ABSTRACT

Over 90% of spinal cord injuries are caused by traumatic accidents and are often associated with secondary tissue damage that can provide a source of continued pain input. Previous work has shown that nociceptive stimulation (pain input) soon after spinal cord injury increases lesion volume, undermines recovery of locomotor function, and induces signs of neuropathic pain. In previous studies, rats received six minutes of intermittent electrical stimulation to the tail twenty-four hours after a moderate lower thoracic spinal cord contusion. The goals of this dissertation were to examine mechanisms that underlie nociceptive stimulation-induced impaired recovery, and to develop a therapeutic strategy that might be capable of reversing the negative effects of acute pain in patients with spinal cord injury.

A clinically relevant spinal cord contusion injury model was used to examine functional outcomes, molecular markers, and histopathological changes associated with acute pain after spinal cord injury. Either pharmacologic or electrical C fiber stimulation after injury was sufficient to undermine functional outcomes. Further, both stimulation paradigms increased signs of inflammation, apoptosis, pyroptosis, and hemorrhage. Hemorrhage was associated with increased formation of SUR1-TRPM4 channels, which is a known pathognomonic indicator of progressive hemorrhagic necrosis. Treatments that target pathologic purinergic signaling to inhibit pyroptosis reduced inflammation, but did not improve functional outcomes. Further, standard treatment with systemic morphine had no impact on acute pain-induced effects. In contrast, spinal block with...
lidocaine blocked the inflammation, apoptosis, pyroptosis, hemorrhage, and impaired functional recovery associated with acute pain.

Experiment results highlight the detrimental impact of acute pain soon after spinal cord injury. Progressive hemorrhagic necrosis was enhanced by nociceptive stimulation and likely triggers increased cell death through a combination of pyroptosis and apoptosis. Further, current clinical treatments for pain management after trauma proved ineffective at reversing the impact of acute pain. However, epidural lidocaine successfully reversed all signs of impaired recovery. Thus, for patients with spinal cord damage, use of lidocaine spinal blocks soon after injury might provide an effective alternative therapy for treatment of acute pain.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Overview

In the United States, approximately 12,000 new patients are diagnosed with spinal cord injury (SCI) each year, and over 250,000 patients currently live with this debilitating condition. Further, spinal cord injury disproportionately impacts young men, particularly athletes and soldiers (Ackery, Tator, & Krassioukov, 2004; Tator, Duncan, Edmonds, Lapczak, & Andrews, 1993; Wyndaele & Wyndaele, 2006). Quality of life is a major concern for individuals with SCI as they often face a future of disability and dependence on caregivers (Anderson, 2004). The direct costs of treating SCI are significant, with even greater indirect costs such as caregiver time and loss of employment (DeVivo, 1997).

Initial mechanical trauma to the spinal cord during injury damages cells and impairs spinal cord function. However, the extent of damage expands rapidly in the following hours in a process called the secondary injury cascade (Hausmann, 2003). Programmed cell death, inflammation, and hemorrhage are hallmarks of this secondary cascade and are major impediments to recovery (Beattie, Farooqui, & Bresnahan, 2000; Simard, Woo, Aarabi, & Gerzanich, 2013). Prior work has shown that nociceptive stimulation soon after SCI amplifies pathways within the secondary injury cascade and contributes to impaired locomotor recovery, enhanced signs of pain, and increased mortality (Grau et al., 2004).
This is important to human individuals with SCI because over 90% of spinal cord injuries occur due to trauma, with motor vehicle collisions, falls, violence, and sports accidents being the most common causes (World Health Organization, 2013). These incidents consistently involve associated injuries such as head and abdominal trauma, chest and pelvic damage, and limb fractures. In addition, abrasions, cuts, and contusions are nearly ubiquitous in SCI patients and provide pain input during recovery. Given prior work, any of these sources of pain input during recovery could amplify the secondary injury cascade and contribute to impaired functional outcomes.

The experiments in this dissertation utilize two models of C fiber input after SCI to examine how nociceptive (pain) input impacts recovery. I show that acute pain after injury impairs recovery. I then explore the neurobiological mechanisms that underlie this effect and some potential therapeutic interventions. In this chapter, I highlight three key molecular mechanisms known to be involved in the secondary injury cascade. Then, I discuss prior work that has examined the impact of nociceptive stimulation on recovery following SCI.

**Molecular mechanisms of cell death after SCI**

*Passive vs active*

Historically, all cell death was thought to be pathological, and cell death in the context of SCI was assumed to be unregulated and energy-independent (passive). Indeed, during spinal cord trauma, pressure waves directly rupture cellular membranes and cause unregulated, energy-independent release of cellular contents and cell death.
Given the time course of spinal cord injury, passive cell death is unavoidable and is thus a poor target for therapeutic interventions.

Today, it is well-accepted that active mechanisms of cell death occur after initial SCI, with further tissue damage and cellular death mediated by the secondary injury cascade. This secondary impact is characterized by activation of regulated, energy-dependent (active) cell death pathways, and is responsible for expansion of the spinal cord lesion (Beattie, Hermann, Rogers, & Bresnahan, 2002). Because the secondary injury cascade is highly regulated and occurs for days after SCI, modifications to the secondary injury cascade provide prime targets for therapeutic intervention.

The following sections will discuss apoptosis and pyroptosis, two forms of active cell death that are known to occur following spinal cord injury. Additionally, a third mechanism, called progressive hemorrhagic necrosis (PHN), will be detailed. The relevance of these three pathways to recovery following SCI will be highlighted.

**Apoptosis**

Apoptosis was the first form of programmed cell death discovered, and has now been well characterized (Beattie et al., 2002). Classically, apoptosis is divided into two forms based on the initiating stimulus. Extrinsic apoptosis of a cell occurs in response to an extracellular signal. In contrast, intrinsic apoptosis occurs in response to intracellular factors.

Extrinsic apoptosis is initiated by one of two extracellular ligands – Fas ligand (FasL) or tumor necrosis factor (TNF). These signaling molecules bind to their cellular receptors cluster of differentiation 95 (CD95) and tumor necrosis factor receptor 1.
(TNFR1), respectively, to initiate the cell death cascade. The simplest pathway occurs in response to FasL stimulation, directly activating caspase-8 through the death domain of the CD95 receptor. Caspase-8 then cleaves the executioner protease, caspase-3, leading to cellular digestion and death (Vucic, Dixit, & Wertz, 2011).

The intrinsic apoptotic cascade is initiated in response to intracellular stimuli. Cellular damage, energy restriction, and withdrawal of growth factors can promote apoptosis without engaging membrane receptors. Initiators of the intrinsic apoptotic cascade are diverse and can act through a number of protein sensors (Vucic et al., 2011).

Both intrinsic and extrinsic pathways of apoptosis converge on apical and executioner caspases to promote protein degradation and cell death. These pathways have been shown to be active following spinal cord injury, primarily in late phases of recovery (Beattie et al., 2000; Crowe, Bresnahan, Shuman, Masters, & Crowe, 1997; Emery et al., 1998; Li et al., 1996). Though cell death is largely presumed to harm functional outcomes, treatments aimed at reducing apoptosis have shown little success (Cittelly, Nesic, Johnson, Hulsebosch, & Perez-Polo, 2008; Cittelly & Perez-Polo, 2007; Thuret, Moon, & Gage, 2006).

**Pyroptosis**

Pyroptosis was originally discovered and studied in circulating macrophages. This type of active cell death shows characteristics of both apoptosis and necrosis (Brennan & Cookson, 2000; Fink & Cookson, 2006). Pyroptosis, like passive cell death, induces a highly pro-inflammatory state that attracts immune cells. However, pyroptosis is engaged by active cellular processes and is highly regulated, thus similar to apoptosis.
The molecular basis of pyroptosis is defined by activation of caspase-1, which leads to increased membrane permeability through recruitment, assembly, and opening of pores, cellular death, DNA fragmentation, and interleukin processing (Bergsbaken, Fink, & Cookson, 2009).

For caspase-1 activation, an inflammasome must be assembled (Schroder & Tschopp, 2010). Activation of inflammasomes is associated with innate immune signaling and typically acts through Nod-Like Receptor (NLR) sensing of damage associated molecular patterns (DAMPs) and apoptosis-associated speck-like protein containing a C-terminal caspase-recruitment domain (ASC) (Hirsiger et al., 2012; Kigerl, de Rivero Vaccari, Dietrich, Popovich, & Keane, 2014; Sagulenko et al., 2013). DAMPs are proposed to enter the pyroptotic cell through cellular pores. DAMPs include nucleic acids, uric acid, adenosine triphosphate (ATP), and molecular patterns found in bacterial products.

After caspase-1 is activated in an assembled inflammasome, it processes the biologically inactive forms of interleukin 1 beta (IL-1β) and interleukin 18 (IL-18), pro-IL-1β and pro-IL-18, to their biologically active, mature forms through proteolytic cleavage (Cerretti et al., 1992; Wilson et al., 1994). These interleukins, are then secreted from the dying cell and contribute to production of a pro-inflammatory environment.

In addition to its role in processing IL-1β and IL-18, caspase-1 has been shown to degrade a number of other targets, including cytoskeletal elements, glycolytic enzymes, and trafficking proteins (Denes, Lopez-Castejon, & Brough, 2012; Shao, Yeretssian, Doiron, Hussain, & Saleh, 2007).
Importantly, caspase-1 and the inflammasome proteins responsible for its activation also exist within cells of the central nervous system. Neurons, astrocytes, and microglia have all been found to express functional inflammasome components that are capable of responding to a wide range of DAMPs (de Rivero Vaccari, Dietrich, & Keane, 2014).

Therapeutics targeting caspase-1 activation and pyroptosis have been developed and include neutralizing antibodies against ASC and NLRP1 (neuronal apoptosis inhibitor protein, major histocompatibility complex class 2 transcription activator, incompatibility locus protein, telomerase-associated protein, leucine-rich repeat, and pyrin domains-containing protein 1). These antibody treatments improved functional recovery and histopathological scores in an animal model of spinal cord injury (Vaccari, Lotocki, Marcillo, Dietrich, & Keane, 2008). However, neutralizing antibodies and other biologicals have limited clinical applicability due to their high cost of production, potential for antigenicity, and long time-to-market. In the future, small molecule inhibitors targeting these pathways may provide improved and more cost-effective treatment.

Problems with clinical application of caspase-1 therapeutics have prompted other laboratories to take alternative approaches, targeting upstream activators of caspase-1 and pyroptosis. Of these pathways, purinergic regulation of inflammasome assembly and caspase-1 activation represents an attractive therapeutic target. Though ATP is typically known for its role in metabolism, extracellular ATP is a potent pro-inflammatory signaling molecule (Antonioli, Pacher, Vizi, & Haskó, 2013). Additionally, signaling
through the purinergic receptor P2X, ligand gated ion channel, 7 (P2X7) and the pannexin-1 channel leads to a positive feedback loop of ATP-induced ATP release, formation of inflammasomes, and activation of caspase-1 (Bernier, 2012). Treatments targeting the P2X7 receptor with brilliant blue G (BBG) have shown promise in a rat model of SCI (Peng et al., 2009).

**Progressive hemorrhagic necrosis**

During spinal cord trauma, mechanical forces shear small capillaries and arterioles, causing primary hemorrhage, extravasation of red blood cells, and cell death. However, in the hours that follow injury, hemorrhage expands in a process called progressive hemorrhagic necrosis (PHN). PHN is defined by the formation and opening of the sulfonylurea receptor (SUR1)- transient receptor potential cation channel subfamily M member 4 (TRPM4) channel (Simard et al., 2013).

In the uninjured animal, expression of SUR1 and SUR1-TRPM4 complexes is undetectable. However, after SCI, SUR1 is up-regulated and forms complexes with TRPM4. SUR1 is up-regulated in response to many pro-inflammatory stimuli, including the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). Following injury, NF-kB expression is increased by upstream signaling pathways including tumor necrosis factor and IL-1β (Simard et al., 2013).

Within the inflammatory environment of the damaged spinal cord, endothelial cells up-regulate the ATP-binding protein SUR1 and promote formation of a SUR1-TRPM4 channel. Activity of the SUR1-TRPM4 channel is tightly linked to endothelial cell death, PHN, and impaired recovery following SCI (Simard et al., 2013).
Because SUR1 is expressed in β-islet cells of the pancreas, and is mechanistically linked to regulation of insulin release, antagonists are already approved by the FDA for treatment of diabetes mellitus type 2. Indeed, glibenclamide is a potent inhibitor of SUR1 and among the most commonly prescribed medications for the treatment of diabetes mellitus type 2 (Balsells et al., 2015; Langer, Conway, Berkus, Xenakis, & Gonzales, 2000). Inhibition of the SUR1-TRPM4 channel with glibenclamide improved recovery after stroke and traumatic brain injury (Patel, Gerzanich, Geng, & Simard, 2010; Simard et al., 2009). After unilateral spinal cord injury, glibenclamide treatment reduced hemorrhage and improved functional outcomes (Kahle, Gerzanich, & Simard, 2010; Simard et al., 2007). However, no effect of glibenclamide was found in a midline contusion injury model (Popovich, Lemeshow, Gensel, & Tovar, 2012; Simard, Popovich, Tsymbalyuk, & Gerzanich, 2012).

**C fiber stimulation in a contusion model**

*Impaired locomotor recovery*

Guided by work in a transection model, it was hypothesized that uncontrollable nociceptive stimulation might impair functional outcomes in a clinically relevant model of spinal cord contusion. Indeed, just six minutes of uncontrollable stimulation administered twenty-four hours following a contusion injury induces signs of neuropathic pain and produces a lasting impairment of functional locomotor recovery (Grau et al., 2004).

Further studies using this model examined the window of vulnerability, and found that stimulation has a more robust effect if given soon after injury. Specifically,
data suggest that the spinal cord is vulnerable for the first four days following injury, and that the impact of stimulation declines after this critical period (Grau et al., 2004).

Additional experiments examining the impact of controllability have shown that only uncontrollable stimulation impacts recovery. As in the transection model of spinal learning, controllable nociceptive stimulation had no adverse effect (Grau et al., 2004).

A limitation of this work is that only one form of C fiber activation has been used. Our laboratory has relied upon an electrical stimulation model of acute pain and C fiber stimulation after SCI. This paradigm allows for the precise temporal and spatial control of C fiber activity, but is not a naturalistic representation of acute pain after SCI. One goal of this work was to extend the results seen in the electrical stimulation paradigm of acute pain into a model of persistent C fiber activity using a peripheral injection of the TRPV1 agonist capsaicin.

