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Defining the Role of Blood-Spinal Cord Barrier Breakdown and Spinal Thrombin Signaling in the Development of Pain Following Neural Trauma

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Defining the Role of Blood-Spinal Cord Barrier Breakdown and Spinal Thrombin Signaling in the Development of Pain Following Neural Trauma

Abstract
Peripheral neural trauma is known to induce blood-spinal cord barrier (BSCB) breakdown, but if and how BSCB breakdown contributes to pain is unknown. The studies in this thesis use rat models of cervical nerve root insults (compressive and inflammatory) to investigate which components of injury contribute to BSCB disruption and if BSCB disruption controls the pain responses. Further, whether serum-derived thrombin extravasates into the spinal parenchyma during BSCB breakdown and contributes to the associated spinal astrocyte activation and pain through its activation of PAR1 is investigated. BSCB breakdown occurs transiently early after root compression exclusively when pain develops; blocking BSCB breakdown with activated protein C prevents pain. During increased BSCB permeability, thrombin acts enzymatically in the spinal cord. By inhibiting spinal thrombin with intrathecal hirudin before a painful compression, and separately by administering exogenous rat thrombin intrathecally to naïve rats, spinal thrombin is found to contribute to BSCB breakdown, spinal astrocyte activation and pain. Additionally, spinal thrombin initiates spinal neuroinflammation and pain through its activation of PAR1, which is determined using pharmacological inhibition of spinal PAR1 with SCH79797. In contrast to mammalian thrombin, studies using the same painful in vivo model and complementary endothelial and astrocytic in vitro experiments also demonstrate that salmon thrombin exerts opposite effects on vascular permeability, astrocytic inflammation, and pain. Using synthetic fluorogenic peptides and in silico protein models, the vascular protection and anti-inflammatory effects of salmon thrombin are hypothesized to be controlled by its unique affinity for protein C over PAR1. Lastly, spinal astrocytic and endothelial expression of the intermediate filament, vimentin, are shown to undergo a delayed increase at day 7 after a painful compression, suggesting that spinal cells may modulate their cellular mechanics in response to a painful neural injury. These studies identify how one pathological response, BSCB breakdown, that occurs in the spinal cord early after painful injury initiates a host of immediate and delayed biochemical and biomechanical responses that contribute to pain development. Findings further establish the powerful, yet diverse, role of spinal thrombin and its ability to initiate pathological or protective effects based on the signaling cascades it activates.

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DEFINING THE ROLE OF BLOOD-SPINAL CORD BARRIER BREAKDOWN AND
SPINAL THROMBIN SIGNALING IN THE DEVELOPMENT OF PAIN
FOLLOWING NEURAL TRAUMA

Jenell R. Smith
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Chapter 1

Introduction & Background

1.1 Introduction & Motivation

In a recent report by the Institute of Medicine of the National Academies, the annual incidence of chronic pain in America is over 100 million adults (Institute of Medicine (US) Committee on Advancing Pain Research 2011). Pain, defined by the International Association for the Study of Pain (IASP), is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage” (Radu et al. 2013). The ability to detect a harmful, or potentially harmful, stimulus is essential for survival; however, when pain persists beyond the potential threat of tissue damage, it becomes problematic. Noxious stimuli, or those that have the potential to cause tissue damage, are encoded through the neural process of nociception (Merksey and Bogduk 1994). Clinically, “acute” pain is pain that resolves within three months; pain is classified as “chronic” when it lasts for longer than three months (Fishbain et al. 2014, Melloh et al. 2011, Wall and Melzack 1994). Depending on the severity of the symptoms, pain can be a debilitating disorder substantially impacting quality of life (Breivik et al. 2006, Merksey and Bogduk 1994, Ramirez-Maestre and Esteve 2013). Each year in the United States, chronic pain accounts for expenditures exceeding $600 billion, which includes the costs associated with both health care and loss of productivity (Institute of
Medicine (US) Committee on Advancing Pain Research 2011). Over 20% of chronic pain cases are reported to have neuropathic characteristics (Bouhassira et al. 2008). According to the IASP, *neuropathic pain* “arises as a direct consequence of a lesion or diseases affecting the somatosensory system” (Jensen et al. 2011).

Nearly 10% of the general population and 20% of the adult population are burdened by chronic pain with neuropathic symptoms (Torrance et al. 2006, van Hecke et al. 2014). Individuals experiencing pain with neuropathic characteristics report that their pain is generally more severe than that of non-neuropathic origin and that their health-related quality of life can be as low as that reported with depression, coronary heart disease or a recent myocardial infarction (Torrance et al. 2014). Neuropathic pain is not itself a disease, but rather it is a complex *syndrome* that can arise from a wide range of disorders in the central or peripheral nervous system (Haanpää and Treede 2010, Jensen et al. 2011). Neuropathic pain arising in the central nervous system (CNS) can result from stroke, spinal cord injury, multiple sclerosis, and other neurological disorders. Painful peripheral neuropathy is most commonly due to lumbar or cervical radiculopathy, postherpetic neuralgia, diabetic neuropathy, human immunodeficiency virus (HIV) related neuropathy, and chronic postsurgical pain (Haanpää and Treede 2010, Jensen et al. 2011).

Radiculopathy is a pathology of the spinal nerve roots with clinical symptoms of pain and numbness that radiate into the extremities (Abbed and Coumans 2007, Caridi et al. 2011). The specific location of the presenting symptoms after root injury depends on which nerve root is affected. The areas of skin that are innervated by a single nerve root exhibit *dermatomes* mapping to each nerve root’s innervation. Injuries to the C7 nerve
root, for example, produce sensitivity and numbness in the arm, forearm, and middle finger (Abbed and Coumans 2007). Injury to the nerve roots can be produced by non-physiological mechanical loading or exposure to chemical and/or inflammatory stimuli (Abbed and Coumans 2007, Garfin et al. 1991, Krivickas and Wilbourn 2000, Olmarker and Myers 1998, Panjabi et al. 2006). The nerve roots are susceptible to mechanical loading from disc herniation or spinal spondylosis, both of which typically apply sustained loads to the roots. In addition to nerve root impingement from disc herniation, there is also often an inflammatory component applied to the nerve root; the nucleus pulposus is secreted from the disc and introduces pro-inflammatory stimuli to the nerve root (Hou et al. 2003, Olmarker et al. 1993). Transient loading the nerve roots via trauma from sports- or automotive-related injury also has the potential to induce long-term radicular pain depending on the profile of the mechanical loading to the root, which depends on the overall kinematics of the spine (Krivickas and Wilbourn 2000, Panjabi et al. 2006, Tominaga et al. 2006).

Quantifiable measures have been developed to assess evoked pain and which provide clinicians and researchers with objective measures of pain (DeLeo and Winkelstein 2002, Loeser and Treede 2008, Merksey and Bogduk 1994). Two types of hypersensitivity are distinguished, allodynia and hyperalgesia. Allodynia is defined as pain in response to a stimulus that is usually not painful, whereas hyperalgesia is the increased response to a stimulus which is normally painful, and includes all conditions of increased pain sensitivity (Baron et al. 2010, Merksey and Bogduk 1994, Winkelstein 2011, Woolf and Mannion 1999). At least 20% of patients with general neuropathic pain have mechanical allodynia and nearly 30% have mechanical hyperalgesia (Baron et al.
2010, Maier et al. 2010). Those behavioral symptoms of sensitivity are perceived on the skin local to the source of pain (primary), as well as in nearby unaffected areas of skin (secondary) (Baron et al. 2010, Maier et al. 2010).

Despite the high prevalence and debilitating nature of neuropathic pain, treatments that effectively eliminate symptoms are still lacking (Baron et al. 2010, O'Connor and Dworkin 2009, Vranken 2009). Less than 50% of patients in neuropathic pain trials achieve satisfactory pain relief with the currently available treatments (O'Connor and Dworkin 2009). The standard set of pharmacologic treatments most commonly recommended for neuropathic pain include tricyclic antidepressants, anticonvulsants, and the 5% lidocaine patch (Dworkin et al. 2003, Finnerup et al. 2010, O'Connor and Dworkin 2009). Each of those treatments only target the neuronal contributions to nociception (Argoff 2000, Gilron 2007, O'Connor and Dworkin 2009, Sindrup et al. 2005). However, it has become widely recognized and accepted that many other cell types within the CNS contribute to the development and maintenance of chronic neuropathic pain (Baron et al. 2010, Milligan and Watkins 2009, Scholz and Woolf 2007, Vallejo et al. 2010, Watkins et al. 2001, Woolf and Mannion 1999). Identifying the contributions of non-neuronal cell types and their pathological processes that contribute to pain can provide important and relevant insight for the design and development of effective treatments for neuropathic pain.

Rat models of painful radiculopathy have defined a host of neuronal and non-neuronal mechanisms that contribute to the development of neuropathic pain (Garfin et al. 1991, Hubbard and Winkelstein 2005, Kawakami et al. 1994). Injury to the nerve root can produce secondary behavioral sensitivity that can be quantified in the forepaw where
the injured afferents innervate (Olmarker and Myers 1998, Olmarker et al. 1993, Rothman and Winkelstein 2007, Winkelstein and DeLeo 2002). Our lab has developed a transient compressive nerve root injury that can produce customizable pain profiles and cellular responses at the location of injury and in the spinal cord where the injured afferents synapse depending on the loading profile, i.e. magnitude and duration of the compression. For example, a root compression that is greater than 38.16mN held for 15 minutes induces mechanical allodynia for up to 7 days (Hubbard et al. 2008a); but a compression greater than that magnitude threshold (98mN) held for only 3 minutes does not induce allodynia (Nicholson et al. 2011, Rothman et al. 2010). In fact, mechanical allodynia lasts for up to 4 weeks after nerve root compression (Rothman et al. 2007). Inflammatory root insults, applied separate or in combination with a mechanical insult, have also been shown to produce mechanical allodynia and hyperalgesia for up to 14 days (Chang and Winkelstein 2011, Rothman et al. 2009, Rothman and Winkelstein 2007, Zhang et al. 2013). These customizable injury scenarios allow for the investigation of, and differentiation between, cellular responses that are specific to pain and those that are produced simply as a result of trauma. Peripheral mechanically-induce neuropathic injuries have been shown to disrupt the blood-spinal cord barrier (BSCB) (Beggs et al. 2010, Cahill et al. 2014, Echeverry et al. 2011, Gordh et al. 2006). However, the components of those injuries that contribute to BSCB breakdown and whether that breakdown contributes to the associated pain is not known.

The studies in this thesis investigate if, and how, BSCB breakdown contributes to the development of pain after a nerve root injury. BSCB breakdown is measured over time after a variety of painful and non-painful insults to the cervical nerve root that are
both mechanical and chemical. In a separate study, BSCB breakdown is pharmacologically blocked with activated protein C (APC) after a painful nerve root compression to define if BSCB breakdown contributes to the onset of pain. Studies then identify whether the serum-derived enzyme, thrombin, enters the spinal parenchyma as a result of BSCB breakdown. The contribution of spinal thrombin and its downstream signaling cascades to the development of pain are then investigated by: (1) blocking spinal thrombin activity prior to a painful nerve root compression, (2) administering rat thrombin to the spinal cord of non-injured naïve rats, and (3) antagonizing the thrombin receptor protease-activated receptor-1 (PAR1) in conjunction with painful root compression or rat thrombin administration. Hirudin is administered intrathecally before a painful compression in order to selectively inhibit thrombin activity in the spinal cord (Narita et al. 2005, Rydel et al. 1990). Lastly, the small molecule, SCH79797, which is a PAR1 antagonist, was given intrathecally before a compression or painful stimulus (Manaenko et al. 2013, Yan et al. 2013), in order to test whether spinal thrombin acts through PAR1 to induce BSCB breakdown, spinal astrocyte activation and pain. Mechanical hyperalgesia, BSCB breakdown and spinal astrocytic activation are quantified in order to identify the role that thrombin and its activation of PAR1 have in pain and spinal endothelial and astrocytic pathology.

In a separate study, the effects of salmon thrombin on mediating pain, vascular permeability, and astrocyte-induced inflammation was measured and compared to human thrombin utilizing complementary in vivo and in vitro studies. The activity of salmon and human thrombin towards different thrombin-activated substrates is also investigated because thrombin mediates cell signaling cascades based on the cell-bound substrate it
activates (Bouwens et al. 2013, Di Cera 2008, Griffin 1995, Komarova et al. 2007, Lane et al. 2005). Lastly, studies establish whether activated spinal astrocytes modulate their cellular mechanics via changes in their expression of vimentin in response to a painful nerve root compression alone or with analgesic treatments of hirudin or salmon thrombin.

The four specific aims of this thesis, the rationale supporting them, and their governing hypotheses are summarized in Chapter 2, which also includes the outline and organization of the other chapters in this thesis. More detailed background information for each study is separately provided within each chapter. The background information in this chapter is intended to provide relevant information about neural anatomy, the cellular populations within the relevant neural tissue and various cellular functions and dysfunctions in response to neuropathic injury that induces pain. In addition, a brief overview of the mechanistic differences between mammalian thrombin and salmon thrombin is introduced.

1.2 Relevant Background

1.2.1. The Cervical Spine & Neural Anatomy

The cervical spine is comprised of seven bony vertebrae (C1-C7) that are connected and stabilized by spinal ligaments and muscles (Figure 1.1) (Antonacci et al. 1998, Crosby et al. 2015). Each pair of bony vertebrae in the cervical spine is separated by the intervertebral disc anteriorly and a pair of bilateral facet joints posteriorly that restricts the motion of the spinal segments (Figure 1.1). The space running longitudinally
through each cervical vertebrae is the spinal canal, which surrounds and protects the spinal cord (Figure 1.1).

![Figure 1.1. Anatomy of the bony structures and soft tissues of a cervical spinal joint. An axial view of the cervical spine in between spinal levels. Adjacent spinal laminae are connected via the articulating intervertebral disc and the bilateral facet joints, which limit motion and transmit loads. The spinal cord is housed within the spinal canal and protected by meninges. The spinal nerve roots exit the cord at the spinal dorsal horn and join distally to the dorsal root ganglion (DRG), which houses the cell bodies of the afferent neurons. The nerve roots exit the spinal canal through the intervertebral foramen and continue to form the peripheral spinal nerves. This figure is adapted from Crosby et al. 2015.](image)

Extending from the spinal cord bilaterally at each spinal level are the dorsal and ventral nerve roots, which exit the spinal column through the intervertebral foramen. The dorsal root carries the afferent axons and the ventral root houses the efferent axons. The efferent axons within the ventral nerve root relay information from the ventral spinal cord, where the efferent somata are housed, to the periphery. In contrast, the afferent axons within the dorsal nerve root originate in the periphery and relay sensory
information in to the spinal cord and CNS (Almeida et al. 2004). The cells bodies of the afferent neurons are clustered together within the bilateral dorsal root ganglia (DRG) at each spinal level (Figure 1.1). Afferent axons synapse in the dorsal horn of the spinal cord with second order neurons that project to the brainstem. In addition to axons, the nerve roots contain blood vessels, which are necessary for supplying adequate amounts of oxygen to the nerve fibers (Rydevik et al. 1984).

Each set of dorsal and ventral nerve roots extend from the spinal cord towards the periphery where they couple together to form the peripheral nerve outside of the spinal column (Figure 1.1) (Martini et al. 2003). Despite there being only seven vertebrae, there are eight levels of the cervical spinal cord and the nerve root that exits the spinal column between the C7 and T1 vertebrae is, by convention, termed the C8 nerve root (Martini et al. 2003). In this thesis, studies focus on insults to the C7 nerve root because clinical studies have found that the C7 nerve root is the most commonly affected cervical root in patients diagnosed with cervical radiculopathy, with a reported incidence rate of around 50% (Carette and Fehlings 2005, Wainer and Gill 2000).

The spinal cord is enclosed by the meninges (Figure 1.1), which are a system of three collagenous membranes (pia mater, arachnoid mater, and dura mater) that separate and protect the spinal cord within the surrounding vertebral column. The dura mater, in particular, resists mechanical tension and provides support to the neural structures that it surrounds (Maikos et al. 2008). The meninges also encase the cerebrospinal fluid (CSF) which serves as a mechanical buffer for the nervous tissue within the CNS. In addition to providing mechanical protection, the meninges of the spinal cord form the first line of defense of the BSCB and protect the CNS from fluctuations in ions, hormones, and other...
signaling molecules in the periphery (Abbott et al. 2010, Ballabh et al. 2004). The dorsal and ventral spinal nerve roots are also encased within CSF, the dura mater, and the arachnoid membrane as they exit the intervertebral foramen and join together to form the peripheral nerves outside of the spinal column (Figure 1.1). This is true in both in the human and in the rat.

In contrast to many other tissues in the body, the brain and spinal cord lack a highly organized extracellular matrix (Gaudet and Popovich 2014, Hynes and Naba 2012, Zimmermann and Dours-Zimmermann 2008). The extracellular space of the CNS encompasses roughly 15-30% of the total CNS volume and is primarily comprised of proteoglycans, hyaluronan and tenascins (Barros et al. 2011, Gaudet and Popovich 2014, Wong et al. 2013, Zimmermann and Dours-Zimmermann 2008). The other 70-85% of the CNS is made up of neurons, glial cells, and endothelial cells. Although arguably the most important cells within the CNS, neurons are outnumbered by astrocytes. Astrocytes comprise the main mechanical support system for neurons and also regulate normal neural functions; astrocytes transport oxygen and nutrients from microvessels to neurons, regulate synaptic transmission, and signal to endothelial cells to maintain the blood-brain barrier (BBB) structure (Abbott et al. 2006, Volterra and Meldolesi 2005, Wong et al. 2013). Microvascular endothelial cells also make up a significant proportion of the cells in the CNS. Each neuronal cell body is typically within 8-20µm of the closest capillary and every neuron in the brain is estimated to have its own capillary (Abbott et al. 2006, Schlageter et al. 1999, Wong et al. 2013, Zlokovic 2008). Other cells within the CNS include microglia, which act as the resident immune cells responding to injury and foreign pathogens (Kreutzberg 1996, Stollg and Jander 1999), and pericytes, which
surround the microvessels helping to regulate vascular tone and to maintain tight junctions (Balabanov and Dore-Duffy 1998, Krueger and Bechmann 2010).

The microvessels in the spinal cord are unique from peripheral blood vessels because they form the BSCB. Endothelial cells that comprise the BSCB face the luminal side of the blood vessels and are bound tightly together by tight junctions (Abbott et al. 2010). Tight junctions are the key feature of the BSCB since they prevent paracellular diffusion of ions and polar solutes between the endothelial cell fenestrations (Abbott et al. 2010). Tight junctions are comprised of adherin junctions, occludins, claudins and junctional adhesion molecules that span the intracellular cleft. Pericytes completely encase the microvascular endothelial cells followed by astrocytic end-feet, both of which contribute to BSCB function and act as a second line of defense to the endothelial structure of the barrier (Balabanov and Dore-Duffy 1998, Wolburg et al. 2009).

1.2.2. Mechanisms of Neuropathic Pain

A number of peripheral and central pathological cellular responses occur after neuropathic injury that contribute to the establishment and maintenance of pain. Altered sensitivity that accompanies neuropathic pain is ultimately controlled by a change in the normal signaling of neurons (Crosby et al. 2015, Vranken 2009). In response to a stimulus, whether mechanical, chemical, or thermal, that is above the nociceptive threshold, the primary afferent neurons transmit nociceptive signals to the spinal dorsal horn (Figure 1.2). Primary afferent neurons can become sensitized and can exhibit a lowered threshold for evoking firing in response to stimuli or an increased rate of firing once activated (Vranken 2009). Sensitization of the primary afferents is induced local to
the injury site by the release of chemicals from the injured axons and vasculature and by a local inflammatory response. Afferent injury produces localized increases in neuropeptides and neurothrophins, such as nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), calcitonin gene-related peptide (CGRP), substance P, bradykinin, as well as nitrous oxide (NO), adenosine triphosphate (ATP) and other molecules that themselves also stimulate the primary afferents (Scholz and Woolf 2007). Macrophages respond almost immediately to peripheral neuropathic injury by migrating to the location of neural trauma and initiating a local inflammatory cascade (Scholz and Woolf 2007). Those macrophages produce cytokines and chemokines that also directly stimulate the afferents and attract additional immune cells to the location of injury (Scholz and Woolf 2007). This peripheral neuroinflammation continuously stimulates afferents which thereby increases their release of excitatory neurotransmitters in the spinal cord where they synapse (Figure 1.2).

Injured afferents synapse in the dorsal horn of the spinal cord and trigger a host of cellular and molecular responses centrally that contribute to central sensitization in response to trauma (Figure 1.2). Central sensitization is a major contributor to dysfunctional pain states and results from changes in neuronal properties such that neurons respond abnormally to non-noxious stimuli or respond with an increased gain to typically noxious stimuli (Latremoliere and Woolf 2009). Although neurons ultimately control aberrant nociceptive signaling in the CNS (Latremoliere and Woolf 2009), the other resident cells within the CNS also have roles in enhancing nociception and contribute to the continued neuronal dysfunction that leads to central sensitization (Inoue 2006, Vallejo et al. 2010, Vranken 2009). As such, the classical view of synaptic...
transmission between two neurons has been adapted to include astrocytes; that “tripartite synapse” consists of the pre-synaptic neuron, the post-synaptic neuron, and the astrocytic encasement of the synapse and controls synaptic transmission (Halassa et al. 2007). With increased information about the physical and trophic interaction between neurons, glia and vasculature in the CNS (Abbott et al. 2006, Hawkins and Davis 2005, Wong et al. 2013), synaptic transmission has also been further expanded to include the microvascular endothelial cells since they contribute to maintaining neuronal function and dysfunction. The functional coupling between synaptic neurons, astrocytes, and endothelial cells makes up the \textit{neurovascular unit} (Figure 1.3) (Hawkins and Davis 2005, Radu et al. 2013, Zlokovic 2008, Zlokovic 2010).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{neurovascular_unit.png}
\caption{Peripheral and central responses initiated after neuropathic injury. Injury to primary afferents induces many cascades in peripheral neural tissue including local nociceptor release of excessive neurotransmitters and neuropeptides and initiation of a complex inflammatory response. Afferents transport excitatory molecules to the spinal dorsal horn, inducing spinal glial activation, immune cell transmigration into the spinal parenchyma, and pro-inflammatory cytokine and chemokine release.}
\end{figure}
1.2.3. The Neurovascular Unit

Microvascular endothelial cells comprise the primary cellular structure of the BBB. The presence of tight junctions distinguishes endothelial cells of the BBB or BSCB from those comprising the blood vessels in the periphery. With the help of pericytes, endothelial cells regulate, and in healthy conditions prevent, the transport of ions and other blood-borne proteins into the CNS parenchyma. Astrocytes act as a physical and a trophic bridge between endothelial cells and nearby neurons. Physically, astrocytic end-feet appose blood vessels and the body of the astrocyte interfaces with the nearby neuronal synapses (Figure 1.3) (Abbott et al. 2006).

**Figure 1.3. The neurovascular unit in healthy and pathological states.** In healthy states, the microvascular endothelial cells are tightly bound together by tight junctions permitting the entrance of serum-derived proteins into the CNS. Astrocytic end-feet appose endothelial cells and provide trophic support maintaining the BBB integrity. Astrocytes also provide essential nutrients to nearby neurons and regulate synaptic concentrations of neurotransmitters. During pathological states of BBB or BSCB breakdown, serum cytokines and proteins extravasate into the CNS along with peripheral immune cells. Astrocytes become activated, release inflammatory molecules, and dysregulate synaptic concentrations.
Under healthy conditions, astrocytes transport essential nutrients from the blood vessels to the neurons, clear neurotransmitters from the synapses, and maintain the BBB/BSCB integrity by secreting molecules, such as transforming growth factor (TGF-β), GDNF, basic fibroblast growth factor (bFGF), and angiopoietin 1, back to the endothelial cells (Abbott et al. 2006, Wong et al. 2013). The neurovascular unit responds to pathological conditions in the CNS. Neurons increase their release of excitatory neurotransmitters, astrocytes become activated, and endothelial junctions are disrupted (Figure 1.3) (Zlokovic 2008). A hallmark of astrocyte activation is the increased astrocytic expression of the intermediate filament, glial fibrillary acidic protein (GFAP) (Haydon and Carmignoto 2006, Pekny and Pekna 2004). Activated astrocytes release pro-inflammatory cytokines, alter their expression of neurotransmitter receptors, and downregulate their production and release of BBB-protecting molecules (Abbott et al. 2006, McMahon et al. 2005, Milligan and Watkins 2009, Watkins et al. 2001, Wolburg et al. 2009). Together, this reactive response exacerbates inflammation within the CNS, leads to even higher synaptic concentrations of excitatory molecules, and exacerbates BBB breakdown. BBB breakdown is caused by a decrease in functional transendothelial tight junctions, which permits the influx of serum molecules that are typically excluded from the CNS. Pro-inflammatory cytokines have been shown to extravasate from the serum into the parenchyma (Echevery et al. 2011, Pan and Kastin 2007, Sharief and Thompson 1992, Szelenyi 2001), but the extent of protein extravasation has not been fully explored. Additionally, whether those serum-derived molecules potentiate astrocytic activation and contribute to the development of pain is not known.
1.2.4. Compressive & Inflammatory Nerve Root Insults


A transient compression of the C7 nerve root that is applied for only 15 minutes induces mechanical behavioral sensitivity that lasts for at least 14 days after injury (Chang and Winkelstein 2011). In contrast, when a compressive load of the same magnitude is applied for only 30 seconds or 3 minutes, mechanical allodynia does not develop (Nicholson et al. 2012, Rothman et al. 2010, Zhang et al. 2013). Nerve root tissue responds differently both mechanically and biologically to a compressive insult depending on the duration of compression (Nicholson et al. 2011, Rothman et al. 2010). A compressive load applied to a nerve root relaxes with increasing time of compression due to the viscoelasticity of neural tissue. By 3 minutes into a compression of
approximately 110mN, the load applied to the root decreases to 84% of the maximum load, but by 15 minutes into the compression the load relaxes to 64% of the peak applied load (Rothman et al. 2010). The nerve root exhibits structural deformation immediately after the removal of the compression only when the compression is applied for 15 minutes, and not 3 minutes (Rothman et al. 2010). The compressed afferent neurons in the root also respond electrophysiologically with increasing time of compression. Evoked neuronal firing in the spinal cord where those compressed neurons synapse is maximally decreased at 6.6±3.0 minutes into a compression of 98mN (Nicholson et al. 2011).

Compressing nerve or nerve root tissue also has been shown to disrupt blood flow through the vasculature depending on the magnitude and time of compression (Garfin et al. 1995, Igarashi et al. 2005, Olmarker et al. 1989, Yoshizawa et al. 1989). In the rabbit, 30mmHg of compression applied to the nerve root is sufficient to block blood flow through the venules, but blood flow through the arterioles is not compromised until pressures reach 60-70mmHg (Garfin et al. 1995, Rydevik et al. 1981, Rydevik et al. 1984). Compressing the cauda equina in pigs with pressures above 40mmHg has been shown to disrupt blood flow in the capillaries of the root (Olmarker et al. 1989). In addition to blocking flow at the location of the compression, a lumbar nerve root compression also induces an immediate drop in blood flow to the spinal cord for the entire time of the 60-minute compression (Igarashi et al. 2005). That study shows that after removal of the 45gf compressive load in dogs, blood flow is restored to baseline values within 1 hour (Igarashi et al. 2005). However, in a rat model, compressing the L5 lumbar nerve root with 45gf for even 2 seconds disrupts blood flow in the DRG that lasts for up to 3 hours after the compression is released (Yoshizawa et al. 1989). Taken
together, these studies demonstrate that nerve root compression introduces ischemia in
the compressed tissue, as well as within the surrounding neural tissue, to an extent that
depends on the magnitude and duration of compression, as well as the species in which
the compression is being administered.

Immediate macroscopic deformation of the nerve root has been shown to be
associated with a longer-term disruption in axonal integrity, as well as the pain that
develops and is maintained. A 15-minute nerve root compression produces an immediate
reduction in the width of the root to 72.4±12.6% of the original geometry (Rothman et al.
2010). By day 7 after that 15-minute compression, expression of neurofilament-200
(NF200) in myelinated axons is decreased and unmyelinated axons exhibit swelling
(Hubbard and Winkelstein 2008, Nicholson et al. 2011). Axonal degeneration is
hypothesized to disrupt the transport of neuropeptides to the spinal dorsal horn. In fact,
the spinal expression of substance P and CGRP is decreased after a 15-minute
compression (Hubbard et al. 2008a, Hubbard et al. 2008b). Mechanical allodynia
develops by day 1 after the 15-minute compression that induces immediate structural
deformation in the root and sustained axonal dysfunction. In contrast, a compression
applied for only 3 minutes does not cause immediate macroscopic deformation of the root
tissue, nor does it induce axonal degeneration at day 7 after injury or produce mechanical
allodynia (Nicholson et al. 2011, Rothman et al. 2010). These studies support the severity
of compressive root injury being associated with the presence of nerve root degeneration
and development of pain.

The magnitude and duration of a nerve root compression also control the degree
of extended glial activation in the spinal cord. A painful 15-minute nerve root
compression induces robust microglial activation in the spinal cord at day 7 after injury; however, non-painful compressions, applied for either 30 seconds or 3 minutes, do not induce microglial activation. Spinal astrocytic activation exhibits a more graded response to compression duration; a 3-minute compression induces an increase in astrocytic activation over normal levels, but a 15-minute compression increases spinal astrocytic activation to levels that are higher than produced by that 3-minute compression. These studies support the further association between compression-induced pain and spinal glial activation following compression.

In addition to compressive insults, inflammatory insults to the spinal nerve roots also induce behavioral sensitivity (Chang and Winkelstein 2011, Cornefjord et al. 2004, Rothman et al. 2009, Rothman and Winkelstein 2007). Our lab has adapted an inflammatory model of nerve injury by applying chromic gut suture to the C7 nerve root (Chang and Winkelstein 2011, Rothman and Winkelstein 2007). Although both a 15-minute compression and an inflammatory insult induce behavioral sensitivity lasting for up to 14 days (Chang and Winkelstein 2011), they have been shown to induce a host of different spinal and nerve root pathologies. For example, within 1 hour, transcription of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6 is increased after a 15-minute painful compression, but only IL-6 transcription is elevated at this time after a painful chromic inflammatory insult (Rothman et al. 2011). Spinal astrocyte and microglia expression are also increased only after a compressive insult, but not an inflammatory insult (Rothman and Winkelstein 2007). Those studies suggest that a painful compression induces more robust immediate and sustained neuroinflammation in the spinal cord compared to the inflammatory insult. Additionally, at day 14, after an
inflammatory insult axonal myelination is not disrupted nor is there robust phagocytotic macrophage infiltration in the nerve root (Chang and Winkelstein 2011). Together, these studies demonstrate that the cellular mechanisms that contribute to nerve root-mediated pain induced by an inflammatory stimulus may be different than those that contribute to pain from a compressive insult.

1.2.5. Thrombin’s Induction of Cell Signaling Cascades

Thrombin is a serine protease that enzymatically activates a vast array of substrates within biological systems, influencing a wide range of biological processes. Thrombin cleaves fibrinogen into fibrin, the main polymeric structure of blood clots, thereby having a crucial role in coagulation (Boon 1993). In addition to activating fibrinogen and other substrates involved in the clotting cascade, thrombin also cleaves and activates, cell-bound receptors to initiate cell signaling cascades (Di Cera 2008, Komarova et al. 2007). Interestingly, in many of those signaling cascades thrombin is capable of activating opposing pathways, including platelet activation, endothelial permeability, and inflammation (Coughlin 2000, Griffin 1995, Komarova et al. 2007). Since thrombin can activate many substrates, and many of which themselves initiate distinct cellular responses, its structure is comprised of regions that interact with co-factors to guide thrombin to activate certain substrates; those regions are known as exosites (Di Cera 2008, Gandhi et al. 2011, Lane et al. 2005). Thrombin activates three separate G-protein coupled receptors (GPCRs) known as the protease-activated receptors (PARs): PAR1, PAR3, and PAR4 (Coughlin 2000, Jacques et al. 2000). Of those, thrombin has the highest affinity for PAR1 due to exosite I of thrombin interacting
closely with an amino acid sequence contained within an extracellular loop of PAR1 (Coughlin 2000). This sequence of amino acids is known as the *hirudin-like* domain of PAR1, due to its remarkable similarity to hirudin; hirudin is a protein derived from leeches and is often used to inhibit thrombin activity because of thrombin’s strong affinity towards it (Jacques et al. 2000, Rydel et al. 1990).

In vascular systems, freely-circulating thrombin primarily activates PAR1 expressed on the endothelial surface, which induces endothelial barrier disruption, cell contraction, and production of pro-inflammatory factors (Figure 1.4) (Bernard et al. 2001, Bouwens et al. 2013, Di Cera 2008, Esmon 1993). However, thrombin can also bind to thrombomodulin, which enables it to activate protein C, bound to the endothelial protein C receptor (EPCR). Thrombin’s activation of endothelial-bound protein C leads to endothelial barrier stability, cytoprotection, and anti-inflammatory events (Bouwens et al. 2013, Esmon 1993). Under normal physiological conditions, thrombin circulates at relatively low concentrations, activating PAR1 and protein C to maintain vasculature stability (Figure 1.4) (Bouwens et al. 2013, Riewald and Ruf 2005). However, under traumatic conditions, thrombin concentration is elevated and thrombin primarily activates PAR1 to disrupt vasculature (Figure 1.4) (Bouwens et al. 2013, Mosnier et al. 2012). Interestingly, APC is an enzyme and produces its vascular protective effects *also* by activating PAR1 (Bouwens et al. 2013, Esmon 1993, Mosnier et al. 2012). However, APC cleaves the extracellular domain of PAR1 at a different residue than thrombin activates (Mosnier et al. 2012). The ability of PAR1 to induce opposite signaling cascades when activated by different agonists is common among many GPCRs and is known as *biased agonism* (Mosnier et al. 2012, Zhao et al. 2014).
In addition to endothelial cells, PAR1 is expressed on a variety of cells within the CNS, including neurons, astrocytes, and microglia (Luo et al. 2007, Shavit et al. 2011, Suo et al. 2002, Vellani et al. 2010, Wang et al. 2002). Under healthy conditions, thrombin is not present in the adult CNS, but the exogenous administration of thrombin into the CNS induces local inflammation (Boven et al. 2003, Fan et al. 2005, Niego et al. 2011, Nishino et al. 1993). For example, thrombin infused into the rat midbrain over 7 days, induces astrocytic upregulation of GFAP and another astrocytic intermediate filament, vimentin, which are both markers of astrocyte activation (Nishino et al. 1993). Thrombin’s activation of astrocytes has also been observed in vitro in mixed hippocampal cultures (Niego et al. 2011). Thrombin induces astrocyte activation and production of pro-inflammatory mediators through PAR1 (Boven et al. 2003, Fan et al. 2005, Nicole et al. 2005, Niego et al. 2011, Nishino et al. 1993, Simmons et al. 2013).

Figure 1.4. Thrombin’s dual regulation of endothelial barrier stability. Thrombin preferentially activates endothelial PAR1, but is also capable of activating protein C into APC. Activation of endothelial-bound protein C induces endothelial barrier stability, whereas PAR1 activation leads to barrier disruption. Under physiological conditions, such as low blood concentration of thrombin, these processes are balanced and vasculature integrity remains intact. During trauma, thrombin concentration is elevated and thrombin preferentially activates PAR1 to disrupt barriers allowing for the passage of serum molecules into the interstitial fluid of nearby tissues.
The role of PAR1 in astrocyte activation is supported by PAR1 null mice exhibiting less astrocyte activation after a cortical stab wound than seen in wild-type mice (Nicole et al. 2005). Thrombin has also been shown to induce mechanical allodynia when injected directly into the spinal cord in mice (Narita et al. 2005). However, the mechanism of thrombin’s entrance into, or production within, the CNS after painful neuropathic injury and whether thrombin contributes to pain have not yet been investigated.

Section 1.2.6. Salmon Thrombin

In contrast to mammalian thrombin, salmon thrombin has been shown to exert distinct effects from human thrombin on certain biological processes. Salmon and human thrombin both retain the same level of activity towards a fibrinogen substrate over a range of concentrations and temperatures (Michaud et al. 2002, Smith et al. 2013). Despite exhibiting indistinguishable enzymatic activity for fibrinogen, salmon thrombin activity is inhibited less effectively by hirudin than is human thrombin (Smith et al. 2013), demonstrating that not all molecular interactions are the same between these two species of thrombin. Salmon thrombin’s lower affinity for hirudin is also suggestive of it having a lower affinity for PAR1, since the hirudin-like sequence on the extracellular domain of PAR1 controls thrombin’s interaction with it (Jacques et al. 2000, Lane et al. 2005). Differences in thrombin’s affinity for PAR1 are hypothesized to manifest as differences in the resulting cell signaling. In fact, salmon thrombin does not activate platelets as strongly as its mammalian counterpart; it takes approximately 10 times as much salmon thrombin to induce platelet aggregation to the same degree as human thrombin in vitro (Michaud et al. 2002). Platelet aggregation is mainly controlled by
thrombin’s activation of platelet-expressed PAR1. Since salmon thrombin has a lower affinity for hirudin and also activates less PAR1-controlled platelet activation than human thrombin, salmon thrombin is hypothesized to have a lower affinity for PAR1.

In neural tissue, salmon coagulation factors have also been shown to induce more favorable effects on neurons and glia compared to mammalian thrombin. Salmon fibrin gels, which are comprised of thrombin and fibrinogen both derived from salmon, have been shown to promote more neurite extension and branching in vitro compared to fibrin gels derived from mammals (Ju et al. 2007). Salmon fibrin applied to the injury site of hemisection of the spinal cord improves locomotor function and bladder control to a greater degree than human thrombin (Sharp et al. 2012). Most importantly, when salmon fibrin or salmon thrombin is applied to the nerve root after a painful 15-minute compression, it inhibits the development of mechanical allodynia for at least 7 days after injury (Weisshaar et al. 2011). That single treatment of salmon thrombin also reduces the infiltration of phagocytotic macrophages into the compressed root at day 7 whereas human thrombin does not (Smith et al. 2013, Weisshaar et al. 2011), implying that salmon thrombin might even exhibit anti-inflammatory properties. Due to the suggested neuroprotective and anti-inflammatory properties of salmon thrombin in neural tissue, as well as its anti-nociceptive effects, studies in this thesis further investigate its role in pain and neuroinflammation following painful nerve root compression.

1.2.7. Cytoskeletal Control of Cellular Mechanics

Many cells respond mechanically to environmental cues and/or respond to the mechanical properties, including stiffness, of their environment (Fletcher and Mullins
2010, Janmey 1998, Janmey and McCulloch 2007). The ability of a cell to resist deformation to applied loads, to transport organelles intracellularly, to change shape, and undergo movements each depends on the integrated organization and movements of the cytoskeleton (Fletcher and Mullins 2010, Janmey 1998, Janmey and McCulloch 2007). The cytoskeleton is comprised of three classes of filaments: microtubules, actin filaments, and intermediate filaments. Actin filaments are crosslinked by the molecular motor, myosin, which displaces those filaments relative to each other. Since actin filaments are also connected to the cell cortex through focal adhesions, their intracellular movements relative to one another translate to extracellular movements of the cell cortex. Focal adhesions physically connect a cell to its surrounding matrix or substrate. The actomyosin/focal adhesion complex enables cells to contract, and detect the stiffness of their matrix and respond accordingly (Fletcher and Mullins 2010). Although actin plays an important role in cell mechanics, microtubules and intermediate filaments play distinct, yet important, roles within a cell as well.

Cytoskeletal filaments are polymeric structures and the three classes of filaments are of different sizes and exhibit different mechanical properties. Microtubules are the largest (25nm) and stiffest of the three filaments, but buckle under compressive loading (Fletcher and Mullins 2010). Because of their inability to resist compressive loads, microtubules do not contribute to the stiffness of a cell. Microtubules form long structures within the cell (up to 5mm) which facilitate trafficking of organelles and molecules along the length of a cell. Actin filaments are the thinnest (8nm) of the three classes of cytoskeletal filaments and undergo rapid polymerization and depolymerization depending as a product of a variety of intracellular signaling cascades (Fletcher and
Mullins 2010). Because of their dynamic nature, actin filaments play a major role in cell movement as they contribute to the propagation of the leading edge of a migrating cell. The size of intermediate filaments is between microtubules and actin filaments (10nm) and only recently have the functions of intermediate filaments begun to be elucidated (Buehler 2013, Fletcher and Mullins 2010, Herrmann et al. 2007). Intermediate filaments are the most stable of the three cytoskeletal components. They resist high tensile loads and therefore have the highest influence on the mechanical integrity of a cell (Buehler 2013, Herrmann et al. 2007).

Vimentin filaments are the most widely expressed intermediate filament in eukaryotic cells and have been shown to control cytoplasmic stiffness (Guo et al. 2013, Janmey et al. 1991, Wang and Stamenovic 2002). Vimentin is expressed in both astrocytes and endothelial cells and plays distinct roles within each cell type. Astrocytes upregulate their expression of vimentin, along with another intermediate filament GFAP, upon their activation (Pekny and Pekna 2004). In vitro, elevated astrocytic expression of vimentin correlates to an increase in cytoplasmic stiffness in vitro (Lu et al. 2011). Although increased expression of astrocytic vimentin is related to their activation and pathology, decreased expression of vimentin in endothelial cells contributes to their dysfunction. In endothelial cells, decreased stability and/or expression of vimentin corresponds to increased vascular endothelial barrier permeability (Buehler 2013, Guo et al. 2013, Liu et al. 2010, Liu et al. 2014). Although vimentin expression plays relevant roles in astrocyte activation and vascular disruption, it is not known if the astrocytic and endothelial expression of vimentin in the spinal cord is modulated after painful nerve root injury.
Cells respond to the mechanics of their substrate in various ways. Neurons, in particular, exhibit increased extension of neurites and more neurite branching on substrates with a softer stiffness (Flanagan et al. 2002, Lamoureux et al. 1998). Certain neuronal functions have also been shown to depend on applied tension and compression, including synaptic vesicle transport along axons and clustering at synapses (Ahmed et al. 2012, Flanagan et al. 2002, Georges et al. 2006, Koch et al. 2012). In the CNS, astrocytes are the main mechanical substrate for neurons (Volterra and Meldolesi 2005). However, it is not known if spinal astrocytes modify their expression of cytoskeletal filaments, such as vimentin, that control their stiffness when they become activated following painful nerve root injury or if changes in their mechanics may contribute to the neuronal dysfunction that maintains nociception.
2.1 Rationale & Context

Winkelstein 2010), highlighting the influence of early spinal inflammation in the development of chronic neuropathic pain.

Spinal glial cells are at least partially responsible for the almost immediate (within 1 hour) upregulation of pro-inflammatory cytokines in the spinal cord after painful nerve root compression (Rothman and Winkelstein 2010). Serum-derived cytokines, along with blood-borne immune cells, can infiltrate into the spinal parenchyma from the periphery and also amplify the spinal neuroinflammatory response induced by neuropathic injury (DeLeo et al. 2004, Echeverry et al. 2011, Radu et al. 2013, Rutkowski et al. 2002, Sweitzer et al. 2002). Under healthy conditions the blood-spinal cord barrier (BSCB), which is made up of fenestrated endothelial cells sealed together by tight junctions, prevents cytotoxic serum components from interacting with spinal neurons (Abbott et al. 2010, Ballabh et al. 2004, Zlokovic 2008). Breakdown of the BSCB occurs in a wide range of neuroinflammatory pathological states (Beggs et al. 2010, Echeverry et al. 2011, Gordh et al. 2006, Hawkins and Davis 2005, Sandoval and Witt 2008, Zlokovic 2010, Zlokovic 2011). Blood-brain barrier (BBB) and BSCB permeability is often identified in animal models by measuring the expression levels of serum proteins, including immunoglobulin G (IgG) and albumin in the CNS parenchyma since they are absent in a healthy state (Beggs et al. 2010, Echeverry et al. 2011, Gordh et al. 2006, Poduslo et al. 1994). Although increased BSCB permeability contributes to inflammation within the spinal cord (Echeverry et al. 2011, Hawkins and Davis 2005, Webb and Muir 2000, Zlokovic 2008), it is not known whether BSCB breakdown is induced after various types of nerve root injuries and if that breakdown facilitates the subsequent pain that develops after neural trauma and induces neuroinflammation. Studies outlined in this thesis
characterize the spatiotemporal distribution of BSCB breakdown after separate painful compressive or inflammatory nerve root insults and establishes the contribution of that breakdown to the spinal and systemic inflammation, as well as pain, that develop after injury.

Although serum-derived inflammatory cytokines can penetrate a compromised BSCB after neuropathic injury (Banks et al. 1995, Echeverry et al. 2011), it is not clear whether other bioactive molecules, such as thrombin, also enter the spinal parenchyma in regions of BSCB breakdown. Thrombin has a crucial role in coagulation by enzymatically cleaving fibrinogen into fibrin which polymerizes to form the main structural component of blood clots (Boon 1993, Di Cera 2008, Griffin 1995). However, thrombin also exerts potent enzymatic effects on a variety of cell types through its catalytic activation of cell receptors, including protease-activated receptors 1, 3 and 4 (PAR1, PAR3 and PAR4), and other cell-bound proteins (Coughlin 2000, Di Cera 2008). Although not typically present in the brain or spinal cord under healthy conditions, mammalian thrombin activates glial cells when injected directly into the midbrain or administered to astrocyte cultures (Niego et al. 2011, Nishino et al. 1993). Thrombin also stimulates astrocytic production of pro-inflammatory cytokines in vitro (Fan et al. 2005, Simmons et al. 2013). Blocking thrombin’s activation of astrocytic PAR1 inhibits thrombin-induced astrocytic inflammation in vitro (Choi et al. 2008), suggesting that thrombin activates astrocytes through PAR1. Further, intrathecal thrombin (1x10^{-12}mol) also induces mechanical behavioral hypersensitivity in naïve mice which persists for up to one week (Narita et al. 2005). Although PAR1 mediates thrombin-induced glial inflammation (Choi et al. 2008, Fan et al. 2005, Nicole et al. 2005, Niego et al. 2011,
Nishino et al. 1993), it not known if spinal thrombin also initiates pain through spinal PAR1. Studies in this thesis test the hypothesis that thrombin extravasates into the spinal parenchyma in regions of BSCB breakdown contributing to spinal glial activation and pain through its activation of spinal PAR1.

Thrombin also regulates vascular permeability. Depending on the substrate to which thrombin is bound it can activate various endothelial cell receptors and cell bound proteins which induce opposing cellular effects (Bouwens et al. 2013, Komarova et al. 2007, Riewald and Ruf 2005). Freely circulating mammalian thrombin preferentially activates endothelial PAR1, which induces vascular leakiness (Coughlin 2000, Griffin 1995, Jacques et al. 2000). However, at low serum concentrations it can also bind to thrombomodulin and activate endothelial-bound protein C into activated protein C (APC), which stabilizes vasculature (Esmon 1993, Griffin 1995, Komarova et al. 2007, Riewald and Ruf 2005). Although the biased agonism of PAR1 is apparent for mammalian thrombin, thrombin that is derived from salmon has unique and opposite effects on a variety of biological processes in mammals while exhibiting an indistinguishable ability to form clots (Laidmae et al. 2006, Wang et al. 2000). Of note, salmon thrombin administered at the nerve root after its transient compression in the rat has prolonged effects on preventing the development of pain, which corresponds to preserving axonal integrity and diminishing the extent of immune cell infiltration within the injured root (Weisshaar et al. 2011). However, the mechanism by which salmon thrombin activates analgesic pathways is unknown and it is also not known if its analgesic effects are unique to salmon thrombin as compared to other species. Several studies in this thesis test whether salmon thrombin exhibits unique specificity for various
thrombin-activated substrates controlling its distinct effects on vascular breakdown and astrocytic inflammation, both of which contribute to pain after neural trauma, in comparison to human thrombin.

CNS tissue, in contrast to many other biological tissues, lacks a prominent fibrous extracellular matrix (ECM) and exhibits a microstructure that is mainly comprised of a well-organized scaffold of cells (Shreiber et al. 2009). Astrocytes make up nearly 2/3 of the cells within the CNS and are the main matrix for the resident neurons (Vallejo et al. 2010). In addition to the biochemical effects that thrombin exerts on a variety of cell types, it also mediates cellular mechanics, also through cell receptor activation. Mammalian thrombin activates endothelial PAR1 which leads to phosphorylation of intracellular myosin light chain and induces cell contraction (Nobe et al. 2005, Satpathy et al. 2004). Endothelial cells increase their cytoskeletal density of actin stress fibers, form larger focal adhesions and increase their cortical stiffness upon contraction (Blum et al. 2008, Cuerrier et al. 2009). In astrocytes, thrombin increases the expression of the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, induces cell spreading and initiates focal adhesion formation (Niego et al. 2011, Suidan et al. 1997), all of which suggest that thrombin also can modify astrocytic mechanics. Since neuronal function depends on substrate stiffness (Flanagan et al. 2002, Georges et al. 2006, Koch et al. 2012), astrocytes that become activated after nerve root compression might modify their stiffness and contribute to sustained neuronal dysfunction and chronic pain.
2.2 Overall Hypothesis & Specific Aims

The objective of the work in this thesis is to use complementary in vivo and in vitro models to elucidate the role of BSCB breakdown and spinal thrombin activity in spinal neuroinflammation and chronic pain. The **overall hypothesis** of this thesis is that compressive nerve root injury disrupts the integrity of the blood-spinal cord barrier allowing for serum molecules, including thrombin, to extravasate into the spinal parenchyma and induce a host of cascades, including spinal PAR1 activation, mechanical modifications in activated spinal glia, and pain. Blocking BSCB breakdown with molecules, such as activated protein C, that stabilize the vascular integrity is hypothesized to prevent nerve root-induced pain; this action is mimicked by salmon thrombin through its unique substrate specificity profile which is distinct from mammalian thrombin. These hypotheses are tested through the following specific hypotheses and specific aims:

**Hypothesis 1.** Painful nerve root compression induces BSCB breakdown, which facilitates the accumulation of serum molecules in the spinal parenchyma in regions of vascular breakdown. The development of nerve root-induced pain is inhibited when that BSCB breakdown is blocked.

**Aim 1:** Characterize BSCB permeability and define its role in pain following different types of injury to the nerve root including, painful compression, non-painful compression, and painful inflammatory insult, separately.
1a. Characterize the time course of BSCB permeability by measuring spinal IgG immunolabeling in the bilateral dorsal horns at the same spinal level as injury at days 1 and 7 after separate painful compression, non-painful compression, and painful inflammatory insults.

1b. Measure serum inflammatory cytokine and chemokine concentrations at time points corresponding to when BSCB breakdown is evident (determined in Aim 1a) following painful and non-painful root injuries.

1c. Block endothelial barrier disruption with APC after painful nerve root injury that induces BSCB leakiness (determined in Aim 1a) and measure behavioral sensitivity.

**Hypothesis 2.** Thrombin acts enzymatically in the spinal parenchyma in regions that undergo BSCB breakdown after painful nerve root compression. Spinal thrombin activates PAR1 leading to nerve root-induced spinal astrocyte activation and pain.

**Aim 2.** Define the role of thrombin-induced spinal PAR1 activation in nerve root-mediated pain, spinal glial activation, and BSCB integrity.

2a. Measure spinal fibrin accumulation in areas of BSCB breakdown by co-immunolabeling for fibrin and fibrinogen, as well as IgG, in the ipsilateral dorsal horn at days 1 and 7 after painful and non-painful nerve root compressions, separately.

2b. Inhibit enzymatic activity of spinal thrombin using hirudin administered intrathecally before painful nerve root injury that induces BSCB leakiness and
evaluate behavioral sensitivity over 7 days. Measure ipsilateral spinal IgG at day 1 and spinal GFAP at day 7 after injury in order to investigate the effects of spinal thrombin on the early BSCB breakdown and sustained spinal glial activation that occurs after painful root compression.

