Role of Tissue Inhibitor of Matrix Metalloproteinase-1 in the Development of Hypersensitivity Using an Animal Model of Cutaneous Inflammation

Brittany Knight

University of Connecticut - Storrs, bknights@uchc.edu

Follow this and additional works at: https://opencommons.uconn.edu/dissertations

Recommended Citation


https://opencommons.uconn.edu/dissertations/2324
Abstract

Persistent pain is a significant clinical problem that affects millions of people worldwide. The severity of acute pain and inflammatory signaling during tissue damage can predispose an individual to develop pain that persists past the normal healing time. In my thesis work, I focused on how molecules involved in the regulation of inflammation and normal wound healing contribute to the progression of pain.

During tissue damage and inflammation, Tissue inhibitor of metalloproteinase-1 (TIMP-1) is upregulated to maintain tissue homeostasis as well as promote normal inflammatory signaling. Recent evidence suggests that TIMP-1 may reduce the development of nerve-injury induced pain, however how TIMP-1 contributes to pain processing required further investigation. Although primarily known as an MMP inhibitor, TIMP-1 has dual functions that can both disrupt ECM proteolysis as well as bind cell-surface receptors to mediate intracellular signaling pathways. In this proposed thesis work, in light of these dual functions, I examined the role of TIMP-1 in the development of hypersensitivity using a model of cutaneous inflammation. Our preliminary data suggested that inflammation induces TIMP-1 expression in inflamed skin in a time course that parallels the development of acute inflammatory pain, and that mice lacking TIMP-1 (T1KO mice) develop rapid onset and heightened sensitivity following inflammation.

In my thesis work, I determined that TIMP-1 plays a significant role in regulating the progression of inflammatory pain. As described in chapter 3, the induction of TIMP-1 during the onset of inflammation is important for reducing peripheral hypersensitivity that may be important for the development of pathological pain-like behaviors. In addition, I discovered that the absence of TIMP-1 resulted in rapid-onset hypersensitivity that persisted for long periods of time in tissues proximal and distal (e.g., mirror image pain) to the site of inflammation. Importantly, administration of full length recombinant TIMP-1, its MMP-inhibiting N-terminal, or its receptor binding C-terminal all attenuate inflammatory hypersensitivity.

Results from the proposed work demonstrate that during tissue damage, TIMP-1 signaling affects the normal progression of pain following inflammation through MMP-dependent and MMP-independent mechanisms.
Role of Tissue Inhibitor of Matrix Metalloproteinase-1 in the Development of Hypersensitivity

Using an Animal Model of Cutaneous Inflammation

Brittany Elizabeth Knight

B.S., Lock Haven University of Pennsylvania, 2013
Ph.D., University of Connecticut, 2019

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut
2019
Doctor of Philosophy Dissertation

Role of Tissue Inhibitor of Matrix Metalloproteinase-1 in the Development of Hypersensitivity Using an Animal Model of Cutaneous Inflammation

Presented by

Brittany Elizabeth Knight, B.S., Ph.D.

Major Advisor

Kyle M. Baumbauer, Ph.D.

Associate Advisor

Erin E. Young, Ph.D.

Associate Advisor

Stephen J. Crocker, Ph.D.

Associate Advisor

Royce Mohan, Ph.D.

University of Connecticut

2019
Table of Contents

Chapter 1. 1

Introduction to pain
I. History and Theories of Pain 3
II. Classification of Pain 6
II. Risk Factors 8
III. Managing pain 12
IV. Modeling Inflammatory Pain 14
V. Assessing hypersensitivity 17
VI. Summary 19

Chapter 2: 30

The Extracellular Environment
I. Inflammation and pain 30
II. Tissue inhibitors of matrix metalloproteinases 34
III. Summary 39

Chapter 3: 45

TIMP-1 attenuates the development of inflammatory pain through MMP-dependent and receptor-mediated cell signaling mechanisms
I. Abstract 46
II. Introduction 47
III. Materials and methods 49
IV. Results 54
V. Discussion 60
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI. Acknowledgements</td>
<td>67</td>
</tr>
<tr>
<td>VII. Figures</td>
<td>68</td>
</tr>
<tr>
<td>Chapter 4:</td>
<td>84</td>
</tr>
<tr>
<td><strong>Discussion and Future Directions</strong></td>
<td></td>
</tr>
<tr>
<td>I. TIMP-1 and inflammatory hyperalgesia</td>
<td>84</td>
</tr>
<tr>
<td>II. Endogenous TIMP-1 expression attenuates pain-like behaviors</td>
<td>87</td>
</tr>
<tr>
<td>III. TIMP-1 and the local inflammatory response</td>
<td>88</td>
</tr>
<tr>
<td>IV. TIMP-1 receptor attenuates hyperalgesia</td>
<td>89</td>
</tr>
<tr>
<td>V. TIMP-1 and glial cells</td>
<td>92</td>
</tr>
<tr>
<td>VI. TIMP-1 and clinical data</td>
<td>95</td>
</tr>
<tr>
<td>VII. Concluding Statement</td>
<td>97</td>
</tr>
<tr>
<td><strong>Bibliography</strong></td>
<td>113</td>
</tr>
</tbody>
</table>
List of Figures and Tables

Chapter 1:
Introduction to Pain

Figure 1-1: Chart representation of four main anatomical areas of pain modulation. 21
Figure 1-2: Simplified representation of the origin of pain published by René Descartes. 23
Figure 1-3: The Stanford pain scale is an updated assessment tool. 25
Figure 1-4: Graph depicting shift in behavioral responding. 27

Chapter 2:
The Extracellular Environment

Figure 2-1: Representation of cutaneous tissue damage and nociception. 42
Figure 2-2: Representation of timp-1 protein structure. 44

Chapter 3:
TIMP-1 attenuates the development of inflammatory pain through MMP-dependent and receptor-mediated cell signaling mechanisms

Figure 3-1: Assessing TIMP-1 expression along peripheral nociceptive circuit following cutaneous inflammation. 69
Figure 3-2: Cellular colocalization of TIMP-1 expression. 71
Figure 3-3: Development of inflammatory hypersensitivity in wt mice. 73
Figure 3-4: Mice lacking TIMP-1 develop thermal and mechanical hypersensitivity following cutaneous inflammation. 75
Figure 3-5: Assessment of cutaneous Timp2 and Timp4 mRNA expression following inflammation. 77
Figure 3-6: Mice lacking TIMP-1 show increased sensitivity in non-inflamed tissues. 79
Figure 3-7: Inflammation does not alter pro-inflammatory molecules in a genotype-specific manner. 81

Figure 3-8: Replacement of TIMP-1 attenuates ongoing inflammatory pain in WT mice. 83

Chapter 4: 84

Discussion and Future Directions

Figure 4-1: Calculation of delta TIMP-1 protein expression. 100

Figure 4-2: Assessment of TIMP-1 in varying levels of inflammation. 102

Figure 4-3: Mice lacking TIMP-1 have altered expression of proresolving mediators. 104

Figure 4-4: Astrocyte-derived TIMP-1 attenuates persistent inflammatory pain. 106

Figure 4-5: Blocking β1 integrin activity attenuates hypersensitivity in T1KO mice. 108

Figure 4-6: 3D surface rendering of SGCs around neurons from naive DRG using CLARITY.110

Figure 4-7: Assessment of mechanical allodynia during dose response curve of rmTIMP-1 administration. 112
List of Abbreviations

ANOVA: Analysis of Variance
BB-94: Batimastat
\( \beta_1 \) Integrin: beta 1 integrin
BFA: brefeldin A
CGRP: calcitonin gene related protein
COMT: catechol-O-methyltransferase
CDC: Center for Disease Control and Prevention
CCI: chronic constrictive injury
CIDP: chronic inflammatory demyelinating polyneuropathy
CPSP: chronic postsurgical pain
CFA: complete Freund’s adjuvant
CPP: conditional place preference
CIP: congenital insensitivity to pain
COX: cyclooxygenase enzyme
K14: cytokeratin 14
EPA: eicosapentaenoic acid
ECM: extracellular matrix
ERK: extracellular signal-regulated kinase
GPCR: G protein-coupled receptors
GWAS: genome wide association studies
GFAP: glial fibrillary acidic protein
HBSS: Hank’s balanced salt solution
IHC: immunohistochemistry
IS: inflammatory soup
IR: infrared
IL-6: Interleukin -6
IL-1β: Interleukin 1 beta
IL-10: Interleukin- 10
i.t.: intrathecal injection
LPS: lipopolysaccharide
MMP: Matrix metalloproteinases
MAPK: mitogen-activated protein kinases
NMDAR: N-methyl-D-aspartate receptor
NP: Neuropathic pain
NSAID: nonsteroidal anti-inflammatory drugs
NF-κB: nuclear factor -κB
NMR: nuclear magnetic resonance
DHA: docosahexaenoic acid
OPRM1: opioid receptor mu 1
OCT: Optimal Cutting Temperature
OA: osteoarthritis
PFA: paraformaldehyde
PWL: paw withdrawal latency
PWT: paw withdrawal threshold
PBS: phosphate buffered saline
PGE2: prostaglandin E2
PsA: psoriatic arthritis
rmTIMP-1: recombinant murine TIMP-1
RvD1: resolvin D1
RvE1: resolvin E1
RA: rheumatoid arthritis
rmTIMP-1 FL: recombinant murine Full length TIMP-1
SGC: satellite glial cells
SNPs: single nucleotide polymorphisms
SES: socioeconomic status
SC: spinal cord
SNL: spinal nerve ligation
s.c.: subcutaneous injection
SG: substantia gelatinosa
MMPI: synthetic MMP inhibitors
TTX: Tetrodotoxin
IASP: The International Association for the Study of Pain
T1KO: TIMP-1 KO
TIMP: tissue inhibitors of matrix metalloproteinase
TLR4: toll-like receptor-4
TRP: transient receptor potential ion channel
TRPV1: transient vanilloid receptor 1
T: transmission cells
TIMP-1(C): truncated C terminus peptide
TIMP-1(N): truncated N terminus peptide
TNFα: Tumor necrosis factor alpha
UV: Ultraviolet radiation
vF: von Frey
WT: wildtype
Chapter 1.

Introduction to pain

Normally, pain produced from an injury will last as long as the tissue undergoes healing and is thus, considered protective (1). However, as stated by John Bonica, the founding father of pain medicine, “in its late phases, when it (2) becomes intractable, it no longer serves a useful purpose and then becomes, through its mental and physical effects, a destructive force” (3). Pain is one of the most common reasons why patients seek medical care (4). In 2016, the Center for Disease Control and Prevention (CDC) approximated that 50 million people (20.4% of U.S. adults) experience pain on most days over the course of 6 months (5). Of those, approximately 19.6 million people (8% of U.S. adults) report having high impact chronic pain, which is defined by restricted mobility and the inability to participate in normal daily activities. This total population is greater than the total number of people diagnosed with diabetes, cancer, and heart disease combined (6). Moreover, chronic pain is associated with various medical conditions including cancer, arthritis, musculoskeletal disorders, neuropathy, and trauma (7). Despite the vast need for chronic pain management, there are no known cures or adequate treatments to alleviate chronic pain symptoms. Long-term inadequate pain relief can negatively affect an individual’s physical and mental health, posing a significant health concern for millions of people. Therefore, chronic pain should be considered as a disease entity in itself and not just a facet of other medical conditions.

Pain is a submodality of somatosensation that is defined as an unpleasant sensory, emotional, and psychological experience that alerts us in the event of actual or potential bodily harm (8, 9).

---

1 Where acute pain can last upwards of a month, pain that persists for at least 3-6+ months is clinical diagnoses as chronic pain.
In the first phase of this process, during exposure to extreme pressures and temperatures, noxious stimulation activates specialized primary sensory neurons called nociceptors. Nociceptors transduce peripheral noxious stimuli into action potentials, which is referred to as nociception, and release neuroactive molecules within the spinal cord. During the second phase, primary afferents release excitatory neurotransmitters that are received by interneurons resulting in signal propagation across the midline and eventually, received by supraspinal modulatory centers within the brain for the perception of pain (Figure 1.1).

The nociceptive circuit provides vital information regarding the status of our peripheral tissues. This status comes in two flavors, escapable pain and inescapable pain (10, 11). Escapable pain is perceived as a bodily threat and engages defensive behavioral programs that promote retraction of potentially damaged limbs to protect them while you escape from the bodily threat (11, 12). Often this pain is localized to somatic tissues that are innervated by nociceptors within in the affected tissue. Inescapable pain originates from internal sources and is associated with more passive behaviors such as, motor quiescence, hyporeactivity, decreased vigilance, reduced responsiveness also referred to as “sickness behavior” for the purpose of survival (11, 13). These internal cues provide insight to the health and function of visceral organs, the meninges, as well as the cerebral vascular tree (10). For instance, visceral pain is a hallmark of many diseases such as, inflammatory bowel disease and pancreatitis as well as less severe medical conditions such as indigestion or reproduction (14). From an evolutionary standpoint, pain is fundamental component to our wellbeing and survival. This becomes apparent in rare hereditary channelopathies, such as congenital insensitivity to pain (CIP) (15), where a mutation in the SCN9A gene results in the production of a nonfunctional alpha subunit of the sodium channel, Nav1.7. The result is a loss of function that prevents nociceptors from generating action potentials, and, consequently the
emergence of pain, despite preservation of other somatosensory functions. Therefore, these patients lack the ability to protect themselves from physical harm and often have unrealized infections, multiple wounds, broken bones, and some even engage in self-mutilation (16). Together, having a fully functional nociceptive circuit decreases an individual’s risk for repeated injuries, inadequate healing, as well as increases their lifespan (15).