Other effects of C fiber stimulation

In addition to negative effects on locomotion, stimulation also impairs functional outcomes in other ways. Subjects receiving C fiber stimulation show delayed recovery of voluntary bladder function, greater mortality, and increased lesion volume (Grau et al., 2004). Further, nociceptive input enhanced mechanical reactivity a week after injury, implying an increase in neuropathic pain following SCI (Garraway et al., 2014).

Molecular mechanisms

Understanding the molecular mechanisms that underlie impaired recovery induced by nociceptive input is critical to acute pain management in conjunction with SCI. Prior work in the transection model of spinal learning highlighted the TNF
signaling pathway as critical to the learning deficit induced by uncontrollable stimulation. Exogenous application of TNF is sufficient to induce a learning deficit, and treatments inhibiting TNF signaling reverse the uncontrollable stimulation-induced learning deficit (Ferguson, Huie, Crown, & Grau, 2012; Huie et al., 2012; Stück et al., 2012).

The TNF signaling pathway has also been examined in the contusion model of spinal cord injury. As in the transection model, uncontrollable stimulation induces a rapid up-regulation in TNF. Additionally, in this model, nociceptive stimulation increased levels of the apoptotic proteins caspase-3 and caspase-8, indicating signaling through TNFR1 (Garraway et al., 2014).

Finally, the pro-inflammatory IL-1β signaling pathway has been shown to be involved in mediating a lipopolysaccharide (LPS) induced learning deficit. Treatment with an IL-1 receptor antagonist (IL-1ra) reversed this learning deficit (Young, Baumbauer, Elliot, & Joynes, 2007).

**Specific aims**

My thesis explores the molecular pathways that contribute to the adverse effect C fiber stimulation has on recovery following SCI. Further, I evaluate a clinically relevant therapeutic intervention that has the potential to prevent these negative effects. The central hypotheses are that C fiber stimulation engages physiological effects that promote cell loss, and that inhibiting C fiber activation will improve functional outcomes.
Aim 1 (Chapter III) compared the impact of pharmacologic and electrical activation of C fibers on behavioral and cellular processes related to functional recovery after SCI. I extend prior work by testing whether a peripheral irritant (capsaicin), that selectively engages C fibers, has the same effect as electrical stimulation.

Aims 2 and 3 (Chapters IV and V) examine whether pyroptosis and progressive hemorrhagic necrosis contribute to impaired recovery in contused rats that have received nociceptive stimulation.

Finally, in Aim 4 (Chapter VI), a novel therapeutic strategy for the treatment of acute pain after SCI was evaluated. This treatment blocked the adverse effect nociceptive stimulation had on recovery.
CHAPTER II

GENERAL METHOD

Animal procedures

Subjects

Adult male Sprague-Dawley rats (100-120 days old) were obtained from Harlan Laboratories (Houston, TX) and acclimated for at least 7 days prior to experimentation. Subjects were dual housed with water and food ad libitum, and maintained on a 12-hour light-dark cycle. Behavioral testing and surgeries were performed during the light portion of the cycle. All experiments were carried out in accordance with NIH standards for the care and use of laboratory animals (NIH publication No. 80-23), and were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Every effort was made to minimize suffering and limit the number of animals used to that which was scientifically necessary.

Spinal contusion

All subjects received a moderate contusion injury at the T10-11 vertebral level using the New York University (NYU) Multicenter Animal Spinal Cord Injury Study (MASCIS) device (Young, 2002). Anesthesia was induced using a mixture of 5% isoflurane in medical oxygen, and maintained at a concentration of 2-3% during surgery. A single longitudinal incision was made through the skin, extending approximately 3 cm rostral and caudal to the injury site. Then, two longitudinal incisions were made on either side of the vertebral column, extending approximately 2 cm rostral and caudal to
the injury site. The T10-11 vertebrae were located by palpation, exposed, and a laminectomy was performed. The dura remained intact. The MASCIS device was then secured around the vertebral column and the 10-gram impactor centered on the lesion site. The drop height was set at 12.5 mm. After surgery, the wound was closed using Michel clips. To prevent urinary tract infection and compensate for fluid loss, subjects received 100,000 units/kg of penicillin and 3 mL of saline after surgery.

Subjects were allowed to recover overnight (18-24 hours) in a temperature-controlled room (25°C) with water and food *ad libitum*. Bladders were expressed twice daily and immediately prior to any experimental procedures. When experimentation was complete, all animals were euthanized with a lethal dose of pentobarbital (100 mg/kg; intraperitoneal [i.p.]).

*Uncontrollable tail shock*

Subjects were loosely restrained in opaque Plexiglas tubes and placed in an acoustic isolation chamber. An electrode was applied to the tail with electrode gel. Shocks were administered on a variable spaced schedule (0.2 – 3.8 second inter-stimulus interval). Shocks lasted 100 ms with 1.5 mA constant current control. One-hundred and eighty stimuli were administered over approximately six minutes. Controls were loosely restrained in the Plexiglas tubes for an equivalent duration, but no shocks were administered (Grau et al., 2004).
Peripheral capsaicin injection

Subjects were loosely restrained in Plexiglas tubes and 0.05 mL of 3% capsaicin was injected into a hind paw using a 27-gauge needle. Controls received an injection of vehicle (7% Tween-20).

Purinergic signaling inhibition

Brilliant Blue G (BBG) and probenecid drug treatments were prepared in 1 mL of sterile phosphate buffered saline (PBS) at pH 7.4. BBG was dissolved directly in PBS at the required concentrations. Probenecid was dissolved in a small volume of 1 M sodium hydroxide (NaOH). Then, PBS was added and the pH adjusted to 7.4 using monobasic potassium phosphate. The drug combination was created by preparing BBG and probenecid solutions at twice the required concentrations, then mixing the two solutions in equal parts. BBG and probenecid drug treatments were administered three, twelve, and twenty-four hours following injury. All drugs were given by intraperitoneal injection.

Systemic opiate treatment

Morphine sulfate was dissolved in sterile normal saline and administered by intraperitoneal injection at a dose of 20 mg/kg. Drug was administered 30 minutes prior to C fiber stimulation (Hook et al., 2011).

Epidural lidocaine

Epidural lidocaine was administered as previously described (Mestre, Pélissier, Fialip, Wilcox, & Eschalier, 1994). Briefly, subjects were anesthetized with isoflurane as described for spinal contusion surgery. Subjects were laid over the edge of the surgical
table to flex the spine and maximize separation between vertebrae. The L5 vertebra was palpated and a 25-gauge needle was inserted just caudal to the vertebra. Twenty-five µL of 15% lidocaine in sterile normal saline was injected, ensuring no resistance to flow throughout the injection. Efficacy of the spinal block was confirmed by tail pinch.

Assessment of locomotor function

A blinded observer assessed locomotor function daily for the first week after spinal cord injury, on the tenth day after injury, and weekly from day 14 after injury. Locomotor function was assessed using the Basso, Beattie, and Bresnahan (BBB) scoring system and converted to a form amenable to parametric analyses (Basso, Beattie, & Bresnahan, 1995; Ferguson et al., 2004).

Following completion of the recovery period, locomotor function was further assessed by blinded observers using beam and ladder tests (Soblosky, Colgin, Chorney-Lane, Davidson, & Carey, 1997; von Euler, Akesson, Samuelsson, Seiger, & Sundstrom, 1996). Briefly, subjects walked across a tapered beam and the width at first misstep was recorded. For the ladder test, subjects were allowed to walk across a horizontal ladder and the number of missed steps and slipped steps were recorded.

Assessment of pain

Mechanical allodynia was assessed by a blinded observer using von Frey filaments applied to the plantar surface of both hind paws. The up-down technique was used to ensure reliable mechanical withdrawal thresholds (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). Thermal reactivity was measured using the IITC Tail Flick Analgesia Meter. At-level pain was determined by counting the number of vocal
responses to a 26-gram mechanical stimulus applied on a four by eleven grid across the girdle region of the rat.

**Analysis of tissue proteins**

*Collection of tissue*

Subjects were injected with a lethal dose of pentobarbital. After the heartbeat had terminated, one centimeter of spinal tissue centered around the lesion was collected, immediately flash frozen in liquid nitrogen, and stored at -80°C until processing. For immunoblotting experiments, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein were extracted and purified using the Qiagen fatty tissue easy RNA kit according to manufacturer’s instructions. For co-immunoprecipitation experiments, protein was extracted with phosphate buffered saline and Complete mini protease inhibitor tablets (Garraway et al., 2011; Garraway et al., 2014).

*Spectrophotometry*

Full spectral analyses were collected for protein extracts. Briefly, 1 μL of protein extract was loaded onto the spectrophotometer and absorbance was measured from 200 to 800 nm. Absorbance at 420 nm was used as a measure of hemoglobin content.

*Immunoblotting*

Protein samples were quantified using a Bradford assay and loaded into 4X Laemmli buffer at a concentration of 3 μg/μL for analysis by immunoblotting. Western blotting was performed using 18- or 26-well pre-cast Criterion gels according to manufacturer’s instructions. Briefly, samples were boiled for ten minutes in 4X Laemmli buffer prior to loading. Thirty μg of each protein sample was loaded into each well and
Electrophoresis was performed at 180 V for approximately one hour and fifteen minutes. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane for one hour at 100 V and blocked in I-Block (caspase proteins) or milk for one hour prior to overnight incubation in primary antibody at 4°C. Following a series of washes in Tris(hydroxymethyl)aminomethane-buffered saline and Tween 20 (TBST), blots were incubated in secondary antibodies for 1 hour at room temperature. Finally, blots were imaged using enhanced chemiluminescence (ECL).

Co-immunoprecipitation

Protein samples were quantified using a Bradford assay and diluted to a final concentration of one μg/μL. Five hundred μL of protein extract was incubated with 50 μL of TrueBlot anti-rabbit immunoprecipitation beads on ice for 30 minutes for pre-clearing. Beads were spun down and the supernatant was retained. Pre-cleared samples were next incubated with primary antibodies on ice for one hour. After incubation with primary antibodies, 50 μL of TrueBlot beads were added and samples were incubated for one hour on a rocking platform. After incubation, samples were spun down and washed with lysis buffer. Finally, 100 μL of 4X Laemmli buffer was added to each sample and the samples were heated for ten minutes at 90-100°C. After heating, samples were spun down and the supernatant retained. The co-immunoprecipitated proteins were then run on 18- or 26-well pre-cast Criterion gels according to manufacturer’s instructions. Samples were boiled for ten minutes in 4X Laemmli buffer prior to loading. Ten μL of each sample was loaded into each well and electrophoresis was performed at 180 V for one hour and 15 minutes. Proteins were then transferred onto a PVDF membrane for one
hour at 100 V and blocked in I-Block (caspase proteins) or milk for 1 hour prior to overnight incubation in primary antibody at 4°C. Following a series of washes in TBST, blots were incubated in secondary antibodies for one hour at room temperature. Finally, blots were imaged using ECL.

**Analysis of tissue by microscopy**

*Collection of tissue*

Subjects were injected with a lethal dose of pentobarbital. After the heartbeat had terminated, subjects were perfused transcardially with ~300 mL of PBS (pH 7.3) followed by ~400 mL of 4% paraformaldehyde (PFA). Two centimeters of spinal tissue centered on the lesion was collected and incubated in 4% PFA for two hours at 4°C. The tissue was rinsed with PBS before cryoprotection in a solution of 30% sucrose in PBS for at least 72 hours. Tissue, embedded in Tissue-Tek optimum cutting temperature compound, was cut into 20 µm sections and collected on Fisherbrand Superfrost Plus microscope slides.

*Hematoxylin and eosin staining*

Slides were subjected to hematoxylin and eosin (H&E) staining according to standard procedures. Briefly, sections were washed with distilled water, then incubated in hematoxylin for 5 minutes. Sections were rinsed with sequential changes of tap water until the water ran clear. Slides were then dipped in 1% hydrochloric acid (HCl) in 70% ethanol (EtOH) twice, and rinsed with tap water. Next, slides were incubated in Scott’s tap water substitute before tap water rinses. Finally, slides were incubated in eosin for 2
minutes, and dehydrated and cleared in ethanol and xylene before mounting with Permount. Images of each slide were collected under light microscopy.

Statistics

All statistics were performed using the SuperANOVA program with $p = .05$ set as the significance. Depending upon the experiment, data were analyzed using a chi-square test, Student’s t-test, analysis of variance (ANOVA), or analysis of covariance (ANCOVA) with or without repeated measures. Post hoc tests used Duncan’s New Multiple Range criteria for assessment of statistical significance.
CHAPTER III
COMPARISON OF PHARMACOLOGIC AND ELECTRICAL ACTIVATION OF C FIBERS AFTER SPINAL CORD INJURY

Prior work has shown that six minutes of intermittent uncontrollable electrical stimulation to the tail is sufficient to undermine recovery of locomotor function, increase lesion volume, and promote cell death following a contusive spinal cord injury (Grau et al., 2004). While detrimental effects of stimulation have been linked to C fiber activation in the spinal transection model of instrumental learning (Grau et al., 2014), we do not know whether C fiber activity also is responsible for the impaired recovery seen after a contusion injury.

After spinal cord contusion, the inflammatory pathways involving TNF and apoptotic pathways involving caspase-3 and caspase-8 are associated with nociceptive input and impaired recovery (Garraway et al., 2014). What remains unknown is whether C fiber activation underlies the detrimental effects of electrical stimulation.

Chapter III examined two different models of C fiber activation in the context of spinal cord contusion. Experiment 1A compared the effects of pharmacologic (capsaicin) and electrical sources of nociceptive stimulation on functional outcomes. Then, Experiment 1B examined the time courses of TNF protein levels and activation of the apoptotic proteases caspase-3 and caspase-8 after both models of nociceptive input.
Exp. 1A: Engaging C fibers impairs recovery after SCI

Experiment 1A compared the impact of electrical stimulation to peripheral capsaicin treatment on locomotor recovery in contused rats. As a positive control, I also evaluated the effect of shock treatment.

Procedure

Subjects in Experiment 1A received a moderate spinal cord injury to the lower thoracic region on day 0. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of four groups (shocked, unshocked, capsaicin, or vehicle) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained in opaque Plexiglas tubes. Subjects in the unshocked and shocked groups had an electrode fastened to the tail. Subjects in the shocked group received six minutes of intermittent electrical stimulation while subjects in the unshocked group received identical treatment without electrical stimulation. Subjects in the capsaicin and vehicle groups received a 0.05 mL injection of capsaicin or vehicle into a single hind paw (randomly assigned). After treatment, all subjects were removed from the Plexiglas tube and returned to their home cages.

