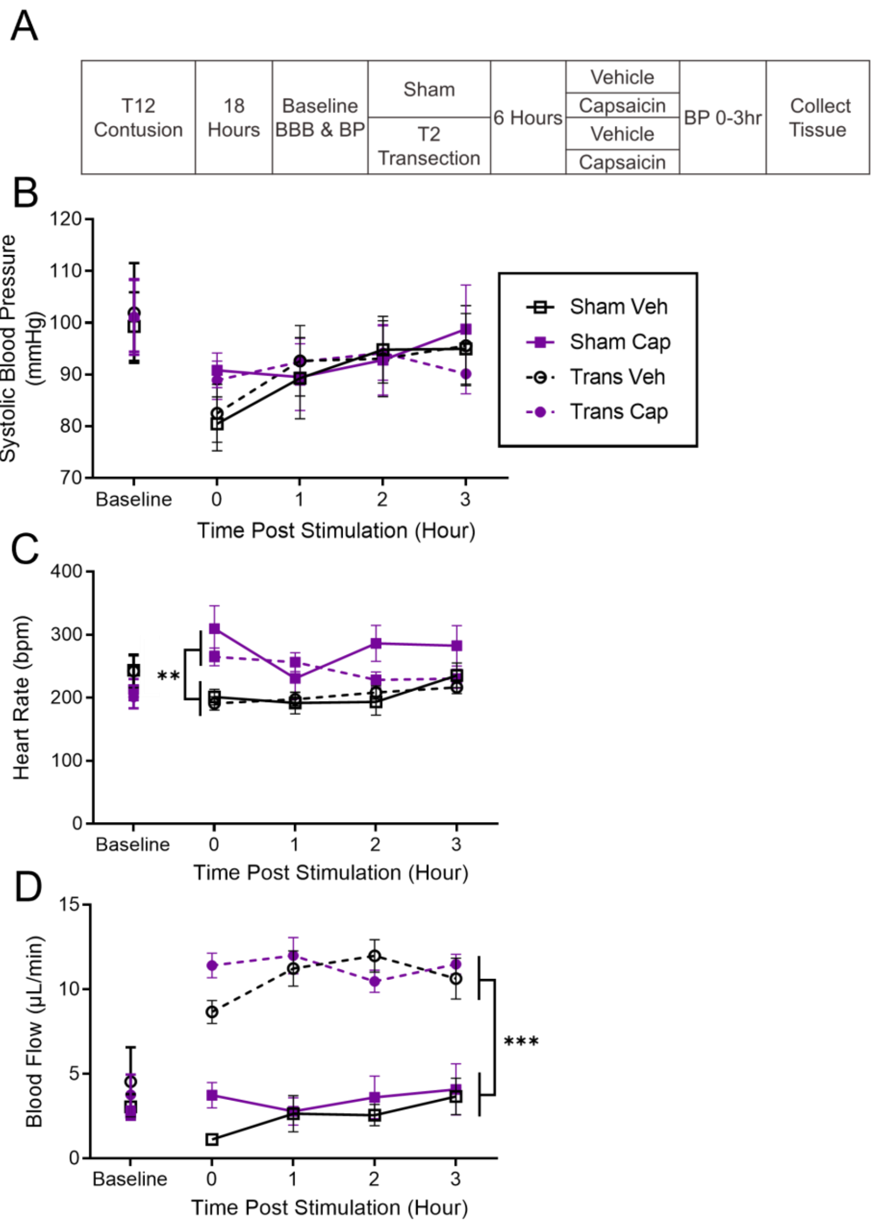


Figure 3. The effect of a rostral transection and capsaicin on cardiovascular function. (A) Experimental design and timeline for experiment 2. (B) Application of capsaicin (Cap) to one hind paw did not induce a change in systolic blood pressure. (C) Capsaicin-treated rats exhibited a higher heart rate throughout the 3 hrs. (D) Only transected rats exhibited a significant rise in blood flow after capsaicin treatment. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Contused rats that had not undergone a spinal cord transection (Sham) exhibited greater absorbance at the wavelength associated with hemoglobin and this effect was blocked by spinal cord transection (Figure 4A). An ANOVA confirmed that the main effects of capsaicin and transection treatment, as well as their interaction, were statistically significant, all Fs > 5.401, $p < 0.0276$) (all $\eta^2 > 0.113$). *Post hoc* comparisons confirmed that the sham group that received capsaicin differed from the other three groups ($p > 0.05$).

A similar pattern of results was obtained with the Drabkin's assay. Again, capsaicin increased hemoglobin concentration at the site of injury in contused rats that were not transected (Sham) but not in contused and transected rats (Figure 4B). An ANOVA confirmed that the main effects of capsaicin and transection treatment, as well as their interaction, were statistically significant, all Fs = 5.75, $p < 0.05$ (all $\eta^2 > 0.096$). *Post hoc* comparisons showed that the sham-operated group that was treated with capsaicin differed from the other three ($p < 0.05$).

Western blotting confirmed that contused rats that received capsaicin had higher concentrations of hemoglobin at the site of injury relative to both the vehicle controls and transected rats that received capsaicin (Figure 4C). Because there was greater variability in behavioral performance after injury in this experiment, we analyzed the data using an analysis of covariance with baseline BBB score entered as a covariate. An ANCOVA revealed that there was a main effect of capsaicin, $F_{(1, 27)} = 6.473$, $p = 0.017$ ($\eta^2 > 0.160$). *Post hoc* comparisons showed that the non-transected (Sham) group that

received capsaicin differed from the other three groups ($p < 0.05$). No other group comparison was significant ($p > 0.05$).

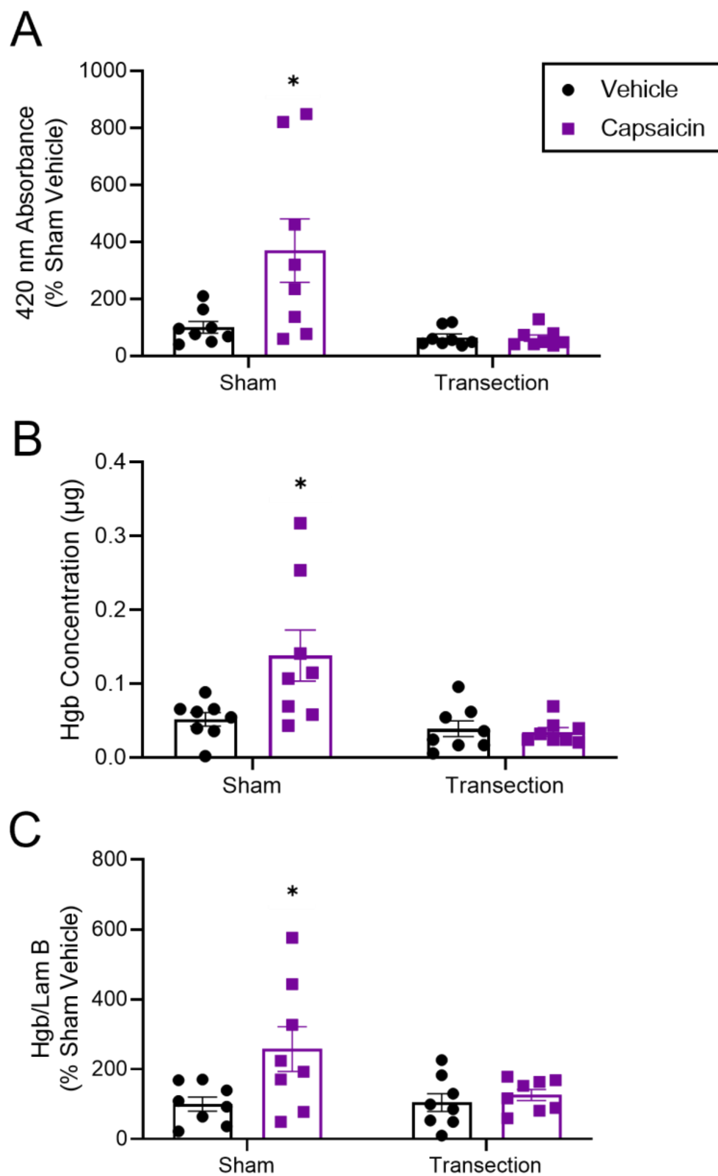


Figure 4. Application of the irritant capsaicin to a hind paw increased hemorrhage after a lower thoracic contusion injury and this effect was blocked by a spinal transection. (A) Sham-operated rats that were treated with capsaicin exhibited greater absorbance at 420 nm for hemoglobin. Transection surgery blocked this effect. (B) Drabkin's assay and western blot (C) showed similar results. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 3: Pharmacologically increasing blood pressure in transected animals does not lead to hemorrhage

We found that noxious electrical stimulation and the irritant capsaicin increase hemorrhage at the site of injury, but only the former effect was accompanied by a rise in BP. The fact that a rostral transection blocked both shock-induced hypertension and hemorrhage suggests that the effects may be related. However, treatment with capsaicin had no discernable effect on systolic BP, but nevertheless increased hemorrhage at the site of injury. To further examine the relationship between BP and hemorrhage, we applied shock to rats that received a contusion injury and a rostral transection, and then pharmacologically induced a rise in BP with a systemic injection of norepinephrine (NE).

Procedure

Thirty-two rats received a contusion injury and were randomly assigned to receive either a rostral transection surgery or sham surgery 18 hours later. Six hours later, systolic blood pressure, heart rate, and blood flow was assessed prior to shock treatment (1.5 mA, 0.2-3.8 second ISI). All the rats in each condition received shock. Next, half of the rats in each condition received a single injection of norepinephrine and the remaining animals were given its vehicle. Cardiovascular function and hemorrhage were assessed at one-hour intervals for three hours (experimental design and timeline in Figure 5A).

After the final cardiovascular assessment, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion

site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized, and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis) and with gel electrophoresis and immunoblotting for hemoglobin. The experiment involved a 2 (Sham vs. Transection) x 2 (NE vs. Vehicle) factorial design (n = 8).

Results

Prior to spinal transection (Baseline), systolic BP ranged from 95.95 ± 5.87 to 103.19 ± 7.83 (mean ± SE) across groups. These group differences were not statistically significant, all Fs < 1.0, $p > 0.05$. Shock induced an increase in BP in vehicle-treated rats that were not transected (Sham Veh) relative to vehicle-treated animals that were transected (Trans Veh) prior to shock treatment (Figure 5B). NE did not affect the shock-induced rise BP in the sham-operated group (Sham NE), but it did produce a robust effect in transected animals (Trans NE). An ANCOVA, with baseline BP serving as the covariate, confirmed that the main effect of NE treatment and its interaction with transection surgery were statistically significant, both Fs > 14.828, $p < 0.0007$ ($\eta^2 >$ 0.253). *Post hoc* comparisons of the group means confirmed that the transected animals that received norepinephrine differed from the other three groups ($p < 0.05$). Additionally, the transected animals that received vehicle had a significantly lower BP throughout the monitoring period than the other three groups ($p < 0.05$). No other group comparisons were significant ($p > 0.05$). Lastly, there was also a three-way interaction

between time, transection, and NE, $F_{(3, 81)} = 2.952$, $p = 0.0375$. No other group comparisons were significant ($p > 0.05$).

Baseline heart rate ranged from 215 ± 19.00 to 262 ± 27.9 (mean ± SE) across groups. These differences were not statistically significant, all Fs < 1.2, $p > 0.05$. Vehicle-treated transected rats (Trans Veh) displayed lower heart rate throughout testing (Figure 5C). An ANCOVA with baseline heart rate serving as a covariate revealed a main effect of transection, $F_{(1, 27)} = 5.370$, $p = 0.0283$ ($\eta^2 = 0.141$). *Post hoc* comparisons of the group means yielded a significant difference between the transected vehicle group from both sham-operated groups ($p < 0.05$). No other comparisons were significant ($p > 0.05$).

Before transection, blood flow ranged from 2.61 ± 1.04 to 3.26 ± 1.27 (mean ± SE) and did not differ between groups, all Fs < 1.0, $p > 0.05$. After shock treatment, sham-operated rats exhibited a rise in blood flow over time and this effect was not increased by NE (Figure 5D). A greater rise in blood flow was observed in vehicle-treated transected rats (Trans Veh) and this effect was amplified by NE (Trans NE). An ANCOVA, with baseline flow serving as the covariate, revealed a main effect of transection surgery and NE treatment, and an interaction between transection and NE treatment, all Fs > 4.655, $p < 0.04$ (all $\eta^2 > 0.043$). *Post hoc* comparisons of the group means showed that the transected group given NE (Trans NE) differed from the other three. In addition, the vehicle-treated transected group (Trans Veh) differed from both sham-operated groups. No other group comparisons were significant ($p > 0.05$). There

was also a within subjects effect of time and an interaction between time and transection treatment, both Fs = 2.839, $p < 0.043$.

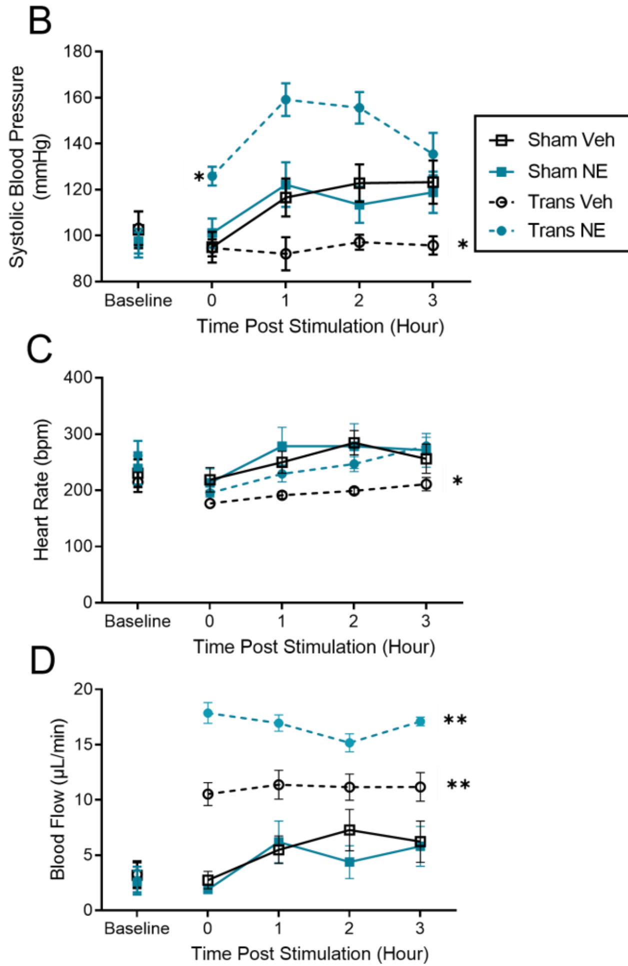


Figure 5. Norepinephrine increased blood pressure in transected but not sham-operated rats. (A) Experimental design and timeline for experiment 3. (B) Sham-operated shocked rats exhibited a rise in systolic blood pressure relative to vehicle-treated shocked animals that were transected. Transected rats that were given NE exhibited the greatest increase in BP. (C) Sham-operated rats exhibited greater heart rate throughout the hrs. Transected rats that received vehicle exhibited the lowest heart rate throughout the 3 hrs. (D) Spinally transected animals exhibited higher levels of blood flow, relative to sham-operated rats. NE increased blood flow in transected, but not sham-operated, rats. Asterisks indicate statistical significance (*p < 0.05, ** p < 0.01, *** p < 0.001, n = 8). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Contused rats that were not transected (Sham) and received shock showed higher peak absorbance at the wavelength associated with hemoglobin. Pretreatment with NE had no effect (Figure 6A). An ANOVA confirmed that the main effect of transection was statistically significant, $F_{(1, 28)} = 11.268$, $p < 0.0023$ ($\eta^2 = 0.272$). No other term approached significance, both *F*s < 2.0, $p > 0.05$.