2c. Administer rat thrombin intrathecally to naïve rats, with and without the PAR1 inhibitor SCH79797, and evaluate behavioral sensitivity for 7 days to determine whether spinal thrombin acts through PAR1 to induce pain. Measure spinal IgG and GFAP at days corresponding to maximal rat thrombin-induced behavioral sensitivity.

2d. Block spinal PAR1 activation with SCH79797 before the BSCB-disrupting painful nerve root injury determined in Aim 1a and evaluate behavioral sensitivity over 7 days. Also measure the ipsilateral spinal expression of IgG at day 1 and GFAP at day 7, in order to determine if spinal PAR1 activation contributes to compression-induced early BSCB breakdown and sustained spinal astrocyte activation.

2e. Quantify and compare astrocytic PAR1 expression in the ipsilateral spinal dorsal horn at days 1 and 7 after painful and non-painful root compression.

**Hypothesis 3.** Salmon thrombin, which initiates cellular signaling cascades distinct from mammalian thrombin, exhibits different rates of activating thrombin-activated substrates. Salmon thrombin’s distinctive substrate affinity translates into unique effects on inflammation and vascular permeability, both of which contribute to its analgesic capabilities after painful nerve root compression.
Aim 3. Define differences between salmon and mammalian thrombin on nerve root-mediated pain and BSCB breakdown in vivo, and astrocytic secretion of inflammatory cytokines and endothelial vessel permeability in vitro. Measure and compare the molecular activation rates of thrombin-activated receptors between those two species of thrombin.

3a. Evaluate and compare behavioral sensitivity and ipsilateral spinal IgG expression at day 1 after painful nerve root compression treated immediately with salmon and human thrombin, in separate groups.

3b. Measure and compare the effects of salmon and human thrombin on cytokine-induced endothelial barrier permeability, with and without protein C, using an in vitro endothelial cell model.

3c. Define how astrocytic inflammatory cytokine production is modulated in response to stimulation by salmon and human thrombin, using in vitro astrocytic cell culture preparations.

3d. Measure and compare the cleavage rate of the thrombin enzymatic substrates, PAR1, PAR3, PAR4 and protein C, by salmon and human thrombin.

Hypothesis 4. Painful nerve root compression activates spinal astrocytes and modifies their expression of intermediate filaments that have a role in cellular mechanical integrity.
Aim 4. Measure changes in astrocytic vimentin expression in the spinal dorsal horn after separate painful and non-painful nerve root compressions at times (days 1 and 7) when astrocytes are activated after root compression.

Rat models of painful nerve root injuries previously developed in our lab (Chang and Winkelstein 2011, Rothman et al. 2010, Rothman and Winkelstein 2007) were used to test the hypotheses guiding each of the Aims in this thesis and were supplemented by complementary in vitro and in silico studies. By quantifying the temporal bilateral spinal expression of IgG immunolabeling after compressive and inflammatory nerve root insults (Aim 1a), BSCB breakdown was determined to occur transiently by day 1 and only after a compression injury that also induces pain. That painful longer 15-minute nerve root compression that induced BSCB breakdown, only exhibited increases in IgG in the ipsilateral spinal dorsal horn, corresponding to the exclusive development of mechanical hyperalgesia in the ipsilateral forepaw after this injury. Together, those findings from the work in Aim 1a were used to guide the subsequent studies. Specifically, since a 15-minute nerve root compression was the only injury to induce ipsilateral BSCB breakdown, and that response peaked at one day after compression, that injury was used in the treatment studies for Aim 1c, as well as studies in Aims 2-4. Inflammatory cytokines and chemokines were profiled at day 1 when BSCB breakdown is a maximum; serum concentrations of the inflammatory analytes were linearly fit to elevations in mechanical hyperalgesia following compressive and inflammatory root injuries (Aim 1b) because systemic inflammation influences both BSCB permeability and pain (Echeverry et al. 2011, Huber et al. 2001, Kraychete et al. 2010, Pedersen et al. 2015, Sharief and
Thompson 1992, Szelenyi 2001). Since APC stabilizes vascular integrity in various neuropathic models (Andreou et al. 2015, Bernard et al. 2001, Zlokovic and Griffin 2011), it was administered intrathecally after painful nerve root compression, but prior to BSCB breakdown, in order to test whether the development of nerve root compression-induced mechanical hyperalgesia depends on the breakdown of the BSCB. Ipsilateral spinal IgG expression was measured at day 1 when applicable throughout the rest of the studies in this thesis since the findings from Aim 1 highlight the importance of early BSCB breakdown in pain induced by nerve root compression.

Studies in Aim 2 were designed to investigate the role of rat thrombin activity in the spinal cord in the development of pain and spinal astrocytic activation. The work in Aim 2a tested whether thrombin extravasates across the BSCB during its breakdown after painful root compression and then exerts enzymatic activity in the spinal parenchyma. As such, fibrin(ogen) was used as a proxy for thrombin activity and co-labeled with IgG in the ipsilateral spinal cord after painful and non-painful root compressions. Results from the studies in Aim 2a suggested that spinal fibrin(ogen) is present only after a 15-minute compression and localizes with spinal regions that also undergo BSCB breakdown. Therefore, the specific thrombin inhibitor, hirudin, used in Aim 2b was administered intrathecally (Narita et al. 2005) prior to a painful 15-minute nerve root compression in order to test whether spinal thrombin activity contributes to pain onset. To further define the role of spinal thrombin in pain, rat thrombin was intrathecally administered to naïve rats to test whether thrombin in the CNS alone is potent enough to induce mechanical hyperalgesia (Aim 2c). In order to determine whether thrombin acts through PAR1 in the spinal cord to induce glial activation and pain, the PAR1 antagonist, SCH79797, was
delivered prior to intrathecal administration of rat thrombin (Aim 2c) or a painful nerve root compression (Aim 2d); mechanical hyperalgesia was monitored for up to 7 days. Studies in Aim 2e measured the temporal expression of astrocytic GFAP and PAR1 using the nerve root injury groups outlined in Aim 1a (painful compression, non-painful compression, inflammatory insult) in order to define whether those common indicators of glial activation are specific to pain or are a result of the type of nerve root insult.

Studies have identified key biological differences between mammalian and salmon thrombin (Michaud et al. 2002, Uibo et al. 2009, Weisshaar et al. 2011). The effects of salmon thrombin on nerve root-mediated BSCB breakdown and pain were measured and compared to those induced by human thrombin (Aim 3a) in order to further define the physiological differences of this enzyme between the two species. Since endothelial cells, and the tight junctions binding them together, control vascular and BBB permeability, studies in Aim 3b investigated whether salmon and human thrombin differentially regulate inflammation-induced endothelial permeability using an in vitro microchannel setup. Sustained glial activation has been shown to be associated with persistent pain after nerve root compression injury (Nicholson et al. 2014, Rothman et al. 2010, Rothman and Winkelstein 2007). Therefore, Aim 3c measured levels of cytokine transcription produced by astrocyte cultures in response to stimulation by both species of thrombin. Since multiple thrombin receptors control the cellular effects elicited by thrombin, the activation rates of PAR1, PAR3, PAR4 and protein C induced by both species of thrombin were measured (in Aim 3d) and supporting in silico studies comparing the protein structures of the two species of thrombin were performed to provide structural support for differences in substrate affinity.
The relationship between glial cell mechanics and nerve root-mediated pain was investigated in studies under Aim 4. The intermediate filament vimentin is expressed in a variety of cell types, including astrocytes and endothelial cells, and levels in its expression within the cytoskeleton partially controls its mechanics and stiffness (Buehler 2013, Guo et al. 2013, Wang and Stamenovic 2002). Vimentin is also upregulated by astrocytes upon their activation, in parallel with GFAP, highlighting the influence of astrocytic vimentin expression not only as a possible indicator of changes in cellular mechanics, but a marker for their activation. Therefore, studies in Aim 4 measured the spinal expression of astrocytic and endothelial vimentin at days 1 and 7 following painful and non-painful nerve root compressions in order to investigate whether painful root compression modulates the cellular mechanics of contractile cells within the CNS.

In summary, the studies presented in this thesis define how nerve root compression-induced modifications in spinal vasculature contribute to persistent spinal neuroinflammation and pain after injury. Studies outlined in the above Aims are presented in separate chapters. Chapter 3 summarizes the in vivo findings that temporally characterize nerve root injury-induced BSCB breakdown, systemic inflammation and pain and establish that their modifications depend on the type of painful mechanical insult. That work highlights the strong influence of increased BSCB permeability on pain development using a pharmacologic strengthener of endothelial junctions. Studies outlined in Aim 2 manipulate the spinal thrombin/PAR1 signaling pathway by administering a variety of biologic and small molecule agents in our models of painful nerve root compression; those findings define how spinal thrombin contributes to nerve root compression-induced spinal glial activation and pain and are presented in Chapter 4.
Studies from Aim 3 are summarized in Chapter 5 and determine that, unlike human thrombin, an analgesic dose of salmon thrombin prevents injury- and inflammation-induced vascular breakdown and does not initiate astrocytic inflammation. Studies in Chapter 5 further define that those contrasting cellular outcomes induced by salmon and human thrombin depend on their distinct substrate specificity profiles. These findings promote the use of salmon thrombin as an effective biologic to treat neuropathic injuries, as well as other BBB-disruptive disorders. The studies under Aim 4 measure painful root compression-induced modifications of spinal vimentin expressed by astrocytes and endothelial cells in order to relate changes in spinal glial cell stiffness to pain after nerve root injury. Those findings are presented in Chapter 6. Lastly, Chapter 7 integrates across the studies in this thesis, the existing literature on spinal thrombin and pain, and the broader implications of those findings in relation to the development of novel biologics to prevent chronic pain.
Chapter 3

Defining Relationships between Painful Compressive & Inflammatory Nerve Root Insults, BSCB Breakdown & Serum Cytokine Expression

Part of this chapter has been adapted from a submitted manuscript:

Smith JR, Galie PA, Slochower DR, Weisshaar CL, Janmey PA, Winkelstein BA. Salmon-derived thrombin inhibits development of chronic pain through an endothelial barrier protective mechanism dependent on APC. Biomaterials, accepted.

3.1. Overview

Cervical radiculopathy is a neuropathic condition that induces long-term pain that radiates from an injured or irritated cervical nerve root to the shoulders and arms (Abbed and Coumans 2007, Wall and Melzack 1994). Cervical disc herniation is a common cause of radicular pain in the cervical spine and often involves both compressive and inflammatory insults to the cervical nerve roots; a bulging disc can physically impinge on the nerve root and also release materials from its nucleus pulposus, which is inflammatory to the root (Abbed and Coumans 2007, Caridi et al. 2011, Wall and Melzack 1994). Rat models imposing compressive or inflammatory nerve root insults separately have established that while both components of injury can individually induce sustained mechanical behavioral sensitivity, these stimuli differentially modulate pathological responses at the site of injury and within the spinal cord (Chang and
Winkelstein 2011, Colburn et al. 1999, Hashizume et al. 2000, Hou et al. 2003, Kawakami et al. 1994, Kawakami et al. 1994, Rothman and Winkelstein 2007). For example, a transient C7 nerve root compression for 15 minutes in the rat upregulates the transcription of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in the spinal cord at 1 hour after compression and activates spinal glia for at least 7 days (Hubbard and Winkelstein 2005, Rothman et al. 2009, Rothman et al. 2010, Winkelstein and DeLeo 2002). An inflammatory insult imposed by chromic gut suture does not induce the same early spinal transcription of IL-1β and TNF-α nor does it lead to the persistent glial activation that is induced after a root compression (Rothman et al. 2009, Rothman and Winkelstein 2007, Rothman and Winkelstein 2010). This same nerve root compression disrupts axonal myelination and promotes phagocytotic macrophage infiltration at the injured root by two weeks after injury, while an inflammatory insult only induces macrophage infiltration and to a much less degree than compression (Chang and Winkelstein 2011). Since both compressive and inflammatory insults to the nerve root produce indistinguishable mechanical hypersensitivity in the ipsilateral forepaw for up to 2 weeks (Chang and Winkelstein 2011, Rothman and Winkelstein 2007), but a compressive insult induces more robust spinal and nerve root pathologies (Chang and Winkelstein 2011, Rothman and Winkelstein 2007), it is hypothesized that different cellular cascades are induced by compressive and inflammatory stimuli to produce and maintain the associated pain responses.

The blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB) are comprised of endothelial cells and act as physical obstacles between the circulatory system and the central nervous (CNS) to inhibit the entrance of blood-borne pro-
inflammatory molecules and cells under healthy conditions (Abbott et al. 2010, Ballabh et al. 2004). Despite being remote from the CNS, chronic nerve ligation injury, which induces sustained pain, disrupts the BSCB for up to 30 days in spinal regions where the injured afferents synapse in both rats and mice (Beggs et al. 2010, Echeverry et al. 2011, Gordh et al. 2006). This increased BSCB permeability facilitates the transmission of radioiodine-labeled IL-1β and green fluorescent protein positive (GFP+) bone marrow-derived monocytes into the spinal cord (Echeverry et al. 2011); both the presence of pro-inflammatory cytokines and peripheral immune cells centrally can exacerbate neuroinflammation. Different types of neural injuries can increase spinal microglial activation through either resident spinal microglial division or through facilitated infiltration of peripheral monocytes that express markers of microglia activation once they are in the spinal cord (DeLeo et al. 2004, Rothman et al. 2009, Rutkowski et al. 2002, Stollg and Jander 1999, Sweitzer et al. 2002). A compressive, but not an inflammatory, insult to the nerve root increases microglia populations in the spinal cord both early (day 1) and at later times (day 7) after injury (Rothman and Winkelstein 2007). It is possible that the lack of spinal microglial activation induced by an inflammatory root insult might be due to its lack of induction of BSCB breakdown preventing peripheral immune cell transmigration into the spinal cord. Studies in this chapter evaluate whether compressive or inflammatory insults to the nerve root induce different extents of BSCB breakdown and whether such barrier breakdown directly mediates pain.

Systemic concentrations of pro- and anti-inflammatory cytokines and chemokines are upregulated in a variety of neuropathic and autoimmune disorders and influence the permeability of the BSCB and BBB (Choi et al. 2008, Kraychete et al. 2010, Pan and
Kastin 2007, Pedersen et al. 2015, Sharief and Thompson 1992). Intravenous pro-inflammatory IL-1β, TNF-α or monocyte chemotactic protein-1 (MCP-1), independently, increase BSCB permeability in normal naïve rats, which also has been shown to permit the extravasation of serum proteins into the spinal parenchyma where the BSCB is compromised (Echeverry et al. 2011, Pan and Kastin 2007, Sharief and Thompson 1992). In contrast, anti-inflammatory transforming growth factor-beta (TGF-β) and IL-10 rescue the BSCB breakdown that is induced after painful sciatic nerve ligation (Echeverry et al. 2011); this increased BSCB integrity is also achieved by anti-MCP-1 given intrathecally over 3 days after ligation (Echeverry et al. 2011). Increased serum levels of some of these BBB-disrupting cytokines are also increased in patients with painful disc herniation (Kraychete et al. 2010, Pedersen et al. 2015); in particular, serum concentrations of IL-6, IL-8 and TNF-α are elevated in those patients experiencing more intense chronic pain compared to those with “mild” or no pain (Kraychete et al. 2010, Pedersen et al. 2015). Despite a strong implication of peripheral inflammation in both BSCB breakdown and pain, the relationship between systemic inflammation at times of increased BSCB permeability after compressive and/or inflammatory nerve root insults and pain has not yet been studied.

This chapter summarizes a multi-part study that tests two separate hypotheses. The first two studies (Section 3.3 & Section 3.4) summarize a subset of the experiments under Aim 1 and test the hypothesis that painful nerve root injury induces BSCB breakdown and systemic inflammation facilitating the accumulation of serum molecules in the spinal parenchyma where the BSCB is compromised (Aim 1a). To test this hypothesis, a nerve root compression model in the rat was used characterize the extent of
BSCB breakdown over time after painful and non-painful nerve root compressions separately (Section 3.3). The comparative effects of compressive and inflammatory nerve root insults on BSCB breakdown and pain were also investigated (Section 3.4). BSCB breakdown was measured in the bilateral spinal cord by immunolabeling for the serum protein, immunoglobulin G (IgG), which is not typically present in the CNS (Echeverry et al. 2011, Poduslo et al. 1994), at times early and later after the neural insults (Aim 1a). To define the relationship between expression of inflammatory serum molecules and pain at times of BSCB breakdown, the concentration of 23 pro- and anti-inflammatory serum cytokines and chemokines was correlated to pain severity after a compressive injury (Section 3.3) or an inflammatory insult (Section 3.4) to the nerve root (Aim 1b). Of the cytokines found to correlate to pain intensity after a nerve root compression, TNF-α expression was immunolabeled in the spinal dorsal horn at the time point when BSCB reaches a maximum after painful and non-painful compression injuries.

The last set of studies in this chapter (Section 3.5) investigates the role of BSCB breakdown in pain development after a nerve root compression and was designed to test the hypothesis that blocking BSCB breakdown can inhibit the development of nerve root compression-induced pain. Activated protein C (APC), a serum protease that strengthens endothelial tight junctions and effectively reduces vascular leakiness in studies of sepsis and ischemic stroke (Bernard et al. 2001, Petraglia et al. 2010, Zlokovic and Griffin 2011), was administered intravenously at 1 hour after a painful nerve root injury that induces BSCB breakdown. The inhibition of BSCB breakdown by APC was confirmed by labeling spinal IgG at day 1; behavioral hypersensitivity also was measured at that
time to determine if BSCB breakdown contributes to the development of nerve root-induced pain (Aim 1c).

3.2. Relevant Background

BBB disruption is characteristic of many neurological disorders, including ischemic stroke, Parkinson’s disease and amyotrophic lateral sclerosis (ALS), and contributes to the associated neuroinflammation and neurodegeneration within the central nervous system (Sandoval and Witt 2008, Winkler et al. 2014, Zlokovic 2011). The neurovasculature of the BBB is comprised of endothelial cells that are trophically coupled to nearby neurons via glial cells, which together, make up the ‘neurovascular unit’ and interact closely to maintain inflammatory dysfunction in disease states (Abbott et al. 2010, Abbott et al. 2006, Hawkins and Davis 2005). The healthy BBB endothelium is bound together by tight junctions, which inhibit the transmission of serum components and blood-borne cells into the CNS (Abbott et al. 2010, Ballabh et al. 2004). Disruption of the BBB permits entrance of neurotoxic factors into the CNS that impair normal neuronal function and exacerbate inflammation (Webb and Muir 2000, Zlokovic 2008). Peripheral nerve injuries themselves have also been shown to increase the permeability of the BSCB at the same spinal level as the injury where the injured afferents synapse (Beggs et al. 2010, Echeverry et al. 2011, Radu et al. 2013). Nerve injuries also induce chronic neuropathic pain which is, at least partially, maintained by spinal neuroinflammation (Echeverry et al. 2011, Rothman and Winkelstein 2007, Watkins et al. 2001). However, it is unclear whether BSCB disruption itself contributes to pain.
Further, if BSCB breakdown is related to development of chronic pain, therapeutics targeting and preventing vascular permeability would be ideal candidates for pain prevention after neuropathic injury.

Systemic inflammation contributes to spinal neuroinflammation both indirectly and directly. Pro-inflammatory molecules indirectly influence spinal homeostasis by stimulating peripheral neuronal receptors (Szelényi 2001). These primary afferents synapse centrally in the spinal dorsal horn and become hyperexcitable due to constant peripheral activation (Szelényi 2001). Circulating pro-inflammatory cytokines, such as TNF-α and IL-1β, also activate endothelial cells leading to increased vascular permeability, which promotes their own entrance into the CNS via a compromised BBB (Echeverry et al. 2011, Hoffmann et al. 2004, Huber et al. 2001, Pan and Kastin 2007, Sharief and Thompson 1992). Once in the CNS, pro-inflammatory cytokines and chemokines can directly interact with and stimulate neurons and glia, which further accentuate nociceptive pathways by increasing synaptic concentration of excitatory neurotransmitters and cytokines (DeLeo and Yezierski 2001, Milligan and Watkins 2009). Of note, independently blocking the actions of either spinal TNF-α or IL-1β attenuates pain after compressive nerve root injury (Rothman et al. 2009), highlighting the strong influence of these pro-inflammatory cytokines in pain signaling. Systemic and central inflammation play important roles in pain and are integrated through BBB permeability (Ballabh et al. 2004, Hawkins and Davis 2005, Huber et al. 2001, Sharief and Thompson 1992, Szelényi 2001, Webb and Muir 2000). Yet, it is not known whether there is a relationship between nerve root-induced pain and systemic levels of inflammatory cytokines or chemokines at times of BSCB breakdown.
Vascular permeability, including that of the BBB and BSCB, is controlled through various endothelial pathways. The serine protease, thrombin, most notably recognized for its role in coagulation, also regulates a variety of endothelial processes including permeability via its enzymatic activation of cell-bound receptors (Coughlin 2000, Di Cera 2008). Thrombin initiates distinct cellular signaling cascades depending on the cofactor it binds, and therefore the substrate it activates (Coughlin 2000, Di Cera 2008, Jacques et al. 2000, Komarova et al. 2007, Riewald and Ruf 2005, Xu et al. 2005). In its unbound state, mammalian thrombin increases vascular permeability by directly cleaving protease-activated receptor-1 (PAR1) on the endothelial surface (Gandhi et al. 2011, Jacques et al. 2000, Komarova et al. 2007). In contrast, when bound to thrombomodulin, thrombin activates endothelial-bound protein C into APC, which stabilizes vascular integrity (Esmon 1993, Fuentes-Prior et al. 2000, Komarova et al. 2007, Xu et al. 2005). Both clinical trials and animal studies have tested APC for its enhancement of endothelial barriers in sepsis and ischemic stroke (Zlokovic and Griffin 2011); but, its strong anticoagulant effects hamper its clinical safety (Bernard et al. 2001, Finfer et al. 2008, Marti-Carvajal et al. 2012). For this reason, a major effort in thrombin mutagenesis and protein engineering has identified peptide domains in thrombin’s structure that control protein C activation which they can manipulate in order to increase thrombin’s innate affinity for protein C instead of PAR1 (Dang et al. 1997, Gibbs et al. 1995, Marino et al. 2010). Although APC is not a clinically relevant treatment option for neural trauma, administering APC after painful nerve root injury to fortify the BSCB enables testing the dependence of nerve root-induced pain on BSCB disruption.
This three-part study was used to test the hypotheses that a painful nerve root compression induces BSCB breakdown, which facilitates the accumulation of serum molecules in the spinal parenchyma in regions of breakdown and that the formation of nerve root-induced pain is inhibited when BSCB breakdown is blocked. Section 3.3 details the methods and results of a characterization study measuring BSCB breakdown after painful and non-painful nerve root compression by immunolabeling IgG, a serum protein that is not expressed in the CNS under normal conditions (Poduslo et al. 1994). In that study, behavioral responses were induced using two previously defined durations of nerve root compression: 15-minutes to induce sustained pain and 3-minutes to serve as a loading control in which the nerve root undergoes injury, but pain does not develop (Nicholson et al. 2012, Rothman et al. 2010). Forepaw mechanical hyperalgesia and spinal IgG were assessed on days 1 and 7 as time points corresponding to the development and maintenance of pain, respectively. Expression of serum cytokines and chemokines were assayed at the time point (day 1) corresponding to BSCB breakdown using a multiplex assay in order to characterize systemic inflammation and its relationship to pain.

The second part of this chapter investigates the influence of the type of pain-inducing nerve root insult (i.e. compressive versus inflammatory) on BSCB breakdown and behavioral hypersensitivity (Section 3.4). A painful inflammatory insult was imposed using a rat model of chromic gut suture application to the nerve root (Chang and Winkelstein 2011, Rothman et al. 2011). Following the same procedures as from the characterization study (Section 3.3), ipsilateral forepaw mechanical hyperalgesia and spinal IgG expression were assessed on days 1 and 7 after an inflammatory root insult to
investigate the comparative effects of compressive and inflammatory nerve root insults on BSCB breakdown (Section 3.4).

The third part of the study defined the role of BSCB breakdown in the development of pain by blocking BSCB after painful root compression injury (Section 3.5). APC was administered after painful nerve root compression to block BSCB breakdown, based on its clinical and pre-clinical effectiveness at reducing vascular disruption (Bernard et al. 2001, Marti-Carvajal et al. 2012). Spinal IgG levels were measured at day 1 after injury with treatment to confirm its effectiveness and then behavioral responses were measured at this early time point. The goal of the studies in this chapter was to identify if BSCB breakdown is induced, and if so, to what spatiotemporal extent within the spinal cord, after both compressive and inflammatory nerve root insults. Further, the influence of nerve-root induced BSCB breakdown on pain development and/or maintenance was investigated. The relationship between serum inflammatory markers and pain severity was also defined at times of increased BSCB permeability.

3.3. Characterization of BSCB Breakdown after Nerve Root Compression

3.3.1. Methods

3.3.1.1. Surgical Procedures for Nerve Root Compression

For all animal studies, male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, IN) were housed under conditions approved by the United State Department of
Agriculture (USDA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) with free access to water and food. All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP) (Zimmermann 1983).

Surgical procedures were performed under inhalation isoflurane anesthesia (4% for induction, 3% for maintenance). Previously defined protocols were used for nerve root compression injury (Rothman et al. 2010). Briefly, rats were placed in a prone position; a midline incision was made along the back of the neck and the paraspinal muscles were removed to expose the C6 and C7 vertebrae. A C6/C7 hemilaminectomy and facetectomy was performed on the right side to expose the right C7 dorsal nerve root. A small incision was made in the dura over the C7 nerve root and a 10gf microvascular clip was applied to the exposed root (Figure 3.1).
Compression was applied to the C7 dorsal nerve root via the clip for 3 minutes (3min, n=12) or 15 minutes (15min, n=10) after which the wound was closed using 3-0 polyester suture and surgical staples. Rats were allowed to recover in room air with continual free access to food and water. Sham operated rats (sham, n=9) underwent identical surgical procedures except they did not undergo nerve root compression. Spinal cord tissue from rats was harvested on day 1 (15min, n=5; 3min, n=7; sham n=5) or day 7 (15min, n=5; 3min, n=5; sham n=4) in order to measure temporal responses of BSCB permeability.

Spinal cord tissue was harvested from rats either on day 1 or day 7 after surgery. Rats received an overdose of sodium pentobarbital (65mg/kg), administered intraperitoneally. Once unconscious, rats were transcardially perfused with 1% phosphate-buffered saline (PBS; Mediatech, Inc.; Manassas, VA) until blood ran clear and were subsequently perfused by 300ml of 4% paraformaldehyde (Sigma; St. Louis, MO). The C7 bilateral spinal cord was exposed via a bilateral C5-T1 laminectomy and facetectomy and harvested en bloc. Spinal tissue was post-fixed overnight in 4% paraformaldehyde, transferred to 30% sucrose for one week at 4°C and then embedded in optical cutting temperature (OCT) compound (Sakura Finetek USA, Inc.; Torrance, CA) for cryosectioning. Fixed spinal cord tissue was sectioned at 14µm along the long-axis of the spinal cord to create cross-sections that were mounted directly onto slides for immunolabeling. Spinal cord tissue at C7 also was harvested from naïve rats (n=2) and included in tissue processing for comparison of expression of IgG in tissue from rats that underwent root compression.
3.3.1.2. Assessment of Mechanical Hyperalgesia

In this study, mechanical hyperalgesia was measured as the response threshold, in grams, of the forepaw to a mechanical stimulus. Mechanical thresholds were measured in the bilateral forepaws for 7 days post-surgery. Prior to each testing round, rats were acclimated for 15 minutes to the testing apparatus, which consisted of an elevated mesh-floored cage with walls providing a separate testing chamber for each rat. A series of calibrated von Frey filaments (1.4g-26g) (Stoelting; Wood Dale, IL) was applied in ascending order to the plantar surface of the forepaw until a filament induced a positive response (Chang and Winkelstein 2011, Chaplan et al. 1994, Lee and Winkelstein 2009). A positive response was defined as a withdrawal of the stimulated forepaw and was often accompanied with a shaking or licking of the paw. Each filament was applied for five consecutive stimulations and the filament strength that elicited a positive response was recorded as the withdrawal threshold if the next consecutive filament also elicited a positive response. If no response was evoked by a filament, then the highest magnitude filament (26g) was recorded as the threshold. Each series of stimulations was repeated three times for each testing round with at least 10 minutes between series; the withdrawal threshold of each forepaw was taken as the average of the three series.

Mechanical hyperalgesia in the bilateral forepaws was assessed prior to surgery on day 0 (baseline) and every other day for 7 days (day 1, 3, 5 and 7) after surgery. Behaviors from rats that were terminated on day 7 were compared over time between a 15-minute compression (15min, n=5), a 3-minute compression (3min, n=5) and sham (n=4). The mechanical threshold of each paw was normalized by the corresponding baseline (day 0) thresholds for each rat on each testing day. Differences between groups
over time in forepaw mechanical hyperalgesia were determined separately for the ipsilateral and contralateral forepaws using two-way repeated measures ANOVAs (group x day) with Tukey’s Honestly Significant Difference (HSD) test.

3.3.1.3. Spinal Immunohistochemistry of IgG Expression

Spinal cord sections at C7 were fluorescently immunolabeled for rat IgG as a proxy for BSCB breakdown since this serum-derived protein is not present in the CNS under normal conditions (Poduslo et al. 1994). Briefly, slide-mounted tissue sections were blocked in 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton-X100 (Bio-Rad Laboratories; Hercules, CA) for 1 hour at room temperature. Slides were then incubated with goat anti-rat IgG Alexa Fluor 568 (1:200; Life Technologies; Carlsbad, CA) for two hours at room temperature and then washed with PBS and cover slipped with fluoro-gel with TRIS buffer (Electron Microscopy Sciences; Hatfield, PA). The ipsilateral and contralateral spinal dorsal horns were digitally imaged at 10x in 2-6 spinal sections for each rat.

Spinal IgG labeling was quantified in uniformly cropped images of the dorsal horn using a custom densitometry MATLAB script (Nicholson et al. 2012, Rothman et al. 2010, Rothman and Winkelstein 2007). The densitometry script quantified the percent of the total tissue pixels that were above a pre-defined threshold; that threshold was chosen using normal naïve spinal tissue to include pixels that represented positive IgG labeling and was kept constant to analyze all images (Nicholson et al. 2012, Rothman et al. 2010, Rothman and Winkelstein 2007). Levels of spinal IgG were then normalized to labeling in the same region from naïve rats and the percent positive IgG in injury groups was
represented as expression relative to normal levels. Differences in normalized percent positive IgG between 15-minute compression, 3-minute compression and sham were tested on day 1 (15min, n=5; 3min, n=7; sham n=5) and day 7 (15min, n=5; 3min, n=5; sham n=4), using two-way repeated measures ANOVA (group x day) with Tukey’s test.

3.3.1.4. Serum Collection & Cytokine Multiplex Assay

Rats were anesthetized with 4% isoflurane anesthesia by inhalation for blood collection procedures. Blood samples were taken from a subset of rats undergoing either a 15-minute (n=6), 3-minute (n=4) or 0-minute (sham, n=3) nerve root compression. Blood was collected (~0.5ml) via a 25G needle syringe from the tail vein on day 0 (baseline) before, and on day 1, after surgery. Whole blood was allowed to clot at room temperature and serum was separated using consecutive spins at 4°C, the first at 1000rcf for 15 minutes and the second at 10,000rcf for 10 minutes. Serum samples were assayed in duplicate for a panel of 23 pro- and anti-inflammatory cytokines and chemokines using a multiplex bead-based Luminex assay kit (#L80-01V11S5; Bio-Rad; Hercules, CA). The analytes measured within this pre-made kit are: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, IL-18, MCP-1, TNF-α, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), keratinocyte-derived chemokines/growth-related oncogene (GRO/KC), interferon-gamma (IFN-γ), macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein-3 alpha (MIP-3α), regulated on activation, normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF). For each rat, the serum concentration of each analyte on day 1 was normalized to baseline levels and
correlated to the normalized paw withdrawal threshold at day 1. Each pair of bivariate data – the normalized withdrawal threshold and the normalized cytokine concentration – was fit with a linear regression and analyzed to identify those cytokines that strongly (R²>0.5) and significantly (p<0.05) correlate to paw withdrawal threshold (Cohen 1988).

3.3.1.5. Spinal Immunohistochemistry for TNF-α

TNF-α was chosen from the four cytokines that were found to strongly correlate to forepaw withdrawal thresholds (IL-7, IL-12, IL-1α, TNF-α) and was co-immunolabeled with IgG in the ipsilateral spinal cord. The IgG protocol described in Section 3.3.1.3 was adapted to include TNF-α in order to visually assess whether its spinal expression co-localizes to spinal regions where there is also BSCB breakdown. Tissue sections harvested from two rats in each of the compressive insult and corresponding control groups (15-minute compression, 3-minute compression, sham) at day 1 were blocked in 5% goat serum in 0.3% TX-100 and incubated over night with rabbit anti-TNF-α (1:200; Cell Signaling; Danvers, MA). Those slides were then fluorescently labeled with goat anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen; Carlsbad, CA) and goat anti-rat IgG Alexa Fluor 568 (1:200; Invitrogen; Carlsbad, CA). The ipsilateral dorsal horn was digitally imaged at 10x and visually inspected for the presence of TNF-α and its co-localization with IgG.

3.3.2. Results

A 15-minute nerve root compression induces a significant decrease (p=0.0002) in the withdrawal threshold in the ipsilateral forepaw, corresponding to an increase in pain,
compared to sham procedures at all 7 days after injury (Figure 3.2A). That decrease in
the withdrawal threshold in the ipsilateral forepaw after a 15-minute compression is also
significant (p=0.0002) when compared to the threshold generated after a 3-minute
compression, which is not different from sham responses (Figure 3.2A). The withdrawal
thresholds in the contralateral forepaw are not different between any of the groups over
time or compared to the respective baseline (day 0) withdrawal thresholds (Figure 3.2A).
The individual paw withdrawal threshold data for each rat in each group are provided in
Appendix A.

Robust spinal IgG labeling is evident on day 1 after the painful 15-minute
compression in the ipsilateral, but not contralateral, spinal dorsal horn (Figure 3.2B). IgG
expression is also minimal, or not present, in the bilateral spinal cord at this time point for
either of the non-painful 3-minute root compression or the sham surgery (Figure 3.2B).
Quantification of spinal IgG reveals that a 15-minute nerve root compression induces a
significant increase in IgG labeling compared to the labeling after both of a 3-minute
compression (p<0.0001) and sham (p<0.0001) at day 1 (Figure 3.2C). The elevation in
spinal IgG induced by a 15-minute compression returns to sham levels by day 7, when
IgG labeling is also significantly lower (p<0.0001) than levels at day 1 (Figure 3.2C).
This early and transient increase in IgG occurs only after painful neural injury. A detailed
summary of the quantification of IgG expression in the bilateral spinal cord for each rat
in this study is provided in Appendix B.
At the same time when BSCB breakdown peaks (day 1) after the painful 15-minute nerve root compression, the withdrawal threshold of the ipsilateral forepaw positively correlates to the serum concentration of four cytokines: IL-7 ($R^2=0.617$; $p=0.0015$), IL-12 ($R^2=0.572$; $p=0.0028$), IL-1α ($R^2=0.558$; $p=0.0033$) and TNF-α ($R^2=0.523$; $p=0.0052$) (Table 3.1; Figure 3.2D). All of these are pro-inflammatory...
mediators and have a coefficient of determination greater than 0.5 (Table 3.1). A 15-minute compression increases TNF-α immunolabeling in the ipsilateral spinal cord at day 1 in regions that are also positively labeled for IgG (Figure 3.2D). Spinal TNF-α is not evident after either the 3-minute compression injury or the sham procedure (Figure 3.2D), both of which do not induce BSCB breakdown or mechanical hyperalgesia at this time either (Figure 3.2). The co-localization of TNF-α with IgG suggests that increased serum concentrations of TNF-α may diffuse into the spinal cord from the circulation during BSCB breakdown. Cytokine and chemokine expression in the serum for all groups is outlined in Appendix C.

Table 3.1. Serum levels of pro- and anti-inflammatory cytokines and chemokines (x) and equations describing their correlation to ipsilateral forepaw withdrawal threshold (y) on day 1 after root compression.

<table>
<thead>
<tr>
<th>cytokine</th>
<th>correlation</th>
<th>R²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-inflammatory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td>y=0.74x+0.77</td>
<td>0.617</td>
<td>0.0015</td>
</tr>
<tr>
<td>IL-12</td>
<td>y=0.73x+1.78</td>
<td>0.572</td>
<td>0.0028</td>
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<td>IL-1α</td>
<td>y=0.57x+1.07</td>
<td>0.558</td>
<td>0.0033</td>
</tr>
<tr>
<td>TNF-α</td>
<td>y=0.72x+1.18</td>
<td>0.523</td>
<td>0.0052</td>
</tr>
<tr>
<td>VEGF</td>
<td>y=0.43x+1.04</td>
<td>0.485</td>
<td>0.0082</td>
</tr>
<tr>
<td>IL-17</td>
<td>y=0.43x+0.98</td>
<td>0.479</td>
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<td>G-CSF</td>
<td>y=0.66x+1.14</td>
<td>0.466</td>
<td>0.0113</td>
</tr>
<tr>
<td>IL-1β</td>
<td>y=0.72x+1.04</td>
<td>0.446</td>
<td>0.0126</td>
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<tr>
<td>MIP-3α</td>
<td>y=0.37x+1.03</td>
<td>0.422</td>
<td>0.0162</td>
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<td>IFN-γ</td>
<td>y=0.56x+1.09</td>
<td>0.376</td>
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<td>IL-2</td>
<td>y=0.41x+1.02</td>
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<td>MCP-1</td>
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<td>0.1459</td>
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<td>GM-CSF</td>
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<td>GRO/KC</td>
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<td>0.163</td>
<td>0.1707</td>
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<tr>
<td>RANTES</td>
<td>y=0.12x+0.98</td>
<td>0.042</td>
<td>0.5031</td>
</tr>
<tr>
<td>anti-inflammatory</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>y=0.68x+1.04</td>
<td>0.496</td>
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<td>IL-10</td>
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<tr>
<td>IL-5</td>
<td>y=0.27x+0.99</td>
<td>0.415</td>
<td>0.0175</td>
</tr>
<tr>
<td>IL-4</td>
<td>y=0.50x+1.02</td>
<td>0.383</td>
<td>0.0241</td>
</tr>
<tr>
<td>EPO</td>
<td>y=0.56x+1.00</td>
<td>0.344</td>
<td>0.0352</td>
</tr>
</tbody>
</table>

Note: Shaded cells indicate R² greater than 0.5. Bolded values represent coefficients of variation greater than 0.5 and p-values <0.05.
3.4. Comparative Effects of Painful Compressive & Inflammatory Nerve Root Insults on BSCB Breakdown

3.4.1. Methods

3.4.1.1. Surgical Procedures for Inflammatory Insult

In order to examine whether BSCB breakdown is altered after an inflammatory insult, a separate group of rats underwent a painful inflammatory insult to the nerve root (chromic, n=11) using previously developed and characterized procedures (Chang and Winkelstein 2011, Rothman and Winkelstein 2007). Identical surgical procedures were followed as those described in Section 3.3.1.1 to expose the right C7 dorsal nerve root in the rat. Following root exposure, four 1mm pieces of 3-0 chromic gut suture (Surgical Specialties; Reading, PA) were placed directly on top the nerve root. Chromic gut suture pieces were kept in contact with the root and the wound was closed using 3-0 polyester suture and surgical staples. On day 1 (n=7) or day 7 (n=4) after surgery, fixed C7 spinal cord tissue was harvested and processed for immunolabeling as described in Sections 3.3.1.1 and 3.3.1.3.

3.4.1.2. Assessment of Mechanical Hyperalgesia

For this study, ipsilateral mechanical hyperalgesia was assessed only on days 0, 1 and 7 after the inflammatory nerve root insult, since those time points correspond to the development and maintence of nerve root-induced pain in this model. Using behavioral hypersensitivity methods described previously (Section 3.3.1.2), mechanical hyperalgesia was measured in the ipsilateral forepaw as measured by the paw withdrawal threshold
normalized to baseline (day 0) levels. The paw withdrawal threshold in rats treated with chromic gut suture were compared to thresholds of rats that received a 15-minute compression or sham operation (see Section 3.3.1). Ipsilateral paw withdrawal thresholds at day 1 (chromic, n=11; 15min, n=11; sham, n=10) and day 7 (chromic, n=4; 15min, n=5; sham, n=4) were normalized to baseline (day 0) thresholds. Differences in the normalized paw withdrawal threshold between groups over time were determined using a repeated measures two-way ANOVA (group x time) with Tukey’s test. This two-way ANOVA also tests for differences between the 15-minute compression group and sham over time, which is also analyzed in the characterization of BSCB breakdown after nerve root compression (Section 3.3).

3.4.1.3. Spinal Immunohistochemistry for IgG

IgG expression was labeled using methods described in Section 3.3.1.3 in the bilateral spinal cord from a subset of rats that underwent inflammatory nerve root injury. In order to assess the influences of injury type on BSCB permeability, spinal IgG expression in tissue from rats receiving an inflammatory insult via chromic gut suture application to the root was compared to IgG expression in the spinal cord from 15-minute compression and sham at day 1 (chromic, n=3; 15min, n=5; sham, n=5) and day 7 (chromic, n=4; 15min, n=5; sham, n=4). A two-way ANOVA (group x day) with Tukey’s test was used to test differences in spinal IgG expression between painful compression and inflammatory injuries. This two-way ANOVA compares IgG expression for the 15-minute compression group and sham over time, which is also analyzed in the characterization of BSCB breakdown after nerve root compression (Section 3.3)
### 3.4.1.4. Serum Collection & Cytokine Multiplex Assay

Procedures for blood draw and elution of serum from whole blood samples were performed according to the methods described in Section 3.3.1.4. For analysis of cytokine levels after an inflammatory nerve root insult, blood was collected from rats on day 1 after exposure to chromic gut suture (chromic, n=7) and assayed. Concentrations of 23 cytokines and chemokines on day 1 were normalized to the corresponding baseline levels and were correlated to the normalized ipsilateral paw withdrawal threshold at day 1 for each rat. Each set of bivariate data (normalized threshold versus normalized cytokine concentration) was fit using a linear regression as described in Section 3.3.1.4.

### 3.4.2. Results

Overall, an inflammatory nerve root insult induces a significant decrease in paw withdrawal threshold compared to sham (p=0.0148), as well as on day 1 compared to sham (p=0.0435) and its own baseline (day 0) levels (p=0.0307) (Figure 3.3A). Similarly, the 15-minute root compression significantly reduces withdrawal threshold in the ipsilateral forepaw compared to sham on days 1 (p<0.0001) and 7 (p=0.0021) and overall (p<0.0001), as well as compared to its own baseline levels (p<0.0001) (Figures 3.2A & 3.3A). The inflammatory and compressive insults do not induce different withdrawal thresholds on any individual day probed (Figure 3.3A); yet, a 15-minute compression does induce a significant drop in withdrawal threshold compared to an inflammatory chromic insult overall (p=0.0159) (Figure 3.3A). The paw withdrawal thresholds for each rat in each group at each day probed are provided in Appendix A.
A painful inflammatory nerve root insult does not induce a substantial increase in spinal IgG labeling and its expression is not different from sham levels (Figures 3.3B & 3.3C). A painful 15-minute compression significantly increases (p<0.0001) ipsilateral spinal IgG expression compared to sham and IgG expression after an inflammatory insult on day 1 (Figures 3.3B & 3.3C). Taken together, both inflammatory and compressive nerve root insults induce behavioral sensitivity (Figure 3.3A), but only the compressive injury also induces BSCB breakdown (Figures 3.3B & 3.3C). A summary of the quantified spinal IgG expression for each rat in this study is detailed in Appendix B.

Figure 3.3. A painful inflammatory nerve root insult does not induce BSCB breakdown at day 1. (A) The normalized paw withdrawal threshold is significantly decreased at 1 day after an inflammatory root insult induced by chromic gut (chromic) compared to sham (**p=0.0435) and baseline (day 0, #p<0.0307). A 15-minute root compression (15min) significantly decreases the withdrawal threshold at days 1 and 7 compared to sham (*p<0.0021) and day 0 (#p<0.0307). (B) Spinal IgG labeling is relatively low at day 1 in the ipsilateral dorsal horn for both sham and a chromic root insult in contrast to the 15min group, which exhibits robust IgG labeling. (C) The percent of IgG labeling normalized to expression in naïve rats in the ipsilateral spinal cord is significantly elevated (*p<0.0001) at day 1 after a 15min compression compared to both sham procedures and a chromic insult. Data are mean±SD.
Serum levels of pro- and anti-inflammatory cytokines and chemokines were assayed at day 1 after an inflammatory nerve root injury in order to determine if inflammatory components also modulate systemic inflammation similarly to a compressive insult. Unlike nerve root compression (Table 3.1), an inflammation-induced nerve root insult does not significantly alter any of the serum factors probed in a manner that correlates to mechanical hyperalgesia (Table 3.2). Serum analyte expression for all rats in each group can be found in Appendix C.

Table 3.2. Serum levels of pro- and anti-inflammatory cytokines and chemokines (x) and equations of their correlation to ipsilateral forepaw withdrawal threshold (y) on day 1 after inflammatory root insult.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Correlation</th>
<th>R²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>y=-0.06x+0.73</td>
<td>0.186</td>
<td>0.5690</td>
</tr>
<tr>
<td>MCP-1</td>
<td>y=0.30x+0.08</td>
<td>0.176</td>
<td>0.3487</td>
</tr>
<tr>
<td>IL-17</td>
<td>y=-0.24x+0.84</td>
<td>0.164</td>
<td>0.3673</td>
</tr>
<tr>
<td>MIP-3α</td>
<td>y=0.15x+0.36</td>
<td>0.042</td>
<td>0.6569</td>
</tr>
<tr>
<td>IL-2</td>
<td>y=0.11x+0.41</td>
<td>0.040</td>
<td>0.6675</td>
</tr>
<tr>
<td>TNF-α</td>
<td>y=0.04x+0.46</td>
<td>0.037</td>
<td>0.6782</td>
</tr>
<tr>
<td>GRO/KC</td>
<td>y=-0.12x+0.67</td>
<td>0.034</td>
<td>0.6914</td>
</tr>
<tr>
<td>RANTES</td>
<td>y=-0.13x+0.66</td>
<td>0.016</td>
<td>0.7890</td>
</tr>
<tr>
<td>IL-1β</td>
<td>y=0.03x+0.49</td>
<td>0.014</td>
<td>0.7982</td>
</tr>
<tr>
<td>IL-7</td>
<td>y=0.05x+0.59</td>
<td>0.011</td>
<td>0.8269</td>
</tr>
<tr>
<td>VEGF</td>
<td>y=0.04x+0.48</td>
<td>0.009</td>
<td>0.8382</td>
</tr>
<tr>
<td>IL-12</td>
<td>y=0.03x+0.50</td>
<td>0.006</td>
<td>0.8645</td>
</tr>
<tr>
<td>G-CSF</td>
<td>y=0.02x+0.50</td>
<td>0.006</td>
<td>0.8648</td>
</tr>
<tr>
<td>IL-18</td>
<td>y=0.03x+0.57</td>
<td>0.004</td>
<td>0.8898</td>
</tr>
<tr>
<td>IL-1α</td>
<td>y=0.01x+0.55</td>
<td>0.001</td>
<td>0.9443</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>y=0.01x+0.55</td>
<td>0.001</td>
<td>0.9375</td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPO</td>
<td>y=0.11x+0.40</td>
<td>0.048</td>
<td>0.6361</td>
</tr>
<tr>
<td>IL-13</td>
<td>y=0.02x+0.50</td>
<td>0.006</td>
<td>0.8655</td>
</tr>
<tr>
<td>IL-10</td>
<td>y=0.04x+0.49</td>
<td>0.005</td>
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<tr>
<td>IL-5</td>
<td>y=0.06x+0.59</td>
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<td>0.9223</td>
</tr>
<tr>
<td>IL-4</td>
<td>y=0.00x+0.54</td>
<td>0.000</td>
<td>0.9772</td>
</tr>
<tr>
<td><strong>Pro- and anti-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>y=0.07x+0.63</td>
<td>0.011</td>
<td>0.8197</td>
</tr>
<tr>
<td>IL-6</td>
<td>y=0.00x+0.54</td>
<td>0.001</td>
<td>0.9618</td>
</tr>
</tbody>
</table>

Note: Shaded cells indicate R² greater than 0.5. Bolded values represent coefficients of variation greater than 0.5 and p-values <0.05.
3.5. The Role of BSCB Breakdown in Pain after Nerve Root Compression

3.5.1. Methods

3.5.1.1. APC Treatment

In order to assess the effects of blocking BSCB breakdown on mechanical hypersensitivity, another group of rats underwent a 15-minute nerve root compression with treatment of the vascular protecting agent APC (Bouwens et al. 2013, Riewald et al. 2002, Zlokovic and Griffin 2011). Human APC (Haematologic Technologies Inc.; Essex Junction, VT) was administered intravenously (0.2mg/kg rat) as a single bolus injection (5ml/kg) given 1 hour after the nerve root compression (15min+APC, n=6). This dosing paradigm was previously optimized in other rat models of sepsis and shown to reduce vascular permeability (Keller et al. 2011, Teke et al. 2008). Since maximal BSCB breakdown was observed at day 1 after painful nerve root compression (Figure 3.2), fixed C7 spinal cord tissue was harvested only on day 1 after APC treatment and a 15-minute compression rats.

3.5.1.2. Spinal IgG Immunolabeling

Methods described above (Section 3.3.1.3) were used for immunolabeling of IgG expression in the bilateral spinal cord of rats that received a 15-minute nerve root compression with APC treatment. To confirm that the APC dose was sufficient to block BSCB breakdown, spinal IgG expression quantified at day 1 was compared between the APC treated 15-minute compression (15min+APC; n=6) and matching tissue from rats.
from the study in Section 3.3 that underwent a 15-minute compression alone (15min; n=5). A two-way ANOVA (group x side) with Tukey’s test was used to determine significant differences in spinal IgG expression between groups.

3.5.1.3. Assessment of Mechanical Hyperalgesia

Behavioral sensitivity after APC-treated compression was measured in the ipsilateral forepaw only on days 0 and 1 since pain develops at day 1 after a 15-minute nerve root compression (Figure 3.2A) and BSCB breakdown reaches a maximum at this time as well (Figure 3.2B & 3.2C). Using methods described in Section 3.3.1.2, the normalized paw withdrawal thresholds measured after the APC-treated 15-minute compression (15min+APC, n=6) was compared to thresholds for the 15-minute compression (15min, n=5) and sham (n=4) groups (Section 3.3). Statistical differences in mechanical hyperalgesia were determined between groups using a two-way repeated measures ANOVA with Tukey’s test.

3.5.2. Results

A painful 15-minute nerve root compression induces a significant increase (p=0.028) in IgG expression in the ipsilateral spinal cord compared to the contralateral spinal cord (Figure 3.4). A single intravenous injection of APC (0.2mg/kg) administered 1 hour after a 15-minute root compression significantly reduces (p<0.0001) IgG expression at 1 day compared to IgG expression for a 15-minute compression alone (Figure 3.4). A detailed summary of the quantification of IgG labeling in the ipsilateral spinal cord for each rat in this study is provided in Appendix B.
In addition, treating a painful nerve root compression with APC prevents the compression-induced decrease in paw withdrawal threshold; rats receiving an APC-treated compression exhibit a significantly higher (p=0.034) threshold at day 1 compared to the untreated compression (Figure 3.4C), suggesting the absence of mechanical hyperalgesia. The individual paw withdrawal thresholds for each rat in each group can be found in Appendix A.