Subjects were examined for four weeks following injury. Locomotor function was assessed using the BBB scoring system, with results converted to a form amenable to parametric analyses (Basso et al., 1995; Ferguson et al., 2004). Weight gain was assessed throughout the recovery period and was used as a measure of general health. Subjects were sacrificed following the four-week recovery period.
Results

Baseline BBB scores ranged from 1.9 (±0.5) to 2.8 (±0.7) and did not differ across groups, all $F$s < 0.39, $p > .05$. Because no differences were found in baseline locomotor function, BBB scores were used as an index of injury severity and as a covariate throughout the rest of this experiment.

Analysis of locomotor function across time demonstrated a significant impairment in subjects that received nociceptive input (Figure 1). An ANCOVA was performed using pre-stimulation locomotor function as the covariate, and injection and C fiber activation as dependent variables, a 2 x 2 factorial design. This ANCOVA yielded significant main effects of time, injection, and C fiber activation, as well as interactions between time and initial locomotor function, time and injection, and time and C fiber activation, all $F$s > 3.44, $p < .05$. These effects emerged because subjects that received electrical stimulation showed impaired recovery when compared with subjects that received no electrical stimulation, and subjects that received capsaicin showed a significant impairment compared with vehicle-treated controls. Additionally, subjects that received injections of any type (capsaicin or vehicle) showed significant impairment when compared with subjects that did not receive peripheral injections (shocked or unshocked).
Figure 1. Locomotor recovery after spinal cord injury and C fiber stimulation. Subjects that received electrical or pharmacologic activation of C fibers showed impaired locomotor recovery when compared to their respective controls. Subjects that received injections also showed impaired recovery when compared to subjects that did not receive peripheral injections. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. 
Assessment of weight gain during recovery showed impairment in subjects that received either electrical or pharmacologic C fiber activation (Figure 2A-B). An ANOVA examining percent change in weight across the recovery period revealed a main effect of time, as well as a significant interaction between time and C fiber activation, both $F_s > 2.30, p < .05$. This effect emerged because subjects treated with either electrical or pharmacologic activation of C fibers showed impaired weight gain compared with their respective controls.
Figure 2. Weight gain after spinal cord injury and C fiber stimulation. Subjects that received electrical (A) or pharmacologic (B) stimulation of C fibers showed impaired weight gain when compared to their respective controls. Error bars represent SEM (n = 6). * indicates significant difference, \( p < .05 \).
Discussion

This experiment evaluated the impact of C fiber activation after spinal cord contusion using the peripheral irritant capsaicin. As a positive control, I included electrical activation of C fibers. This provides converging evidence that C fiber activation underlies impairment in recovery previously generated by electrical stimulation.

Interestingly, subjects that received any peripheral injection (capsaicin or vehicle) showed impaired recovery when compared with subjects that received no injection (shocked or unshocked). The vehicle used in this study was 7% Tween-20 in sterile normal saline. The injection procedure caused local swelling for approximately one hour after injection; however, the swelling resolved rapidly and was undetectable after a day. This effect of injection has potential clinical relevance because patients receive many treatments after SCI that might increase nociceptive input and could further impair functional outcomes. Relatively mild nociceptive input, as would be expected from a single peripheral injection, might be sufficient to significantly impair recovery. In the clinic, providers should consider management of nociceptive stimulation for all procedures, even if the patient is not able to consciously perceive pain.

Taken together, the results from this experiment show that C fiber activation after SCI undermines functional outcomes. Further, this experiment suggests that minimal activation of C fibers is sufficient to undermine recovery, while strong stimulation of C fibers severely impairs recovery.
Exp. 1B: Nociceptive stimulation engages TNF inflammatory and apoptotic pathways

Experiment 1A showed that acute nociceptive input, elicited by either noxious shocks or a peripheral irritant (capsaicin), impairs recovery after a contusion injury. Prior work suggests that nociceptive stimulation adversely affects recovery because it enhances programmed cell death (apoptosis). The current experiment further explores this issue by evaluating the expression of apoptosis-related proteins (TNF, caspase 3 and 8) over time in subjects that have received shock or capsaicin treatment.

Procedure

Subjects in Experiment 1B received a moderate spinal cord injury to the lower thoracic region on day 0. Twenty-four hours later, function was assessed using the BBB locomotor scale. In the first group of animals, subjects were then randomly assigned to one of two treatment conditions (shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in the shocked group received six minutes of intermittent uncontrollable electrical stimulation while subjects in the unshocked group received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

A second group of animals was treated similarly, but received C fiber activation through capsaicin injection. Briefly, subjects received a moderate contusion to the lower
thoracic spinal cord on day 0. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two treatment conditions (capsaicin or vehicle) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained in an opaque Plexiglas tube. Subjects received a 0.05 mL injection of capsaicin or vehicle into a single hind paw (randomly assigned). After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

In both groups of animals, subjects were sacrificed at one of three time points (1, 3, or 24 hours after stimulation). Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Protein was extracted from whole tissue lysate and diluted to a final concentration of 3 mg/mL in 4X Laemmli buffer. Proteins were separated by gel electrophoresis and transferred onto a PVDF membrane before overnight incubation with primary antibodies for TNF, caspase-3, caspase-8, and lamin B at 4°C. Quantification was performed using ECL and normalized to lamin B as a loading control for nucleated cell protein.

Results

For subjects that received electrical C fiber stimulation, baseline transformed BBB scores ranged from 3.3 (±0.9) to 5.8 (±1.4) and did not differ across treatment conditions, all $F_s < 2.40, p > .05$. This indicated that injury severity was comparable across all conditions studied.
A similar result was obtained for subjects that received pharmacologic activation of C fibers. Transformed baseline BBB scores ranged from 2.5 (±0.6) to 5.5 (±0.8) and did not differ across treatment groups, all Fs < 0.70, p > .05. This indicated that injury severity was similar across all conditions.

Immunoblotting for TNF revealed that electrical stimulation increased levels of the pro-inflammatory cytokine in shocked subjects (Figure 3A). An ANOVA yielded significant main effects of stimulation and time point, all Fs > 7.06, p < .05. The interaction between time point and stimulation was not significant, F = 1.88, p > .05, which indicated that TNF signaling was increased similarly at all time points examined.

Comparable results were obtained in subjects treated with capsaicin. Immunoblotting for TNF revealed that capsaicin injection increased levels of the pro-inflammatory cytokine (Figure 3B). An ANOVA yielded significant main effects of condition and time point, all Fs > 5.46, p < .05. The interaction of time point and condition was not significant, F = 1.61, p > .05, and indicated that TNF processing was altered similarly across the time points examined.
Figure 3. TNF protein levels after electrical stimulation and capsaicin injection. (A) Subjects that received electrical stimulation showed increased levels of TNF soon after electrical stimulation. (B) Subjects that received capsaicin injection showed increased levels of TNF soon after injection. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. 
Immunoblotting for caspase-3 revealed that electrical stimulation increased levels of the active form of this apoptotic protease in shocked subjects (Figure 4A). An ANOVA yielded a significant main effect of stimulation, $F = 4.22, p < .05$. Neither the main effect of time point, nor the interaction between time point and stimulation were significant, $Fs < 2.18, p > .05$. This indicated that caspase-3 signaling was increased similarly at all time points examined.

In contrast to the increased activation of caspase 3 observed following electrical stimulation, immunoblotting for caspase-3 revealed that capsaicin injection had no effect on levels of the active form of this apoptotic protease (Figure 4B). An ANOVA yielded no statistically significant effects, all $Fs < 2.34, p > .05$, though activated caspase 3 was elevated at the three-hour time point.
Figure 4. Caspase-3 protein levels after electrical stimulation and capsaicin injection. (A) Subjects that received electrical stimulation showed increased levels of the apoptotic protease. (B) Subjects that received capsaicin injection showed similar levels of caspase-3 compared to control subjects. Error bars represent SEM (n = 6). * indicates significant difference, p < .05.
Immunoblotting for caspase-8 revealed electrical stimulation increased levels of the protein in shocked subjects (Figure 5A). An ANOVA yielded a significant main effect of stimulation, $F = 8.44, p < .05$. Neither the main effect of time point, nor the interaction between time point and stimulation were significant, $Fs < 1.12, p > .05$. This indicated that caspase-8 signaling was increased similarly at all time points examined.

Again, while electrical stimulation caused a robust increase in caspase-8, pharmacologic activation of C fibers did not reproduce these same effects. Immunoblotting for caspase-8 revealed that levels of this apoptotic protein increased over time in subjects that received capsaicin injection (Figure 5B). An ANOVA yielded a significant main effect of time, $F = 3.82, p < .05$. However, neither the main effect of condition nor the interaction between stimulation and time point were found to be statistically significant, $Fs < 2.42, p > .05$. 
Figure 5. Caspase-8 protein levels after electrical stimulation and capsaicin injection. (A) Subjects that received electrical stimulation showed increased levels of caspase-8 soon after electrical stimulation. (B) Subjects that received capsaicin injection showed similar levels of caspase-8 after injection. Error bars represent SEM (n = 6). * indicates significant difference, p < .05.
Discussion

Subjects that received electrical stimulation showed increased levels of the pro-inflammatory cytokine TNF, and increased concentrations of the active form of two proteases tightly linked to the apoptotic cell death cascade. Both TNF and caspase-8 were rapidly engaged following electrical stimulation. This experiment extended prior work by detailing protein expression between 1 and 24 hours and comparing the effect of two forms of nociceptive stimulation.

Subjects that received capsaicin injection showed increased levels of the pro-inflammatory cytokine TNF. This highlighted that pharmacologic and electrical activation of C fibers caused a similar activation of this pro-inflammatory pathway. Additionally, whereas levels of TNF peaked three hours after electrical stimulation of C fibers, levels of TNF after capsaicin injection rose by three hours and were maintained at high levels even twenty-four hours after stimulation. This finding correlated well with data from Experiment 1A that showed capsaicin injection impaired recovery to a greater extent than electrical stimulation.

In contrast to pro-inflammatory pathway results, findings related to active forms of apoptotic proteins were less clear cut. Activation of apoptosis due to electrical C fiber activation was robust, but pharmacologic activation of C fibers with the TRPV1 agonist capsaicin did not cause a statistically significant increase in caspase-3 and caspase-8. Several potential explanations for why capsaicin did not engage apoptotic cascades to a greater extent than vehicle injection include (1) pharmacologic activation of C fibers did not induce apoptosis, (2) induction of apoptosis by the vehicle injection was sufficiently
high as to cause a ceiling effect, or (3) pharmacologic activation of C fibers caused a delayed activation of the apoptotic cascades that would only have been captured at later time points. Because vehicle injection alone was found sufficient to undermine locomotor recovery in Exp. 1A, it would not be surprising that levels of apoptotic caspases were elevated in response to the vehicle injection. Future experiments should examine whether a single injection of vehicle is sufficient to increase inflammation and apoptosis within the lesion, as this could be especially relevant to clinical application.

Taken together, the results from this chapter demonstrated that C fiber activation soon after spinal cord injury engaged harmful pro-inflammatory and pro-cell death pathways that undermined locomotor recovery and impaired weight gain after spinal cord injury. This chapter provided converging evidence that the impaired recovery seen following electrical stimulation is due to activation of C fibers.
CHAPTER IV
PATHOLOGIC PURINERGIC SIGNALING AND PYROPTOSIS AFTER ACUTE PAIN AND SPINAL CORD INJURY

In the transection model of spinal learning, pro-inflammatory pathways involving TNF and IL-1β are critically involved in mediating the detrimental effects of uncontrollable nociceptive stimulation (Huie et al., 2012; Young et al., 2007). What we do not know is whether these same pathways are involved in the spinal contusion model, and how these pro-inflammatory pathways are engaged by nociceptive stimulation. Of particular interest is the protease responsible for processing IL-1β into its mature, biologically active form, caspase-1 (Denes et al., 2012).

Chapter IV examined the activation of caspase-1, a protease linked to a pro-inflammatory programmed cell death pathway called pyroptosis. In Experiment 2A, I first examined whether electrical stimulation increased activation of caspase-1 and processing of the pro-inflammatory cytokines IL-1β and IL-18. Next, in Experiment 2B, I examined whether these same markers were increased after peripheral injection of capsaicin. Because I found that these pathways were rapidly engaged by nociceptive stimulation, in Experiment 2C I tested whether upstream inhibition of pathologic purinergic signaling might reduce caspase-1 activation and cytokine processing. Finally, in Experiment 2D I evaluated inhibition of pathologic purinergic signaling as a potential treatment for nociceptive stimulation after contusive SCI.
Exp. 2A: Electrical stimulation activates caspase-1 after SCI

Experiment 2A examined the time course of changes in caspase-1 activation and pro-inflammatory cytokine processing within the lesion site. I started by examining the impact of electrical stimulation because it produced a more robust effect in Experiment 1B. As discussed earlier, activation of caspase-1 and processing of the pro-inflammatory cytokines IL-1β and IL-18 are linked to pyroptosis, a form of programmed inflammatory cell death.

Procedure

The protein isolates used in Experiment 2A were obtained from the same subjects utilized in Experiment 1B. Briefly, subjects received a moderate spinal cord injury to the lower thoracic region. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two groups (shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in the shocked group received six minutes of intermittent uncontrollable electrical stimulation while subjects in the unshocked group received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

Subjects were sacrificed at one of three time points (1, 3, or 24 hours after stimulation). Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.
Tissue was homogenized and extracted for total protein, then examined by gel electrophoresis and immunoblotting for caspase-1, IL-1β, and IL-18. Because IL-1β and IL-18 are both proteolytically cleaved into their mature forms by caspase-1, these immunoblots provided independent measures of caspase-1 levels and caspase-1 activity.

Results

Immunoblotting for caspase-1 revealed that electrical stimulation increased levels of the active form of caspase-1 in shocked subjects (Figure 6). An ANCOVA (ANCOVA) that used pre-stimulation locomotor function as the covariate yielded a significant main effect of stimulation, $F = 6.00, p < .05$. The interaction between time point and stimulation was not significant, and indicated that caspase-1 was increased across all time points examined.
Figure 6. Caspase-1 activation after electrical stimulation. Subjects that received electrical stimulation showed increased activation of caspase-1 soon after electrical stimulation. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. Representative blot from the 24-hour time point showed increased caspase-1 activation after electrical stimulation.
Immunoblotting for IL-1β demonstrated increased processing for subjects that received electrical stimulation (Figure 7). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded significant main effects of stimulation and time, all $F_s > 13.70, p < .05$. The interaction between time point and stimulation was not significant, and indicated that IL-1β processing was affected similarly at all time points.
Figure 7. **IL-1β processing after electrical stimulation.** Subjects that received electrical stimulation showed prolonged processing of the pro-inflammatory cytokine IL-1β. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. Representative blot from the 24-hour time point showed increased IL-1β processing after electrical stimulation.
Immunoblotting for IL-18 demonstrated altered processing for subjects that received electrical stimulation (Figure 8). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded significant main effects of stimulation and time, all $F_s > 3.76, p < .05$. The interaction between time point and stimulation was not significant, $F = 1.91, p > .05$. 
Figure 8. IL-18 processing after electrical stimulation. Subjects that received electrical stimulation show increased processing of the pro-inflammatory cytokine IL-18. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. Representative blot from the 24-hour time point showed increased IL-18 processing after electrical stimulation.
Discussion

In general, subjects that received electrical stimulation showed increased activation of caspase-1 and increased processing of the pro-inflammatory cytokines IL-1β and IL-18. Because IL-1β and IL-18 are processed from their immature, biologically inactive, forms into their mature (cleaved) forms by caspase-1, this experiment established that caspase-1 was activated by nociceptive stimulation and that this activation was biologically relevant.