I. History and Theories of Pain

For most of human history, philosophers, clinicians, and scientists, alike, have tried to explain the normal progression of pain in pursuit of identifying better treatments for alleviating pain symptoms. Some of the oldest theories attribute pain to demonic possession that causes an unpleasant experience which helped reconcile why pain exists with accidental injury, it did not, however, provide a satisfying interpretation of how pain manifests from within the body, such as occurs from disease (16). In the Treatise of Man (1664), René Descartes describes how he imagined contact with noxious heat caused a withdrawal reflex:

“The small rapidly moving particle of fire moves the skin of the affected spot causing a thin thread to be pulled. This opens a small valve in the brain [pineal gland] and through it animal spirits are sent down to the muscles which withdraw the foot. Just as by pulling at one end of a rope one makes to strike at the same instant a bell which hangs at the other end.”

This proposed mechanism suggested that the transmission of pain arises from a modality-specific “labeled-line” that directly connects the skin to the brain. Therefore, leading to the hypothesis that pain could be extinguished if the specific “labeled-line” was both, identified and then, severed (Figure 1.2) (16). For the majority of the last 300 years this concept underestimated the physiological complexity of pain. Eventually over time, clinical presentations of nerve injury

2 Excerpt from Tractatus De Homine (Treatise of Man) published by René Descartes (1596-1650) in 1664.
revealed a contradictory phenomenon that damaged or severed neurons resulted in exacerbated pain and not the alleviation of pain. Despite knowing this, in extreme cases and if pharmacological interventions do not sufficiently alleviate symptoms, patients may opt for surgical treatment to partially remove or destroy nerves however, pain surgery to this day still “suffers from a lack of evidence” that supports the use of surgery to manage pain (17). Moreover, we know from cases of limb amputation that approximately 40-80% of patients report that they still experience pain in the removed limb, referred to as phantom limb pain, following surgery (18). It was from these clinical cases of abnormal pain that challenged how we conceive of pain transmission.

Throughout the 20th century, the field of pain science progressed with the establishment of new theories that ultimately challenged existing dogma. Arguably the most influential theory was published in 1965 by Ronald Melzack and Patrick Wall. In their 1965 paper, the authors explained that surgery is an inadequate method for pain management and debunked René Descartes “labeled-line” theory by suggesting that the severance of nerves serves as a stimulus that causes nerve impulses that lead to the “Gate Control Theory of Pain” (19, 20). The theory explains that the painfulness of a stimulus is determined by the balance between different populations of afferents, involved in normal touch sensation (large fibers) and nociception (small fibers). Both populations send projections to the dorsal horn of the spinal cord and synapse onto cells in the substantia gelatinosa (SG) as well as central transmission (T) cells. Increased activity of large fibers causes presynaptic inhibition of T cells by SG cells and closes the gate to all incoming noxious input (20). However, activation of small nociceptive fibers converges onto SG cells and causes disinhibition of T cells thus, eliciting presynaptic activity and opening the gate. The gate represents a central control mechanism that preferentially allows non-noxious input, such as touch, to suppress noxious input from ascending to the brain. This theory was tested on a small cohort of patients, half of
which reported neuropathic pain, by using high frequency stimulation of the large fibers of the affected nerve to elicit pain abolition (21). Regardless of preexisting nerve trauma, patients reported transient pain relief that was explained by the selective stimulation of large fibers which closed the gate to nociception, but removal of stimulation eventually led to the gradual opening of the gate by ongoing nociceptive activity. Eventually later studies showed that input from small afferents actually enhanced, and not decreased, SG cell activity (22, 23). Moreover, persistent activation of small fibers resulted in increased spinal neuron excitability, also referred to as wind-up, which was explained by the theory as a summation of presynaptic inputs (22, 23). Eventually, wind-up was determined to result from the release of excitatory neurotransmitters (i.e. glutamate) within the spinal cord, that could prolong synaptic transmission, and which could, be prevented with N-methyl-D-aspartate receptor (NMDA) antagonists (24-26). Within the spinal cord, windup allows enhanced processing of nociceptive input that can eventually lead to central sensitization, a separate process that causes reduced thresholds for firing action potentials (27, 28). Over time as more observations were made on afferent physiology, debates on whether gate control theory is an accurate interpretation of actual physiology led to research on the 1) sensitization of both peripheral and central nervous systems and 2) the plasticity of the nervous system, both of which are thought to contribute to chronic pain. Overall, it is agreed that Melzack and Wall’s theory formulated one of the first biological mechanisms of pain that suggested small and large fibers engage different spinal effects however, the effect of small fiber nociceptive input turned out to be vastly different than was proposed. If it was not for the re-evaluation of pain mechanisms, impart through the deconstruction of Melzack and Wall’s theory, the field would not have grown to incorporate interesting studies on pain genetics as well as environmental and affective components
of pain. The questions now become, what are the underlying mechanisms that cause the
development of pathological pain and how do we prevent chronic pain from occurring?

I. Classification of Pain

Of all physician visits, 15-20% are accounted for by patients seeking medical care for pain
symptoms (29, 30). The assessment of pain and other symptoms are essential to medical diagnoses
as well as to guide treatment. The International Association for the Study of Pain (IASP), which
focuses on understanding the biological basis of chronic pain in order to improve current
treatments, recognizes three common neurobiological etiologies of chronic pain: neuropathic,
nociceptive, and nociplastic pain (2, 31). Determining the class of pain requires several parameters
be taken into account: physiology (neuropathic, nociceptive, and inflammatory), intensity (using
a 0-10 numeric scale; refer to Figure 1.3), duration (acute or chronic), tissue affected (skin, muscle,
viscera, joints), and medical history (cancer, surgery, migraine). However, classifying pain is not
always straight forward. Many pathological conditions are composites of more than one
mechanism of pain within one patient, for example, following nerve injury mechanisms of
inflammatory pain may be superimposed on mechanisms of neuropathic pain (32).

Neuropathic pain (NP) is caused by lesion or disease of the somatosensory nervous system.
Acute and chronic NP is commonly associated with stroke, diabetes, herpes zoster infection, and
cancer (33-35). Diagnosing NP often requires access to a patient’s medical history which may
provide information on previous surgeries, medical conditions, or infections that may provide
evidence for nerve trauma (33-35). More definitive diagnoses may require one or more of the
following, imaging, biopsy, neurophysiological and/or laboratory tests to determine if nerve

---

3 IASP is comprised of scientists, clinicians, health-care providers, and policymakers that work together to support
pain research with the goal to improve pain relief worldwide. IASP, Classification of chronic pain
descriptions of chronic pain syndromes and definitions of pain terms. (IASP Press, Seattle, Washington ed. second
1986).
damage is centrally or peripherally located (36). Symptoms of NP are burning sensation and/or irregular bouts of “electric shock-like” shooting sensation as well as paresthesias\(^4\) and dysesthesias\(^5\) that are often reported as crawling, numbness, itching, and tingling on the body (37). These symptoms may be spontaneous and/or evoked-pain by everyday stimuli such as light touch, pressure of clothing, wind, sitting, or temperature. In addition, pain may be greater than expected in response to a noxious stimuli (hyperalgesia) or results from normally innocuous stimuli (allodynia).

Approximately 50-80% of chronic pain conditions arise in the absence of nerve trauma (38). The most common type of this pain originates from the activity of neurons following actual or potential tissue damage, referred to as nociceptive pain (2). This type of pain commonly arises from chemical, mechanical, or thermal injuries of peripheral afferents associated with arthritis, surgery, temporomandibular joint disorder, and sports injuries (38-40). Symptoms are typically reported as being “sharp” and localized to a specific bodily region (40). However, the majority of individuals diagnosed with chronic low back pain, about 90%, also present symptoms similar to NP (allodynia and dysesthesia) although physically they do not show evidence of nerve trauma (41). Suggesting that chronic inflammation associated with low back pain may lead to maladaptive plasticity of the nervous system.

Most types of pain are idiopathic and do not originate from a particular known injury or anatomical defect (38). For instance, fibromyalgia, complex regional pain syndrome, and visceral pain disorders are commonly associated with wide-spread pain without any obvious pathophysiological determinant (31). Individuals in this separate class were often misunderstood.
by clinicians and their symptoms were reported vaguely as “pathological,” “centralized,” or “dysfunctional”. In an attempt to improve consistency of terminology and provide more defined understanding of this type of decentralized pain, the IASP recently established a new class, called nociplastic pain which arises from altered nociceptive function (2, 31, 42). The IASP hopes the effort to provide these definitions will help guide pain management strategies for individuals suffering from more than one type of pain (31).

II. Risk Factors

Most diseases are a composite of several factors, and chronic pain is not an exception. Factors that may contribute to individualized differences in pain thresholds may result from biological influences such as inherited genetic susceptibility. Advances within the field of genetics in conjunction with ‘genome wide association studies’ (GWAS), ‘linkage analysis’, and twin studies have revealed several ‘pain genes’ (43). Some of the most commonly associated genes are ion channels, broadly categorized as voltage gated and ligand gated. Examples include sodium ion channels (SCN9a and SCN10a), potassium ion channels (KCNS1), and calcium ion channels (CACNA2D3 and CACNG2). Other genes associated with altered pain thresholds include IL-6, ADRB2, H2TRA, COMT, OPRM1, TRPV1, and GCH1 (43, 44). Individual variations in pain thresholds may also result from epigenetic mechanisms or single nucleotide polymorphisms (SNPs). For instance, chronic postsurgical pain (CPSP) intensity has been associated with SNPs within the catechol-O-methyltransferase (COMT) and opioid receptor mu 1 (OPRM1) genes (45). Individual differences in pain thresholds cannot be entirely explained by genetics. For instance, two individuals that undergo a similar surgical procedure may report contrasting intensities of

---

6 A ‘pain gene’ is a gene that has one or more polymorphisms and affects the expression or the functioning of its protein product in a way that affects pain response (43). S. James, Human pain and genetics: some basics. Br J Pain 7, 171-178 (2013).
post-operative pain based on their age, sex, or cultural background. The 2016 household health survey of the civilian noninstitutionalized U.S. population revealed several demographic factors that also increase an individual risk for developing chronic pain: sex (female), age (>65 years), race/ethnicity, as well as socioeconomic status (unemployed, impoverished, access to public health insurance, and rural residence) (5).

**Gender/ Sex**

Population based studies have consistently shown that pain is more commonly reported among women compared to men. Women are more likely to report daily and chronic widespread pain relative to their male counterparts. This is a consistent finding across geographical regions (46). In addition, we know from clinical studies that women are overrepresented in clinical populations diagnosed with fibromyalgia, migraine, irritable bowel syndrome, temporomandibular disorders, as well as interstitial cystitis (47). Extensive evidence suggests that women have increased pain sensitivity and risk for developing clinical pain however, understanding why women experience pain more often than men and the underlying physiological has been a challenge for the field (48). In part this is due to sex bias in experimental studies that primarily include male subjects but also, could be the result of psychosocial and cultural factors that impact sex differences in pain perceptions and coping strategies that are difficult to test experimentally (49).

Although, few papers have attempted to investigate this disparity, current studies suggest that sex hormones and immune cells contribute to sex specific mechanisms in pain (48-51). The immune system plays a major component in the induction and maintenance of pain. Moreover, sex hormones interact with their receptors expressed on immune cells and can determine cellular behaviors (52). Female hormones in particular are reported to have a potent effect on immune cell
production and function, referred to as immune dimorphism (53). In a paper by Sorge and Colleagues (50), they showed that between male and female mice, macrophages and microglia or T cells, respectively, regulate hypersensitivity following peripheral nerve injury. Essentially, this means that between males and females different immune mechanisms contribute to pathological pain. Moreover, because majority of pain research has been conducted in male mice, these immune-driven sex differences may explain the discrepancies in pain management outcomes between male and female populations. Understanding these underlying sex differences may help direct sex-specific pain treatments with regards to managing chronic pain as well as other diseases which may be impacted by sex hormones and the immune system.

**Age**

A famous study conducted by Crook in the 1980’s was first to demonstrate that there is increasing prevalence of pain in the aging population (54). Reasons for this age-dependent increase may be from pathologic load which may contribute to comorbidities as well as pain (55). For instance, adults become more hyperglycemic with age which may contribute to diabetic neuropathy and result in neuropathic pain (39). Time, by way of aging, may also sustain a disease and allow it to progress which can cause long-term effects to affect the function of the nervous system.

**Race / Ethnicity**

Studies that assess large groups of people provide insight to the distribution and prevalence of chronic pain to ultimately improve methods for limiting pain severity and minimizing disability that may differ among populations. Population studies have shown that race7 and ethnicity8 can

---

7 Typically, race is determined by populations of people with similar ancestral heritage and biological disposition.
8 Ethnicity is defined by behavioral, cultural, biological, as well as physical characteristics shared by individuals from a particular nation.
contribute to vast differences in the frequency and severity of chronic pain conditions. For instance, McNeilly and Zeichner (1989), showed that African-American participants, compared to Caucasian participants, had higher heart rates and blood pressure during intravenous catheterization, suggesting that hypertension may increase an individual’s reactivity to pain (56). In a recent comprehensive review of experimental pain sensitivity, ethnic/racial minorities were more common to report higher pain sensitivity and lower pain tolerance compared to non-white Hispanics (57). These characteristics may be accounted for by intrinsic variations in genetics that may have resulted from early human migration and the diversification of our species as we settled in various regions of the planet. Or, pain severity and duration associated with underrepresented populations may also be the result of chronic stress associated with discrimination, racism, unfair treatment, as well as access to healthcare, and distrust of the medical establishment (58). Biologically, exposure to high amounts of stress can increase sympathetic activation and psychological fatigue.