A similar pattern was observed with the Drabkin's assay. Sham-operated rats showed a higher peak absorbance than transected rats, and NE had no effect (Figure 6B). An ANOVA revealed a significant main effect of transection surgery, $F_{(1, 28)} = 4.248$, $p = 0.0487$ ($\eta^2 = 0.128$). No other term approached significance, both *F*s < 2.0, $p > 0.05$.

Western blot confirmed the spectrophotometry analyses, demonstrating that shocked contused rats that had not received a transection (Sham) had higher concentrations of hemoglobin at the site of injury relative to the transected groups (Figure 6C). An ANOVA yielded a main effect of transection, $F_{(1, 28)} = 37.357$, $p < 0.001$ ($\eta^2 = 0.538$). Neither NE treatment, nor its interaction with transection, was statistically significant, both *F*s < 2.27, $p > 0.05$. *Post hoc* comparisons of group means confirmed that Transected groups differed from Sham groups ($p < 0.05$).

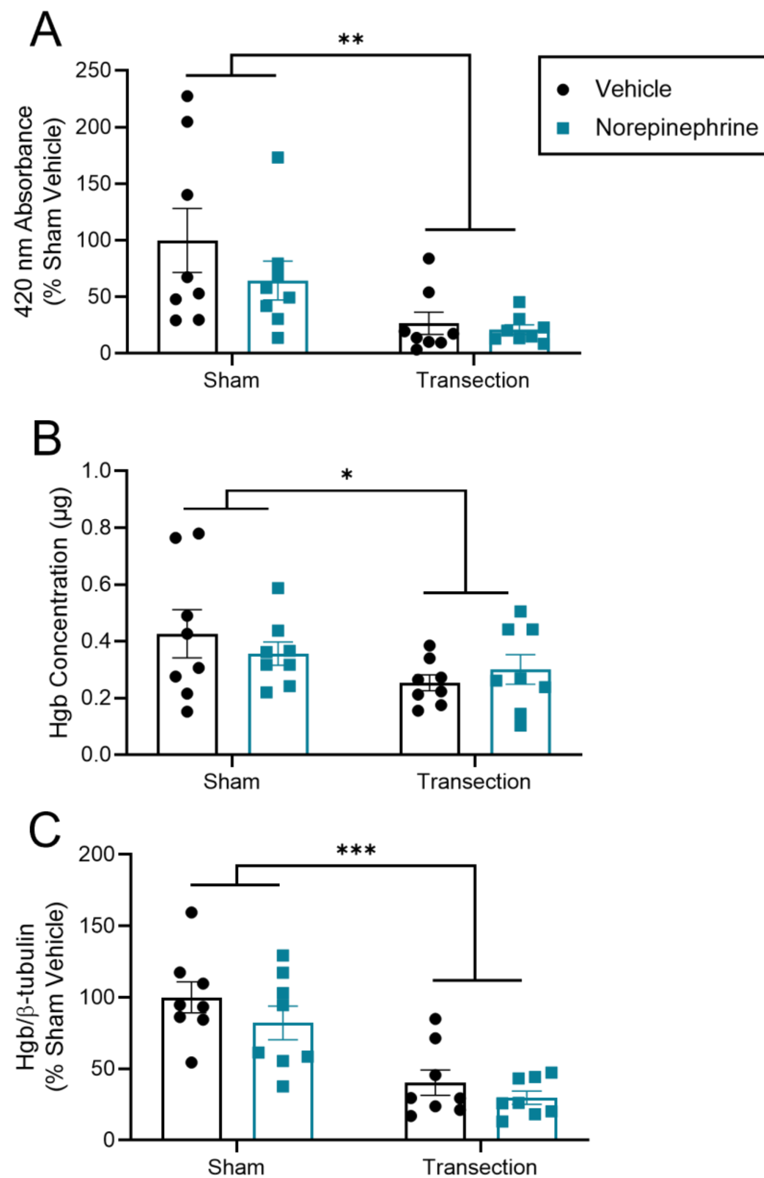


Figure 6. Norepinephrine did not induce hemorrhage in transected rats exposed to noxious tail shock. (A) Spectrophotometry results at 420 nm for hemoglobin revealed that sham-operated shocked rats exhibited greater absorbance, relative to the transected animals. NE had no effect. (B) Drabkin's assay and western blot (C) showed a similar pattern. Only sham-operated rats showed an increase in hemoglobin content and expression at the injury site. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n = 8). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Summary

Experiment 1 explored the role of the brain in the shock-induced rise in BP and hemorrhage. I replicated previous results and found that electrical stimulation induces a rise in systolic BP, heart rate and blood flow. I built upon these results by showing that disrupting communication with the brain with a rostral T2 transection blocks the significant rise in BP and heart rate. Interestingly, I found that the T2 transection led to a rise in blood flow, comparable to that of the sham-operated rats that received shock.

In addition to cardiovascular effects, I replicated previous results (Turtle et al., 2019) and showed that sham-operated rats that received shock treatment exhibited an increase in hemorrhage at the T12 lesion site. This effect was generalized across three separate hemorrhage analyses, spectrophotometry, Drabkin's assay, and immunoblot. I also showed that cutting supraspinal signals blocked the significant rise in hemorrhage.

Experiment 2 assessed the effect of capsaicin treatment on blood pressure and hemorrhage in T2 transected rats. I found that capsaicin treatment had no effects on systolic blood pressure over three hours, replicating previous results (Misty M. Strain et al., under review). I also found that rats given a transection surgery exhibited a reduced heart rate compared to sham-operated animals. On the other hand, transection surgery resulted in a significant rise in blood flow compared to sham-operated controls.

Despite the lack of changes in systolic blood pressure due to capsaicin, the sham-operated rats that received capsaicin treatment exhibited a greater amount of hemorrhage compared to vehicle controls. Additionally, transection surgery blocked this effect in all three analyses of hemorrhage.

Experiment 3 examined if inducing a pharmacological rise in BP would be sufficient to induce hemorrhage in shock-treated rats. In sham operated rats that received noxious stimulation, norepinephrine did not induce an increase systolic BP, heart rate, or blood flow compared to vehicle controls. However, in transected rats, norepinephrine induced a rise in systolic BP even greater than that of sham-operated rats. In contrast to systolic BP, norepinephrine treatment induced a rise in heart rate comparable to that of sham-operated rats. Lastly, norepinephrine treatment in transected rats induced a rise in blood flow that was greater than that of vehicle controls. Despite norepinephrine's effect on cardiovascular measures, it failed to induce a rise in hemorrhage in transected rats. Only sham-operated rats displayed a rise in hemorrhage in response to shock treatment.

CHAPTER IV

IS BRAIN-DEPENDENT HEMORRHAGE RELATED TO AN INCREASE IN BLOOD SPINAL CORD BARRIER PERMEABILITY?

The BSCB functions to protect the spinal cord from foreign molecules from penetrating the spinal cord. Previous work in our laboratory suggests that exposure to noxious stimulation increases the permeability of the BSCB, allowing blood borne contents to enter the neural tissue and expanding the area of tissue loss. My next set of experiments examine whether noxious stimulation increases BSCB permeability after SCI. Given positive evidence, I then tested whether this effect depends upon communication with the brain.

Experiment 4: Nociception after spinal cord injury increases blood spinal cord barrier permeability

Previous work has shown that noxious input after SCI exacerbates secondary injury, including hemorrhage, inflammation, and cell death (Turtle et al., 2019; Turtle et al., 2018). More specifically, pain input after SCI has been associated with progressive hemorrhagic necrosis with evidence of an upregulation of Sur1-Trpm4 and capillary fragmentation (Turtle et al., 2019). Several studies have linked indices of PHN with increased blood – brain/spinal cord – barrier permeability in models of neurotrauma and stroke (Jiang et al., 2017; Lee et al., 2014; Lee, Choi, et al., 2015; Lee, Choi, Park, Ju, & Yune, 2018; Park et al., 2019; Yao et al., 2018). Here, I examined if pain-induced hemorrhage is due to increased BSCB permeability.

Procedure

Twenty rats received a moderate contusion at T12. Twenty-four hours later, baseline locomotor scores were assessed using the BBB locomotor score system. Then, half of the rats (n = 10, randomly assigned) received six minutes of shock (1.5 mA, 0.2-3.8 second ISI) or an equal period of restraint. Immediately after shock treatment, all animals received an injection of Evans blue dye. Then, locomotor scores were assessed at hourly intervals for three hours (experimental design and timeline in Figure 7A).

After the last locomotor score was collected, rats were sacrificed with a lethal dose of pentobarbital, perfused with cold saline, and then a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for Evans blue spectrophotometry analysis. To prepare for analysis, the spinal cord tissue was homogenized and centrifuged, and the supernatant was then diluted. The samples were analyzed for Evans blue concentration using spectrophotometry.

Results

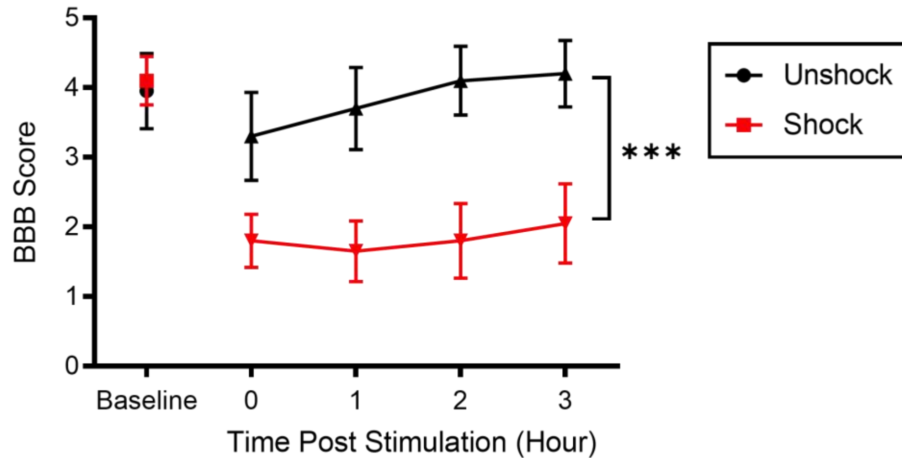
Locomotor scores were collected at hourly intervals to confirm the effect of shock treatment on contused rats (Figure 7B). Similar to previous studies, exposure to noxious electrical stimulation disrupted locomotor performance. An ANCOVA revealed a significant effect of shock treatment, $F_{(1,17)} = 28.0$, $p < 0.001$.

Rats that received shock showed higher Evans blue concentration in their spinal cords after spectrophotometry analysis (Figure 7C). A simple group comparison (*t*-test) confirmed that this difference was statically significant, $p < 0.05$.

A

T12 Contusion	24 hours	BBB Baseline	Unshock / Shock	Evans Blue	BBB 0-3 hr	Collect Tissue

B



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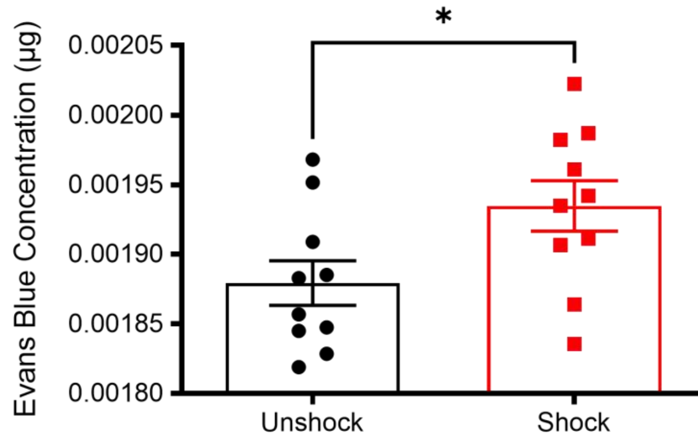

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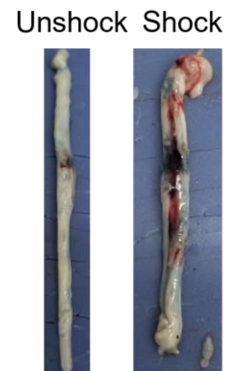

Figure 7. Electrical stimulation increases BSCB permeability. (A) Experimental design and timeline. (B) Electrical stimulation induced an acute locomotor deficit. (C) Electrical stimulation increased Evans blue concentration (an indicator for BSCB permeability) in the spinal cord. (D) Representative cords from the experiment. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 10$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 5: Transection block pain-induced increase in blood spinal cord barrier disruption

The previous experiment showed that exposure to noxious stimulation increases BSCB permeability. My earlier experiments showed that nociception-induced secondary injury is dependent on brain systems. Given this, I hypothesized that blocking communication with the brain will block the pain-induced increase in BSCB permeability. I tested this by assessing infiltration of Evans blue at the site of injury in animals that received both noxious stimulation and a spinal transection.

Procedure

Forty rats received a moderate contusion at T12 and then immediately after a complete transection at T2 or a sham surgery. Twenty-four hours later, half of the rats in each group (randomly assigned) received six minutes of shock (1.5 mA, 0.2-3.8 second ISI) or an equal period of restraint. Immediately after shock treatment, all rats received an injection of Evans blue dye (experimental design and timeline in Figure 8A).

Three hours later, rats were sacrificed with a lethal dose of pentobarbital, perfused with cold saline, and then a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for Evans blue spectrophotometry analysis. To prepare for analysis, the spinal cord tissue was homogenized and centrifuged, and the supernatant was then diluted. The samples were analyzed for Evans blue concentration using spectrophotometry. The experiment involved a 2 (Sham vs. Transection) x 2 (Unshock vs. Shock) factorial design (n = 10).

Results

Sham operated animals that received shock displayed higher Evans blue dye in their spinal cords relative to unshocked controls. A transection surgery blocked this effect (Figure 8B). An ANOVA revealed significant main effects of shock treatment and transection surgery, Fs > 5.582, $p < 0.05$. *Post hoc* comparisons of the group means confirmed that the sham rats that received shock were different from the other three groups ($p < 0.05$).