Figure 3.4. Blocking BSCB breakdown with intravenous APC inhibits mechanical hyperalgesia development after neural injury. (A) Spinal IgG is reduced after APC treated 15-minute compression (15min+APC) compared to compression alone (15min). (B) A 15min compression significantly increases (**p=0.028) spinal IgG in the ipsilateral dorsal horn compared to the contralateral dorsal horn on day 1. The IgG in the ipsilateral spinal cord for the 15min+APC group is significantly lower (*p<0.0001) than after the 15min compression on day 1 and is not different from respective contralateral values. (C) The Forepaw withdrawal threshold at day 1 is significantly reduced (*p=0.028) by a 15min compression compared to sham, whereas the 15min+APC group exhibits a significantly higher (**p=0.034) withdrawal threshold than 15min, which is not different sham. All data are represented as mean±SD.
3.6. Discussion

The immunohistochemical and behavioral studies in this chapter demonstrate that the development of pain after a nerve root compression depends on the breakdown of the BSCB that occurs transiently, and early, after injury (Figures 3.2 & 3.4). Behavioral sensitivity and BSCB breakdown are induced in parallel within 1 day of a 15-minute nerve root compression (Figure 3.2), and are both evident only after that longer compressive injury and only in the ipsilateral spinal cord, supporting their association. Since pain is still present on day 7 after that compression, when BSCB permeability has returned to normal (Figure 3.2), BSCB breakdown may be associated with the onset, rather than the maintenance, of nerve root compression-induced pain. Early breakdown in the BSCB is unique to compression-mediated pain and not pain in general, since the inflammatory nerve root insult does not increase BSCB permeability at any of the time points probed, despite inducing pain (Figure 3.3). The 15-minute nerve root compression utilized in these studies has been shown to deform the macroscopic nerve root structure immediately after compression and to decrease the structural integrity of the compressed axons within the root for up to 2 weeks (Chang and Winkelstein 2011, Hubbard et al. 2008b, Nicholson et al. 2011). Neither a 3-minute compression nor an inflammatory insult to the root induces the axonal disruption that is typically observed at later times after the painful compression (Chang and Winkelstein 2011, Nicholson et al. 2011, Rothman et al. 2010). The lack of BSCB breakdown induced by both a non-painful compression and an inflammatory insult suggests that BSCB breakdown might be controlled by the mechanical components of neural injury. Further, blocking the early
compression-induced BSCB breakdown with APC completely prevents pain from developing (Figure 3.4C), suggesting that the rapid targeting of the APC pathway after neural injury might eliminate the need for chronic use of analgesics later.

The treatment window for blocking BSCB is expected to be on the order of hours to a day after the initial injury since increased BSCB permeability is observed by day 1 after painful nerve root compression (Figure 3.2). In agreement, a painful chronic sciatic nerve ligation induces BSCB breakdown in the lumbar spinal cord between 6 and 24 hours (Beggs et al. 2010, Echeverry et al. 2011). However, that nerve ligation induces breakdown for up to 30 days in some cases (Echeverry et al. 2011), whereas spinal IgG levels return to normal by day 7 after a transient root compression (Figure 3.2). This discrepancy in the duration of BSCB breakdown after a nerve root compression and a sciatic nerve ligation is most likely due to duration of local mechanical injury; the nerve root compression is only applied transiently (for 15 minutes) in contrast to a nerve ligation, which imposes a sustained neural compression.

Deforming neural tissue compromises both neuronal and vascular integrity transiently during the applied compression (Igarashi et al. 2005, Rothman et al. 2010, Rydevik et al. 1981, Yoshizawa et al. 1989). Compression of the nerve root blocks blood flow through the root inducing a transient state of ischemia (Igarashi et al. 2005, Olmarker et al. 1989, Rydevik et al. 1981). Although blood flow is restored within hours after the compression is removed (Igarashi et al. 2005, Yoshizawa et al. 1989), evidence of decreased axonal integrity is still present for up to 2 weeks (Chang and Winkelstein 2011). Since neural compression-induced ischemia occurs transiently (Yoshizawa et al. 1989), but axonal degeneration persists for much longer (Chang and Winkelstein 2011,
Hubbard et al. 2008b), it is possible that the transient disruption in the BSCB is due to the ischemic, rather than neuronally-mediated effects. A nerve root compression applied for a longer duration, which may be more clinically relevant due to a bulging disc or spinal stenosis, might induce BSCB breakdown that lasts longer, and, therefore, may offer a longer treatment window after compression. It is also possible that chronic compression of the nerve root may induce BSCB breakdown that is so robust that a single treatment or even repeat treatments with APC would not adequately fortify the vasculature. Administering APC or some other vascular protecting drug repeatedly would provide more information about the regulatory mechanism of root-induced BSCB disruption.

A limitation of the current study is that the mechanism by which a painful nerve root compression, which is remote from the spinal cord, is capable of disrupting spinal vasculature was not investigated. The nerve root, where the compression is applied, is physically connected to the spinal cord through afferent axons and the vasculature, suggesting that injury to, and therefore dysfunction of, axons or vasculature might mediate the breakdown. The effect of the neural compression duration on spinal pathology is important. By day 7, a painful 15-minute compression induces axonal degeneration in the nerve root, whereas a 3-minute compression does not induce any nerve root pathology (Nicholson et al. 2011, Rothman et al. 2010). In the current study, BSCB breakdown occurs only after the longer 15-minute, and not the shorter 3-minute, compression (Figure 3.2). Electrophysiological activity in the ipsilateral dorsal horn is maximally reduced at 6.6±3.0 minutes after the start of root compression (Nicholson et al. 2011). That threshold of altered spinal neuronal signaling falls between the two durations of compression examined in the current study (3-minutes and 15-minutes).
Since spinal IgG expression increases exclusively after a compression that is held for longer than that threshold required to immediately disrupt spinal neuronal firing (Figure 3.2), it is possible that BSCB breakdown may also be influenced by early changes in neuronal signaling. Yet, neuronal hyperexcitability in the spinal cord and axonal degeneration in the nerve root remain at day 7 after a 15-minute compression (Zhang et al. 2013) when BSCB integrity has recovered (Figure 3.2), suggesting that the breakdown is not entirely controlled by neuronal signaling.

Compression applied to the nerve root, and the vasculature within, disrupts blood flow to the spinal cord and dorsal root ganglia (Igarashi et al. 2005, Kobayashi et al. 2008, Olmarker et al. 1989, Yoshizawa et al. 1989), which may also influence BSCB breakdown experienced after a compressive injury. This study shows that only the 15-minute compression induces changes in permeability of the spinal vasculature (Figure 3.2). Previous studies have reported that compression of the lumbar nerve roots or cauda equina also induce duration-dependent changes in blood flow. Blood flow within the root is reduced within 10 minutes of compression and continuously decreases as the duration of compression increases (Igarashi et al. 2005, Olmarker et al. 1989), suggesting that only the 15-minute compression might disrupt blood flow to the spinal cord. The compression-induced blockage of blood flow in the nerve root is similar to ischemic stroke models that transiently block cerebral arteries for different periods of time to induce different patterns of cerebral ischemia and reperfusion (Dobbin et al. 1989, Ek et al. 2015, Sage et al. 1984). Occluding the carotid artery for 15 minutes induces transient BBB breakdown as soon as 3 hours that lasts for up to 24 hours (Dobbin et al. 1989, Ek et al. 2015), paralleling the transient BSCB breakdown that occurs within 1 day of painful root
compression (Figure 3.2). A compressed root undergoes more structural deformation and load-relaxation after 15 minutes of compression than after just 3 minutes (Rothman et al. 2010); it is possible that the shorter duration of compression may not deform the root tissue to an extent that induces physical compression of the vasculature within, thereby not blocking blood flow to the spinal cord and not inducing ischemia. An inflammatory insult to the root similarly does not mechanically disrupt the nerve root and, therefore, does not block blood flow to the spinal cord. Since an inflammatory injury also does not induce BSCB breakdown (Figure 3.3), BSCB breakdown may be at least partially controlled by ischemia due to physical impingement of the root vasculature.

Systemic inflammation may be one mechanism by which compression-induced BSCB breakdown facilitates pain. Serum concentrations of IL-7, IL-12, IL-1α and TNF-α correlate to the severity of pain at day 1 (Table 3.1), which is when BSCB breakdown is most robust (Figure 3.2). Of these cytokines, spinal IL-1α and TNF-α have been shown to mediate pain after the nerve root compression injury examined in this chapter (Rothman et al. 2009, Rothman and Winkelstein 2010). Painful nerve root compression increases IL-1α and TNF-α transcription in the ipsilateral spinal cord as early as 1 hour after compression; both are likely produced by glial cells since IL-1α expression is exclusively localized to astrocytes in the spinal cord at 1 hour of compression (Rothman and Winkelstein 2010). By day 1 after compression, IL-1α and TNF-α transcription levels in the spinal cord return to normal (Rothman et al. 2009), but at that time spinal TNF-α protein expression is elevated (Figure 3.2D). Since cells within the spinal parenchyma are not transcribing TNF-α by day 1 after nerve root compression (Rothman et al. 2009) and TNF-α expression co-localizes with IgG in the spinal cord (Figure 3.2D) (Smith et al.
peripheral TNF-α is likely transported from the blood into the spinal parenchyma rather than being produced by cells within spinal cord at this time.

The early breakdown of the BSCB after painful compression may also promote the extravasation of peripheral immune cells into the spinal parenchyma. Spinal microglial expression is elevated by day 7 exclusively after a painful compression, and not a non-painful compression (Rothman et al. 2010). Increases in spinal microglia can be attributed to the migration of microglia from other locations within the CNS, division of local microglia, or through the transmigration of peripheral immune cells into the CNS (Inoue 2006, McMahon et al. 2005, Milligan and Watkins 2009, Stollg and Jander 1999, Sweitzer et al. 2002, Vallejo et al. 2010, Watkins et al. 2001). The early BSCB breakdown induced after a 15-minute compression may facilitate the extravasation of peripheral cells into the spinal parenchyma and contribute to the increase in spinal microglia that is evident by day 7. In agreement, a chromic gut-induced inflammatory nerve root insult, which does not induce BSCB breakdown (Figure 3.3), also does not increase the spinal microglia population at day 7 after insult (Rothman and Winkelstein 2007). Although that chromic gut nerve root insult is inflammatory in nature, systemic concentrations of inflammatory cytokines are not elevated at day 1 when pain is present (Table 3.2; Figure 3.3). Since pro-inflammatory serum cytokines can induce BSCB breakdown de novo (Echeverry et al. 2011, Huber et al. 2001, Pan and Kastin 2007), the lack of systemic inflammation produced after an inflammatory root insult may be responsible for the lack of BSCB breakdown after that painful inflammatory insult (Figure 3.3).
Although the roles of TNF-α and IL-1β in the development of radicular pain are well established, the contributions of IL-7 and IL-12 to pain are controversial (Chen et al. 2013, Heitzer et al. 2012, Matute Wilander et al. 2014, Zhang et al. 2015). Clinically, IL-7 transcription and expression were shown to be elevated in intervertebral disc cells from patients experiencing low back pain (Zhang et al. 2015). However, serum concentrations of IL-7 in patients with cancer-related pain increase within 3 hours of an analgesic opioid treatment (Heitzer et al. 2012). Additionally, serum levels of IL-12 are elevated in females with work-related musculoskeletal pain (Matute Wilander et al. 2014); in contrast, administration of IL-12 subcutaneously reduces mechanical allodynia and hyperalgesia for up to 4 hours when administered 9 days after a painful chronic constriction of the sciatic nerve (Chen et al. 2013). In the current study, serum concentrations of both IL-7 and IL-12 positively correlate to the severity of pain at day 1 following root compression (Table 3.1). Together with previous literature, it is clear that the roles of serum IL-7 and IL-12 in pain severity likely depend on the cause of pain. Studies blocking IL-7 and IL-12 systemically following painful root injury would provide more information on whether elevated serum concentrations of those cytokines contribute to the development of behavioral sensitivity.

The studies presented in this chapter emphasize the significance of BSCB breakdown in pain and support targeting this early injury-induced phenomenon as a novel and promising therapeutic route. The vascular-stabilizing agent, APC, was administered at 1 hour after a compressive-injury that induces BSCB breakdown (Figure 3.4). This time point was chosen as a conservative estimate based on a previous study reporting that BSCB permeability is not induced until at least 6 hours after peripheral nerve crush
(Beggs et al. 2010). In a complementary pilot study (n=3 rats), BSCB breakdown was measured at 6 hours after a painful nerve root compression in order to investigate whether BSCB breakdown occurs prior to day 1. Spinal cord tissue was harvested from rats at 6 hours a painful nerve root compression, immunolabeled for IgG and compared to tissue harvested at day 1 after a painful compression or a sham surgery. IgG labeling was not apparent in the ipsilateral spinal cord at 6 hours after a painful compression and does resemble expression in sham tissue at day 1 (Figure 3.5). The marked increase in IgG labeling by day 1 after a painful compression (Figures 3.1 & 3.5) suggests that the BSCB is disrupted between 6 and 24 hours after a 15-minute root compression. Since blood flow to the surrounding neural tissue is still not completely re-established by 3 hours after a 2-second root compression (Igarashi et al. 2005), it is possible that the 15-minute compression reduces blood flow to the spinal parenchyma on the order of hours after injury. Blood reperfusion after cerebral ischemia induces a breakdown of the BSCB for up to 1 day (Dobbin et al. 1989, Ek et al. 2015, Sage et al. 1984). It is possible that reperfusion of the ipsilateral spinal cord does not become completely restored until after 6 hours following a painful root compression and that such reperfusion contributes to BSCB breakdown, similar to the ischemia, and subsequent reperfusion, that is observed in parallel with the BBB breakdown after ischemic stroke.
Although treating a painful compression with intravenous APC completely inhibits pain development (Figure 3.4), this enzyme is a potent anticoagulant (Bernard et al. 2001, Finfer et al. 2008, Marti-Carvajal et al. 2012) and, therefore, is clinically unsafe for use after traumatic injuries such as a nerve root compression. Determining a treatment modality that activates the APC pathway to rescue vascular permeability, while at the same time promotes coagulation would be an ideal candidate to block BBB disruption and prevent development of pain after traumatic neural injury. This hypothesis is further explored in Chapter 5 through investigations of salmon thrombin’s effects on painful nerve root compression-induced BSCB through its preferential activation of protein C.

3.7. Integration & Conclusions

The studies presented in this chapter are the first to demonstrate that nerve root compression injury induces an early, but delayed, transient opening of the BSCB at the same spinal level and same side as the injury (Figure 3.2). The timing of BSCB breakdown parallels the timing of pain development (Figure 3.2A). Previous studies have
reported that other painful mechanical-based nerve injuries induce BSCB disruption (Beggs et al. 2010, Echeverry et al. 2011, Gordh et al. 2006). Nerve root compression, with or without inflammatory components, imposed chronically disrupts spinal vasculature as evidenced by increased spinal expression of endothelial cellular adhesion markers by 1 week after injury (Rutkowski et al. 2002). Increased Evans Blue extravasation into the ipsilateral spinal dorsal horn is still evident at 3 weeks after the initiation of nerve root compression (Kobayashi et al. 2008). Although these chronic mechanical nerve root injuries increase spinal vascular permeability, the studies in this chapter are the first to show that a transient compression of the nerve root induces BSCB disruption (Figure 3.2) and contributes to the onset of pain (Figure 3.4). In contrast, the inflammatory nerve root insult used in these studies does not disrupt the BSCB, despite producing pain (Figure 3.3).

Because an inflammatory insult does not mechanically disrupt the structures, whether neuronal or vascular, within the nerve root and also does not induce BSCB breakdown, the mechanical root deformation applied during compression may facilitate the BSCB breakdown, which is remote from the injury. The greater extent of spinal vascular permeability that is induced by a compressive, rather than an inflammatory, insult may also contribute to the downstream spinal glial activation that is induced to a greater extent by compression than a chronic gut insult (Rothman and Winkelstein 2007). The concentration of four pro-inflammatory cytokines (IL-7, IL-12, IL-1α, TNF-α) correlates to the behavioral sensitivity at day 1 after different durations of root compression (Table 3.1; Figure 3.2D). At this same time, TNF-α expression is elevated in the ipsilateral spinal dorsal horn in anatomical regions that also express the serum-
The findings from this chapter provide multiple layers of clinical relevance for the early identification and intervention of radicular pain. Since only compression-induced pain correlates to pro-inflammatory cytokine expression in the serum and only that painful compression induces BSCB breakdown, it is possible that elevations in serum IL-7, IL-12, IL-1α and TNF-α may enhance BSCB breakdown after neural injury. Further, serum levels of these cytokines may have potential as serum biomarkers for painful neural compression that induces BSCB breakdown. Further studies evaluating if these cytokines are only upregulated when the BSCB is compromised, or whether their elevation follows a different temporal profile than does BSCB breakdown, would be advantageous to inform clinicians on whether the BSCB is compromised. By day 1 after a painful compression, pro-inflammatory cytokine concentrations are elevated in the serum, the BSCB is disrupted, and pain is developed. As such, measuring all three of
these responses, i.e. serum cytokine concentration, BSCB permeability, and behavioral sensitivity, at time points earlier than 1 day after compression would provide a more complete picture of the contribution of systemic cytokines and BSCB breakdown to the development of pain.

Therapies that combine the administration of neuroprotective agents, which are typically prevented from reaching the CNS due to an intact BBB (Wong et al. 2013), at times of BSCB breakdown followed by agents that restore BSCB integrity might be ideal for preventing the development of neuropathic pain. A shortcoming of the current studies is that IgG immunolabeling only reveals the accumulation of that serum protein in the CNS. Therefore, robust spinal IgG immunolabeling at day 1 indicates that the BSCB was disrupted at, or before, day 1. In order to understand the dynamic responses of BSCB permeability, administration of visually absorptive or fluorescent tracers that do not normally cross the BBB, such as Evans Blue or NaFlu (Beggs et al. 2010, Echeverry et al. 2011), at early time points between 6 hours and 1 day after painful root compression would provide a more detailed temporal profile for BSCB breakdown and, so, a better understanding of the most effective timing for therapeutic intervention after injury. The current studies were focused on measured the accumulation of serum components, rather than the real-time permeability of the BSCB after compression, in order to establish whether proteins from the serum do in fact interact with cells in the spinal parenchyma and possibly contribute to the early pathologies and development of pain at day 1.

Since TNF-α expression increases in both the blood and the spinal cord (Figure 3.2D), serum factors likely cross a compression-induced compromised BSCB. Studies in Chapter 4 investigate whether thrombin, a bioactive enzyme expressed in the
bloodstream, can also cross the BSCB after a painful root compression allowing it to interact with cells within the CNS and to contribute to nociceptive processes. Those studies test the hypothesis that endogenous thrombin extravasates into the spinal parenchyma in areas that undergo BSCB breakdown after painful nerve root compression (Chapter 4). Thrombin activity is measured by immunolabeling for the expression of fibrinogen, the enzymatic polymeric product of thrombin (Boon 1993, Di Cera 2008), in the spinal cord after painful and non-painful compressions. The half-life of a fibrin clot ranges from hours to days at physiological temperature depending on the conditions (Shainoff and DiBello 2003). Although the early BSCB breakdown after compression returns to normal levels by day 7 (Figure 3.2), fibrinogen might still be present in the CNS at times later than when IgG is cleared (Cooper et al. 2013). As such, spinal fibrinogen is measured at both days 1 and 7 after painful and non-painful nerve root compression.

Previous studies have shown that thrombin-activated central PAR1 can induce astrocyte activation (Choi et al. 2008, Nicole et al. 2005, Niego et al. 2011) and behavioral sensitivity (Narita et al. 2005). In order to determine whether compression-induced spinal thrombin activity may lead to spinal astrocyte activation and pain development, thrombin activity is blocked with the specific inhibitor hirudin (Narita et al. 2005) prior to a 15-minute compression. The effect of thrombin activity in the spinal cord on the development of pain without any compression-induced biomechanical inputs was investigated by administering rat thrombin intrathecally to naïve rats. Studies in Chapter 4 also begin to investigate the role of the thrombin-activated spinal PAR1 in the development of pain and spinal glial activation by administering the PAR1 antagonist,
SCH79797 (El Eter and Aldrees 2012, Manaenko et al. 2013), with either an injection of rat thrombin or a painful 15-minute compression. Together the studies in Chapter 4 investigate one mechanism by which BSCB breakdown may facilitate the development of compression-induced pain and begin to define a novel role for spinal thrombin, and its activation of PAR1 centrally, in the development of neuropathic pain and spinal glial activation.
Chapter 4

Defining the Contribution of Spinal Thrombin to Compression-Induced Spinal Astrocyte Activation & Pain through PAR1

Parts of this chapter are adapted from:

Smith JR, Winkelstein BA. Spinal thrombin activity is elevated following nerve root injury in the rat and initiates pain through the protease-activated receptor-1. Pain, submitted.

4.1. Overview

Breakdown of the blood-brain barrier (BBB) or blood-spinal cord barrier (BSCB) is a pathological response in the central nervous system (CNS) that is induced by many types of traumatic injuries and neuropathic disorders, including spinal cord injury, traumatic brain injury, ischemic stroke, multiple sclerosis, Parkinson’s disease and Alzheimer’s disease (Hawkins and Davis 2005, Hirsch and Hunot 2009, Maikos and Shreiber 2007, Popovich et al. 1996, Ryu and McLarnon 2009, Sandoval and Witt 2008, Zlokovic 2008). Recently, studies have identified that BSCB breakdown can also occur after peripheral nerve injuries that are remote from the spinal cord itself, such as a sciatic nerve ligation or transection (Beggs et al. 2010, Cahill et al. 2014, Echeverry et al. 2011). The studies in Chapter 3 demonstrate that BSCB breakdown occurs transiently within 1
day of a painful nerve root compression and contributes to the development of pain after that injury (Smith et al. 2015). Increased permeability of the BSCB often complicates spinal pathologies by facilitating the extravasation of pro-inflammatory cytokines and chemokines into the spinal parenchyma (Banks et al. 1995, Echeverry et al. 2011, Pan et al. 1997, Pan and Kastin 2007, Sharief and Thompson 1992). Of note, tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), both of which have been reported to cross a compromised BSCB barrier (Banks et al. 1995, Echeverry et al. 2011, Pan and Kastin 2007), also contribute to pain once they are active in the spinal cord after nerve root injury (de Souza Grava et al. 2012, Rothman et al. 2009, Rothman and Winkelstein 2010). Findings from the studies in Chapter 3 suggest that an increase in spinal TNF-α expression at day 1 after painful root compression is at least partially attributed to its extravasation across the BSCB since TNF-α co-localizes with the serum protein immunoglobulin G (IgG) (Figure 3.2) (Smith et al. 2015). However, it is not known whether other blood-borne proteins or enzymes also extravasate into the spinal parenchyma and contribute to spinal pathologies and pain following injury.

Of particular interest, thrombin is a serum enzyme that is not found in the CNS under normal healthy conditions but has been implicated in CNS pathology. For example, endogenous thrombin contributes to BBB breakdown after ischemic stroke and potentiates the inflammation and edema in the brain that are associated with injury and unfavorable patient outcomes (Junge et al. 2004, Kawai et al. 2001, Lee et al. 1997, Liu et al. 2010). When thrombin is exogenously administered into the CNS it can induce astrocyte activation (Liu et al. 2010, Nishino et al. 1993); separately thrombin has been shown to produce pain (Narita et al. 2005). Together, these pro-inflammatory effects of
thrombin in the CNS highlight a potential pathological role for this enzyme in painful neuropathic disorders. Thrombin is a versatile enzyme and can activate a large number of substrates, many of which are cell bound receptors that when activated initiate cell signaling cascades (Di Cera 2008, Lane et al. 2005). Thrombin-induced activation of astrocytes has largely been attributed to its cleavage of the protease-activated receptor-1 (PAR1) (Choi et al. 2008, Fan et al. 2005, Nicole et al. 2005). As such, endogenous thrombin is hypothesized to extravasate into the spinal parenchyma during the BSCB breakdown that occurs after painful nerve root injury and contributes to astrocyte activation and pain through its activation of spinal PAR1.

The studies presented in this chapter focus on addressing Aim 2 and are broken into two major sections. Studies in Section 4.3 measure whether thrombin is present in the spinal cord after separate painful and non-painful nerve root compressions and also determine the contribution of spinal thrombin to nerve root-mediated pain through its activation of PAR1. Studies outlined in Section 4.4 investigate whether spinal thrombin activity contributes to spinal astrocytic activation, which has been previously documented at day 7 after a painful nerve root compression injury (Hubbard and Winkelstein 2005, Rothman et al. 2010, Rothman and Winkelstein 2007). Because thrombin alone has been shown also to induce vascular leakiness (Komarova et al. 2007, Liu et al. 2010), the role of spinal thrombin in BSCB breakdown after painful root compression is investigated in parallel studies (Section 4.4).

Studies in Sections 4.3 and 4.4 utilize the same nerve root compression injury model in the rat as presented in Chapter 3. By administering various pharmacologic agents to manipulate the thrombin/PAR1 signaling pathway centrally, the studies
investigate whether spinal thrombin contributes to BSCB breakdown, spinal glial activation, and pain following painful nerve root compression. The selective thrombin inhibitor, hirudin, was administered intrathecally prior to a painful root compression in order to block spinal thrombin; the effects of hirudin on spinal thrombin activity, via immunolabeling for fibrin(ogen), and the development of mechanical hyperalgesia was measured over 7 days (Section 4.3). The effects of hirudin on compression-induced BSCB breakdown and spinal astrocyte activation were also measured by immunolabeling for IgG at day 1 and for glial fibrillary acidic protein (GFAP) at day 7 after injury (Section 4.4). These time points were selected because BSCB breakdown is evident for 1 day after compression and spinal GFAP expression is robust by day 7, respectively. Exogenous rat thrombin was administered in naïve rats in order to measure its capability of inducing mechanical hyperalgesia (Section 4.3). BSCB breakdown and spinal astrocyte activation were measured by immunolabeling for IgG and GFAP at the time (day 1) when behavioral sensitivity is most robust after injection (Section 4.4). Lastly, in order to determine whether thrombin acts through PAR1 in the spinal cord to induce pain, BSCB breakdown and astrocyte activation, a PAR1 antagonist, SCH79797, was administered prior to an injection of rat thrombin and prior to a painful compression, separately (Sections 4.3 and 4.4). Together, these studies begin to define if there is a pathophysiologic role of spinal thrombin activation of PAR1 in the BSCB breakdown and glial activation that are associated with nerve root-mediated pain.
4.2. Relevant Background

Chronic pain affects over 100 million adults in the United States each year, which is greater than the numbers of people affected by cancer, heart disease and diabetes combined (Institute of Medicine (US) Committee on Advancing Pain Research 2011, Tsang et al. 2008). Despite the relatively high prevalence of painful disorders, current available treatments are not effective at treating all of the presenting symptoms (Arner and Meyerson 1988, Baron et al. 2010, Dworkin et al. 2003, Finnerup et al. 2010), partly owing to the fact that the biochemical mechanisms maintaining pain after neuropathic injuries within the peripheral or CNS (Baron et al. 2010, Scholz and Woolf 2007, Woolf 2004, Woolf and Mannion 1999) are not fully understood. Such insults have been shown to induce a host of cellular and biochemical cascades within the CNS that contribute to both the initiation and maintenance of pain (Milligan and Watkins 2009, Scholz and Woolf 2007, Watkins et al. 2001). Previous studies have shown that mechanical injury to either a peripheral nerve or nerve root increases the permeability of the BSCB (Beggs et al. 2010, Echeverry et al. 2011, Gordh et al. 2006, Kobayashi et al. 2008), which facilitates the transmission of serum molecules into the CNS where they interact with, and activate, resident neuronal and glial cells (Ballabh et al. 2004, Radu et al. 2013, Webb and Muir 2000). We recently showed that BSCB breakdown occurs exclusively after nerve root compression that also induces pain and that blocking early BSCB breakdown prevents pain from developing (Smith et al. 2015). Yet, it is not known if certain serum proteins that extravasate into the CNS during increased BSCB permeability contribute to the development of nerve root induced pain.
Thrombin is a bioactive blood-borne enzyme that does not interact with neural cells under normal healthy conditions, but when it is introduced into the CNS, it induces cellular cascades that promote pain (Garcia et al. 2010, Niego et al. 2011, Nishino et al. 1993). Human thrombin that is continuously infused at high concentrations over 7 days into the rat midbrain induces elevated vimentin-positive astrocytes (Nishino et al. 1993). Although that study did not measure behavioral sensitivity, spinal astrocyte activation has been shown to occur in parallel with pain induced by a variety of neuropathic pain conditions in animal models (Hubbard and Winkelstein 2005, Rothman et al. 2010, Rothman and Winkelstein 2007). Spinal thrombin also has been related to the development of pain; intrathecal administration of thrombin induces tactile allodynia in naïve mice that lasts for up to 10 days and blocking spinal thrombin activity prior to a sciatic nerve ligation produces less pain than an untreated injury for up to 7 days (Narita et al. 2005). Together, these studies suggest that spinal thrombin may induce mechanical allodynia and may contribute to that pain that develops after neural injury. Yet, it is not known how thrombin is produced in the CNS or whether and how it may enter the CNS after injury; further, it remains unknown if such a response is specific to painful injury.

Although thrombin is a key enzyme in clot formation by cleaving fibrinogen into fibrin, it also initiates a variety of subcellular signaling cascades by activating cell surface receptors or cell bound substrates (Coughlin 2000, Di Cera 2008). Of the known thrombin activated receptors, mammalian thrombin preferentially activates protease-activated receptor-1 (PAR1), which is expressed in multiple cell types within the CNS, including neurons, astrocytes and microglia (Shavit et al. 2011, Suo et al. 2002, Vellani et al. 2010). PAR1 activation in the CNS has been implicated in glial activation and related
inflammatory responses (Bunnett 2006, Fan et al. 2005, Nicole et al. 2005, Suo et al. 2002). Administration of the synthetic peptide, TFLLR, which selectively activates PAR1, into the striatum of mice induces local astrocyte activation and proliferation (Nicole et al. 2005). Not only does selective activation of PAR1 induce neuroinflammatory cascades, but blocking PAR1 activation or suppressing PAR1 expression also attenuates thrombin-induced inflammation (Choi et al. 2008, Scarisbrick et al. 2012). For example, blocking PAR1 activation with the small molecule inhibitor, SCH79797, in glial cultures inhibits protease-induced astrocytic activation as well as astrocytic production of the pro-inflammatory proteins, IL-6 and matrix metalloproteinase-9 (MMP-9), (Choi et al. 2008, Scarisbrick et al. 2012). Further, when microglia from mice lacking PAR1 are stimulated by thrombin they produce significantly less TNF-α compared to wild type microglia (Suo et al. 2002), suggesting that thrombin acts through PAR1 to initiate this pro-inflammatory cascade. Because PAR1−/− mice exhibit less astrocyte activation in the regions of a wound after a cortical incision (Nicole et al. 2005), it is possible that injury-induced astrocytic activation may depend on PAR1 expression. Despite PAR1’s implication in glial-controlled inflammatory processes and the well-established role of gliosis in pain (McMahon et al. 2005, Milligan and Watkins 2009, Vallejo et al. 2010, Watkins et al. 2001), no study has defined the contribution of thrombin’s activation of spinal PAR1 pain.

The first set of studies in this Chapter test the hypothesis that thrombin extravasates into the spinal parenchyma in areas that undergo BSCB breakdown after painful nerve root injury, inducing behavioral sensitivity through its enzymatic activation of PAR1 (Section 4.3). Complementary studies utilized a controlled nerve root
compression injury in the rat and a variety of pharmacological interventions. First, the ability of thrombin to act enzymatically in the spinal cord after a nerve root compression that induces mechanical hyperalgesia was examined. Spinal immunolabeling for fibrin(ogen), which labels fibrin and fibrinogen, was evaluated as a proxy of spinal thrombin activity and was co-immunolabeled with the serum-derived IgG to reveal regions where thrombin is localized to areas of increased BSCB permeability. Spinal thrombin activity was then inhibited using hirudin prior to a painful nerve root compression and mechanical hyperalgesia was monitored in the forepaw ipsilateral to injury over time to determine whether spinal thrombin contributes to the development of compression-induced pain. The last part of these studies tested whether spinal thrombin acts through PAR1 to produce pain using the PAR1 inhibitor, SCH79797, administered intrathecally. Rat thrombin was delivered to naïve rats, with or without pre-treatment with SCH79797, and bilateral mechanical hyperalgesia was measured for up to 7 days. SCH79797 was also given prior to a painful nerve root compression; mechanical hyperalgesia was measured in the ipsilateral forepaw for 7 days and spinal fibrin(ogen) and IgG were labeled at days 1 and 7 after that injury to investigate whether spinal PAR1 is requisite for development of radicular pain, even under conditions of elevated spinal thrombin activity.

The second set of studies in this chapter expands the first set of studies and tests the hypothesis that increases in spinal thrombin after painful root compression contribute to spinal astrocyte activation through its activation of spinal PAR1 (Section 4.4). Because thrombin is a known inducer of vascular permeability (Komarova et al. 2007, Liu et al. 2010) and BSCB breakdown is robust at day 1 after a painful compression (Figure 3.2)
(Smith et al. 2013), the contribution of spinal thrombin to that early compression-induced BSCB was also investigated. The expression of PAR1 in spinal astrocytes after painful and non-painful compressions was measured over time in order to determine whether modulations in astrocytic PAR1 expression occur exclusively in response to a painful root injury. The contribution of compression-induced spinal thrombin on BSCB breakdown and spinal astrocyte activation was investigated by using a hirudin pre-treatment before injury and immunolabeling for IgG at day 1 and GFAP at day 7 after injury. The direct action of thrombin, absent any other stimuli, on both of these spinal pathologies was then measured in order to investigate the sole effects of spinal thrombin without any other injurious inputs. Rat thrombin was given intrathecally to naïve rats and spinal IgG and GFAP were measured at day 1 when mechanical hyperalgesia is most robust (Smith and Winkelstein 2015). Lastly, in order to investigate the involvement of spinal PAR1 in BSCB breakdown and astrocyte activation in the spinal cord, SCH79797 was administered prior to a painful rat thrombin administration or painful nerve root compression and spinal IgG and GFAP expression were measured. The collective findings from both of the complementary studies investigating the role of spinal thrombin and its activation of PAR1 on the induction of pain, spinal astrocyte activation, and BSCB breakdown are discussed and integrated in Sections 4.5 and 4.6.
4.3. Defining the Contribution of Spinal Thrombin & PAR1 Activation to Pain

4.3.1. Study Design

4.3.1.1. Assessing Compression-Induced Spinal Thrombin Effects on Pain

In order to determine whether endogenous thrombin is enzymatically active in the spinal cord after neural injury, immunohistochemical techniques were used to label spinal fibrin(ogen) expression after different durations of nerve root compression that either do or do not induce pain (Rothman et al. 2010). A 15-minute (15min, n=10), 3-minute (3min, n=10) or 0-minute (sham, n=11) compression was separately applied to the nerve root using previously described surgical procedures on day 0 (Rothman et al. 2010). Mechanical hyperalgesia was measured in the ipsilateral forepaw in rats on day 0 before surgery (baseline) and on days 1, 3, 5 and 7 after surgery. Time-dependent differences in paw withdrawal threshold between 15min (n=5), 3min (n=5) and sham (n=6) were determined using a two-way repeated measures ANOVA (group x day) with Tukey’s Honestly Significant Difference test.

Spinal cord tissue at C7 was harvested on day 1 (15min, n=5; 3min, n=5; sham, n=5) or day 7 (15min, n=5; 3min, n=5; sham, n=6) in separate groups, in order to evaluate the temporal spinal fibrin(ogen) immunolabeling extent and pattern. Spinal fibrin(ogen) expression in the ipsilateral dorsal horn was quantified using densitometry (Nicholson et al. 2012, Rothman et al. 2010), and compared to fibrin(ogen) expression in matching spinal tissue from normal naïve rats. Significant differences were evaluated using a two-way ANOVA (group x day) with Tukey’s test. The spinal tissue taken from
the rats undergoing a 15-minute compression was also immunolabeled for fibrin(ogen) with the serum component, IgG, in order to determine whether spinal thrombin activity is localized to spinal regions that undergo BSCB breakdown.

In a complementary study, spinal thrombin activity was blocked prior to imposing the painful 15-minute compression in order to assess whether nerve root-induced spinal thrombin contributes to the development of pain after that injury. Rats received an intrathecal injection of hirudin, a selective thrombin inhibitor, 1 day prior to nerve root compression (hir+15min, n=6). Mechanical hyperalgesia was measured in the ipsilateral forepaw before hirudin (day -1), after hirudin but before surgery (day 0), and on alternating days (1, 3, 5, and 7) after injury. The ipsilateral paw withdrawal threshold was compared between hir+15min and 15min (n=5) and statistical differences in withdrawal threshold between hir+15min and 15min were determined using a two-way repeated measures ANOVA (group x day) with Tukey’s test.

At day 7, spinal cord tissue was harvested from rats that were given hirudin intrathecally with a 15-minute compression and immunolabeled for fibrin(ogen) and IgG. An additional group of rats received an intrathecal injection of hirudin before undergoing a 15-minute root compression; spinal cord tissue was harvested from those rats at day 1 after injury (n=5) and immunolabeled for fibrin(ogen). Quantification of fibrin(ogen) expression for hir+15min was compared to fibrin(ogen) expression in comparable tissue from rats undergoing a 15-minute compression (day 1, n=5; day 7, n=6) but not receiving hirudin treatment; spinal fibrin(ogen) expression was normalized to expression in naïve tissue and differences were compared using a two-way ANOVA (group x day) with Tukey’s test.
4.3.1.2. Investigating the Effects of Thrombin-Activated Spinal PAR1 on Pain

In order to determine the effects of spinal thrombin on non-injury induced pain, rat thrombin was administered via a single intrathecal injection to naïve rats (RTh, n=6). Mechanical hyperalgesia was measured in the bilateral forepaws on day 0 before, and on days 1, 3, 5 and 7 after, thrombin injection; the left and right paw withdrawal thresholds were averaged for each rat since this treatment is ubiquitous and not expected to have preferential effects on hyperalgesia in the bilateral forepaws. The average paw withdrawal threshold at days 1, 3, 5, and 7 were compared to baseline (day 0) thresholds using separate two-tailed paired t-tests in order to determine if the threshold on each of those days is changed from baseline.

A separate complementary study was performed to assess whether spinal thrombin acts through PAR1 to induce pain. Rats received a single injection of rat thrombin (RTh, n=6) at day 0 and mechanical hyperalgesia was measured in the bilateral forepaws at day 0 before, and at day 1 after, the thrombin injection. In a separate group of rats, the small molecule PAR1 inhibitor, SCH79797, was injected intrathecally 1 day before the rat thrombin administration (SCH+RTh n=6). Mechanical hyperalgesia was measured bilaterally prior to SCH injection (day -1), after SCH treatment but before rat thrombin injection (day 0), and on day 1 after rat thrombin administration. Differences in the average bilateral paw withdrawal threshold between the SCH+RTh and RTh groups were detected using a two-way repeated measure ANOVA (group x day) with Tukey’s test.
4.3.1.3. Determining the Effects of Spinal PAR1 Activation on Compression-Induced Pain

In order to examine whether PAR1 activation contributes to the development of pain after a compressive nerve root injury, SCH79797 was administered a day before a 15-minute nerve root compression (SCH+15min, n=7) in a separate group of rats. Since the vehicle for SCH79797 contains dimethyl sulfoxide (DMSO), which is not biologically inert (Galvao et al. 2014, Qi et al. 2008), control groups were included in which DMSO was administered intrathecally at 1 day before either a 15-minute root compression (DMSO+15min, n=5) or a sham surgical procedure (DMSO+sham, n=5). Ipsilateral forepaw mechanical hyperalgesia was measured on the day before (day -1) SCH79797 or vehicle injection, on day 0 after injection but before surgery, and on days 1, 3, 5 and 7 after surgery. Differences in withdrawal threshold between SCH+15min, DMSO+15min and DMSO+sham were compared with a two-way repeated measures ANOVA (group x day) with Tukey’s test.

In order to determine whether thrombin remains active in the spinal cord over time despite the SCH79797 treatment, spinal tissue was harvested on day 1 from separate groups of rats receiving a 15-minute compression with either a SCH79797 or a DMSO vehicle pre-treatment. Fibrin(ogen) labeling was measured in spinal cord tissue at day 1 (SCH+15min, n=6; DMSO+15min, n=4) and day 7 (SCH+15min, n=7; DMSO+15min, n=5; DMSO+sham, n=5) and compared using a two-way ANOVA (group x day) with Tukey’s test.
4.3.2. Specific Detailed Methods

4.3.2.1. Animals

All studies were performed using adult male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, IN). Rats were housed under conditions approved by the United State Department of Agriculture (USDA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in a temperature- and light-controlled room with free access to water and food. All operational and experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP) (Zimmermann 1983).

4.3.2.2. Surgical Procedures for Nerve Root Compression

Surgical procedures were performed under inhalation isoflurane anesthesia (4% for induction, 3% for maintenance). Previously reported protocols were followed for administering nerve root compression injury (Chang and Winkelstein 2011, Nicholson et al. 2012, Rothman et al. 2010). Briefly, rats were placed in a prone position, a midline incision was made along the back of the neck and the paraspinal muscles were removed to expose the C6 and C7 vertebrae. A C6-C7 hemilaminectomy and partial facetectomy was performed on the right side to expose the right C7 dorsal nerve root. A small incision was made in the dura over the C7 root and a calibrated 10gf microvascular clip (World Precision Instruments; Sarasota, FL) was applied to the exposed root. The root was compressed for either 3 or 15 minutes after which the clip was removed and the wound
was closed by 3-0 polyester suture and surgical staples. Rats were allowed to recover in room air with continual free access to food and water. Sham operated rats underwent identical surgical procedures except did not undergo nerve root compression.

4.3.2.3. Optimization of Dosing of the Pharmacologic Agents

Three separate drugs were administered in the studies in this chapter: hirudin, rat thrombin, and SCH79797. Initial doses for each agent were chosen based on previous studies in the literature and review of the doses that had behavioral effects. Hirudin was administered prior to a 15-minute nerve root compression and its ability to inhibit the development of pain over 7 days was evaluated. Only one previous study was identified that administered hirudin intrathecally (Narita et al. 2005). In that study, hirudin was administered intrathecally at a dose of 30pmol/day for a 25g mouse for 8 days after a sciatic nerve ligation; that dose prevented mechanical allodynia from developing during the dosing period and for 8 days after the cessation of treatment (Narita et al. 2005). It is important to note that 1 NIH unit (U) of thrombin is equivalent to 8.85±2.01pmol of thrombin (Hemker 2012), and 1U of hirudin neutralizes 1U of thrombin at 37°C. Adjusting for the weight of a rat being approximately 15 times as large as a mouse, hirudin was chosen to be initially administered only once at a dose of 4.2U/rat (n=6), injected intrathecally at 1 day prior to a painful nerve root compression. Since that initial dose was sufficient to prevent the development of pain for at least 7 days in the rat, hirudin was given at 4.2U/rat for all studies in this chapter.

Rat thrombin was administered intrathecally to naïve rats and its ability to produce mechanical hyperalgesia was tested in the bilateral forepaws over 7 days. A
previous study reported that a single intrathecal injection of thrombin (1pmol/mouse) in naïve mice produced mechanical allodynia for at least 8 days (Narita et al. 2005). Further, rat thrombin infused into the midbrain of rats at a rate of 0.5U/hour induces a significant increase in local astrocyte activation after 7 days of constant infusion (Nishino et al. 1993). Based on those reports, three doses of rat thrombin were chosen for pilot studies in order to measure their effect on mechanical hyperalgesia: 0.04U/rat (RTh 0.04, n=4), 0.4U/rat (RTh 0.4, n=5) and 4U/rat (RTh 4, n=6). Mechanical hyperalgesia was measured on day 0 before injection (baseline) and on days 1, 3, 5, and 7 after injection. Significant differences in hyperalgesia were evaluated using individual paired t-tests comparing the withdrawal threshold at each day to corresponding baseline thresholds, for each dose separately. Of the pilot doses, only the highest (4U/rat) produced a significant drop in the paw withdrawal threshold, which was apparent at days 1 and 3 after injection (Figure 4.1). The two lower doses (0.04 and 0.4U/rat) did not alter the threshold at day 1, although the 0.4U/rat dose did induce a delayed significant drop in forepaw withdrawal threshold that was evident at day 3 (Figure 4.1). As such, a dose of rat thrombin for 4U/rat was chosen for the rest of the studies in this chapter.
Spinal PAR1 was blocked with the small molecule inhibitor, SCH79797, administered intrathecally 1 day before a painful compression. A previous study found that an intrathecal injection of 25µg/kg of SCH79797 was effective at reducing edema in the brain within 1 day after a surgically-induced brain injury, whereas a dose of 1µg/kg did not (Manaenko et al. 2013). In another study, SCH79797 was administered intraperitoneally at doses over a range of 10-250µg/kg, with 25-100µg/kg of SCH79797 reducing brain water content at day 1 after subarachnoid hemorrhage (Yan et al. 2013). The effects of two different intrathecal doses of SCH79797 (25µg/kg, n=4; 50µg/kg, n=7) on mechanical hyperalgesia when given intrathecally prior to a painful compression were measured over 7 days. A vehicle control containing dimethyl sulfoxide (DMSO; 0µg/kg, n=5) in phosphate buffered saline (PBS) was also included for comparison purposes. Mechanical hyperalgesia was measured on day 0 before injection (baseline) and on days...
1, 3, 5, and 7 after root compression. Differences in hyperalgesia were determined using a two-way ANOVA (group x day) with Tukey's test comparing between both doses of SCH79797 and the vehicle.

As expected, the vehicle treatment did not prevent the development of mechanical hyperalgesia and induced a significant drop in withdrawal threshold for the ipsilateral forepaw at day 1 compared to baseline (day 0) (Figure 4.2). Of the treatment doses tested, only 50µg/kg of SCH79797 was sufficient to inhibit the production of mechanical hyperalgesia at day 1 after compression (Figure 4.2); therefore, that dose (50µg/kg) of SCH79797 was used for the studies in this chapter.
4.3.2.4. Preparation & Intrathecal Administration of Agents

Thrombin from rat plasma (Sigma Aldrich; St. Louis, MO) was dissolved in sterile phosphate-buffered saline (PBS; Mediatech, Inc.; Manassas, VA) and stored at -80°C until further use, avoiding repeated freeze-thaw cycles. Rat thrombin was administered to rats via intrathecal injection at a final dose of 4U/rat (Narita et al. 2005). Hirudin (Sigma Aldrich; St. Louis, MO) was dissolved in sterile PBS, stored at -20°C and administered intrathecally (4.2U/rat) 1 day prior to any surgical procedures (day -1) (Narita et al. 2005).

SCH79797 dihydrochloride (N³-Cyclopropyl-7-[[4-(1-ethylethyl)phenyl]methyl]-7H-pyrrolo[3,2-f]quinazoline-1,3-diamine dihydrochloride; Tocris; Bristol, United Kingdom) was fully dissolved in DMSO (Sigma Aldrich; St. Louis, MO) at a stock concentration of 10mg/ml and was stored at -20°C. When ready for use, the DMSO SCH79797 solution was diluted with sterile PBS and administered intrathecally (50µg/kg) 1 day before surgery or rat thrombin administration (El Eter and Aldrees 2012, Manaenko et al. 2013). A DMSO vehicle control was also included for comparison purposes; DMSO alone was diluted in sterile PBS (1:15) and intrathecally delivered to rats 1 day prior to surgery.

All drug solutions were administered in the intrathecal space between the L4 and L5 vertebrae via a lumbar puncture via a 25G needle and syringe (Rothman and Winkelstein 2010). Lumbar puncture volume was the same (30µl) injection for each treatment. The paw withdrawal thresholds for rats in the dosing studies for rat thrombin and SCH79797 are detailed in Appendix A.

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4.3.2.5. Behavioral Assessment of Mechanical Hyperalgesia

Behavioral sensitivity was defined as the response threshold of the rat forepaw to an applied mechanical stimulus. Mechanical thresholds were measured using previously defined methods applying a series of von Frey filaments of increasing strength to the forepaw (Chang and Winkelstein 2011, Chaplan et al. 1994, Lee and Winkelstein 2009). On each testing day, rats were acclimated for 15 minutes to the testing apparatus, which consisted of an elevated mesh-floored cage. A series of calibrated von Frey filaments (1.4, 2, 4, 6, 8, 10, 15, and 26 g) (Stoelting; Wood Dale, IL) was applied in ascending order to the plantar surface of the forepaw until stimulation by a filament induced a positive response, which consisted of the rat withdrawing its forepaw and often was accompanied with shaking or licking of the paw (Chang and Winkelstein 2011, Chaplan et al. 1994, Lee and Winkelstein 2009). On each testing day rats underwent three consecutive rounds of testing. In each round, the filaments were applied for five stimulations until a positive response was elicited. If a rat responded to two consecutive filaments, the lower strength filament was recorded as the paw withdrawal threshold. If no filament elicited a response, then the highest magnitude filament (26g) was recorded as the threshold. On each testing day, the mechanical threshold was measured three times for each forepaw tested and averaged for each rat across the rounds.

For groups that received intrathecal injection(s) but did not undergo surgical procedures (i.e. RTh and SCH+RTh), the forepaw withdrawal threshold was measured in both the left and the right forepaws on each testing day. The withdrawal thresholds were then averaged between the bilateral forepaws for each rat on each testing day so that only one threshold value was included for each rat for analysis. For groups that underwent
surgery to expose the right C7 nerve root (for either a compression injury or a sham procedure, with or without treatments), the withdrawal threshold was measured only in the forepaw on the side ipsilateral to surgery and were averaged across the three testing rounds on each day.

4.3.2.6. Tissue Harvest, Immunofluorescent Labeling & Quantification of Fibrinogen

Rats were deeply anaesthetized with an overdose of sodium pentobarbital (Oak Pharmaceuticals Inc.; Lake Forest, IL) administered intraperitoneally at a dose of 65mg/kg in order to prepare for tissue harvest. Rats were transcardially perfused with 1% PBS until blood ran clear and then with 300ml of 4% paraformaldehyde (Sigma; St. Louis, MO). The C7 spinal cord was exposed via a C6-C7 full laminectomy and facetectomy and harvested en bloc. Cervical spinal tissue was post-fixed overnight in 4% paraformaldehyde, transferred to 30% sucrose for one week at 4°C and then embedded in OCT medium (Sakura Finetek USA, Inc.; Torrance, CA) for cryosectioning. Fixed spinal cord tissue was sectioned axially at a 14µm thickness along the long-axis and mounted directly onto slides for immunolabeling. C7 spinal cord tissue was also harvested from naïve rats (n=2) and included in tissue processing for normal comparison.

Spinal cord sections at the C7 level were fluorescently co-immunolabeled for fibrinogen, as a proxy for thrombin activity (Cortes-Canteli et al. 2012), and immunoglobulin G (IgG), as an indicator of BSCB breakdown (Echeverry et al. 2011, Poduslo et al. 1994). Briefly, slide-mounted tissue sections were blocked in 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton X-100 (Bio-Rad
Laboratories; Hercules, CA) for 1 hour at room temperature. Slides were incubated overnight at 4°C with FITC-labeled rat fibrin(ogen) (1:100; Dako; Carpinteria, CA) followed by a 2-hour incubation at room temperature with goat anti-rat IgG Alexa Fluor 568 (1:200; Life Technologies; Carlsbad, CA). Slides were then washed with PBS and water and cover slipped with fluoro-gel with TRIS buffer (Electron Microscopy Sciences; Hatfield, PA).