**Socioeconomic status**

Socioeconomic status (SES) is often used to understand the relationship between non-biological factors and the morbidity associated with chronic pain. Socioeconomic status is determined by a summary score based on income, occupational status, and education level. Educational level is most often used to determine socioeconomic disadvantage because it’s a relatively easy parameter to obtain, as opposed to income level, is usually constant after early adulthood, and is not affected by health status (59). Poleshuck and Green describe a few reasons for this, higher education may improve critical thinking skills to allow individuals to more

9 A systematic review of human studies (2001-2016) that experimentally assessed pain sensitivity to thermal, cold, pressure, ischemic, mechanical cutaneous, electrical, and chemical modalities.
effectively navigate healthcare systems as well as establish effective interactions with healthcare providers and staff (59). Individuals with higher levels of education may have more knowledge of practical skills that can prevent them from engaging in risky behaviors. Nonetheless, education level and economic return of education varies greatly across social groups based on gender, race, ethnicity. Consequently, understanding the relationships between socioeconomic factors could provide important information for clinicians and pain scientists attempting to improve treatment for individuals that are socioeconomically disadvantaged.

III. Managing pain

Over the course of human history, pain treatments have both vastly changed and stayed the same. In ancient times, natural remedies such as herbane, mandrake10, and Solanaceae11 were commonly used to treat pain. Whereas, salicylates12, applications of heat and cold, and electrical stimulation13 that have been around for centuries are still commonly used to treat pain. In a 2006 study of ambulatory visits, it was revealed that nonsteroidal anti-inflammatory drugs (NSAIDs) (i.e. acetylsalicylic acid and naproxen) were the most commonly prescribed analgesics, with approx. 99.1 million mentions14 a year, followed by narcotic analgesics and nonnarcotic analgesics (4, 60). NSAIDs are primarily used to treat pain, lower fever, and reduce inflammation associated with arthritis, bursitis and tendonitis. Over-the-counter NSAIDs antagonize the synthesis of proinflammatory molecules downstream of cyclooxygenase (COX) enzymes, COX-1 and COX-2. Specific COX-2 inhibitors may be prescribed to directly inhibit COX-2 which is involved in the

10 A mandrake is taken from the root of Mediterranean plants from the genus Mandragora.
11 Solanaceae, also referred to as nightshades, are a family of flowering plants that constitute 3,000 species.
12 The willow tree was also harvested for its oils which contain salicylates. Salicylates are derived from salicylic acid and have anti-inflammatory properties and are still used to treat pain and fever.
13 In Egypt, Greece, and Rome, electroichthyologic treatment (e.g. electricity) was used to manage joint stiffness and headaches. This method required placing the afflicted extremity in a bowl with live electric fish or torpedo fish (i.e. electric ray).
14 Drug mention refers to a physician entry of a pharmaceutical agent on a Patient’s Record Form (PRF).
inflammatory process whereas COX-1 is primarily involved in homeostatic functions such as protecting the stomach lining from acids and digestive chemicals.

Narcotics are the second most commonly prescribed analgesics (4). This class of psychoactive drug is often prescribed for short-term relief of intense pain, such as post-surgical pain. Opioids are more effective for acute pain and long-term use is actually known to exacerbate pain. In Egypt (1500-1300 B.C.; XVIII. Dynasty) the poppy plant (i.e. Papaver Somniferum) was commonly cultivated for its naturally occurring opiates (plant alkaloids) to treat toothaches (16). Today, the pharmaceutical industry capitalizes on several synthetic analogs of opiates (i.e. opioids), classified as schedule II drugs, for various medical uses. Opioids bind and activate opioid receptors expressed by neurons to prevent nociception from reaching the brain. The opioid receptor family consists of four (μ, δ, κ, and opioid receptor like-1) G protein-coupled receptors (GPCRs) that are expressed throughout the peripheral and central nervous systems (61, 62). However, most opioids activate the μ receptor at 3 potential sites to produce potent analgesia, binding to free-nerve endings of sensory neurons in peripheral tissue, inhibiting neural transmission within the spinal cord, or inhibiting descending cortical pathways (63). Activation of any of the four opioid receptors causes the dissociation of Ga and Gβγ subunits and decreases calcium conductance, which is believed to impart, to decrease voltage activation of the opioid channel pore opening (64, 65). With increasing knowledge about the side-effects of opioids including tolerance and addiction, opioid use led to greater problems than just relieving pain for millions of people, in part due to the rising rate of opioid prescriptions for various medical ailments and the devastating number of overdose-induced deaths caused by opioid abuse (66). Therefore, treating pain with opioids has resulted in a socioeconomic crisis, that must be resolved. The importance of discovering effective non-opioid

---

15 Schedule II drugs are defined as drugs that have severe psychological or physical dependence and have a high potential for abuse.
pain treatments for chronic conditions is paramount to decreasing the dependence on opioids as well as improve the future of chronic pain treatment.

IV. Modeling Inflammatory Pain

Animal models have been used since the early 19th century to mimic specific characteristics of clinical conditions in human populations (67). Inflammation plays an integral role in the pathogenesis of most types of pain and is therefore a biological risk factor for developing chronic pain. Often, preclinical animal models of inflammatory pain are used to investigate mechanisms of persistent pain because they allow researchers to assess the contribution of various factors, involved in tissue damage, to the induction and maintenance of nociception as well as the emergence of pain-like behavior. Thus, understanding and interfering with how inflammation contributes to pathological pain is integral for disrupting the abnormal transition from acute pain to chronic pain.

Typically, inflammation is induced with an injection of an inflammatory agent or irritant to produce tissue injury and hyperalgesia in skin, muscle, joints, or visceral organs (68, 69). Common irritants include complete Freund’s adjuvant (CFA), inflammatory cytokines, carrageenan, zymosan, mustard oil, formalin, capsaicin, Melittin (bee venom), hypertonic saline, lipopolysaccharide (LPS). Other models used to induce inflammation and subsequent hyperalgesia include burning, freezing, or Ultraviolet radiation (UV) (refer to Table 1.1)(70). Depending on the site of injection, cutaneous versus muscle or joint injections, may cause different behavioral outcomes of the inflammatory agent (67, 71). For instance, intravenous injection of CFA into the base of a rat’s tail causes polyarthritis and persistent pain whereas, subcutaneous injection of CFA produces a localized response that only affects the injected limb (72, 73). Therefore, tissue-

---

16 Polyarthritis is any type of arthritis that affects 5 or more joints simultaneously.
specific differences may account for potential discrepancies between behavioral outcomes of models as well as their mechanisms. Although no one model can simulate all the characteristics of chronic pain, they can provide important insights to mechanisms that can led to better pain management therapies (70).

**Carrageenan Model**

Carrageenan is a common model of local inflammatory pain. Following subcutaneous injection, carrageenan produces hyperalgesia within 1 hour and is typically resolved by 24 hours (71). However, injection of carrageenan into the paw, muscle, or joint can produce acute inflammation that develops into chronic inflammation 2 weeks following injection. Within the model, hyperalgesia occurs in two phases, the first phase occurs within the first 30 minutes and the second lasts for about three hours post-injection. Carrageenan models primary and secondary hyperalgesia which is indicative of human sprains, strains, and myositis.

**Formalin Model**

The formalin model is utilized to study complex response patterns. Within approximately one hour following subcutaneous injection of formalin into the footpad, two-phases of nocifensive behavior can be observed (74, 75). Within the first 5-15 minutes post-injection, animals typically avoid weight-bearing on the affected limb, presumably due to persistent inflammatory stimulation of nociceptive free-nerve endings produced by the irritant. During the second phase, 15-60 minutes post-injection, animals present a variety of pain behaviors such as flinching as well as guarding, flicking, and shaking the afflicted limb (76). The mechanism of these second-phase behaviors is generally agreed to result from ongoing peripheral activity during the first phase and increased excitability of spinal neurons during the second phase (77, 78).

**Capsaicin Model**
Capsaicin, an extract of cayenne pepper, is used to model neurogenic inflammation and hyperalgesia (79). Intradermal injections of capsaicin activate transient vanilloid receptor 1 (TRPV1) on nociceptors and results in dose-dependent changes in threshold local to the site of injection (primary hypersensitivity) as well as produces a flare reaction and subsequent hyperalgesia to light touch (alldynia) in secondary zones. In primate studies, capsaicin injections have been used to investigate peripheral and spinal mechanisms of neurogenic inflammation (79, 80). In murine studies, capsaicin has been used to mechanisms of behavioral responses to thermal and mechanical stimulation (68, 81). Capsaicin causes transient increases in nocifensive responses to heat and to mechanical stimulation that last for a few hours post-injection (70).

**Complete Freund’s Adjuvant Model**

CFA is the heat-inactivated and dried form of *Mycobacterium butyricum* in adjuvant (82), and is commonly used to induce inflammatory responses in experimental models (71). The CFA model produces a dose-dependent inflammatory response (30-200ug *Mycobacterium butyricum*) that activates local immune cells, dendritic cells and macrophages, within minutes to hours following injection and peak inflammation usually occurs with 5-8 hours post-injection (71, 72). The resultant infiltration of immune cells to the site of injection causes paw edema to develop within 24 hours post-injection. Although, subcutaneous injection of emulsified CFA (1:1; 50 ug) into the hindpaw typically results in hyperalgesia within a few hours, and is resolved by 1-2 weeks following injection, this model does not interfere with normal locomotor activity, grooming, or cause weight loss (83). CFA-induced hyperalgesia and alldynia in preclinical models are consistent with those seen in to humans that received unintentional injections of CFA (84). CFA causes a more persistent inflammatory phenotype indicative of rheumatoid arthritis and tendonitis (67).
V. Assessing hypersensitivity

Stimulation of inflamed paw typically results in decreased response thresholds (i.e. increased sensitivity) \((70, 85)\) (Refer to Figure 1.4). To assess this, paw withdrawal latency (PWL) or paw withdrawal threshold (PWT) are often used to assess hyperalgesia following injection of inflammatory agent or vehicle control. During exposure to noxious mechanical or thermal (or radiant heat) stimulation animals withdraw their limb reflexively to the stimuli as well as exhibit more complex behaviors such as paw licking and limb guarding \((68)\). The presence of pain during inflammation is inferred by increased responding to a noxious stimulus (hyperalgesia) or nocifensive responding to an innocuous stimulus, that normally is not perceived as painful (allodynia). In humans, cutaneous hypersensitivity is often reported following nerve injury, surgical procedures, and inflammatory conditions. Reflexive testing to assess thermal and mechanical sensitivity is often used to understand underlying mechanisms of cutaneous hyperalgesia and allodynia. Assays such as the flick test\(^{17}\), hot plate test\(^{18}\), and/or Hargreaves apparatus\(^{19}\) \((67, 68)\) are used to measure heat sensitivity whereas, von Frey filaments are often used to assess mechanical hypersensitivity as well as allodynia\(^{20}\).

**Reflexive/Evoked behavior**

Over the past century, reflexive behaviors and withdrawal thresholds have helped pain scientists to identify various mechanisms of pain. These behavioral assessments are used to study the underlying mechanisms of allodynia and hyperalgesia by evoking behavioral responses following the application of heat, cold, mechanical force, or electrical stimulation. One of the merits of reflexive tests is the ability to apply specific intensities of different sensory modalities

---

\(^{17}\) Heat stimulus is applied to tail and latency to remove tail is recorded. Tail flick is modulated by spinal reflex.

\(^{18}\) Heat stimulus is applied to hind paws and latency to remove paw or licking paw is recorded.

\(^{19}\) Developed to deliver more controlled and localized heat stimulus. Latency to remove paw is measured.

\(^{20}\) Thermal allodynia is not typically reported in human populations.
and to record leftward shifts (Figure 1.4) in the functional response to noxious or innocuous stimuli. These studies helped identify important neurotransmitters, protein receptors, intracellular signaling molecules, genes, pharmacological and nonpharmacological therapies.

Physiologically, evoked behavioral tests activate peripheral nociceptors at the site of testing (i.e. at the source of stimulation) and triggers a localized stereotypic motor response. Behavioral outcomes from these tests can occur in the absence of supraspinal activation. However ascending noxious neurotransmission can be modulated by descending efferents. In addition, reflexive tests can be used to assess sensitivity at the site of insult or outside the region of insult. The location closest to the source of insult (i.e. injury or injection of an irritant) is called primary hyperalgesia and is accounted for by increased nociceptive signaling by primary afferents (also referred to as sensitization) (85). In comparison, sensitivity assessed outside the region of insult is referred to as secondary sensitivity. Secondary sensitivity is however presented as persistence of pain without the presence of injury or direct tissue damage. In human chronic pain conditions, patients often report pain in both primary and secondary zones. Pain in secondary zones is also reported to spread to additional regions across the body. As a result, primary and secondary hyperalgesia have different underlying mechanisms and understanding the location of the original insult or injury is important for the interpretation of data.

Nonreflexive behaviors

Nonreflexive, or spontaneous, behaviors are commonly used to assess additional features of pain that cannot be captured with reflexive testing. This is important because spontaneous pain is a hallmark of centralized pain. Non-reflexive tests include the evaluation of paw licking and paw guarding or weight bearing following administration of an irritant or injury. In addition, behavioral paradigms that include self-administration of analgesics, such as NSAIDs, opioids, or
cannabinoid agonists, are used to probe pain reduction or pain producing effects of various drugs using animal models. Pain relief, similar to humans, involves the reward pathway. To capture whether an animal is in pain, paradigms that assess preference for analgesics (i.e. conditional place preference; CPP) are used to determine the presence of recurrent pain (86). CPP is assessed using a 3-chamber box which animals are placed into and following an injection of an irritant, one of the chambers is paired with the administration of an analgesic and chamber preference in measured. In theory, the animal would prefer the chamber paired with the analgesic if its effective against the irritant. This is a powerful tool to essentially “ask” an animal subject to reveal whether they are in pain based on their motivation to seek pain relief.