Exp. 2B: Pharmacologic activation of C fibers increases caspase-1 activation

Experiment 1A demonstrated that nociceptive stimulation in the form of electrical or pharmacologic C fiber activation was sufficient to undermine locomotor recovery following injury. Experiment 2A extended these results, and showed that electrical stimulation was sufficient to rapidly induce caspase-1 activity and prolong pro-inflammatory cytokine processing. Experiment 2B examined whether pharmacologic C fiber activation with capsaicin causes increased caspase-1 activation and pro-inflammatory cytokine processing.

Procedure

The protein isolates used in Experiment 2B were obtained from the same subjects utilized in Experiment 1B. Briefly, subjects received a moderate spinal cord injury at to the lower thoracic region. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two groups (capsaicin or vehicle) such that the average BBB score across groups was balanced (randomized block design). Subjects in the capsaicin group received a single injection of capsaicin to
a hind paw while subjects in the vehicle group received an injection of vehicle. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

Subjects were sacrificed at one of three time points (1, 3, or 24 hours after stimulation). Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Tissue was homogenized and extracted for total protein, then examined by gel electrophoresis and immunoblotting for caspase-1, IL-1β, and IL-18. Because caspase-1, IL-1β, and IL-18 are all proteolytically cleaved into their mature forms, both total protein content and processing was assessed in a single assay.

**Results**

Immunoblotting for caspase-1 revealed that capsaicin injection produced a trend towards increased levels of the active form of caspase-1 in subjects when compared to vehicle injected controls (Figure 9). An ANCOVA that used mean baseline locomotor function scores yielded no significant effects. However, the main effect of stimulation and the interaction between time point and stimulation did approach statistical significance, all $Fs > 3.36, p < .059$. 

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Figure 9. Caspase-1 activation after capsaicin injection. Subjects that received peripheral capsaicin injection showed a trend towards increased activation of caspase-1 after peripheral capsaicin injection. Error bars represent SEM (n = 6). Representative blot from the three-hour time point that showed a trend towards increased levels of caspase-1.
Immunoblotting for IL-1β demonstrated increased duration of processing for subjects that received capsaicin injection compared to vehicle injected controls (Figure 10). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded a significant main effect of time point, as well as a significant interaction between time point and stimulation, all $F_s > 6.28, p < .05$. Comparisons of IL-1β processing at each time point revealed that stimulation had a significant effect on IL-1β processing at the three-hour time point.
**Figure 10. IL-1β processing after capsaicin injection.** Subjects that received peripheral capsaicin injection showed prolonged processing of the pro-inflammatory cytokine IL-1β. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. Representative blot from the one-hour time point showed increased processing of IL-1β.
Immunoblotting for IL-18 demonstrated increased processing of the pro-inflammatory cytokine over time for subjects that received capsaicin injection compared to vehicle injected controls (Figure 11). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded a significant main effect of time, $F = 12.20$, $p < .05$. This effect emerged because IL-18 processing increased over time, but did not depend upon stimulation condition.
Figure 11. IL-18 processing after capsaicin injection. Subjects showed changes in IL-18 processing over time. Error bars represent SEM (n = 6). Representative blot from the three-hour time point.
Discussion

Experiment 2B showed that C fiber activation with a single peripheral injection of capsaicin was sufficient to alter pro-inflammatory cytokine processing. The differences seen between capsaicin and vehicle treated subjects, however, did not appear as robust as those seen in Experiment 2A between unshocked and shocked subjects. As discussed earlier, a smaller difference may have been observed because an injection per se provides nociceptive stimulation. Further work is needed to explore this possibility.

Taken together, the results of Experiments 2A and 2B demonstrate that either electrical stimulation or capsaicin injection are sufficient to induce caspase-1 activity as measured by pro-inflammatory cytokine processing. Thus, C fiber activity is sufficient to induce pyroptosis and inflammation following SCI. This represents a potential therapeutic target for the treatment of acute pain after spinal cord injury.
**Exp. 2C: Inhibition of pathologic purinergic signaling reduces caspase-1 activity**

Experiments 2A and 2B demonstrated that C fiber stimulation engaged caspase-1, the pro-inflammatory cell death protease. A known upstream activating pathway of caspase-1 is pathologic purinergic signaling (Bernier, 2012). This pathway is particularly intriguing because it provides a positive feedback loop that can drive pathologically high levels of extracellular ATP, engaging the P2X7 receptor to induce release of additional ATP through pannexin-1 channels and the activation of caspase-1 (Wicki-Stordeur & Swayne, 2014). Experiment 2C examined whether inhibition of the P2X7 receptor with BBG and the pannexin-1 channel with probenecid would reduce activation of caspase-1 and pro-inflammatory cytokine processing. Nociceptive stimulation was provided using electrical stimulation because it had a more robust effect on caspase-1 and IL-1β expression.

**Procedure**

Subjects in Experiment 2C received a moderate spinal cord injury to the lower thoracic region on day 0. Two hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of eight groups (four injection conditions that included BBG (100 mg/kg), probenecid (100 mg/kg), combination, or vehicle, which were crossed with shocked or unshocked states) such that the average BBB score across groups was balanced (randomized block design). These doses were chosen based on previously published results in rat models (Peng et al., 2009) or adapted to a rat model from *in vitro* studies (Adamczak et al., 2014). Subjects received the first intraperitoneal injection three hours after injury, and subsequent
injections at twelve and twenty-four hours after injury. Thirty minutes after the injection at the twenty-four-hour time point, all subjects had electrodes fastened to the tail and were restrained for six minutes in an opaque Plexiglas tube. Subjects in the shocked groups received six minutes of intermittent uncontrollable electrical stimulation while subjects in the unshocked groups received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

Subjects were sacrificed three hours after stimulation. Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Tissue was homogenized and extracted for total protein, then examined by gel electrophoresis and immunoblotting for caspase-1, IL-1β, and IL-18. Because caspase-1, IL-1β, and IL-18 are all proteolytically cleaved into their mature forms, both total protein content and processing was assessed in a single assay.

Results

Immunoblotting for caspase-1 revealed that electrical stimulation produced increased levels of the active form of caspase-1 in shocked subjects, and that treatment with probenecid unexpectedly increased caspase-1 activation (Figure 12). An ANOVA yielded significant main effects of stimulation, probenecid, and BBG, as well as significant interaction effects between probenecid and BBG, probenecid and stimulation, BBG and stimulation, as well as the three-way interaction between probenecid, BBG, and stimulation, all $F$s $> 53.58$, $p < .05$. These results emerged because treatment with
the combination of probenecid and BBG caused a massive increase in caspase-1 activation after electrical stimulation.

Figure 12. Caspase-1 activation after electrical stimulation and purinergic inhibition. Subjects that received electrical stimulation showed increased activation of caspase-1 soon after electrical stimulation. Treatment with BBG and probenecid combined exacerbated this effect. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. 
Immunoblotting for IL-1β showed that electrical stimulation increased expression and that this effect was partially blocked by treatments targeting purinergic signaling (Figure 13A). An ANOVA yielded significant main effects of probenecid, BBG, and stimulation, as well as significant interactions between probenecid and BBG, BBG and stimulation, and the three-way interaction between probenecid, BBG, and stimulation, all $F_s > 5.00$, $p < .05$. These effects emerged because subjects that received electrical stimulation showed increased processing of IL-1β that was reversed by any treatment (BBG, probenecid, or combination).

Immunoblotting for IL-18 demonstrated increased cytokine processing for subjects that received electrical stimulation (Figure 13B). An ANOVA yielded significant main effects of BBG and stimulation, both $F_s > 4.15$, $p < .05$. All other effects were statistically insignificant. These effects emerged because subjects that received electrical stimulation showed increased processing of IL-18 that was not reversed by treatment.
Figure 13. IL-1β and IL-18 processing after electrical stimulation and purinergic inhibition. (A) Subjects that received electrical stimulation showed increased processing of the pro-inflammatory cytokine IL-1β that was reversed by treatments that targeted purinergic signaling. (B) Subjects that received electrical stimulation showed increased processing of the pro-inflammatory cytokine IL-18. Error bars represent SEM (n = 6). * indicates significant difference p < .05.
Discussion

Experiment 2C replicated prior work showing that subjects given electrical stimulation exhibited increased activation of caspase-1, as well as increased processing of the pro-inflammatory cytokines IL-1β and IL-18.

Additionally, Experiment 2C examined the efficacy of inhibition of pathologic purinergic signaling in preventing activation of caspase-1 and pro-inflammatory cytokine processing. While BBG, probenecid, and combination therapies all showed some efficacy in reducing pro-inflammatory cytokine processing, the combination of probenecid and BBG unexpectedly increased caspase-1 activation. This result is surprising based on current models of P2X7 and pannexin-1 activity, and suggests that strong inhibition of ATP-induced ATP release may activate powerful compensatory mechanisms in the context of SCI. This unexpected result provides a potential avenue for further exploration. However, due to this detrimental outcome, combination therapy with probenecid and BBG was eliminated from future experiments.

Given the problem with combination therapy, treatment with either BBG or probenecid seemed to have the greatest potential for preventing the pyroptotic cascade following electrical stimulation. Because BBG has previously been shown to improve functional recovery after SCI alone (Peng et al., 2009), I focus on this drug treatment in the next experiment.
Exp. 2D: BBG treatment does not block the adverse effect of acute pain on recovery after SCI

Experiment 2D explored whether inhibition of pathologic purinergic signaling through the P2X7 receptor with BBG improved functional recovery following SCI and nociceptive input.

Procedure

Subjects in Experiment 2D received a moderate SCI to the lower thoracic region on day 0. Two hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of four groups (BBG or vehicle, crossed with shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). Subjects that underwent BBG treatment received the first intraperitoneal injection three hours after injury, with subsequent injections at twelve and twenty-four hours after injury. Vehicle-treated subjects received saline injections at concurrent time points. Thirty minutes after the twenty-four-hour injection, all subjects had an electrode fastened to the tail and were restrained for six minutes in an opaque Plexiglas tube. Subjects in shocked groups received six minutes of intermittent uncontrollable electrical stimulation while subjects in unshocked groups received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and returned to their home cages.

Subjects were examined for six weeks following injury. Locomotor function was assessed using the BBB scoring system. Weight gain was assessed daily throughout the experiment. Finally, locomotor function and measures of pain were further assessed at
completion of the experiment. Subjects were sacrificed following the six-week recovery period.

Results

Baseline BBB scores ranged from 4.1 (±0.5) to 4.3 (±0.3) and did not differ across groups, all $Fs < 0.07, p > .05$. Because no differences were found in baseline BBB scores, the results were used as an index of injury severity and a covariate throughout the rest of this experiment.

Analysis of locomotor function across time demonstrated significant impairment in subjects that received nociceptive input that was not reversed by BBG treatment (Figure 14A). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded main effects of stimulation, time, and initial locomotor function, as well as significant interactions between time and initial locomotor function, and between time and stimulation, all $Fs > 1.89, p < .05$. These effects emerged because subjects that received electrical stimulation showed impaired recovery of locomotor function regardless of drug treatment.

My analyses of locomotor scores employed a conversion designed to improve the metric properties of the scale (Ferguson et al., 2004). Interestingly, inspection of the non-transformed data revealed an effect of drug treatment per se (Figure 14B). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded significant main effects of initial locomotor function and time, as well as the time by drug interaction, all $Fs > 5.72, p < .05$. Comparisons of locomotor function at each time
point showed that subjects treated with BBG had better function at the 28-day and 42-day time points.

To further evaluate whether BBG treatment affected recovery in the absence of shock stimulation, I compared the unshocked vehicle and BBG groups using a non-parametric test (Mann-Whitney U). This test revealed a statistically significant difference between vehicle- and BBG-treated subjects on terminal (day 42) locomotor function scores, $U = 12, p < .05$. This provides converging statistical evidence suggesting that BBG treatment improved locomotor outcomes in subjects that did not receive electrical stimulation.
Figure 14. Locomotor function after electrical stimulation and BBG treatment. (A) Subjects that received electrical stimulation showed impaired locomotor recovery that was not reversed by BBG treatment. (B) Subjects that did not receive electrical stimulation showed improved raw BBB scores with BBG treatment. Error bars represent SEM (n = 8). * indicates significant difference $p < .05$. 
Assessment of weight gain during recovery showed impairment in subjects that received C fiber activation (Figure 15). An ANOVA that examined percent change in weight across the recovery period revealed significant main effects of time, stimulation, and the time by stimulation interaction, all $F > 15.51$, $p < .05$. These effects emerged because subjects that received electrical stimulation showed impaired weight gain irrespective of BBG treatment.

**Figure 15. Weight gain after electrical stimulation and BBG treatment.** Subjects that received electrical stimulation showed impaired weight gain that was not reversed by brilliant blue treatment. Error bars represent SEM ($n = 8$). * indicates significant difference $p < .05$. 
Examination of subject tactile reactivity at 42 days revealed that C fiber stimulation enhanced reactivity to mechanical stimulation (Figure 16A). An ANOVA revealed a significant main effect of stimulation, $F = 5.58, p < .05$. This effect emerged because subjects that received C fiber stimulation showed reduced mechanical withdrawal threshold that was not impacted by drug treatment. Subjects that received BBG treatment without electrical stimulation showed reduced responsiveness to mechanical stimulation, however this reduction was not statistically significant.

Examination of thermal hyperalgesia with a tail flick test yielded no significant effects (Figure 16B). An ANOVA revealed no significant effects.