The ipsilateral dorsal horn was digitally imaged at 10x for 2-6 spinal sections for each rat. Fibrin(ogen) labeling was quantified in images that were uniformly cropped to include the dorsal horn using a custom densitometry MATLAB script (Rothman et al. 2010). A pixel intensity threshold was set in order to include positive fibrin(ogen) labeling in C7 spinal cord tissue from normal naïve rats; this threshold was kept constant for all analyses. The percent of the total pixels in the tissue area that was above that threshold of positive labeling for fibrin(ogen) in normal tissue was taken as the percent positive pixels in each tissue section (Nicholson et al. 2012, Rothman and Winkelstein 2007). Percent positive fibrin(ogen) was then normalized to labeling in tissue from naïve rats and data were represented as expression relative to normal tissue. Differences in fibrin(ogen) labeling in the spinal cord was determined between groups (as outlined in Section 4.3.1) using separate two-way ANOVAs (group x day) with a Tukey’s test.
4.3.3. Results

4.3.3.1. Painful Nerve Root Compression Promotes Extravasation of Spinal Thrombin that Contributes to Pain

A 15-minute nerve root compression significantly reduces the paw withdrawal threshold of the ipsilateral forepaw compared to baseline levels, reflecting an increase in pain, that is induced by day 1 (p<0.001) (Figure 4.3A). That reduction in threshold from baseline is maintained until day 7 (p<0.001) (Figure 4.3A). At day 1, the rats undergoing a 15-minute compression also exhibit a significantly lower (p<0.001) withdrawal threshold than those undergoing a corresponding sham procedure and this difference is also detected until day 7 (Figure 4.3A). In contrast, a 3-minute compression does not induce any change in paw withdrawal threshold from baseline at any time point probed (Figure 4.3A). However, when comparing the overall group responses, rats undergoing a 3-minute compression do exhibit significantly lower (p=0.028) withdrawal thresholds compared to those exposed to a sham surgery, and overall, the withdrawal threshold is not different compared to rats undergoing a 15-minute compression (Figure 4.3A).

In parallel with the production of pain at day 1, a 15-minute compression also induces a marked increase in fibrin(ogen) immunolabeling in the ipsilateral spinal dorsal horn (Figure 4.3B). The 3-minute compression exhibits less spinal fibrin(ogen) labeling compared to a 15-minute compression and only a slight increase when compared to sham levels, which are virtually absent (Figure 4.3B). When quantified, a 15-minute compression induces a significant increase (p<0.001) in spinal fibrin(ogen) compared to sham at day 1 (Figure 4.3C). At day 7, spinal fibrin(ogen) decreases to sham levels for both of the compression durations (Figure 4.3B). Spinal fibrin(ogen) expression is
significantly reduced (p=0.003) at day 7 after a 15-minute compression as compared to the levels of fibrin(ogen) at day 1 (Figure 4.3C). When assessing overall differences between groups, spinal fibrin(ogen) expression induced by a 15-minute compression is significantly higher than levels induced by either a 3-minute compression (p=0.0015) or a sham procedure (p=0.0001) (Figure 4.3C).

Figure 4.3. Spinal fibrin(ogen) expression increases early after painful nerve root compression in areas of BSCB breakdown. (A) A 15-minute compression (15min) significantly reduces the withdrawal threshold at days 1 and 7 relative to corresponding responses at day 0 (*p<0.001) and to sham responses (*p<0.001) at both days 1 and 7. (B) Fibrin(ogen) is robustly expressed at day 1 in the spinal dorsal horn (dotted white outline) for 15min, but is minimally expressed for sham and 3min. (C) The trends are similar for both days, with 15min significantly increasing (*p<0.001) the percent fibrin(ogen) compared to levels after sham at day 1. By day 7 after 15min, percent fibrin(ogen) is significantly reduced (#p=0.003) compared to its expression at day 1. (D) Spinal fibrin(ogen) and IgG are highly co-localized (indicated by yellow) on day 1 after 15min. All data are mean±SD.
The robust expression of fibrin(ogen) labeling in the ipsilateral spinal cord induced at day 1 after the painful 15-minute compression strongly co-localizes with IgG labeling (Figure 4.3D). The co-localization of fibrin(ogen) with the serum-derived IgG suggests that thrombin likely extravasates into the spinal parenchyma with other serum proteins at times when BSCB breakdown occurs, which happens only after neural injury that also induces pain (Figure 3.2).

4.3.3.2. Inhibiting Spinal Thrombin Prevents Nerve Root Pain

Spinal fibrin(ogen) is elevated by day 1 after a painful 15-minute compression; this increase in fibrin(ogen) significantly decreases (p<0.001) by day 7 after that injury (Figures 4.3B & 4.4). A single intrathecal administration of hirudin substantially reduces the compression-induced increases in fibrin(ogen) and IgG labeling that are evident at day 1 in the ipsilateral spinal cord (Figure 4.4A). When quantified, hirudin treatment significantly reduces (p=0.006) spinal fibrin(ogen) expression at day 1 compared to an untreated 15-minute compression (Figure 4.4B). By day 7, the spinal fibrin(ogen) expression pattern is not different between an untreated 15-minute compression and a compression that was pre-treated with hirudin (Figure 4.4A). Indeed, the quantified spinal fibrin(ogen) expression is not different between those two groups at day 7 (Figure 4.4B). These results confirm that hirudin pre-treatment of nerve root compression prevents the enzymatic activity of thrombin in the spinal cord early after injury.
The withdrawal threshold in the ipsilateral forepaw is significantly reduced (p<0.001) from the corresponding baseline levels by day 1 and for all subsequent days tested after a 15-minute nerve root compression (Figure 4.5). Pre-treating with hirudin 1 day before that painful compression prevents the compression-induced reduction in the forepaw withdrawal threshold (Figure 4.5). Rats undergoing compression alone exhibit a significantly lower (p<0.011) threshold than rats receiving hirudin intrathecally at day 1 prior to a compression for all days tested (Figure 4.5).
4.3.3.3. Spinal Thrombin Initiates Pain through PAR1 Activation

Rats receiving a single intrathecal injection of rat thrombin (4U/rat) exhibit a significant decrease (p=0.032) in the withdrawal threshold at day 1 (Figure 4.6A). The paw withdrawal threshold remains significantly lower (p=0.018) than baseline levels at day 3 after an injection of rat thrombin, but returns to baseline levels by day 5 (Figure 4.6A). A single intrathecal injection of rat thrombin induces a significant reduction (p<0.001) in the bilateral withdrawal threshold on day 1 compared to the threshold at day 0 (Figure 4.6B). However, blocking spinal PAR1 with intrathecal SCH79797 prior to the rat thrombin administration prevents the development of the mechanical hyperalgesia at day 1 that is produced by rat thrombin alone. The withdrawal thresholds at day 1 in rats treated with SCH79797 prior to rat thrombin are not different from the thresholds before the thrombin administration (at day 0) (Figure 4.6B).
4.3.3.4. Blocking PAR1 Inhibits Nerve Root-Induced Pain

Intrathecally blocking PAR1 with SCH79797 prior to a 15-minute compression prevents compression-induced mechanical hyperalgesia. By day 3, SCH79797 treatment prevents (p<0.0065) the drop in withdrawal threshold that is evident with a 15-minute compression receiving vehicle pre-treatment, which is sustained for up to 7 days after injury (Figure 4.7). Overall, SCH79797 reduces hyperalgesia to sham levels; the same is evident each testing day except for day 7, when a compression treated with SCH79797 exhibits a significantly lower (p=0.020) threshold compared to a sham procedure treated...
with vehicle (p=0.020) (Figure 4.7). Of note, at day 1, the withdrawal thresholds are not different for rats undergoing root compression with vehicle treatment and those receiving a sham operation with vehicle treatment (Figure 4.7). In contrast, the respective untreated compression and sham groups at this same time point exhibit a significant difference in mechanical hyperalgesia (Figure 4.3A). However, by day 3 after a compression with a vehicle treatment, the typical development of a significantly lower (p<0.001) withdrawal threshold than sham responses are evident (Figure 4.7). The increased sensitivity relative to sham responses is maintained until day 7 (Figure 4.7).

Spinal fibrin(ogen) expression is robustly increased over normal levels at day 1 after a 15-minute compression with a vehicle injection (Figure 4.8A). This response parallels the increase in fibrin(ogen) expression that is evident in the ipsilateral spinal
cord at day 1 after an untreated compression (Figure 4.3). However, unlike SCH79797 treatment which returns mechanical hyperalgesia to sham levels (Figure 4.7), blocking spinal PAR1 with SCH79797 does not attenuate the compression-induced elevation in fibrin(ogen); spinal fibrin(ogen) expression is similar at day 1 for a compression treated with either SCH79797 or the vehicle (Figure 4.8). By day 7, spinal fibrin(ogen) expression is reduced to normal expression levels following a compression with either SCH79797 or vehicle treatment (Figure 4.8A). Elevated spinal fibrin(ogen) expression at day 1 after the compression with treatment or vehicle are significantly reduced (p<0.001) by day 7 (Figure 4.8B). Fibrin(ogen) expression in the ipsilateral dorsal horn is not different for any groups probed at day 7 (Figure 4.8B). The paw withdrawal thresholds for each rat included in Section 4.3.3 are summarized in Appendix A. Appendix B summarizes quantification of spinal fibrin(ogen) expression for each rat in these studies.
4.4. Evaluating the Effects of Spinal Thrombin & PAR1 Activation on BSCB Breakdown & Astrocyte Activation

4.4.1. Study Design & Surgical Groups

4.4.1.1. Quantifying Spinal Astrocytic PAR1 Expression

Since compression-induced spinal thrombin activity is hypothesized to activate astrocytes in the spinal cord through PAR1, the spinal astrocytic expression of PAR1 was measured over time after root compression injuries. Briefly, a 15-minute (15min, n=9), 3-minute (3min, n=8) or 0-minute (sham, n=10) compression was separately applied to the right C7 nerve root using previously described surgical procedures on day 0 (Section...
4.3.2.2) (Rothman et al. 2010). Spinal cord tissue at C7 was harvested on day 1 (15min, n=5; 3min, n=4; sham, n=4) or day 7 (15min, n=4; 3min, n=4; sham, n=6) in separate groups, in order to evaluate the temporal expression of the co-localization of GFAP and PAR1 in the ipsilateral spinal dorsal horn. Spinal GFAP was co-immunolabeled with PAR1 and the co-localization was quantified; co-localized GFAP and PAR1 labeling was normalized to labeling in naïve tissue and differences between groups over time were determined using a two-way ANOVA (group x day) with Tukey’s test.

4.4.1.2. Measuring the Effects of Compression-Induced Spinal Thrombin Activity on BSCB Breakdown & Astrocyte Activation

In order to assess the effect of compression-induced spinal thrombin activity on BSCB breakdown and glial activation, thrombin was blocked intrathecally with hirudin prior to imposing a painful 15-minute nerve root compression. The methods for the intrathecal injection of hirudin and for the surgical methods of the root compression injury are detailed in Section 4.3.2.4. Briefly, rats received an intrathecal injection of hirudin (4.2U/rat) 1 day prior to a 15-minute C7 nerve root compression (hir+15min, n=6). In order to measure BSCB breakdown, fixed C7 spinal cord tissue was harvested at day 1 since BSCB breakdown is evident at day 1 after a 15-minute compression (Figure 3.2) (Smith et al. 2015). IgG immunolabeling was quantified in the ipsilateral spinal cord and was compared between compression with hirudin treatment and matching tissue from that same time point after an untreated 15-minute compression (15min, n=5). Spinal IgG labeling was normalized to expression in naïve tissue and differences between the
hir+15min and 15min groups were determined using a one-way ANOVA with Tukey’s test.

A separate group of rats receiving hirudin treatment and a painful root compression were perfused at day 7 (hir+15min, n=5) to measure astrocyte activation, since spinal GFAP is robustly elevated at day 7 after painful nerve root compression (Rothman et al. 2010, Rothman and Winkelstein 2007). GFAP expression in the ipsilateral spinal cord was immunolabeled and quantified. Details about the specific methods for the GFAP immunolabeling are the same as described in Section 4.4.2.3. Spinal GFAP expression was normalized to expression in naïve tissue and compared to normalized GFAP expression in tissue from matching rats at day 7 after undergoing a 15-minute compression (15min, n=6) without hirudin treatment. Differences in spinal GFAP labeling between groups were determined using a one-way ANOVA with Tukey’s test.

4.4.1.3. Measuring the Effects of Spinal PAR1 on BSCB Breakdown & Astrocyte Activation

In order to determine the effects of spinal thrombin and its activation of PAR1 on non-injury induced, but painful, BSCB breakdown and astrocyte activation, rat thrombin was intrathecally administered (4U/rat; RTh, n=5) to naïve rats or to rats receiving an intrathecal injection of the PAR1 inhibitor, SCH79797 (50µg/kg) at 1 day earlier (SCH+RTh, n=6). Additionally, rats receiving an intrathecal injection of a PBS vehicle were included for immunohistochemical comparisons (PBS, n=2). Because rat thrombin induces the most substantial increase in mechanical hyperalgesia at day 1 (Figures 4.1 & 4.6), BSCB breakdown and spinal astrocyte activation were both measured at day 1.
Bilateral C7 spinal cord tissue was harvested and immunolabeled for IgG and GFAP. IgG and GFAP expression were separately quantified and compared between the RTh, SCH+RTh, and PBS groups using separate one-way ANOVAs with Tukey’s test for each label of neuroinflammation.

4.4.1.4. Defining the Role of PAR1 in Compression-Induced Spinal Astrocyte Activation

In order to determine if spinal PAR1 activation contributes to BSCB breakdown at day 1 or astrocyte activation at day 7 after a painful nerve root compression, SCH79797 (50µg/kg) was administered 1 day before a 15-minute nerve root compression (SCH+15min, n=13). Vehicle controls were included in which DMSO in PBS (1:15) was administered intrathecally at 1 day before either a 15-minute root compression (DMSO+15min, n=8) or a sham surgery (DMSO+sham, n=4). Methods for the surgeries and the intrathecal injections are detailed in Section 4.3.2.4. Fixed C7 spinal cord tissue was harvested at day 1 (SCH+15min, n=6; DMSO+15min, n=4) or day 7 (SCH+15min, n=7; DMSO+15min, n=4; DMSO+sham, n=4) and immunolabeled for IgG and GFAP, respectively. Details about the methods for the quantification of spinal IgG and GFAP expression can be found in Sections 4.4.2.2 and 4.4.2.3, respectively. IgG expression in the ipsilateral spinal cord at day 1 after injury was compared for a compression with SCH79797 or vehicle treatment using a one-way ANOVA with Tukey’s test. Spinal GFAP labeling was measured at day 7 after injury and compared between a compression treated with SCH79797 or vehicle, and a sham operation with vehicle treatment, using a one-way ANOVA with Tukey’s test.
4.4.2. Detailed Immunohistochemical Techniques

4.4.2.1. Immunohistochemistry for Co-localization of GFAP & PAR1

Fixed spinal cord sections at the C7 level were fluorescently co-immunolabeled for PAR1 and GFAP. Slide-mounted tissue was blocked in 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton X-100 (Bio-Rad Laboratories; Hercules, CA) for 1 hour at room temperature. Slides were incubated over night at 4°C with rabbit anti-PAR1 (1:100; Abcam; Cambridge, UK) and mouse anti-GFAP (1:750; Dako; Carpinteria, CA) followed by a 2-hour incubation at room temperature with goat anti-rabbit Alexa Fluor 488 (1:250; Life Technologies; Carlsbad, CA) and goat anti-mouse Alex Fluor 568 (1:1000; Life Technologies; Carlsbad, CA). Slides were then washed with PBS and water, and cover slipped with fluoro-gel with TRIS buffer (Electron Microscopy Sciences; Hatfield, PA). The ipsilateral dorsal horn of 2-6 sections from each rat was digitally imaged at 10x. Spinal astrocytic PAR1 was quantified using a custom co-localization MATLAB script (Dong et al. 2013). The percent of co-localized pixels labeling PAR1 and GFAP in spinal sections was further normalized to the percent co-localization in spinal cord tissue from naïve rats (n=2).

4.4.2.2. Immunofluorescent Labeling & Quantification of IgG

Slide-mounted spinal cord tissue was fluorescently immunolabeled for rat IgG by blocking with 0.3% Triton-X100 (Bio-Rad Laboratories; Hercules, CA) for 1 hour at room temperature and then incubating with goat anti-rat IgG Alexa Fluor 568 (1:200; Life Technologies; Carlsbad, CA) for 2 hours at room temperature. Slides were washed with PBS and cover slipped with fluoro-gel with TRIS buffer (Electron Microscopy Sciences; Hatfield, PA).
Sciences; Hatfield, PA). The ipsilateral spinal dorsal horn was digitally imaged at 10x in 2-6 spinal sections for each rat. Total spinal IgG was quantified using a custom MATLAB script (Nicholson et al. 2012, Rothman et al. 2010). The percent of pixels labeling IgG in tissue sections was further normalized to the percent positive IgG labeling in spinal cord tissue from normal naïve rats (n=2).

4.4.2.3. Immunofluorescent Labeling & Quantification of GFAP

For GFAP immunolabeling, the same procedures were followed as described in Section 4.4.2.1, except without the PAR1 primary antibody and matching secondary. The ipsilateral dorsal horn was imaged at 20x in 2-6 spinal sections per rat. Total spinal GFAP expression was quantified using a custom MATLAB script (Nicholson et al. 2012, Rothman et al. 2010). The percent of pixels labeling GFAP in spinal cord sections was normalized to the percent positive labeling in spinal cord tissue from naïve rats (n=2).

4.4.3. Results

4.4.3.1. Spinal Astrocytic PAR1 Increases after Painful Compression

Spinal PAR1 co-localizes with GFAP in all groups at all times probed (Figure 4.9A), bolstering the fact that spinal astrocytes express PAR1. A nerve root compression, applied for either 3- or 15-minutes, induces a qualitative increase in the co-localization of PAR1 with GFAP in the spinal cord on the side ipsilateral to injury (Figure 4.9A). That increase in astrocytic PAR1 is significant for both the non-painful 3-minute (p=0.018) and the painful 15-minute (p=0.001) compressions compared to sham. Yet, only a painful 15-minute nerve root compression induces a significant increase in spinal astrocytic
PAR1 expression at day 7; that increase is over both sham expression at day 7 (p<0.001) and the expression at day 1 that is evident after a 15-minute compression (p=0.024) (Figure 4.9B). The quantification of the spinal co-localization of PAR1 and GFAP in the ipsilateral spinal cord for each rat in this study is provided in Appendix B.

Figure 4.9. Spinal astrocytic PAR1 increases by day 7 after a 15-minute nerve root compression. (A) Spinal PAR1 (green) and GFAP (red) co-localize (yellow) in the ipsilateral spinal cord to a greater degree at day 7 after either a 3-minute (3min) or a 15-minute (15min) compression. (B) Only a 15-minute compression induces a significant increase in percent spinal PAR1 co-localization with GFAP at day 7 after compression; this is significant compared to sham levels at that same day (*p<0.001) and expression levels at day 1 for 15min (#p=0.024). Data are presented as mean±SD.
4.4.3.2. Hirudin Prevents Compression-Induced BSCB Breakdown & Spinal Astrocyte Activation

Blocking spinal thrombin activity with a single intrathecal injection of hirudin at day 1 before a painful root compression significantly reduces (p=0.002) the spinal expression of IgG that is robustly increased at day 1 after a painful compression (Figure 4.10A & 4.10B). By day 7, hirudin also significantly decreases (p=0.021) spinal GFAP expression in comparison to expression of GFAP at that time point after a compression without any treatment (Figure 4.10). Quantification of the spinal IgG expression at day 1 and GFAP expression at day 7 for each rat in this study is detailed in Appendix B.

Figure 4.10. Intrathecal hirudin prevents spinal IgG accumulation at day 1 and spinal astrocyte activation at day 7 after painful root compression. (A) IgG expression in the ipsilateral spinal cord at day 1 and GFAP expression at day 7 are markedly reduced following treatment with hirudin after compression (hir+15min) compared to compression alone (15min). (B) Quantified IgG expression relative to expression in normal rats is significantly reduced (*p=0.002) for hir+15min compared to 15min at day 1. (C) Hirudin significantly decreases (*p=0.021) spinal GFAP expression at day 7 compared to expression after a compression alone. Data are presented as mean±SD.
4.4.3.3. Spinal Thrombin Induces BSCB Breakdown through PAR1

A single intrathecal injection of rat thrombin increases the spinal expression of GFAP and IgG at day 1 after injection compared to an injection of PBS (Figure 4.11A). Blocking PAR1 activation in the spinal cord with SCH79797 given prior to the administration of the rat thrombin reduces both spinal GFAP and IgG labeling compared to rat thrombin alone (Figure 4.11A). Intrathecal thrombin significantly increases spinal IgG at day 1 compared to a PBS injection; blocking PAR1 prior to injecting the rat thrombin prevents that increase in IgG (Figure 4.11B). In contrast, an injection of rat thrombin does not significantly increase spinal GFAP expression at day 1 (Figure 4.11C). There is no difference in GFAP expression between any group despite intrathecal rat thrombin inducing a slight, but not significant (p=0.054), elevation in spinal GFAP expression compared to the PBS injection (Figure 4.11C). Quantification of the IgG and GFAP expression at day 1 in the bilateral spinal cord for each rat in this portion of the study is detailed in Appendix B.
4.4.3.4. Blocking Spinal PAR1 does not Reduce Spinal IgG or GFAP Expression after Compression

A painful compression with vehicle treatment (given 1 day before compression) increases IgG expression in the ipsilateral spinal cord at day 1 over normal levels (Figure 4.12A). Blocking spinal PAR1 with SCH79797 prior to the root compression induces an even more marked increase in spinal IgG expression (Figure 4.12A). In fact, spinal IgG expression at day 1 is significantly greater (p<0.001) for a compression treated with SCH79797 compared to IgG labeling observed in tissue after a compression without
treatment (Figure 4.12A). Spinal GFAP expression appears similar between all groups at day 7 and is not different when quantified (Figure 4.12C). Detailed quantification of the IgG expression at day 1 and GFAP expression at day 7 in the ipsilateral spinal cord for each rat in this study is detailed in Appendix B.

**Figure 4.12. Intrathecal SCH79797 does not prevent compression-induced elevations in spinal IgG at day 1 or spinal GFAP at day 7.** (A) IgG expression in the ipsilateral spinal cord is robust at day 1 after a 15-minute compression pre-treated with a DMSO vehicle (DMSO+15min) compared to expression in normal tissue. Spinal IgG labeling after pre-treating with SCH79797 (SCH+15min) is even more robust. Normalized spinal IgG expression is significantly increased (*p<0.001) at day 1 after SCH+15min compared to DMSO+15min. (B) Spinal GFAP expression is minimal for all groups (DMSO+sham, DMSO+15min, SCH+15min) at day 7. Data are mean±SD.

### 4.5. Discussion

This is the first study to demonstrate that a compressive nerve root injury increases spinal thrombin activity, which contributes to spinal astrocyte activation and the mechanical hyperalgesia that both develop. Additionally, spinal thrombin at least partially contributes to early compression-induced BSCB breakdown and the
development of pain through its activation of PAR1 (Figures 4.5 & 4.11). Thrombin activity, as evaluated by positive labeling for fibrin(ogen), is increased in the ipsilateral spinal cord at day 1 only after a transient nerve root compression that also induces pain (Figure 4.3). That expression of fibrin(ogen) is present in spinal regions where BSCB breakdown also occurs (Figure 4.3D). A single intrathecal dose of hirudin prevents the painful compression-induced increases in spinal fibrin(ogen) and IgG at day 1 (Figures 4.4 & 4.10) and the increase in spinal GFAP at day 7 (Figure 4.10). Blocking spinal thrombin with hirudin is also sufficient to prevent the development of pain after a 15-minute compression (Figure 4.5). These findings suggest that thrombin activity in the spinal cord is requisite for the onset of pain after nerve root injury and may contribute to sustained astrocyte activation in the spinal cord.

The role of spinal thrombin in pain development is further supported by the findings that a single intrathecal injection of rat thrombin is potent enough to produce behavioral sensitivity for up to 3 days in naïve rats (Figure 4.6A). That single administration of exogenous thrombin also induces BSCB breakdown at day 1 when hyperalgesia is maximal (Figure 4.11). Blocking PAR1 activation with the antagonist, SCH79797, prior to injection of rat thrombin prevents the development of thrombin-induced mechanical hyperalgesia (Figure 4.6B), indicating that thrombin may act centrally through PAR1 to modulate nociceptive mechanisms. SCH79797 is also sufficient to block rat thrombin-induced BSCB at day 1 (Figure 4.6). Further supporting the PAR1 pathway in thrombin-mediated pain, blocking spinal PAR1 activation prior to a painful compression also prevents the development of pain after injury (Figure 4.7). However, blocking PAR1 with SCH79797 does not mitigate the spinal fibrin(ogen) and
IgG deposition that is evident at day 1 after painful compression (Figures 4.8 & 4.12). Thrombin still extravasates into the spinal parenchyma even when blocking PAR1 with SCH79797, suggesting that thrombin is capable of activating substrates in the spinal cord in addition to PAR1. Therefore, thrombin’s activation of other substrates within the spinal cord does not contribute to radicular pain. Since SCH79797 and its vehicle (DMSO) each separately attenuate spinal astrocyte activation at day 7 after painful compression (Figure 4.12), it is possible that spinal thrombin does not activate astrocytes through PAR1 activation. For example, astrocytes also express PAR3 and PAR4 (Wang et al. 2002), which are activated by thrombin. Activation of PAR4 in astrocytes in vitro even exerts toxic effects (Wang et al. 2002), suggesting that thrombin may contribute to spinal astrocyte activation following painful compression through PAR4. Despite SCH79797 and DMSO both attenuating spinal astrocyte activation, intrathecal SCH79797 also attenuates pain, whereas the DMSO vehicle does not (Figure 4.7). Therefore, spinal PAR1 activation does contribute to nociception following root compression and might do so through non-glial mechanisms. Since neurons also express PAR1 and activation of neuronal PAR1 has been shown to sensitize nociceptive neurons (Vellani et al. 2010), SCH79797 might reduce the neuronal hyperexcitability that is evident by day 7 after compression.

Circulating thrombin has been previously hypothesized to cross a compromised BSCB and thereby contribute to neuroinflammatory processes in the CNS (Suo et al. 2004). Yet, this is the first in vivo study demonstrating that thrombin enters the CNS in areas of BSCB breakdown that appear after nerve root compression injury and that thrombin is required for pain (Figure 4.3). Spinal thrombin activity is elevated exclusively
after a painful nerve root compression and is abundant at day 1 when pain is first evident (Figure 4.3) (Hubbard and Winkelstein 2005, Rothman et al. 2010). Spinal fibrin(ogen) expression increases along the same time course as the breakdown of the BSCB after this painful root injury (Smith et al. 2015); spinal fibrin(ogen) and IgG (Smith et al. 2015) expression both increase by day 1 and return to normal levels by day 7 (Figure 4.3). In fact, fibrin(ogen) is co-expressed with the serum protein IgG, suggesting that thrombin enters the spinal parenchyma from the periphery. Additionally, the spinal expression of fibrin(ogen), the polymeric enzymatic product of thrombin, suggests that thrombin is capable of exerting enzymatic activity once in the spinal parenchyma. Yet, fibrin(ogen) is used in the current study as a proxy for thrombin activity, elevation in fibrin(ogen) in regions of BSCB breakdown could also be due to fibrin or fibrinogen crossing the BSCB and accumulating in the spinal cord. However, exogenous fibrin in the brain does not adversely affect neuronal health, nor does it induce local inflammation (Hultman et al. 2014). Therefore, it is not likely that the increases in spinal fibrin(ogen) that are evident after a compression injury are responsible for those pathologic mechanisms that are involved in the development of pain. Blocking fibrin’s enzymatic precursor, thrombin, centrally prior to compression prevents pain (Figure 4.5) and further supports thrombin’s presence in the spinal parenchyma as contributing to pain after injury. Although previous studies have demonstrated that pain-related pro-inflammatory cytokines can cross the BSCB when its integrity is compromised (Echeverry et al. 2011, Pan et al. 1997), this is the first report that serum-derived enzymes can also enter the spinal parenchyma and appear to initiate nociceptive cascades.
The transient early elevation in spinal thrombin activity after a painful compression (Figure 4.3) contributes to the spinal astrocytic activation that is evident by day 7 after that injury since blocking spinal thrombin with hirudin prevents astrocyte activation at day 7 (Figure 4.10) (Rothman and Winkelstein 2007). Further, although only a trend, intrathecal rat thrombin also increases spinal GFAP expression at day 1 compared to a PBS vehicle (Figure 4.11); however, the PBS vehicle group only represents spinal cord tissue from 2 rats and the statistical test did not have enough power. In order to reach a power of 0.8 the PBS vehicle group would need to be expanded out to at least 3 rats. This is a limitation of that study and one rat should have been added to the PSB vehicle group in order to accurately test whether spinal GFAP expression was different between the vehicle group and the group that received an injection of rat thrombin. Rat astrocytes express multiple thrombin-activated receptors (Figure 4.9) (Shavit et al. 2011, Wang et al. 2002), and activation of astrocytic PAR1, in particular, induces glial activation and proliferation (Fan et al. 2005, Nicole et al. 2005, Wang et al. 2002). Following this same painful root compression, spinal GFAP has been reported to be marginally elevated by day 1 and more robustly increased by day 7 (Rothman and Winkelstein 2007). Thrombin activity is elevated in the spinal parenchyma by day 1 and is no longer present in the spinal cord by day 7 (Figures 4.3) when both pain and astrocytic activation persist (Rothman et al. 2010, Rothman and Winkelstein 2007). Therefore, the early spinal thrombin activity induced by a painful compression may be an initiator of sustained spinal astrocyte activation.

Although these studies confirm a role for thrombin activity in both nerve root-mediated glial activation and pain, evaluating the expression of fibrin(ogen) only captures
the accumulation of thrombin’s enzymatic products and does not provide information on the \emph{dynamic} expression profile of thrombin activity. A recent study reported a method by which thrombin activity can be quantitatively measured in vitro within brain slices excised after an ischemic insult via the middle cerebral artery (Bushi et al. 2013). By applying a fluorescent thrombin substrate on fresh brain slices after ischemia, thrombin activity was found to increase by 24 hours after ischemia, but only on the side ipsilateral to the insult (Bushi et al. 2013). By taking fresh slices of C7 spinal cord tissue at 1 day after a painful nerve root compression, this fluorogenic method for detecting thrombin activity could be used to determine whether thrombin is actually active in the spinal parenchyma at day 1 when fibrin(ogen) accumulation is evident (Figure 4.3).

To investigate whether spinal thrombin contributes to nerve root compression-induced pain, we selectively blocked thrombin activity prior to a painful injury. When hirudin is given before the compression it prevents the deposition of fibrin(ogen) in the spinal parenchyma (Figure 4.4), confirming that hirudin is sufficient to block thrombin from enzymatically producing fibrin(ogen) in the spinal cord. That single treatment of hirudin is potent enough to completely inhibit the development of pain for up to 1 week after compression (Figure 4.5), which is in agreement with a previous study showing that intrathecal hirudin prevents mechanical and thermal sensitivity after a sciatic nerve ligation in the mouse during and after the treatment period (Narita et al. 2005). However, in contrast to that study in which repetitive hirudin dosing marginally suppresses pain (Narita et al. 2005), a single dose of hirudin completely prevents pain after a root compression (Figure 4.5). A sciatic nerve ligation also induces breakdown of the BSCB lasting for up to 30 days (Beggs et al. 2010, Echeverry et al. 2011), in contrast to a 15-
minute nerve root compression which only induces BSCB breakdown for a single day (Smith et al. 2015). It is possible that a sciatic nerve injury permits thrombin extravasation into the spinal parenchyma for a much longer duration (up to 30 days) than does the transient root compression, and hence allows thrombin to exert sustained activity within the spinal cord. Since hirudin is relatively stable in vivo and retains 60-80% of its activity after 24 hours (Kaiser et al. 2012), it is still active in the spinal cord at the time when the root undergoes compression, and likely remains active after the breakdown of the BSCB. Spinal thrombin is likely only active is the spinal parenchyma for approximately the same duration as the BSCB permeability (1 day) after compression since its half-life is on the order of hours (Vogel et al. 1979). A single dose of hirudin likely remains active for the entire duration of increased thrombin activity and, therefore, likely fully blocks spinal thrombin’s contribution to pain.

In contrast to hirudin’s completely preventing nerve root-induced pain, pain is only partially attenuated by inhibiting the action of TNF-α or IL-1 in the spinal cord prior to injury (Rothman and Winkelstein 2007, Rothman and Winkelstein 2010). Once in the CNS, thrombin induces glial production of pro-inflammatory cytokines (Choi et al. 2008, Fan et al. 2005, Simmons et al. 2013). Thrombin is an upstream inducer of pro-inflammatory cytokine production (Simmons et al. 2013, Suo et al. 2004). Therefore, blocking the action of just one pro-inflammatory cytokine in the CNS might not attenuate pain to the same degree as does blocking thrombin since thrombin contributes to spinal inflammation. Blocking thrombin in the spinal cord may also attenuate the local inflammatory responses that contribute to the development of pain, further enhancing its anti-nociceptive effects. Future studies should examine whether blocking spinal thrombin
activity explicitly reduces spinal pro-inflammatory cytokine expression exhibited after painful root compression.

Spinal thrombin’s contribution to nerve root-induced pain is supported by the findings that a single intrathecal injection of rat thrombin induces pain for up to 3 days in the absence of any other injury-induced inputs (Figure 4.6A). Yet, more robust behavioral sensitivity has been reported in mice receiving a single injection of thrombin, with mechanical allodynia persisting for up to 10 days (Narita et al. 2005). Since the current study administered a comparable amount of thrombin in rats, the disparity in the duration of pain following the central administration of thrombin might depend on the species being treated in comparison to the species in which the exogenous thrombin is derived. Narita et al. (2005) did not specify which species of thrombin was used; it is possible that thrombin derived from a non-murine species was administered. Administering “non-self” proteins, including thrombin, induces a complex immune response (Ballard et al. 2010, Laidmae et al. 2006, Lawson 2006), and if administered to the CNS might exacerbate neuroinflammation and contribute to the extended period of mechanical allodynia that was observed in the murine study.

Blocking spinal thrombin significantly blunts BSCB breakdown at day 1 after a painful compression (Figures 4.4A & 4.10). Although BSCB breakdown is requisite for thrombin extravasation into the spinal parenchyma, it is possible that hirudin also prevents spinal thrombin’s prolonged activation of neurovascular endothelial cells thereby reducing the extent of compression-induced BSCB breakdown. Exogenous rat thrombin administered intrathecally significantly increases spinal IgG within 1 day (Figure 4.11), further implicating that spinal thrombin activity in the induction of BSCB
breakdown, even without any other injury inputs. The ability of thrombin to induce vascular permeability in the spinal cord at least partially depends on its activation of PAR1; blocking activation of spinal PAR1 with SCH79797 significantly inhibits BSCB breakdown induced after a spinal injection of rat thrombin (Figure 4.11). However, blocking PAR1 prior to a painful compression does not attenuate injury-induced BSCB breakdown (Figure 4.12), suggesting that thrombin is not the only contributor to the breakdown of BSCB after painful nerve root compression. For example, microvascular permeability is controlled by intracellular actomyosin contractility that can be induced by activated neutrophils, TNF-α, and histamine, in addition to thrombin (Nooteboom et al. 2002, Rigor et al. 2013). Of those inducible factors, TNF-α expression is increased in the spinal cord on the side ipsilateral to the root injury by 1 hour after compression (Rothman et al. 2009, Rothman and Winkelstein 2010), and also likely contributes to the BSCB breakdown that is also evident in that spinal region.

Blocking spinal PAR1 activation prior to a nerve root injury produces similar behavioral results as does pre-treating with hirudin (Figures 4.5 & 4.7); both treatments prevent nerve root-induced pain for at least 7 days. Yet, in contrast to hirudin, SCH79797 does not alter pain outcomes immediately (by day 1) (Figure 4.7). Unlike hirudin, which was administered in an inert saline vehicle, the vehicle (DMSO) used to deliver SCH79797 is not biologically inert and has been shown to be neurotoxic in some cases in a dose-dependent manner (Galvao et al. 2014, Qi et al. 2008). In fact, pre-treatment with the DMSO vehicle in the sham group in this study appears to induce a slight increase in mechanical hyperalgesia at day 1 (Figure 4.7), which is not typical for sham operated rats (Figure 4.3A) (Hubbard and Winkelstein 2005, Rothman and Winkelstein 2007, Smith
and Winkelstein 2015). A recent study demonstrated that treating CA1 neurons in hippocampal brain slices with DMSO for up to 5 hours reduces the threshold for action potential firing in response to an electrical stimulus (Tamagnini et al. 2014). In the current study, DMSO also might decrease the threshold for neuronal firing in the spinal cord, thereby contributing to central sensitization. That pro-nociceptive effect of DMSO on neurons might outweigh the anti-inflammatory effects that it produces via modulating spinal glia (Figure 4.12) (Lapuente Chala et al. 2013), and contribute a painful root compression treated with the DMSO vehicle inducing behavioral sensitivity but at the same time reducing spinal astrocyte activation. Although hirudin and SCH79797 both inhibit pain (Figures 4.5 & 4.7), SCH79797 is not sufficient to prevent early nerve-root induced spinal fibrin(ogen) deposition or BSCB breakdown (Figure 4.8). Since SCH79797 reduces pain without also reducing spinal fibrin(ogen) expression, fibrin(ogen) is not likely an initiator of pain. In fact, the use of fibrin gels to treat spinal cord injury has been widely investigated since fibrin matrices promote neuronal regrowth (Itosaka et al. 2009, Uibo et al. 2009, Winer et al. 2009). Since this polymeric enzymatic product of thrombin does not actively contribute to nociception, spinal thrombin’s activation of PAR1 and inflammation likely promotes its role in pain development after nerve root compression.

This study establishes a novel contribution for spinal thrombin activity in the pain that develops after neural injuries that are associated with BSCB breakdown. Previous reports emphasize the extravasation of pro-inflammatory cytokines and the transmigration of immune cells into the CNS as contributing to neuroinflammation and pain (Ballabh et al. 2004, Echeverry et al. 2011, Webb and Muir 2000). The findings of
the current study extend existing models implicating the contribution of BSCB breakdown to pain by demonstrating that bioactive thrombin also enters the spinal parenchyma after nerve root injury and is requisite for pain onset. Blocking the enzymatic activity of thrombin in the spinal cord before a nerve root compression completely prevents the development of pain and substantially reduces the longer-term spinal astrocytic activation (Figures 4.5 & 4.10). Findings from this study support the notion that spinal thrombin induces pain through its activation of PAR1 centrally (Figures 4.6 & 4.7); yet, defining which CNS cell type(s) express PAR1 and are most likely to contribute to pathological mechanisms that do contribute to nociception are needed. Nonetheless, these findings have implications not only for the treatment of painful neuropathic injuries, but also for the host of other neuropathic disorders that induce, and are exacerbated by, BSCB breakdown like ischemic stroke, Alzheimer’s disease, Parkinson’s diseases and multiple sclerosis, among others (Hirsch and Hunot 2009, Sharief and Thompson 1992, Zlokovic 2008, Zlokovic 2011).

4.6. Integration & Conclusions

BSCB breakdown after a nerve root compression is only evident when behavioral sensitivity is also present (Figure 3.2), and that breakdown contributes to pain (Figure 3.4). Root compression-induced BSCB breakdown facilitates the spinal extravasation of TNF-α (Figure 3.2), which when expressed in the spinal cord has been shown to contribute to the development of mechanical allodynia after root trauma (Rothman and Winkelstein 2010). The studies in this chapter demonstrate that endogenous thrombin
also extravasates into the spinal parenchyma where there is BSCB breakdown from a painful compression (Figure 4.3). Once in the spinal cord, thrombin contributes to the development of pain, both after a 15-minute nerve root compression and after exogenous rat thrombin administration into the spinal cord (Figures 4.5 & 4.6). Although intrathecal rat thrombin only induces marginal spinal astrocytic activation at day 1 when pain was present (Figure 4.11), blocking thrombin activity with hirudin does reduce spinal astrocyte activation at a later time (i.e. day 7) after painful compression (Figure 4.10). Spinal thrombin activity is likely greater following a root compression than an injection of rat thrombin since BSCB permeability increases after painful compression for at least 1 day (Figure 3.2); therefore, thrombin may enter the spinal parenchyma over that entire day. The different degrees of spinal astrocyte activation after those insults likely depends on the amount of thrombin activity. Since blocking spinal PAR1 with SCH79797 prevents pain from developing after either insult (Figures 4.6 & 4.7), spinal thrombin is taken to mediate pain through PAR1 regardless of how it is induced. Although PAR1 is important for thrombin’s induction of nociception in the CNS, these studies do not determine whether thrombin activates astrocytes through PAR1. Antagonizing PAR1 prior to a painful compression reduces the spinal astrocyte activation that is typically evident at day 7, but the vehicle treatment also reduces spinal GFAP (Figure 4.12). Since SCH79797 blocks compression-induced pain but DMSO does not (Figure 4.7), this spinal astrocytic activation is not likely the sole regulator of nociception following painful root compression. Regardless, endogenous thrombin activity in the spinal cord appears to play an important role in nerve root-mediated pain by contributing to spinal astrocytic activation and exacerbating BSCB breakdown.
Although these studies focus on the role of rat thrombin in pain, thrombin derived from non-mammalian species has been shown to influence pain outcomes differently. For example, treating a painful root compression with salmon thrombin at the injury site prevents the development of behavioral sensitivity (Weisshaar et al. 2011). Thrombin controls cellular processes through its enzymatic activation of many cell receptors, including PAR1, PAR3, PAR4, and endothelial-bound protein C (Coughlin 2000, Di Cera 2008, Lane et al. 2005). In many cellular cascades, including coagulation, inflammation, and endothelial permeability, thrombin’s activation of PAR1 exerts the exact opposite cellular effects than its activation of endothelial-bound protein C (Bouwens et al. 2013, Di Cera 2008, Griffin 1995, Komarova et al. 2007, Vergnolle et al. 1999). For example, here we demonstrate that spinal rat thrombin activates PAR1 and induces vascular leakiness (Figure 4.11); but, mammalian thrombin can also stabilize endothelial barriers when it is bound to thrombomodulin by activating endothelial-bound protein C (Esmon 1993, Xu et al. 2005, Yang et al. 2006). Thrombin’s enzymatic activity is guided towards particular substrates through a variety of molecular events, such as binding to a cofactor or altered sodium concentration, which modulate the chemistry and three-dimensional structure of thrombin (Gandhi et al. 2011, Jacques et al. 2000, Lane et al. 2005). Since various species of thrombin have naturally-evolved structures that are slightly different (Dang et al. 1997, Doolittle 2011, Tort et al. 2003), this diversity might influence the innate affinity of each species of thrombin for its substrates. Salmon thrombin is hypothesized to initiate pain-related pathways that are opposite from those of mammalian thrombin because it might exhibit different activation rates of thrombin-cleavable cell receptors. The studies in Chapter 5 investigate the differential effects
between salmon and human thrombin on BSCB breakdown and pain induced after a painful nerve root compression, as well as on endothelial barrier permeability and astrocyte activation in vitro.
Chapter 5

Differential Effects of Salmon & Human Thrombin on Vascular Permeability, Astrocyte-Induced Inflammation, & Pain

Parts of this chapter are included in both a published paper and a submitted manuscript:


Smith JR, Galie PA, Slochower DR, Weisshaar CL, Janmey PA, Winkelstein BA. Salmon-derived thrombin inhibits development of chronic pain through an endothelial barrier protective mechanism dependent on APC. *Biomaterials*, accepted.

5.1. Overview

Less than 50% of patients in neuropathic pain trials achieve satisfactory pain relief with currently available treatments (O’Connor and Dworkin 2009). According to the neuropathic pain special interest group (NeuPSIG) of the International Association for the Study of Pain (IASP) the standard set of FDA approved treatments that are first recommended for neuropathic pain patients include tricyclic antidepressants (e.g. norepinephrine and serotonin reuptake inhibitors), anticonvulsants (such as gabapentin and pregabalin) and the 5% lidocaine patch (Dworkin et al. 2003, Finnerup et al. 2010, O’Connor and Dworkin 2009). All of these treatments selectively target the neuronal
aspects of nociception; they either modulate synaptic concentration of monoamine neurotransmitters (e.g. norepinepherin and serotonin) (Sindrup et al. 2005), increase the neurotransmitters in synapses that inhibit excitatory neuronal signaling by binding to voltage gated calcium channels (i.e. gamma-Aminobutyric acid, GABA) (Gilron 2007, O'Connor and Dworkin 2009), or block sodium channel function (Argoff 2000, Meier et al. 2003). Although the classic view of nociception involves aberrant neuronal signaling, it has become widely accepted that glia, including astrocytes and microglia, are also powerful modulators of the propagation and maintenance of pain (Inoue 2006, McMahon et al. 2005, Vallejo et al. 2010, Watkins et al. 2001). Glial cells contribute to nociception by releasing pro-inflammatory cytokines, which further stimulate neurons, and by modifying their expression of various neurotransmitter receptors, thereby dysfunctionally modulating the synaptic concentration of neurotransmitters (DeLeo and Yezierski 2001, Inoue 2006, Latremoliere and Woolf 2009, Milligan and Watkins 2009, Rothman et al. 2009, Rothman and Winkelstein 2010, Scholz and Woolf 2007, Vallejo et al. 2010, Volterra and Meldolesi 2005, Vranken 2009). Therapeutic strategies for treating chronic neuropathic pain that dually target both the neuroinflammatory cascades involved in pain and the neuronal dysfunction are promising replacements for current first-line treatments.

The studies presented in Chapters 3 and 4 together demonstrate that blood-spinal cord-barrier (BSCB) breakdown induced by painful nerve root compression is responsible for the infiltration of endogenous thrombin into the spinal parenchyma, and that blocking an initiator (BSCB breakdown) or outcome (thrombin activity in the spinal cord) of this cascade prevents the development of radicular pain. Although studies in Chapter 3 establish that activated protein C (APC), a known protector of vascular
breakdown, is effective at blocking the BSCB breakdown that is induced by day 1 after a painful nerve root compression injury (Figure 3.4) (Smith et al. 2015), this enzyme is not an ideal clinical treatment because its inherent anticoagulant properties prevent the clotting that is necessary to stop bleeding (Bernard et al. 2001, Finfer et al. 2008, Martí-Carvajal et al. 2012). For traumatic nerve root compression, which mechanically disrupts axonal and vascular integrity within the root (Garfin et al. 1995, Olmarker et al. 1989, Yoshizawa et al. 1989), APC is even less ideal since internal bleeding is also evident at the site of trauma to the root. Activating the APC pathway without hampering the coagulation that promotes local healing, or administering APC in conjunction with a procoagulant agent would be ideal alternate strategies for inhibiting BSCB breakdown. Recent approaches in protein therapy have focused on modifying the inherent structure of mammalian thrombin in order to direct its affinity towards protein C instead of the protease-activated receptors (PARs), in order to stabilize endothelial barriers while maintaining the ability to form clots (Dang et al. 1997, Marino et al. 2010). Interestingly, native thrombin produced in non-mammalian species exhibits slightly modified protein structures from thrombin derived from mammals (Dang et al. 1997, Laidmae et al. 2006, Manseth et al. 2004) and potentially induces different cell signaling cascades because of the modified substrate affinities (Michaud et al. 2002, Sharp et al. 2012, Weisshaar et al. 2011). As such, the studies outlined in this chapter investigate the whole body, cellular, and enzymatic effects of salmon thrombin compared to human thrombin in relation to pain, BSCB breakdown and spinal inflammation.

The work presented in this chapter focuses on the experiments that are outlined in Aim 3 and test the specific hypotheses that salmon thrombin exhibits unique effects on
vascular permeability and glial inflammation, both of which contribute to its analgesic capabilities after painful neural trauma. It is further hypothesized that salmon thrombin’s effects on endothelial cells and astrocytes depend on its rates of substrate activation that are distinct from human thrombin. An in vivo model of painful nerve root compression in the rat was used to investigate different effects of salmon and human thrombin on the compression-induced development of behavioral hypersensitivity and early BSCB breakdown (Section 5.3). For those in vivo studies, salmon thrombin was applied directly to the nerve root since we have previously shown that salmon thrombin is analgesic when administered this way (Weisshaar et al. 2011). To determine whether salmon thrombin acts directly on endothelial cells to promote vascular integrity, studies in Section 5.3 also include in vitro microchannel experiments designed to measure human endothelial cell barrier permeability in the presence of an inflammatory inducer of vascular leakiness, tumor necrosis factor-alpha (TNF-α) (Galie et al. 2014, Nooteboom et al. 2002, Sharief and Thompson 1992). Section 5.4 details studies measuring the comparative effects of salmon and human thrombin on the maintenance of nerve root compression-induced pain. They further utilize mixed glial-neuronal cortical cultures to investigate the ability of salmon thrombin to induce astrocytic production of inflammatory cytokines that are involved in pain; effects are compared to those of human thrombin. Lastly, a variety of fluorogenic peptide assays were designed and utilized to quantify the comparative cleavage rates of PAR1, PAR3 and PAR4 (Section 5.3), as well as the rate of APC generation (Section 5.4), in order to provide comparative measures of the substrate selectivity profiles for salmon and human thrombin. A combined background is provided (Section 5.2) for the effects of both species of thrombin on vascular protection and glial-
induced inflammation. Yet, the methods and results investigating how salmon thrombin influences vascular integrity (Section 5.3) and glial inflammation (Section 5.4) are presented as separate sections because they are induced by different thrombin-mediated cellular signaling cascades. This chapter concludes with a presentation of discussion and conclusions that integrate findings from each of the separate studies.

5.2. Relevant Background

Neck pain is estimated to affect up to 70% of the general adult population, with substantial annual physical burdens, health care costs, and associated disabilities (Cote et al. 2004, Haldeman et al. 2009, Strine and Hootman 2007). Neuropathic pain is classified as pain resulting from a direct insult, whether mechanical or inflammatory, to neural tissue, which can become chronic and persist long after the initial injury due to a variety of biochemical changes in the central nervous system (CNS) (Scholz and Woolf 2007, Woolf and Mannion 1999). The cervical dorsal nerve roots, which are comprised of sensory axons, are especially susceptible to injurious mechanical loading. The nerve roots bridge the peripheral and the central nervous systems and are physically surrounded by cerebrospinal fluid and enclosed within the dura (Maikos et al. 2008, Ommaya 1968). Although the dura and cerebrospinal fluid (CSF) protect the nerve root from the normal physiological motions of the surrounding bony structures that make up the intervertebral foramen, under pathological conditions normal neck motions can become injurious (Reid 1960). Further, when the spine undergoes rapid, non-physiological movements the roots can be compressed, even transiently, and can produce pain or numbness that radiates

Using a model of cervical nerve root compression, our lab has defined many neuronal and glial mechanisms that contribute to nerve root-mediated pain. A transient (15-minute) compression applied to the nerve root induces immediate and long-term cellular modifications at the site of injury, as well as in the spinal dorsal horn where the injured afferents synapse (Chang and Winkelstein 2011, Hubbard et al. 2008a, Hubbard and Winkelstein 2005, Rothman et al. 2009, Winkelstein and DeLeo 2002). Within 1 hour after a painful root compression, pro-inflammatory cytokines, including TNF-α, interleukin-1 beta (IL-1β) and IL-6, are transcribed and secreted by cells within the spinal cord on the side ipsilateral to the injured root (Rothman et al. 2009, Rothman and Winkelstein 2010). Independently blocking the actions of IL-1 or TNF-α prior to that nerve root compression attenuates pain for up to 1 week after injury (Rothman et al. 2009, Rothman and Winkelstein 2010). Although the early (within hours) inflammatory response that is produced by cells within the CNS subsides within 1 day, spinal microglia and astrocytes are already activated at day 1 (Rothman et al. 2009, Rothman and
Winkelstein 2007, Winkelstein and DeLeo 2002). Glial activation in the spinal cord remains elevated at 1 week after injury (Hubbard and Winkelstein 2005, Rothman et al. 2010); at that same time, the axons within the injured nerve root exhibit degeneration and their transport of neuropeptides, such as substance P and calcitonin gene-related protein (CGRP), to the dorsal horn is disrupted (Hubbard et al. 2008a, Hubbard and Winkelstein 2008, Kangrga and Randic 1990, Smullin et al. 1990). Axonal degeneration after painful nerve root compression is accompanied by infiltration of macrophages around the injured area, both of which persist for at least 2 weeks, paralleling the presence of pain (Chang and Winkelstein 2011). Based on the complicated timing of inflammation and neuronal dysfunction after painful nerve root injury, mitigating both the early inflammatory response(s) and promoting nerve root health after neural injury has the potential to attenuate the associated persistent pain that also develops.