VI. Summary

Although pain is a highly individual experience and is affected by many factors, understanding the underlying biology of pain conditions will provide insight to potential targets that may improve the quality of life of individuals living with chronic pain. Moreover, the vast differences across human pain conditions and preclinical animal models, suggest that finding underlying commonalities between models may have more potential for therapeutic targets.

Inflammation is an integral physiological component of our bodies defense machinery and is common factor across many diseases and pain conditions. Therefore, understanding the underlying mechanisms of inflammatory pain and the molecular mediators that regulate the inflammatory process may provide ample opportunity to prevent the abnormal transition from acute to chronic pain.
AREAS OF PAIN PROCESSING

Cortex

Amygdala

Cingulate cortex

Third ventricle

Hypothalamus

Somatosensory cortex

Thalamus

Insular cortex

Periaqueductal gray

Midbrain

Rostral ventral medulla

Spinal Cord

Dorsal root ganglion

Nociceptive C fiber

Dorsal horn

Gray matter

Supraspinal Modulation

Peripheral Modulation

Pain Source

Spinal Modulation
Figure 1-1: Chart representation of four main anatomical areas of pain modulation. Starting with the peripheral nociception of pain source, spinal modulation, and supraspinal modulation for the perception of pain (87).
Figure 1-2: Simplified representation of the origin of pain published by René Descartes. Published in the Tractatus De Homine (Treatise of Man) in 1677 (88), this schematic is the first known explanation of how pain is transmitted from a single channel from the skin to the brain (16).
## COMPARATIVE PAIN SCALE CHART (Pain Assessment Tool)

<table>
<thead>
<tr>
<th>No Pain</th>
<th>Minor Pain</th>
<th>Moderate Pain</th>
<th>Severe Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain Free</td>
<td>Feeling perfectly</td>
<td>Nagging, annoying, but</td>
<td>Interferes significantly with</td>
</tr>
<tr>
<td></td>
<td>normal</td>
<td>doesn't interfere</td>
<td>daily living activities. Requires</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with most daily living</td>
<td>lifestyle changes but patient</td>
</tr>
<tr>
<td></td>
<td></td>
<td>activities. Patient able</td>
<td>remains independent. Patient</td>
</tr>
<tr>
<td></td>
<td></td>
<td>to adapt to pain</td>
<td>unable to adapt pain.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>psychologically and</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>with medication or</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>devices such as</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cushions.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1-3: The Stanford Pain Scale is an updated assessment tool. This tool ranks pain on a numeric 0-10 scale. This clinical tool is primarily unidimensional and focuses on pain intensity (89-91).
Figure 1-4: Graph depicting shift in behavioral responding. Following injury or inflammation, behavioral responsiveness is shifted leftward indicating less stimulus intensity is required to elicit a response (hyperalgesia) or the gain in responsiveness to normally innocuous stimuli can occur (allodynia) (92).
Table 1  Comparison of inflammatory models of pain and hyperalgesia

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Hyperalgesia</th>
<th>Allodynia</th>
<th>Time of onset</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA*</td>
<td>Yes</td>
<td>Yes</td>
<td>2-6 hr</td>
<td>1-2 wk</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>Yes</td>
<td>Yes</td>
<td>1 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>Mustard oil</td>
<td>Yes</td>
<td>Yes</td>
<td>5 min</td>
<td>&lt; 1 hr</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>Yes</td>
<td>Yes</td>
<td>1 min</td>
<td>&lt; 1 hr</td>
</tr>
<tr>
<td>Formalin</td>
<td>NA*</td>
<td>NA</td>
<td>&lt; 1 min</td>
<td>5-10 min</td>
</tr>
<tr>
<td>Phase I</td>
<td>NA</td>
<td>NA</td>
<td>10 min</td>
<td>1 hr</td>
</tr>
<tr>
<td>Phase II</td>
<td>NA</td>
<td>NA</td>
<td>30 min</td>
<td>24 hr</td>
</tr>
<tr>
<td>Zymosan</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CFA, complete Freund's adjuvant; NA, not applicable.
Table 1-1: **Summary table of inflammatory models of pain.** Depending on the inflammatory irritant, the temporal development of hyperalgesia and/or allodynia can vary (70).
Chapter 2:

The Extracellular Environment

Managing chronic pain conditions often requires balancing effective analgesia with associated risks of analgesic therapies. For instance, life-long pain conditions such as osteoarthritis (OA) and rheumatoid arthritis (RA) encompass inflammatory and neuropathic pain mechanisms that must be accounted for in pain treatment however, commonly prescribed NSAIDs and opioids are not conducive to long-term use because of their increased risk for developing adverse side-effects such as, stomach pain and bleeding, heart attack, tolerance and addiction (93-99). The lack of effective therapies that are not associated with substantial risk have created barriers in pain management and violates the fundamental human right to effective analgesia (100-102). Therefore, discovering novel treatments that improve patient safety and minimalize side-effects are both necessary and crucial for the future of medicine.

I. Inflammation and pain

Inflammation is a physiological mechanism for isolating determinants of tissue damage, ensuring tissue regeneration, and restoring normal tissue function. Inflammation is typically defined by five cardinal symptoms of which one is pain. Normally, when inflammation resolves and tissues return to homeostasis, pain also resolves. Failure to eliminate a determinant of tissue damage or immune response can prolong both, inflammation and pain. Over time, persistent activation of the nociceptive pathway can contribute to the development of pathological pain. However, the relationship between inflammation and tissue damage is convoluted. In some conditions, the amount of inflammation associated with tissue damage or disease is correlated with

21 The five cardinal symptoms of inflammation include rubor (redness), calor (increased heat), tumor (swelling), function laesa (loss of function), and dolor (pain).
pain intensity (34, 60). In an epidemiological study of arthritic knee patients, it was shown that levels of self-reported pain were proportional to disease severity, as determined by radiography (103, 104). This study suggested that identifying molecules involved in the regulation of inflammation may provide therapeutic targets that can attenuate arthritic pain. However, this is not the rule. A study that compared pain ratings and functional scores of people that underwent a typical surgical intervention with those that received a sham surgery, revealed that regardless of surgical intervention, similar pain ratings were reported in both groups (105). Therefore, severity of tissue damage is not necessarily indicative of pain severity but, understanding which molecules are involved in the development of pain during inflammation may provide insight to targets that can interfere with the abnormal transition to chronic pain (38, 60, 106-109).

Cytokines

Exposure to extreme pressure or temperatures causes the release of algogenic molecules, such as cytokines and growth factors, aid in both repairing the damaged tissue as well as protecting the body from further damage (85). The first evidence that cytokines were important for the development of inflammatory hyperalgesia came from studies that directly administered intraplanar Interleukin-1 beta (IL-1\(\beta\)) or Tumor necrosis factor alpha (TNF\(\alpha\)) into the hindpaw of the rat (110). Cytokines can bind to their respective receptors on nociceptors and can increase their excitability by activating of Tetrodotoxin (TTX)-resistant sodium channels, non-selective cation channels, or by initiating intracellular signaling cascades such as, mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-\(\kappa\)B pathways (Figure 2.1)(111, 112). Studying the mechanism of IL-1\(\beta\), both in vitro and in vivo model systems, revealed that IL-1\(\beta\) is a fast-acting neuroactive molecule that can alter heat evoked release of calcitonin gene related protein (CGRP), which is released by nociceptors and is involved in neuroinflammation (108, 113).
Clinically, the advent of cytokine-targeted therapies has drastically improved the management of chronic inflammatory diseases. Specifically, Etanercept, a TNFα blocker, revolutionized the treatment of RA and psoriatic arthritis (PsA) (114). Rodent studies show that Etanercept ameliorates inflammation and pain associated with arthritic flares and bone metastasis (115, 116). Subsequently, clinical studies have shown consistent analgesic properties of Etanercept in populations with head and neck pain associated with intervertebral disc disease (117) as well as metastatic bone-cancer (115, 118). All this being said, TNFα inhibition also does not cure pain and is not an effective treatment for everyone. One study showed that baseline pain severity was a predictive factor for discontinuing TNFα-inhibitors within the first year, which suggests that elevated levels of initial pain may decrease long term effectiveness of the treatment (119). Likewise, TNF blockers are associated with serious side effects such as central and peripheral demyelination (120). Clinical trials of TNFα-inhibitors for the treatment of multiple sclerosis revealed that Etanercept actually worsened disease by causing central lesions and increasing the occurrences of relapses (121). Additional studies on the role of TNFα in the nervous system gave insight to the multiple functions of TNFα that are, in the grand scheme of inflammation, essential and at certain levels, can also be protective (122). Therefore, shifting the “conventional dichotomous” convention of perceiving the effects of cytokines as beneficial and deleterious to essential and desirable. Rather than suppressing the immune system, reestablishing appropriate levels or the balance between algogenic cytokines and antinociceptive molecules may be an effective strategy to achieve tissue homeostasis and resolve pain associated with inflammation (123).

Matrix Metalloproteinases
Matrix metalloproteinases (MMPs) are a class of secreted and membrane-bound zinc-dependent proteinases with known roles in tissue remodeling, inflammation, and pain. Currently, there are more than twenty MMPs identified in mammals of which are subdivided into 3 classes: collagenases, stromelysins, and gelatinases. These enzymes are secreted as zymogens and require proteolytic cleavage of a pro-peptide domain at the N-terminus to become active degraders of the extracellular matrix (ECM). Although primarily known as bulk degraders of ECM proteins, MMPs also interact with various immune-related substrates that regulate several inflammatory signaling cascades. Normally, MMP activity is spatially and temporally controlled through interactions with α2-macroglobulin, in plasma, or tissue inhibitors of matrix metalloproteinases (TIMPs). However, during disease conditions, dysregulated MMP activity can contribute to tumor development and metastasis, blood brain barrier leakage, and neuroinflammation associated with neuropathic pain, cancer pain, chronic inflammatory demyelinating polyneuropathy (CIDP), non-systemic vasculitic neuropathy, visceral pain, and post-operative pain. Identifying approaches to regulate MMP activity may provide a novel therapy for attenuating chronic pain.

Of the 3 subclasses of MMPs, the gelatinase family consists of two of the most studied MMP proteins, MMP-2 and MMP-9, which have known roles in neuropathic pain. Whereas, MMP-2 is constitutively expressed in central nervous system tissues, MMP-9 is primarily induced by TNFα and IL-1β in peripheral and central tissues. While MMP-9 was previously shown to contribute to the induction of neuropathic pain MMP-2 was shown to facilitate maintenance of pain. Both phases were associated with MMP-driven IL-1β cleavage and

---

22 MMPs are members of the metzincin family of proteases.
23 The ECM is a dynamic network of macromolecules (collagens, fibronectin, laminin and proteoglycans) that is important for maintaining issue integrity and function.
glial activation in the spinal cord which could be prevented with pharmacological inhibition of glial cells with the administration of minocycline and paeoniflorin (136, 137).

In humans, synthetic MMP inhibitors (MMPIs) such as Batimastat (BB-94) and marimastat (BB-2516) are used to treat arthritis, atherosclerosis, periodontitis, and cancer (126). However, despite the impending success demonstrated by preclinical animal models, MMPIs also caused inflammation, skeletal muscle joint stiffness, cancer, and pain therefore, demonstrating their inappropriateness for pain management (138, 139). Recent studies suggest that endogenous inhibitors of MMPs may provide an alternative approach to regulating MMP activity as well as other mechanisms of inflammatory and neuropathic pain (140, 141).

II. Tissue inhibitors of matrix metalloproteinases

Tissue inhibitors of matrix metalloproteinases are key inhibitors of MMPs in tissues. The TIMP family consists of four conserved proteins (TIMP-1, -2, -3, -4). Their expression in tissues and different cell types are controlled to balance MMP-dependent degradation of extracellular matrix components. All four TIMPs temporally and spatially regulate the activity of all MMPs through non-covalent 1:1 stoichiometric interaction with active MMPs (142, 143). Of the four TIMPs, TIMP-1 and TIMP-2 have been previously shown to play roles in pain processing (135, 144). Specifically, administration of recombinant TIMP-1 1 day following spinal nerve ligation (SNL) attenuated the onset of mechanical allodynia but did not if administered 10 days following SNL. In comparison, administration of recombinant TIMP-2 10 days following SNL reversed persistent mechanical allodynia by suppressing IL-1β cleavage and activation of the MAPK/extracellular signal-regulated kinase (ERK) pathway in spinal astrocytes (135). The results of this paper suggest that TIMP-1 during the onset of tissue damage may be important for gating the emergence of neuropathic pain but how TIMP-1 produced analgesia or antinociception remained
unknown. Remaining questions include whether the of TIMP-1 in the development of neuropathic pain can be broadly applied to the induction of inflammatory pain. If so, does TIMP-1 function as a protective signaling molecule during the synthesis of pain following tissue damage and what occurs when it is absent? Third, what are the functions of TIMP-1 signaling during inflammation? My thesis focused on understanding how TIMP-1 delays the onset of mechanical allodynia by answering these questions.

**Tissue inhibitor of matrix metalloproteinase-1**

TIMP-1 is a relatively small glycoprotein comprised of about 184 amino acid residues (25-31 kDa) (145). Crystallographic and nuclear magnetic resonance (NMR) studies show that all TIMP proteins have 12 conserved cysteine residues that form 6 disulfide bonds that fold the protein into N- and C- subdomains (Refer to Figure 2.2) (146, 147). The N terminus consists of three highly conserved disulfide bonds, of which the first cysteine residue, Cys1, is vital for the displacement of the catalytic Zinc2+ ion from the active cleft of the MMP molecule (142, 146, 148, 149). TIMP-1 is the broadest regulator of MMP activity, inhibiting (or antagonizing) about 14 MMPs (143, 148, 150, 151).