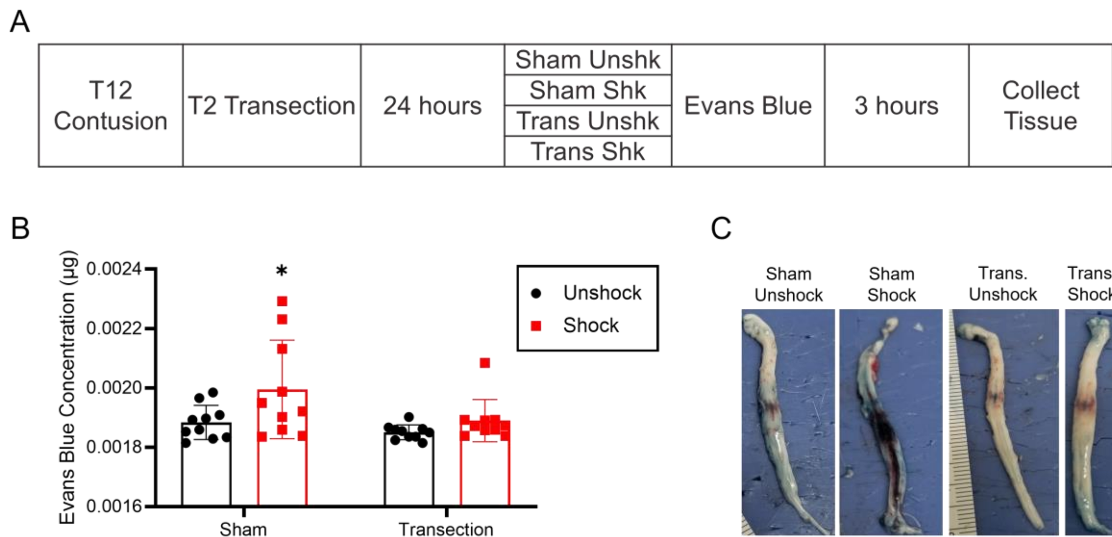

Figure 8. Transection blocks shock-induced increase in BSCB permeability. (A) Experimental design and timeline. (B) Evans blue concentration in the injured tissue was higher after shock treatment. This effect was blocked by spinal transection. (C) Representative pictures from the experiment. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 10$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Summary

Experiment 4 assessed BSCB permeability in response to electrical stimulation. I replicated previous results (M. M. Strain et al., 2019; Turtle et al., 2019) and found that electrical stimulation induced an acute locomotor deficit. I also found that BSCB permeability, measured by the infiltration of the large molecule dye Evans blue, increased in response to nociceptive input.

Experiment 5 explored the role of supraspinal systems in BSCB permeability. I replicated my earlier observation and showed that sham-operated rats that received electrical stimulation exhibited a significant rise in Evans blue dye within the spinal cord, suggesting a rise in permeability. Additionally, I found that a subsequent T2 transection surgery blocked this effect.

CHAPTER V

IS BRAIN-DEPENDENT PAIN SUFFICIENT TO DRIVE HEMORRHAGE?

The previous chapters have shown that the brain is involved the development of hemorrhage at the injury site. Data from Experiments 1-3 suggest that a brain-dependent rise in BP and flow, while elevates in response to electrical stimulation, is not sufficient to induce hemorrhage. It is currently unknown what brain-dependent processes underlie this effect. The current chapter explores whether brain-dependent pain is sufficient to induce hemorrhage. To test this, I first assess the relationship between morphine and BP/flow, showing that morphine fails to block the rise in BP observed after noxious electrical stimulation as well as hemorrhage. I then explore whether engaging brain processes with noxious stimulation applied above the injury site is sufficient to induce hemorrhage.

Experiment 6: Anesthetic dose of morphine fails to protect against pain-induced rise in blood pressure and hemorrhage

I showed above that the nociception-induced rise in hemorrhage and blood pressure/flow depends on brain systems. Because electrical stimulation only induces hemorrhage when it is set at a level that engages defensive behavior (M. M. Strain et al., 2019), and because activation of pain (C) fibers induces hemorrhage after SCI (Turtle et al., 2019), I naturally hypothesized that the rise in blood pressure and increased hemorrhage was tied to brain-dependent pain. Contrary to this hypothesis, past work suggests that pretreatment with an analgesic (morphine) does not attenuate shock-

induced hemorrhage (Turtle et al., 2017). In the present experiment, I sought to replicate this finding and test whether morphine lacks a protective effect because it does not block the brain-dependent rise in blood pressure.

Procedure

Twenty-eight rats received a moderate contusion at T12. Twenty-four hours later locomotor scores were assessed using the BBB scoring system. Then, rats were given an injection of morphine (i.p.) or its vehicle. Fifteen minutes later, half the animals in each group received either six minutes of shock (1.5 mA, 0.2-3.8 second ISI) or an equal period of restraint. Systolic blood pressure, heart rate, and blood flow were measured prior to morphine injection and at hourly intervals for 3 hours after shock treatment (experimental design and timeline in Figure 9A).

After obtaining the last three-hour cardiovascular measurement, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis) and with gel electrophoresis and immunoblotting for hemoglobin. The experimental design involved a 2x2 factorial with four groups, Vehicle Unshock (n=6), Vehicle Shock (n=8), Morphine Unshock (n=6), and Morphine Shock (n=8).

Results

Analysis of BBB scores showed that animals that received shock displayed a lower BBB score throughout the three hours (Figure 9B). An ANOVA, with baseline BBB score serving as the covariate, confirmed that the main effects of shock and morphine treatment were statistically significant, all Fs > 7.279, $p < 0.013$. There was also a within subjects effect of time on morphine $F_{(3, 69)} = 3.648$, $p = 0.017$.

A

T12 Contusion	24 Hours	Baseline BBB & BP	Vehicle	15 Minutes	Unshock	BP & BBB 0-3hr	Collect Tissue
					Shock		
			Morphine		Unshock		
					Shock		

B

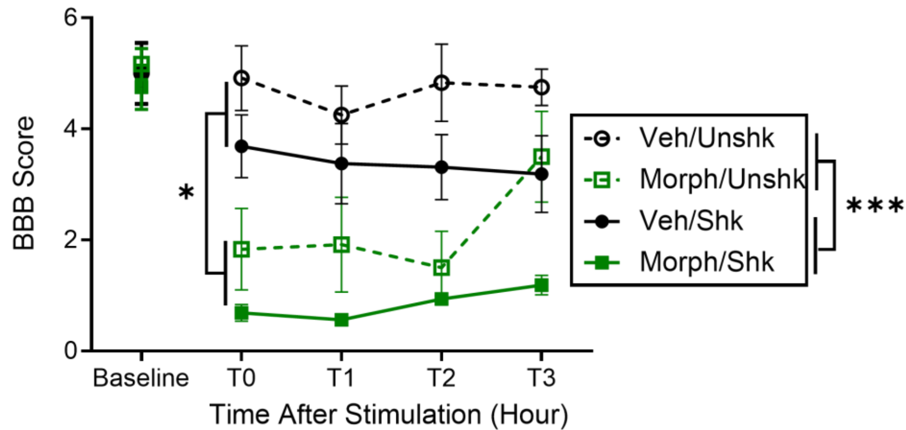

Figure 9. Systemic morphine does not block the acute disruption in locomotor performance observed after noxious electrical stimulation. (A) Experimental design and timeline. (B) Electrical stimulation induced a locomotor deficit. Animals treated with morphine displayed significantly lower locomotor scores than vehicle-treated animals. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Prior to shock and morphine treatment, systolic BP ranged from 106 ± 7.96 to 109 ± 10.7 (mean ± SE) across groups. These differences were not statistically significant, all *F*s < 1.0, $p > 0.05$. Analysis of BP revealed that rats that received morphine and pain input exhibited a higher BP after shock treatment (Figure 10A). An ANCOVA, with baseline BP serving as the covariate, confirmed that the main effects of shock and morphine, and their interaction, were statistically significant, *F*s > 6.93, $p < 0.015$. *Post hoc* comparisons of the group means showed that the rats that received morphine and shock treatment differed from the other three groups ($p < 0.05$). The within subjects term time, and its interactions with morphine treatment, were also significant, *F*s > 2.59, $p < 0.039$.

Prior to shock and morphine treatment, heart rate ranged from 263 ± 22.0 to 317 ± 41.7 (mean ± SE) across groups. These differences were not statistically significant, all *F*s < 1.633, $p > 0.05$. Analysis of heart rate after nociceptive stimulation revealed that shock-treated rats exhibited a higher heart rate across the three hours of testing (Figure 10B). An ANCOVA confirmed a main effect of shock treatment $F_{(1, 23)} = 9.090$, $p = 0.006$. There was also a within subjects interaction between time and morphine treatment, $F_{(3, 69)} = 5.007$, $p = 0.003$. No other comparisons were significant.

Prior to shock and morphine treatment, blood flow ranged from 4.37 ± 1.78 to 6.72 ± 1.78 (mean ± SE) across groups. These differences were not statistically significant, all *F*s < 1.0, $p > 0.05$. Analysis of flow after nociceptive stimulation revealed that shock-treated rats exhibited significantly higher flow throughout the three hours (Figure 10C). An ANCOVA confirmed a main effect of shock treatment $F_{(1, 23)} = 5.492$,

$p = 0.028$. The within subjects term time, and its interaction with morphine treatment, were also significant, both Fs > 3.094, $p < 0.033$. No other comparisons were significant.

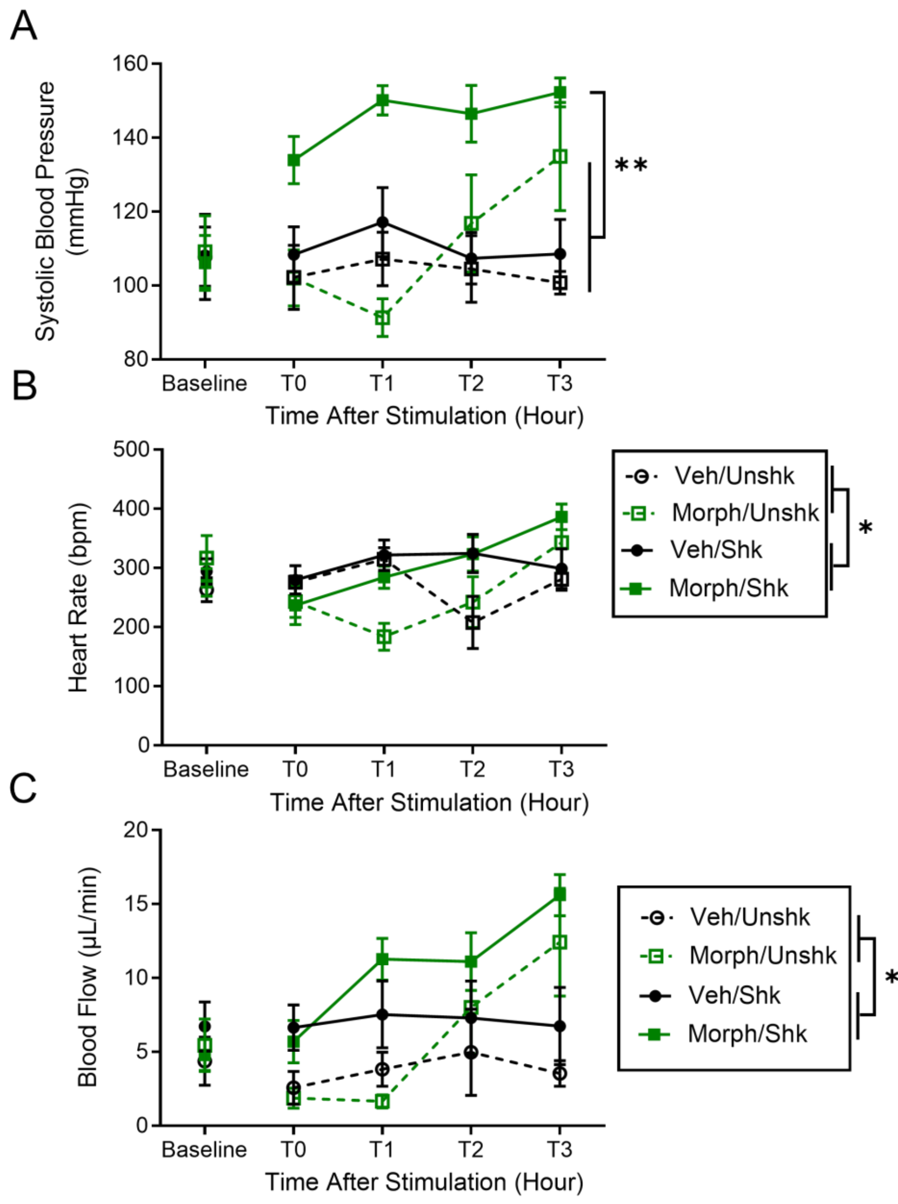

Figure 10. Morphine and electrical stimulation elevated blood pressure. (A) Systolic BP for Morphine-treated rats that received electrical stimulation displayed higher systolic BP than the other three groups. (B) Electrical stimulation induced a rise in heart rate and (C) flow. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Spectrophotometric analysis of the spinal protein samples yielded no significant results based on morphine or shock treatment (Figure 11A). An ANOVA confirmed no main effects or interactions between morphine and shock conditions, $Fs < 2.3$, $p > 0.05$.

Hemoglobin concentration obtained with the Drabkin's assay revealed that electrical stimulation increased hemoglobin in the spinal cord lesion site, relative to unshock controls (Figure 11B). Treatment with morphine had no effect. An ANOVA confirmed the main effect of shock treatment, $F_{(1, 24)} = 6.707$, $p = 0.016$. No other comparisons were significant.

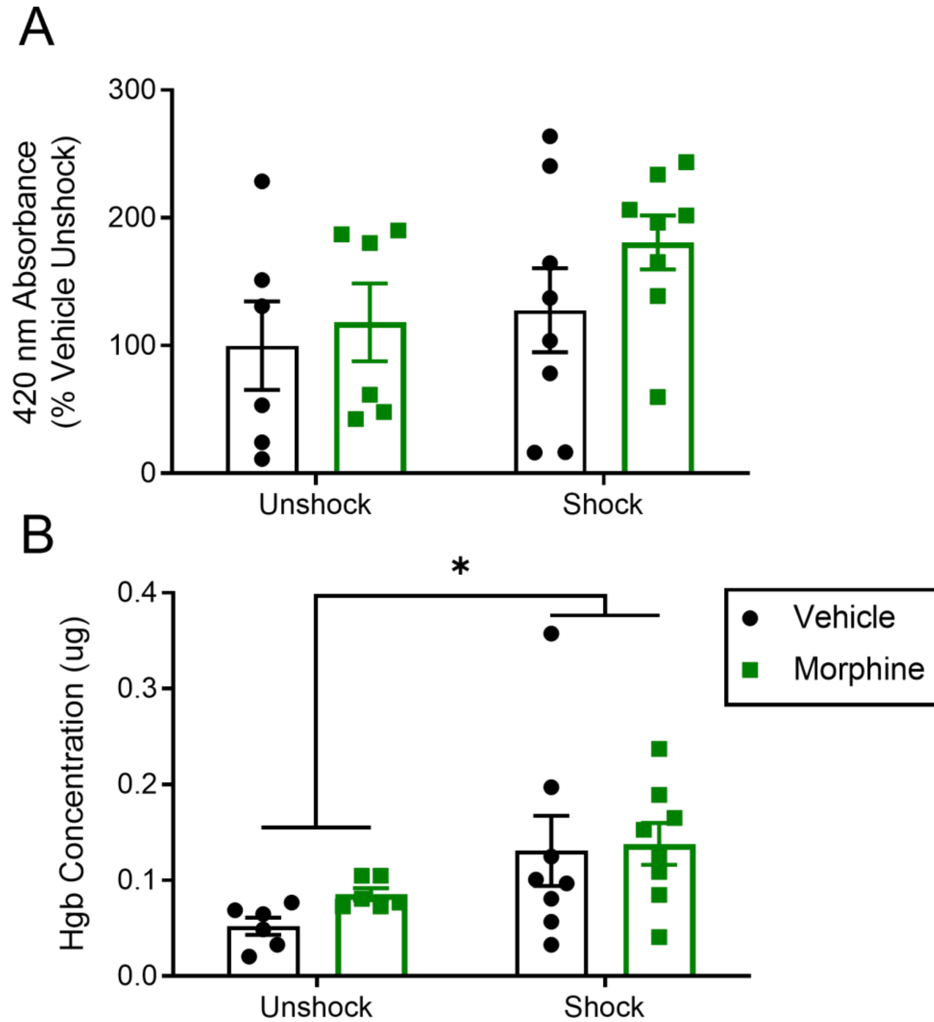

Figure 11. Treatment with electrical stimulation increased hemorrhage. (A) Spectrophotometry at 420 nm did not yield significant effects. (B) Drabkin's assay revealed that electrical stimulation induced an increase in hemoglobin in the spinal cord. Morphine failed to block this effect. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 7: Only shock below the injury induces hemorrhage at the lesion site

Experiments 1-3 have shown that the pain-induced rise in hemorrhage is dependent on brain systems. It is unclear whether brain processes modulate (necessary, but not sufficient) or mediate (sufficient) the effect of noxious stimulation on hemorrhage. To explore this issue, electrical stimulation was delivered either above or below the injury. If the brain-dependent effects are mediating the induction of hemorrhage, stimulation above the injury should be sufficient to activate the brain-dependent hemorrhage at the injury site. Given that prior work has shown that nociceptive stimulation below the injury leads to local effects within the spinal cord akin to central sensitization and EMR, I hypothesized that nociception must travel through the injury site to initiate local mechanisms that participate in the development of secondary injury.