Blood-brain barrier (BBB) disruption is characteristic of many neurological disorders, including stroke, Parkinson’s disease and multiple sclerosis, and contributes to the associated neuroinflammation and neurodegeneration in those diseases (Sandoval and Witt 2008, Winkler et al. 2014, Zlokovic 2008, Zlokovic 2011). Recent studies have demonstrated that BSCB breakdown also is induced early after a mechanical injury to a peripheral nerve or nerve root (as reported in Chapter 3) (Beggs et al. 2010, Echeverry et al. 2011, Smith et al. 2015). Disruption of the BBB permits the extravasation of neurotoxic factors into the CNS that can impair the normal neuronal function and exacerbate inflammation (Webb and Muir 2000, Zlokovic 2008). Blocking the BSCB breakdown that occurs early and transiently after a 15-minute nerve root compression via APC administration early after injury completely prevents pain development (Chapter 3).
(Smith et al. 2015), highlighting BSCB breakdown as a promising target for preventing not only the inflammation localized to the spinal cord, but also the pain that develops as a result of compression-induced BSCB breakdown.

The protease thrombin, most recognized for its role in the coagulation cascade, also regulates a variety of endothelial processes via its enzymatic activation of cell-bound receptors or proteins (Di Cera 2008). Thrombin can initiate distinct signaling cascades depending on the cofactor it binds and the substrate it cleaves (Bouwens et al. 2013, Di Cera 2008, Griffin 1995, Komarova et al. 2007). Thrombin can activate PAR1, PAR3 and PAR4, but has the highest affinity for PAR1 due to a hirudin-like sequence of amino acids positioned near the extracellular cleavage site that interacts with exosite I of thrombin (Jacques et al. 2000, Luo et al. 2007). In its unbound state, mammalian thrombin increases vascular permeability by directly activating PAR1 on the endothelial surface (Coughlin 2000, Di Cera 2008). In contrast, when bound to thrombomodulin, thrombin cleaves endothelial-bound protein C into APC, which stabilizes vascular stability (Esmon 1993, Griffin 1995, Ye et al. 1992). Intravenously administered APC after a painful nerve root compression completely prevents BSCB breakdown (Smith et al. 2015), emphasizing the potency of the APC pathway in protecting vascular integrity and its effectiveness in treating BSCB disruptive disorders. Although clinical trials have tested APC for its enhancement of endothelial barriers in sepsis, stroke and traumatic brain injury (Bernard et al. 2001, Petraglia et al. 2010, Zlokovic and Griffin 2011), its strong anticoagulant effects hamper its clinical safety (Bernard et al. 2001). For this reason, mutagenesis and protein engineering studies manipulate specific peptide domains within thrombin’s structure to increase its innate affinity for protein C instead of PAR1,
while maintaining its ability to cleave fibrinogen (Dang et al. 1997, Gibbs et al. 1995, Marino et al. 2010, Zlokovic and Griffin 2011).

In addition to endothelial responses, thrombin can also mediate glial responses that contribute to inflammation and even contribute to nociception within the CNS (Garcia et al. 2010, Narita et al. 2005, Niego et al. 2011, Nishino et al. 1993). Cleavage of astrocytic PAR1 contributes to astrocyte activation and production of inflammatory cytokines (Boven et al. 2003, Fan et al. 2005, Nicole et al. 2005, Niego et al. 2011, Nishino et al. 1993, Simmons et al. 2013). Similarly, human thrombin induces the transcription and secretion of IL-1β, TNF-α and IL-6 by astrocytes in culture as early as 6 hours and lasting for up to 24 hours (Boven et al. 2003, Fan et al. 2005, Simmons et al. 2013). In addition to PAR1 activation inducing inflammation, PAR1−/− mice exhibit significantly less glial fibrillary acidic protein (GFAP) expression after a cortical stab wound compared to wild type mice (Nicole et al. 2005), suggesting that astrocyte expression of PAR1 in the CNS might be requisite for astrocytic activation.

Thrombin’s role in pain is controversial since this enzyme is capable of both initiating and attenuating pain depending on its concentration and the site of its administration (Asfaha et al. 2002, Fang et al. 2003, Narita et al. 2005, Weisshaar et al. 2011). At low concentrations (1.5x10^{-17}–1.5x10^{-14}mol/mouse), intrathecally-administered human thrombin attenuates the behavioral sensitivity that is typically induced by a painful N-Methyl-D-aspartic acid (NMDA) intrathecal stimulus (Fang et al. 2003); but, it also induces sustained mechanical allodynia when administered intrathecally alone at a higher concentration (1x10^{-12} mol/mouse) (Narita et al. 2005). Peripherally, a single injection of human thrombin in the rat forepaw transiently decreases the mechanical
algesia (i.e. sensitivity to mechanically-induced pain) that is evoked by a noxious stimulus (Asfaha et al. 2002). Together, these studies imply that thrombin may contribute to the pro-inflammatory responses within the CNS that potentiate pain states. Yet, it is not known if a thrombin-like enzyme that activates different substrates compared to human thrombin, would also induce those same inflammatory and algesic responses.

Interestingly, fish differ significantly from mammals in their inflammatory responses and produce naturally-evolved enzymes with slightly distinct capabilities from their mammalian counterparts (Doolittle 2011). Specifically, thrombin derived from salmon exhibits nearly indistinguishable clotting capabilities, but initiates different cell signaling cascades compared to human thrombin (Doolittle 2011, Michaud et al. 2002, Smith et al. 2013, Weisshaar et al. 2011). The salmon coagulation factor, fibrin, which is formulated by fibrinogen and thrombin, both derived from salmon, has been shown to attenuate pain when administered at the nerve root immediately after its painful compression (Weisshaar et al. 2011). This same treatment by salmon fibrin also prevents the infiltration of phagocytotic macrophages that are normally evident at the root after its compression (Weisshaar et al. 2011), supporting the anti-inflammatory capabilities of salmon coagulation factors. In comparison to its human counterpart, salmon fibrin promotes more locomotor and bladder function recovery after traumatic spinal cord injury without intensifying pain, suggesting that it is also neuroprotective (Sharp et al. 2012). Although salmon fibrin has already been shown to have analgesic properties, it is not known if salmon thrombin alone can attenuate pain. Further, it is unknown if salmon thrombin exhibits a different affinity for protein C and the PARs compared to mammalian thrombin, which might promote its abilities to protect spinal vascular
integrity and to reduce the inflammation that occurs after root compression, both of which are known to contribute to the development of pain (DeLeo and Yezierski 2001, Smith et al. 2015, Watkins et al. 2001).

The first set of studies in this chapter investigates the potential of salmon thrombin as a vascular protecting agent using both in vivo and in vitro approaches in comparison to the effects of human thrombin on vascular protection (Section 5.3). A painful nerve root compression model in the rat was used to define the differential effects of salmon and human thrombin on mediating the nerve root compression-induced BSCB breakdown at day 1. Supplementary in vitro studies were performed to define salmon thrombin’s effect on inflammation-induced endothelial permeability, with and without serum components, in comparison to human thrombin. In order to establish whether salmon thrombin activates protein C with a higher affinity than does human thrombin, APC generation rates produced by salmon and human thrombin were measured and compared using a fluorogenic peptide assay (Section 5.3). These complementary studies integrate in vivo and in vitro assays and are augmented by an in silico study using protein modeling of fish and human thrombin to identify mechanistic differences that may explain the experimental observations (Section 5.5).

The second portion of studies in the chapter was designed to test the hypothesis that salmon thrombin exhibits unique analgesic properties compared to its human analog that are dependent on its activation profile of PARs and its induction of glial-controlled inflammation. In order to evaluate salmon thrombin’s effects on glial-induced inflammation and pain (Section 5.4) rats undergoing a 15-minute nerve root compression were immediately treated with either salmon or human thrombin applied directly to the
nerve root. Following injury and treatment, mechanical allodynia (i.e. pain produced in response to a normally non-noxious mechanical stimulus) was measured over 7 days. Mixed cultures of astrocytes and neurons derived from embryonic rat brain cortices were used to define if the two species of thrombin differentially control IL-1β and TNF-α transcription early after thrombin treatment (Section 5.4). Proteolytic differences between salmon and human thrombin were measured using fluorogenic synthetic peptides corresponding to the cleavage sequences of PAR1, PAR3 and PAR4 (Section 5.4). Together, the results from Sections 5.3 and 5.4 demonstrate that salmon thrombin exhibits the distinct capability of activating protein C which leads to its inhibition of vascular disruption while only weakly activating PAR1, thereby preventing it from inducing inflammation; these findings are discussed and integrated in Sections 5.5 and 5.6.

5.3. Evaluating Salmon Thrombin as a Vascular Protecting Agent

5.3.1. Methods

5.3.1.1. Study Objectives & Design

The purpose of this study was to evaluate the effectiveness of salmon thrombin at preventing the BSCB breakdown and pain that are evident after a 15-minute nerve root compression (as shown in Figure 3.2) and to determine whether it acts through protein C to protect endothelial barriers, integrating in vivo and in vitro methods. For the in vivo portion of this study (Section 5.3.1.2), rats undergoing painful nerve root compression were treated with salmon thrombin, human thrombin, or neurobasal media as a vehicle
control. Since findings from studies in Section 3.3 identified day 1 as the time when BSCB breakdown is maximal (Figure 3.2), spinal IgG was labeled and quantified on day 1 after the injury and with the different treatments. To test whether salmon thrombin directly protects endothelial barriers, an in vitro microchannel setup was used to measure the relative effects of salmon and human thrombin on inflammation-induced vascular permeability (Section 5.3.1.3). Those experiments were performed in the presence of serum to expose the endothelial surface to protein C, as well as under serum-free conditions in order to determine if thrombin acts through surface receptors or serum components to modulate permeability. The production rate of APC also was compared between salmon and human thrombin using a fluorogenic peptide assay to determine if the two species exhibit different protein C activation rates (Section 5.3.1.4). Section 5.3.1.5 of this methods section describes modeling methods used to perform comparative protein modeling of fish and human thrombin.

5.3.1.2. Compression-Induced BSCB Breakdown & Mechanical Allodynia

Salmon thrombin (0.04U/rat; Sea Run Holdings; Freeport, ME) or human thrombin (0.04U/rat; Sigma Aldrich; St. Louis, MO) was dissolved in 20µl of neurobasal media and separately administered to treat a 15-minute nerve root compression. Salmon thrombin (15min+STh, n=5) or human thrombin (15min+HTh, n=4) solutions were delivered directly on top of the C7 nerve root immediately after removal of the 10-gram force clip that had applied compression (Chapter 3.3.1.1) (Rothman and Winkelstein 2010, Smith et al. 2013, Weisshaar et al. 2011). A control group received 20µl of the neurobasal media alone to account for the effects of the compression and the vehicle.
treatment (15min+veh, n=5). A separate group of rats also underwent a sham operation (n=3), as described in Chapter 3, to serve as surgical controls.

Rats underwent behavioral testing prior to surgery (day 0) and on day 1 after the procedures. Mechanical allodynia was used to quantify behavioral sensitivity in the forepaw ipsilateral to the injury and was measured by counting the number of forepaw withdrawals in response to 30 stimulations by a 4g von Frey filament (Stoelting Co., Wood Dale, IL) (Rothman et al. 2010, Smith et al. 2013). The number of paw withdrawals was averaged for each group on each day; differences were compared using a two-way repeated measures ANOVA (group x day) with Tukey’s HSD test. At day 1, spinal cord tissue at C7 on the side ipsilateral to the injury was harvested and fixed for immunolabeling of spinal IgG, as described in Section 3.3.1.3. Differences in ipsilateral spinal IgG between groups were determined using a two-way ANOVA (group x side) with Tukey’s HSD test.

5.3.1.3. Human Umbilical Vein Endothelial Cell Culture & Microchannel Experiments

Human Umbilical Vein Endothelial Cell (HUVEC)-lined microchannels were created as previously described (Galie et al. 2014). HUVECs were graciously provided as a gift from the Janmey lab. A stainless steel acupuncture needle was used to create a 400µm diameter channel within a central chamber of the microfluidic device filled with collagen (BD Biosciences; Franklin Lakes, NJ) at a concentration of 2mg/ml (Figure 5.1). HUVECs were injected into the channel (10,000 cells/cm²) and tight junctions were allowed to form by applying flow (5µl/min) for 24 hours. After sealing the vessels, FITC-
labeled dextran (70kDa; Sigma Aldrich; St. Louis, MO) and TNF-α (100ng/ml; Sigma Aldrich; St. Louis, MO) were perfused through the HUVEC-lined channels. Separate cell-lined channels were treated with dextran and TNF-α and separately stimulated with either salmon thrombin (1U/ml) or human thrombin (1U/ml). A separate set of tests were performed with the HUVECs exposed to serum free flow for 4 hours before stimulation by TNF-α, with or without either species of thrombin. Fluorescent dextran permeation into the collagen was tracked over 10 minutes by taking a fluorescent image every minute at 4x magnification. The diameter of the flow-front was measured at three points along the channel length in each image using ImageJ software, averaged and normalized to the channel diameter (Galie et al. 2014). The slope of the normalized front diameter over time was divided by the slope of the change in diameter over time that was induced by TNF-α alone in order to directly compare the effects of the two species of thrombin on the flux. Experiments were run in triplicate and differences in normalized dextran flux were compared between groups using a one-way ANOVA.

**Figure 5.1. Schematic of microfluidic in vitro setup.** The microfluidic device is comprised of UV-cured polydimethylsiloxane with an inner chamber of collagen. A cylindrical channel (400µm) is created within the collagen construct and HUVECs are seeded on the inner lumen of the chamber. Flow was applied (5µl/ml) to form vessels with the HUVECs (Galie et al. 2014).
5.3.1.4. Activated Protein C Substrate Assay

Salmon and human thrombin (1U/ml) were separately added to solutions containing protein C (19µg/ml; Haematologic Technologies Inc.; Essex Junction, VT) and a fluorogenic peptide mapping the cleavage site of the substrate for APC (Leu-Ser-Thr-Arg; 50µg/ml; Sigma Aldrich; St. Louis, MO) (Ohno et al. 1981). The fluorescent intensity of the solutions was read at 380nm/460nm (ex/em) every 10 seconds for 300 seconds. Controls included salmon and human thrombin separately in solution with the APC substrate in order to account for any substrate activation by either species of thrombin alone. Experiments were run in triplicate and differences in the rate of APC production induced by salmon and human thrombin were measured using a t-test.

5.3.1.5. Comparative Protein Modeling of Fish & Human Thrombin

In a complementary study, the protein structures of fish and human thrombin were compared to determine if the specific regions within the thrombin structure that control substrate affinity differ between the two species of thrombin. The human thrombin autolysis loop (residue 146 to 149e) was absent from the crystal structure of thrombin. Therefore, the autolysis loop (UniProt: P00734) was modeled into a previously validated crystal structure for human thrombin bound to a short peptide of protein C (PDB: 4DT7 chain B). The thrombin model containing the autolysis loop was created by generating 100 potential human thrombin models, and the final structure was the average of the ten models with the lowest overall Discrete Optimized Protein Energy score (Eswar et al. 2006, Fiser et al. 2000, Marti-Renom et al. 2000, Sali 1995). The sequence for trout thrombin (UniProt: Q5NKF9) (Figure 5.2), which is the most similar fully sequenced
protein to salmon thrombin (Griffin 1995), was then threaded into the human thrombin crystal structure.

A model for protein C was created by submitting the full peptide sequence for protein C (UniProt: P04070) to the Protein Homology/analogy Recognition Engine V2.0 (Phyre 2) webserver (Kelley and Sternberg 2009). Using PDB code 4O03 as a template, 89% of the residues were modeled at >90% confidence. The full protein C model was refined using a steepest descent minimization in the Gromacs simulation (Pronk et al.

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**Figure 5.2. Comparison of the amino acid sequences for thrombin derived from human (Homo sapien) and rainbow trout (Salmo gairdneri).** The primary structure of human thrombin (HTh, top line) exhibits strong agreement with the structure of fish thrombin (FTh, bottom line), except for in the autolysis loop (boxed in blue), which partially controls thrombin’s interaction with protein C.
2013) software in the presence of water and ions. A short (100ps) NVT simulation was used to further equilibrate the protein structure. The full protein C was then aligned with the short protein C peptide (QEDQVDPR) in the human thrombin structure (PDB: 4DT7) with an RMSD of 3.0 Å over 65 atoms.

5.3.2. Results

Salmon thrombin that is applied directly to the nerve root immediately after a 15-minute compression significantly reduces (p=0.0148) mechanical allodynia, quantified as the number of paw withdrawals, elicited by a 4g stimulus on day 1 compared to responses for compression treated with human thrombin (Figure 5.3A). Treatment of a 15-minute compression with human thrombin induces a significantly greater (p=0.016) number of paw withdrawals compared to sham operated rats and is not different from the responses after the neurobasal media vehicle treatment (Figure 5.3A). The individual paw withdrawal data for each rat in each group are provided in Appendix A.

Paralleling the pain responses, salmon thrombin induces significantly less (p<0.0009) IgG labeling in the ipsilateral spinal cord on day 1 after compression compared to treatment with either human thrombin or vehicle (Figure 5.3B). Both the human thrombin and vehicle treated compression injuries induce a significant increase (p<0.0065) in IgG in the ipsilateral spinal cord compared to the levels in the contralateral spinal cord (Figure 5.3B). A detailed summary of the quantification of IgG expression in the ipsilateral spinal cord for each rat in this study is provided in Appendix B.
TNF-α induces endothelial barrier permeability in HUVEC-lined microchannels, which is evident by the flux of fluorescent dextran out of the channel in the presence or absence of serum (Figure 5.3C). The addition of salmon thrombin with TNF-α in the HUVEC vessels blunts the propagation of the fluorescent front into the collagen, but only in the presence of serum (Figure 5.3C). In contrast, human thrombin with TNF-α only exacerbates the rate of dextran flux out of the channel (Figure 5.3C); this elevation in dextran flow rate over TNF-α alone induced by human thrombin occurs both in the
presence and the absence of serum. Salmon thrombin significantly reduces (p=0.0073) the TNF-α-induced dextran flux through the HUVEC channels compared to human thrombin, but only in HUVEC cultures exposed to serum. The normalized rates of dextran diffusion for each treatment group in both serum containing and serum free conditions are detailed in Appendix D.

Salmon thrombin produces APC at a rate approximately 45-times faster than does human thrombin (Figure 5.3D); indeed, that increase in rate is significant (p=0.008). The reactions within this experiment are coupled; thrombin activates protein C within the test solution and the generated APC activates the fluorogenic substrate. The rate of substrate cleavage in a solution containing human thrombin with protein C is not different than the rate produced by a control solution of human thrombin alone with the substrate, suggesting that human thrombin is incapable of generating APC. Appendix E summarizes the detailed rates of production of APC by both species of thrombin.

The predicted model of fish thrombin is highly aligned with the crystal structure for human thrombin with an RMSD=0.51Å over the 265 residues (Figure 5.4). The peptide sequence that makes up the autolysis loop within the thrombin structures varies between the two species (Figures 5.2 & 5.4). That difference in sequence of amino acids is translated into a predicted geometric difference in crystal structures between the two species of thrombin (Figure 5.4). Introducing the protein C crystal into the crystalized structures of human and fish thrombin shows that the autolysis loop for both species of thrombin interacts closely with protein C, but that human thrombin may sterically inhibit protein C interaction more than salmon thrombin (Figure 5.4).
Fig. 5.4. Protein structure for thrombin derived from fish and from human differs in the autolysis loop, which partially controls thrombin’s specificity for protein C. Aligned amino acid sequences for the B chains of human thrombin (HTh) and fish thrombin (Salmo gairdneri, FTh) exhibit a key divergence in the autolysis loop (blue). A crystal structure for HTh (magenta) bound to a small peptide of protein C (PDB:4DT7) and the created homology model for FTh (orange) have strong alignment (RMSD=0.51Å over 265 residues) shown in ribbon representation. The autolysis loop (in blue) does not retain the same structure between species. The residues within both autolysis loops are represented below as sticks to visualize predicted close interactions with protein C (green cage structure).
5.4. Evaluating Salmon Thrombin’s Effects on Glial-Induced Inflammation & Pain

5.4.1. Methods

5.4.1.1. Formulation of Human & Salmon Thrombin

Thrombin from human plasma (Sigma Aldrich; St. Louis, MO) lyophilized from a sodium citrate buffer was reconstituted in sterile water to a concentration of 100U/ml, according to manufacturer protocols. Salmon thrombin (Sea Run Holdings; Freeport, ME) was prepared from precipitates derived from anticoagulated salmon blood, as previously described (Michaud et al. 2002, Smith et al. 2015, Weisshaar et al. 2011, Winer et al. 2009, Zhang et al. 2013). Salmon thrombin was reconstituted in a buffer consisting of 1M NaCl, 1mM EGTA, 20mM Tris, pH 7.0 and 0.6mg/ml sucrose, at a concentration of >1000U/ml (Weisshaar et al. 2011). Stock solutions of human and salmon thrombin were aliquoted and stored at -80°C until used for testing. The activity of human and salmon thrombin was determined prior to each experiment using either the fluorogenic thrombin substrate III (Calbiochem; San Diego, CA) diluted in PBS or the chromogenic substrate Chromozym TH (Roche; Nutley, NJ); this procedure ensured that equivalent activity between thrombin species were used in each study.

5.4.1.2. Surgical Protocol & Behavioral Assessment

In vivo experiments were performed on male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, IN), weighing 358 ± 26g at the start of the study. Rats were housed under conditions compliant with the U.S. Department of Agriculture and Association for
Assessment and Accreditation of Laboratory Animal Care including a 12–12 hour light–dark cycle and free access to food and water.

A transient compression of the right C7 dorsal nerve root was applied for 15 minutes (Hubbard and Winkelstein 2005, Rothman et al. 2009, Weisshaar et al. 2011). Briefly, surgical procedures were performed under inhalation anesthesia with the rat in the prone position (4% isoflurane for induction, 2% for maintenance). An incision was made from the base of the skull to the T2 spinous process. Along the dorsal region of the spine, the posterior bones and the facet joint on the right side of the C6/C7 spinal levels were removed in order to expose the right C7 dorsal nerve root. A hole was made in the dura and the cervical nerve root was compressed for 15 minutes with a calibrated 10gf microvascular clip (World Precision Instruments; Sarasota, FL). Following clip removal, any blood was cleared from the compressed nerve root and 20µl of either salmon (15min+STh, n=13) or human (15min+HTh, n=11) thrombin (2U/ml in neurobasal media) was added to the nerve root. A separate control group received a vehicle treatment (15min+veh, n=12) of only 20µl of neurobasal media. Sham operated rats (sham, n=5) were included to serve as a surgical control, underwent all surgical procedures except for compression of the nerve root, and did not receive vehicle or treatment.

Behavioral sensitivity was assessed in the forepaw by measuring mechanical allodynia on days 1, 3, 5 and 7 after injury (Hubbard and Winkelstein 2005, Weisshaar et al. 2011). Allodynia was also measured for each rat before any surgical procedures in order to establish baseline responses (day 0). Prior to each testing session rats were placed in elevated cages with mesh bottoms and allowed to acclimate for 15 minutes. Mechanical allodynia was measured by stimulating the plantar surface of the forepaw on
the side ipsilateral to the injury, using 1.4, 4 and 10g von Frey filaments (Stoelting Co.; Wood Dale, IL). Testing sessions consisted of three rounds of 10 stimulations with each filament, separated by 10 minutes of rest. A positive response was defined as a paw withdrawal and was often accompanied by licking or shaking of the paw. The number of paw withdrawals elicited by each filament in a session was counted, averaged and reported as the measurement of mechanical allodynia for that day. Two-way repeated measures ANOVAs (group x day) with Tukey’s test determined statistical differences in mechanical allodynia between a nerve root compression treated with vehicle, human thrombin, or salmon thrombin, and sham for each testing filament.

5.4.1.3. Culture of Dissociated Brain Cortices

Primary cortical cultures were isolated from embryonic day 18 rat pup brains (Miller et al. 2009). The meninges were removed and the remaining cortices were dissected and dissociated at 37°C in neurobasal media (Invitrogen Corp.; Carlsbad, CA) with trypsin (0.3mg/ml; Sigma-Aldrich; St. Louis, MO) plus DNase I (0.2mg/ml; Amersham Biosciences; Piscataway, NJ). After 20 minutes, soybean trypsin inhibitor (0.5mg/ml; Gibco; Grand Island, NY) was added and the tissue was broken apart by manual pipetting. Cell solutions were centrifuged at 1000rpm for 5 minutes and the remaining pellet was re-suspended in DMEM with Glutamax (Gibco; Grand Island, NY) supplemented with fetal bovine serum (FBS; Gibco; Grand Island, NY). Cells were filtered through 60µm and 28µm Nitex meshes sequentially and plated at a density of 4x10^6 cells/ml on T75 tissue culture flasks treated with poly-D-lysine (PDL; Sigma Aldrich; St. Louis, MO). Cultures were maintained at 37°C and 5% CO₂ and the media
was replaced every 3 or 4 days. Mixed cultures were re-plated at a concentration of $2 \times 10^6$ cells/ml onto 60mm PDL-coated culture dishes at 14 days in vitro (DIV).

### 5.4.1.4. Real Time RT-PCR for Cytokine mRNA

Early upregulation of spinal pro-inflammatory cytokine (IL-1β, TNF-α, and IL-6) production has been directly implicated in nerve root-mediated pain, with blocking either IL-1β or TNF-α after a painful compression significantly reducing behavioral sensitivity (Rothman et al. 2009, Rothman and Winkelstein 2010). For this reason, levels of transcription of these two pro-inflammatory cytokines induced in primary cortical cultures by salmon and human thrombin were measured using real time reverse transcriptase polymerase chain reaction (RT-PCR). In order to distinguish the direct effect of both species of thrombin on inflammatory cytokine production, separate mixed cultures were treated with salmon (STh, n=5) or human (HTh, n=5) thrombin (1U/ml) at 20 DIV (day 0). At 4 hours after treatment, cells were washed with PBS and RNA was harvested using Qiagen’s RNeasy mini kit (Qiagen; Valencia, CA). The concentration and quality of the RNA samples were measured using a NanoDrop spectrometer (NanoDrop Technologies; Wilmington, DE). RNA samples (0.5µg total RNA) were treated with DNTP (Invitrogen; Carlsbad, CA) and random primers (Invitrogen; Carlsbad, CA) for 5 minutes at 65°C before they were reverse transcribed according to manufacturer protocols using Superscript III reverse transcriptase (Invitrogen; Carlsbad, CA) and the RNase inhibitor, RNaseOUT (Invitrogen; Carlsbad, CA), at 50°C for 45 minutes.
Synthesized cDNA was used for real-time PCR with specific primer sequences for the pro-inflammatory cytokines, IL-1β (Fwd: 5’-CAC CTC TCA AGC AGA GCA CAG-3’, Rev: 5’-GGG TTC CAT GGT GAA GTC AAC-3’) and TNF-α (Fwd: 5’-ATC ATC TTC TCA AAA CTC GAG TGA CAA-3’, Rev: 5’-CTG CTC CTC TGC TTG GTG CAA-3’) (Rothman et al. 2009). Each reaction contained equal amounts of synthesized cDNA, appropriate primers, and SYBR green master mix (Applied Biosystems; Foster City, CA). Real-time PCR was performed using an ABI-7300 system (Applied Biosystems; Foster City, CA) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 minutes and 60°C for 1 minute. All samples were run in duplicate and a no-cDNA standard was included for each run.

Expression of the target genes were normalized to levels of the housekeeping gene, cyclophilin-A (Fwd: 5’-TAT CTG CAC TGC CAA GAT CTA GTG-3’, Rev: 5’-CTT CTT GCT GGT CTT GCC ATT CC-3’). The expression of the target gene in the groups treated with thrombin were normalized to levels in untreated controls (UT, n=8) from each cortical dissociation using the ∆∆Ct method (Rothman et al. 2009). Gene expression levels were reported as the fold-difference compared to untreated control levels. Values were averaged within groups and differences between HTh treated, STh treated and untreated cultures were tested using a one-way ANOVA, with post-hoc Tukey HSD test.

5.4.1.5. PAR-Based Synthetic Peptide Cleavage

Since PAR1, PAR3 and PAR4 are the main receptors through which thrombin modulates cellular responses (Bunnett 2006, Coughlin 2000, Jacques et al. 2000, Luo et
al. 2007), the relative cleavage rates of PAR-based peptides by salmon and human thrombin were measured in order to determine if salmon thrombin preferentially activates one receptor over another. Fluorogenic synthetic peptide substrates were designed corresponding to the amino acid sequence at the human PAR1, 3 and 4 cleavage sites at greater than 85% purity (Abgen; San Diego, CA). The peptides were comprised of a hydroxyl group, the three amino acids terminal to the native receptor’s extracellular cleavage site and an amido-4-methylcoumarin (AMC) functional group that fluoresces when hydrolyzed from the peptide (Table 5.1).

<table>
<thead>
<tr>
<th>receptor</th>
<th>peptide sequence</th>
<th>fluorogenicity</th>
</tr>
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<tbody>
<tr>
<td>PAR1</td>
<td>Asp-Pro-Arg (DPR-AMC)</td>
<td>AMC</td>
</tr>
<tr>
<td>PAR3</td>
<td>Pro-Ile-Lys (PIK-AMC)</td>
<td>AMC</td>
</tr>
<tr>
<td>PAR4</td>
<td>Ala-Pro-Arg (APR-AMC)</td>
<td>AMC</td>
</tr>
<tr>
<td>PAR1-hir</td>
<td>(DNP)-WFPEYKDNPNRLLFSRPDL-Abz</td>
<td>FRET</td>
</tr>
</tbody>
</table>

The peptide substrates (40µM) were separately added to PBS at 37°C and vortexed. Human (HTh, n=2) or salmon (STh, n=2) thrombin at 1U/ml was added to each solution, vortexed and immediately placed in the fluorimeter. The substrate solutions were excited at 346nm and the fluorescent intensity was measured at 446nm and recorded over a period of 5 minutes. Substrate hydrolysis was reported as the slope of 4 minutes of the intensity versus time data. Differences in cleavage rates for each PAR-based substrate were determined between salmon and human thrombin using a Student’s t-test.

A peptide sequence on an exodomain of PAR1, also known as the hirudin-like sequence, interacts with exosite I of thrombin (see Figure 5.4) and facilitates mammalian
thrombin’s cleavage of PAR1 in vivo (Jacques et al. 2000, Seeley et al. 2003). A modified PAR1-based fluorescence resonance energy transfer (FRET) peptide (PAR1-hir) was designed to include the hirudin-like amino acid sequence in order to more accurately model the native PAR1 receptor (Table 5.1). The PAR1-hir FRET substrate (40µM) was diluted in PBS at 37°C and 1U/ml of each species of thrombin (n=4/group) was added, separately, to the solutions. Immediately following the addition of thrombin, the mixture was excited at 298nm and the fluorescence at 355nm was recorded for 60 seconds. All substrate cleavage data are reported as the slope of the intensity-time plot fitted to 20 seconds of linear data. The difference between STh and HTh was determined using a Student’s t-test.

5.4.2. Results

A 15-minute nerve root compression treated with neurobasal media vehicle induces a significant increase (p<0.012) in the number of ipsilateral paw withdrawals compared to sham responses overall, for each filament strength tested (1.4, 4 and 10g) (Table 5.2; Figure 5.5). Salmon thrombin treatment of a nerve root compression significantly reduces the number of paw withdrawals elicited by all of the filament strengths overall relative to vehicle treatment (p<0.028) (Table 5.2; Figure 5.5). In fact, when considering the overall response, treating a painful compression with salmon thrombin reduces the number of paw withdrawals to sham levels in response to all filaments (Table 5.2; Figure 5.5). When human thrombin is applied to the compressed nerve root, allodynia responses are not different from those induced by compression with
the vehicle and are significantly higher (p<0.047) than the responses for salmon thrombin treatment (Table 5.2; Figure 5.5).

![Figure 5.5. Salmon thrombin attenuates mechanical allodynia in the ipsilateral forepaw after a painful nerve root compression.](image)

The number of withdrawals by the ipsilateral forepaw is significantly elevated (p<0.012) after a 15-minute compression treated with the vehicle (15min+veh), compared to sham overall, for all filament strengths (1.4, 4, 10g). Treating a compression with salmon thrombin (15min+STh) reduces (p<0.028) the number of paw withdrawals compared to 15min+veh and is not different from sham overall, for all filaments. Treating a compression with human thrombin (15min+HTh) does not alter mechanical allodynia from vehicle treatment and the overall allodynia response is significantly elevated over the sham (p<0.009) and the 15min+STh (p<0.047) groups, for each filament. The 15min+veh group exhibits significantly more paw withdrawals than the sham group on day 1 for all filament strengths (#p<0.010) and this elevation is maintained until day 3 (#p=0.036) for testing with the 10g filament. Salmon thrombin significantly reduces the number of paw withdrawals compared to vehicle treatment on day 3 for the 10g filament (&p=0.006). Compared to responses after 15min+HTh, the 15min+STh group exhibits significantly fewer paw withdrawals on day 1 for the 1.4g filament (**p=0.003) and on each individual testing day for the 10g filament (***p<0.027). The number of paw withdrawals is significantly higher for 15min+HTh than sham on day 1 for the 1.4g filament (*p<0.001), for days 1 and 3 for testing with the 4g filament (*p<0.041), and for all days for testing with the 10g filament (*p<0.027). Data are represented as mean±SD.
The differences in mechanical allodynia between a compression with vehicle treatment and a sham operation are most robust at day 1 (Figure 5.5), with the number of paw withdrawals elicited by each filament being significantly higher ($p<0.010$) for the compression with vehicle treatment than a sham response at day 1 (Table 5.2; Figure 5.5). Yet, overall a painful compression treated with human thrombin does not prevent the increase ($p<0.009$) in the number of withdrawals for testing with all three filament strengths (Table 5.2; Figure 5.5). The number of paw withdrawals elicited by a 10g filament for compression with human thrombin treatment is significantly higher ($p<0.027$) than sham on each day of testing (Table 5.2; Figure 5.5). Further, salmon thrombin significantly attenuates ($p<0.027$) compression-induced allodynia on every testing day compared to human thrombin in response to stimulation by the 10g filament (Table 5.2; Figure 5.5). All of the mechanical allodynia data for each rat on each test day in response to the 1.4, 4 and 10g von Frey filaments are summarized in Appendix A.

**Table 5.2.** Statistical differences in the number of paw withdrawals between a 15-minute compression treated with salmon thrombin (STh), human thrombin (HTh), or vehicle, and sham procedures summarized by the von Frey filament strength and day, as well as overall.

<table>
<thead>
<tr>
<th></th>
<th>1.4g</th>
<th>4g</th>
<th>10g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>day 1</strong></td>
<td>$p=0.003$ STh &lt; HTh</td>
<td>$p&lt;0.001$ HTh &gt; sham</td>
<td>$p=0.001$ STh &lt; HTh</td>
</tr>
<tr>
<td></td>
<td>$p=0.010$ vehicle &gt; sham</td>
<td>$p=0.006$ HTh &gt; sham</td>
<td>$p&lt;0.001$ STh &lt; HTh</td>
</tr>
<tr>
<td><strong>day 3</strong></td>
<td>none</td>
<td>$p=0.041$ HTh &gt; sham</td>
<td>$p=0.004$ STh &lt; HTh</td>
</tr>
<tr>
<td></td>
<td>$p=0.006$ HTh &gt; sham</td>
<td>$p=0.006$ STh &lt; vehicle</td>
<td>$p=0.027$ HTh &gt; sham</td>
</tr>
<tr>
<td><strong>day 5</strong></td>
<td>none</td>
<td>none</td>
<td>$p&lt;0.001$ STh &lt; HTh</td>
</tr>
<tr>
<td></td>
<td>$p=0.006$ HTh &gt; sham</td>
<td>$p=0.006$ HTh &gt; sham</td>
<td>$p=0.013$ HTh &gt; sham</td>
</tr>
<tr>
<td><strong>day 7</strong></td>
<td>none</td>
<td>none</td>
<td>$p=0.027$ STh &lt; HTh</td>
</tr>
<tr>
<td></td>
<td>$p=0.012$ vehicle &gt; sham</td>
<td>$p=0.047$ STh &lt; HTh</td>
<td>$p=0.004$ STh &lt; HTh</td>
</tr>
<tr>
<td><strong>overall</strong></td>
<td>$p=0.024$ STh &lt; HTh</td>
<td>$p=0.028$ STh &lt; vehicle</td>
<td>$p=0.004$ STh &lt; HTh</td>
</tr>
<tr>
<td></td>
<td>$p=0.009$ HTh &gt; sham</td>
<td>$p=0.022$ STh &lt; veh</td>
<td>$p=0.014$ STh &lt; veh</td>
</tr>
<tr>
<td></td>
<td>$p=0.007$ HTh &gt; sham</td>
<td>$p=0.007$ HTh &gt; sham</td>
<td>$p=0.001$ HTh &gt; sham</td>
</tr>
<tr>
<td></td>
<td>$p=0.004$ vehicle &gt; sham</td>
<td>$p=0.004$ vehicle &gt; sham</td>
<td>$p=0.003$ veh &gt; sham</td>
</tr>
</tbody>
</table>
The IL-1β and TNF-α mRNA levels produced by mixed cortical cultures are significantly upregulated at 4 hours after treatment with human thrombin compared to untreated controls (p<0.001) (Figure 5.6). In contrast to the pro-inflammatory responses induced by human thrombin, mixed cultures treated with salmon thrombin express IL-1β and TNF-α mRNA at the same levels as untreated cultures and at levels significantly lower than in the cultures treated with human thrombin (p<0.002) (Figure 5.6). The specific expression levels of IL-1β and TNF-α mRNA induced by human and salmon thrombin in astrocytic cultures is detailed in Appendix F.

Figure 5.6. Salmon thrombin does not increase pro-inflammatory cytokines in cortical cultures at 4 hours after treatment. IL-1β and TNF-α mRNA are significantly upregulated at 4 hours after treatment with human thrombin (HTh) at 1U/ml compared to untreated cultures (UT) (*p<0.001). Salmon thrombin (STh), at the same concentration, induces levels of IL-1β and TNF-α transcription that are significantly less than human thrombin treatment (HTh) (**p<0.002) and not different from levels in UT. Data are mean±SD.

Salmon thrombin hydrolyzes the fluorogenic PAR1 peptide mapping the cleavage site of PAR1 (Table 5.1) significantly slower (p=0.004) than does human thrombin (Figure 5.7A). Salmon thrombin also cleaves the PAR1 peptide including the cleavage site and the hirudin sequence (Table 5.1) at a significantly slower rate than does human
thrombin (p<0.001) (Figure 5.7A). The peptides corresponding to the PAR3 and PAR4 cleavage sites are cleaved at rates that are indistinguishable between salmon and human thrombin (Figure 5.7B) and that are substantially slower than that for either PAR1 peptide (Figure 5.7). Appendix E contains the summary of specific studies quantifying the activation rate of these PAR-based peptides by both species of thrombin.

5.5. Discussion

Collectively, these results are the first to show that salmon thrombin attenuates behavioral sensitivity generated by a painful nerve root compression and suggest that unique cellular and sub-cellular mechanisms promote its analgesic properties. Even a single dose of salmon thrombin placed directly in contact with the injured nerve root inhibits the development of pain (Figure 5.3A), an effect that is sustained for up to 7 days.
after the initial injury and is not evident with human thrombin (Figure 5.5). Salmon thrombin also blocks the nerve root-induced BSCB disruption that is typically observed at day 1 when pain is also observed (Figure 5.3). Of note, salmon thrombin actively blocks inflammation-induced endothelial barrier disruption, but only in the presence of serum (Figure 5.3C), and does not induce astrocytic transcription of TNF-α or IL-1β (Figure 5.6). The biological differences between these two species of thrombin can be explained by their differences in substrate activation rates; specifically, salmon thrombin activates PAR1 slower, and generates APC faster, than does human thrombin (Figures 5.3D & 5.7). Since salmon thrombin actively prevents vascular disruption without stimulating glial-induced inflammation, this therapeutic is also promising for other neurodegenerative disorders that are exacerbated by BBB breakdown (Hawkins and Davis 2005, Hirsch and Hunot 2009, Sandoval and Witt 2008, Sharief and Thompson 1992, Winkler et al. 2014, Zlokovic 2008, Zlokovic 2011).

Salmon thrombin given immediately after a painful nerve root compression attenuates pain by day 1 and is effective for at least 7 days after injury, whereas human thrombin does not reduce behavioral sensitivity at this dose and administration route (Figures 5.3A & 5.5). Salmon and other teleosteans, or bony fish, evolved in parallel to humans and, in turn, exhibit less complicated clotting and inflammation cascades than mammals (Doolittle 2011, Tort et al. 2003). Because inflammation and pain are highly integrated biological cascades (DeLeo and Yezierski 2001, Scholz and Woolf 2007), it is possible that salmon produce coagulation proteins that do not elicit the pro-inflammatory and pro-nociceptive responses that are induced by their human counterparts. Salmon fibrin, which is the product of thrombin and fibrinogen, also attenuates pain after this
same root compression (Weisshaar et al. 2011), confirming the analgesic properties of multiple coagulation factors derived from salmon.

Unlike fish thrombin, mammalian (rodent) thrombin has been shown to transiently induce pain and its inhibition in the CNS by intrathecal hirudin prior to neural injury reduces sensitivity for up to 8 days after a peripheral nerve (Narita et al. 2005) or a nerve root injury (Figure 4.5). Interestingly, human thrombin has been reported to both induce and attenuate pain depending on the concentration and the anatomical location of its administration (Asfaha et al. 2002, Chang et al. 2010, Fang et al. 2003, Narita et al. 2005). In the current study, both salmon and human thrombin were administered at 2U/ml, a concentration at which their enzymatic activity toward fibrinogen is not different (Smith et al. 2013). Further, both species of thrombin were administered in the same location directly on top of the compressed nerve root, which removes the potential variability due to the site of administration. Thrombin’s enzymatic activity is directed by its chemical and structural interaction with its substrates (Dang et al. 1997, Gandhi et al. 2011, Seeley et al. 2003), and in turn controls which substrate it activates (Fuentes-Prior et al. 2000, Jacques et al. 2000, Xu et al. 2005). Salmon thrombin exhibits high affinity for protein C and low affinity for PAR1 (Figures 5.3D & 5.7), which is likely controlled by the differences in protein structure between the two species that is revealed in the comparative protein model (Figure 5.4). Therefore, the observed differences in analgesia produced by these two species of thrombin are independent of both concentration and location and likely depend on their different inherent substrate affinity profiles and the induced downstream cellular signaling cascades.
This work is the first to show that salmon thrombin innately blocks endothelial vessel permeability – both in the spinal cord after painful nerve root compression (Figure 5.3B) and in HUVEC microvessels induced by TNF-α stimulation (Figure 5.3C). Interestingly, the effects of salmon thrombin on endothelial cells are potent enough to combat those effects that are induced by the relatively high dose of TNF-α used to disrupt HUVEC barriers (Figure 5.3C). Stimulation of HUVEC monolayers with TNF-α at even 0.5ng/ml induces significant monolayer permeability (Nooteboom et al. 2002), whereas the dose of TNF-α (100ng/ml) used in this study was 200-times greater. The fact that salmon thrombin only prevents TNF-α-induced vascular disruption in the presence of serum (Figure 5.3C) suggests that its favorable effects on endothelial cells occur indirectly through serum proteins, likely serum-derived protein C (Riewald et al. 2002), and not via endothelial cell-expressed receptors, such as the PARs. In biological systems, mammalian thrombin is also capable of stabilizing endothelial barriers, but only when bound to thrombomodulin (Bouwens et al. 2013, Riewald et al. 2002). However, mammalian thrombin preferentially binds to, and activates, PAR1 over thrombomodulin which causes it to primarily disrupt vasculature (Coughlin 2000, Esmon 1993, Ye et al. 1992). This endothelial-disrupting effect of human thrombin is demonstrated by the in vivo and in vitro studies in this chapter as well (Figure 5.3C). Since salmon thrombin is capable of producing APC in the absence of thrombomodulin (Figure 5.3D), it may be implied that the innate chemistry and structure of salmon thrombin does not inhibit its interaction with protein C, which is typically observed for mammalian-derived thrombin. Therefore, naturally-derived salmon thrombin might be a promising alternative to
synthetically modified versions of mammalian thrombin that exhibit a higher affinity for protein C.

In addition to exerting distinct effects on endothelial cells, salmon thrombin does not appear to contribute to pro-inflammatory cytokine production in mixed neuronal-glial cultures, which is in contrast to the effects of human thrombin (Figure 5.6). Human thrombin induces the transcription of IL-1β and TNF-α at 4 hours after treatment (Figure 5.6), which is consistent with other reports that glial cultures produce IL-1β, TNF-α, IL-6 and a variety of other cytokines by 8 hours after stimulation by human thrombin (Boven et al. 2003, Choi et al. 2008, Fan et al. 2005, Simmons et al. 2013). In the same painful radiculopathy model used in the current work (Figures 5.3 & 5.5), rat thrombin has been shown to extravasate into the spinal parenchyma by day 1 (Figure 4.3) (Smith and Winkelstein 2015), where it can interact with both neurons and astrocytes. That painful root compression also induces an early increase in the spinal transcription and expression of TNF-α and IL-1β, both of which contribute to pain development (Rothman et al. 2009, Rothman et al. 2011). Since human thrombin increases the transcription of TNF-α and IL-1β in neural populations in vitro (Figure 5.6), it is possible that rat thrombin stimulates spinal cells to produce these cytokines when it is present in the spinal cord after root injury, thereby contributing to the production of pain. Since salmon thrombin does not initiate inflammation in cultures derived from the CNS at the concentration tested in this study (Figure 5.6), using salmon thrombin as a treatment for neural injury is not expected to exacerbate inflammatory states that contribute to pain.

Salmon thrombin may also prevent pain development after root compression by promoting neuronal health. By day 14 after a 15-minute nerve root compression, the
injured root exhibits disruption in the normal striated patterning of myelin basic protein (MBP) and axonal degeneration (Chang and Winkelstein 2011, Hubbard and Winkelstein 2005, Hubbard and Winkelstein 2008). Those hallmarks of axonal demyelination are also accompanied by increases in the phagocytotic macrophage population and sustained pain (Chang and Winkelstein 2011). However, salmon thrombin prevents that axonal demyelination and significantly reduces the macrophage infiltration at the root (Figure 5.8) (Smith et al. 2013). In agreement, a previous study showing that thrombin and fibrin, both derived from salmon, also reduce nerve root compression-induced macrophage infiltration at the injured root at an earlier time point (day 7) (Weisshaar et al. 2011). Yet, human thrombin may actually exacerbate both myelin disruption and macrophage infiltration (Figure 5.8) (Smith et al. 2013), which may be a mechanism by which it further contributes to neurodegeneration after trauma. Indeed, mammalian thrombin has been shown to induce neurite retraction and apoptosis in neuronal cultures when given at concentrations above 10U/ml for 24 hours (Garcia et al. 2015, Striggow et al. 2000). Although human thrombin was administered at a lower dose (2U/ml) after nerve root compression (Smith et al. 2015, Smith et al. 2013), it is possible that in combination with the rat thrombin that is likely present at the site of injury due to the bleeding from the tissue trauma, the combined increase in mammalian thrombin is sufficient to exacerbate the axonal degeneration in the rat (Figure 5.8). Distinct from the effects of human thrombin, salmon thrombin has also been shown to reduce spinal neuronal hyperexcitability at day 7 after painful nerve root compression (Smith et al. 2013). Salmon thrombin’s protection of axonal structure in the compressed root and suppression
of hyperexcitability centrally (Smith et al. 2013), further support salmon thrombin as a neuroprotective treatment for pain.

The unique cellular effects exerted by salmon thrombin are likely due to its distinct substrate activation profile from that of human thrombin: its higher activation rate of protein C and its slower activation rate of PAR1 (Figure 5.3D & 5.7A). The cleavage rates of the PAR3 and PAR4 peptides by either species of thrombin are much slower than for PAR1 (Figure 5.7B), suggesting that activation of PAR3 and PAR4 is not responsible for the different cellular outcomes induced by the two species of thrombin. In biological
systems, mammalian thrombin exhibits the highest affinity for PAR1 because exosite I of thrombin is highly attracted to a sequence of amino acids on an extracellular loop of PAR1 that resembles hirudin (Gandhi et al. 2011, Jacques et al. 2000, Seeley et al. 2003). Hirudin is a natural peptide that is secreted from leeches and is a potent and specific inhibitor of the enzymatic activity of mammalian thrombin (Rydel et al. 1990). The studies in this chapter identify that salmon thrombin is substantially less effective than human thrombin at activating PAR1, even when that hirudin-like sequence is included in the fluorogenic peptide (Figure 5.7A). This reduced rate of PAR1 activation suggests that salmon thrombin might exhibit a lower affinity than human thrombin for hirudin and peptides with similar sequences. In fact, salmon thrombin, at the same concentration used in this study, is inhibited less effectively by hirudin than human thrombin (Smith et al. 2013), further supporting that exosite I of salmon thrombin might not induce the same high affinity for hirudin that human thrombin does. The amino acid sequence of exosite I for salmon thrombin is slightly modified compared to that of human thrombin (Figure 5.2); those amino acid substitutions might prevent salmon thrombin from interacting with the hirudin-like sequence in PAR1 and contribute to the reduction in PAR1 activation rate compared to human thrombin that is observed here (Figure 5.7A). Mammalian thrombin induces astrocyte activation and pro-inflammatory cytokine release through its cleavage of PAR1 expressed in astrocytes (Nicole et al. 2005, Niego et al. 2011, Nishino et al. 1993). Since salmon thrombin activates PAR1 less effectively (Figure 5.7A) and induced less astrocytic production of TNF-α and IL-1β (Figure 5.6) compared to human thrombin, it can be inferred that salmon thrombin may not induce inflammation because of its inability to activate PAR1.
Salmon thrombin also produces APC more effectively than human thrombin, even in the absence of thrombomodulin (Figure 5.3D). Thrombomodulin is requisite for mammalian thrombin’s activation of protein C; it binds to exosite I preventing thrombin from activating PAR1 and other substrates recognized by exosite I (Fuentes-Prior et al. 2000, Pineda et al. 2004). There is evidence that thrombomodulin might also alter the shape of the thrombin epitope that interfaces with protein C, thereby changing the confirmation of residues that sterically inhibit the interaction in the unbound confirmation (Xu et al. 2005, Ye et al. 1992). Salmon thrombin exhibits amino acid substitutions in exosite I compared to human thrombin that change the charges of those regions (Figure 5.2). Specifically the Threonine and Tyrosine contained in exosite I of human thrombin are amino acids that contain neutral and hydrophobic side chains, respectively (Figure 5.2). Those residues are substituted with a hydrophobic Alanine and a very hydrophobic Phenylalanine in salmon thrombin (as shown in Figure 5.2), which are non-conservative amino acid substitutions and might modify the native structure of salmon, compared to human, thrombin. Since thrombomodulin sterically changes the structure of human thrombin in order to bind to protein C (Xu et al. 2005, Ye et al. 1992), salmon thrombin’s already modified structure might enable it to bypass that requisite thrombomodulin binding exhibited by mammalian thrombin and directly interact with protein C (Fuentes-Prior et al. 2000, Rezaie and Yang 2003, Xu et al. 2005, Yang et al. 2006).