For many years following the initial NMR and crystallography studies, the physiologic function of the small C-terminus (6-8 kDa) remained a mystery. However, studies on the role of TIMP-1 in the tumor invasion and malignancies led to mounting evidence that TIMPs could facilitate trophic functions through interactions with growth factor receptors (152) and cell adhesion molecules (153-156). Eventually, mutagenic studies determined that two N-terminal residues (His7Ala and Gln9Ala) effectively facilitate MMP inhibition without affecting its trophic function as previously shown (145, 157). This led to the hypothesis that TIMP-1 facilitates growth

\[ \text{The MMP inhibitory function of TIMP proteins is conducted by the highly conserved VIRAK sequence (Cys1-thr2-cys3-val4) between amino acids 18-22 on the N terminus.} \]
promoting functions through the C terminus domain although a cell-surface binding protein had yet to be discovered. Jung and colleagues, however, determined that the trophic functions of TIMP-1 were similar to the downstream signaling effects of tetraspanin receptors and their complexes with cell adhesion molecules (147, 158-161). Specifically, using a breast epithelial MCF10A cell line this group showed that TIMP-1 interacts with tetraspanin receptor CD63 to induce a conformational change in β1 integrin to promote cell survival signaling and prevent apoptosis (156). Therefore, TIMP-1 may exert trophic support and antinociception (i.e. blocking the detection of noxious stimuli by sensory neurons) by modulating the CD63/β1 integrin complex, which, depending on the cell type, can affect downstream MAPK/PI3K or Wnt/β catenin pathways (158, 162). Currently, TIMP-1 has been shown to also interact with LDL-receptor related protein 1 (LRP-1) and CD82, although these interactions are less understood (141, 163)(Refer to Figure 2.2). Therefore, the complexity of TIMP-1 protein structure supports interactions with many substrates, contributing to a larger protein interactome, as previously reported (141).

Understanding the role of TIMP-1 in disease processes, such as inflammatory and neuropathic pain, must consider the versatility of TIMP-1.

The expression of TIMP-1 has been implicated in several chronic pain conditions including NP, back pain, RA, and temporomandibular pain (164-167). Inflammation and injury alter the expression of proteins in the DRG and spinal cord that can influence the development and maintenance of neuropathic pain (168, 169). Similar to the induction of immune-related factors, TIMP-1 expression is induced by growth factors and cytokines during tissue damage (170-173). Microarray analysis comparing gene expression profile in the DRG and spinal cord of rats that received subcutaneous injections of CFA or subjected to chronic constriction injury (CCI) revealed that both inflammation and nerve injury caused significant increases in TIMP-1 expression in
DRG, however only CCI increased TIMP-1 mRNA expression in the spinal cord (168). Comparison between the two injury types suggests that elevated expression of TIMP-1 following CCI may reflect the function of TIMP-1 during nerve regeneration, possibly pain, even pain severity (174-176). Although the bias in the field is to characterize TIMP-1 as a protease inhibitor, the complexity of TIMP-1 signaling and its receptor-mediated affects, suggests that TIMP-1 may facilitate antinociception through a yet to be defined mechanisms that is independent of MMP-inhibition.

Often, TIMP-1 is regarded as a protective molecule that promotes recovery and supports cellular growth (169, 172, 177-179). For instance, examination of the risk factors involved the pathogenesis of corneal ulceration in patients with rheumatoid arthritis, revealed significantly reduced or absent TIMP-1 in diseased corneas compare to controls, as well as had increased collagenase staining and active immune response, indicative of tissue damage (165). This protective phenotype has also been shown in animal model of cutaneous (i.e. UVB) photodamage whereby overexpression of TIMP-1 caused less severe tissue damage through suppression of ECM degradation and TNFα signaling (173). In knockout studies, mice lacking TIMP-1 had higher infiltrated immune cells, hemorrhage, and vascular permeability during bleomycin-induced acute lung injury (180). And in a model of CNS myelin injury, mice lacking TIMP-1 exhibited poor myelin recovery, decreased astrogliosis, and persistent macrophage activation (181). However, this phenotype was reversed with replacement of recombinant murine TIMP-1 (rmTIMP-1), TIMP-1 KO (T1KO) mice had improved differentiation of oligodendrocytes and astrocyte proliferation that was not recapitulated with a potent broad-spectrum MMP inhibitor (GM6001) (182). In a later study, conducted by the same group, they showed that TIMP-1 facilitated oligodendrocyte differentiation through interactions with CD63/β1 integrin complex. Supporting
this, exogenous application of recombinant TIMP-1 in a mouse model of retinal degeneration protected photoreceptors by suppressing apoptosis and delaying rod degeneration. In other studies, TIMP-1 suppressed apoptotic pathways independent of MMP-inhibition (160, 183, 184). Together, these data suggest that during inflammation TIMP-1 may produce antinociception through a novel signaling mechanism that affects the inflammatory response and limits the severity of tissue damage.

How TIMP-1 may attenuate nociception through cell-receptor mediated is not understood. In a model of kainate-induced seizures in rats, TIMP-1 was mRNA levels were upregulated in hippocampal neuronal layers, which was not prevented with administration of cycloheximide, an inhibitor of protein synthesis (185). In a subsequent paper, administration of recombinant TIMP-1 protected hippocampal neurons against glutamate-induced calcium influx and excitotoxic stress (184). Although these papers did not show the physical interaction of TIMP-1 with hippocampal neurons, non-neuronal cells are known to upregulate TIMP-1 during inflammatory conditions as well as release factors that can augment nociception (82, 132, 186-189). These cell types include astrocytes and oligodendrocytes, endothelial cells, fibroblasts, mast cells, and keratinocytes (172, 173). TIMP-1 expression can also be induced with experimental autoimmune encephalomyelitis in spinal astrocytes (172, 190). Damage to the sciatic nerve increases TIMP-1 expression in neuronal cell bodies and satellite glial cells, a peripheral analog to astrocytes, in the DRG (144). With regards to cutaneous inflammation, keratinocytes upregulate TIMP-1 to facilitate protection during photodamage, and we also know that keratinocyte-derived factors interact with primary afferent nerve endings to influence their activity (191-193). In another paper, CD63 positive extracellular vesicles were shown to bind to primary afferent cell bodies and their central terminals and attenuated neuropathic pain (194). Therefore, we hypothesize that in response to inflammation,
non-neuronal cells upregulate and release TIMP-1 to attenuate hyperalgesia through receptor mediated affects.

III. Summary

Animal studies implicate TIMP-1 is a potential target for attenuating the inflammatory response, limiting tissue damage, and preventing nerve injury-induced pain. Although we don’t fully understand how TIMP-1 contributes to antinociception, we do know that TIMP-1 facilitates multiple functions that may provide protection and subsequent analgesia during different injury conditions. However, current knowledge about TIMP-1 in pain processing is limited to neuronal damage. In order to determine how TIMP-1 may provide analgesia it is imperative to determine whether TIMP-1 can prevent the induction of inflammatory hypersensitivity, in the absence of frank tissue damage. Understanding the potential efficacy of TIMP-1 under inflammatory conditions may provide a novel target that augment nociception without interfering with the normal inflammatory process. In addition, identifying the role of TIMP-1 in the development of inflammatory pain is a key step in determining the functional implications for attenuating persistent pain.

Using the CFA model, we will characterize how TIMP-1 expression is altered in nervous system tissues following subcutaneous inflammation. To characterize the contribution of TIMP-1 in the normal progression of pain we will assess hyperalgesia in WT and T1KO mice following CFA as well as determine the contributions of both MMP-regulator and trophic functions of TIMP-1 by administering individual recombinant N and C peptides in inflamed T1KO mice. Ultimately the goal of this research is to identify a molecular target that regulates the normal progression of pain during acute tissue damage to better understand how to augment persistent pain. In this thesis I will address four questions: 1) how does inflammation regulate TIMP-1 expression; 2) does
endogenous TIMP-1 expression affect the normal progression of pain following cutaneous inflammation; 3) how does TIMP-1 impact the local expression of inflammatory molecules; and 4) does exogenous replacement of TIMP-1 attenuate hypersensitivity? By answering these questions, we can determine whether TIMP-1 expression is affected in the inflamed environment, potentially regulating ECM remodeling and the pro-inflammatory milieu compared to more distal tissues, such as DRG and SC, which may suggest TIMP-1 is released by glial cells to decrease to affect subsequent excitation of nociceptors or second order afferents in the dorsal horn.
Figure 2-1: Representation of cutaneous tissue damage and nociception. During tissue damage the release of algogenic molecules can directly bind to their receptors on peripheral afferents thus, influencing their activity (195).
**Figure 2-2: Representation of TIMP-1 protein structure.** TIMP-1 is comprised of 2 subdomains, the N terminal domain binds and inhibits proteases and the C terminal domain can interact with cell-surface receptors (I41).
Chapter 3:
TIMP-1 attenuates the development of inflammatory pain through MMP-dependent and receptor-mediated cell signaling mechanisms

Knight, B.E.3, Kozlowski, N.,1 Havelin, J.7, 8, King T.6, 7, 8, Crocker, S.J.3, 5, Young, E.E.1, 2, 4, 5, Baumbauer, K.M.1, 2, 3, 5, 9

1School of Nursing, University of Connecticut, Storrs, CT, USA
2The Center for Advancement in Managing Pain, School of Nursing, University of Connecticut, Storrs, CT, USA
3Department of Neuroscience, UConn Health, Farmington, CT, USA
4Genetics and Genome Sciences, UConn Health, Farmington, CT, USA
5Institute for Systems Genomics, UConn Health, Farmington, CT, USA
6College of Osteopathic Medicine, University of New England, Biddeford, ME, USA
7Center for Excellence in the Neurosciences, University of New England, Biddeford, ME, USA
8Graduate School of Biomedical Science and Engineering, University of Maine, Orono, ME, USA
9Rita Allen Foundation, Princeton, NJ, USA

Author Contributions
BEK – experimental design, data collection and analysis, mouse breeding and care, manuscript preparation
NK – data collection
JH – collection of conditioned place preference data
TK – experimental design and analysis of data from conditioned place preference experiments, manuscript preparation
SJC - generation of T1KO mouse line, experimental design, manuscript preparation
EEY – experimental design, data analysis, manuscript preparation
KMB – experimental design, data analysis, manuscript preparation, experimental oversight
I. Abstract

Unresolved inflammation is a significant predictor for developing chronic pain, and targeting the mechanisms underlying inflammation offers opportunities for therapeutic intervention. During inflammation, matrix metalloproteinase (MMP) activity contributes to tissue remodeling and inflammatory signaling and is regulated by tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 and -2 have known roles in pain, but only in the context of MMP inhibition. However, TIMP-1 also has receptor-mediated cell signaling functions that are not well understood. Here, we examined how TIMP-1-dependent cell signaling impacts inflammatory hypersensitivity and ongoing pain. We found that hindpaw injection of complete Freund’s adjuvant (CFA) increased cutaneous TIMP-1 expression that peaked prior to development of mechanical hypersensitivity, suggesting that TIMP-1 inhibits the development of inflammatory hypersensitivity. To examine this possibility, we injected TIMP-1 knockout (T1KO) mice with CFA and found that T1KO mice exhibited rapid onset thermal and mechanical hypersensitivity at the site of inflammation that was absent or attenuated in WT controls. We also found that T1KO mice exhibited hypersensitivity in adjacent tissues innervated by different sets of afferents, as well as skin contralateral to the site of inflammation. Replacement of recombinant murine (rm)TIMP-1 alleviated hypersensitivity when administered at the site and time of inflammation. Administration of either the MMP inhibiting N-terminal or the cell signaling C-terminal domains recapitulated the antinociceptive effect of full-length rmTIMP-1, suggesting that rmTIMP-1 inhibits hypersensitivity through MMP inhibition and receptor-mediated cell signaling. We also found that hypersensitivity was not due to genotype-specific differences in MMP-9 activity or expression, nor to differences in cytokine expression. Administration of rmTIMP-1 prevented mechanical hypersensitivity and ongoing pain in WT mice, collectively suggesting a novel role for TIMP-1 in the attenuation of inflammatory pain.
II. Introduction

Tissue inhibitors of matrix metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) are released during tissue damage to facilitate tissue remodeling through degradation and reorganization of the extracellular matrix (ECM) (140, 146, 179). During this process MMPs also engage an inflammatory response through proteolytic maturation of cytokines, and both of these activities are regulated through a 1:1 stoichiometric interaction with one of four tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, -4) (144). The interaction between MMPs and TIMPs is tightly controlled, but research has shown that during tissue damage, imbalance between MMPs and TIMPs can lead to pathological conditions such as arthritis, multiple sclerosis, Parkinson’s Disease, cancer, and even chronic pain (143, 196-199). Studies examining the role of MMPs in pain specifically have shown that increased MMP-2 and -9 activity contribute to increased pain-related behavior in response to injury that can be reversed by MMP antagonism (111, 135, 200, 201). These findings contributed, in part, to the development of several small molecule drugs that directly target and inhibit MMP activity. However, more than 50 clinical trials examining the efficacy of these drugs were discontinued due to the emergence of adverse events, including musculoskeletal pain (138, 202). While the results of these trials indicated that specific targeting of MMP activity alone is not an effective strategy for pain treatment, they also suggest that additional mechanisms related to MMP activity may contribute to pain and its inhibition, and that endogenous inhibitors of MMPs, such as TIMP-1, may attenuate pain-related behavior.