Finally, assessment of at-level pain with the girdle tactile test revealed a statistically significant decrease in the number of vocalizations in subjects that received BBG treatment (Figure 16C). An ANOVA revealed a main effect of drug, $F = 5.70, p < .05$. The interaction between drug and stimulation was not significant, $F = 0.91, p < .05$. This result indicated that BBG treatment reduced at-level pain.
Figure 16. Tactile, thermal, and girdle reactivity after electrical stimulation and BBG treatment. (A) Subjects that received electrical stimulation showed a reduction in tactile withdrawal threshold that was not impacted by BBG treatment. (B) Neither electrical stimulation nor BBG affected reactivity to thermal stimuli. (C) Subjects that received BBG treatment showed reduced vocalizations to girdle stimulation. Error bars represent SEM (n = 8). * indicates significant difference $p < .05$. 
Discussion

Experiment 2D replicated prior work that showed six minutes of intermittent electrical stimulation was sufficient to undermine locomotor function weeks after spinal cord contusion. Further, subjects that received nociceptive input showed impaired weight gain and reduced mechanical withdrawal threshold. None of these effects were reversed by treatment with BBG. However, treatment with BBG reduced signs of at-level neuropathic pain in all subjects, irrespective of nociceptive stimulation. Additionally, examination of BBB scores prior to transformation revealed that BBG treatment produced improved recovery of coordination in subjects that did not receive nociceptive stimulation. This improvement was small and therefore lost when BBB scores were transformed.

The results from this experiment replicate previous findings from other labs showing that BBG treatment improves recovery after spinal cord injury without acute pain (Peng et al., 2009). Additionally, BBG treatment reduced signs of neuropathic pain in all subjects. This suggests that the dose of BBG was appropriate.

Taken together, results from this experiment suggest that purinergic signaling may contribute to the development of at-level pain. However, BBG treatment did not prevent the detrimental impact of nociceptive stimulation. Because BBG treatment was successful at reducing pro-inflammatory signaling, alleviating signs of at-level pain, and improving locomotor outcomes in subjects that did not experience acute pain, but was unsuccessful at reversing the impact of acute pain on recovery, pathological purinergic signaling appears to be a downstream mediator of pain.
As mentioned earlier, BBG treatment was chosen for this experiment because I believed this therapy would be more likely than probenecid to block the adverse effects of nociceptive stimulation. As BBG treatment failed to block the behavioral effects of acute pain after SCI, future work could examine the impact of probenecid. However, BBG and probenecid had relatively weak effects on cellular indicators of pyroptosis (Exp. 2C) and both target the same ATP-induced ATP release pathway. Given this, I would expect probenecid would have a similar effect on recovery.
CHAPTER V
PROGRESSIVE HEMORRHAGIC NECROSIS AFTER ACUTE PAIN AND SPINAL CORD INJURY

In the process of collecting tissue for prior experiments, I noticed that protein isolates from subjects that received nociceptive stimulation were much darker than from controls. In Chapter V, I characterized the increased darkness of tissue samples and examined a potential mechanism.

Many processes of cell death are engaged following spinal cord injury. However, one of the most destructive is called progressive hemorrhagic necrosis (PHN). This pathway has been linked to injury severity and recovery following injury (Aarabi et al., 2012). The PHN pathway is defined by engagement of a SUR1-TRPM4 channel in neurovascular endothelial cells, apoptosis of these cells, capillary fragmentation, and massive hemorrhage (Gerzanich et al., 2009; Simard et al., 2007, 2013).

In Experiments 3A-B, I first identified hemoglobin as the protein that was the source of the dark red coloration using full spectral analysis and immunoblotting. Further, I characterized the time course of hemorrhage after nociceptive input. Finally, in Experiment 3C, I examined whether nociceptive stimulation increased the formation of SUR1-TRPM4 channels.
**Exp. 3A: Electrical stimulation increases hemoglobin within the lesion**

Casual examination of protein isolates from Experiment 1B revealed a drastic increase in the redness of samples recovered from subjects that received C fiber stimulation. Experiment 3A examined whether this increase in redness could be best explained by an increase in hemoglobin within the lesion.

*Procedure*

The protein isolates used in Experiment 3A were obtained from the same subjects utilized in Experiment 1B. Briefly, subjects received a moderate spinal cord injury to the lower thoracic region. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two groups (shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in the shocked group received six minutes of intermittent uncontrollable electrical stimulation while subjects in the unshocked group received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

Subjects were sacrificed at one of three time points (1, 3, or 24 hours after stimulation). Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Protein was extracted from whole tissue lysate and spectral analysis was performed using a Nanodrop spectrophotometer. Triplicates were obtained for each
sample and averaged to obtain the sample reading. For confirmation, immunoblotting was performed with hemoglobin alpha as the target.

An additional group of subjects was used to examine the histologic changes associated with nociceptive input. These subjects received a moderate spinal cord injury to the lower thoracic region on day 0. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two groups (shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in the shocked group received six minutes of intermittent uncontrollable electrical stimulation while subjects in unshocked groups received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

Subjects were sacrificed three hours following electrical stimulation or control. Briefly, a lethal dose of pentobarbital was administered through intraperitoneal injection. After death, the subject was flushed with phosphate buffered saline through transcardial perfusion, followed by a 4% paraformaldehyde fixative. The spinal cord was harvested and post-fixed in 4% paraformaldehyde overnight. Following a rinse in PBS, the spinal cord was cryoprotected in 30% sucrose for at least one week before sectioning and staining with hematoxylin and eosin.
Results

Group differences in sample color were apparent to the naked eye (Figure 17). Subjects in the shocked condition universally showed dark protein isolate color, whereas subjects in the unshocked condition typically showed much lighter protein isolate color. This increase in opacity and redness peaked around three hours, but was also present in tissue collected just one hour after stimulation.
Figure 17. Visible change in color of protein isolates after electrical stimulation. Protein isolates from subjects that did not receive electrical stimulation typically were clear yellow solutions. Samples from subjects that received electrical stimulation three hours earlier were opaque with a dark red coloration.
To quantify the increase in opacity and color, spectrophotometry was performed. Spectral analysis revealed a peak in absorbance around 420 nm (Figure 18A). This peak in absorbance is linked to the absorption peak for hemoglobin (Prahl, 1999).

The magnitude of the peak in absorbance at 420 nm was examined for all samples. Subjects that received electrical stimulation showed increased absorbance at this wavelength (Figure 18B). An ANOVA revealed significant main effects of stimulation and time point, as well as a significant interaction between stimulation and time point, all $F_s > 5.23$, $p < .05$. These effects emerged because subjects that received electrical stimulation showed increased absorbance at the one- and three-hour time points, but not at the twenty-four-hour time point.
Figure 18. Absorbance peak at 420 nm detected by full spectral analysis. (A) Full spectral analysis of protein isolates showed a clear peak in absorbance near 420 nm. (B) Subjects that received electrical stimulation showed increased absorbance at 420 nm. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. 
To reinforce these results, and demonstrate that hemoglobin was increased following nociceptive stimulation, immunoblotting of the protein extracts was performed. Immunoblotting for hemoglobin alpha revealed that electrical stimulation increased levels of hemoglobin α within the tissue (Figure 19). An ANOVA revealed main effects of stimulation and time point, as well as a stimulation by time point interaction, all $F_s > 5.27$, $p < .05$. Comparisons between shocked and unshocked subjects at each time point revealed that the three-hour time point showed a significant difference in levels of hemoglobin alpha between subject groups ($p < .05$). No other time points showed a statistically significant effect of stimulation.
Figure 19. Hemoglobin alpha protein levels after electrical stimulation. Subjects that received electrical stimulation showed increased levels of hemoglobin alpha three hours after stimulation. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. Representative blot from the three-hour time point.
Hematoxylin and eosin stained sections showed hemorrhage and identifiable extravascular red blood cells in subjects that received electrical stimulation (Figure 20A-B). Low power images revealed the extent of extravascular red blood cells. High powered images showed identifiable red blood cells.
Figure 20. Hematoxylin and eosin staining of spinal cord sections after electrical stimulation. (A) High numbers of extravascular red blood cells were apparent after electrical stimulation and were indicative of hemorrhage. (B) Prototypical anuclear, biconcave red blood cells were apparent in high power images.
Typical stained sections from subjects that received electrical stimulation appeared grossly deformed and hemorrhagic (Figure 21A-B). Sections from subjects that received SCI alone showed hemorrhage that was largely limited to the dorsal white matter; however, subjects that received spinal cord injury in conjunction with C fiber stimulation showed extensive hemorrhage in gray matter areas and dorsal white matter. Further, sections from subjects that received electrical stimulation showed large areas of missing tissue, and healthy neurons were nonexistent.
Figure 21. Hematoxylin and eosin sections after electrical stimulation. (A) Sections from subjects that did not receive C fiber input showed hemorrhage limited to the dorsal white matter. (B) Sections from subjects that received C fiber input were grossly deformed and showed extensive hemorrhage in both white and gray matter.
**Discussion**

Experiment 3A demonstrated that protein isolates from subjects that received electrical stimulation were darker and redder than those from subjects that did not receive electrical stimulation. Further, the protein responsible for increased redness and darkness of the purified protein extracts was hemoglobin. This highlighted the possibility that hemorrhage might be the mechanism responsible for impaired recovery caused by C fiber stimulation after spinal cord injury.

This experiment also provided some evidence that the source of hemoglobin was hemorrhage of red blood cells into the lesion. The presence of identifiable red blood cells coupled with massive destruction of tissue architecture was strongly indicative of hemorrhage. Quantitative assessment of hemorrhage area by Brumley (2016) has confirmed that shock treatment has a statistically significant effect.

One pathway known to occur following spinal cord injury is progressive hemorrhagic necrosis. The extent of PHN has been shown to predict the eventual extent of the glial scar, and to correlate strongly with recovery (Simard et al., 2013). Therapeutics aimed at inhibiting PHN have been shown to reduce hemorrhage following injury and improve functional outcomes (Simard et al., 2008). Interestingly, PHN may depend upon damage to afferent signaling pathways (Popovich et al., 2012; Simard et al., 2012).
Exp. 3B: Pharmacologic activation of C fibers increases lesion hemoglobin content

Experiment 3A demonstrated that electrical stimulation of C fibers after spinal cord injury increased hemoglobin content within the lesion site. Experiment 3B examined whether pharmacological activation of C fibers had the same effect. If pharmacologic activation of C fibers had the same effect, it would provide converging evidence that nociceptive stimulation induced hemorrhage, and discount the possibility that the effect was due to stimulation-induced movement.

Procedure

The protein isolates used in Experiment 3B were obtained from the same subjects utilized in Experiment 1B. Briefly, subjects received a moderate spinal cord injury at to the lower thoracic region. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two groups (capsaicin or vehicle) such that the average BBB score across groups was balanced (randomized block design). Subjects in the capsaicin group received a single injection of capsaicin to a hind paw while subjects in the vehicle group received an injection of vehicle. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until they were sacrificed.

Subjects were sacrificed at one of three time points (1, 3, or 24 hours after stimulation). Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Protein was extracted from whole tissue lysate and spectral analysis was performed using a Nanodrop spectrophotometer. Triplicates were obtained for each
sample and averaged to obtain the sample reading. For confirmation, immunoblotting was performed with hemoglobin alpha as the target.

Results

The magnitude of the peak in absorbance at 420 nm was examined at 1, 3 and 24 hours after treatment. Subjects that received capsaicin showed increased absorbance at this wavelength (Figure 22). An ANOVA revealed a significant main effect of stimulation, $F = 7.93, p < .05$. Neither the main effect of time point, nor the interaction between time point and capsaicin treatment were significant, $Fs < 1.44, p > .05$. This indicated that pharmacologic activation of C fibers increased hemoglobin within the lesion similarly at all time points examined.
Figure 22. Absorbance at 420 nm after capsaicin injection. Subjects that received capsaicin injection showed increased absorbance at 420 nm. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. 
Further evidence that capsaicin treatment increased hemoglobin alpha content was obtained using immunoblotting (Figure 23). An ANOVA revealed a significant main effect of stimulation, $F = 5.23, p < .05$. Again, neither the main effect of time point nor the interaction between time point and stimulation were statistically significant, $Fs < 1.23, p > .05$. This indicated increased hemorrhage at all time points studied.
Figure 23. Hemoglobin alpha protein levels after capsaicin injection. Subjects that received capsaicin injection showed increased levels of hemoglobin alpha. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. Representative blot from the three-hour time point.
Discussion

As was seen with electrical stimulation, pharmacologic activation of C fibers induced a significant increase in hemoglobin content of the lesion site. This finding provided converging evidence that afferent pain processing fibers (C fibers) were critically involved in hemorrhage initiation following spinal cord injury. Further, because capsaicin treatment elicits little motor activity, the results imply that the magnitude of hemorrhage is unrelated to stimulation-induced movement.
**Exp. 3C: Electrical stimulation increases SUR1-TRPM4 interactions**

Nociceptive input increased hemoglobin within the lesion site by inducing hemorrhage of red blood cells into spinal parenchyma. However, what remained unknown was the molecular mechanism that engaged the hemorrhage. Experiment 3C examined the role that the SUR1-TRPM4 channel and progressive hemorrhagic necrosis (PHN) played in mediating detrimental effects of nociceptive stimulation. The best evidence for PHN requires identification of the specific SUR1-TRPM4 protein-protein complex (Simard et al., 2013). Co-immunoprecipitation was used in Experiment 3C to demonstrate increased formation of SUR1-TRPM4 protein-protein complexes in response to acute pain after SCI.

*Procedure*

Subjects in Experiment 3C received a moderate spinal cord injury to the lower thoracic region on day 0. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of two groups (shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). All subjects were restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in shocked groups received six minutes of intermittent uncontrollable electrical stimulation while subjects in unshocked groups received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.
Subjects were sacrificed one hour after electrical stimulation. Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Protein was extracted from whole tissue lysate under non-denaturing conditions and prepared for co-immunoprecipitation using an agarose bead preparation. Briefly, protein samples were precleared with agarose beads. Next, proteins were incubated with primary antibody against SUR1, followed by agarose beads. Proteins complexed to the SUR1 antibody and agarose beads were spun down, and the resulting protein complexes were subsequently denatured and examined by gel electrophoresis and immunoblotting against TRPM4.

**Results**

Protein levels of TRPM4 captured from SUR1 co-immunoprecipitation showed a significant increase in SUR1-TRPM4 protein complexing just one hour after stimulation (Figure 24). A significant difference between shocked and unshocked subjects was revealed using a t test, $p < .05$. 
Figure 24. SUR1-TRPM4 protein complexes after electrical stimulation measured by co-immunoprecipitation. Subjects that received electrical stimulation showed increased formation of SUR1-TRPM4 protein complexes. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. Representative blot showed increased TRPM4 protein levels in subjects that received electrical stimulation.
Discussion

Co-immunoprecipitation of SUR1 and TRPM4 showed a rapid and significant increase in SUR1-TRPM4 protein complexes. As expected, subjects that received spinal cord injury alone showed detectable levels of SUR1-TRPM4 complexes as measured by co-immunoprecipitation. Subjects that received electrical stimulation soon after spinal cord injury showed a 165 percent increase in SUR1-TRPM4 protein-protein interactions.