The comparative thrombin structural models show that salmon thrombin exhibits the most radical changes from human thrombin in the autolysis loop region (Figure 5.4). Part of that loop in human thrombin is comprised of Threonine, Alanine, Asparagine and Valine, which are amino acids that contain side chains that are neutral, hydrophobic,
hydrophilic and very hydrophobic, respectively (Figures 5.2 & 5.4). In contrast, that region of the autolysis loop of *salmon* thrombin is one amino acid shorter and made up of a trimer of Serine, which is neutrally charged (Figures 5.2 & 5.4). Removing the nine amino acids that make up the autolysis loop can effectively inhibit thrombin’s activation of PAR1, allowing it to activate protein C more efficiently (Dang et al. 1997). Although the autolysis loop in salmon thrombin is only one amino acid shorter than its human counterpart, its composition of all neutral residues may facilitate salmon thrombin’s interaction with protein C. The comparative protein structural model even suggests that the autolysis loop of human thrombin may sterically inhibit proper binding with protein C more so than that loop salmon thrombin (Figure 5.4). Synthetically modifying the amino acid sequence of the autolysis loop in human thrombin to match that of salmon thrombin and measuring its rate of protein C would confirm if the autolysis loop directs protein C binding and would identify how to manipulate that regions of thrombin in order to facilitate protein C binding while maintaining clotting capabilities.

The studies in this chapter assume that salmon thrombin’s vascular protective effects depend on protein C because salmon thrombin only blocks vascular permeability when endothelial cells are exposed to protein C-containing serum and because it activates protein C at a high rate (Figure 5.3). Administering an antibody to block protein C activation in conjunction with salmon thrombin, either at the root in vivo or in HUVEC microvessels exposed to TNF-α and serum, would confirm whether salmon thrombin prevents vessel permeability through protein C. Pre-treating serum-starved HUVEC channels with protein C prior to TNF-α and salmon thrombin would also verify that salmon thrombin activates protein C to prevent barrier disruption. Relatedly, these in
vitro studies are only able to suggest that human thrombin-induced astrocytic inflammation depends on its activation of PAR1, because human thrombin both activates PAR1 more effectively and induces more pro-inflammatory cytokine production than does salmon thrombin. To investigate whether human thrombin indeed acts through PAR1 to initiate the transcription of IL-1β and TNF-α, future studies should administer the PAR1 blocker SCH79797 in conjunction with human and salmon thrombin to astrocyte cultures. However, administering salmon thrombin directly to the nerve root would not be pragmatic clinically. Since intravenous APC is effective at blocking compression-induced BSCB breakdown (Figure 3.4), investigating whether a similar route of intravenous salmon thrombin can also prevent BSCB breakdown and pain development would determine if salmon thrombin is still analgesic when administered in this more clinically-favorable route. Further, since BSCB breakdown does not occur for at least 6 hours after a painful root compression injury (Figure 3.5) (Beggs et al. 2010), dosing studies that systematically manipulate the administration time of salmon thrombin would provide more information about the window of time in which that treatment is effective at inhibiting pain development.

In summary, the results of this study highlight the analgesic, vascular protecting, and anti-inflammatory properties of salmon thrombin that do not exist with human thrombin. Salmon thrombin ameliorates BSCB breakdown (Figure 5.3B), in parallel with its ability to combat inflammation-induced vascular disruption in vitro (Figure 5.3C), likely through its activation of the APC pathway (Figure 5.3D). That in vivo treatment of salmon thrombin also completely inhibits the pain that develops after a nerve root compression injury. Taken together, all of these vascular-related findings support salmon
thrombin as an ideal early treatment for neuropathic injuries that induce BBB breakdown and lead to pain. Further, salmon thrombin itself does not potently activate PAR1 or induce inflammation (Figures 5.6 & 5.7), in contrast to its human counterpart. Yet, salmon thrombin does exhibit the same enzymatic ability to cleave fibrinogen (Smith et al. 2013) and to form fibrin clots (Michaud et al. 2002) as human thrombin. Salmon thrombin’s clotting capabilities is a key distinguishing feature between it and APC as potential therapeutics for BBB disruptive disorders since APC prevents clot formation in vivo (Bernard et al. 2001, Finfer et al. 2008, Marti-Carvajal et al. 2012). Additionally, salmon thrombin has been reported to have an increased stability compared to mammalian thrombin during gamma irradiation for sterility (Laidmae et al. 2006), supporting potential safety for this protein therapy in treating various disorders in humans. Salmon thrombin contains a naturally evolved structure that enables it to innately activate vascular protective pathways, while not inducing the same inflammatory cascades as mammalian thrombin. Salmon thrombin is not only a promising therapy for painful neural injury, but its naturally modified structure from human thrombin will inform future mutagenesis studies aiming to direct thrombin’s affinity towards protein C without severely preventing its ability to form clots.

5.6. Integration & Conclusions

Studies in Chapter 4 provide evidence that rat thrombin, either endogenously or exogenously introduced into the rat spinal cord, initiates behavioral sensitivity and is, at least partially, responsible for nerve root compression-induced pain and spinal astrocyte
activation. Unlike rat thrombin that induces pain when administered in the CNS (Figure 4.6) (Narita et al. 2005), salmon thrombin inhibits nerve root compression-induced pain (Figures 5.3A & 5.5) (Weisshaar et al. 2011). Also, distinct from human thrombin, salmon thrombin prevents compression-induced BSCB breakdown (Figure 5.3B) and attenuates TNF-α-induced vascular disruption (Figure 5.3C), likely through its unique activation of protein C (Figure 5.3D). Salmon thrombin also does not initiate astrocyte transcription of pro-inflammatory cytokines (Figure 5.6) and actively protects against the axonal degeneration and infiltration of immune cells that are typically observed within the compressed nerve root in pain states (Figure 5.8). This anti-inflammatory response is in contrast to human thrombin’s induction of the astrocytic transcription of pro-inflammatory cytokines in vitro and promoting macrophage infiltration at the nerve root (Figures 5.6 & 5.8). Thrombin induces inflammatory cascades in astrocytes and immune cells through its activation of PAR1 (Choi et al. 2008, Fan et al. 2005). Since salmon thrombin activates PAR1 less effectively than does human thrombin (Figure 5.7), this likely contributes to the lack of pro-inflammatory cytokine transcription induced in astrocytes by salmon thrombin (Figure 5.6). The studies in this chapter demonstrate that salmon and human thrombin exert distinct effects on pain, vascular permeability and astrocyte activation (Figures 5.3, 5.5, 5.6 & 5.8), and that those differences are likely controlled by the substrate specificity of each species of thrombin (Figures 5.3D and 5.7).

Salmon thrombin, administered to the injury site immediately after a 15-minute compression, prevents the BSCB breakdown (Figure 5.3B) that is typically evident by day 1 (Figure 3.2). Similar vascular protecting effects are also evident when APC is administered intravenously at 1 hour after a root compression (Figure 3.4), suggesting
that those two treatments (salmon thrombin and APC) might activate the same cell signaling pathway(s). Further supporting this hypothesis, salmon thrombin produces APC markedly faster than human thrombin (Figure 5.3D), and likely prevents endothelial barrier disruption indirectly through its activation of protein C within the serum (Figure 5.3C). Thrombin, derived from salmon and other species, degrades on the order of hours under physiological conditions (Laidmae et al. 2006, Michaud et al. 2002). Yet, salmon thrombin given only once and immediately after root compression is not only sufficient to prevent BSCB breakdown early after injury, but also promotes neuronal structural health within the root (Figure 5.8), reduces spinal neuronal hyperexcitability (Smith et al. 2013), and attenuates pain (Figure 5.5) for up to 1 week. Identifying if those longer-term physiologically protecting effects depend on the unique effects of salmon thrombin or purely the reduction in the early breakdown of the BSCB, would further define the pathophysiological mechanisms induced after compression-induced BSCB breakdown.

Despite being a natural protein, salmon thrombin has been shown to be safe in vivo (Laidmae et al. 2006), supporting its potential as a therapeutic agent. Two main concerns of protein therapy include: (1) contamination with infectious agents that could potentially infect the patient and (2) immunogenicity reactions within the patient (Barbosa and Celis 2007, De Groot and Scott 2007). Naturally-derived proteins are often exposed to heat-treatments or gamma-irradiation in order to rid them of infections agents prior to human use, but sometimes these procedures are not effective at removing all viruses, such as the human parvovirus (Hino et al. 2000, Kawamura et al. 2002). The human body temperature is 37°C and as low as -2°C in cold water fish, depending on the temperature of the water (Tort et al. 2003). Viruses in salmon are non-hazardous to
humans because of the difference in basal body temperature between these species, thereby removing the threat of infectious agent transmissibility into humans from purified salmon thrombin (Wang et al. 2000). Further, treating salmon thrombin with gamma irradiation does not reduce its enzymatic activation of fibrinogen into fibrin (Laidmae et al. 2006), further supporting the use of thrombin, or other proteins, derived from salmon for various human treatments.

In addition to contamination, patient immune response to foreign proteins has the potential to be very problematic. Antibodies towards the epitope of the foreign protein are produced when introduced into a foreign body, which can cause tolerance to and reduced efficacy for, future treatments with that protein (Barbosa and Celis 2007, De Groot and Scott 2007). For example, a single intraperitoneal administration of salmon thrombin or fibrin in mice or rabbits elevates the serum concentration of antibodies for both foreign proteins, a response that is even more robust after a second administration 30 days later (Laidmae et al. 2006). Therefore, tolerance to salmon thrombin might develop, thereby reducing its suitability for multiple treatments over time. However, just one administration of salmon thrombin is sufficient to prevent pain development, block BSCB breakdown, and attenuate neural injury and inflammation (Figures 5.3, 5.5 & 5.8), which eliminates the need for repeated dosing and reduces the tolerance-based issues in relation to salmon thrombin’s as a therapeutic (Barbosa and Celis 2007, De Groot and Scott 2007). Even more severe immune reactions occur when antibodies towards foreign proteins cross-react with an analogous endogenous protein (Barbosa and Celis 2007, De Groot and Scott 2007). Antibody recognition of endogenous thrombin would be especially harmful because of its major role in coagulation (Boon 1993, Di Cera 2008);
antibodies produced in response to bovine thrombin used in surgery can result in a prolonged clotting time clinically (Ortel et al. 2001). However, immune studies with salmon thrombin and fibrin show that antibodies towards those proteins do not cross-react with the proteins from the host species in either case (Laidmae et al. 2006), suggesting that salmon thrombin would induce long-term clotting complications when used as an analgesic treatment.

In addition to the thrombin-induced endothelial and astrocytic effects examined in the studies in this chapter, mammalian thrombin is a known regulator of cellular mechanics (Cuerrier et al. 2009, Suidan et al. 1997). Thrombin administered to endothelial cells at 10nM in vitro increases their expression of actin stress fibers, corresponding to an almost doubling in cellular stiffness after only 40 minutes of stimulation (Cuerrier et al. 2009). Astrocytes also exhibit changes in their mechanical responses after thrombin exposure: 20 minutes of thrombin stimulation (0.2nM) induces astrocyte spreading and formation of focal adhesions in vitro (Suidan et al. 1997). Studies in Chapter 4 demonstrate that rat thrombin extravasates into the spinal parenchyma across a compromised BSCB after painful root compression (Smith and Winkelstein 2015), which allows for it to interact with cells within the CNS. Thrombin within the spinal cord may also modify the mechanics of the resident contractile cells, including astrocytes, which make up a majority of mechanical substrate for neurons (Shreiber et al. 2009, Vallejo et al. 2010), as well as endothelial cells. In fact, spinal astrocytes become activated at days 1 and 7 after this same painful nerve root trauma (Rothman et al. 2010); since neurons respond to the stiffness of their mechanical substrate (Betz et al. 2011, Flanagan et al. 2002, Koch et al. 2012, Lamoureux et al. 1998), it is hypothesized that in
astrocyte mechanics are altered in response to neural injury. Accordingly, the studies outlined in Chapter 6 investigate whether a painful nerve root compression induces changes in astrocytic and endothelial expression of the intermediate filament vimentin, which is a known mediator of cell shape and cytoplasmic stiffness (Buehler 2013, Guo et al. 2013, Janmey and McCulloch 2007, Lu et al. 2011, Wang and Stamenovic 2002).
Chapter 6

Effects of Root Compression on Spinal Astrocytic Activation & Endothelial Vimentin Expression

6.1. Overview

The tissue in the central nervous system (CNS) is structurally unique from other tissues in that it lacks an extensive extracellular matrix (ECM) (Gaudet and Popovich 2014, Hynes and Naba 2012, Zimmermann and Dours-Zimmermann 2008). Instead, the CNS is comprised of a loose meshwork of hyaluronan, sulfated proteoglycans, and tenascin-R surrounding a hierarchical structure of neurons and support cells, including glia and endothelial cells (Barros et al. 2011, Gaudet and Popovich 2014, Wong et al. 2013). Because of its structure, the bulk mechanical properties of CNS tissue are heavily influenced by those mechanical properties of cells within it (Shreiber et al. 2009). At the cellular level, astrocytes create a mechanical barrier between the neurons and the vascular endothelial cells and provide the main mechanical substrate for those neurons (Volterra and Meldolesi 2005). These three cell types (neurons, astrocytes, and endothelial cells) interact closely and together form the “neurovascular unit”, which regulates the normal function and pathological dysfunction within the CNS (Abbott et al. 2006, Halassa et al. 2007, Hawkins and Davis 2005, Zlokovic 2010). While much is known about the
physiological relationship between these cells, it is not known if they alter their mechanics in response to insults outside of the CNS.

The mechanical response of a cell can be described by measuring its stiffness and/or viscoelastic response, and many other parameters (Janmey and McCulloch 2007). Cellular mechanics are controlled intracellularly by the cytoskeleton (Fletcher and Mullins 2010), which is an integrated structure comprised of filamentous actin, intermediate filaments and microtubules that act as scaffolding to support the cell membrane. Since the cytoskeletal filaments are dynamic structures, changes in the intracellular mechanics also contribute to cell. The roles of actin and microtubules in cellular mechanics have been well established; because these polymeric filaments are highly dynamic they contribute to changes in cell shape, cell motility or migration, and transport of organelles within a cell (Buehler 2013, Fletcher and Mullins 2010, Janmey and McCulloch 2007, McBeath et al. 2004). Only recently has the roles of the intermediate filaments in cellular mechanics begun to be elucidated.

Since the intermediate filaments are the most stable of the cytoskeletal components, they directly influence cellular integrity (Buehler 2013, Herrmann et al. 2007). One intermediate filament in particular, vimentin, has been shown to control cytoplasmic stiffness (Guo et al. 2013, Janmey et al. 1991, Wang and Stamenovic 2002). An ischemia-induced increase in astrocytic vimentin expression corresponds to an increase in cytoplasmic stiffness in vitro (Lu et al. 2011). Because vimentin filaments can withstand significantly more mechanical deformation than microtubules, actin, and other intermediate filaments (Guo et al. 2013, Janmey et al. 1991), vimentin has been suggested to have an important role in regulating cellular stiffness. Many neuronal functions have
been shown to depend on mechanical cues, such as applied tension, applied compression, and substrate stiffness (Ahmed et al. 2012, Flanagan et al. 2002, Georges et al. 2006, Koch et al. 2012). Because astrocytes are the main mechanical substrate for neurons in the spinal cord (Volterra and Meldolesi 2005), changes in their stiffness might directly modulate neuronal function. Due to the role of sustained activation of spinal astrocytes in the maintenance of traumatic radicular pain (Rothman et al. 2010, Rothman and Winkelstein 2007), it is hypothesized that spinal astrocytes modulate their expression of vimentin after a painful nerve root compression.

The studies in this chapter address Aim 4 of this thesis and measure changes in spinal astrocytic vimentin expression following painful and non-painful nerve root compressions. The experiments test the general hypothesis that painful nerve root compression modifies the astrocytic expression of an intermediate filament that has a role in cellular mechanical integrity (Section 6.3). Astrocytic vimentin was measured by co-immunolabeling the ipsilateral spinal cord for vimentin with the astrocytic marker, glial fibrillary acidic protein (GFAP), at days 1 and 7 following painful and non-painful nerve root compressions, separately. In order to investigate whether spinal thrombin mediates compression-induced changes in astrocytic vimentin expression, spinal thrombin was blocked with hirudin prior to a painful compression and the co-localization of vimentin and GFAP was quantified at day 7. The effects of hirudin and salmon thrombin, which separately inhibit the development pain after compression (Chapters 4 and 5) (Smith et al. 2015, Smith and Winkelstein 2015), on spinal astrocytic vimentin expression was also investigated at day 7 after a painful compression in order to determine if changes in astrocytic vimentin are specific to pain-related behaviors.
Vimentin is also expressed in endothelial cells and partially regulates the integrity of endothelial monolayers. Vimentin helps to maintain cellular integrity when expressed as long stable filaments dispersed throughout the cytoplasm (Buehler 2013, Guo et al. 2013). Phosphorylation of vimentin filaments disassembles their intracellular organization and causes those filaments to aggregate around the nucleus (Grin et al. 2012). Decreases in the stability of endothelial vimentin, via phosphorylation, has been shown to correspond to increased endothelial barrier permeability (Liu et al. 2014, Liu et al. 2010). The permeability of the blood-spinal cord barrier (BSCB) transiently increases early after a painful nerve root compression and stabilizes by day 7 (Chapter 3) (Smith et al. 2015), suggesting microvascular endothelial function to be compromised in response to neural compression. In the studies measuring astrocytic vimentin expression (Section 6.3), a majority of the vimentin labeling was found to localize to spinal vasculature, so additional studies were performed also to measure endothelial vimentin expression in response to root compression (Section 6.4). Vimentin was co-immunolabeled with von Willebrand factor (VWF), as a proxy for endothelial cells, at days 1 and 7 after separate painful and non-painful nerve root compressions. The effects of treating a painful compression with hirudin or salmon thrombin on spinal endothelial vimentin expression were also quantified at day 7 in order to determine if these analgesic treatments rectify compression-induced changes in endothelial vimentin. The responses of both astrocytic and endothelial vimentin expression to nerve root compression insults are discussed and integrated with the other studies in this thesis in Sections 6.5 and 6.6.
6.2. Relevant Background

Intermediate filaments perform many roles within cells; they maintain cell shape, resist tensile loads, transmit mechanotransductive signals, act as a substrate for phosphorylation reactions in the cytoplasm and stabilize connectivity between cells within a tissue (Guo et al. 2013, Herrmann et al. 2007, Janmey et al. 1991). The structure of intermediate filaments is rich in α-helices allowing the filament to undergo a range of molecular unfolding with applied stretch (Buehler 2013). This structural feature enables intermediate filaments to resist much higher tensile loads than other cytoskeletal filaments (Buehler 2013, Guo et al. 2013, Janmey et al. 1991). One intermediate filament in particular, vimentin, greatly influences the mechanical integrity of the cell cytoplasm; fibroblasts derived from vimentin deficient (vim−/−) mice exhibit half the cytoplasmic stiffness compared to wild type fibroblasts (Guo et al. 2013). Vimentin is expressed in a variety of cell types within the CNS including astrocytes and endothelial cells, and its expression within those cells is modulated after pathological insults (Liu et al. 2010, Lu et al. 2011, Pekny and Pekna 2004). Since neuronal function and dysfunction at least partially depends on the stiffness of the local environment (Ahmed et al. 2012, Flanagan et al. 2002, Koch et al. 2012), it is hypothesized that changes in the stiffness of astrocytes, which comprise much of the mechanical substrate for neurons in the CNS, may facilitate aberrant signaling by neurons and other cells in the spinal cord.

Although the expression of vimentin, along with GFAP, has been shown recently to correlate positively with astrocyte stiffness in vitro (Lu et al. 2011), increases in astrocytic vimentin expression is more often investigated as an indicator of glial
activation (Calvo et al. 1991, Pekny and Pekna 2004). Both vimentin and GFAP are upregulated in the spinal cord by day 7 after chronic constriction of the sciatic nerve and those increases last for up to 28 days in parallel with pain persistence (Cao et al. 2015). However, astrocytic expression of GFAP and vimentin does not always follow the same temporal profile. Ischemic injury induced in the middle cerebral artery increases numbers of cells positive for GFAP and numbers of cell positive for vimentin in nearby cortical regions within 48 hours (Barreto et al. 2012). However, only the number of GFAP-positive cells remains elevated for 30 days, whereas the number of cells positive for vimentin returns to normal by that time (Barreto et al. 2012). Although both intermediate filaments are established markers of astrocyte activation, it is not known if, or how, vimentin and GFAP expression in astrocytes is differentially regulated in response to different parameters of neural injury, such as the duration of a transient neural compression injury. Further, it is not known if vimentin expression within astrocytes is associated with pain from that injury.

Endothelial vimentin controls a variety of endothelial processes (Gonzales et al. 2001, Liu et al. 2014, Tsuruta and Jones 2003). Disruption of endothelial vimentin with withaferin A increases endothelial monolayer permeability in vitro (Liu et al. 2014), suggesting that vimentin expression and stability maintains integrity of the endothelial barriers. This is further supported by a previous study showing that vascular integrity is disrupted in vim<sup>−/−</sup> mice as evidenced by a substantial increase in the paracellular transmigration of blood mononuclear cells into the lymph nodes and spleen (Nieminen et al. 2006). When endothelial cells express a form of vimentin that resists phosphorylation, and therefore disruption, it prevents the breakdown of endothelial monolayers that is
induced by lipopolysaccharide (LPS) (Liu et al. 2014). This stabilization of endothelial vimentin preventing endothelial layer permeability supports a role for vimentin in augmenting vascular integrity. Vascular breakdown occurs in the spinal cord within a day of a painful root compression and is restored by 1 week (Chapter 3) (Smith et al. 2015). Yet, the structural changes that endothelial cells undergo in order to facilitate that breakdown and subsequent recovery are not fully understood. Modifications in endothelial vimentin expression over time might contribute to the changes in BSCB integrity that are induced by a painful nerve root compression injury (Chapter 3) (Smith et al. 2015).

The different durations of nerve root compression that have been investigated in the studies in this thesis induce varying levels of BSCB permeability, spinal astrocyte activation and pain along different time courses (Nicholson et al. 2011, Rothman et al. 2010, Smith et al. 2015). A 15-minute compression induces a substantial breakdown in the ipsilateral BSCB which occurs at the same time as the development of mechanical hyperalgesia by day 1 (Chapter 3). BSCB breakdown is induced after the shorter, 3-minute compression and pain responses are less robust than those induced by a 15-minute compression (Chapters 3 & 4) (Nicholson et al. 2012, Rothman et al. 2010, Smith et al. 2015). By day 7, spinal GFAP expression is robustly elevated after a 15-minute compression when pain is still present (Rothman et al. 2010). Although mechanical allodynia is still not evident at day 7 after a 3-minute compression, there is a moderate increase in GFAP expression in the spinal cord, albeit to a lesser degree than that induced by the 15-minute compression (Rothman et al. 2010). Together, these findings suggest that early compression-induced BSCB breakdown occurs exclusively with the
development of pain after injury, but that sustained spinal glial activation may be induced by any mechanical compression, regardless of whether it produces pain. Due to the apparent roles of vimentin in both astrocytic activation and vascular permeability (Barreto et al. 2012, Gonzales et al. 2001, Haseloff et al. 2006, Liu et al. 2014, Liu et al. 2009, Lu et al. 2011), the studies in this chapter were designed to measure the response of vimentin expression in spinal astrocytes (Section 6.3) and endothelial cells (Section 6.4) after painful and non-painful injuries to the nerve root, in order to investigate whether modifications in spinal vimentin expression are specific to compression that induces pain.

The first set of studies in this chapter was designed to test the hypothesis that painful nerve root compression activates spinal astrocytes and modifies their expression of intermediate filaments that have a role in cellular mechanical integrity. Because of the apparent role of vimentin in controlling cellular stiffness and since increases in astrocytic vimentin correlate to increased cell stiffness (Lu et al. 2011), vimentin expression was quantified in spinal astrocytes in response to a root compression. Astrocytic vimentin was measured by co-immunolabeling for vimentin and GFAP in the ipsilateral spinal cord separately at days 1 and 7 after a 15-minute and a 3-minute compression. Studies in Chapter 4 demonstrate that blocking spinal thrombin activity with hirudin prevents painful compression-induced BSCB breakdown at day 1 and spinal astrocyte activation at day 7 (Smith et al. 2015, Smith and Winkelstein 2015). As such, the effects of blocking spinal thrombin with hirudin on spinal astrocytic vimentin expression are also measured in order to determine whether thrombin might contribute to the changes in astrocyte mechanics (Section 6.3). In contrast to hirudin, salmon thrombin also induces analgesia but does not mitigate the compression-induced spinal astrocytic activation at day 7.
Therefore, the effects of salmon thrombin on spinal astrocytic vimentin expression are also measured at day 7 (Section 6.3).

Since immunolabeling for spinal vimentin revealed that expression of this intermediate filament is evident in vascular structures, studies were extended to quantify spinal endothelial vimentin expression at days 1 and 7 following the same nerve root compressive insults (Section 6.4). Vimentin was co-immunolabeled with the marker for endothelial cells (VWF) at days 1 and 7 after a painful 15-minute and a non-painful 3-minute compression. Since hirudin and salmon thrombin each prevent spinal vascular disruption at day 1 after the painful compression (Chapter 4) (Smith and Winkelstein 2015), spinal endothelial vimentin was measured also at day 7 after compression with those treatments (Section 6.4).

6.3. Measuring Total Spinal Vimentin & Astrocytic Vimentin Expression after Root Compression

6.3.1. Methods

6.3.1.1. Root Compression & Tissue Harvest Procedures

Surgical procedures for these studies were performed as described in Section 3.3.1.1 of this thesis (Nicholson et al. 2011, Rothman et al. 2010). Briefly, adult male Holtzman rats were anesthetized using inhalation isoflurane anesthesia and the right C6 and C7 vertebrae were cleared of the paraspinal muscle. The right C7 dorsal nerve root was then exposed via C6/C7 hemilaminectomy and partial facetectomy. That nerve root was compressed for either 15 minutes (15min, n=12) or 3 minutes (3min, n=9) with a
Separate rats underwent the same surgical procedures, but did not receive a compression (sham, n=9) to serve as a surgical control. Spinal cord tissue from the C7 spinal level was harvested from rats on day 1 (15min, n=7; 3min, n=5; sham n=4) or day 7 (15min, n=5; 3min, n=4; sham n=5) after surgery and was fixed with 4% paraformaldehyde and cryosectioned for subsequent immunohistochemical processing and analysis (as described in Section 3.3.1.1).

### 6.3.1.2. Spinal Cord Immunohistochemistry for Vimentin & GFAP

In order to investigate the expression of spinal vimentin and astrocytic vimentin over time, vimentin was co-immunolabeled with GFAP. Fixed spinal cord tissue was cryosectioned axially at 14µm and mounted on slides. Spinal cord sections were incubated in a blocking solution consisting of 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton-X100 (Bio-Rad Laboratories; Hercules, CA) in phosphate buffered saline (PBS) for 1 hour at room temperature. Slides were then incubated with goat-anti-vimentin raised in rabbit (1µg/ml; Abcam; Cambridge, MA) and goat-anti-GFAP raised in mouse (1:750; Millipore; Billerica, MA) overnight at 4°C. After rinses with PBS, tissue sections were incubated in goat-anti-rabbit Alexa Flour 488 and goat-anti-mouse Alexa Fluor 568 (1:1000; Life Technologies; Carlsbad, CA) for 2 hours at room temperature. Slides were washed with PBS and cover slipped with TRIS buffer (Electron Microscopy Sciences; Hatfield, PA) for imaging.

The ipsilateral dorsal horn was imaged at 20x for 2-6 spinal sections for each rat. Total spinal vimentin was quantified using a custom MATLAB densitometry script.
(Nicholson et al. 2012, Rothman et al. 2010) to measure the percent of the total pixels that were positively labeled for vimentin. The percent of pixels labeling vimentin in tissue sections was further normalized to the percent positive vimentin labeling in spinal cord tissue from normal naïve rats (n=2). Differences in the normalized percent vimentin expression were determined between a 15-minute compression, a 3-minute compression and sham at day 1 and day 7 using two-way ANOVAs (group x day) with Tukey’s honestly significant difference test.

Spinal astrocytic vimentin was also measured in the same tissue sections using a MATLAB script that quantified the percent of co-localized pixels that were labeled for both vimentin and GFAP (Dong et al. 2013). The percent of co-localized vimentin and GFAP pixels was then compared to the percent of co-localized pixels in spinal tissue from normal rats (n=2). Differences in the normalized percent vimentin co-localized with GFAP were determined between groups at days 1 and 7 using two-way ANOVAs (group x day) with Tukey’s test.

6.3.1.3. Hirudin Pre-treatment Prior to Painful Root Compression

In order to investigate whether compression-induced spinal thrombin activity contributes to changes in astrocytic vimentin expression, spinal thrombin was inhibited by administering hirudin intrathecally 1 day prior to a 15-minute nerve root compression using methods described previously (Section 4.3.2.4) (Smith and Winkelstein 2015). Briefly, hirudin (4.2U/rat; Sigma Aldrich; St. Louis, MO) was injected via lumbar puncture with a 25G needle (Smith and Winkelstein 2015). At 24 hours after hirudin administration, rats underwent a 15-minute C7 nerve root compression (hir+15min, n=6).
Spinal cord tissue was harvested from rats at day 7 after the compression and prepared for immunohistochemistry as described in Section 3.3.1.1. Spinal cord tissue from matching rats that received a 15-minute compression without any treatment (15min, n=5) were included for immunohistochemical comparison. Using methods described in Section 3.3.1.1, ipsilateral C7 spinal cord tissue from rats receiving a 15-minute compression with or without hirudin was co-labeled for vimentin and GFAP. The percent of pixels labeled positively for vimentin and the percent of pixels labeled positively for vimentin co-localized with GFAP, were each separately normalized to respective levels in tissue from normal naïve rats. Differences in normalized vimentin and normalized co-localization of vimentin and GFAP between hir+15min and 15min were determined using one-way ANOVA with Tukey’s test.

6.3.1.4. Salmon Thrombin Treatment

In order to investigate whether an analgesic treatment of salmon thrombin prevents compression-induced modifications in astrocytic vimentin expression, salmon thrombin was administered after a 15-minute nerve root compression as described previously (Section 5.3.1.2) (Smith et al. 2013, Smith et al. 2015, Weisshaar et al. 2011). Briefly, salmon thrombin (0.04U/rat; Sea Run Holdings; Freeport, ME) was dissolved in 20µl of neurobasal media and administered directly on top of the right C7 nerve root immediately after its compression for 15 minutes (15min+STh, n=7). A separate group of rats received a treatment of human thrombin (0.04U/rat; Sigma Aldrich; St. Louis, MO) dissolved in neurobasal media applied to the nerve root immediately after a 15-minute compression (15min+HTh, n=4).
Spinal cord tissue was harvested from rats at day 7 after compression with treatment and prepared for immunohistochemistry as described in Section 3.3.1.1. Spinal cord tissue from matching rats receiving a sham operation (sham, n=6) was included for immunohistochemical comparison as well. Fixed C7 tissue was co-immunolabeled for vimentin and GFAP, as described in Section 6.3.1.2. The ipsilateral dorsal horn was imaged and the percent of pixels positively labeled for vimentin, as well as the percent of pixels that was co-labeled for vimentin and GFAP each were quantified using MATLAB scripts and reported relative to expression in normal tissue. Differences in the normalized percent vimentin and the normalized percent co-localization of vimentin and GFAP at day 7 between the 15min, 15min+STh and 15min+HTh groups were compared using a one-way ANOVA with Tukey’s test.

6.3.2. Results

Vimentin is expressed in the ipsilateral spinal cord in all groups probed at day 1 and day 7 and is localized to the vasculature (Figure 6.1A). Each of a painful 15-minute and a non-painful 3-minute nerve root compression induces a robust increase in vimentin expression in the ipsilateral spinal cord at day 7 after compression (Figure 6.1A). Spinal vimentin labeling is significantly elevated after a 15-minute compression (p<0.001) and a 3-minute compression (p<0.001) compared to labeling after a sham procedure, both overall and at day 7 (Figure 6.1B). The increase in spinal vimentin that is observed at day 7 after a 3-minute compression is significant compared to its own expression at day 1 (p<0.001), but spinal vimentin at day 7 after a 15-minute compression is not significantly increased compared to its expression at day 1 (Figure 6.1). At day 1, all groups, including
sham, exhibit approximately a 2-fold increase in spinal vimentin over normal levels (Figure 6.1).

Figure 6.1. Spinal vimentin increases at day 7 following nerve root compressions of different durations, but spinal astrocytic vimentin only increases after a painful compression. (A) Vimentin (green) is more robustly expressed in the ipsilateral spinal dorsal horn at day 7 after a 3-minute (3min) or 15-minute (15min) compression compared to sham levels. GFAP (red) and vimentin co-localization (yellow) is slightly elevated after a 3min compression, but is more evident after a 15min compression. (B) Spinal vimentin expression normalized to expression in normal tissue is significantly higher (*p<0.001) than sham at day 7 after each of a 3min and 15min compression. Total vimentin expression at day 7 after a 3min compression is also significantly increased (#p<0.001) compared to its expression levels at day 1. (C) Co-localization of vimentin and GFAP normalized to levels in naïve tissue, is significantly increased (*p=0.024) over sham only after a 15min compression at day 7. Data are mean±SD.

Quantification of the percent of pixels that are positively labeled for both vimentin and GFAP, as a proxy for astrocytic vimentin, reveals that both a 15-minute
compression (p<0.001) and a 3-minute compression (p=0.024) induce significant increases in astrocytic vimentin compared to sham overall (Figure 6.1C). A 3-minute compression induces a 5.3-fold increase over normal in spinal astrocytic vimentin expression at day 7, but that is not different from expression at that day after a sham operation or a 15-minute compression (Figure 6.1C). Only a 15-minute compression, which elevates astrocytic vimentin 5.8-fold over normal, induces a significant increase (p=0.023) in astrocytic vimentin over sham at day 7, although with a large degree of variability (Figure 6.1C). Astrocytic vimentin expression is close to normal levels on both day 1 and day 7 after a sham operation (Figure 6.1).

Blocking spinal thrombin with hirudin 1 day before a painful nerve root compression prevents the development of pain for up to 7 days following injury (Figure 4.5) (Smith and Winkelstein 2015). A 15-minute compression induces a robust increase in total and astrocytic vimentin in the ipsilateral spinal dorsal horn at day 7 over normal levels (Figure 6.2A). Spinal astrocytic vimentin expression at day 7 is significantly reduced (p=0.048) when treating a painful compression with hirudin compared to an untreated compression (Figure 6.2). In fact, intrathecal hirudin reduces astrocytic vimentin to normal levels at day 7 (Figure 6.2C). Interestingly, total vimentin expression in the ipsilateral spinal cord after a 15-minute compression treated with hirudin is even more robust than after an untreated compression (Figure 6.2A). Indeed, total spinal vimentin labeling is significantly increased (p<0.001) in rats receiving a 15-minute compression with hirudin than in rats receiving only a compression (Figure 6.2B).
Salmon thrombin, administered to the nerve root immediately after a painful compression, prevents the development of mechanical allodynia for up to 7 days; that anti-nociceptive effect is not evident when treating the compression with human thrombin (Figures 5.3 & 5.5) (Smith et al. 2013, Smith et al. 2015). Vimentin expression in the ipsilateral spinal cord is higher than normal and sham levels at day 7 when the 15-minute compression is treated with salmon thrombin (Figure 6.3A). This apparent elevation in spinal vimentin at day 7 after a compression treated with salmon
thrombin is significant over both a sham procedure (p<0.001) and a compression treated with human thrombin (p=0.004) (Figure 6.3B). Spinal astrocytic vimentin is elevated 4-fold over normal levels for a salmon thrombin treated compression. Despite this fold-increase over normal, astrocytic vimentin expression is not significantly different for a 15-minute compression treated with salmon thrombin than sham at day 7 (Figure 6.3C). Only the human thrombin treated compression exhibits significantly more (p=0.018) spinal astrocytic vimentin than sham at day 7 (Figure 6.3C).

Figure 6.3. Salmon thrombin does not reduce the compression-induced increase in spinal astrocytic vimentin at day 7. (A) Spinal vimentin (green) expression is more robust in the ipsilateral spinal cord at day 7 after a 15-minute compression treated with human thrombin (15min+HTh) than after a sham procedure or in normal tissue. Vimentin labeling is greater for treatment with salmon thrombin (15min+STh) than with human thrombin. The co-localization (yellow) of vimentin and GFAP (red) labeling is slightly more robust in the 15min+HTh group than in the other groups. (B) Normalized quantification of spinal vimentin expression is significantly greater for a 15min+STh than each of the sham (**p<0.001) and 15min+HTh (&p=0.002) groups at day 7. The 15min+HTh group also exhibits significantly more spinal vimentin labeling than sham at day 7 (*p=0.004). (C) Co-localization of vimentin and GFAP expression is significantly elevated (*p=0.018) after 15min+HTh compared to sham levels. Astrocytic vimentin expression for 15min+STh is not different from sham or 15min+HTh. Data are presented as mean±SD.
A detailed summary of the quantification of spinal vimentin expression and astrocytic vimentin expression in the ipsilateral spinal cord for each rat in the studies in Section 6.3 is provided in Appendix B.

6.4. Measuring Spinal Endothelial Vimentin after Root Compression

6.4.1. Methods

6.4.1.1. Root Compression & Treatment Procedures

Surgical procedures for the nerve root compression with and without treatment, are detailed in Section 6.3.1. Three separate groups of rats received surgery. In order to determine the effect of painful and non-painful compression on changes in spinal endothelial vimentin expression, rats received a compression to the C7 nerve root via a 10gf microvascular clip for either 15 or 3 minutes (as described in Section 3.3.1.1) (Nicholson et al. 2011, Rothman et al. 2010). Separate rats underwent sham procedures to serve as a surgical control.

A separate group of rats was used in order to investigate the effects of blocking spinal thrombin on compression-induced changes in endothelial vimentin expression in the ipsilateral spinal cord at day 7. Briefly, rats received an intrathecal injection of hirudin (4.2U/rat in 30µl PBS) 1 day before receiving the painful C7 nerve root compression for 15-minutes (Sections 3.3.1.3 and 6.3.1.1). Separate rats underwent a compression without pre-treatment for comparison purposes.

The comparative effects of salmon and human thrombin on spinal endothelial vimentin expression after painful compression also were investigated using another set of
rats. Briefly, the C7 nerve root was compressed for 15 minutes and was immediately treated with either salmon thrombin (0.04U/rat) or human thrombin (0.04U/rat) in 20µl neurobasal media (see Sections 5.4.1.2 & 6.3.1.4) (Smith et al. 2013, Smith et al. 2015). Sham operated rats were also included to serve as a surgical control and did not receive any treatment. Methods for the compression surgery and thrombin treatments are described in more detail in Section 6.3.1.4.

6.4.1.2. Spinal Cord Immunohistochemistry for Vimentin & VWF

Spinal cord tissue was harvested after perfusion with paraformaldehyde (as in Section 3.3.1.1). Tissue was cryosectioned at 14µm and mounted on glass slides (see Section 3.3.1.1) (Smith et al. 2013, Smith and Winkelstein 2015). Slides were blocked in a solution consisting of 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton-X100 (Bio-Rad Laboratories; Hercules, CA) in PBS for 1 hour at room temperature and then incubated with goat-anti-vimentin raised in rabbit (1µg/ml; Abcam; Cambridge, MA) and goat-anti-VWF raised in mouse (1:75; Abcam; Cambridge, MA) overnight at 4°C. Tissue sections were then rinsed and incubated in goat-anti-rabbit Alexa Flour 488 (1:1000; Life Technologies; Carlsbad, CA) and goat-anti-mouse Alexa Fluor 568 (1:500; Life Technologies; Carlsbad, CA) for 2 hours at room temperature. The ipsilateral dorsal horn was imaged at 20x in 2-6 spinal sections from each rat. Spinal endothelial vimentin expression was quantified using a custom co-localization MATLAB script, as described in Section 6.3.1.2 (Dong et al. 2013). The percent of co-localized vimentin and VWF pixels was normalized to the percent of co-localized pixels in spinal tissue from naïve rats (n=2).
Statistical differences in the normalized percent of co-localized vimentin and VWF pixels was determined in the separate combinations of groups outlined in Section 6.4.1.1. Differences in the percent of co-localized pixels at days 1 and 7 after a 15-minute compression (15min; day 1, n=8; day 7, n=4), a 3-minute compression (3min; day 1, n=5; day 7, n=4) and a sham surgery (day 1, n=4; day 7, n=6) were determined using a two-way ANOVA (group x day) with Tukey’s honestly significant difference test. Differences in the percent co-localization at day 7 after a 15-minute compression alone (15min, n=4) and a compression pre-treated intrathecally with hirudin (hir+15min, n=6) was determined using a one-way ANOVA with Tukey’s test. Lastly, differences in endothelial vimentin expression were compared at day 7 after a painful 15-minute compression treated with salmon thrombin (15min+STh, n=8), or with human thrombin (15min+HTh, n=4), and a sham procedure (n=6) using a one-way ANOVA with Tukey’s test.

6.4.2. Results

The co-localization of vimentin with VWF, to indicate endothelial vimentin, is elevated at day 7 for both compression durations (i.e. 15-minute or 3-minute) in comparison to sham at that time and respective levels at day 1 (Figure 6.4A). Yet, only a 3-minute compression induces a significant increase (p<0.001) in spinal endothelial vimentin compared to sham, at day 7 and overall (Figure 6.4B). That elevation in spinal endothelial vimentin induced by a 3-minute compression at day 7 is also significant (p<0.001) compared to the corresponding expression at day 1 (Figure 6.4). Despite not exhibiting significant differences on the individual days probed, a 15-minute compression
induces a 2.2-fold increase (p<0.001) in endothelial vimentin in the ipsilateral spinal cord, which is significant compared to sham overall (Figure 6.4B). Expression of endothelial vimentin at days 1 and 7 after sham procedures is at normal levels (Figure 6.4). Values for vimentin co-localization with VWF for each rat is included in Appendix B.

Figure 6.4. Nerve root compression induces an increase in spinal endothelial vimentin at day 7. (A) Spinal co-localization (yellow) of vimentin (green) and VWF (red) is qualitatively increased at day 7 after a 3-minute (3min) or a 15-minute (15min) compression compared to sham expression at day 7 or expression by any group at day 1. (B) The percent co-localization of vimentin and VWF normalized to expression in naïve tissue is significantly elevated only at day 7 after a 3min compression and only over sham at day 7 (*p<0.001) and its corresponding expression at day 1 (#p<0.001). Data are mean±SD.
Intrathecally treating with hirudin before a 15-minute nerve root compression increases the co-localization of vimentin and VWF labeling at day 7 relative to levels observed for an untreated 15-minute compression and normal tissue (Figure 6.5). Spinal endothelial vimentin is elevated 4.5-fold over normal after hirudin treatment and that increase is significant (p<0.001) at day 7 compared to expression levels after compression alone (Figure 6.5). Appendix B includes a detailed summary of the quantification of the co-localization of vimentin with VWF expression in the ipsilateral spinal cord for each rat in this study.

Figure 6.5. Intrathecal hirudin elevates compression-induced spinal endothelial vimentin at day 7. The co-localization (yellow) of vimentin (green) and VWF (red) labeling is more robust in the ipsilateral spinal cord at day 7 after a compression that is pre-treated with hirudin (hir+15min) compared to compression alone (15min). That increase in endothelial vimentin is significant (*p<0.001). Values are mean±SD.
Endothelial vimentin expression increases in the ipsilateral spinal cord at day 7 after a 15-minute compression treated with either salmon or human thrombin, separately, over both normal and sham levels (Figure 6.6). However, spinal endothelial vimentin expression increases nearly 3-fold over normal levels; this increase is significantly greater (p<0.001) than expression at day 7 after sham (Figure 6.6). A detailed summary of the quantification of the co-localization of vimentin and VWF in the ipsilateral spinal cord for each rat is provided in Appendix B.

Figure 6.6. Salmon thrombin increases endothelial vimentin expression in the ipsilateral spinal cord at day 7 after a 15-minute compression. There is more co-localized (yellow) vimentin (green) and VWF (red) at day 7 after a 15-minute compression with human thrombin (15min+HTh) compared to sham; yet, this increase is not significant. Spinal endothelial vimentin is significantly elevated (*p<0.001) over sham levels after treating a 15-minute compression with salmon thrombin (15min+STh). Data are mean±SD.
6.5. Discussion

Spinal astrocytic vimentin is increased after mechanical trauma to the nerve root (Figure 6.1), paralleling the extent of mechanical hyperalgesia that is produced after compressive injury (Figure 4.3) (Smith and Winkelstein 2015). A 15-minute compression induces mechanical hyperalgesia and allodynia by day 1, both of which are maintained for at least 7 days (Figures 3.2 & 4.3) (Chang and Winkelstein 2011, Rothman et al. 2010, Smith et al. 2015, Smith and Winkelstein 2015). That painful compression also increases spinal expression of astrocytic vimentin over sham expression (Figure 6.1). The behavioral studies in Chapter 4 demonstrate that a 3-minute compression also induces a decrease in paw withdrawal threshold compared to sham overall (Figure 4.3), corresponding to an increase in hyperalgesia. But, withdrawal thresholds induced by a 3-minute compression are not statistically different from thresholds for sham or a 15-minute compression either at day 1 or day 7 (Figure 4.3) (Smith and Winkelstein 2015), suggesting that the pain response is graded depending on the duration of compression. Overall, spinal astrocytic vimentin expression is increased over sham, but that elevation is not different from sham or a 15-minute compression at either day 1 or day 7 (Figure 6.1). This lack of difference parallels the mechanical hyperalgesia profile that is seen after a 3-minute compression (Figure 4.3). Further supporting the association between spinal astrocytic vimentin and behavioral responses, treating a painful compression with hirudin reduces astrocytic vimentin expression to normal levels (Figure 6.2) and prevents the development of mechanical hyperalgesia (Figure 4.5).
The 3-minute compression, which induces a marginal increase in mechanical hyperalgesia, does not induce a significant increase in mechanical allodynia (Nicholson et al. 2012, Rothman et al. 2010). A previous study measured mechanical allodynia for up to 7 days using a 4g filament, which is typically a non-noxious stimulus, and found that allodynia did not develop at any day after a 3-minute root compression (Rothman et al. 2010). Although that 3-minute compression induces a drop in paw withdrawal threshold by up to 26% from baseline levels, the thresholds at days 1 and 7 are 10.4±5.3g and 14.2±9.2g, respectively (Figure 4.3) (Smith and Winkelstein 2015). Those mechanical thresholds are both at least one standard deviation above the 4g stimulus used to measure mechanical allodynia (Nicholson et al. 2012, Rothman et al. 2010). As such, it can be inferred that behavioral sensitivity would not be evident by testing with a 4g stimulus. Spinal astrocytic vimentin expression is graded following a root compression depending on the duration of compression (Figure 6.1), similar to hyperalgesia induced after those compressive insults (Figure 4.3) (Smith and Winkelstein 2015). Because the shorter, 3-minute, compression does not induce mechanical allodynia (Rothman et al. 2010), astrocytic vimentin expression seems to parallel mechanical hyperalgesia, and not allodynia, suggesting that the amount of spinal astrocytic activation may contribute to the severity of sustained pain following root compression.

Interestingly, salmon thrombin prevents the development of mechanical allodynia after a 15-minute compression (Chapter 5) (Smith et al. 2013, Smith et al. 2015, Weisshaar et al. 2011). The current studies demonstrate that astrocytic vimentin expression is elevated 4-fold over normal when treating a painful compression with salmon thrombin, albeit those responses are not different from sham levels at day 7
(Figure 6.3), thus suggesting that salmon thrombin does not entirely prevent the spinal astrocytic activation produced by painful compression, albeit preventing allodynia. Of note, mechanical hyperalgesia has not been measured following salmon thrombin treatment. Since salmon thrombin does not completely prevent the compression-induced elevation in spinal astrocytic vimentin, it is possible that salmon thrombin also does not entirely prevent compression-induced mechanical hyperalgesia. It is possible that the withdrawal threshold after a painful compression treated with salmon thrombin is reduced relative to sham, but not below 4g, and as such the rat would not respond when the paw is stimulated by a mechanical force that is 4g or lower, thereby not inducing measurable mechanical allodynia.

In contrast to spinal astrocytic vimentin, endothelial vimentin expression is elevated after either duration of nerve root compression at day 7, regardless of whether that compression also induces mechanical hyperalgesia (Figure 6.4). In fact, the 3-minute compression, which produces slight behavioral sensitivity (Figures 3.2 & 4.3) (Smith et al. 2015, Smith and Winkelstein 2015), induces an even more robust increase in endothelial vimentin compared to the painful 15-minute compression, although this difference is not significant (Figure 6.4). Both analgesic treatments used in this chapter (hirudin and salmon thrombin) also induce marked increases in spinal endothelial vimentin expression at day 7, both of which are over three times greater than normal (Figures 6.5 & 6.6). Hirudin and salmon thrombin prevent pain-related behaviors from developing but induce substantial elevations in spinal endothelial vimentin expression, suggesting that increased endothelial vimentin might be protective and/or promoting of anti-nociception. Since expression of vimentin in vascular endothelial cells contributes to
vascular integrity (Liu et al. 2014, Stasek et al. 1992), the increase in spinal endothelial vimentin may help to protect the BSCB from being disrupted.

The increases in spinal astrocytic and endothelial vimentin evident after a painful nerve root compression (Figures 6.1 and 6.4) suggest that a variety of spinal cell types respond to a neural injury, despite being remote from the tissue injury itself. Compression of nerve root tissue compromises the integrity of the axons and vasculature within the injured tissue; in the case of nerve root compression both the injured axons and vasculature directly connect to the spinal parenchyma. The nerve root recovers to only 75% of its original width after 15 minutes of compression (Rothman et al. 2010). That macroscopic structural response is associated with the longer-term breakdown of the structural integrity of axons in the root by day 7 (Hubbard et al. 2008b, Hubbard and Winkelstein 2008, Nicholson et al. 2011). Disruption of neurofilament expression and demyelination of axons, both of which are evident at day 7 after a painful compression, are further associated with altered transport of neuropeptides to the spinal dorsal horn (Hubbard et al. 2008a, Hubbard et al. 2008b). Spinal astrocytes that are localized to injured afferent synapses respond to synaptic fluctuations of neuropeptides such as substance P, glial cell line-derived neurotrophic factor (GDNF), and ATP, and become activated (Milligan and Watkins 2009, Nakagawa and Kaneko 2010, Watkins et al. 2001). In contrast to a 15-minute compression, the nerve root width is almost completely restored to its unloaded geometry after 3 minutes of compression (Rothman et al. 2010), and there is no compromise of structural integrity at day 7 (Nicholson et al. 2011). Thus, the less injurious 3-minute compression likely does not disrupt spinal neuropeptide concentrations or transport to the same extent as a 15-minute compression, thereby not
inducing astrocyte activation to as great of a degree. Astrocytic vimentin is also more robustly elevated after a 15-minute, than a 3-minute, compression (Figure 6.1). In contrast, endothelial vimentin is upregulated robustly after both durations of compression (Figure 6.4), suggesting that astrocytic regulation of vimentin is more sensitive to root trauma than endothelial vimentin. The lack of apparent association between endothelial responses and synaptic concentrations of neuropeptides is supported by the configuration and functionality of the neurovascular unit. Astrocytes act as a physical and trophic buffer between neuronal synapses and the microvascular endothelial cells (Radu et al. 2013, Zlokovic 2010). As such, fluctuations in synaptic peptides, which are evident exclusively after a painful compression by day 7 (Hubbard et al. 2008a), might not directly affect endothelial cells due to the physical encasement of synapses by astrocytes.