TIMP-1 is best characterized as an inhibitor of MMP activity. Indeed, TIMP-1 regulates 14 of the 24 known MMPs (135, 146, 179, 203), and has been shown to prevent the development of mechanical and thermal hypersensitivity following nerve damage (135, 202). However, this identified role was characterized purely in the context of MMP inhibition. TIMP-1 inhibits MMP
activity through the binding of its N-terminus with the targeted MMP, resulting in chelation of Zn$^{2+}$ from the enzyme active site (204). Interestingly, there is now mounting evidence that the C-terminal domain can bind to membrane bound receptors, including CD63 (146). The binding of TIMP-1 to CD63 engages intracellular signaling events that allow TIMP-1 to function as a trophic factor and initiate cellular migration and differentiation (169, 172, 177-179). Because TIMP-1 and MMPs can be up-regulated simultaneously during tissue damage and repair, such as in peripheral nerve injury (144, 168, 173, 176), disentangling how TIMP-1 regulates tissue remodeling/repair from the induction of pain, per se, is challenging.

Inflammation is a core component of the nerve injury process (107), and, in general, is a significant predictor of pain chronicity (34, 38, 205). Therefore, we used a model of cutaneous inflammation to examine the effects of TIMP-1 signaling on pain in the absence of frank tissue damage. We found that hindpaw injection of complete Freund’s adjuvant (CFA) induced TIMP-1 expression in keratinocytes prior to the emergence of hypersensitivity in wildtype (WT) mice, suggesting that the release of TIMP-1 at the site of inflammation prevented the development of hypersensitivity. Supporting this conclusion, we found that TIMP-1 knockout (T1KO) mice exhibited robust hypersensitivity to stimulation of tissues local and distal to the site of inflammation. This phenotype was prevented by the administration of recombinant murine (rm)TIMP-1, as well as the individual N- and C-terminal constructs at the time of CFA-injection. These results suggest that cell-signaling mechanisms may also contribute to the antinociceptive effects of TIMP-1. In addition, inflammation in WT and T1KO mice did not result in the genotype-specific activation or expression of cutaneous MMP-9 or cytokines. Finally, we found that the administration of rmTIMP-1 prevented ongoing inflammatory pain and evoked mechanical
hypersensitivity in WT mice. Collectively, our data suggests that TIMP-1 regulates the algogenic properties of inflammation and that TIMP-1 may be a target for improving pain management.

III. Materials and methods

Animals: Experiments were conducted using 8-12-week-old (20-30 g) male WT (C57BL/6; Jackson Laboratories, Bar Harbor, ME) and T1KO mice that were group housed, and maintained in a temperature-controlled environment on a 12 hr light-dark cycle with free access to food and water. TIMP-1 knockout (T1KO) mice (206) were backcrossed onto a C57BL/6 background for greater than 13 successive generations and bred in-house as a homozygous line (181). All studies were approved by the UConn Health Institutional Animal Care and Use Committee and treated in accordance with published NIH standards.

Complete Freund’s Adjuvant (CFA): To produce an acute, local inflammatory response, we subcutaneously (s.c.) injected the right hindpaw of mice with emulsified (50% in 10 μL) CFA (Sigma, St. Louis, MO). To assess primary hypersensitivity (i.e. at the site of inflammation) we administered CFA into the glabrous skin or ventral surface of the right hindpaw. Conversely, secondary hypersensitivity was assessed in skin that was adjacent or contralateral to the site of inflammation. All samples were compared to naïve controls because in a pilot experiment we found that vehicle injection alone caused increased sensitivity in T1KO mice. While this result is interesting and suggests that subtle perturbations cause robust alterations in sensory thresholds, adding saline-treated mice confounds our ability to examine inflammatory hypersensitivity. Therefore, to interpret the effects of inflammation per se, naïve mice were used as comparison controls. The literature is also mixed on the use of vehicle controls in experiments using CFA, and our experiments are in line with previously published work (207-209).
Recombinant Murine TIMP-1 Administration: WT and T1KO mice received injections (s.c.) of recombinant murine (rm)TIMP-1 (10 ng/μL, 10 μL; R&D Systems; Minneapolis, MN) immediately following CFA injection (10 μL) into the right hindpaw. In subsequent experiments, T1KO mice received equimolar concentrations of the truncated C-terminus peptide (TIMP-1(C); 6.3kDa; Peptide 2.0 Inc., Chantilly, VA) that retains cell signaling function or the truncated N-terminus peptide (TIMP-1(N); 20 kDa; Abcam, Cambridge, UK) that retains MMP-inhibitory function and no cell-signaling ability, immediately following CFA injection.

von Frey (vF) testing: All mice were place into transparent Plexiglas chambers (radius = 32 mm, height = 108 mm) on an elevated mesh screen and were allowed to acclimate for a minimum of 1 hr before testing. To assess mechanical sensitivity, the plantar surface of the right hindpaw was stimulated using von Frey filaments using the up-down method previously described by (210). Nocifensive responses were counted as robust flexion responses, paw shaking, or paw licking and subtracted from individual baseline threshold to account for inter-subject variability. Data are presented as paw withdrawal thresholds (PWT; in grams).

Thermal hyperalgesia: Thermal hyperalgesia to radiant heat was assessed using a Hargreaves apparatus (Harvard Apparatus; Holliston, MA) (68). Briefly, all mice were placed in transparent Plexiglas chambers (radius = 32 mm, height= 108 mm) on top of a framed glass panel and were allowed to acclimate for a minimum of 1 hr before testing. Following the acclimation period, an infrared (IR) beam was aimed at the plantar surface of each hindpaw in an alternating fashion. The intensity of the IR beam was chosen to produce average baseline paw withdrawal latency (PWL) of 15-20 seconds. Stimuli were presented 5 times in an alternating fashion between each hindpaw with 5-minute intervals between successive stimulus exposures. A 30 seconds exposure cutoff was
employed to prevent tissue damage. PWLs collected from each paw were then averaged and analyzed.

**Conditioned Place Preference (CPP):** CPP was used to assess ongoing pain in WT mice. A 3-day single trial protocol was used. On day 1, all mice freely explored a 3-chamber CPP box for 15 minutes prior to injection with CFA or saline. Preconditioning (baseline) behavior was analyzed using automated software (ANYMAZE, Stoelting) to ensure there were no baseline differences in the time spent in any of the chambers. On day 2 (conditioning day), all mice received an intrathecal (i.t.) injection of saline (5 μL volume) under isoflurane anesthesia and upon waking (within 2 min) were confined into the pre-assigned pairing chamber for 30 min. They were then returned to their home-cages for 4 hr. All mice then received an intrathecal (i.t.) injection of clonidine (2 μg/μL; 5 μL volume) through lumbar puncture and upon waking were confined to the opposite pairing chamber for 30 min. Vehicle and clonidine paired chambers were randomly assigned and counterbalanced between animals. On day 3 (test day), mice were returned to the CPP apparatus and allowed to freely explore all chambers across 15 min. The total time spent in each chamber was assessed using automated software (ANYMAZE). Conditioning day was 20-24 hr following CFA injection as previous research indicates ongoing pain is observed at this time-point (Okun et al, He et al). A total of 17 mice were used, 8 were treated with rTIMP-1 and 9 with vehicle.

**Tissue collection:** Mice were anesthetized with a lethal dose of ketamine and xylazine mixture (90/10 mg/kg, respectively) and intracardially perfused with ice cold 0.9% saline prior to the dissection of ipsilateral hairy skin, L2 -L3 dorsal root ganglia (DRG), and lumbar spinal cord segments (L2 -L3). Tissues were collected following the completion of behavior or at designated time-points for molecular analysis.
**Enzyme-linked immunosorbent assay (ELISA):** Protein was extracted through homogenization in ice-cold RIPA buffer/protease inhibitor cocktail and spun for 20 min at 4°C at 18,000 rcf. Each sample’s total protein concentration was determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). The following ELISAs, TIMP-1 (R&D Systems, Minneapolis, MN), MMP-9 (R&D Systems; Minneapolis, MN), IL-6 (Invitrogen Carlsbad, CA), TNF-α (ThermoFisher Scientific, Waltham, MA), IL-10 (ThermoFisher Scientific, Waltham, MA), and IL-1β (R&D Systems, Minneapolis, MN) were run according to manufacturer’s instructions. All samples were run in duplicate and absorbance ratios were read at 450nm.

**Immunohistochemistry (IHC):** Hairy skin, glabrous skin, and DRG from WT mice were excised and incubated in 0.06% brefeldin A (BFA) in serum free Hank’s balanced salt solution (HBSS) for 20 min at room temperature. Half of the samples were incubated in inflammatory soup (IS) (10uM; bradykinin triacetate, histamine dihydrochloride, serotonin hydrochloride, prostaglandin E2 (PGE2) dissolved in normal cerebral spinal fluid, pH 6.0) or serum-free media (211, 212). Incubation in IS or serum-free media occurred for 24 hr (211). Spinal cords from inflamed or naïve WT mice were isolated 24 hr following CFA treatment or from designated naïve controls. Prior to tissue collection, mice were intracardially perfused with 0.06% BFA in 0.9% saline for 20 min and then perfused with 4% paraformaldehyde. All samples were post-fixed overnight in 4% paraformaldehyde (PFA), cryoprotected overnight in 30% sucrose, and later embedded in Optimal Cutting Temperature (OCT). Samples were cut into 30 µm cross sections using a cryostat. Tissue sections were briefly washed with sterile phosphate buffered saline (PBS) and incubated with staining buffer (0.05% triton and 30% fetal bovine serum in PBS) solution for 40 min at room temperature. Slices were then incubated with primary unconjugated antibodies for 48hr at 4°C. The following primary antibodies were diluted in staining buffer: monoclonal anti-mouse
cytokeratin 14 (K14; Abcam, Cambridge, United Kingdom; 1:300 dilution), polyclonal anti-goat TIMP-1 (R&D Systems; Minneapolis, MN; 1:300 dilution), monoclonal anti-mouse microtubule-associated protein 2 (MAP2; Millipore Sigma, Burlington, MA; 1:1000 dilution), and monoclonal anti-mouse primary conjugated-cy3 glial fibrillary acidic protein (GFAP; Abcam, Cambridge, United Kingdom; 1:500 dilution). Tissue slices were then incubated with secondary antibodies for 2-3 hr at 4°C. The following secondary antibodies were diluted in staining buffer: polyclonal rabbit anti-mouse Alexa-488 (Life Technologies, Carlsbad, CA, 1:000), polyclonal donkey anti-goat Alexa-568 (Life Technologies, Carlsbad, CA, 1:000), and polyclonal goat anti-mouse Alexa-568 (Life Technologies, Carlsbad, CA; 1:1000 dilution). Slides with DRG and spinal cord slices were incubated with 300um DAPI prior to cover-slippering to visualize nuclei of satellite glial cells and astrocytes, respectively.

**MMP-9 colorimetric activity:** Protein was extracted from the hindpaw of WT and T1KO mice 1 day post-CFA injections (s.c., 10μL) or from designated naïve controls for a high throughput screening of MMP-9 activity. Gelatinase activity was measured using the SensolyteGeneric MMP colorimetric assay kit (Anaspec, Fremont, CA). Samples were run in duplicate and end-point enzymatic activity was analyzed using a glutathione reference standard.

**RT-qPCR:** Total RNA from WT and T1KO was extracted from naïve and inflamed samples 1 day post CFA injection using a RNeasy Mini Kit (Qiagen, Venlo, Netherlands). To quantify cutaneous TIMP-2 and TIMP-4 mRNA expression, equal amounts of cDNA were synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and mixed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and 2μM of both forward and reverse primers (see Table 1). GAPDH was amplified as an internal control. The threshold crossing value was noted for each transcript and normalized to the internal control.
The relative quantitation of each transcript was performed using the \( \Delta \Delta \text{Ct} \) method and presented as fold change relative to naïve WT expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timp2</td>
<td>5’- CCAGAAGAGAGCCTGAACCA-3’</td>
<td>5’- GTCCATCCAGAGGCACCTCATC-3’</td>
</tr>
<tr>
<td>Timp4</td>
<td>5’- TGCAGAGGGAGAGCCTGAA-3’</td>
<td>5’- GGTACATGGCACTGCATAGCA-3’</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5’- ATGAATACGGCTACAGCAACAGG-3’</td>
<td>5’- CTCTTGCTCAGTGTCCTTGCTG-3’</td>
</tr>
</tbody>
</table>

Table 2: Primer sequences for qPCR

Statistical analysis: All data were analyzed using one-way or mixed designs Analysis of Variance (ANOVA). Post hoc analyses were performed using Tukey’s HSD, and statistical significance was determined using a \( p<.05 \). Statistical analysis was performed using SPSS (Version 25). Since ANOVAs rely on linear relationships among data, and not all effects can be resolved using linear based statistical tests, we used trend analyses (e.g., contrasts) to test for significant nonlinear relationships in some of our behavioral analyses. An added benefit of this approach is that trend analyses are more robust than ANOVAs (213). Clonidine induced CPP was assessed using a 2-way repeated measures ANOVA with post-hoc analysis of pre vs post conditioning time spent in the clonidine paired chamber for each treatment group using Sidak’s multiple comparisons test. Between groups analysis was performed on difference scores calculated as post-conditioning (-) pre-conditioning time spent in the clonidine paired chamber.