Procedure

Thirty-six rats received a moderate contusion at T12 and twenty-four hours later locomotor scores were assessed using the BBB scoring system. Then, all the rats received an injection of morphine (i.p.) and fifteen minutes later, half the rats in each group received either six minutes of shock (1.5 mA, 0.2-3.8 second ISI) to the hindlimb, forelimb or an equal period of restraint. BBB, systolic blood pressure, heart rate, and blood flow were measured prior to morphine injection and at hourly intervals for 3 hours after shock treatment (experimental design and timeline in Figure 12A).

After obtaining the last cardiovascular measurement, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered

on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized, and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis) and with gel electrophoresis and immunoblotting for hemoglobin. The experiment involved a 2 (Unshock vs. Shock) x 2 (Hindlimb vs. Forelimb) factorial design. The Unshock groups were collapsed into a single group as no differences were found in the outcome measures reported for this study between the two groups, Fs < 4.14, $p > 0.05$ (n=6). Both shock groups had an n of 12.

Results

Analysis of BBB scores across the three hours revealed no differences between groups (Figure 12B). An ANCOVA, with the baseline BBB score serving at the covariate, confirmed no significant differences due to shock treatment to the hindlimb or forelimb relative to unshock controls, Fs < 0.108, $p > 0.05$.

A

T12 Contusion	24 Hours	SCI Baseline	Morphine Injection	15 Minutes	Electrode Insertion	Unshock Hindlimb Shk Forelimb Shk	BP & BBB 0-3hr	Collect Tissue

B

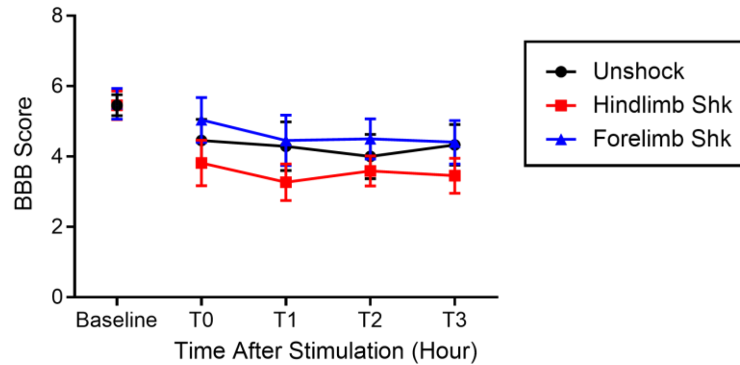

Figure 12. Electrical stimulation to either the hindlimb or forelimb did not induce a change in locomotor performance. (A) Experimental design and timeline. (B) Locomotor scores over three hours (T0-T3) after electrical stimulation. Shock treatment to the hindlimb or forelimb failed to induce any acute changes in BBB scores. Error bars represent the standard error of the mean (SEM, n= 12).

Prior to shock and morphine treatment, systolic BP ranged from 98.2 ± 6.44 to 102 ± 5.42 (mean ± SE) across groups. These differences were not statistically significant, all $F_{(2, 33)} = 0.102$, $p > 0.05$. There were no differences between groups across the three hours (Figure 13A). An ANCOVA, with baseline BP serving as the covariate, confirmed no significant differences, Fs < 2.48, $p > 0.05$.

Prior to shock and morphine treatment, heart rate ranged from 77.0 ± 5.33 to 222 ± 21.4 (mean ± SE) across groups These differences were statistically significant with the Unshock group exhibiting a higher heart rate, all $F_{(2, 29)} = 55.7$, $p < 0.001$. *Post hoc* comparison of the means confirmed that the rats that received no electrical stimulation were different from the other two groups ($p < 0.05$). There were no differences between groups across the three hours (Figure 13B). An ANCOVA, with baseline heart rate serving as the covariate, confirmed no significant effects, Fs < 1.742, $p > 0.05$.

Prior to shock and morphine treatment, flow ranged from 3.31 ± 0.94 to 4.74 ± 1.60 (mean ± SE) across groups. These differences were not statistically significant, $F_{(2, 33)} = 0.357$, $p > 0.05$. There were no differences between groups across the three hours (Figure 13C), Fs < 1.696, $p > 0.05$. There was, however, a significant effect of time, $F_{(3, 60)} = 10.057$, $p = 0.0001$.

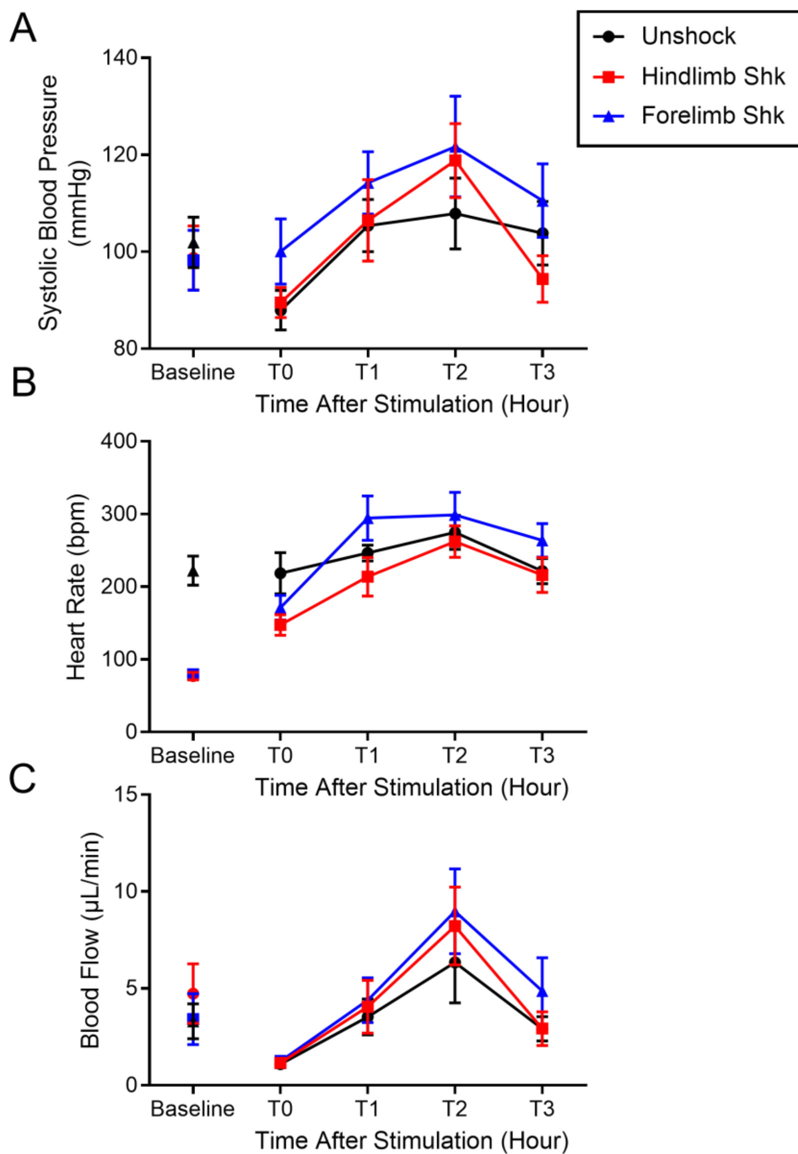

Figure 13. Electrical stimulation to the limbs did not have a significant effect on cardiovascular function. There were no differences between groups in systolic BP (A), heart rate (B), or flow (C). Error bars represent the standard error of the mean (SEM, n = 12).

Analysis of hemoglobin content with spectrophotometry suggests that hindlimb shock had no effect on hemorrhage (Figure 14A). An ANOVA confirmed no significant effects, $F_{(2, 33)} < 1.0$, $p > 0.05$.

However, hemoglobin content, measured by Drabkin's assay, revealed that shock treatment below the injury increased hemoglobin in the spinal cord lesion site (Figure 14B). An ANOVA confirmed the significant difference between groups, $F_{(2, 33)} = 3.597$, $p = 0.0386$. *Post hoc* comparison of the means revealed that the Hindlimb Shock group differed from the other two ($p < 0.05$).

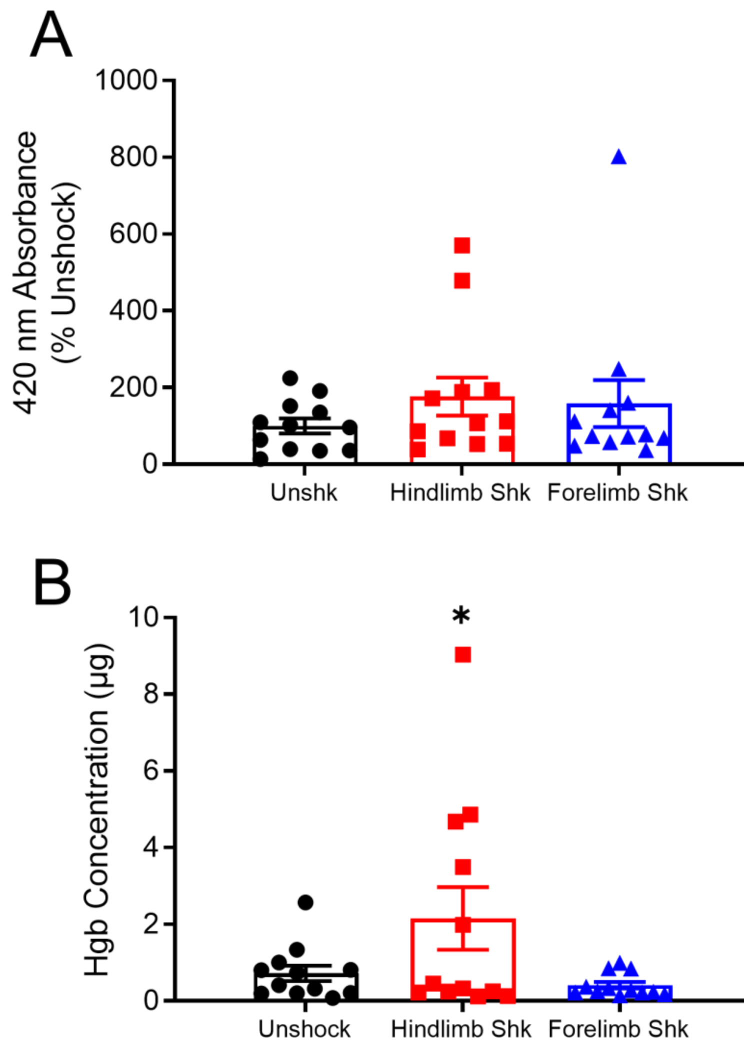

Figure 14. Only stimulation to the hindlimb induced hemorrhage. (A) Spectrophotometry at 420nm for hemoglobin. There were no differences between groups. (B) Hemoglobin concentration measured by Drabkin's assay. Rats that received hindlimb shock exhibited greater hemorrhage than the other two groups. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 12$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 8: Only capsaicin below the injury induces hemorrhage at the lesion site

Experiment 7 showed that shock above the injury fails to amplify hemorrhage after SCI. To explore the generality of this effect with a more clinically relevant pain model, I tested the effects of the irritant capsaicin. Because Experiment 2 showed that treatment with capsaicin does not impact our measures of cardiovascular function, these were not assessed in the present experiment.

Procedure

Thirty-six rats received a moderate contusion at T12 and twenty-four hours later locomotor scores were assessed using the BBB scoring system. Animals were then given a single injection of capsaicin or its vehicle into the hind paw or forepaw (randomly assigned) (experimental design and timeline in Figure 15A).

After obtaining the final BBB score, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized, and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis) and with gel electrophoresis and immunoblotting for hemoglobin. The experiment involved a 2 (Vehicle vs. Capsaicin) x 2 (Hind paw vs. Forepaw) factorial design (n=10). The Vehicle groups were collapsed into a single group as no

differences were found in the outcome measures reported for this study between the two groups, Fs < 1.27, $p < 0.05$.

Results

Analysis of BBB scores across the three hours revealed no differences between groups (Figure 15B). An ANCOVA, with the baseline BBB score serving at the covariate, confirmed no significant group differences due to capsaicin treatment to the hindlimb or forelimb relative to vehicle controls, Fs < 0.332, $p > 0.05$. There was a significant (within subjects) difference of time, $F_{(6, 108)} = 3.99$, $p < 0.001$.

A

<table>
<tr><td rowspan="4">T12 Contusion</td><td rowspan="4">24 Hours</td><td rowspan="4">Baseline BBB</td><td rowspan="2">Vehicle</td><td>Forepaw</td><td rowspan="4">3 hours BBB 0-3hr</td><td rowspan="4">Collect Tissue</td></tr>
<tr><td>Hind paw</td></tr>
<tr><td rowspan="2">Capsaicin</td><td>Forepaw</td></tr>
<tr><td>Hind paw</td></tr>
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B

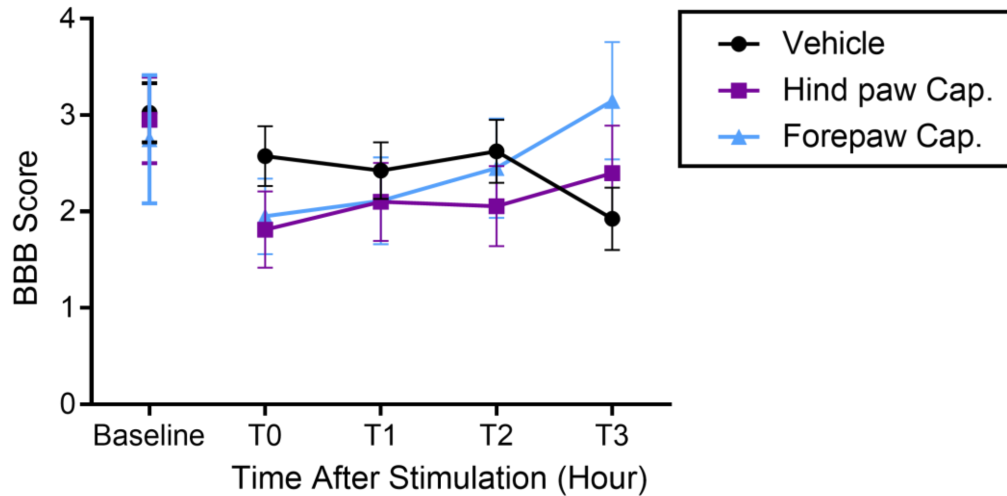


Figure 15. Capsaicin treatment to hind paw or fore paw did not impact locomotor performance. (A) Experimental design and timeline. (B) Locomotor scores over three hours (T0-T3) following capsaicin injection. Capsaicin treatment failed to induce any BBB changes throughout the three hours. Error bars represent the standard error of the mean (SEM, n = 12).