In addition to neuronal input, cells within the spinal cord also respond to vascular changes such as ischemia, which is a reduction in blood flow thereby producing an inadequate oxygen supply. Transient compression of the nerve root has been shown to induce an immediate drop in blood flow to the spinal cord (Kobayashi et al. 2008, Olmarker et al. 1989, Yoshizawa et al. 1989), creating an ischemic environment transiently in the spinal cord. Although the duration of ischemia after compression depends on the magnitude of compression (Rydevik et al. 1981, Rydevik et al. 1984), a previous study demonstrated that blood flow within the nerve root remains compromised for up to 3 hours after just 2 seconds of 45gf compression (Igarashi et al. 2005). Although the magnitude of compression was lower in this study (10gf), that load was the same for both of the compression durations suggesting that both a 15-minute compression and a 3-minute compression may induce the same level of spinal ischemia, at least during
compression. In vitro, although endothelial cells decrease their expression of vimentin transiently when exposed to hypoxia for 30 minutes, endothelial vimentin expression is restored to levels even higher than pre-hypoxic ones within 1 hour (Liu et al. 2010). Since the spinal endothelium is likely exposed to ischemia during, and maybe after, a nerve root compression, and either duration of compression in this study leads to an increase in spinal endothelial vimentin at day 7 (Figure 6.4), spinal endothelial vimentin likely responds to ischemic inputs that result from nerve root compression.

The compression-induced increase in endothelial vimentin might contribute to restoring BSCB stability, which is evident at day 7 after either compression duration (Figure 3.2). BSCB breakdown is evident at day 1 exclusively after a 15-minute nerve root compression, but not a 3-minute compression (Figure 3.2). At that same time, spinal endothelial vimentin is not different for either compression duration from normal levels (Figure 6.4). An increase in endothelial vimentin expression has been previously associated with strengthened endothelial barrier integrity in vitro (Liu et al. 2010, Liu et al. 2014). Since endothelial vimentin is not altered after a 15-minute compression (Figure 6.4), vimentin likely does not control vascular permeability at day 1 after compression. Instead a reduction in endothelial tight junctions might contribute to the decreased integrity of the spinal vasculature at day 1 after a 15-minute compression (Ballabh et al. 2004, Echeverry et al. 2011, Hawkins and Davis 2005). At day 7, however, spinal endothelial vimentin is increased 2- to 2.5-fold over normal levels after either compression duration (Figure 6.4). Yet, at that time the BSCB integrity is restored and the BSCB integrity remains intact after the 3-minute compression (Figures 3.2 & 4.3) (Smith et al. 2015, Smith and Winkelstein 2015). Taken together, the delayed increase in
endothelial vimentin might result from a downstream effect of compression-induced ischemia and might help to stabilize endothelial barriers after neural compression.

The association between increased spinal endothelial vimentin expression and BSCB integrity at day 7 after painful compression is supported by the effects of both of the pharmacologic treatments used in the current study. Intrathecally pre-treating a 15-minute compression with hirudin prevents the breakdown of the BSCB, which is evident at day 1 after compression (Figure 4.4) (Smith and Winkelstein 2015). Hirudin is hypothesized to prevent BSCB breakdown by inhibiting the activity of spinal thrombin after compression, since thrombin is an inducer of vascular leakiness (Coughlin 2000, Komarova et al. 2007). Thrombin applied at 1U/ml to endothelial cultures induces the phosphorylation of vimentin within 15-30 seconds (Bormann et al. 1986). Since phosphorylation of vimentin decreases the stability of the filament and induces endothelial layer permeability (Grin et al. 2012, Liu et al. 2010), blocking the early elevation in spinal thrombin after compression with hirudin might partially prevent BSCB breakdown by stabilizing the integrity of vimentin filaments in the endothelium. Similarly, salmon thrombin also prevents BSCB breakdown at day 1 (Figure 5.3), and, as such, likely reduces spinal thrombin extravasation after compression. The early vascular protection induced by both hirudin and salmon thrombin also leads to a longer lasting increase in endothelial vimentin at day 7 (Figures 6.5 & 6.6). The association between the early stability of spinal vasculature and the later increase in endothelial vimentin suggests that protecting BSCB breakdown early after neural compression might facilitate sustained vascular integrity.
The fact that expression of vimentin increases in both spinal astrocytes and endothelial cells after painful nerve root compression suggests that spinal cells may respond by changing their mechanical properties after a neural injury, even when that trauma is remote from the spinal cord itself. Increases in astrocytic and endothelial vimentin have been shown to independently correlate with increases in cytoplasmic stiffness (Guo et al. 2013, Lu et al. 2011). Of those two cells, astrocytes comprise a majority of the cellular makeup of the spinal cord and significantly contribute to the tensile stiffness of the spinal cord (Shreiber et al. 2009, Vallejo et al. 2010). The elevation in astrocytic vimentin observed at day 7 (Figure 6.1) may contribute to an increase in the stiffness of the spinal cord after a painful nerve root compression. In contrast, compressive stiffness of the spinal cord decreases for up to 8 weeks after a hemisection injury (Saxena et al. 2012). A hemisection injury compromises the local cellular and extracellular integrity of the spinal cord itself and the lesion is repopulated by activated astrocytes, oligodendrocytes, immune cells, and connective tissue elements that collectively form a glial scar (Fitch and Silver 2008, Hausmann 2003). Yet, the macroscale changes in mechanical integrity of the spinal parenchyma after spinal cord injury are robust and likely dominate any contributions from changes in the mechanical properties of the resident cells. Since a nerve root compression is remote from the spinal cord and does not produce bulk structural changes to the spinal cord, any mechanical changes in the spinal parenchyma must be due to changes of the mechanical responses of the cells, or of the ECM proteins, in the spinal cord. Since spinal astrocytes are activated, i.e. express more GFAP and vimentin, at day 7 after a painful root compression (Figure

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(Hubbard and Winkelstein 2005, Rothman et al. 2010), they may also influence the bulk mechanics of the spinal parenchyma at that time.

Spinal astrocytes are the main mechanical substrate for nearby neurons (Volterra and Meldolesi 2005). External mechanical inputs control a variety of neuronal functions, such as growth cone formation and propagation, neurite extension and synaptic vesicle transport within axons (Ahmed et al. 2012, Flanagan et al. 2002, Koch et al. 2012, Siechen et al. 2009). A change in neuronal substrate stiffness, i.e. astrocyte stiffness, would modulate neuronal intracellular tension and, therefore, might disrupt or enhance neurotransmitter vesicle trafficking within those neurons (Ahmed et al. 2012, Siechen et al. 2009). Modifying neuronal vesicle movement also would affect synaptic concentration of neurotransmitters, which would modulate the hyperexcitability of spinal neurons (Latremoliere and Woolf 2009, Scholz and Woolf 2007). Neuronal hyperexcitability increases in the spinal cord by day 7 after this painful nerve root compression (Zhang et al. 2013). Although no study to date has measured the electrophysiological responses of neurons to substrate stiffness, it is possible that the increase in astrocytic vimentin, and possibly astrocytic stiffness, may, at least in part, contribute to the hyperexcitability that is evident in the spinal cord at day 7 after compressive injury by modulating neuronal intracellular transport.

There are several limitations associated with the current study. Measuring vimentin expression alone does not provide information about the functionality of this intermediate filament. For example, the subcellular organization of vimentin, as well as the state of its phosphorylation, both contribute to this intermediate filaments control over cytoplasmic stiffness (Buehler 2013, Li et al. 2006, Liu et al. 2014). Phosphorylation of
vimentin can be induced with the steroidal lactone, Withaferin A, which disrupts the stability of vimentin filaments (Grin et al. 2012, Liu et al. 2014). Administering Withaferin A intrathecally at day 7 after painful compression, when astrocytic and endothelial vimentin are upregulated (Figures 6.1 & 6.2), would determine whether the upregulation in vimentin influences pain and the associated cellular responses in the spinal cord at that time. Also, the mechanical properties of the spinal parenchyma were not measured directly. Microscopic techniques to measure compressive stiffness, such as microindentation or atomic force microscopy (AFM), allow measurement of regional mechanical properties of biological tissues (Levental et al. 2010, Lin and Horkay 2008). Measuring the micromechanics of the spinal cord in regions that exhibit increased astrocytic vimentin expression at day 7 after painful nerve root compression, would determine whether the spinal parenchyma undergoes stiffness changes that correspond to changes in cell mechanics. Pilot studies did utilize a custom microindentation device to measure the stiffness of the spinal parenchyma in anatomical regions and at times in which astrocyte activation is evident after painful compression. However, the variability in measurements produced by that approach was greater than any trending differences between groups. As such, a more sensitive indentation device or AFM might be more suitable for detecting any differences in spinal cord stiffness after painful nerve root compression.

Nevertheless, this is the first study to investigate the response of the intermediate filament, vimentin, within spinal astrocytes and endothelial cells after a peripheral neural injury. Astrocytic vimentin undergoes a delayed elevation at day 7 after nerve root compression (Figure 6.1), along the same profile as spinal GFAP (Rothman et al. 2010,
Rothman and Winkelstein 2007), and is associated with the mechanical hyperalgesia that is produced (Figure 4.3). Spinal hirudin prevents both compression-induced elevations in astrocytic vimentin and mechanical hyperalgesia for up to 7 days (Figures 4.5 & 6.2), further supporting the association between increased spinal astrocytic vimentin and pain. In contrast to astrocytic vimentin responses, endothelial vimentin is increased in the spinal cord at day 7 after a nerve root compression, regardless of whether pain develops or not (Figure 6.4). Endothelial vimentin is further upregulated at day 7 after compression with either of the analgesic treatments investigated in this study (i.e. hirudin and salmon thrombin) (Figures 6.5 & 6.6), suggesting that an increase in endothelial vimentin might even be protective.

6.6. Integration & Conclusions

This study did not test the original hypothesis that the stiffness of regions in the spinal parenchyma that exhibit elevated astrocyte activation following painful nerve root compression is modified. This hypothesis was explored in pilot studies by utilizing a custom microindentation device with a 255µm diameter probe (Levental et al. 2010). The relatively small size of the indentation probe allowed for regional measurements since it could spatially resolve the ipsilateral and contralateral sides of the spinal cord. However, the sensitivity of the device, and therefore variation within and between samples, did not produce reliable data. As such, the original hypothesis was adapted to quantify an aspect of the microscale mechanical response of astrocytes and with the aim of investigating whether spinal astrocytes respond mechanically when activated after a painful nerve root
compression. Measuring the astrocytic expression of the intermediate filament, vimentin, was chosen because of vimentin’s already-established association with astrocyte activation and its independent role in contributing to cytoplasmic stiffness (Guo et al. 2013, Lu et al. 2011, Pekny and Pekna 2004). By measuring astrocytic vimentin after each of a 15-minute and a 3-minute compression, that induce different levels of mechanical hyperalgesia (Figure 4.3) (Smith and Winkelstein 2015), this study suggests that spinal astrocytes may alter their mechanical response to a peripheral neural injury (Figure 6.1), in association with pain production.

Immunolabeling spinal vimentin as the means to quantify its expression allowed for the observation that vimentin is highly localized to vasculature within the spinal cord. Endothelial vimentin expression fortifies endothelial barriers (Gonzales et al. 2001, Liu et al. 2014, Lu et al. 2011). Because studies in Chapters 3 and 4 show that the spinal vasculature increases its permeability to both IgG and thrombin transiently at day 1 after a painful nerve root compression (Figures 3.2 & 4.3) (Smith et al. 2015, Smith and Winkelstein 2015), the current study also quantified changes in endothelial vimentin expression over time after compressive injury. Endothelial vimentin increases by day 7 in the spinal cord after both a 15-minute and a 3-minute compression (Figure 6.4), a time when the BSCB integrity is either re-established or maintained, respectively (Figure 3.2) (Smith et al. 2015). Since a nerve root compression, regardless of the duration, may directly induce ischemia in the spinal cord by disruption of the root vasculature, the delayed increase in endothelial vimentin is likely due to vascular ischemia. However, this was not explicitly investigated. Additionally, both hirudin and salmon thrombin, which prevent the development of behavioral sensitivity for up to 7 days after painful
compression (Figures 4.5 & 5.5) (Smith et al. 2013, Smith and Winkelstein 2015), and also induce substantial elevations in endothelial vimentin at this time (Figures 6.5 & 6.6). Thus, increased spinal endothelial vimentin likely plays a protective, rather than a pathological, role in the spinal cord after painful nerve root compression.
Chapter 7
Synthesis & Future Work

7.1. Introduction

Approximately 116 million adults in the United States are affected by chronic pain each year with an associated annual national economic cost of more than $600 billion (Institute of Medicine (US) Committee on Advancing Pain Research 2011). Despite the astounding number of lives impacted by chronic pain in our country and across the world (Bouhassira et al. 2008, Breivik et al. 2006, Hasselstrom et al. 2002), the available treatments options still are often ineffective at mitigating symptoms (Dworkin et al. 2003, Finnerup et al. 2010, O’Connor and Dworkin 2009). The pharmacologic agents that are FDA-approved and recommended to treat chronic pain, such as tricyclic antidepressants, anticonvulsants and the lidocaine patch, each target only a single neuronal mechanism that contributes to nociception (Argoff 2000, Dworkin et al. 2003, Finnerup et al. 2010, Gilron 2007, Meier et al. 2003, O’Connor and Dworkin 2009, Sindrup et al. 2005). Although aberrant neuronal signaling does ultimately control nociception (Latremoliere and Woolf 2009, Woolf and Mannion 1999), the pathophysiological contributions of spinal glial and endothelial cells influence neuronal signaling and contribute to central sensitization, which maintains pain. Spinal astrocytes
become activated after neuropathic injury that leads to pain; they stimulate neurons by releasing pro-inflammatory molecules and increase synaptic excitatory neurotransmitter concentration by downregulating neurotransmitter receptors expression at spinal synapses (Abbott et al. 2006, Haydon and Carmignoto 2006, Radu et al. 2013, Volterra and Meldolesi 2005). Although astrocytes have been established as contributing to nociception in a variety of ways, the pathological roles of endothelial cells, which comprise the blood-spinal cord barrier (BSCB), and their contribution to pain after peripheral neural injury were not well defined prior to the studies in this thesis. As increased research defines the non-neuronal mechanisms that contribute to and maintain pain (Halassa et al. 2007, Inoue 2006, McMahon et al. 2005, Milligan and Watkins 2009, Radu et al. 2013, Scholz and Woolf 2007, Vallejo et al. 2010), new pharmacologic treatments that target both neuronal and non-neuronal cascades may likely provide better control against nociception and lead to effective pain relief.

Sustained nerve and nerve root injuries that produce chronic pain-related behaviors in rodent models have been shown to induce a breakdown of the BSCB early after neural injury, despite those injuries being remote from the spinal cord itself (Beggs et al. 2010, Cahill et al. 2014, Echeverry et al. 2011, Gordh et al. 2006, Kobayashi et al. 2008). However, if and how BSCB breakdown contributes to the development of pain after those injuries had not been defined before. The studies in this thesis demonstrate that BSCB breakdown occurs by 1 day after a transient nerve root compression, only if that compression also induces pain (Figure 3.2) (Smith et al. 2015). Compression-induced BSCB breakdown, albeit even if only transient, is necessary for the development of pain after a compressive neural injury (Figure 3.4) (Smith et al. 2015). BSCB breakdown after
nerve root compression facilitates the extravasation of serum components including immunoglobulin G (IgG), tumor necrosis factor alpha (TNF-α), and thrombin into the spinal parenchyma (Figures 3.2 & 4.3) (Smith et al. 2015, Smith and Winkelstein 2015). Of those, spinal thrombin has been shown to induce mechanical allodynia and astrocyte activation when exogenously administered directly into the central nervous system (CNS) (Narita et al. 2005, Nishino et al. 1993). Yet, prior to the studies presented in this thesis it was not known if thrombin crosses a compromised BSCB and if it contributes to the development of pain after a nerve root injury.

The studies in Chapter 4 demonstrate that thrombin is elevated in the spinal cord following a painful root compression and is both sufficient and necessary for the development of pain and spinal astrocyte activation after that injury (Smith and Winkelstein 2015). Elevated thrombin activity in the spinal cord also exacerbates BSCB breakdown at day 1 and facilitates the longer-term glial activation that is evident at day 7 after a painful nerve root injury (Figures 4.10 & 4.11). Further, spinal thrombin contributes to the development of BSCB breakdown and pain following neural injury at least partially through its activation of the protease-activated receptor-1 (PAR1) in the spinal cord (Figures 4.7 & 4.11). In contrast to rat thrombin, thrombin derived from salmon has been shown to initiate distinct and favorable outcomes on pain, vascular permeability, and astrocyte-induced inflammation due to its reduced ability to activate PAR1 and its heightened affinity for protein C (Chapter 5). The contribution of neural compression to modifications in the spinal expression of the intermediate filament, vimentin, was also established. Astrocytic vimentin exhibits a delayed upregulation after a painful nerve root compression and is associated with the resulting sustained
mechanical hyperalgesia (Figure 6.1). In contrast, spinal endothelial vimentin is elevated at day 7 after any root compression, regardless of the behavioral hypersensitivity pattern that develops – pain or otherwise.

The key findings of this thesis are that BSCB breakdown and spinal thrombin activity, which is facilitated by BSCB breakdown, both contribute to the development of pain following mechanical neuropathic injury (Figures 3.2, 3.4, 4.3 and 4.5). Those findings are discussed in the context of the broader literature related to nerve root compression, the mechanisms of root compression that lead to neurovascular unit dysfunction in the spinal cord, and nociception (Section 7.2). This chapter concludes in Section 7.3 with a discussion of the limitations of this work and areas for future work that would supplement the findings from this thesis. The broad hypothesis of this thesis was that compressive nerve root injury disrupts the integrity of the blood-spinal cord barrier allowing for serum molecules, including thrombin, to extravasate into the spinal parenchyma and induce a host of cascades, including spinal PAR1 activation, mechanical modifications in activated spinal glia, and pain. Blocking BSCB breakdown with molecules, such as activated protein C, that stabilize the vascular integrity prevents nerve root-induced pain; this action is mimicked by salmon thrombin through its unique substrate specificity profile which is distinct from mammalian thrombin. The studies in this thesis support the general hypotheses and are further described in Section 7.2.
7.2. Summary & Synthesis of Major Findings

Macroscopic deformation of the nerve root tissue mechanically compromises the axons and vasculature within the neural tissue (Figure 7.1). Afferent conduction, blood flow and nutrient flow are all reduced within the nerve root during the compression and the extent of their reduction increases with increasing time of compression (Garfin et al. 1995, Igarashi et al. 2005, Olmarker 1991, Pedowitz et al. 1992, Yoshizawa et al. 1989). The afferent axons within the root synapse in the spinal dorsal horn and the blood vessels in the dorsal nerve root directly supply oxygen and nutrients to the spinal parenchyma (Almeida et al. 2004, Antonacci et al. 1998, Martini et al. 2003). Because of the anatomical apposition of the nerve root and the spinal cord, mechanically compromising the axonal and vascular structures in the nerve root also manifests immediately in the spinal cord. For example, only 6.6 minutes into a 10gf compression of the nerve root, spinal electrophysiological activity is substantially reduced (Nicholson et al. 2011), and just 2 seconds of 45gf root compression reduces blood flow to the spinal cord for up to 3 hours (Igarashi et al. 2005). Within the spinal cord, afferent synapses and microvascular endothelial cells are physically separated, yet trophically coupled, by spinal astrocytes. Spinal astrocytes respond to fluctuations in synaptic neurotransmitter concentrations and local ischemia rapidly (del Zoppo 2009, Scholz and Woolf 2007, Takano et al. 2009), suggesting that the alterations in spinal neuronal activity and spinal ischemia induced during and after a painful nerve root compression likely both contribute to spinal astrocytic responses.
The permeability of the spinal vasculature increases between 6 hours and 1 day after a transient nerve root compression along the same temporal profile as the development of pain (Figures 3.2 & 3.5) (Smith et al. 2015). BSCB permeability develops at the same spinal level as the root injury and is only evident on the side
ipsilateral to injury (Figures 3.2 & 7.2), supporting the spatial association between a mechanical injury to the nerve root and the resulting vascular dysfunction. Spinal ischemia is induced for at least the duration of that painful nerve root compression (15 minutes), and might be sustained for up to 1-3 hours following the release of the compression (Igarashi et al. 2005, Yoshizawa et al. 1989). Ischemia in the brain produced by transiently occluding the middle cerebral artery produces blood-brain barrier (BBB) breakdown immediately upon reperfusion of the cerebral tissue (Dobbin et al. 1989, Sage et al. 1984). It is possible that reperfusion of the spinal vasculature that originates in the nerve root, is delayed for up to 3 hours after the root compression (Igarashi et al. 2005). Assuming that the BSCB and the BBB respond similarly to ischemia and subsequent reperfusion, the BSCB would be compromised at a maximum of 3 hours after the root compression. However, BSCB breakdown is not yet evident even by 6 hours after a painful compression (Figure 3.5). Therefore, spinal ischemia induced by a transient nerve root compression does not entirely control the BSCB breakdown that is evident at day 1 following a painful nerve root compression (Smith et al. 2015).

Immediate altered afferent activity in the spinal cord from compression of the nerve root may contribute to BSCB breakdown indirectly through spinal astrocytic responses. Increases in synaptic concentrations of glutamate, gamma-Aminobutyric acid (GABA) and ATP released from synaptic terminals after axonal injury initiate intracellular calcium oscillations in nearby astrocytes (del Zoppo 2009, Takano et al. 2009). Astrocytic calcium fluctuations lead to a host of intracellular cascades within astrocytes including an increase in their transcription of the pro-inflammatory cytokines TNF-α, interleukin-1 beta (IL-1β), and IL-6 (Hansson and Ronnback 2003, Norris et al. 2005).
Following a painful root compression spinal astrocytes upregulate their production of each of TNF-α, IL-1β, and IL-6 within 1 hour (Figure 7.1) (Rothman et al. 2009, Rothman and Winkelstein 2010). This almost instantaneous activation of spinal astrocytes following a root compression that is remote from the spinal cord might be a direct result from the altered afferent activity. Both TNF-α and IL-1β separately induce permeability of the spinal vasculature (Echeverry et al. 2011, Hoffmann et al. 2004, Nootoboom et al. 2002, Pan and Kastin 2007, Sharief and Thompson 1992). TNF-α increases myosin light chain phosphorylation within endothelial cells which causes actin stress fiber formation, cell contraction and ultimately vascular disruption (Petrache et al. 2001). TNF-α-induced vascular permeability is not induced until 4 hours after endothelial stimulation and persists for 48 hours (Petrache et al. 2001). Since astrocytic production of TNF-α begins within 1 hour in the spinal cord after a painful root compression, that increase in spinal TNF-α may contribute to the local BSCB breakdown that is initiated between 6 and 24 hours after compression. The proposed causality between spinally-produced TNF-α and BSCB breakdown is further supported by the chromic gut inflammatory root insult not inducing BSCB breakdown (Figures 3.4 & 7.2), and also not increasing the spinal transcription of TNF-α (Rothman et al. 2009).

Since TNF-α potently induces vascular permeability (Nootoboom et al. 2002, Pan and Kastin 2007, Sharief and Thompson 1992), the systemic upregulation of TNF-α at day 1 after a painful compression also likely contributes to the robust breakdown in the BSCB that is observed by day 1 after painful compression (Figures 3.2 & 7.2). Increased BSCB permeability that develops after a painful compression is only evident at the same spinal level as the root injury and is only evident on the side ipsilateral to injury (Figures 228
3.2 & 7.2) (Smith et al. 2015). The increase in TNF-α in the serum at day 1 is a systemic increase and, therefore, would likely reach vasculature of the entire body including the spinal cord on both the ipsilateral and contralateral sides of the compression. Although TNF-α is a known inducer of vascular permeability (Nootboom et al. 2002, Pan and Kastin 2007, Sharief and Thompson 1992) and is increased systemically early after compression, BSCB breakdown occurs exclusively in the spinal cord on the side ipsilateral to injury, suggesting that serum elevation of TNF-α is not the sole cause of BSCB breakdown after painful compression.

Collectively, the studies in this thesis demonstrate that systemic and spinal inflammation, which are typically separated by an intact BSCB, are integrated with each

![Figure 7.2](image-url)

**Figure 7.2.** The transient increase in BSCB permeability that occurs after a painful nerve root compression promotes the extravasation of serum proteins into the spinal parenchyma in association with compressive pain. Spinal expression of IgG, fibrinogen (as a proxy for thrombin) and TNF-α are upregulated at day 1 only after a compressive nerve root injury that also produces pain; expression of those proteins is not evident after a painful inflammatory insult or a non-painful compression. Also at day 1, serum pro-inflammatory cytokine levels are also increased exclusively after a painful root compression and levels of TNF-α, IL-1α, II-7, and IL-12 correlate with pain severity. By day 7, spinal IgG and thrombin levels return to normal in all groups, despite the pain still being present.
other during periods when the permeability of the BSCB is elevated after a painful compression. The integration of peripheral and central inflammatory cascades is evident by the increase in serum-exclusive proteins (i.e. IgG and thrombin) in the spinal parenchyma, as well as the upregulation of TNF-α systemically and spinally (Chapters 3) (Figure 7.1) (Smith et al. 2015, Smith and Winkelstein 2015). Although TNF-α transcription returns to pre-injury levels by day 1 after a painful root compression (Rothman et al. 2009, Rothman and Winkelstein 2010), the studies in this thesis is the first to implicate systemic TNF-α as extravasating into the spinal parenchyma after a painful compressive root injury (Chapter 3) (Smith et al. 2015). The early activity of spinal TNF-α, along with IL-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1), have been previously established as contributing to the development of pain from nerve or nerve root injury (Choi et al. 2015, Jeon et al. 2009, Kawasaki et al. 2008, Olmarker et al. 2003, Rothman and Winkelstein 2010). Although the systemic expression of MCP-1 does not strongly correlate with mechanical hyperalgesia at day 1 when BSCB breakdown occurs after painful root compression (Table 3.1), MCP-1 can independently induce vascular leakiness when it is elevated in the circulatory system (Echeverry et al. 2011, Sharief and Thompson 1992). Because MCP-1 is produced downstream of TNF-α and IL-1β by astrocytes in culture (Lu et al. 2014), the early upregulation of those cytokines in the spinal parenchyma might lead to increased MCP-1 at times later than day 1. If spinal MCP-1 peaks later than day 1, it is possible that the spinal vasculature remains permeable for longer than just 24 hours.

In fact, in pilot studies related to Aim 1 of this thesis, Evan’s Blue dye, which binds to albumin and is excluded from the CNS when administered intravenously, was
evident in the brain at day 3 after a painful nerve root compression (pilot data based on n=1 rat/group) (Figure 7.3). That increase in BBB permeability at times later than day 1 that is implied by Evan’s blue staining in the midbrain at day 3 has multiple implications. Although BSCB breakdown was not measured at times between days 1 and 7, those pilot studies suggest that CNS vasculature may remain permeable for longer than 1 day after a painful root compression. IgG is cleared from the CNS through its efflux across the BBB via the FcRn receptor (Cooper 2013, Zhang and Padridge 2001). That IgG clearance from the CNS has a half-life of 10-12 hours in the rat (Zhang and Padridge 2001), suggesting that a majority of IgG would be cleared from the spinal parenchyma within 1 day after the BSCB is reestablished. Because spinal IgG labeling is at sham levels by day 7 after a painful compression (Figure 3.2), it is possible that the BSCB is still compromised at day 3, and possibly for up to 6 days, after a painful compression. Additionally, the presence of Evans blue dye in the midbrain (Figure 7.3), suggests that a peripheral neural injury may induce a widespread breakdown of the BSCB and the BBB.

Figure 7.3. Evan’s Blue extravasation in the midbrain at day 3 after a 15-minute compression or sham surgery. Evan’s blue, which binds to albumin in the circulation, extravasates into the midbrain of rats at day 3 after undergoing a 15-minute nerve root compression (15min). Evan’s blue is less pronounced after a sham operation.
In addition to pro-inflammatory cytokines in the CNS having well-established roles in inducing vascular permeability and promoting pain, the studies in this thesis demonstrate that endogenous thrombin in the spinal cord contributes to neuroinflammation, BSCB breakdown and the development of pain following a transient root compression (Figure 7.4) (Chapter 4) (Smith and Winkelstein 2015). In the circulatory system, thrombin is activated from its zymogen prothrombin at locations of vascular trauma (Boon 1993). Circulating thrombin likely stays close to the location of injury due to its high affinity for substrates involved in coagulation and receptors localized to the endothelial surface of blood vessels (Boon 1993, Coughlin 2000, Di Cera 2008, Esmon 1993, Jacques et al. 2000). Because the nerve root is anatomically adjacent to the spinal cord, it is probable that thrombin is transported from the root vasculature to the microvasculature of the spinal dorsal horn early after injury. Similar to TNF-α, thrombin induces vascular monolayer breakdown by stimulating the phosphorylation of the myosin light chain which leads to cellular contraction via the actomyosin complex (Cuerrier et al. 2009, Nobe et al. 2005, Rigor et al. 2013, Satpathy et al. 2004). Therefore, the elevation of thrombin in the blood stream of the spinal vasculature might contribute to BSCB breakdown. The ratio of hirudin to thrombin for effectively blocking thrombin activity is 1:1 (Hemker 2012). As such, since blocking spinal thrombin activity with 4.2U of hirudin attenuates compression-induced BSCB breakdown (Figures 4.10 & 7.4), suggests that approximately 4U of thrombin is expressed in the spinal cord following painful compression. In fact, rat thrombin administered at 4U directly to the spinal cord, which would allow for its interaction with the spinal microvasculature, induces BSCB permeability within 1 day after administration (Figures 4.11 & 7.4). Together, these
studies indicate that a concentration of thrombin on the order of 1-10U is sufficient to induce BSCB breakdown and contributes to the increased BSCB permeability that is evident following painful nerve root compression.

Thrombin extravasates into the spinal parenchyma early during periods of BSCB breakdown exclusively after a compressive insult that also induces behavioral sensitivity (Figures 4.3 & 7.4). Arguably even more importantly, blocking spinal thrombin activity with a single pre-treatment of hirudin prior to painful compression is potent enough to completely prevent the development of nerve root compression-induced pain (Figure 4.5). Based on this, thrombin appears to play a role in nociceptive mechanisms contributing to neuropathic pain. Spinal astrocytes express PAR1 (Figure 4.9) and prior studies have localized PAR1 expression to astrocytic end-feet (Junge et al. 2004, Shavit et al. 2011), which tightly appose spinal the vasculature. As such, thrombin, which extravasates through the microvascular fenestrations during BSCB breakdown, would immediately interact with astrocytes through PAR1. Thrombin, at concentrations on the same order as 4U, induces astrocytic production of nitrous oxide (NO), inducible NO synthase (iNOS), and pro-inflammatory cytokines through its activation of PAR1 (Boven et al. 2003, Fan et al. 2005, Malik and Fenton 1992, Meli et al. 2001, Suo et al. 2002). Those compounds are known to directly stimulate neurons and may lead to thrombin’s enhancement of nociceptive processing (Scholz and Woolf 2007, Watkins and Milligan 2001). In vitro, thrombin (1-10U) also enhances the release of NO induced by either TNF-α or interferon-gamma (IFN-γ) (Meli et al. 2001, Suo et al. 2004), suggesting that spinal thrombin potentiates neuroinflammation following a painful root compression.
In addition to interacting with endothelial cells and astrocytes in the neurovascular unit, spinal thrombin would also directly interact with spinal neurons. Thrombin has been shown to produce a biphasic response in neurons through its activation of PAR1, but in a concentration-dependent manner (Fang et al. 2003, Gingrich et al. 2000, Suo et al. 2004). At very low concentrations (3pM, or approximately 0.3U) thrombin attenuates NMDA-induced biting and scratching, which is indicative of thrombin producing anti-nociceptive behaviors, when administered intrathecally in mice (Fang et al. 2003). Yet, at a 10-fold higher concentration (3U/ml), thrombin potentiates
NMDA-induced electrical currents in hippocampal brain slices (Gingrich et al. 2000), indicating that it enhances glutamate signaling at higher concentrations in the CNS. The concentration of thrombin in the spinal cord after painful root compression is likely between 1 and 10U, since 4.2U of hirudin blocked spinal thrombin-induced astrocyte activation and pain (Chapter 4). The concentration of thrombin being between 1 and 10U in the spinal cord implies that thrombin is likely more pro-nociceptive than anti-nociceptive after a painful compression. Thrombin controls neuronal signaling through its activation of neuronal PAR1 (Fang et al. 2003, Gingrich et al. 2000, Shavit et al. 2011, Vellani et al. 2010). Within the dorsal root ganglion (DRG) in the mouse, PAR1 localizes to approximately 15% of the neuronal cell bodies, and is mainly expressed in small diameter nociceptive neurons of the peptidergic subtype (Vellani et al. 2010). Since the afferents of the DRG synapse in the spinal dorsal horn, it is likely that the nociceptive afferent synaptic terminals in the spinal cord also express PAR1, thereby being susceptible to thrombin-induced stimulation. Thrombin expressed in the spinal cord early after a painful compression may directly stimulate PAR1-expressing neurons, sensitizing them to increased synaptic glutamate.

Salmon thrombin only weakly activates PAR1 and is much less efficient at activating PAR1 than human thrombin at the same concentration (1U/ml) (Figures 5.7 & 7.5). Because mammalian thrombin is both pro-inflammatory and vascular disrupting through its activation of PAR1 (Di Cera 2008, Lee et al. 1997, Nicole et al. 2005, Nishino et al. 1993, Simmons et al. 2013), salmon thrombin would not initiate those same neuroinflammatory responses within the CNS. In fact, salmon thrombin does not induce TNF-α and IL-1β transcription in mixed neural cultures at 1U/ml, when human thrombin
does (Figures 5.6 & 7.5) (Smith et al. 2013). When salmon thrombin is applied directly on top of a compressed nerve root and the exposed spinal parenchyma, it likely interacts with many cells within those anatomical regimes. In vitro, salmon thrombin actively blocks the vascular disruption that is induced by TNF-α (Figures 5.3 & 7.5) (Smith et al. 2015). Although the use of HUVECs in the in vitro study did not closely mimic the complex interactions of spinal microvascular endothelial cells and other support cells of the BSCB, the implications of salmon thrombin blocking vascular disruption can be assumed to be maintained in spinal vasculature. Salmon thrombin is administered via a bath application to the nerve root and spinal cord; as such, salmon thrombin is expected to directly contact the endothelial cells of the spinal microvasculature. Because TNF-α is transcribed by spinal astrocytes almost immediately after a painful compression (Rothman et al. 2009, Rothman and Winkelstein 2010), local increases in spinal TNF-α are hypothesized to stimulate microvascular endothelial cell contraction and contribute to the breakdown of the BSCB within 24 hours (Figure 7.1). Salmon thrombin applied to the spinal tissue likely inhibits the TNF-α-induced disruption of the BSCB following painful compression. Indeed, a single treatment of salmon thrombin substantially inhibits the breakdown of the BSCB following painful compression (Figure 3.2 & 7.5) (Smith et al. 2015). Salmon thrombin protects endothelial barriers by potently activating protein C in the absence of thrombomodulin (Figure 5.3) (Smith et al. 2015). Because salmon thrombin activates protein C to protect vasculature and also does not initiate neuroinflammation through PAR1, salmon thrombin is a very promising therapeutic for vascular disrupting disorders within the CNS, including ischemic stroke, Parkinson’s
The studies in this thesis demonstrate that spinal endothelial cells and astrocytes respond mechanically to a painful compressive nerve root injury, both early and later after that injury (Figure 7.6). Between 6 hours and 1 day after a painful mechanical nerve root injury, BSCB permeability increases (Figure 7.1) (Smith et al. 2015). A breakdown of the spinal vasculature is likely due to the microvascular endothelial cells contracting...
and compromising the integrity of the transendothelial junctions (Riewald et al. 2002, Rigor et al. 2013, Satpathy et al. 2004). By day 7 after a painful compression, BSCB permeability is reduced along with upregulation of endothelial and astrocytic vimentin. Vimentin intermediate filaments act to sustain cytoplasmic integrity and, in turn, when expressed within endothelial cells, fortify endothelial barriers (Bormann et al. 1986, Buehler 2013, Liu et al. 2014). By day 7 after a painful root compression spinal endothelial vimentin expression is upregulated and the BSCB integrity is re-established (Figures 6.4, 7.1 & 7.6). As such, elevated endothelial vimentin may contribute to the increased mechanical integrity of the spinal vasculature at that time. A 3-minute compression, which does not induce BSCB breakdown (Figures 3.2, 7.2 & 7.6) (Smith et al. 2015), induces a delayed increase in spinal endothelial vimentin, similar to that which is evident after the painful compression (Figure 6.4 & 7.6). Since the two durations of compression induce different levels of BSCB breakdown but the same robust elevation in spinal endothelial vimentin, microvascular endothelial vimentin is taken as not being modulated as a downstream effect of the early alterations in endothelial function controlling vascular permeability. In contrast to endothelial vimentin, spinal astrocytic vimentin is exclusively elevated following a painful nerve root compression (Figures 6.1 & 7.6). Increased astrocytic vimentin correlates to increased cellular stiffness (Lu et al. 2011), which may suggest that spinal astrocytes increase their stiffness following a painful neural trauma. Preventing the early increase in spinal thrombin activity with hirudin prior to a painful compression reduces astrocytic vimentin expression at day 7 to levels of the less painful 3-minute compression (Figure 7.6). In combination with the anti-nociceptive and BSCB-protective effects of hirudin, these studies collectively
suggest that early exposure of the spinal parenchyma to thrombin after compression injury disrupts spinal endothelial barriers, induces longer term changes in astrocyte activation and mechanics, and contributes to the development of pain.

7.2. Limitation & Future Work

The studies presented in this thesis demonstrate that pain symptoms that are initiated and sustained by mechanical loading of the nerve root are complex responses controlled by neuronal, astrocytic and endothelial dysfunction within the neurovascular unit of the spinal cord. Although the findings from the studies in this thesis represent a
substantial step towards understanding the role of vascular endothelial cell dysfunction and the downstream effects of that in the development of neuropathic pain, there are several assumptions and limitations that must be considered for integrating these findings. As such, this section addresses several critical limitations and also outlines areas for future investigation that would enhance the understanding of how thrombin influences neuronal and non-neuronal cells leading to the aberrant nociceptive mechanisms that contribute to chronic pain.

A wide range of spinal responses occur within hours following a painful nerve root compression, and many of those have been shown to independently contribute to the development of pain (Nicholson et al. 2011, Rothman et al. 2009, Rydevik et al. 1984). Measuring BSCB breakdown at day 1 and day 7 provides only brief snapshots of a very complex spinal response. The BBB is known to undergo a biphasic breakdown in response to transient ischemia; studies report a slight breakdown of the BBB within minutes that is re-established and then more robustly compromised at 12-48 hours following an ischemic insult (Dobbin et al. 1989, Sage et al. 1984). Although a painful compression was shown to induce BSCB breakdown by day 1, it is also possible that the BSCB undergoes increased permeability at times even earlier than 1 day. If BSCB breakdown, even if only marginal, occurs within minutes of the ischemia that may be induced following a painful nerve root compression, it is possible that the extravasation of serum factors would contribute to the early activation of spinal neurons and glia. Additionally, pain is maintained for up to 4 weeks following a mechanical nerve root injury (Rothman et al. 2007), so investigating BSCB breakdown at later times would also provide a more complete picture of how spinal vascular dysfunction contributes to pain.
A recent study utilized dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) to define the spatiotemporal profile of BSCB breakdown after spared nerve injury (Cahill et al. 2014). That non-invasive technique allows for the dynamic measurement of BSCB permeability over time instead of selecting multiple termination points requiring the use of multiple rats. That study showed that BSCB breakdown spanned from the lumbar spinal cord into the thoracic segments of the spinal cord in a transient manner, peaking between 24 and 36 hours (Cahill et al. 2014). Employing DCE-MRI in conjunction with a painful nerve root compression, which transiently induces pain, would provide more information about the time course of BSCB breakdown in relation to the development of pain. Additionally, BSCB breakdown was not confirmed to induce pain in absence of a compressive nerve root insult. Radiation (2-50Gy) administered to the cervical spinal cord has been shown to induce BSCB breakdown within 24 hours, which gradually returns to pre-radiation permeability over the course of 2 weeks (Li et al. 2004). Inducing BSCB permeability in the cervical spinal cord via the application of radiation would help to determine whether BSCB breakdown itself can induce pain without the other injurious inputs that are associated with painful nerve root compression.

Although spinal thrombin activity was suggested to increase, via elevated spinal fibrin(ogen) labeling, following painful nerve root compression (Smith and Winkelstein 2015), thrombin activity was not explicitly measured. The activity of thrombin in the spinal cord could be measured by utilizing a fluorogenic thrombin activity assay (Bushi et al. 2013). Slices of spinal cord tissue taken at times between 6 and 24 hours after a painful root compression could be bathed with the fluorogenic thrombin substrate and imaged to determine the spatiotemporal patterning of spinal thrombin enzymatic activity.
Additionally, the studies in this thesis only infer that spinal thrombin is derived from the periphery. Circulating thrombin becomes inactive by circulating thrombin inhibitors within hours after it is originally activated from the prothrombin zymogen (Boon 1993, Vogel et al. 1979). Because the enzymatic product of thrombin – fibrin – is present in the spinal parenchyma 24 hours after painful root compression (Figure 4.3) (Smith and Winkelstein 2015), it is possible that thrombin is continually produced in the circulatory system for up to 1 day after an injury. Measuring thrombin activity in the serum and spinal parenchyma after painful compression would determine if there is an association between thrombin concentrations in the periphery and the CNS. In order to confirm that spinal thrombin originates systemically after painful compression, fluorescently-labeled thrombin could be administered intravenously and fluorescence could be measured in the spinal cord at times after a painful compression.

The studies of this thesis did not consider the interaction of thrombin with other cells resident within the CNS. Activation of microglial PAR1 by thrombin or a PAR1 activating peptide induces microglial proliferation (Suo et al. 2002). However, thrombin stimulation of microglial cells does not induce microglial production of TNF-α (Suo et al. 2002), suggesting that thrombin stimulation of microglial cells may not directly contribute to their production of pro-inflammatory cytokines. Although spinal microglia potentiate neuropathic pain through their production of pro-inflammatory molecules, spinal thrombin may not directly contribute to their activation. Measuring microglial activation and production of pro-inflammatory cytokines following intrathecal thrombin injection or after painful nerve root injury pre-treated with hirudin would determine whether spinal thrombin contributes to spinal inflammation through its activation of
spinal microglia. Additionally, thrombin is a known activator of platelets within the circulatory system, inducing platelet aggregation with other platelets as well as with other inflammatory cells in the blood stream (Langer and Chavakis 2013). During increased BSCB permeability, endothelial cells upregulate their expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Rutkowski et al. 2002), which enables the adhesion of platelets to the endothelial cell surface. Endothelial-bound platelets interact with circulating monocytes and facilitate the transmigration of monocytes into the spinal parenchyma (Cicmil et al. 2000, Langer and Chavakis 2013), which further potentiates inflammation. Because BSCB breakdown likely occurs in parallel with the extravasation of thrombin into the spinal parenchyma (Chapters 3 and 4), thrombin-activated platelets bound to vasculature walls might facilitate monocyte infiltration into the spinal parenchyma and contribute to the development of pain. Studies investigating the effect of spinal thrombin on platelet and microglial activation are needed in order to fully characterize the influence of thrombin on spinal cell populations that are involved with nociception.

The interaction between neurons, astrocytes, and endothelial cells of the neurovascular unit plays an important role in the development and maintenance of nociceptive states induced by painful nerve root compression. However, the specific mechanisms by which thrombin disrupts or enhances such crosstalk to promote sensitization were not established by the studies here. It is hypothesized that mechanically injuring the afferent axons within the nerve root immediately increases the synaptic concentration of glutamate and ATP to stimulate the local astrocytes. Measuring synaptic concentrations of these two excitatory neuropeptides within the first hour after a
painful root compression and in parallel with measuring ERK phosphorylation or calcium signaling within astrocytes, as measures of their activation, would confirm a causal relationship between the two responses. Additionally, the astrocytic production of TNF-α was hypothesized to produce endothelial barrier disruption, facilitating thrombin’s diffusion into the spinal parenchyma. Measuring spinal thrombin activity and levels of TNF-α and IgG would provide a more complete picture of the sequence in which those responses occur.

A major limitation of the studies in this thesis is that mechanical properties of the spinal parenchyma were not directly measured in areas that undergo spinal astrocyte activation following painful root compression. It is nearly impossible to measure the stiffness of individual cells as they natively reside within a tissue. Since astrocytes contribute to the macroscopic mechanical stiffness of the spinal cord (Shreiber et al. 2009), measuring the compressive stiffness of the spinal cord in regions that undergo astrocytic activation following painful compression would help to define the response of spinal micromechanics to root injury. The original hypothesis was explored in pilot studies by using a custom microindentation device with a 255µm diameter probe (Levental et al. 2010). The relatively small size of the indentation probe allowed for regional measurements since it could spatially resolve the ipsilateral and contralateral sides of the spinal cord. However, the sensitivity of the device, and therefore variation within and between samples, prevented that technique from producing reliable data. Atomic force microscopy using cantilevers that have been adapted to measure stiffness on the micrometer scale (Mackay and Kumar 2013), might better capture slight modulations in mechanical changes of the spinal parenchyma at that scale. That
technique was not utilized because an atomic force microscope that exhibited a wide enough range in the z-axis to account for spinal cord slice thickness and indentation depth (together at least 1mm) was not available. As such, measuring astrocytic vimentin expression as a proxy for changes in cell mechanics was explored. Because astrocytes robustly upregulate their expression of vimentin by day 7 after painful compression, probing the stiffness of the spinal parenchyma at this later time point would be ideal because the changes in astrocytic mechanics at that time are more likely to modulate spinal parenchymal stiffness.

Modifications in spinal cord stiffness could also occur because of extracellular matrix (ECM) protein deposition or degradation. It has been previously shown that the dorsal root entry zone of the spinal parenchyma undergoes deposition of chondroitin 6-sulfate proteoglycans and tenascin at 2 weeks after a nerve root lesion (Pindzola et al. 1993). Those ECM molecules are hypothesized to create a biomechanical barrier to axonal re-growth by modulating the stiffness of the spinal parenchyma. Because cell mechanics are modulated in response to changes in substrate stiffness (Ahmed et al. 2012, Discher et al. 2005, Flanagan et al. 2002), changes in cell mechanics may possibly parallel changes in ECM stiffness. Measuring the expression of proteoglycans and tenascin within the spinal cord in parallel with measuring changes in cellular mechanics would provide a more complete picture of the whether the mechanical microenvironment of the spinal ECM and the cells within might contribute to spinal nociceptive signaling.

This thesis did not investigate how changes in the mechanical properties of astrocytes might contribute to nociceptive signaling. Manipulating the mechanics of cells within the spinal cord after painful compression would begin to elucidate whether
changes in astrocytic cellular mechanics do, in fact, contribute to pain. Pharmacologically disrupting vimentin filament stability with Withaferin A (Grin et al. 2012) after a painful root compression at times when astrocytic vimentin is upregulated, would determine if increases in astrocytic vimentin expression functionally contribute to nerve root-mediated pain. Yet, changes in the expression of the intermediate filament vimentin does not fully capture the wide range of mechanical changes that cells of the spinal cord might undergo after painful nerve root compression. Of note, dynamic cellular contraction, which also contributes to cell stiffness, is controlled by the actomyosin complex (Fletcher and Mullins 2010, Maekawa et al. 1999, McBeath et al. 2004). Phosphorylation of RhoA and Rho associated kinase (ROCK) occur upstream of actomyosin-controlled cell contraction (Maekawa et al. 1999). Blocking ROCK with an intrathecal injection of H-1152 at day 7 after painful L5 spinal nerve transection attenuates mechanical hyperalgesia for at least 45 minutes (Tatsumi et al. 2005), suggesting that actively blocking cell contraction may be anti-nociceptive at least transiently. Administering H-1152 at times before pain onset would provide useful information on whether changes spinal cellular mechanics contribute to the development of nerve root-induced pain.

Salmon thrombin was shown to prevent the development of mechanical allodynia following a compressive nerve root injury (Chapter 5). In contrast to the other treatments used throughout this thesis, i.e. activated protein C (APC), hirudin and SCH79797, the effectiveness of salmon thrombin preventing compression-induced mechanical hyperalgesia was not investigated. Changes in the withdrawal threshold of the forepaw to a mechanical stimulus is a more sensitive metric for measuring evoked mechanical sensitivity and as such should have been measured following a painful compression
treated with salmon thrombin. Additionally, salmon thrombin’s effects on thermal hyperalgesia was not measured. Activating PAR1 on peptidergic neurons expressing transient receptor potential vanilloid subfamily 1 (TRPV1), which transmits nociceptive signals in response to noxious heat stimuli, sensitizes their response to electrical stimulation (Vellani et al. 2010). Salmon thrombin prevents BSCB breakdown and, thus, the extravasation of thrombin into the spinal parenchyma. The absence of spinal thrombin after painful compression would, in turn, prevent the activation of neuronal PAR1; therefore, it is possible that salmon thrombin would also prevent thermal sensitivity after a painful root compression.

Although salmon thrombin was shown to be a suitable treatment for neuropathic pain by targeting and preventing BSCB breakdown following painful root compression (Chapter 5), a few barriers remain in the way of its clinical success. Administering salmon thrombin immediately after injury and directly on top of the nerve root and spinal cord was the only dosing method investigated in these studies and is not clinically feasible. Administering salmon thrombin via intrathecal injection would be a viable delivery route and would remove the need for surgery. However, lumbar punctures are not routinely performed except for in rare cases, including delivery of nerve blocks during childbirth and diagnosing diseases of the CNS (Boon et al. 2004). Therefore intrathecal delivery of salmon thrombin is also not a preferred delivery route. Injecting salmon thrombin intravenously would also facilitate its delivery to spinal vasculature, but this delivery mode is potentially dangerous since excess thrombin could lead to blood clots (Boon 1993, Griffin 1995). Since salmon thrombin was shown to be effective at blocking BSCB breakdown and pain when administered at the site of injury immediately
after root compression, its clinical application might be better suited as a treatment that is administered at the site of surgery within the central nervous system as a prevention of post-surgical pain.

Salmon thrombin is hypothesized to be analgesic if administered at any time before the breakdown of the BSCB after injury since it prevents BSCB breakdown to attenuate pain (Chapters 3 & 5). APC was administered intravenously at 1 hour after painful compression and was sufficient to prevent BSCB breakdown and pain at day 1 after injury (Figure 3.4), suggesting that salmon thrombin could also be administered at least at 1 hour after a transient compression and still possibly prevent pain development. Yet, pilot studies performed in our lab determined that administering salmon thrombin at 1 day after a painful 15-minute compression demonstrated that salmon thrombin does not prevent behavioral sensitivity when administered at that time. Therefore, salmon thrombin might in fact need to be delivered very early after an injury in order to be effective. The requisite immediate administration of salmon thrombin is a major limitation to using this treatment after injury because patients may not seek treatment until after pain has developed and persisted for some time. However, a patient may be admitted to the hospital in cases of traumatic injury to the neck or spine, which would be an appropriate application for salmon thrombin might as a pain treatment. Additionally, the nerve root compression injury examined in this thesis was only transient and induced transient BSCB breakdown. A disc herniation or spinal stenosis may impinge a nerve root chronically, which might induce BSCB breakdown that lasts for a longer time period; as such, the therapeutic window could be longer than 1 day for those pathologies and would allow targeting BSCB breakdown with salmon thrombin a more viable option.
Another clinical barrier to salmon thrombin is that it is a natural protein and, thus presents a risk for immunogenicity issues including tolerance. This could hinder salmon thrombin as a potential therapeutic because it may only be effective when administered the first time. In vivo studies have defined that antibodies to salmon thrombin develop by day 20 after a single injection in mice (Laidmae et al. 2006), suggesting that a second dose administered after antibodies towards salmon thrombin have already developed may not be as effective. The effectiveness of salmon thrombin at preventing BSCB breakdown and pain after multiple doses should be explored using pre-clinical animal studies prior to its use in clinical studies. If salmon thrombin only retains effectiveness with one dose, this would greatly hamper its clinical potential at treating BBB disruptive disorders.