IV. Results

3.1 Cutaneous TIMP-1 expression is upregulated prior to the onset of inflammatory hypersensitivity

To determine whether cutaneous inflammation alters the expression of TIMP-1 in tissues along the peripheral sensory circuit, we injected emulsified CFA (10 \( \mu \text{L}, \text{s.c.} \)) into the hairy skin
of the ipsilateral hindpaw and collected spinal cord (SC; L2-L3), dorsal root ganglia (DRG; L2-L3), and hairy skin over the course of 7 days. We found that inflammation did not alter the overall expression of TIMP-1 protein in SC or DRG, all $F$s > 1.13, $p$>.05 (Figure 1 A, B). However, we observed a significant increase in cutaneous TIMP-1 protein 1, 3, 5, and 7 days following CFA administration, $F(4,19) = 37.54, p$<.01 (Figure 1C).

3.2 Cellular colocalization of TIMP-1 expression.

To confirm the above results, and to localize the cellular source of TIMP-1 expression, immunohistochemistry (IHC) was performed on DRG and skin samples incubated in vitro with or without inflammatory soup (IS)(211), as well as spinal cords following in vivo inflammation. Although overall TIMP-1 expression levels were unaltered in the spinal cord and DRG following inflammation, we found that TIMP-1 co-localized with glial fibrillary acidic protein (GFAP) expressing cells following inflammatory stimulation, demonstrating that astrocytes (Figures 2A) and satellite glial cells (Figure 2B) appear to express TIMP-1 during inflammation (144, 188). We also found that TIMP-1 expression was upregulated in K14-positive keratinocytes in both hairy and glabrous skin following inflammatory stimulation (Figure 2C; glabrous skin data not shown).

3.3 Development of inflammatory hypersensitivity in WT mice.

To associate the expression of cutaneous TIMP-1 with the development of mechanical hypersensitivity, we assessed paw withdrawal thresholds (PWT) on the plantar surface of the hindpaw for 7 days following CFA injection into the dorsal, hairy skin. We found that TIMP-1 protein levels peaked 3 days following CFA administration (see Figure 1C), at a time when mice developed mechanical hypersensitivity, $F(2,12) = 43.94, p$< 0.05, (Figure 3). Together, these data indicate that cutaneous inflammation induces the expression of TIMP-1 in keratinocytes at the time of inflammation and prior to the onset of mechanical allodynia.
3.4 Mice lacking TIMP-1 exhibit hyperalgesia in inflamed and uninflamed cutaneous tissues.

To determine whether endogenous TIMP-1 expression is important for the normal progression of hypersensitivity, we used a global TIMP-1 knockout (T1KO) mouse strain. We first assessed behavioral responsiveness to radiant heat on the plantar surface of the hindpaw following s.c. administration of a diluted CFA solution. We chose to use a diluted CFA solution because our preliminary experiments suggested that exposure to slight challenges significantly altered sensitivity in T1KO. To ensure that any potential differences in responding to inflammatory stimulation were not due to preexisting differences in sensory thresholds between mouse strains, we measured baseline responding to radiant heat and found no significant differences in paw withdrawal latencies (PWL), $F(1, 31) = .47, p > .05$ (Figure 4A). Interestingly, while we did not observe any significant differences in PWL between naïve and WT mice that received diluted (e.g., subthreshold) CFA, we did find that inflamed T1KO mice exhibited thermal hyperalgesia that persisted for 29 days in response to diluted CFA injection, all $F_s < 2.30, p < .05$ (Figure 4B).

Next, we assessed mechanical response thresholds (von Frey) following diluted CFA administration. Analysis of baseline responses to mechanical stimulation did not reveal any significant differences between genotypes $F(1, 36) = .34, p < .05$ (Figure 4C). We did find that CFA administration reduced mechanical response thresholds in both genotypes $F(1, 34) = 17.61, p < .01$. However, T1KO mice exhibited greater mechanical hypersensitivity 1 day following CFA treatment, compared to WT controls, all $F_s < 4.59, p < .05$ (Figure 4D). Therefore, we concluded that the up-regulation of TIMP-1 following inflammation delays the onset of hypersensitivity, and that in the absence of TIMP-1, the normal development of hypersensitivity is altered.

3.5 Assessment of cutaneous Timp2 and Timp4 mRNA expression following inflammation.
To determine whether the rapid emergence of inflammatory hypersensitivity in T1KO mice was due to compensatory expression of \textit{Timp2} or \textit{Timp4} mRNA, we examined cutaneous expression of each transcript in naïve and inflamed T1KO mice. We found no significant differences in the basal expression of either transcript in WT or T1KO mice. However, \textit{Timp2} and \textit{Timp4} expression decreased in T1KO mice following inflammation (Figure 5A, B), suggesting that genetic deletion of TIMP-1 did not result in a compensatory response from other TIMPs, all $F_s < 11.65, p < 0.01$.

3.6 Mice lacking TIMP-1 show increased sensitivity in non-inflamed tissues.

Our current data demonstrate that cutaneous TIMP-1 is an early emergent protein following inflammation, and that the absence of TIMP-1 alters the normal development of hypersensitivity. Therefore, TIMP-1 signaling may have important implications for regulating the development of inflammatory hypersensitivity in tissue adjacent to the site of inflammation that is innervated by afferent terminals that are different from those that innervate inflamed skin. To test this possibility, we assessed the development of hypersensitivity in the glabrous skin following injection of diluted CFA into hairy skin in both T1KO and WT following baseline assessment of sensitivity. Again, we observed no genotype-specific differences in baseline reactivity, and because of this consistent finding, we will no longer present data depicting baseline behavioral reactivity. Analysis using an ANOVA revealed that T1KO mice, relative to WT mice, exhibited increased sensitivity to mechanical stimulation on the plantar surface of the hindpaw following inflammation of hairy skin that was not temporally dependent, all $F_s < 4.41, p < .05$, (Figure 6A, B). However, trend analyses revealed that inflamed T1KO mice exhibited increased inflammatory hypersensitivity 1 day following CFA treatment when compared to all other mice, $F(1,57) = 11.55, p < .01$ (Figure 6A).
To examine whether administration of recombinant murine (rm)TIMP-1 prevented inflammatory hypersensitivity, and the potential mechanism by which this effect occurs, separate groups of T1KO mice received a single injection of recombinant full-length rmTIMP-1 [TIMP-1(FL)], the truncated N terminus peptide [TIMP-1(N)] that retains MMP inhibitory function but no cell signaling capacity, or the truncated C terminus peptide [TIMP-1(C)] that lacks MMP inhibitory capacity but retains its cell signaling function at the time of CFA administration. To limit the complexity of our experimental design, and to determine the optimal dose for the administration of each TIMP-1 construct, we conducted a pilot experiment using a small cohort of T1KO mice given 1, 10, or 100 ng/μL of TIMP-1 at the time of inflammation. We found that 10 ng/μL was effective at reducing inflammatory hypersensitivity (data not shown). We then administered a separate cohort of T1KO mice 10 ng/μL of TIMP-1(FL), TIMP-1(N), or TIMP-1(C) at the time of CFA administration. Mechanical hypersensitivity was assessed 24 hr later. While inflamed T1KO mice exhibited a significant reduction in mechanical thresholds, T1KO mice treated with the rmTIMP-1 peptide constructs did not. Moreover, we observed no significant differences in the response thresholds between mice given TIMP-1(FL), TIMP-1(N), or TIMP-1(C), all $Fs > 4.54$, $p < .01$ (Figure 6C), demonstrating that TIMP-1 attenuates inflammatory hypersensitivity through MMP-dependent and MMP-independent signaling mechanisms.

The above data show that inflammation in one somatic region could lead to mechanical hypersensitivity in tissue distal to the site of inflammation, reminiscent of “mirror image pain” (36, 85). To test this possibility, we inflamed one hindpaw and measured mechanical sensitivity on the opposite hindpaw for 7 days following CFA administration. We also examined whether treatment with rmTIMP-1 at the site and time of inflammation affected sensitivity. We found that inflamed T1KO mice exhibited contralateral mechanical hypersensitivity over the course of 7 days.
following CFA-injection relative to WT mice (Figure 5D). Interestingly, this contralateral hypersensitivity was prevented by treatment with rmTIMP-1 in T1KO mice, $F (4, 43) = 5.52, p < 0.05$ (Figure 6D).

### 3.7 The lack of TIMP-1 does not alter the expression of local inflammatory molecules

TIMP-1 is primarily known as a broad-spectrum MMP inhibitor, and because MMPs are known to contribute to hypersensitivity, we hypothesized that the absence of TIMP-1 may cause hypersensitivity due to elevated activity and expression of cutaneous MMP-9 ($135, 143$). Examination of hairy skin collected 1 day following CFA from WT and T1KO mice demonstrated that there was an inflammation-induced increase in both MMP-9 expression and activity, all $Fs > 7.61, p < .05$ but that these effects were not genotype-specific, all $Fs < 2.05, p > .05$ (Figure 7A, B). The TIMP/MMP axis also regulates the proteolytic maturation of inflammatory molecules which can cause hypersensitivity ($190$). We next assessed whether the absence of TIMP-1 during inflammation caused elevated cytokine expression in the skin. Using ELISAs, we assessed the expression of cutaneous IL-1β, IL-6, TNFα, and IL-10 at 1 day following CFA-injection. Analysis revealed an inflammation-induced increase in IL-1β and IL-6 expression, but this increase in expression was not different between genotypes, all $Fs > 12.94, p < .05$ (Figure 7C, D). In comparison, analysis of TNF-α and IL-10 did not reveal any significant differences following inflammation, all $Fs < 4.64, p > .05$ (Figure 6E, F). These data suggest that TIMP-1 does not affect the emergence of hypersensitivity through differences in inflammatory cytokine expression.

### 3.8 Administration of recombinant TIMP-1 attenuates ongoing pain in WT mice.

Previous experiments demonstrate that the administration of rmTIMP-1 attenuates evoked mechanical and thermal hypersensitivity in T1KO mice. Here, we examined whether the administration of rmTIMP-1 also attenuated ongoing pain in WT mice using CPP as previous
described (86, 214). Analysis of pre- compared to post-conditioning time spent in the conditioning chamber indicate an effect of drug, $F(1, 30) = 7.269, p<0.05$), and post-hoc analysis confirmed an increase in post-conditioning time spent in the clonidine paired chamber compared to pre-conditioning time in vehicle treated mice ($p<0.01$) but not in rmTIMP-1 treated mice ($p>0.05$) (Figure 8). These observations indicate that WT mice administered CFA and TIMP-1 did not demonstrate clonidine-induced CPP (Figure 8). Because clonidine only produces CPP in the state of injury (86), these results further suggest that treatment with rmTIMP-1 attenuated ongoing inflammatory pain.

V. Discussion

The balance between TIMPs and MMPs is important for maintaining tissue homeostasis and preventing pathological conditions. Following tissue damage and inflammation, TIMP-1 is expressed in a variety of cell types that can modulate neuronal function and wound healing, including, astrocytes, oligodendrocytes, Schwann cells, endothelial cells, mast cells, and keratinocytes (171-173). Because TIMP-1 is broadly expressed, TIMP-1 may also regulate neuroinflammation and neuropathic pain (111, 135, 144, 215). Although the predominant view is that TIMP-1 exerts these functions by inhibiting MMPs, emerging evidence suggests that TIMP-1 may also facilitate these functions by binding cell-surface receptors and mediating their subsequent downstream signaling pathways (158, 160, 163, 215). Therefore, the present set of studies was designed to investigate the role of TIMP-1 in the development of inflammatory hypersensitivity and the mechanisms of its action.

To determine how inflammation affected the expression of TIMP-1 in tissues proximal and distal to the site of CFA-injection, we examined TIMP-1 expression in skin, DRG, and spinal cord over the course of 7 days. While we did not observe changes in TIMP-1 expression in the DRG or
spinal cord, we did localize its expression to cells positive for GFAP, suggesting that TIMP-1 is expressed by satellite glial cells and astrocytes, respectively. When we examined skin, we found that CFA induced an 8.27-fold increase in TIMP-1 protein expression within 24 hr of inflammation, and that this temporal upregulation in TIMP-1 was observed in basal keratinocytes. Given that keratinocytes augment nociceptive signaling through the release of neuroactive molecules (192, 193), the release of TIMP-1 from keratocytes may attenuate pronociceptive behavior caused by inflammation (171, 173, 216). To test this hypothesis, we examined the temporal expression pattern of TIMP-1 in relationship to the development of cutaneous hypersensitivity in WT mice. We found that the largest change in TIMP-1 expression, within 24 hr of injection, preceded the onset of cutaneous hypersensitivity, and that at 3 days when TIMP-1 expression peaked, behavioral sensitivity was the greatest in WT mice. This result suggests that peak TIMP-1 expression may in some way signal the onset of hypersensitivity. Alternatively, it is also possible that the relationship between TIMP-1 expression and the onset of hypersensitivity is determined by a relative change in expression between two consecutive time points. In the case of the current experiments the largest change in expression is observed between baseline and 24 hr following inflammation, where we observe an 8.27-fold change in expression. Conversely, we only detect a 1.43 fold increase in expression between Day 1 and Day 3, implying that it is not the absolute level of TIMP-1 expression, per se, that contributes to the delay in hypersensitivity, but rather the extent to which TIMP-1 expression changes relative to previous levels of expression over time. Consequently, it may be possible that as TIMP-1 expression increases during the first 24 hr of inflammation the emergence of pain-related behavior is attenuated. However, the overall change in TIMP-1 expression over the next 48 hr is no longer sufficient to prevent the emergence of hypersensitivity. Supporting this, we found that the replacement of recombinant TIMP-1 within
24 hr of CFA-injection, which causes a significant increase in expression from baseline expression in the skin, prevented the onset of hypersensitivity in mice lacking TIMP-1. Taken together, these data suggest that the immediate induction and release of TIMP-1 from basal keratinocytes attenuates inflammatory hypersensitivity.