Findings from Experiment 3C represent some of the strongest evidence for progressive hemorrhagic necrosis, and provide promising direction for future studies. While therapeutics aimed at PHN have had some difficulty with replication, Experiment 3C suggests that these therapeutics might be especially effective in subjects that have damage to or activation of afferent C fiber pathways.

Additionally, protein-protein interactions between SUR1 and TRPM4 represent an active biological process that is an attractive therapeutic target for intervention. These data also discount the possibility that increased hemorrhage is occurring in response to hypertension alone. If the destruction of blood vessels and hemorrhage associated with acute pain were caused by hypertension, it is unlikely that an active biological process would be seen.
Chapter VI

Therapeutic Interventions for Acute Pain After Spinal Cord Injury

Having demonstrated that nociceptive stimulation engages cell death pathways, induces hemorrhage, and impairs behavioral recovery, the next key question is: How can we block these adverse effects to reduce secondary injury and promote recovery? One possibility involves the administration of an opiate analgesic, such as morphine. In Experiment 4A, I tested whether treatment with systemic morphine would block the hemorrhagic effect of nociceptive stimulation. Consistent with prior work (Hook et al., 2007, 2009), morphine treatment had no protective effect. Next, in Experiment 4B, I examined whether epidural lidocaine, a clinically translatable therapeutic strategy, was more effective at blocking the hemorrhage, inflammation, and cell death associated with nociceptive stimulation. Finding that epidural lidocaine successfully prevented all cellular indices of nociceptive stimulation, Experiment 4C examined whether epidural lidocaine blocks the effect of nociceptive stimulation on behavioral recovery following a contusion injury.
Exp. 4A: Systemic morphine does not reverse acute pain-induced hemorrhage

Treatment with a high dose of morphine blocks shock-induced motor reactivity and attenuates brain-dependent pain/stress. It should also reduce blood pressure spikes in response to acute pain (Mahinda, Lovell, & Taylor, 2004). If stimulation-induced hemorrhage and cytokine expression is related to any of these processes, morphine treatment should attenuate the effects of shock.

Procedure

Subjects received a moderate spinal cord injury to the lower thoracic region. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of four groups (vehicle or morphine, crossed with shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). Subjects then received a single intraperitoneal injection of either morphine (20 mg/kg) or vehicle. This dose was selected because it has been previously shown to induce robust antinociception in rats with spinal cord injury (Hook et al., 2007). Subjects were returned to their cages for thirty minutes. All subjects were then restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in shocked groups received six minutes of intermittent uncontrollable electrical stimulation while subjects in unshocked groups received identical treatment without electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.
Subjects were sacrificed three hours after stimulation. Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80°C until processing.

Protein was extracted from whole tissue lysate and spectral analysis was performed using a Nanodrop spectrophotometer. Triplicates were obtained for each sample and averaged to obtain the sample reading. For confirmation, immunoblotting was performed with hemoglobin alpha as the target.

*Results*

The magnitude of the peak in absorbance at 420 nm was examined using a spectrophotometer. Subjects that received electrical stimulation showed increased absorbance that was not affected by morphine treatment (Figure 25A). An ANOVA revealed a significant main effect of stimulation, $F = 17.64, p < .05$. No other effects were statistically significant, $Fs < 1.51, p > .05$. This indicated that electrical stimulation increased hemorrhage and that the increase was unaffected by morphine treatment.

Immunoblotting for hemoglobin was performed to confirm the source of coloration. Subjects that received electrical stimulation showed increased levels of hemoglobin alpha within the tissue, and this increase was not impacted by morphine treatment (Figure 25B). An ANOVA revealed a main effect of stimulation, $F = 10.46, p < .05$. Again, no other effects were significant, $Fs < 1.01, p > .05$. These results indicated that electrical stimulation increased hemorrhage and that the increase was not reversed by morphine treatment.
Figure 25. Absorbance at 420 nm and hemoglobin alpha protein levels after electrical stimulation and systemic morphine treatment. (A) Subjects that received electrical stimulation showed increased absorbance at 420 nm irrespective of morphine treatment. (B) Subjects that received electrical stimulation showed increased levels of hemoglobin alpha three hours after stimulation that was not reversed by morphine treatment. Error bars represent SEM (n = 6). * indicates significant difference, $p < .05$. 
Immunoblotting for IL-1β demonstrated increased processing in subjects that received electrical stimulation that was not blocked by morphine treatment (Figure 26A). An ANOVA yielded the significant main effect of stimulation, $F = 9.80, p < .05$. The interaction between stimulation and drug treatment was not significant, $F = 0.03, p > .05$, and indicated that IL-1β processing was not impacted by morphine treatment.

Immunoblotting for IL-18 demonstrated increased processing for subjects that received electrical stimulation that was not blocked by morphine treatment (Figure 26B). An ANOVA yielded the significant main effect of stimulation, $F = 15.42, p < .05$. The interaction between stimulation and drug treatment was not significant, $F < 1.00, p > .05$, and indicated that IL-18 processing was not impacted by morphine treatment.
Figure 26. IL-1β and IL-18 processing after electrical stimulation and systemic morphine treatment. (A) Subjects that received electrical stimulation showed increased processing of the pro-inflammatory cytokine IL-1β that was not reversed by morphine treatment. (B) Subjects that received electrical stimulation showed increased processing of the pro-inflammatory cytokine IL-18 that was not reversed by morphine treatment. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. 
Discussion

Morphine treatment did not reverse the effect of stimulation on hemorrhage. This experiment is clinically relevant because morphine is very commonly used to control pain following trauma. Here, using a dose of morphine that induces a profound analgesia, and that blocks motor reactivity to shock, we found no effect of drug treatment on shock-induced hemorrhage. This implies that the hemorrhage effect is unrelated to both psychological pain and stimulation-induced movement.

Additionally, Experiment 4A provides some evidence that hemorrhage does not depend upon stress or cardiovascular changes. While nociceptive stimulation is expected to increase heart rate and blood pressure, morphine treatment normally attenuates these effects (Mahinda et al., 2004). Yet, morphine was completely ineffective in reversing signs of hemorrhage.

Experiment 4A also showed that morphine did not reverse the increased processing of IL-1β and IL-18. Taken together, these results highlight the need for better treatment of acute pain following spinal cord injury. Behavioral reactivity, emotional aspects of pain processing, and increased hemorrhage should all be considered in the pursuit of effective care for SCI patients.
**Exp. 4B: Inhibiting neural activity with epidural lidocaine blocks cellular indices of acute pain**

Experiment 4A showed that nociceptive input increased hemoglobin within the lesion site and that a commonly used analgesic was ineffective at reversing this effect. Experiment 4C examined if a different clinically relevant analgesic therapy might be effective at reversing hemorrhage after electrical stimulation and spinal cord injury.

Spinal and nerve blocks are frequently performed clinically, and are very useful for pain management. Local and regional anesthesia are effective in the management of pain during labor, Caesarian delivery, and lower limb surgery (Albright & Forster, 1999; Ben-David, Miller, Gavriel, & Gurevitch, 2000; Burke, Kennedy, & Bannister, 1999). These techniques rely upon the basic principle that neurons carry information through action potentials. Using sodium channel blockers, neurons are unable to propagate action potentials, and information flow is interrupted. Lidocaine is the first discovered sodium channel blocker and is still in use clinically (Wildsmith, 2011). Recent evidence suggests that lidocaine also has some anti-inflammatory properties (Caracas, Maciel, de Souza, & Maia, 2009; Hollmann & Durieux, 2000; Van Der Wal et al., 2015). In the following experiment, lidocaine was administered caudal to the lesion to inhibit neural activity in response to nociceptive stimulation.

**Procedure**

Subjects in Experiment 4B received a moderate spinal cord injury to the lower thoracic region on day 0. Twenty-four hours later, function was assessed using the BBB locomotor scale. Subjects were then randomly assigned to one of four groups (lidocaine...
or vehicle, crossed with shocked or unshocked) such that the average BBB score across groups was balanced (randomized block design). All subjects were lightly anesthetized with isoflurane, then an acute injection of lidocaine (25 µL, 15%) or vehicle was administered. Thirty minutes later, all subjects were restrained for six minutes in an opaque Plexiglas tube and had an electrode fastened to the tail. Subjects in shocked groups received six minutes of intermittent uncontrollable electrical stimulation while subjects in unshocked groups received identical treatment without electrical stimulation. An observer blind to the treatment condition of each animal recorded the presence of motor and vocal responses to electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and kept in a temperature-controlled room until sacrifice.

Subjects were sacrificed three hours after stimulation. Spinal cords were then rapidly harvested and flash frozen in liquid nitrogen. Cords were kept at -80ºC until processing.

Protein was extracted from whole tissue lysate and spectral analysis was performed using a Nanodrop spectrophotometer. Triplicates were obtained for each sample and averaged to obtain the sample reading.

Results

Baseline BBB scores ranged from 3.2 (±0.7) to 3.7 (±1.3) and did not differ across groups, all $Fs < .01$, $p > .05$. This indicated that injury severity was similar across all groups.
Subjects that did not receive lidocaine treatment showed motor and vocal responses to electrical stimulation (3/3). No subjects that received lidocaine treatment showed motor or vocal responses to electrical stimulation (0/3). As expected, in the absence of stimulation subjects did not exhibit a motor or vocal responses (0/6). A chi-square test revealed a statistically significant increase in motor and vocal responses in subjects that received electrical stimulation and vehicle treatment, $\chi^2 = 9.00, p < .05$.

The magnitude of the peak in absorbance at 420 nm was examined using a spectrophotometer. Subjects that received electrical stimulation showed increased absorbance at this wavelength. This effect was completely reversed by lidocaine treatment (Figure 27). An ANOVA revealed significant main effects of stimulation and drug treatment, as well as a significant interaction between stimulation and drug treatment, all $F_s > 57.82, p < .05$. These effects emerged because vehicle-treated subjects that received electrical stimulation showed increased absorbance compared to vehicle-treated subjects that did not receive electrical stimulation. Subjects that received lidocaine treatment showed no difference in hemoglobin content after electrical stimulation.
Figure 27. Absorbance at 420 nm after electrical stimulation and epidural lidocaine. Subjects that received electrical stimulation showed increased absorbance at 420 nm that was completely reversed by lidocaine treatment. Error bars represent SEM (n = 3). * indicates significant difference, p < .05.
Immunoblotting for IL-1β revealed that electrical stimulation produced increased processing of the pro-inflammatory cytokine in shocked subjects, and that lidocaine treatment blocked this increase (Figure 28A). An ANOVA yielded a significant main effect of drug, as well as a significant interaction between drug and stimulation, all \( F_s > 5.43, p < .05 \). These effects emerged because a statistically significant difference existed between shocked subjects that received vehicle and lidocaine treatments.

Immunoblotting for IL-18 showed similar results to those for IL-1β (Figure 28B). An ANOVA yielded significant main effects of drug and stimulation, as well as the drug with stimulation interaction, all \( F_s > 12.81, p < .05 \). Again, these effects emerged because lidocaine treatment blocked C fiber stimulation-induced processing of the pro-inflammatory cytokine.
Figure 28. IL-1β and IL-18 processing after electrical stimulation and epidural lidocaine. (A) Subjects that received electrical stimulation showed increased processing of IL-1β that was reversed by lidocaine treatment. (B) Subjects that received electrical stimulation showed increased processing of IL-18 that was reversed by lidocaine treatment. Error bars represent SEM (n = 3). * indicates significant difference p < .05.
Immunoblotting for TNF revealed that epidural lidocaine reduced levels of the pro-inflammatory cytokine in shocked subjects (Figure 29). An ANOVA yielded a significant main effect of drug, $F = 9.25, p < .05$. Both the main effect of stimulation and the interaction of drug and stimulation approached statistical significance, both $F_s > 5.06, p < .055$.

Figure 29. TNF protein levels after electrical stimulation and epidural lidocaine. Subjects that received epidural lidocaine showed reduced levels of TNF. Error bars represent SEM (n = 3). * indicates significant difference $p < .05$. 

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Immunoblotting for caspase-3 revealed that electrical stimulation produced increased levels of the active form of this apoptotic protease in shocked subjects, and that lidocaine treatment blocked this increase (Figure 30A). An ANOVA yielded significant main effects of drug and stimulation, as well as a significant interaction between drug and stimulation, all $F_s > 6.58, p < .05$. These effects emerged because a statistically significant difference existed between shocked subjects that received vehicle and lidocaine treatments.

Immunoblotting for caspase-8 revealed a similar pattern (Figure 30B), but the group differences did not reach statistical significance, all $F_s < 2.01, p > .05$. 
Figure 30. Caspase-3 and caspase-8 protein levels after electrical stimulation and epidural lidocaine. (A) Subjects that received electrical stimulation showed increased levels of caspase-3 that were reversed by lidocaine treatment. (B) Subjects did not show differences in caspase-8 protein levels after electrical stimulation and lidocaine treatment. Error bars represent SEM (n = 3). * indicates significant difference $p < .05$. 
Discussion

Experiment 4B replicated prior work that showed vehicle-treated subjects that received electrical stimulation showed massive increases in hemorrhage within the spinal cord lesion. Epidural lidocaine blocked this effect. Because the extent of hemorrhage correlates well with eventual recovery, this experiment provided compelling evidence to support spinal lidocaine as a potential therapeutic for acute pain after spinal cord injury.

I also examined the impact of epidural lidocaine on pro-inflammatory cytokine expression. Epidural lidocaine blocked stimulation-induced increases in IL-1β and IL-18 processing. Lidocaine also blocked stimulation-induced TNF signaling and activation of apoptotic proteases.

Whereas opiate medications coopt a biological pathway responsible for adjusting responses to pain, epidural lidocaine quells neuronal activity in response to nociceptive stimulation. Additionally, opiate medications lead to a number of major problems including dependence, addiction, and paradoxical pain (Angst, Koppert, Pahl, Clark, & Schmelz, 2003; Lee, Silverman, Hansen, & Patel, 2011). In contrast, side effects of spinal blocks are minimal (Auroy et al., 1997; Carpenter, Caplan, Brown, Stephenson, & Wu, 1992). Thus, while both morphine and lidocaine are used clinically for pain treatment, only epidural lidocaine blocked the detrimental impact of C fiber stimulation on inflammation, hemorrhage, apoptosis, and pyroptosis.
Exp. 4C: Epidural lidocaine blocks the adverse effect of acute pain on recovery after SCI

Experiments 1A-B demonstrated that C fiber activation undermines functional recovery after spinal cord injury, and that pro-inflammatory and apoptotic cascades are engaged. Experiments 2A-B showed that C fiber stimulation activated caspase-1 and increased the processing of pro-inflammatory cytokines. Experiments 3A-C revealed that nociceptive stimulation increased hemorrhage after spinal cord injury. Finally, Experiment 4B demonstrated that epidural lidocaine protected subjects from the electrical stimulation-induced activation of pro-inflammatory signaling, apoptotic cascades, pyroptosis, and hemorrhage. Experiment 4C examined whether epidural lidocaine also blocks the adverse effect acute nociceptive stimulation has on the recovery of locomotor function.