Those studies only suggest that salmon thrombin protects vasculature through its preferential activation of protein C (Figure 5.3) (Smith et al. 2015); this hypothesis was not explicitly tested. Using HUVEC microchannels, salmon thrombin was shown to prevent TNF-α disruption when the cells were exposed to serum, which contains protein C, prior to running the experiment (Figure 5.3). Blocking the activation of protein C with antibodies in conjunction with administering salmon thrombin to HUVEC channels would establish whether salmon thrombin does indeed protect vascular integrity through protein C. At the molecular level, APC prevents vascular disruption by enzymatically cleaving PAR1 at Arg-46, which is at a different location on the extracellular domain of PAR1 than mammalian thrombin activates (Arg-42) (Mosnier et al. 2012). By non-canonically activating PAR1 (i.e. at Arg-46 and not Arg-42), APC initiates a distinct and opposite signaling pathway than mammalian thrombin’s activation of PAR1, which is also referred to as biased agonism (Zhao et al. 2014). It is also possible that salmon
thrombin activates the APC pathway in part by directly activating PAR at PAR1 at Arg-46. Creating a fluorogenic peptide corresponding to the Arg-46 cleavage site of PAR1 would be an initial step towards fully understanding the unique enzymatic actions of salmon thrombin towards protein C. Because protein C must be bound to the endothelial protein C receptor (EPCR) in order to induce vascular protection (Bouwens et al. 2013, Esmon 1993, Xu et al. 2005), using knock out mice that do not express EPCR would provide valuable insight into whether salmon thrombin protects vascular integrity in vivo through its activation of endothelial-bound protein C.

An important question that was not addressed in the studies of this thesis was whether salmon thrombin out-competes the effects of mammalian thrombin at the site of root injury to provide analgesic effects. Because of the inherent bleeding that occurs at the surgical site during the administration of a compressive nerve root insult, rat thrombin would contact the nerve root and spinal cord during and after the injury. Salmon thrombin is administered at the site of injury, after the compression, thereby acting in the same location as the rat thrombin. Yet, this is the case also for sham conditions; which were not investigated in these studies. In order to test whether the anti-inflammatory and vascular protective effects of salmon thrombin counteract those pro-inflammatory and vascular disruptive effects induced by mammalian thrombin, separate in vitro studies with astrocytic cultures and HUVEC microchannels could be performed with equivalent doses of salmon and rat thrombin administered simultaneously. Both TNF-α and mammalian thrombin stimulate vascular permeability through the phosphorylation of the myosin light chain (Cuerrier et al. 2009, Nobe et al. 2005, Nooteboom et al. 2002, Komarova et al. 2007, Satpathy et al. 2004). Because salmon thrombin actively prevents
TNF-α-induced vascular disruption, it is hypothesized that salmon thrombin may also attenuate the vascular disruptive effects of mammalian thrombin.

The structural comparison between fish and human thrombin defined the autolysis loop as having the most radical residue substitutions (Figure 5.4), which translated into differences in the three-dimensional structure of that region of the molecule (Smith et al. 2015). In order to confirm that this region of salmon thrombin contributes to its preferential activation of protein C over PAR1, mutagenesis studies could substitute the fish thrombin sequence for the autolysis loop into the human thrombin structure and measure the activation rate produced by that mutated thrombin towards protein C and PAR1. It is also possible that salmon thrombin preferentially activates protein C through the collective amino acid substitutions throughout its entire structure. Mutagenesis studies have defined other regions within human thrombin’s structure that contribute to its preferential activation of PAR1 versus protein C. For example, a double point mutation of W215 and E217, each replaced by alanine, increases the anti-inflammatory properties of human thrombin through its increased affinity for protein C (Verbout et al. 2015). However, W215 and E217 are conserved between the structures of human and trout thrombin, suggesting that those residues do not enhance salmon thrombin’s affinity for protein C. Within human thrombin, R35 has been identified to sterically inhibit human thrombin’s interaction with protein C when unbound from thrombomodulin (Yang et al. 2006). Although trout thrombin expresses lysine and not arginine at the 35th residue location (Figure 5.2), both amino acids are hydrophilic indicating that this substitution is conservative and also likely does not control the difference in substrate affinity between salmon and human thrombin. In any case, the salmon thrombin molecule should be fully
sequenced and more thoroughly compared to the structure of human thrombin in order to define the substituted residues that play the largest role in controlling the substrate affinity for salmon thrombin.

In summary, the studies presented in this thesis define the contribution of BSCB breakdown, and its subsequent facilitation of thrombin extravasation into the spinal parenchyma, to mechanically-induced nerve root-mediated pain. Behavioral sensitivity induced by painful root compression can be initiated through sensitization of compressed afferents and is, at least partially, maintained by contributions from endothelial cells and astrocytes. Excessive release of excitatory neurotransmitters at spinal afferent synapses may initiate astrocyte activation almost immediately and is evident by their transcription of pro-inflammatory and pro-nociceptive cytokines. Of those cytokines, TNF-α, not only enhances nociceptive activity in neurons, but it might also directly contribute to a disruption in the spinal vasculature within hours following a compressive root injury. That breakdown of the BSCB permits the transmission of thrombin from the periphery into the spinal parenchyma allowing thrombin to directly activate spinal endothelial cells, glia and neurons. Certainly, additional studies are needed to define the exact cellular targets of spinal thrombin and how thrombin contributes to dysfunctional nociceptive signaling of the neurovascular unit. Yet, the studies in this thesis help establish an integral role of spinal endothelial dysfunction in the development of pain and support the consideration of endothelial cells as having a role in contributing to the neuroinflammatory responses in the spinal cord that initiate and maintain pain after neural trauma.
Appendix A

Mechanical Behavioral Sensitivity

This appendix contains the mechanical sensitivity responses for all rats included in the behavioral studies that are presented in Chapters 3-5. Measurements of mechanical hyperalgesia and mechanical allodynia were used to quantify behavioral sensitivity in response to a stimulus throughout the studies in this thesis. Each table in this appendix summarizes the behavioral data arranged by each study, with the tables arranged by the order that the studies are presented in the chapters.

The baseline responses (day 0) and corresponding post-surgery responses (up to day 7) were measured. An increase in mechanical hyperalgesia is defined as a drop in the mechanical threshold (in grams) for a rat to withdraw its paw. The withdrawal thresholds were determined by applying a discrete series of von Frey filaments to the specified paw in an ascending order of force (1.4g-26g). The filament that evoked the paw to be withdrawn, was recorded as the threshold if the next consecutive filament also elicited a response (see Section 3.3.1.2 for further details). An elevation in mechanical allodynia corresponds to an increase in the number of paw withdrawals elicited in response to a typically non-noxious stimulation; to measure allodynia, the 1.4g, 4g and 10g strength von Frey filaments were used in these studies. A total of 10 stimulations by
each filament was applied to the paw for each rat three separate times and averaged on each testing day (see Section 5.4.1.2 for details).

Tables A.1.1-A.1.4 summarize the paw withdrawal threshold data that are included in the studies characterizing blood-spinal cord barrier (BSCB) breakdown after painful and non-painful nerve root insults in Chapter 3. Additionally, the withdrawal thresholds on days 1, 3, 5 and 7 are normalized to corresponding baseline thresholds (at day 0), which is how those data are presented in that chapter. Table A.1.1 details the withdrawal thresholds for the ipsilateral forepaw for each rat on days 1, 3, 5 and 7 after a sham operation, a 3-minute compression (3min), and a 15-minute compression (15min). The nerve root compression was applied unilaterally to the C7 dorsal nerve root with a calibrated 10gf microvascular clip (see Section 3.3.1.1 for details). Sham operated rats underwent the same surgical procedures with no applied compression. The corresponding withdrawal thresholds for the forepaw contralateral to injury side for each rat after sham, 3min and 15min are itemized in Table A.1.2. Since hyperalgesia developed only in the forepaw ipsilateral to a 15-minute nerve root compression (Figure 3.2), the subsequent studies measured the withdrawal threshold only in the forepaw ipsilateral to injury, unless otherwise specified. Mechanical hyperalgesia on days 1 and 7 after an inflammatory insult produced by application of chromic gut suture to the nerve root (chromic) is detailed in Table A.1.3. That table also includes the corresponding thresholds from rats at day 1 and day 7 after a 15-minute compression (15min) and a sham surgery. For details about the surgical procedures for the chromic gut insult are provided in Section 3.4.1.1. Lastly, Table A.1.4 summarizes the withdrawal thresholds for rats receiving an
intravenous injection of activated protein C (APC) (0.2mg/kg rat) at 1 hour after a 15-minute compression (15min+APC) (described further in Section 3.5.1.1), as well as the corresponding thresholds from rats at day 1 after an untreated compression.

Studies in Chapter 4 utilized pharmacological agents to manipulate various aspects of the thrombin/protease-activated receptor-1 (PAR1) pathway in the spinal cord and measured the effects on mechanical hyperalgesia. Tables A.2.1-A.2.7 summarize those withdrawal threshold data and appear in the order in which those data are presented in Chapter 4. Rat thrombin was administered intrathecally in order to measure the effects of spinal thrombin on pain. Withdrawal thresholds were measured in the bilateral forepaws and averaged between paws for each rat since this insult is not expected to favor either side. In pilot studies optimizing a dose that was sufficient to induce behavioral sensitivity, withdrawal thresholds were measured at day 0, before and at days 1, 3, 5, and 7 after, an intrathecal injection of rat thrombin at doses of 0.04U/rat, 0.4U/rat, and 4U/rat (Table A.2.1). The PAR1 antagonist, SCH79797, was administered as a single intrathecal injection to block spinal PAR1 activation prior to a 15-minute nerve root compression. Two doses of SCH79797 were piloted in dosing studies, 25ug/kg and 50ug/kg, as well as a vehicle which contained no SCH79797 (0µg/kg). Withdrawal thresholds in the ipsilateral forepaw were measured at day -1 prior to SCH79797 injection, at day 0 after the injection, and at days 1, 3, 5 and 7 after a 15-minute compression (provided in Table A.2.2). See Section 4.3.2.3 for more details on the dosing of intrathecal rat thrombin and SCH79797.
Studies in Chapter 4 measured spinal fibrinogen as a proxy for thrombin activity at days 1 and 7 following a sham operation, a 3-minute compression or a 15-minute compression. Mechanical hyperalgesia was measured prior to surgery (day 0), and at each of day 1 and day 7 after sham surgery or compression; those behavioral data are presented in Table A.2.3. Hirudin, a selective inhibitor of thrombin activity, was intrathecally administered (4.2U/rat) 1 day before a 15-minute compression in order to block spinal thrombin (hir+15min). To assess the effects of blocking spinal thrombin on compression-induced pain, mechanical hyperalgesia was measured prior to hirudin (day -1), prior to compression (day 0) and over 7 days after the compression (Table A.2.4). Exogenous rat thrombin was separately administered intrathecally (4U/rat) to naïve rats and mechanical hyperalgesia was measured in the bilateral forepaws and averaged between paws because that intrathecal injection provides a bilateral insult. Average paw withdrawal thresholds for days 0 through 7 following intrathecal rat thrombin are summarized in Table A.2.5.

In order to investigate the role of spinal PAR1 activation in thrombin-induced and nerve root-induced pain, SCH79797 was separately administered at 1 day prior to intrathecal injection of rat thrombin or painful root compression. Since intrathecal rat thrombin produced the most robust behavioral responses at day 1 after injection, mechanical hyperalgesia was only measured prior to SCH79797 injection (day -1), before compression (day 0) and at day 1 after compression, for both rat thrombin alone and rat thrombin together with SCH79797 administration (Table A.2.6). Lastly, SCH79797 was given intrathecally at 1 day before a 15-minute compression (SCH+15min). Because the
vehicle (DMSO) is not inert, those studies also included separate groups that received a vehicle treatment before sham (DMSO+sham) or a compression (DMSO+15min). Behavioral responses were measured prior to the pre-treatment (day -1), at day 1 after treatment but before compression (day 0), and for up to 7 days after the compression (Table A.2.7). Please see Section 4.3.2.4 for more details on the intrathecal dosing and administration of all of the pharmacologic agents administered in the studies presented in Chapter 4.

The studies in Chapter 5 define the role of salmon thrombin in painful compression-induced mechanical allodynia by administering salmon thrombin directly to the nerve root after its compression (Section 5.4.1.2). Mechanical allodynia was chosen as the metric to quantify behavioral sensitivity for those studies based on a previous study showing that salmon thrombin prevents alldynia for up to 7 days after a nerve root compression (Weisshaar et al. 2011). The ability of salmon thrombin to prevent early BSCB breakdown and development of behavioral sensitivity after a painful compression was investigated at day 1 (Table A.3.1). As such, mechanical allodynia was measured prior to the surgery (day 0) and on day 1 after a sham operation with no treatment (sham), and a 15-minute compression treated with either a vehicle (15min+veh), salmon thrombin (15min+STh), or human thrombin (15min+HTh); testing was performed only using a 4g von Frey filament (Table A.3.1). The longer term effects of salmon thrombin on nerve root health was also measured at day 7 after injury. As such mechanical allodynia produced after those same treatment groups was measured for up to 7 days using the 1.4g (Table A.3.2), 4g (Table A.3.3) and 10g (Table A.3.4) strength filaments.
Table A.1.1. Withdrawal thresholds (g) and thresholds normalized to baseline in the ipsilateral forepaw following sham, 3-minute compression (3min), and 15-minute compression (corresponding to studies in Section 3.3).

<table>
<thead>
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<th>group</th>
<th>withdrawal threshold (g)</th>
<th>withdrawal threshold (fold over baseline)</th>
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</thead>
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<td>day 1</td>
</tr>
<tr>
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</tr>
<tr>
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<td>115</td>
<td>26.00</td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>18.67</td>
</tr>
<tr>
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<td>159</td>
<td>22.33</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>85</td>
<td>8.17</td>
</tr>
<tr>
<td></td>
<td>113</td>
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<tr>
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<td>22.33</td>
</tr>
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Table A.1.2. Withdrawal thresholds (g) and thresholds normalized to baseline in the contralateral forepaw following sham, 3-minute compression (3min), and 15-minute compression (corresponding to studies in Section 3.3).

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<td>day 1</td>
</tr>
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</tr>
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<td>18.67</td>
</tr>
<tr>
<td>3min</td>
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<td>83</td>
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</tr>
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Table A.1.3. Withdrawal thresholds (g) and thresholds normalized to baseline in the ipsilateral forepaw on day 1 and day 7 after sham, chromic gut inflammatory insult (chronic), and 15-minute compression (15min) applied to the nerve root (corresponding to studies in Section 3.4).

<table>
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<th>Day 1</th>
<th>Day 7</th>
<th>Day 0 (Fold)</th>
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<th>Day 7 (Fold)</th>
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<td>0.31</td>
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</table>

Note: For this study, rats were followed for 1 or 7 days after injury in order to characterize the early and late BSCB permeability following these root insults. A dash (“-”) at day 7 indicates those rats for which the study was terminated at day 1.
Table A.1.4. Withdrawal thresholds (g) and thresholds normalized to baseline in the ipsilateral forepaw at day 1 after sham, 15-minute compression (15min), and compression treated with APC (15min+APC) (corresponding to studies in Section 3.5).

<table>
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<tr>
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<th>withdrawal threshold (fold over baseline)</th>
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</thead>
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<td></td>
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<td>day 1</td>
</tr>
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<td>sham</td>
<td></td>
<td></td>
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<td>26.00</td>
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<td>13.33</td>
</tr>
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</tr>
<tr>
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<td>11.00</td>
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<tr>
<td>15min+APC</td>
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<td>18.67</td>
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Table A.2.1. Average withdrawal thresholds (g) in the bilateral forepaws in response to intrathecal rat thrombin (RTh) given at doses of 0.04U/rat, 0.4U/rat, and 4U/rat (corresponding to studies in Section 4.3.2.3).

<table>
<thead>
<tr>
<th>group</th>
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<th>day 0</th>
<th>day 1</th>
<th>day 3</th>
<th>day 5</th>
<th>day 7</th>
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</thead>
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<td>7.33</td>
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<td>9.83</td>
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Table A.2.2. Withdrawal thresholds (g) in the ipsilateral forepaw after a 15-minute compression (15min) with SCH79797 at doses of 0µg/kg, 25µg/kg, and 50µg/kg (corresponding to studies in Section 4.3.2.3).

<table>
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<th>day 1</th>
<th>day 3</th>
<th>day 5</th>
<th>day 7</th>
</tr>
</thead>
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<td>3.33</td>
</tr>
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<td>218</td>
<td>11.50</td>
<td>9.33</td>
<td>4.67</td>
<td>5.33</td>
<td>6.67</td>
<td>6.00</td>
</tr>
<tr>
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<td>230</td>
<td>13.33</td>
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<td>5.33</td>
<td>6.67</td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>231</td>
<td>17.00</td>
<td>15.00</td>
<td>6.67</td>
<td>6.00</td>
<td>7.33</td>
<td>5.33</td>
</tr>
<tr>
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<td>232</td>
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<td>4.00</td>
<td>4.67</td>
</tr>
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<tr>
<td>SCH(50)+15min</td>
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Table A.2.3. Withdrawal thresholds (g) in the ipsilateral forepaw following sham, 3-minute compression (3min), and 15-minute compression (15min) (corresponding to studies in Section 4.3.3.1).

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<th>day 1</th>
<th>day 7</th>
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<td>2.47</td>
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<td></td>
<td>157</td>
<td>22.33</td>
<td>4.67</td>
<td>-</td>
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</tbody>
</table>

Note: A dash (“-”) at day 7 indicates those rats for which the study was terminated at day 1.
Table A.2.4. Withdrawal thresholds (g) in the ipsilateral forepaw following a 15-minute compression (15min) and a compression pre-treated with hirudin (hir+15min) (corresponding to studies in Section 4.3.3.2).

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<td>109</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>158</td>
</tr>
<tr>
<td>hir+15min</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>214</td>
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Table A.2.5. Bilateral withdrawal thresholds (g) over 7 days following intrathecal rat thrombin (RTh) (corresponding to studies in Section 4.3.3.3).

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</thead>
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<td>220</td>
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<tr>
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Table A.2.6. Bilateral withdrawal thresholds (g) following intrathecal rat thrombin (RTh) and rat thrombin pre-treated with SCH79797 (SCH+RTh) (corresponding to studies in Section 4.3.3.3).

<table>
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<th>day 0</th>
<th>day 1</th>
</tr>
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<td>263</td>
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<td>15.00</td>
<td>3.00</td>
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</tr>
<tr>
<td></td>
<td>SCH+RTh</td>
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<td></td>
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Table A.2.7. Withdrawal thresholds (g) in the ipsilateral forepaw following sham with DMSO pre-treatment (DMSO+sham) and a 15-minute compression pre-treated with either DMSO (DMSO+15min) or SCH79797 (SCH+15min) (corresponding to studies in Section 4.3.3.4).

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Table A.3.1. Number of withdrawals of the ipsilateral forepaw in response to a 4g filament at day 1 after a sham or 15-minute compression treated with vehicle (15min+veh), salmon thrombin (15min+STh), or human thrombin (15min+HTh) (corresponding to studies in Section 5.3.2).

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Table A.3.2. Number of withdrawals of the ipsilateral forepaw in response to a 1.4g filament for 7 days after a sham or 15-minute compression treated with vehicle (15min+veh), human thrombin (15min+HTh), or salmon thrombin (15min+STh) (corresponding to studies in Section 5.4.2).

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Table A.3.3. Number of withdrawals of the ipsilateral forepaw in response to a 4g filament for 7 days after a sham or 15-minute compression treated with vehicle (15min+veh), human thrombin (15min+HTh), or salmon thrombin (15min+STh) (corresponding to studies in Section 5.4.2).

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Table A.3.4. Number of withdrawals of the ipsilateral forepaw in response to a 10g filament for 7 days after a sham or 15-minute compression treated with vehicle (15min+veh), human thrombin (15min+HTh), or salmon thrombin (15min+STh) (corresponding to studies in Section 5.4.2).

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Appendix B

Quantified Immunohistochemistry of the Spinal Cord & Nerve Root

This appendix summarizes the immunolabeling of target proteins in the dorsal horn of the spinal cord or nerve root that was quantified in the studies presented in Chapters 3-6 of this thesis. The tables provide values for each rat used in each study and are listed in the order that they appear in the thesis. Images are identified by the rat number (ID) followed by the image number (ID-number). For all studies, densitometry was performed using Matlab to quantify the positively-labeled pixels in each image (Densitometry.m). For some studies, quantification of the co-localization of pixels positively labeling two different target proteins was performed using a specialized co-localization Matlab script (colocalizationKJN.m). See Appendix G for both Matlab scripts. All data are represented as percent expression of a target protein relative to the expression of that protein in tissue from normal naïve rats. For all studies, the expression of each target protein is quantified for the spinal dorsal horn on the side ipsilateral to injury or surgery. A selection of studies in Chapters 3 and 4 quantified protein labeling in the bilateral spinal cord; for those cases, the side of the spinal cord is indicated in the tables below. Spinal cord tissue was harvested at days 1 and/or 7 and 1-6 tissue section(s) per rat were included for each study, as indicated by each table.
Studies in Chapter 3 measured the spinal expression of immunoglobulin G (IgG), a serum protein not typically found in the CNS, as a proxy for blood-spinal cord barrier (BSCB) breakdown at days 1 and 7 after the different nerve root insults. Quantified spinal IgG for each rat in those studies is listed in Tables B.1.1-B.1.3. Table B.1.1 summarizes the expression of IgG in the ipsilateral (ipsi) and contralateral (con) spinal dorsal horns at days 1 and 7 after a sham procedure, a 3-minute compression (3min), and a 15-minute compression (15min). The methods of the surgical and immunohistochemical procedures are provided in Section 3.3.1. Table B.1.2 provides the normalized IgG expression in the ipsilateral and contralateral spinal cord at days 1 and 7 after a sham procedure, a painful inflammatory chromic gut insult (chromic), and a painful compression (15min) (see Section 3.4.1). BSCB breakdown was blocked with activated protein C (APC) given intravenously (0.2mg/kg) in order to determine if BSCB contributes to the development of pain. Table B.1.3 details the individual expression of IgG in the ipsilateral spinal cord after a painful 15-minute compression with or without APC given intravenously at 1 hour after injury (Section 3.5.1.1).

The expression of fibrin(ogen), which is the enzymatic product of thrombin, was immunolabeled and quantified in the ipsilateral spinal cord after painful compression with various pharmacologic treatments; those data are summarized in Tables B.2.1-B.2.3 and refer to studies in Chapter 4. Fibrin(ogen) expression was immunolabeled in the ipsilateral spinal cord at days 1 and 7 following a sham operation, a 3-minute compression (3min), and a 15-minute compression (15min) in the studies from Chapter 4 (Table B.2.1); see Section 4.3.2.6 for details on the immunohistochemistry methods. In
order to determine if intrathecal hirudin given a day before compression (hir+15min) blocks spinal thrombin activity, spinal fibrinogen was labeled in the ipsilateral spinal cord at 1 day after a painful compression with or without hirudin treatment (Table B.2.2). Spinal fibrinogen was also measured at 1 day after compression pre-treated with the protease-activated receptor-1 (PAR1) inhibitor SCH79797 given 1 day before a 15-minute compression (SCH+15min); corresponding tissue from rats receiving a DMSO vehicle prior to compression were also included for comparison (Figure B.2.3).

In order to measure if spinal astrocytes modulate their expression of PAR1 following various compressive insults to the nerve root, PAR1 and GFAP were co-immunolabeled in the ipsilateral spinal cord after sham, a 3-minute compression (3min), and a 15-minute compression (15min) in studies from Chapter 4 (Table B.2.4). For the remaining studies in Chapter 4, BSCB breakdown and spinal astrocyte activation were quantified after treatments targeting the spinal thrombin/PAR1 signaling cascade in association with root compression. Spinal IgG was measured at day 1 after a painful compression pre-treated with the thrombin inhibitor hirudin (hir+15min) in order to determine if spinal thrombin contributes to BSCB breakdown early after compression (Table B.2.5). Relatoly, the effect of compression-induced spinal thrombin on spinal astrocyte activation was quantified by immunolabeling GFAP at day 7 after compression with and without intrathecal hirudin (Table B.2.6). Since mechanical hyperalgesia is maximum at day 1 after an intrathecal injection of rat thrombin in naïve rats (RTh), IgG and GFAP expression were co-immunolabeled in the bilateral spinal cord at day 1 after rat thrombin injection. Additionally, spinal PAR1 was blocked with SCH79797 prior to
rat thrombin (SCH+RTh) and IgG and GFAP expression were quantified at day 1 and compared with the rat thrombin injection alone (Table B.2.7). Since the thrombin injection did not favor one side, IgG and GFAP expression was measured in the left and right spinal cord and averaged between the two sides. Images are identified in applicable tables as the rat ID followed by the spinal cord section number followed by the side, either left (L) or right (R) (ID-number-side). SCH79797 was also administered at 1 day before a 15-minute compression (SCH+15min) to block spinal PAR1. IgG was measured in the ipsilateral spinal cord at day 1 (Table B.2.8) and spinal GFAP was measured using the densitometry Matlab code at day 7 (Table B.2.9) after compression with SCH79797 (SCH+15min) or the DMSO vehicle (DMSO+15min). For expression of GFAP at day 7, a vehicle treated sham group was also included (DMSO+sham).

Studies in Chapter 5 investigate the effect of salmon thrombin on nerve root-induced pain, on BSCB breakdown and on macrophage infiltration at the nerve root. Salmon thrombin (15min+STh), human thrombin (15min+HTh), or the vehicle neurobasal media (15min+veh) was applied to the root immediately after a 15-minute compression (see Section 5.3.1.2 for more details). BSCB breakdown was measured in rats in those groups by immunolabeling the bilateral spinal cord for IgG at day 1 after compression (Table B.3.1). At day 7 after a 15-minute compression treated with vehicle, salmon thrombin or human thrombin, the extent of macrophage infiltration in the nerve root was measured by immunolabeling for Iba1 (Section 5.5). Iba1 expression was quantified in the ipsilateral nerve root using the Matlab densitometry script; those data are detailed in Table B.3.2.
Data from the studies in Chapter 6 measuring spinal vimentin, spinal astrocytic vimentin, and spinal endothelial vimentin after a compressive nerve root injury with various treatments are presented in Tables B.4.1-B.4.6. Vimentin expression and the co-localized expression of vimentin and GFAP in the ipsilateral spinal cord were measured at day 1 and day 7 after sham, a 3-minute compression (3min), and a 15-minute compression (15min) (Table B.4.1). Spinal vimentin expression was quantified using the Matlab densitometry script and the astrocytic vimentin was quantified using the co-localization code in Matlab. Table B.4.2 details the quantified expression of vimentin and astrocytic vimentin in the ipsilateral spinal cord at day 7 after compression pre-treated with hirudin at 1 day before compression (hir+15min) and compression alone (15min). Spinal vimentin and astrocytic vimentin expression at day 7 after sham or compression treated with human thrombin (15min+HTh) or salmon thrombin (15min+STh) are itemized in Table B.4.3. Spinal vimentin expression co-localized with von Willebrand factor (VWF), as a proxy for endothelial cells, at days 1 and 7 after sham, a 3-minute compression (3min), and a 15-minute compression (15min) is provided in Table B.4.4. The co-localization of positive pixels for vimentin and VWF was measured using the custom Matlab co-localization script (see Appendix G). The expression of endothelial vimentin in the ipsilateral spinal cord at day 7 was also quantified at day 7 following a 15-minute compression with intrathecal hirudin given 1 day before compression (hir+15min) (Table B.4.5). Lastly, endothelial vimentin was labeled and quantified in the ipsilateral spinal cord at day 7 after sham or a compression treated with human thrombin (15min+HTh) or salmon thrombin (15min+STh).
Table B.1.1. Normalized IgG labeling in the ipsilateral (ipsi) and contralateral (con) spinal cord at days 1 and 7 after sham, 3-minute compression (3min), or 15-minute compression (15min) (corresponding to studies in Section 3.3.2).

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Note: Rat ID numbers that begin with a "K" are rats that received surgery by Kristen Nicholson, a former member of our lab (K251, K253, and K258).
Table B.1.2. Normalized IgG labeling in the ipsilateral (ipsi) and contralateral (con) spinal cord at days 1 and 7 after sham, chromic gut insult (chromic), and 15-minute compression (15min) (corresponding to studies in Section 3.4.2).

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|     | 192-05 | 2.530 |

| 193 | 193-01 | 33.209 |
|     | 193-02 | 29.730 |
|     | 193-03 | 6.642 |
|     | 193-04 | 3.479 |
|     | 193-05 | 4.428 |
|     | 193-06 | 2.647 |

| 109 | 109-01 | 2.902 |
|     | 109-02 | 17.605 |
|     | 109-03 | 47.296 |
|     | 109-04 | 4.377 |
|     | 109-05 | 260.698 |
|     | 109-06 | 206.360 |

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|     | 110-03 | 136.178 |
|     | 110-04 | 82.982 |
|     | 110-05 | 38.874 |

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Table B.1.3. Normalized IgG labeling in the ipsilateral (ipsi) and contralateral (con) spinal cord at day 1 after a 15-minute compression (15min) and a compression treated with APC (15min+APC) (corresponding to studies in Section 3.5.2).

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Table B.2.2. Normalized fibrin(ogen) labeling in the ipsilateral spinal cord at days 1 and 7 after 15-minute compression (15min) and compression pre-treated with hirudin (hir+15min) (corresponding to studies in Section 4.3.3.2).

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Table B.2.3. Normalized fibrin(ogen) labeling in the ipsilateral spinal cord at days 1 and 7 after 15-minute compression pre-treated with DMSO (DMSO+15min) or SCH79797 (SCH+15min) and at day 7 after DMSO treatment with sham (DMSO+sham) (corresponding to studies in Section 4.3.3.4).

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Table B.2.4. Normalized co-localization of PAR1 and GFAP in the ipsilateral spinal cord at days 1 and 7 after sham, 3-minute compression (3min), and 15-minute compression (15min) (corresponding to studies in Section 4.4.3.1).

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**Sham**

**Day 7**

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Note: Rat ID numbers that begin with a “J” are rats that received surgery by Christine Weisshaar, another member of the lab (J23, J28).
Table B.2.5. Normalized IgG expression in the ipsilateral spinal cord at day 1 after a 15-minute compression (15min) and compression with hirudin (hir+15min) (corresponding to studies in Section 4.4.3.2).

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Table B.2.6. Normalized GFAP expression in the ipsilateral spinal cord at day 7 after a 15-minute compression (15min) and compression with hirudin (hir+15min) (corresponding to studies in Section 4.4.3.2).

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Table B.2.7. Normalized IgG and GFAP expression in the bilateral spinal cord at day 1 after intrathecal PBS, rat thrombin (RTh) and SCH79797 pre-treated rat thrombin (SCH+RTh) (corresponding to studies in Section 4.4.3.3).

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**Table B.3.1. Normalized IgG expression in the ipsilateral (ipsi) and contralateral (con) spinal cord at day 1 after 15-minute compression treated with vehicle (15min+veh), salmon thrombin (15min+Sth), or human thrombin (15min+HTh) (corresponding to studies in Section 5.3.2).**

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Table B.3.2. Normalized Iba1 expression in the ipsilateral nerve root at day 7 after 15-minute compression treated with vehicle (15min+veh), human thrombin (15min+HTh), or salmon thrombin (15min+STh) (corresponding to findings in Section 5.5).

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Note: Images with artefact in the specified fluorescent channel were not included and are indicated with a dash ("-").
Table B.4.2. Normalized vimentin and vimentin co-localized with GFAP in the ipsilateral spinal cord at day 7 after 15-minute compression (15min) and compression treated with hirudin (hir+15min) (corresponding to studies in Section 6.3.2).

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Note: Images with artefact in the specified fluorescent channel were not included and are indicated with a dash (“-”).
Table B.4.3. Normalized vimentin and vimentin co-localized with GFAP in the ipsilateral spinal cord at day 7 after sham and 15-minute compression treated with human thrombin (15min+HTh) or salmon thrombin (15min+STh) (corresponding to studies in Section 6.3.2).

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Table B.4.5. Normalized vimentin co-localized with VWF in the ipsilateral spinal cord at day 7 after 15-minute compression (15min) and compression treated with hirudin (hir+15min) (corresponding to studies in Section 6.3.3).

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Table B.4.6. Normalized vimentin co-localized with VWF in the ipsilateral spinal cord at day 7 after sham and 15-minute compression treated with human thrombin (15min+HTh) or salmon thrombin (15min+STh) (corresponding to studies in Section 6.3.3).

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Appendix C

Correlation of Serum Cytokine/Chemokine Concentrations & Mechanical Hyperalgesia

This appendix summarizes the concentration of 23 pro- and anti-inflammatory cytokines and chemokines in the serum from individual rats at day 1 after a variety of nerve root insults (painful compression, non-painful compression, inflammatory insult) as described in Chapter 3. Blood samples were collected from rats at day 0 (baseline) prior to, and at 1 day after, the insult. Serum was isolated via consecutive centrifugation steps and processed using a multiplex bead Luminex assay (#L80-01V11S5; Bio-Rad; Hercules, CA). The Luminex assay measures the following analytes: interleukin-1 alpha (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, IL-18, monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), keratinocyte-derived chemokines/growth-related oncogene (GRO/KC), interferon-gamma (IFN-γ), macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein-3 alpha (MIP-3α), regulated on activation, normal T cell expressed and secreted (RANTES) and vascular endothelial growth factor (VEGF). The concentration of each analyte in serum samples at day 1 after surgery was normalized to the baseline concentration for each rat. Additionally, the normalized
concentration of each cytokine was correlated the corresponding withdrawal thresholds for each rat at day 1. Similar to the serum data, the withdrawal threshold (g) at day 1 of the forepaw ipsilateral to injury was normalized to the withdrawal threshold at day 0 for each rat. Please see Chapter 3 for further details (Smith and Winkelstein 2015).

Table C.1.1 summarizes the normalized serum expression of the 23 analytes at day 1 after a sham surgery, a 3-minute compression (3min), and a 15-minute compression (15min) (Section 3.3). For each rat in Table C.1.1, the corresponding normalized withdrawal threshold for the ipsilateral forepaw at day 1 are also included. Similarly, Table C.1.2 summarizes the concentration of the 23 analytes at day 1 after a chromic gut inflammatory insult, with normalized data for the corresponding analytes and withdrawal thresholds for each rat detailed (Section 3.4).
Table C.1.1. Normalized cytokine and chemokine concentrations in the serum and corresponding normalized withdrawal thresholds in the ipsilateral forepaw at day 1 after sham, 3-minute compression (3min), and 15-minute compression (15min) (corresponding to studies in Section 3.3).

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Note: Serum samples that did not produce a positive reading (were undetected) for an analyte are indicated with a dash (“-”).
This appendix contains the data from the human umbilical vein endothelial cell (HUVEC) microchannel experiment presented in Chapter 5. HUVECs were seeded in the inner lumen (400µm diameter) of a microfluidic device filled with collagen. Those luminal HUVEC monolayers form vessels that are impermeable to transluminal flow when flow is applied at a rate of 5µl/ml (Galie et al. 2014). In order to determine the effects of salmon thrombin on vessel permeability, initially impermeable channels were stimulated with TNF-α (100ng/ml) to induce barrier opening. Separate channels were treated with TNF-α with salmon thrombin (1U/ml) (TNF-α+STh) or with human thrombin (1U/ml) (TNF-α +HTh). In order to track endothelial barrier breakdown, FITC-labeled dextran was added to the channel flow and its diffusion into the surrounding collagen was tracked for 10 minutes, at 1 minute intervals. Detailed methods for the microfluidic setup and experiments are provided in Section 5.3.1.3. The diameter of the dextran front at each minute was normalized to the diameter of the inner lumen of the channel to obtain values for the normalized dextran diameter.

Table D.1 contains the normalized dextran diameter for each group in each trail (n=3 each). These experiments were performed in conditions containing serum, as well as
in serum free conditions, in which the cultures were starved of serum for 4 hours prior to testing. Using serum and serum-free conditions was done in order to determine if salmon thrombin acts to protect vasculature stability directly through an endothelial receptor or indirectly through a serum component. Additionally, the slope of the change in the dextran front over time produced by each test group in each of the three trials was then normalized to the slope produced by TNF-α alone with serum. The normalized dextran flux data are presented in Section 5.3.2 and included in Table D.2 below.
Table D.1. Normalized dextran front in HUVEC microchannels exposed to TNF-α and TNF-α with salmon thrombin (TNF-α+STh) or human thrombin (TNF-α+HTh) in serum containing or serum free conditions (corresponding to studies in Section 5.3.2).

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Note: Two trials were disrupted due to microchannel failure prior to the 10 minute endpoint. In those cases values are represented as a dash (“-“).
Table D.2. Slope of the dextran front over time, normalized to the slope produced in cultures exposed to serum and TNF-α, in HUVEC channels exposed to TNF-α and TNF-α with salmon thrombin (TNF-α+STh) or human thrombin (TNF-α+HTh) in serum containing or serum free conditions (corresponding to studies in Section 5.3.2).

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Appendix E

Fluorogenic Synthetic Peptide Cleavage

This appendix presents data for studies presented in Chapter 5 that measured and compared the rate of cleavage of various fluorogenic substrates produced by salmon thrombin (STh) and human thrombin (HTh). The ability of salmon and human thrombin to produce activated protein C (APC) was investigated using solutions of protein C in its zymogen form and the fluorogenic substrate for APC (Leu-Ser-Thr-Arg; Sigma Aldrich; St. Louis, MO) with either species of thrombin. The coupled reaction produced in those solutions is thrombin activating protein C into APC and then APC activating the substrate. Since thrombin is itself an enzyme and may also activate the fluorogenic APC substrate, the rates of substrate activation produced by thrombin alone was subtracted from the rate produced in solutions containing the respective species of thrombin with protein C (see Section 5.3.1.4 for more details on methods). The fluorescent signals generated over 290 seconds produced by salmon thrombin, with protein C (STh+PC) or without (STh), and human thrombin, with protein C (HTh+PC) or without (HTh), in each of three trials are provided in Table E.1. The fluorescent intensity over time was taken as the rate of APC substrate cleavage for each trial. Table E.2 provides the rates of APC
substrate cleavage produces by STh+PC and HTh+PC, as well as those rates corrected by subtracting the respective rates of substrate cleavage generated by STh or HTh alone.

In order to directly compare the rates of activation by salmon thrombin (STh) and human thrombin (HTh) on protease-activated receptor-1 (PAR1), PAR3 and PAR4, three fluorogenic peptides were designed (Abden; San Diego, CA) mapping the cleavage sites of those receptors (see Section 5.4.1.5). Additionally a fluorescence resonance energy transfer (FRET) peptide mapping the PAR1 cleavage site and containing the hirudin-like sequence (PAR1-hir) was designed and tested, because mammalian thrombin has the highest affinity for PAR1 due to that hirudin-like sequence on the extracellular domain of PAR1 (Jaques et al. 2000, Seeley et al. 2003). In all cases, salmon or human thrombin (1U/ml) was added to the substrate and fluorescence was measured over time. The slopes of the fluorescent signal intensity over time are provided in Table E.3.
Table E.1. Fluorescent signal generated in solutions containing a fluorogenic substrate for APC with salmon thrombin and protein C (STh+PC), human thrombin and protein C (HTh+PC), or either species of thrombin without protein C (corresponding to studies in Section 5.3.2).

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Table E.2. APC generation by salmon thrombin (STh) and human thrombin (HTh) (corresponding to studies in Section 5.3.2).

<table>
<thead>
<tr>
<th>group</th>
<th>trial</th>
<th>slope (generated by thrombin with PC)</th>
<th>slope (slope generated by thrombin alone subtracted)</th>
</tr>
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<tbody>
<tr>
<td>STh</td>
<td>trial 1</td>
<td>33.918</td>
<td>18.657</td>
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<td>trial 2</td>
<td>40.796</td>
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<td>trial 3</td>
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<td>19.032</td>
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<td>HTh</td>
<td>trial 1</td>
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<td>1.019</td>
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<td></td>
<td>trial 2</td>
<td>17.832</td>
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<tr>
<td></td>
<td>trial 3</td>
<td>16.222</td>
<td>-1.764</td>
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Table E.3. Slope of fluorescence over time produced by salmon thrombin (STh) and human thrombin (HTh) using fluorogenic peptides for PAR1, PAR1-hir, PAR3, and PAR4 (corresponding to studies in Section 5.4.2).

<table>
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<th>fluorescence-time slope</th>
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<td></td>
<td>trial 2</td>
<td>0.0311</td>
</tr>
<tr>
<td></td>
<td>HTh</td>
<td>trial 1</td>
<td>0.1122</td>
</tr>
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<td></td>
<td></td>
<td>trial 2</td>
<td>0.1118</td>
</tr>
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<td>PAR1-hir</td>
<td>STh</td>
<td>trial 1</td>
<td>0.0106</td>
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<td>trial 2</td>
<td>0.0101</td>
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<td>trial 3</td>
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<td></td>
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<tr>
<td></td>
<td>HTh</td>
<td>trial 1</td>
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<td></td>
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<td>trial 2</td>
<td>0.0269</td>
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<td>trial 3</td>
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</tr>
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<td>trial 4</td>
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<td></td>
<td></td>
<td>trial 2</td>
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<td>trial 2</td>
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</tr>
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<td>HTh</td>
<td>trial 1</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trial 2</td>
<td>-0.0005</td>
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Appendix F

Mixed Cortical Culture mRNA for IL-1β & TNF-α

This appendix contains the gene expression data for the transcription of IL-1β and TNF-α by mixed astrocyte and neuron cortical cultures after their stimulation with salmon or human thrombin (studies are presented in Chapter 5). Primary cultures of dissociated rat brain cortices were treated for 4 hours with human thrombin (HTh, 1U/ml) or salmon thrombin (STh, 1U/ml) and RNA was harvested. Four separate trials were run, each using cultures from a different brain dissociation; two untreated controls (UT) were included from each dissociation for consistency with normalization. Using reverse transcriptase real time-polymerase chain reaction (RT-PCR), the expression of the genes for IL-1β (Fwd: 5’-CAC CTC TCA AGC AGA GCA CAG-3’, Rev: 5’-GGG TTC CAT GGT GAA GTC AAC-3’) and TNF-α (Fwd: 5’-ATC ATC TTC TCA AAA CTC GAG TGA CAA-3’, Rev: 5’-CTG CTC CTC TGC TTG GT-3’) were measured and compared to levels of the housekeeping gene, cyclophilin-A (Fwd: 5’-TAT CTG CAC TGC CAA GAC TGA GTG-3’, Rev: 5’-CTT CTT GCT GGT CTT GCC ATT CC-3’). Expression of the respective genes in untreated cultures (UT) using the ∆∆Ct method (Rothman et. Al 2009). For more details on RNA harvest, reverse transcription, RT-PCR and subsequent analysis, please see Section 5.4.1.4. The normalized increase in IL-1β mRNA
is summarized in Table F.1 and data for the normalized increase in TNF-α mRNA is presented in Table F.2.
Table F.1. Fold increase in IL-1β mRNA produced by mixed cultures treated with salmon thrombin (STh) or human thrombin (HTh) normalized to production in untreated cultures (UT) (corresponding to studies in Section 5.4.2).

<table>
<thead>
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<th>IL-1β mRNA (fold increase over UT)</th>
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<td>UT</td>
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<tr>
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<td>HTh</td>
<td>0.491</td>
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<td>STh</td>
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<td>trial 8</td>
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</tr>
<tr>
<td></td>
<td>HTh</td>
<td>1.051</td>
</tr>
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<td></td>
<td>STh</td>
<td>5.544</td>
</tr>
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<td>HTh</td>
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<td>STh</td>
<td>5.428</td>
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<td>HTh</td>
<td>0.855</td>
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Table F.2. Fold increase in TNF-α mRNA produced by mixed cultures treated with salmon thrombin (STh) or human thrombin (HTh) normalized to production in untreated cultures (UT) (corresponding to studies in Section 5.4.2).

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<td>1.378</td>
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<tr>
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<td>6.918</td>
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Appendix G

Matlab Codes for Densitometry & Co-localization

The Matlab code that was used to quantify the percent positive pixels in fluorescently labeled tissue sections throughout this thesis and summarized in Appendix B is provided here (Section G.1). Prior to running this script, the threshold (0-250) is defined and entered as “pos_thresh” in the code. Once the script is finished computing the percent positive pixels for all images, typing “Iname” into the command window will return a list of all the images that were processed. Entering “percpos” into the command window will return the percent positive pixels that were calculated for the corresponding images returned by the Iname command.

Also included in this appendix is the Matlab code that was used to quantify the percent of pixels within an image that co-localized between two different fluorescent labels. Prior to running that script, a threshold (0-250) is separately defined for each fluorescent label and entered as “red_thresh” and “green_thresh” in the code. Once the script is finished computing the percent positive pixels for all images, typing “Iname” into the command window will return a list of all of the images that were processed. Entering “percpos” into the command window returns the percent positive pixels from the red channel that were calculated for the corresponding images returned by the Iname
command; entering “percpos_green” will do the same for the green channel. Typing “perc_co” into the command window will return a list of the percent of co-localized red and green positive pixels calculated for the corresponding images returned by the Iname command. To run either the densitometry or the co-localization script, the user must save the code and the images in the same file directory.
Section G.1. Matlab Code for Densitometry

%% This script was written to calculate and visualize percent positive pixels per image. To run the file, u want to create an excel file that has the detailed information abt the images to be analyzed, then number those images with a common name that has an ordered numerical (1, 2...) ending. NOTE: for the excel file, please have the following columns (in order): rat #, tissue type, injury type, image no., threshold, raw % results, normalized results. Number normals last.

%% requires MATLAB 7.0 (or higher) and imaging toolbox.


clear all;
close all;

D = dir('*.tif');%specify which images to analyze (* = wildcard character)

for k=1:length(D);

%reads file in
file=D(k).name;

% Load the image
imag_orig = imread(file);
% converts to grayscale
imag_orig = imag_orig(:,:,1);%grab the red labeled image
imag_orig = rgb2gray(imag_orig);
imag = imag_orig;
invImag = 255-imag;

%calc number of pixels
[a b]=size(imag);
tsize=a*b;
low=double(min(imag(:)));
high=double(max(imag(:)));
whiteSpace = 0.996*high;
pos_thresh = 215; %input based on normal run, remember for DAB staining
% higher value corresponds to a higher +ive threshold.

backg=sum(sum(imag>whiteSpace));
posp=sum(sum(imag<pos_thresh));
% calc percent of positive pixels in tissue
percpos(k) = posp/(tsize-backg);
tpost(k) = posp;
Iname(k) = {file};

% map out pos and neg pixels
pmap=(imag<pos_thresh);
nmap=(imag>whiteSpace);

% make figure for each image, if you are processing a bunch of images, you
% may want to comment this part out

% make positive pixels more green, and background pixels less blue
imag1(:,:,1)=double(imag)/255;
imag1(:,:,2)=(1-pmap).*double(imag)/255+pmap;
imag1(:,:,3)=double(imag)/255.*(1-nmap);

h = figure;
subplot(3,1,3);
subimage(imag);
axis image
axis off
subplot(3,1,2);
subimage(imag1);
image(imag1);
axis image
axis off
colormap gray
subplot(3,1,1);
subimage(imag_orig)
axis image
axis off
drawnow

clear imag imag1
end
Section G.2. Matlab Code for Co-localization

%% This script was written to calculate and visualize percent positive pixels per image. To run the file, you want to create an excel file that has the detailed information about the images to be analyzed, then number those images with a common name that has an ordered numerical (1, 2...) ending. NOTE: for the excel file, please have the following columns (in order): rat #, tissue type, injury type, image no., threshold, raw results, normalized results. Number normals last.

%% requires MATLAB 7.0 (or higher) and imaging toolbox.

%% Written by Ling Dong (modified from K. Quinn) on Feb 2, 2009.

clear all;
close all;
clf;

D = dir('*.tif'); % common prefix for all images
% name the excel output file
% IPfname = 'PAR1-GFAP_colocalization_8-25-11(2)';
% OPfile = strcat(IPfname, '.xls');
% fid = fopen(OPfile, 'w');

red_thresh = 185; % input based on normal run
green_thresh = 180; % higher value corresponds to more positive pixels
h1 = waitbar(0, 'Please wait...');

for k=1:length(D);
    file=D(k).name;

    % Load the image
    imag_orig = imread(file);
    imag_red = imag_orig(:,:,1); % grab the red labeled image
    imag_green = imag_orig(:,:,2); % grab the green labeled image

    % take the inverse of the image
    imag_red = uint8(-double(imag_red) + 255);
    imag_green = uint8(-double(imag_green) + 255);

    % calc number of pixels
    [a b]=size(imag_red);
tsize=a*b;
low=double(min(imag_red(:)));
high=double(max(imag_red(:)));
whiteSpace = 0.99*high;
backg = sum(sum(imag_red > whiteSpace));
posp = sum(sum(imag_red < red_thresh));
% calc percent of positive pixels in tissue for red signal
percpos(k) = posp/(tsize-backg);

backg_green = sum(sum(imag_green > whiteSpace));
posp_green = sum(sum(imag_green < green_thresh));
% calc percent of positive pixels in tissue for green signal
percpos_green(k) = posp_green/(tsize-backg_green);

% map out pos and neg pixels
pmap_red = {imag_red < red_thresh};
nmap = {imag_red > whiteSpace};
pmap_green = {imag_green < green_thresh};

% make positive pixels green, and background pixels blue
ima1(:, :, 1) = zeros(a, b);
ima1(:, :, 2) = pmap_red;
ima1(:, :, 3) = zeros(a, b);
ima2(:, :, 1) = zeros(a, b);
ima2(:, :, 2) = pmap_green;
ima2(:, :, 3) = zeros(a, b);

%% set all non-green/red labeled pixels in the green image to zero
and
%% create a new image for the co-localization
[R, C] = find(pmap_red == 0);
ima_co = ima2;
for ind = 1:length(R)
    ima_co(R(ind), C(ind), 2) = 0;
end

%% calculate the number of green positive pixels relative to total
% number of pixels that express red(gfap/map2, etc.)
tot_green(k) = length(find(pmap_green == 1));
tot_co(k) = length(find(ima_co(:, :, 2) == 1));
col_per_red(k) = tot_co(k)/posp; % amount col per red area
perc_co(k) = tot_co(k)/(tsize-backg_green);
Iname(k) = {file};

%% calculate the intensity of the green-labeled cells
and
[R, C] = find(ima_co(:, :, 2) == 1);
for ind = 1:length(R)
    ints(ind) = ima_green(R(ind), C(ind));
end
Ave_ints(k) = mean(ints);
clear ints
fprintf(fid, '%s
', D(k).name);
fprintf(fid, 'percent green : %7.4d
', percpos_green(k));
fprintf(fid, 'percent red : %7.4d
', percpos(k));
fprintf(fid, 'percent colocal : %7.4d
', perc_co(k));
fprintf(fid, 'colocal per red area : %7.4d
',
col_per_red(k));

h = figure(k);
subplot(3,2,1);
subimage(imag_red);
axis image; axis off
title('RED in gray scale')
subplot(3,2,2);
subimage(imag_green);
axis image; axis off
title('GREEN')

subplot(3,2,3);
subimage(imag1);
axis image; axis off;
title('RED positive')
subplot(3,2,4)
subimage(imag2);
axis image; axis off;
title('GREEN positive')

subplot(3,2,5);
blue = zeros(a,b); red = zeros(a,b);
imag_orig(:,:,3) = red; imag_orig(:,:,3)=blue;
subimage(imag_orig)
axis image; axis off
title('original image')

subplot(3,2,6);
subimage(imag_co)
axis image; axis off

title('colocalized pixels')
drawnow

%saveas(h, ['colocalize-' D(k).name], 'jpg')
waitbar(k/length(D))
clear imag imag1 imag2 R C

end
close(h1)
status = fclose(fid);
Bibliography


Rothman, S. M., Ma, L. H., Whiteside, G. T., Winkelstein, B. A. (2011) Inflammatory cytokine and chemokine expression is differentially modulated acutely in the dorsal root