Analysis of the hemorrhage through spectrophotometry revealed no significant differences across groups (Figure 16A). An ANOVA yielded no significant results, $F_{(2, 37)} < 1.0$, $p > 0.05$.

Hemorrhage analyzed by Drabkin's assay showed that capsaicin treatment below the injury increased hemoglobin in the spinal cord lesion site (Figure 16B). Because there was some variability in our assay results across the first and second half (blocks) of the animals, this term was entered as a factor in an ANCOVA. The analysis revealed a significant effect of capsaicin treatment, $F_{(2, 36)} = 3.188$, $p = 0.0531$. Orthogonal contrasts confirmed that the group treated with capsaicin to the hind paw differed from the other two ($p < 0.05$).

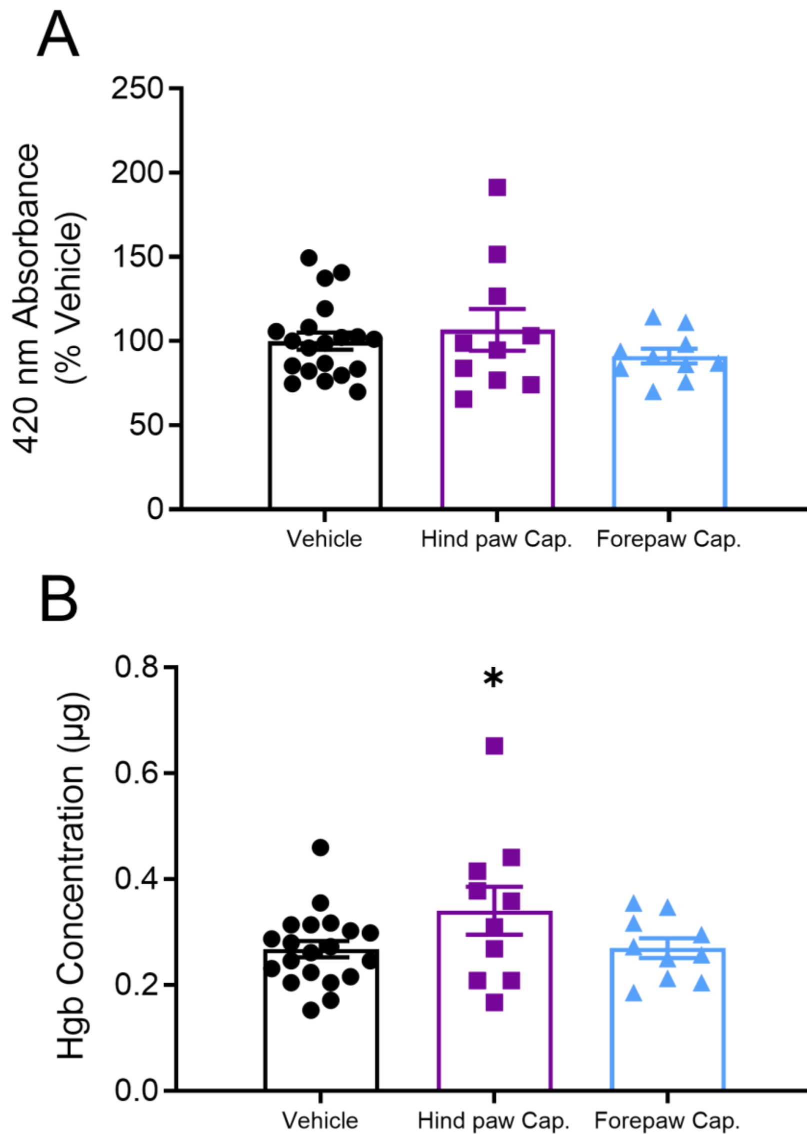


Figure 16. Capsaicin administered to the hind paw induced hemorrhage. (A) Spectrophotometry measured at 420 nm for hemoglobin. Spectrophotometric analysis did not find any group differences. (B) Hemoglobin concentration obtained by Drabkin's assay. Rats treated with capsaicin to the hind paw exhibited greater hemorrhage in the spinal cord. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 12$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Summary

Experiment 6 explored the effects of morphine on BP and hemorrhage. As previously reported (REF), morphine did not block nociception induced hemorrhage at the site of injury. I extended this observation by showing that the drug also does not block the effect of shock treatment on locomotor function. Morphine also failed to attenuate the nociception-induced rise in systolic BP, heart rate and flow. In fact, rats treated with morphine and electrical stimulation exhibited significantly higher systolic BP that all other groups.

Experiment 7 examined whether electrical stimulation administered above the site of injury is sufficient to induce hemorrhage. I found that neither stimulation to the hindlimb nor forelimb was sufficient to induce an acute locomotor deficit. Similarly, electrical stimulation to the forelimb and hindlimb did not induce any changes in cardiovascular measures. Despite the lack of locomotor deficit and changes in BP, noxious stimulation induced hemorrhage when applied to a hindlimb.

CHAPTER VI

IMPACT OF STIMULATION AT PAIN THRESHOLD

The current method of electrical stimulation to the tail has been developed through prior work showing that electrical shock delivered at 1.5 mA is sufficient to activate C-fibers, induce an acute and long-term locomotor deficit, and promote hemorrhage (Baumbauer et al., 2008; Grau et al., 2004; Turtle et al., 2019). The previous chapter explored the role of brain-dependent pain in the development of hemorrhage using electrical stimulation the limbs. While I was able to establish an effect of pain-induced hemorrhage when shock was delivered below the injury, I failed to establish a locomotor deficit or brain-dependent increase in BP, implying that electrical stimulation to the limbs does not have an effect comparable to tailshock.

Electrical stimulation to the limbs is delivered through wire electrodes inserted into the skin whereas tail shock is applied using cutaneous electrodes. It is possible that shock applied to the limbs through wire electrodes failed to induce a change in behavioral/physiological function because it was less aversive. To address this issue, I developed a procedure that equated the aversive, brain-dependent, response to stimulation. This was achieved by establishing the shock intensity required to elicit a vocalization response. I first evaluate whether intermittent shock at this intensity induces an acute disruption in locomotor performance and hemorrhage. I then use this procedure to evaluate the effect of stimulation applied to hindlimb and forelimb.

Experiment 9: Electrical stimulation at vocalization threshold induces comparable hemorrhage to electrical stimulation at 1.5 mA

In the current experiment, I examined whether shock at a threshold that evokes a vocalization response has an effect comparable to our usual shock procedure, that involves the application of constant current shock at an intensity of 1.5 mA. My hypothesis was that nociceptive stimulation to the tail at an intensity that elicits a vocalization response would have a comparable effect on locomotor performance and hemorrhage.

Procedure

Thirty-two rats received a moderate contusion at T12 and twenty-four hours later locomotor scores were assessed using the BBB scoring system. Then, half of the rats had their vocalization thresholds measured using a procedure based on (King et al., 1996). The other half rested in the tubes for an equal period. After thresholds were collected, rats were shocked either at the intensity of their threshold or at 1.5 mA (0.2-3.8 second ISI). Unshocked controls experienced an equal period of restraint during this time. Locomotion performance was measured immediately after shock treatment and at hourly intervals for three hours (experimental design and timeline in Figure 17A).

After obtaining the final BBB score, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized, and the protein was extracted. Then, the protein was analyzed for evidence of

hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis). The experiment involved four groups: Unshock, Shock, Unshock_Test, and Vocal_Test (n=8).

Results

Animals that received six minutes of electrical stimulation exhibited a significant locomotor deficit relative to the Unshock controls (Figure 17B). While the threshold test does involve electrical stimulation, it was not sufficient to induce any locomotor deficit in the Unshocked_Test group. Rats shocked at vocalization threshold exhibited a locomotor deficit that was significantly different from the Unshock controls without the threshold test, while shocking at 1.5 mA induced a deficit that was significantly different from both Unshock control groups. An ANCOVA, with baseline BBB score serving as the covariate, yielded a significant group difference, $F_{(3, 27)} = 8.70$, $p < 0.001$. *Post hoc* comparison of the means confirmed significant differences between Unshock and both shock groups (Shock and Vocal_Test), and between the Unshock_Test and the Shock groups ($p < 0.05$).

A

T12 Contusion	24 Hours	BBB Baseline	No Threshold	Unshk_No Test	BBB 0-3hr	Collect Tissue
				Shk_No Test		
			Shock Threshold	Unshk_Test		
				Vocal_Test		

B

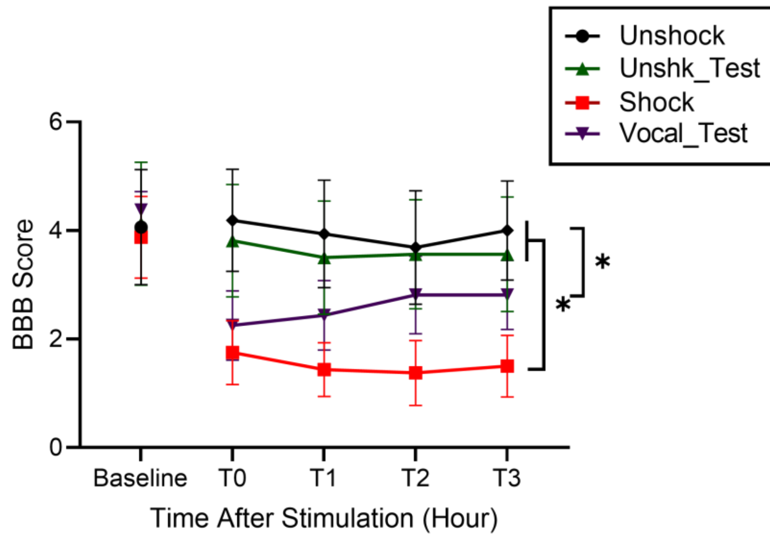


Figure 17. Electrical stimulation at the threshold for eliciting a vocalization response induced an acute locomotor deficit. (A) Experimental design and timeline. (B) Locomotor scores three hours (T0-T3) after electrical stimulation. Rats that received six minutes of electrical stimulation exhibited a locomotor impairment compared to Unshock controls. Rats shocked at 1.5 mA displayed a greater impairment than rats shocked at vocalization threshold relative to Unshock_Test controls. Asterisks indicate statistical significance (*p < 0.05, ** p < 0.01, *** p < 0.001, n = 8). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Spectrophotometry conducted on the protein samples revealed that six minutes of shock increased hemorrhage at the lesion site (Figure 18A). An omnibus ANOVA test with *a priori* comparisons of the shocked groups to the unshocked controls confirmed that electrical stimulation had a significant effect, $F_{(1, 28)} = 5.777$, $p = 0.023$.

Hemoglobin content measured by Drabkin's assay revealed no significant differences between groups (Figure 18B). Shock to the tail at vocalization threshold or 1.5 mA failed to induce a significant increase in hemorrhage. An ANOVA revealed no statistical difference across the groups, $F_{(3, 28)} = 1.91$, $p > 0.05$.

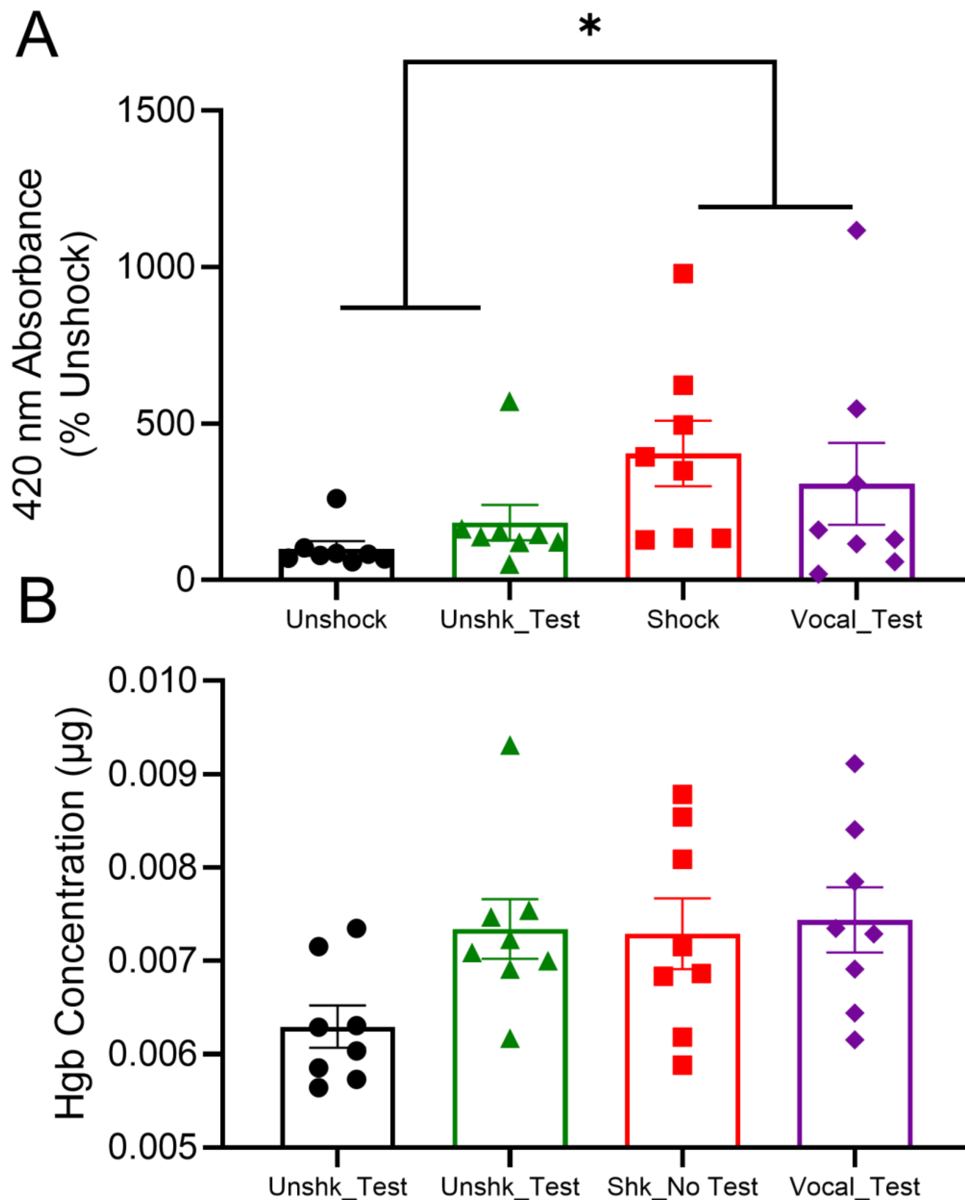


Figure 18. Electrical stimulation at pain threshold is sufficient to induce comparable hemorrhage. (A) Spectrophotometry at 420nm for hemoglobin. Rats that received shock treatment at pain threshold or 1.5 mA exhibited greater hemorrhage than Unshock controls. (B) Hemoglobin concentration collected by Drabkin's assay displayed no group differences. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 10: Electrical stimulation to the tail and hindlimb at vocalization threshold leads to comparable hemorrhage

The previous experiment confirmed that nociceptive stimulation at an intensity that elicits a vocalization response is sufficient to induce hemorrhage. While I showed above that shock to a hindlimb induces hemorrhage, the effect was relatively weak, possibly because it was less aversive than tailshock. To address this issue, I compared the effect of noxious stimulation to the tail and hindlimb when the aversive quality was equated, by applying the stimulation at both sites at an intensity that elicited a vocalization response. I hypothesized that shocking at vocalization threshold will be sufficient to induce hemorrhage independent of the site of stimulation.