Procedure

Subjects in Experiment 4C were treated identically to subjects in Experiment 4B, but were examined over a six-week recovery period. Briefly, subjects received a moderate spinal cord injury on day 0. Twenty-four hours later, an acute injection of lidocaine or vehicle was administered. Thirty minutes after the injection, all subjects were restrained and had an electrode fastened to the tail. Subjects in shocked groups received electrical stimulation. After treatment, all subjects were removed from the Plexiglas tube and returned to their home cages.

Subjects were examined for six weeks following injury. Locomotor function was assessed using the BBB scoring system. Weight gain was assessed daily throughout the
experiment. Finally, additional measures of locomotor function (beam and ladder tests) and pain (tactile reactivity, thermal tail flick, and girdle tests) were taken at the end of the recovery period.

One subject was sacrificed after 21 days due to extensive autophagia. Because locomotor recovery asymptotes within 21 days, this subject’s terminal score was carried forward in our statistical analyses.

Results

Baseline BBB scores ranged from 2.8 (±0.5) to 3.4 (±0.6) and did not differ across groups, all $F$s < 0.52, $p > .05$. Because no differences were found in baseline BBB scores, the results were used as an index of injury severity and a covariate throughout the rest of this experiment.

Subjects that did not receive lidocaine treatment showed motor and vocal responses to electrical stimulation (6/6). No subjects that received lidocaine treatment showed motor or vocal responses to electrical stimulation (0/6). No subjects that did not receive electrical stimulation showed motor or vocal responses (0/12). A chi-square test revealed a statistically significant increase in motor and vocal responses in subjects that received electrical stimulation and vehicle treatment, $\chi^2 = 18.00, p < .05$.

Analysis of locomotor function across time demonstrated a significant impairment in subjects that received nociceptive input. This effect was reversed by lidocaine treatment (Figure 31). An ANCOVA that used pre-stimulation locomotor function as the covariate yielded main effects of stimulation and time, as well as significant interactions between drug and stimulation, time and stimulation, and time by
drug by stimulation, all $Fs > 2.95$, $p < .05$. These effects emerged because subjects that received electrical stimulation without lidocaine showed impaired locomotor recovery compared to all other groups.

Figure 31. Locomotor function after electrical stimulation and epidural lidocaine. Subjects that received electrical stimulation showed impaired locomotor recovery that was reversed by epidural lidocaine. Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. 

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Baseline weights ranged from 294 (±4.2) to 325 (±14.6) and did not differ across groups, all $F_s < 3.78, p > .05$. Because no differences were found in baseline weights, the values were used as a covariate in analysis of weight gain.

Assessment of weight gain during recovery showed impairment in subjects that received C fiber activation. This effect was reversed with lidocaine treatment (Figure 32A-B). An ANCOVA that examined percent change in weight across the recovery period revealed significant main effects of initial weight and time, as well as significant interactions between drug and stimulation, time and initial weight, and time by drug by stimulation, all $F_s > 2.12, p < .05$. These effects emerged because subjects that received electrical stimulation without lidocaine showed impaired weight gain compared to subjects that received lidocaine treatment.
Figure 32. Weight gain after electrical stimulation and epidural lidocaine. Subjects that received electrical stimulation showed impaired weight gain (A) that was blocked by epidural lidocaine (B). Error bars represent SEM (n = 6). * indicates significant difference $p < .05$. 
I sought to further evaluate locomotor function using beam and ladder assessments. However, no subjects that received C fiber stimulation without lidocaine met the minimum locomotor requirements necessary to complete these tests. A chi-squared test examining the fraction of subjects within each group that were capable of completing the beam and ladder assessments revealed significance, $p < .05$, indicating that subjects that received electrical stimulation without lidocaine had significantly worse locomotor function compared to all other groups.

Further statistical analysis was performed using a one-way ANOVA for the remaining three groups. Subjects that received lidocaine and electrical stimulation were statistically indistinguishable on both beam and ladder tests from subjects that did not receive electrical stimulation (Figure 33A-B). ANOVAs analyzing data from the beam and ladder tests did not detect any significant effects, all $F_s < 0.43$, $p > .05$. 
Figure 33. Beam and ladder performance after electrical stimulation and epidural lidocaine. (A) Subjects that received electrical stimulation and epidural lidocaine were indistinguishable on beam performance from subjects that did not receive electrical stimulation. (B) Subjects that received electrical stimulation and epidural lidocaine were indistinguishable on ladder performance from subjects that did not receive electrical stimulation. Error bars represent SEM (n = 6 for Veh-Unshk, Lid-Shk; n = 5 for Lid-Unshk).
Examination of subject tactile reactivity at 42 days revealed no differences between groups (Figure 34A). An ANOVA revealed no statistically significant effects; however, subjects that received C fiber stimulation without lidocaine did show a reduced withdrawal threshold.

Examination of thermal hyperalgesia with a tail flick test yielded similar results to tactile reactivity findings (Figure 34B). An ANOVA revealed no significant effects, but the main effect of stimulation did approach significance, $F = 4.23, p = .054$. Subjects in the group that received electrical stimulation without lidocaine showed the shortest latency to tail withdrawal.

Finally, assessment of at-level pain using the girdle test revealed a statistically significant increase in the number of vocalizations in subjects that received lidocaine (Figure 34C). An ANOVA revealed a main effect of drug, $F = 5.17, p < .05$. 
Figure 34. Tactile, thermal, and girdle reactivity after electrical stimulation and epidural lidocaine. (A) Subjects that received electrical stimulation without lidocaine showed a statistically insignificant reduction in tactile withdrawal threshold. (B) Subjects that received electrical stimulation without lidocaine showed a reduction in thermal withdrawal latency that was not statistically significant. (C) Subjects that received epidural lidocaine showed increased vocalizations to girdle stimulation. Error bars represent SEM (n = 6 for Veh-Unshk, Veh-Shk, Lid-Shk; n = 5 for Lid-Unshk). * indicates significant difference $p < .05$. 
Discussion

This experiment replicated prior work that showed six minutes of intermittent electrical stimulation was sufficient to undermine the recovery of locomotor function after spinal cord contusion. Further, subjects that received nociceptive input showed impaired weight gain.

Experiment 4C demonstrated that epidural lidocaine reversed the detrimental effects of C fiber stimulation. This provides further evidence that epidural lidocaine treatment soon after spinal cord injury might be therapeutically relevant for treatment of pain due to associated injuries.

Interestingly, subjects that received lidocaine treatment also showed increased vocalizations in response to girdle stimulation. This suggests that lidocaine treatment may have the side effect of increasing at-level neuropathic pain. Alternatively lidocaine treatment could enhance vocalization to stimulation because it reduced the loss of ascending sensory fibers.

Taken together, data from Experiment 4C suggest that nociceptive stimulation after spinal cord injury undermines functional outcomes, and that epidural lidocaine might be a therapeutically relevant treatment.
CHAPTER VII
GENERAL DISCUSSION

Using a clinically relevant model of spinal cord injury, this dissertation examined the impact of acute pain on recovery. Two distinct models of C fiber activation were presented in Chapter III, with the relative importance of pyroptosis and progressive hemorrhagic necrosis characterized in Chapters IV and V, respectively. Chapter VI examined possible therapeutic interventions for acute pain associated with spinal cord injury.

First, either electrical or pharmacologic activation of C fibers were found to similarly engage known inflammatory and apoptotic pathways, and to impair functional outcomes in a long-term recovery study. Then, another cellular pathway associated with inflammatory programmed cell death was shown to be amplified following nociceptive stimulation. Pyroptosis, a known impediment to recovery following spinal cord injury alone, was shown to be engaged by acute pain. While inhibition of pathologic purinergic signaling was found to be moderately successful at reversing caspase-1 activation and reducing pro-inflammatory cytokine processing, treatment with BBG did not improve functional outcomes.

In the process of evaluating the role of pyroptosis on impaired recovery from acute pain, protein extracts from subjects that received C fiber activation were observed to be much darker than controls. Further study revealed that acute pain after spinal cord
injury increased hemorrhage, and that formation of SUR1-TRPM4 channels were the likely cause.

Finally, an effective therapeutic approach for treatment of acute pain in animals with spinal cord injury was explored. Whereas treatment with morphine did not reverse the detrimental impact of acute pain, and actually exacerbated hemorrhage, acute spinal block with lidocaine prevented inflammatory, apoptotic, pyroptotic, hemorrhagic, and behavioral effects of acute pain after spinal cord injury. These experimental results provide compelling evidence that acute pain after spinal cord injury is a major impediment to successful recovery, and that the use of opiates for treatment of acute pain after spinal cord trauma requires further examination.

**Acute pain-induced impaired recovery**

Prior work in the laboratory has shown how a wide number of different forms of C fiber and inflammatory stimuli impact spinal plasticity in a transection model of spinal learning. Electrical stimulation applied to either the tail or leg, capsaicin injection, formalin injection, and LPS injection all have been relatively well-characterized and found to undermine spinal plasticity (Grau et al., 2014; E. E. Young et al., 2007). However, only electrical stimulation has been explored in the more clinically relevant model of spinal cord contusion. In this model, just six minutes of electrical stimulation was sufficient to undermine locomotor recovery, increase lesion volume, and induce inflammation.

In this dissertation, a single peripheral injection of capsaicin was found to engage similar processes to electrical activation of C fibers. A single injection of capsaicin given
24 hours after spinal cord injury prevented nearly all recovery of locomotor function over a four-week recovery period. When compared with subjects given a vehicle injection, capsaicin impaired recovery. These subjects also showed impaired weight gain, which is an indicator of general health. Taken together, these data provide converging evidence that C fiber activation is the primary source of stimulation-induced impaired recovery.

Interestingly, subjects that received a vehicle injection alone showed impaired recovery when compared with subjects that did not receive an injection. This suggests that even relatively little C fiber stimulation may be sufficient to be biologically relevant, which might be especially important in the clinical population.

Experiments also showed that electrical and pharmacologic activation of C fibers activated the same pro-inflammatory pathway. Tumor necrosis factor (TNF), the same cytokine known to mediate many of the detrimental impacts of stimulation on spinal plasticity, was rapidly upregulated following electrical stimulation or capsaicin injection. Signaling through TNF receptors has been shown to engage the NF-κB pathway and apoptosis. This has been studied extensively in the transection model of spinal learning and is known to involve activation of TNFR1. Downstream targets of TNF signaling remain an interesting area of study in the contusion model of spinal cord injury.

**Role of pyroptosis**

In this dissertation, acute pain soon after spinal cord injury was shown to induce pro-inflammatory pathways and apoptosis that undermine recovery of locomotor function. Recent work has demonstrated that a form of pro-inflammatory programmed
cell death called pyroptosis is a key pathway involved in the secondary injury cascade after spinal cord injury (Vaccari et al., 2008). Consequently, if pyroptosis were exacerbated following spinal cord injury, recovery would be impaired.

Experiments presented in Chapter IV showed that nociceptive input after spinal cord injury enhanced markers of pyroptosis. Protein levels of the active form of caspase-1 were increased soon after stimulation, and were maintained at high levels for at least 24 hours. Further, for both electrical and pharmacologic activation of C fibers, processing of the pro-inflammatory cytokines IL-1β and IL-18 was increased and prolonged, highlighting the biological relevance of caspase-1 activity.

Correlation between caspase-1 activity and acute pain after spinal cord injury was found to be very strong. Thus, the potential for inhibition of this pathway to reverse the detrimental impact of nociceptive input was examined. While inhibition of caspase-1 activation with BBG was successful at reducing pro-inflammatory cytokine processing, this treatment was completely ineffective at improving functional outcomes. However, treatment with BBG was shown to be successful at reducing signs of at-level pain.

Other work has shown that treatment with BBG improves locomotor recovery following spinal cord injury alone (Peng et al., 2009). Experiments in this dissertation showed only a modest improvement in coordination with BBG treatment in subjects that did not receive electrical stimulation. Thus, other treatment strategies should be pursued.

Because BBG treatment was successful at reducing pro-inflammatory cytokine processing, but failed to improve functional outcomes, pyroptosis and caspase-1 activity are most likely downstream pathways involved in the impaired recovery seen after
nociceptive stimulation and spinal cord injury. Future work could examine a more powerful caspase-1 inhibitor, but other evidence from this thesis suggests that pyroptosis is not the primary cause of the observed impairment. Thus, alternative treatment strategies that target different pathways may have a greater probability of success.

**Role of progressive hemorrhagic necrosis**

The serendipitous observation of drastic color changes in protein isolates from subjects that received nociceptive stimulation provided an interesting avenue of research. To identify the protein responsible for these color changes, full spectral analysis was used. The finding of an absorbance peak around 420 nm indicated increased hemoglobin concentration. Subsequent immunoblotting showed that nociceptive stimulation increased hemoglobin content within the lesion. Determination of the hemoglobin source was then undertaken using standard histopathologic assessment with H&E staining. Nociceptive stimulation was found to cause a massive increase of hemorrhage at the lesion epicenter, as well as rostral and caudal to the epicenter. Taken together, this evidence suggests that hemorrhage was engaged by nociceptive stimulation.

One mechanism of hemorrhage described after spinal cord injury is progressive hemorrhagic necrosis (PHN). As discussed earlier, hemorrhage within the lesion is one of the best predictors of eventual recovery. PHN is defined by formation of a SUR1-TRPM4 channel in neurovascular endothelial cells, death of these endothelial cells, and massive hemorrhage into the spinal cord (Gerzanich et al., 2009; Simard et al., 2007, 2013). In Experiment 3D co-immunoprecipitation studies were performed to demonstrate that nociceptive stimulation increased the formation of SUR1-TRPM4
channels. These results highlighted the relevance of acute pain in the defined PHN pathway.