Procedure

Thirty-two rats received a moderate contusion at T12. Twenty-four hours later locomotor scores were assessed using the BBB scoring system. Then, half of the rats had wire electrodes inserted into the skin in their hindlimbs. After electrode insertion, vocalization thresholds were collected for all shock rats and half the unshock rats. After thresholds were collected, rats were shocked at the intensity of their threshold with 100ms shocks given on a variable schedule (0.2-3.8 second ISI). Unshocked controls experienced an equal period of restraint. There were no differences in any of the outcome measures between unshock groups for this experiment, Fs < 1.0, $p > 0.05$, and for this reason, these groups were collapsed into a single group. Locomotion performance was measured immediately after shock treatment and at hourly intervals for three hours (experimental design and timeline Figure 19A).

After obtaining the final BBB score, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized, and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis). The experiment involved three groups: Unshock (n=8), Tail Shock (n=12), and Hindlimb Shock (n=12).

Results

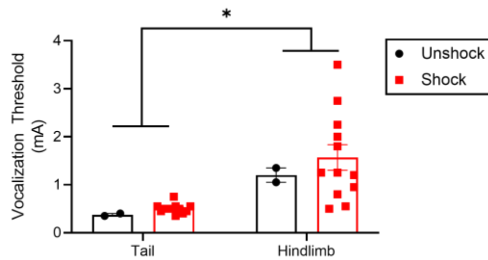
More intense stimulation was required to elicit a vocalization response when it was applied through a wire electrode on a hind limb (Figure 19B). An ANOVA revealed a significant main effect of electrode site, $F_{(1, 24)} = 7.735$, $p = 0.01$.

Rats that received electrical stimulation at vocalization thresholds exhibited a deficit in locomotor function throughout the three hours (Figure 19C). An ANCOVA, with baseline BBB score serving as the covariate, revealed a significant group effect, $F_{(2, 20)} = 5.96$, $p = 0.009$. *Post hoc* comparisons of the group means confirmed a significant difference between the unshock controls and the two shock groups ($p < 0.05$).

A

T12 Contusion	24 Hours	BBB Baseline	No Threshold	Unshock	Tail	BBB 0-3hr	Collect Tissue
					Hindlimb		
			Shock Threshold	Unshock	Tail		
					Hindlimb		
				Shock	Tail		
					Hindlimb		

B



C

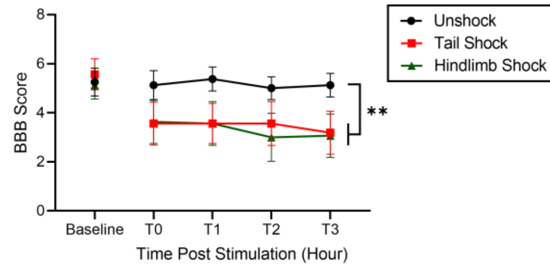


Figure 19. Electrical stimulation to the hindlimb induced a comparable locomotor deficit to tail shock. (A) Experimental design and timeline. (B) Pain thresholds collected from electrical stimulation to the tail or hindlimb. Electrical stimulation to the hindlimb required significantly higher pain thresholds. (C) Locomotor scores across three hours (T0-T3) after shock treatment. Electrical stimulation induced an acute impairment in BBB scores regardless of location (tail or hindlimb). Asterisks indicate statistical significance (*p < 0.05, ** p < 0.01, *** p < 0.001, n = 8 for Unshock and n = 12 for Shock groups). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Spectrophotometric analysis of the protein samples revealed no differences across groups. Shock treatment failed to increase hemorrhage regardless of electrode type (Figure 20A). An ANOVA confirmed no statistical differences between groups, $F_{(2, 29)} = 1.21$, $p > 0.05$.

Hemoglobin concentration, obtained by the Drabkin's assay, revealed that shock treatment increased hemoglobin content at the spinal cord lesion site (Figure 20B). A linear regression model that built upon the *a priori* that shock treatment would induce hemorrhage independent of electrode location/type yielded a significant difference between the unshock control group and both shock groups ($p < 0.05$).

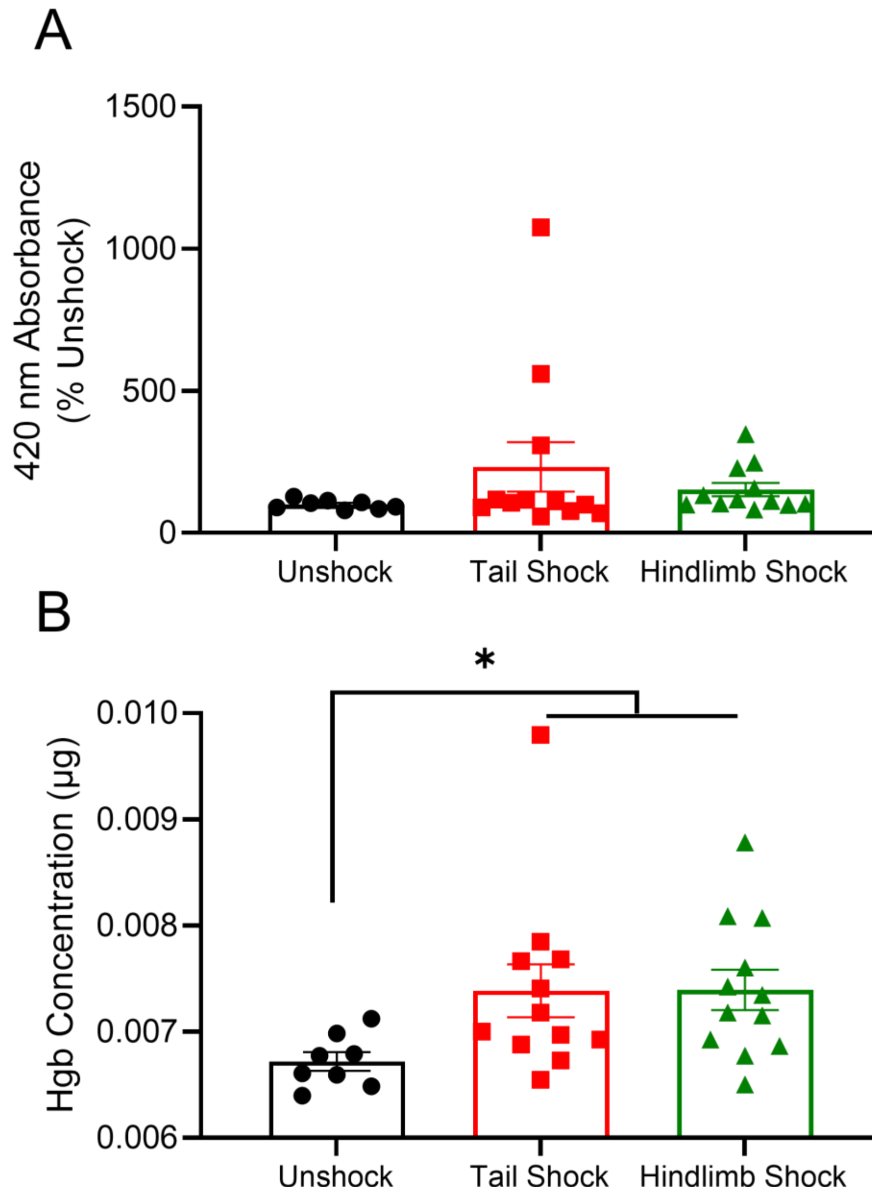


Figure 20. Electrical stimulation to the hindlimb or tail at vocalization threshold induced comparable hemorrhage. (A) Spectrophotometry at 420nm for hemoglobin showed no differences between groups. (B) Hemoglobin concentration from Drabkin's assay exhibited greater hemorrhage in rats that received electrical stimulation to the tail or hindlimb. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$ for Unshock and $n = 12$ for Shock groups). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Experiment 11: Forelimb shock fails to induce a rise in hemorrhage or systolic blood pressure

The previous experiment confirmed that hindlimb shock at an intensity that evokes a vocalization response is sufficient to induce hemorrhage. Given this, I anticipated that stimulating above the injury at an intensity that evokes the same response should elicit a comparable cardiovascular response. The key question is whether it would also increase the area of hemorrhage.

Procedure

Forty rats received a moderate contusion at T12. Twenty-four hours later locomotor scores were assessed using the BBB scoring system. Then, wire electrodes were inserted either into the hindlimb or in the forelimb. After electrode insertion, vocalization threshold was measured in the shock treatment groups as described above. After thresholds were collected, rats were shocked at the intensity of their threshold or experienced an equal period of restraint. Two unshock control groups were added, one with electrode insertion and one without electrode insertion, to account for the pain input from the electrodes. None of the unshock controls experienced the vocal threshold test. Locomotor performance was assessed immediately after shock treatment and at hourly intervals for three hours (experimental design and timeline in Figure 21A).

After obtaining the final BBB score, rats were sacrificed with a lethal dose of pentobarbital and a one-centimeter section of spinal cord tissue centered on the lesion site was collected and flash frozen. Spinal cords were kept at -80°C until processed for hemorrhage analysis. To prepare for analysis, the spinal cord tissue was homogenized,

and the protein was extracted. Then, the protein was analyzed for evidence of hemorrhage with spectrophotometry (420nm for observational analysis and 540nm for Drabkin's analysis). The experiment involved four groups: Unshock, Unshock_Elect., Hindlimb Shock, and Forelimb Shock (n=10).

Results

When electrical stimulation was applied to the forelimb, less intense shock was needed to elicit a vocalization response (Figure 21B). An ANOVA confirmed this effect of group, $F_{(1, 18)} = 18.8$, $p < 0.001$. No other comparisons were significant.

Replicating previous results, shock treatment at vocalization threshold to the hindlimb resulted in a locomotor deficit for three hours following treatment. Shock to the forelimb, however, failed to induce a locomotor deficit (Figure 21C). An ANCOVA, with baseline BBB score serving as the covariate, revealed a significant effect of group, $F_{(3, 35)} = 5.42$, $p = 0.004$. *Post hoc* comparison of the means revealed that the Hindlimb Shock group was significantly different from the other three groups ($p > 0.05$).

A

<table>
<tr><td rowspan="4">T12 Contusion</td><td rowspan="4">24 hours</td><td rowspan="4">BBB & BP Baseline</td><td rowspan="2">No Threshold</td><td>Unshock</td><td rowspan="4">BBB & BP 0-3hr</td><td rowspan="4">Collect Tissue</td></tr>
<tr><td>Unshock_Elect.</td></tr>
<tr><td rowspan="2">Shock Threshold</td><td>Hindlimb Shk</td></tr>
<tr><td>Forelimb Shk</td></tr>
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B

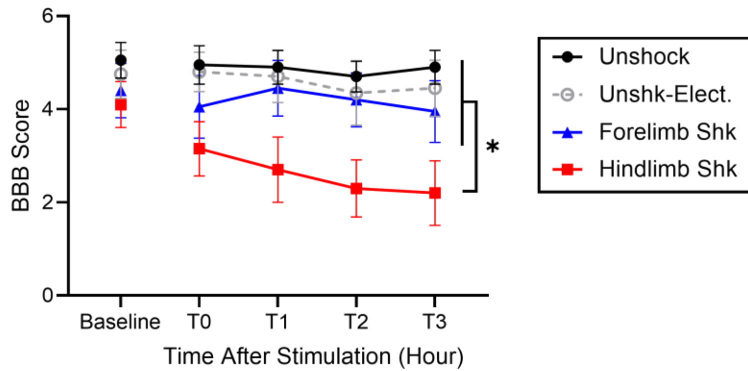

Figure 21. Shock to hindlimb, but not the forelimb, induced acute locomotor deficit. (A) Experimental design and timeline. (B) Locomotor scores across three hours (T0-T3) after shock treatment. Electrical stimulation to the hindlimb induced an acute BBB impairment compared to the other three groups. Asterisks indicate statistical significance (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 10$). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Prior to shock treatment, systolic BP ranged from 84.8 ± 4.71 to 103 ± 7.91 (mean ± SE) across groups. These differences were not statistically significant, all $F_{(3, 36)} = 1.65$, $p > 0.05$. Analysis of BP across time revealed that neither shock nor location of electrodes had any effect on systolic BP (Figure 22A). An ANCOVA, with baseline BP serving as the covariate, confirmed no differences between groups, Fs > 1.757, $p > 0.05$.

Prior to shock treatment, heart rate ranged from 97.9 ± 42.7 to 130 ± 43.8 (mean ± SE) across groups. These differences were not statistically significant, all $F_{(3, 36)} < 1.0$, $p > 0.05$. Analysis of heart across time yielded no differences between groups due to shock treatment or location of electrodes (Figure 22B). An ANCOVA, with baseline heart rate serving as the covariate, confirmed no significant differences Fs < 1.5, $p > 0.05$.

Prior to shock treatment, blood flow ranged from 0.956 ± 0.182 to 3.16 ± 1.27 (mean ± SE) across groups. These differences were not statistically significant, $F_{(3, 36)} = 1.27$, $p > 0.05$. Analysis of flow across the three hours revealed no differences between groups (Figure 22C). An ANCOVA, with baseline flow serving as the covariate, confirmed no between subjects effects, $F_{(3, 35)} < 1.0$, $p > 0.05$. There was, however, a within subjects effect of time, $F_{(3,105)} = 3.180$, $p = 0.027$. No other comparisons were significant.