Glibenclamide is an inhibitor of the SUR1-TRPM4 channel and is already FDA approved for treatment of diabetes mellitus type II. This drug has been shown to reduce hemorrhage following spinal cord injury (Simard et al., 2008). However, later work showed difficulty in replicating these original findings (Popovich et al., 2012). One potential problem noted when performing these replication studies was that the drug showed a larger beneficial effect when given in a unilateral model of spinal cord injury (Simard et al., 2012). Data from this dissertation further suggest that nociceptive stimulation engages neural circuits that may be especially prone to development of PHN. Thus, while Popovich and Simard hypothesize that the initial hemorrhage size determines the efficacy of glibenclamide treatment (Simard et al., 2012), it could be that a neural circuit approach might prove valuable in defining regions more prone to PHN. Further, neurovascular units within the dorsal horns might be especially important targets for glibenclamide treatment, due to their role in pain processing.

Additional work from our laboratory has quantified the extent of hemorrhage after SCI and nociceptive stimulation. Electrical stimulation increased hemorrhage across the extent of the lesion by nearly three-fold (293%), confirming that acute pain after SCI increases hemorrhage (Brumley, 2016). Current work is examining capillary fragmentation as additional evidence for progressive hemorrhagic necrosis.
Other models of polytrauma

Work in our laboratory suggests that the interaction between damage to the nervous system and associated injuries is especially important in determining outcomes. The impact of polytrauma on recovery after traumatic brain injury has been examined as early as 1990 and is relatively well-studied (Groswasser et al., 1990). However, to my knowledge, no other laboratories have published research that examines the impact of polytrauma on recovery from spinal cord injury.

Early work in traumatic brain injury has shown that while a single source of peripheral tissue damage is not associated with impaired recovery, multiple sources of peripheral tissue damage undermines functional outcomes in human patients (Groswasser et al., 1990). However, recent studies have found opposite results. In fact, veterans with increased peripheral tissue damage show reduced post-concussive symptoms and reduced incidence of post-traumatic stress disorder (French et al., 2012, Lange et al., 2014). More must be done to further elucidate the impact of associated injuries on recovery from nervous system trauma.

Other laboratories have begun to explore the effect of polytrauma in other animal models of nervous system damage. In a model of multi-trauma associated with traumatic brain injury (TBI), tibial fracture significantly impaired functional outcomes and increased cell loss (Shultz et al., 2015). Mice that received a mild TBI showed no impairment in behavior five weeks after injury. In contrast, subjects that received a mild TBI in conjunction with a unilateral tibial fracture showed behavioral abnormalities and increased ventricular volume. This indicated that repair mechanisms were capable of
promoting full functional recovery in subjects that received a mild nervous system injury. However, when associated injuries were present, these same repair mechanisms were unable to restore function fully. Together, these data indicate that associated injuries drastically alter repair processes that occur in response to nervous system damage.

**Brain-dependent mechanisms of acute pain-induced impaired outcomes**

Recent work has demonstrated that spared fibers play a role in mediating the detrimental effect of nociceptive stimulation after spinal cord injury. Using a rostral transection model in combination with the lower thoracic contusion model, animals that received both transection and contusion injuries were protected from the hemorrhagic and inflammatory properties of acute pain (Reynolds, 2016). This demonstrates that spared fibers are involved in these effects. However, which descending brain pathways are responsible for hemorrhage and inflammation in response to nociceptive stimulation is unknown.

**Stress**

One brain-dependent pathway that has been shown to be involved in recovery following a diverse set of injury models is stress. Across models of multiple sclerosis, cancer, and infection, stress has been shown to impact disease outcomes (Ben-Eliyahu, Yirmiya, Liebeskind, Taylor, & Gale, 1991; Johnson et al., 2006; Marsland, Bachen, Cohen, Rabin, & Manuck, 2002). Interactions between the nervous and immune systems are extensive and reciprocal. Stressors, both chronic and acute, engage neuronal circuits that induce synthesis and release of hormones including cortisol, prolactin, and growth
hormone. Each of these hormones engages a number of downstream signaling pathways that exert dramatic effects on inflammation (Glaser & Kiecolt-Glaser, 2005).

However, prior work from our laboratory suggests that stress does not underlie the effects of acute pain on recovery. First, the inability of morphine treatment to reverse the impact of nociceptive stimulation provides weak evidence against this hypothesis. Other experiments provide stronger evidence. If nociceptive stimulation is administered below the site of the lesion (to the hind limb), locomotor recovery is undermined. If, on the other hand, nociceptive stimulation is administered above the lesion (to the forelimb), locomotor recovery is not impaired. In fact, subjects that received stimulation to the forelimb showed improved locomotor recovery compared with subjects that received no stimulation (unpublished). This provides stronger evidence that stress is not the critical pathway engaged by nociceptive input. Future work will examine the extent to which cortisol signaling is engaged by our treatments, a stronger test of whether stress underlies the nociceptive stimulation-induced impaired recovery.

*Blood pressure*

As discussed above, Experiment 4A convincingly showed that brain-dependent systems play a role in the acute pain-induced hemorrhage and impaired recovery. One potential explanation for how acute pain impairs recovery is by increasing blood pressure. Recent studies in animal models of spinal cord injury and analyses of patient charts after traumatic brain injury have shown that hypertension soon after injury impairs functional outcomes (Nielson et al., 2015; Sellmann et al., 2012).
Using a big-data analysis approach that examined health records from multiple laboratories, blood pressure during injury was identified as a strong predictor of eventual recovery. Increases in mean arterial blood pressure above 140 mmHg around the time of injury correlated with impaired recovery. The effect size for hypertension soon after injury was even greater than that shown by many experimental therapeutics, indicating the great potential for harm that hypertension may represent (Nielson et al., 2015).

Work in polytrauma models of traumatic brain injury has shown similar effects. Mortality is significantly increased in human patients with systolic blood pressures greater than 160 mmHg prior to admission (Sellmann et al., 2012). In conjunction with data from the spinal cord injury literature, hypertension soon after nervous system injury represents a clinically relevant therapeutic target. What remains in question is the role that hypertension plays in mediating the detrimental effect of acute pain after spinal cord injury.

Though blood pressure would be expected to increase in the context of acute pain, treatment with morphine at doses that are sufficient to reverse behavioral reactivity to pain failed to reverse both hemorrhagic and behavioral effects. This dose of morphine would be expected to blunt, if not reverse, the increase in blood pressure seen in response to acute pain (Mahinda et al., 2004). However, if anything, systemic treatment with morphine increased hemorrhage and inflammation in response to nociceptive stimulation. Further, as mentioned above, nociceptive stimulation provided above the level of the injury failed to reproduce the detrimental effects.
Future work will examine changes in blood pressure in response to acute pain. I hypothesize that nociceptive stimulation will increase blood pressure. Further, additional studies will examine the impact of morphine and lidocaine treatments on these blood pressure changes. I expect that either treatment with morphine or lidocaine will reverse the changes in blood pressure. If correct, this will suggest that while blood pressure is an important mediator of damage around the time of spinal cord injury, hypertension is likely not the primary cause of increased hemorrhage and impaired functional recovery.

If, however, morphine does not reverse the hypertension associated with acute pain, then blood pressure may be a primary cause. Thus, future work may examine whether anti-hypertensive or anxiolytic medications will reverse both blood pressure changes and hemorrhage. This could represent an important therapeutic target as human patients likely experience increased stress and potentially increased blood pressure after spinal cord injury.

**Systemic inflammation**

Another mechanism that may play an important role in mediating the impact of nociceptive stimulation is systemic inflammation. This dissertation and earlier work from the laboratory have shown massive increases in inflammation following SCI, and that this inflammation was amplified by nociceptive stimulation. Evidence for local inflammation is strong, including increased processing and release of cytokines, increased apoptosis, and increased pyroptosis. However, evidence of systemic inflammation is somewhat more limited.
Early work in the laboratory has shown that spleen weights are significantly reduced soon after nociceptive input (Washburn, 2007). This decrease in spleen weight may be associated with release of leukocytes in response to systemic inflammation. Future work will examine the extent of systemic inflammation in response to nociceptive input.

**Clinical implications**

*Need to evaluate importance of polytrauma in human patients*

The results presented in this dissertation, coupled with work from other labs, suggest that polytrauma may impair patient outcomes, especially in situations with damage to nervous tissue. Because the cause of most spinal cord injuries is traumatic, the prevalence of associated injuries is assumed to be very high. However, to my knowledge, no published studies have examined the prevalence and severity of associated injuries in patients with spinal cord injury. A retrospective review of patient charts would be relatively simple to perform and would generate specific data identifying the prevalence of polytrauma in patients after spinal cord injury.

In addition to examinations of the prevalence of polytrauma in patients with spinal cord injury, additional studies must be performed to examine whether recovery in human patients with spinal cord injury is affected by associated tissue injuries. Again, retrospective analysis of patient charts at admission to determine the severity of associated tissue damage could be correlated with functional outcomes. Alternatively, to generate more predictive data, a prospective study could be performed to determine the predictive value of associated tissue damage in eventual recovery outcomes. I
hypothesize that the extent and severity of polytrauma would serve as a predictor of eventual outcome.

A potential pitfall that may arise in the attempt to perform this work is the relative paucity of appropriate controls. It is expected that very few patients with spinal cord injury will present with no associated tissue damage. In fact, nearly every patient presenting to emergency departments after injury will receive a battery of mildly noxious procedures. Data from Experiment 1A showed that minimal C fiber activation was sufficient to undermine recovery. If it is the case that minimal C fiber activation undermines recovery in human patients, a floor effect may emerge, where all patients receive sufficient stimulation to fully undermine recovery. Thus, patients with severe polytrauma may be no worse than patients with limited polytrauma. Nonetheless, a retrospective study predicting functional outcomes with extent and severity of polytrauma would be relatively simple and inexpensive to conduct and would serve as an adequate first test of our working hypothesis.

*Failure of morphine for the treatment of acute pain*

Because of the detrimental effects of nociceptive input on recovery, our laboratory has sought to develop an effective treatment for acute pain. In an early set of experiments, systemic morphine was given in attempt to reverse the effects of C fiber input. After spinal cord injury, high doses of morphine were required to reverse behavioral reactivity to electrical stimulation. Even when behavioral reactivity to electrical stimulation was reversed, morphine did not prevent the shock-induced attenuation of locomotor recovery. In fact, rats that received morphine and electrical
stimulation showed higher mortality rates and impaired functional outcomes compared to rats that did not receive morphine. In rats that did not receive electrical stimulation, morphine alone was sufficient to undermine locomotor recovery and increase lesion volume (Hook et al., 2007).

Many studies have examined the mechanisms of morphine-induced impaired recovery after spinal cord injury. Work has linked these effects to the κ-opioid receptor (κOR) and the non-classical opioid receptor TLR4 (Aceves, Mathai, & Hook, 2016; Hook et al., 2009; Hutchinson et al., 2008). Despite the fact that inhibition of TLR4 or κOR in conjunction with morphine treatment may reverse the morphine-induced impaired recovery and successfully prevent conscious perception of pain, morphine remains a non-ideal treatment for pain following injury.

Morphine has been shown to create dependence and paradoxically induce pain (Angst et al., 2003; Lee et al., 2011). These effects have been linked to numerous pathways, and more work is needed to optimize morphine treatment into an effective and safe analgesic after spinal cord injury. For these reasons, morphine has moved from a first-line treatment for pain after spinal cord injury to a second-line treatment.

Treatment of acute pain after spinal cord injury

For patients presenting to emergency departments after trauma, the current standard of care for treatment of acute pain is poorly defined (Alpen & Morse, 2001). Because of their ease of use and commonality, opiate medications are a mainstay for pain treatment in these emergency settings. However, Experiment 4B reinforced prior work demonstrating that while opiate medications are effective at reversing behavioral
responses and conscious perception of pain, these medications do not reverse the detrimental effects of acute pain on recovery and, in fact, may be detrimental to recovery themselves.

In this dissertation, a new therapeutic strategy for the treatment of acute pain after spinal cord injury was examined. Spinal block with lidocaine effectively blocked all behavioral responses to acute pain. Subjects showed no vocalization or movement in response to stimulation. Further, all cellular and molecular indicators of acute pain were effectively prevented with the spinal block. Signs of inflammation, apoptosis, pyroptosis, and progressive hemorrhagic necrosis were effectively reduced to levels seen in subjects that did not receive acute pain. Further, in behavioral studies, treatment with lidocaine prevented stimulation-induced impaired locomotor recovery and also prevented impaired weight gain. Additional tests showed that subjects that received spinal block with lidocaine showed locomotor behavior, peripheral tactile pain responses, and thermal pain responses that were indistinguishable from subjects that did not receive acute pain. One potential side effect of this treatment was an increase in responsiveness to at-level mechanical stimulation. However, the magnitude of increase seen in this test was relatively small compared with effect sizes seen in other experiments and may reflect preserved sensory function. Replication studies will help build understanding about epidural lidocaine. Thus, due to relatively mild side effects and robust reversal of the acute pain-induced impaired recovery, spinal block with lidocaine should be considered in treatment of acute pain following traumatic spinal cord injury.
Treatment with lidocaine for acute pain after spinal cord injury is a plausible option because this technique is available for use in nearly every hospital in the United States. Over 60% of laboring patients in the United States receive some form of epidural analgesia (Michelle, Osterman, & Martin, 2011). Further, local anesthesia with either spinal or nerve blocks has been successfully used for anesthesia in lower limb surgical procedures and post-operative pain after abdominal surgeries (Burke et al., 1999; McDonnell et al., 2007).

If future work examining polytrauma in human patients demonstrates that polytrauma undermines recovery, spinal block with lidocaine should be considered as a potential analgesic strategy. Whereas opiates and other analgesic medications have side effects including dependence, addiction, and paradoxical pain (Angst et al., 2003; Lee et al., 2011), spinal and nerve blocks have very low incidences and mild severity of side effects (Auroy et al., 1997; Carpenter et al., 1992). Treatment with nerve blocks in the emergent control of acute pain after spinal cord trauma may be an effective way to maintain high patient satisfaction of pain control, minimize side effects, and improve outcomes.
REFERENCES


Brumley, M. A. (2016, May). *Nociceptive stimulation increases hemorrhage after spinal cord injury* (Undergraduate Thesis). Texas A&M University, College Station, TX.


Kahle, K. T., Gerzanich, V., & Simard, J. M. (2010). Molecular mechanisms of microvascular failure in CNS injury – Synergistic roles of NKCC1 and
http://doi.org/10.1016/j.expneurol.2014.01.001

http://doi.org/10.1056/NEJM200010193431601


http://doi.org/10.1038/jcbfm.2008.120


Reynolds, J. A. (2016, May). *Spared fibers mediate the detrimental effects of nociceptive input after spinal cord injury* (Undergraduate Thesis). Texas A&M University, College Station, TX.


Van Der Wal, S., Vaneker, M., Steegers, M., Van Berkum, B., Kox, M., Van Der Laak, J., … Scheffer, G. J. (2015). Lidocaine increases the anti-inflammatory cytokine