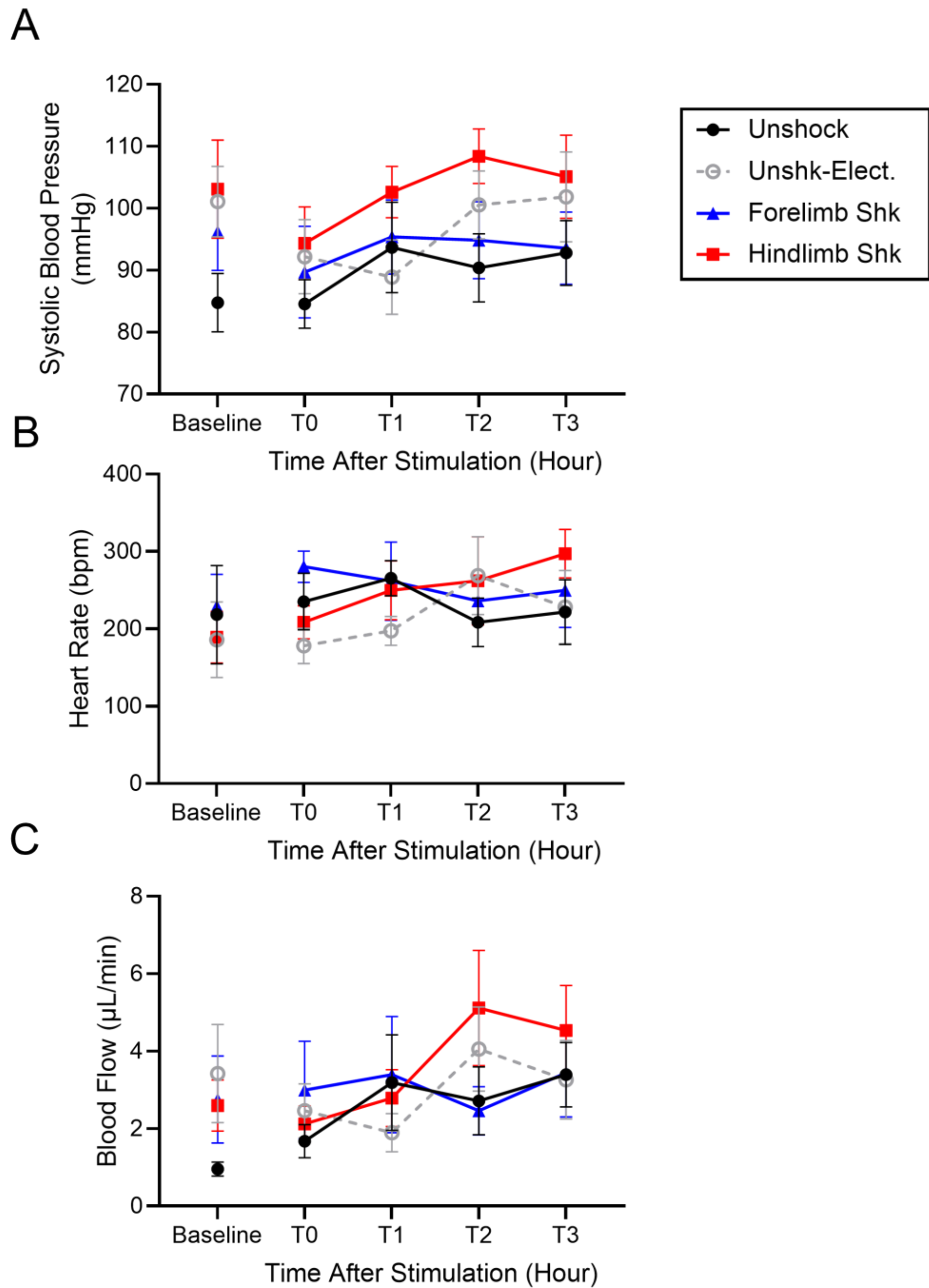

Figure 22. Electrical stimulation to the limbs at pain threshold did not induce a significant cardiovascular response. Electrical stimulation to the hindlimb or forelimb failed to induce any group differences in systolic BP (A), heart rate (B) or flow (C). However, there was a time-dependent effect of hindlimb shock on flow. Error bars represent the standard error of the mean (SEM, n = 10).

Despite the hindlimb shock inducing a locomotor deficit, there were no differences in hemorrhage compared to control groups, measured by spectrophotometry at 420nm. Indeed, any shock treatment, whether to the hindlimb or forelimb, failed to increase blood content at the lesion site (Figure 23A). An ANOVA confirmed no significant differences between groups, $F_{(3, 36)} = 1.10$, $p > 0.05$.

Additionally, to spectrophotometry results, hemoglobin content measured by Drabkin's assay yielded similar results. Hemoglobin content did not increase due to shock treatment or location of that shock treatment (Figure 23B). An ANOVA confirmed no significant differences across groups, $F_{(3, 36)} < 1.0$, $p > 0.05$.

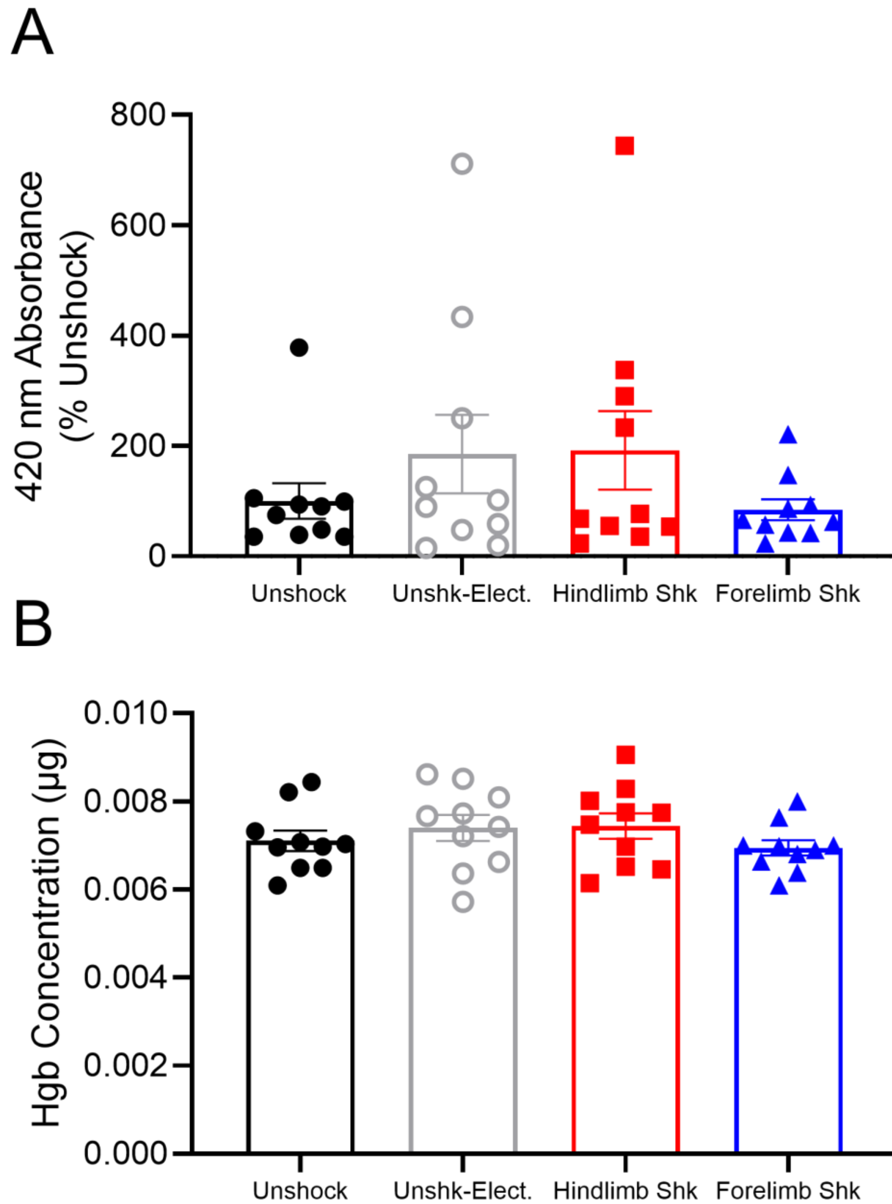

Figure 23. Electrical stimulation to the limbs at vocalization threshold failed to induce hemorrhage. Electrical stimulation to the forelimbs and hindlimbs at vocalization threshold did not induce hemorrhage as analyzed with spectrophotometry (A) or Drabkin's assay (B) compared to unshock controls. Error bars represent the standard error of the mean (SEM, n = 10).

Summary

Experiment 9 examined whether electrical stimulation delivered to the tail at pain threshold induces hemorrhage comparable to that of shock at 1.5 mA. I replicated previous results and showed that electrical stimulation at 1.5 mA impairs acute locomotor recovery compared to unshocked controls. I also showed that the vocalization threshold test *per se* does not induce a locomotor deficit. Finally, I showed that electrical stimulation, at an intensity that induces a vocalization response causes an acute locomotor deficit comparable to that of rats shocked at 1.5 mA.

In addition to the locomotor deficit, I found that shock delivered at vocalization threshold is sufficient to drive hemorrhage in the spinal cord. This effect was comparable to that of rats shocked at 1.5 mA.

Experiment 10 built upon previous results and examined if electrical stimulation at vocalization threshold increases hemorrhage when delivered to the hindlimb. I found that vocalization thresholds were significantly higher in hindlimb rats than tail rats. I also found that shock delivered at this intensity resulted in an acute locomotor deficit comparable to that observed in rats given stimulation to the tail. Lastly, I found that both tail shock and hindlimb shock delivered at vocalization threshold increases hemorrhage in the spinal cord.

Experiment 11 examined if shock delivered at vocalization threshold to the forelimb is sufficient to induce hemorrhage. I found that only electrical stimulation to the hindlimb led to an acute locomotor deficit. I replicated previous results from Experiment 7 and found that there were no group differences in systolic BP, heart rate,

and blood flow. Finally, and in contrast to previous results found in Experiment 10, I found that electrical stimulation to neither the forelimb nor hindlimb at vocalization threshold was sufficient to induce hemorrhage.

CHAPTER VII

GENERAL DISCUSSION

Summary

Using a clinically relevant rodent model of contusion SCI, the current dissertation examined the role of spared fibers in the development of nociception-induced hemorrhage. I began in Chapter III by assessing whether cutting communication with the brain blocks hemorrhage because it blocks the pain-induced rise in BP. Next, I further characterized the effect of pain on secondary injury by examining BSCB permeability in Chapter IV. Then, in Chapter V, I determined if brain-dependent pain is sufficient to induce hemorrhage by examining the effects of morphine and noxious stimulation above the site of injury. Finally, I explored how nociception at threshold affects acute hemorrhage and locomotor function.

I found that disrupting brain/spinal cord communication reduced hemorrhage and the pain-induced rise in BP. However, pain input induced by capsaicin revealed no changes in BP, although the subsequent T2 transection did induce a rise in flow. Despite the lack of change in systolic BP, hemorrhage induced by capsaicin was blocked by T2 transection. Lastly, I found that pharmacologically inducing a rise in BP with norepinephrine led to a rise in BP in transected rats. However, this elevation was not sufficient to induce hemorrhage.

In Chapter IV, I examined if shock increases BSCB permeability by systemically injecting the rats with Evans blue dye. Evans blue, while considerably low in molecular

weight (961 Da), readily binds to serum albumin. This transforms the dye to a high molecular weight (69,000 Da) permeability marker (Ahishali & Kaya, 2021; Saunders, Dziegielewska, Møllgård, & Habgood, 2015; Wolman et al., 1981). When the blood brain barrier or BSCB is disrupted, the dye leaks into the compromised region and stains the area blue. Pain input is known to increase the infiltration of blood cells into the spinal tissue (M. M. Strain et al., 2019; Turtle et al., 2019). In Experiment 4, I found that electrical stimulation increases BSCB permeability to allow the passage of toxic blood cells. In Experiment 5, I determined that the effect of shock on BSCB permeability is dependent on spared fibers.

In Chapter V, I confirmed that an anesthetic dose of morphine does not protect against the adverse effects of noxious input on hemorrhage and locomotor performance. I also found that morphine treatment resulted in elevated BP and flow. When evaluating the effects of noxious stimulation above the injury to engage brain-dependent pain processes, I found that neither electrical stimulation nor capsaicin induced an acute locomotor deficit. I also found no changes in BP due to electrical stimulation. However, both treatments resulted in hemorrhage, but only when the treatment was delivered below the injury site, not above.

Finally, in Chapter VI, I found that noxious stimulation administered at pain (vocalization) threshold is sufficient to induce hemorrhage that is comparable to hemorrhage induced at 1.5 mA. I also found that electrical stimulation delivered to the hindlimb through wire electrodes, while requires a higher pain threshold, also results in hemorrhage comparable to that of tailshock. Lastly, I confirmed that delivery of

electrical stimulation at pain threshold above the site of injury is not sufficient to fuel hemorrhage.

Taken together, this work demonstrates that spared fibers and brain systems are involved in the development of nociceptive-induced hemorrhage. The brain processes that drive hemorrhage do not appear to be linked to perceived pain because attenuating this state with morphine had no effect. Likewise, while a rise in BP may amplify the adverse effects of noxious stimulation, the fact exposure to capsaicin induces hemorrhage but has little effect on blood pressure/flow suggests it is not necessary. Likewise, pharmacologically inducing an increase in blood flow/pressure was not sufficient to drive hemorrhage. Further, engaging a brain-dependent response with stimulation applied rostral to injury failed to produce hemorrhage, which suggests that engaging brain processes is not sufficient.

Pain-Induced Secondary Injury

Prior work has shown that nociceptive input administered soon after injury impairs functional recovery and increases tissue loss and inflammation (Grau et al., 2004; M. M. Strain et al., 2019; Turtle et al., 2017; Turtle et al., 2018). Subsequent research has expanded on these findings and established the circumstances under which these effects are observed. For example, the spinal cord is only vulnerable to the adverse effects of pain input within the first few days (1-4 days) after SCI (Grau et al., 2004). In an electrical stimulation model, stimulation is delivered at 1.5 mA for six minutes because it has been found to induce a learning impairment in a transection model (Ferguson et al., 2006) and functional impairments in a contusion model (Grau et al.,

2004). However, recent research has found that only 72 seconds of stimulation and stimulation delivered at 0.5 mA is sufficient to induce an acute and long-term locomotor deficit as well as expand the hemorrhagic lesion (M. M. Strain et al., 2019).

Importantly, work in our laboratory has also found that hemorrhage indued by noxious pain is associated with the upregulation of Sur1-Trpm4 and capillary fragmentation, indices indicative of progressive hemorrhagic necrosis (Turtle et al., 2019). I expanded on this work by demonstrating noxious stimulation increases BSCB permeability (Experiment 4) and that this effect is brain-dependent (Experiment 5).

Work outside the laboratory have linked indices of progressive hemorrhagic necrosis with breakdown of the BSCB (Lee et al., 2014; Lee et al., 2018; Lee, Kang, & Yune, 2015; Park et al., 2020; Yao et al., 2018). The BSCB provides protection against large molecules such as hemoglobin. Damage to the BSCB is a universal consequence of SCI, found in both humans and animal models (Bartanusz, Jezova, Alajajian, & Digicaylioglu, 2011). It is also the main hallmark of secondary injury, as it has been shown to increase in permeability as soon as five minutes after SCI (Maikos & Shreiber, 2007), remains compromised as long as 56 days after SCI (Cohen et al., 2009), and is responsible for allowing the infiltration of toxic materials such as red blood cells.

The disruption of the microvasculature of the BSCB allows the progression of PHN and tissue loss. In the secondary injury literature, PHN has been closely linked to the excessive expression and activation of matrix metalloproteases (MMPs, specifically MMP9) after SCI, leading to the numerous pathologies of secondary injury (de Castro, Burns, McAdoo, & Romanic, 2000; Gerzanich, Kwon, Woo, Ivanov, & Simard, 2018;

Lee et al., 2014; Noble, Donovan, Igarashi, Goussev, & Werb, 2002). Additionally, MMPs have been linked to neuropathic pain (Kawasaki et al., 2008). Because we have linked pain input to the upregulation of Sur1-Trpm4, capillary fragmentation, hemorrhage, and BSCB permeability, it is possible that MMPs play a pivotal role within our own model. Future studies also need to examine the effects of capsaicin on BSCB permeability.

Role of Brain-Dependent BP in SCI

Rise in BP is Brain-Dependent

Prior work has shown that electrical stimulation administered 24 hours after spinal cord injury leads to a rise in systolic BP for up to three hours and that this effect is associated with hemorrhage (Misty M. Strain et al., under review). It was also found that pharmacologically blocking the nociception-induced rise in BP and flow with prazosin attenuates the shock-induced rise in hemorrhage. On the other hand, pharmacologically inducing an acute rise in BP with norepinephrine in the absence of pain input impaired acute locomotor recovery but did not drive hemorrhage. Interestingly, capsaicin treatment did not induce a rise in systolic BP but instead elevated blood flow. This acute rise in blood flow was associated to a rise in hemorrhage. Taken together, this work suggests that hemorrhage is dependent, in part, on a rise in systolic BP/flow.

I replicated these results in Experiment 1 and found a rise in systolic BP/flow after electrical stimulation. I also found this effect to be associated with a rise in hemorrhage. I then expanded upon previous research and showed that the shock-induced

rise in BP/flow is blocked by T2 transection, suggesting that the pain-induced rise in BP and hemorrhage is brain-dependent.

However, I did not find a rise in blood flow in the capsaicin-treated rats in Experiment 2. Other work has found that capsaicin has an inconsistent effect on blood pressure (Chahl & Lynch, 1987). It is possible that the effect of 3% capsaicin on BP in Experiment 2 was not sufficient to induce a sufficient rise in BP. However, despite the lack of elevated BP, capsaicin treatment induced hemorrhage in sham-operated rats, suggesting that a rise in BP and flow is not necessary for the expansion of hemorrhage in a capsaicin model.

While prior work with norepinephrine explored the effects of the drug on hemorrhage, it was performed in the absence of pain input. I found that norepinephrine induces a rise in BP in transected rats however, there was no synergistic effect of norepinephrine on sham-operated rats that received shock treatment. I also found that norepinephrine treatment was not sufficient to induce hemorrhage in the transected rats, suggesting that a rise in BP is not sufficient to induce hemorrhage. These results are consistent with previous statistical models suggesting that pain input has a direct and indirect effect on hemorrhage, and the ladder is dependent on BP (more specifically, blood flow).

Finally, I found an overall effect of transection on blood flow, showing a rise in blood flow after a transection. This effect was independent of pain input. It is well known that high thoracic injuries lead to a dysregulation of blood pressure due to the disruption of sympathetic fibers (Eldahan & Rabchevsky, 2018), and this effect seen in

Experiments 1-3 could be a reflection of that. However, hemorrhage was consistently blocked by spinal transection, suggesting that communication with the brain is necessary for the development of hemorrhage.

Effect of Morphine on BP and Hemorrhage

Previous research has shown that the expansion of hemorrhage is dependent on the activation of C-fibers rather than "psychological pain" (Turtle et al., 2017). Supporting this, attenuating pain with the opioid analgesic morphine failed to block the expansion of hemorrhage and tissue loss. Experiment 6 extended these results by testing the effect of morphine treatment on BP and locomotor performance. Consistent with its effects on hemorrhage, morphine failed to block the pain-induced rise in BP and acute disruption in locomotor performance. While these data are generally consistent with the view that a nociception-induced rise in BP/flow plays a role, they call into question the putative link to perceived pain.

Effect of Electrical Stimulation to the Limbs on BP

Experiments 7, 10, and 11 employed a new method of applying electrical stimulation to the limbs through wire electrodes as opposed to shock to the tail through a cutaneous electrode. Other work in our laboratory, examining stimulation-induced plasticity in spinally transected rats, has used electrical stimulation applied to the hind limb and shown that this form of stimulation can induce a form of nociceptive sensitization that impairs adaptive learning (Baumbauer et al., 2008; Ferguson et al., 2006). The current study was the first to use this technique to explore nociception-induced hemorrhage in contused rats.

In Experiments 7 and 11, I found that electrical stimulation to the limbs did not result in any group differences in BP. While there was a time dependent effect on flow in Experiment 11, this effect was independent of shock treatment. Although there was no effect of limb shock on BP, hindlimb shock induced hemorrhage in all three experiments. Further work is needed to elucidate the effect of electrical stimulation of the limbs on BP. A potential problem with the procedure is that the insertion of the wire electrodes induces some stress/noxious stimulation that could potentially drive hemorrhage. While we examined this possibility in Experiment 11 and found little evidence that the procedure used to prepare and/or baseline test the animals had an effect, we did observe increased variability in the unshocked animals which undermined our ability to resolve an effect of shock treatment.

Autonomic Dysreflexia

A major outcome measure for this dissertation was BP in response to pain input. Cervical injuries or high thoracic injuries can lead to dysregulated BP due to the damage to the autonomic nervous system, a syndrome called autonomic dysreflexia (AD). While previous work has found increased BP due to pain input (Misty M. Strain et al., under review), the injury in this model was in the lower thoracic region (T11- T12), avoiding areas innervated by the sympathetic nervous system. The current experiments in Chapter III explored if a high thoracic transection, normally used in models of AD, resulted in hemodynamic changes. I found that a T2 transection did not result in any changes to systolic BP or flow due to pain input. While blood flow did rise as a result of transection, this effect had no effect on hemorrhage.

A hypothesis explored in Chapter III was that the brain-dependent process responsible for the expansion of hemorrhage was a rise in BP and flow, similar to dysregulation found in AD. However, lack of hemodynamic changes in response to pain input found in the transected rats coupled with the absence of hemorrhage suggests that AD, or AD-like symptoms, are not the underlying mechanism. Other characteristics of the effect suggest that this is not dependent on AD. For example, while previous work has found a rise in BP, this effect does not extend past three hours after pain input and 24 hours after SCI (Misty M. Strain et al., under review). Autonomic dysreflexia, on the other hand, manifests itself in the chronic phase of SCI, often 3-6 months after injury in humans (Lindan et al., 1980) and 3-4 weeks in rodent models (Marsh & Weaver, 2004; Mayorov, Adams, & Krassioukov, 2001). Additionally, data from Experiment 3 showed that inducing hypertension in transected rats did not drive hemorrhage.

Brain-dependent Processes in SCI

The central hypothesis of this dissertation was that brain-dependent processes are involved in the expansion of secondary injury. I consistently found that disrupting spared fibers rostral to the SCI blocked nociception-induced hemorrhage (Experiments 1-3). In addition, a rostral transection blocked the shock-induced rise in BSCB permeability (Experiment 5). My findings are broadly consistent with on-going research showing that cutting communication with the brain, either surgically or by infusing the anesthetic lidocaine at T2, blocks hemorrhage (Davis et al., 2020; Reynolds et al., 2019). Further, a pharmacological transection blocks the adverse effect pain input has on long-term

recovery (Davis et al., 2020). It is currently unknown however, what fiber pathways mediate these effects.

Descending Circuits

Prior work has examined the modulation of spinal cord plasticity by descending fibers. In a transection model of instrumental learning, it has been found that the spinal cord can learn to maintain the hindleg in a flexed position to minimize net shock exposure (Crown & Grau, 2001). This effect is not observed if the noxious stimulation occurs independent of leg position (uncontrollable). Further, exposure to uncontrollable stimulation induces a lasting learning impairment (Crown et al., 2002b). Subsequent work found that administration of uncontrollable shock prior to spinalization fails to disrupt learning, suggesting that the brain exerts a protective effect (Crown & Grau, 2005). Later, it was found that descending serotonergic fibers residing in the DLF protect the spinal cord against the adverse effects of noxious input. Indeed, lesions to the DLF and 5-HT antagonists emulate the effect of spinalization and block the brain's protection against uncontrollable stimulation (Crown & Grau, 2005). Further work is needed to determine whether serotonergic fibers within the DLF contribute to nociception-induced hemorrhage.

Serotonergic fibers within the DLF could potentially enable hemorrhage by modifying the action of the neurotransmitter GABA. In an adult uninjured spinal cord, the release of GABA dampens neural excitability within the dorsal horn in the event of nociceptive stimulation. This is evidenced with the application of GABA agonists resulting in antinociception (Hwang & Yaksh, 1997; Kaneko & Hammond, 1997;

Roberts, Beyer, & Komisaruk, 1986). Conversely, local application of a GABA-A antagonist results in pronociception and promotes the development of nociceptive sensitization (Baba et al., 2003; Dougherty & Hochman, 2008; Roberts et al., 1986; Sivilotti & Woolf, 1994; Zhang, Hefferan, & Loomis, 2001). However, in the injured cord, GABA transforms from having an inhibitory (hyperpolarizing) effect to an excitatory effect (depolarizing), due to a downregulation in membrane-bound K+-Cl-cotransporter 2 (KCC2), producing over-excitability within the dorsal horn (Boulenguez et al., 2010; Cramer et al., 2008; Drew, Siddall, & Duggan, 2004; Hasbargen et al., 2010). In these studies, a change in GABA function was tracked by testing the effect of a GABA-A antagonist (bicuculline), which was shown to have an antinociceptive (rather than pronociceptive) effect after spinal transection. The switch in GABA function was related to a loss of descending serotonergic fibers within the DLF. Lesions to the DLF or the local administration of 5-HT antagonists were sufficient to induce a downregulation of KCC2 and promote nociceptive sensitization (Huang & Grau, 2018). Additionally, work with a place conditioning task with bicuculline showed that animals that received a DLF lesion preferred the bicuculline chamber, implying that the GABA-A antagonist had a paradoxical antinociceptive effect. Taken together, the results suggest that the loss of descending serotonergic fibers maintain homeostasis within the spinal cord through the regulation of KCC2.

Other work has shown that a down regulation of KCC2 contributes to the development of spasticity and chronic pain after a contusion injury (Boulenguez et al., 2010; Cramer et al., 2008; Hasbargen et al., 2010). It is not known whether a change in

GABA function contributes to nociception induced hemorrhage. Nor is it known whether the adverse effect brain systems have on tissue loss after a contusion injury depends upon fibers within the DLF. Given that prior work suggests that these fibers normally exert a protective effect (Crown & Grau, 2005), I hypothesize that brain processes fuel hemorrhage by means of an alternative fiber pathway or a systemic process.

Stress Systems

As discussed above, stress factors, such as corticosterone and immune cells are potential contributors to the expansion of secondary injury (Popovich et al., 2001). We have shown that six minutes of uncontrollable stimulation increases corticosterone for up to 72 hours, elevates markers for IL-1β, and decreases spleen weights (Washburn, 2007). Work in this dissertation suggest that systemic stress activated by pain input may not be sufficient to drive hemorrhage.

Experiments 7 and 8 explored the effect of brain-dependent pain on hemorrhage. I tested this by administering stimulation above the site of injury, effectively activating brain-dependent pain processes without the signal traveling through the spinal cord. However, neither electrical stimulation to the forelimb nor capsaicin injection to the forepaw was sufficient to induce hemorrhage. However, it is possible that the method of electrical stimulation through wire electrodes failed to produce hemorrhage because it is less aversive than shock delivered through a cutaneous electrode. To address that issue and equate the two shock delivery methods, I showed that electrical stimulation to the hindlimb at vocalization threshold produces comparable hemorrhage to tail shock at

vocalization threshold (Experiment 10). I then showed that electrical stimulation delivered to the forelimb with this method was not sufficient to induce hemorrhage (Experiment 11). It could be argued that the lack of hemorrhage was due to the significantly less intense shock that was required to induce a vocalization. However, when the intensity was equated to that of tail shock at 1.5 mA in Experiment 7 (which is above the average vocalization threshold for the hindlimb at 1.02 mA, data not shown), shock to the forelimb still did not increase hemorrhage. Finally, in another study that examined the effects of electrical stimulation to the limbs on long-term recovery, only hindlimb shock resulted in a locomotor deficit compared to unshocked controls (unpublished). Further work is needed to examine the effect of treatments that target the stress response after injury. For example, would tight restraint amplify the effect of noxious stimulation? Would blocking components of the stress response have a protective effect?

Future Directions

A potential limitation to the experiments in Chapter VI is that shocking at vocalization threshold requires a vocalization threshold test, which could provide a form of pain input. Data from Experiments 9 and 10 suggest that the vocalization test was not sufficient to induce hemorrhage. This appears to not be the case however because Experiment 11 displayed no differences in hemorrhage between groups. This could be because shock to the hindlimb, while sufficient to induce an acute locomotor deficit, was not sufficient to induce hemorrhage. This is in contrast with data from Experiments 7 and 10, which provides evidence that shock to the hindlimb results in hemorrhage.

However, to avoid the issue, data from Experiments 9 and 10 can provide reason to avoid the threshold test in future experiments and provide electrical stimulation at an intensity found in Experiment 10. This could eliminate the potential of additional pain input from the vocalization test that could potentially be masking any BP or hemorrhage effects.

Another limitation is the inconsistent hemorrhage results from Experiments 6-10. While I used two measures of hemorrhage for these experiments, only one assay yielded significant results while the other did not. There are some potential explanations for this. In 4 out of the 5 experiments, the use of wire electrodes could have added variability due to the pain input from the placement itself and the movement of the limbs afterwards that could aggravate the area of skin surrounding the electrode. This stimulation could lead to hemorrhage in the Unshocked rats. Another explanation is that some rats were more vocal during the threshold test than others which led to nociceptive stimulation at a lower intensity than their pain threshold. This was made apparent when their vocalizations ceased part-way through the electrical stimulation session (behavior recorded but not shown). This was probably due to the electrode insertion done a few minutes before the vocalization threshold test, leaving the rats in a sensitive state. Receiving nociceptive stimulation at an intensity less than their true vocalization threshold could lead to a reduced hemorrhage effect. In the future, the electrodes could be placed during the time of surgery while under anesthesia or cutaneous electrodes could be employed to reduce the stress and pain from wire electrodes.

Implications

The findings of this dissertation highlight the importance of spared fibers in the expansion of secondary injury. It is clear that spared afferent fibers trigger a brain-dependent response that can amplify tissue loss at the site of injury. Further, brain processes are required to engage the rise in BP/flow after noxious electrical stimulation. The results suggest that brain systems play an essential role. It does not appear, however, that engaging a brain-dependent response to noxious stimulation is sufficient to drive hemorrhage. Nor is artificially inducing a rise in BP/flow sufficient. Finally, I found that blocking a brain-dependent response (vocalization) to noxious stimulation did not attenuate hemorrhage. While presenting noxious stimulation at an intensity that engages a vocalization response caudal to injury fostered hemorrhage, I found no evidence that engaging pain fibers rostral to injury has an adverse effect. The results suggest that nociception-induced hemorrhage may depend upon two effects: (1) a local modification driven by the sensory stimulus; and (2) a brain-dependent process. Further work is needed to delineate how the brain fosters hemorrhage, whether this depends upon descending fibers or a systemic process, and the brain processes/states that drive this adverse effect.

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