If the release of TIMP-1 is important for delaying the onset of inflammatory hypersensitivity, it is also possible that hypersensitivity is exacerbated in the absence of TIMP-1. To test this, we compared mechanical hypersensitivity and thermal hyperalgesia at the site of inflammation in WT and T1KO mice. While we observed a robust reduction in mechanical thresholds in WT and T1KO mice, hypersensitivity persisted for a longer duration in T1KO mice relative to WT controls. Interestingly, when we examined thermal hyperalgesia, we observed a significant reduction in paw withdrawal thresholds in T1KO mice, while WT mice appeared to be unaffected. This result suggests that TIMP-1 may differentially regulate the processing of thermal and mechanical stimulation. In particular, in WT mice, the presence of TIMP-1 appears to delay the onset and persistence of mechanical sensitivity while having no effect on thermal reactivity during mild inflammation. More broadly, others have argued that mechanical sensitivity is a hallmark sign of pathological pain states (36, 85), and our results imply that the dysregulation in TIMP-1 signaling may contribute to this process by influencing hypersensitivity to mechanical stimulation.

Given that thermal hyperalgesia and prolonged mechanical hypersensitivity were observed in T1KO, but not WT mice, following mild inflammation, our data suggest that the absence of TIMP-1 increases susceptibility to subtle perturbations that would otherwise be considered innocuous. Indeed, we found that injections of normal physiological saline produced mechanical hypersensitivity in T1KO mice, which was not observed in WT controls. This hypersensitivity
could be due to a number of plausible factors, including hypertonicity (217-220), the disruption of cutaneous integrity from needle insertion, cutaneous distention following injection, or the induction of some inflammatory process. Whatever the cause of hypersensitivity is following saline injection, it is tempting to postulate that when TIMP-1 is not present, the physiological processes in the periphery become dysfunctional and sensory stimulation is amplified.

Pathological pain is also characterized by increased sensitivity in tissues adjacent to, and distal from, the site of inflammation, as is the case with “mirror image” pain (221, 222). When we assessed sensitivity in tissue adjacent to the site of inflammation and that are innervated by different sets of afferent terminal endings (e.g., hairy vs. glabrous skin), we found that T1KO mice exhibited mechanical hypersensitivity. Interestingly, we observed hypersensitivity in the uninflamed (contralateral) hindpaw, and the sensitivity occurred in a different dermatome from the inflamed dermatome. Administration of rmTIMP-1 at the site of inflammation alleviated hypersensitivity on both the inflamed and uninflamed paws. These data suggest that inflammation-induced TIMP-1 expression occurs in a coordinated fashion that influences the normal progression of inflammatory sensitivity in both inflamed and uninflamed tissues, which may attenuate afferent input and prevent the development of central sensitization.

TIMP-1 is well known as an inhibitor of MMPs, and we know that MMPs contribute to pain following various injury and inflammatory conditions (135, 200). We hypothesized that disrupting the balance between TIMP and MMP expression and activity would exacerbate hypersensitivity in T1KO mice due to elevated MMP activity and pro-inflammatory cytokine expression. However, we did not detect any genotype-specific differences in the activity of MMP-9 or pro-inflammatory cytokines proximal to the site of CFA administration. Prior work indeed shows that the inhibition of MMPs reduces pain (111, 135), and our current results demonstrate
that administration of the N-terminal domain of TIMP-1, the domain responsible for MMP inhibition, attenuated hypersensitivity. We also show that administration of TIMP-1(C), the domain responsible for engaging receptor-mediated cell signaling events (159-161), also attenuates hypersensitivity following inflammation, suggesting that TIMP-1 may also delay the emergence of hypersensitivity through a novel receptor-mediated mechanism. Consequently, TIMP-1 may attenuate the development of pain through both pathways. This latter point may help to explain, at least in part, why small molecule inhibitors of MMP activity have limited efficacy (138). By understanding how both subdomains alleviate hypersensitivity, we may be able to effectively manage pain progression.

While our results show that TIMP-1 attenuates pain and hypersensitivity through both MMP inhibition and receptor-mediated signaling, the precise mechanisms by which TIMP-1 acts are not known. Our data suggest that the amount of TIMP-1 present at the site of inflammation may determine functional outcomes, which is consistent with previously published work (141). The function of TIMP-1 is also determined by the specific interactions TIMP-1 has with its binding partners, which includes both proteases and membrane-bound receptors. We have yet to identify which receptor is responsible for the antinociceptive properties of TIMP-1, but it is known that TIMP-1 binds and activates the CD63/β1 integrin receptor complex (158-161). Interestingly, prior work has shown that interfering with the interaction between β1 integrin and versican attenuates inflammatory and neuropathic pain, as well as nociceptor activity(223, 224). Consequently, keratinocyte-derived TIMP-1 may bind to CD63/β1 integrin expressed on cutaneous nerve endings ultimately, attenuating primary afferent function. While primary afferents are known to express β1 integrin, it is unclear whether they also express CD63. If neurons do not express CD63 there may be an indirect pathway involving other cell types, such as mast cells or keratinocytes, where
TIMP-1 may bind to CD63 to alter the release pronociceptive molecules that influence neuronal function (192, 193, 225). Interestingly, disrupting the activity of β1 integrin prevents persistent pain in a model of hyperalgesic priming while leaving the acute phase of sensitivity intact (224). Therefore, stimulating TIMP-1 release, or the direct delivery of TIMP-1, may prevent the emergence of pathological pain, through β1 integrin activation, while leaving the capacity to detect normally painful stimuli unaffected.

Finally, demonstrating that TIMP-1 alleviates ongoing pain in WT mice we show that TIMP-1 is a potential clinical target for therapeutic intervention. It may be possible that TIMP-1 acts as a physiological “brake” on the nociceptive system to prevent overexcitation of primary afferents and the development of centralized pain states. Consequently, targeting TIMP-1 may have therapeutic benefit for both peripheral and central pain. For example, while we did not directly assess hypersensitivity at somatic regions beyond the contralateral hindpaw, our data may have implications for understanding the underlying mechanisms of widespread pain syndromes, such as fibromyalgia. Our data may also have implications for understanding metastatic processes in nonpainful forms of cancer. Indeed, TIMP-1 has been studied extensively in cancer (160, 226, 227) and is therefore of significant interest for determining how metastases develop without producing pain. It is not yet clear whether painful and nonpainful cancers differentially express TIMP-1 or whether TIMP-1 receptor binding kinetics are altered in painful and non-painful cancers. Understanding these dynamics may have clinical implications for developing early cancer detection strategies, especially if TIMP-1 is considered a regulator of pain state and not just one involved in tissue remodeling. Finally, if TIMP-1 can be utilized as a target for attenuating pathological pain, TIMP-1 may serve as an alternative to opioid-based medicines. This possibility is intriguing given our data showing that peripheral administration of TIMP-1 has antinociceptive
properties and prevents the spread of sensitivity to uninflamed somatic regions. However, these conclusions should be taken with some caution as research has shown that excess TIMP-1 expression, specifically through genetic overexpression, may lead to unintended adverse events (141). Therefore, future research should focus on illuminating the mechanisms by which TIMP-1 attenuates pain and hypersensitivity and how targeting this system may be therapeutically beneficial.
VI. Acknowledgements

Research was supported by the National MS Society grant ‘(SJC).

This manuscript has been released as a Pre-Print at bio-Rxiv (doi: https://doi.org/10.1101/540724).

We would like to thank Ms. Nicole Glidden and Ms. Jessica Yasko for their technical assistance.
VII. Figures

A

Spinal Cord

TIMP-1 (ng/mL)

BL 1 3 5 7

Days Post Inflammation

B

DRG

TIMP-1 (ng/mL)

BL 1 3 5 7

Days Post Inflammation

C

Hairy Skin

TIMP-1 (ng/mL)

BL 1 3 5 7

Days Post Inflammation
Figure 3-1: Assessing TIMP-1 expression along peripheral nociceptive circuit following cutaneous inflammation. (A) Cutaneous inflammation does not alter overall TIMP-1 protein expression in lumbar spinal cord or (B) DRG but does increase protein expression in (C) hairy skin. n=4/condition, * indicate significant differences compared to naïve controls, $p<0.05$, and error bars depict SEM.
Figure 3-2: Cellular colocalization of TIMP-1 expression. (A) IHC (20X) of naïve and inflamed lumbar spinal cord 24 hr following inflammation. TIMP-1 (green) expression is localized to cells positive for GFAP (red). n=3/condition, scale bar 20 μm. (B) IHC (20X) of naïve and inflamed lumbar DRG 24 hr following inflammation. TIMP-1 (green) expression is colocalized with cells positive for GFAP (red). n=3/condition, scale bar 20 μm. (C) IHC (40X) of hindpaw hairy skin shows cells positive for K14 (red) upregulate TIMP-1 (green) 24hr following inflammation compared to naïve control. n=3/condition, scale bar 50 μm.
Mechanical allodynia

![Graph showing mechanical allodynia over days post inflammation. The graph compares WT Naive and WT CFA groups.](image)
Figure 3-3: Development of inflammatory hypersensitivity in WT mice. Assessment of mechanical hypersensitivity over 7 days following s.c. administration of CFA. Inflamed mice show a significant reduction in mechanical thresholds relative to naïve mice 3 days following inflammation (n=6/condition). * indicate significant differences compared to naïve controls, p<0.05, and error bars depict SEM.
Figure 3-4: Mice lacking TIMP-1 develop thermal and mechanical hypersensitivity following cutaneous inflammation. (A) No differences in baseline thermal PWL are exhibited between T1KO and WT mice (n=16/condition). (B) Inflamed T1KO mice exhibit significantly reduced PWLs compared to inflamed WT mice and naïve WT and T1KO mice (n=8/condition). (C) Baseline assessment of mechanical PWTs revealed no genotypic differences between T1KO (n=20) and WT mice (n=18). (D) T1KO mice develop significantly reduced PWTs 1, 5, and 7 days following CFA administration compared to WT controls (n=8-10/condition). $p<0.05$, and error bars depict SEM.
A  

**Timp2**  

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>T1KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

B  

**Timp4**  

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>T1KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 3-5: Assessment of cutaneous Timp2 and Timp4 mRNA expression following inflammation. (A) Timp2 mRNA expression is decreased in T1KO mouse skin 1 day following inflammation relative to WT controls. (B) Timp4 mRNA expression is decreased 1 day following CFA compared to WT inflamed mice. n=4/condition, * indicate significant differences compared to naïve controls, p<0.05, and error bars depict SEM.
**Figure 3-6: Mice lacking TIMP-1 show increased sensitivity in non-inflamed tissues.** (A) Injection of CFA into the hairy skin causes mechanical hypersensitivity on the plantar surface of the paw to develop 1 day following inflammation in T1KO, but not WT, mice. (B) Graph depicting mechanical responsiveness following inflammation collapsed across time. Inflamed T1KO mice greater mechanical sensitivity overall following cutaneous inflammation. (C) Administration of TIMP-1(FL), TIMP-1(N), or TIMP-1(C) into the hairy skin at the time of inflammation prevents the development of mechanical hypersensitivity in T1KO mice. (D) Hindpaw administration of CFA produces mechanical hypersensitivity on the paw contralateral to inflammation in T1KO relative to WT mice. Treatment with rmTIMP-1 attenuated contralateral hypersensitivity in T1KO mice. PWT are presented as change from baseline. n=8/condition, * represent significant differences relative to naïve controls, $p<0.05$, and error bars depict SEM.
Figure 3-7: Inflammation does not alter pro-inflammatory molecules in a genotype-specific manner. A) Cutaneous inflammation significantly increases MMP-9 protein expression in WT and T1KO skin 1 day following CFA administration (n=7/condition). B) Cutaneous inflammation increases MMP-9 activity in WT and T1KO hairy skin 1 day following CFA administration. C) Cutaneous inflammation significantly increases IL-1β protein expression in WT and T1KO hairy skin 1 day following inflammation. D) Cutaneous inflammation significantly increases IL-6 protein expression in WT and T1KO hairy skin 1 day following CFA administration. E) Cutaneous inflammation does not affect expression of TNF-α following CFA administration. F) Cutaneous inflammation does not affect expression of IL-10 protein in WT and T1KO skin following CFA administration. n=4/condition, * represent significant differences relative to naïve controls, \( p<0.05 \), and error bars depict SEM.
Figure 3-8: Replacement of TIMP-1 attenuates ongoing inflammatory pain in WT mice.

Comparison of pre-conditioning and post-conditioning time spent in the clonidine paired chamber show a significant increase in the post-conditioning time in the Vehicle/CFA treated mice, but not the rTIMP-1/CFA treated mice. *p<0.01 vs pre-conditioning time. Sample size CFA/rTIMP-1 = 9; CFA/Veh = 8. Error bars = S.E.M. In collaboration with T. King and J. Havelin.
Chapter 4:
Discussion and Future Directions

Chronic inflammatory pain is a pathological condition that can be accelerated by changes that occur in the microenvironment of damaged tissues. TIMP-1 is a highly inducible protein involved in wound healing and inflammation, and recent evidence suggests that TIMP-1 expression may also delay the emergence of pain (135). Yet, only a few reports have investigated the functional role of TIMPs in the peripheral nervous system. This dissertation focuses on characterizing how inflammation-induced TIMP-1 expression affects the progression of pain. My data suggests that following CFA-injection, TIMP-1 is induced in a temporal and spatial dependent-manner to reduce inflammatory hyperalgesia. Using TIMP-1 KO (T1KO) mice, I showed that endogenous TIMP-1 expression reduces primary hyperalgesia which may affect the development of pathological pain-like behaviors such as alldynia and contralateral hypersensitivity. Further, the replacement of recombinant TIMP-1 at the site of inflammation reduced both alldynia and contralateral pain. Although, I did not detect any genotype-specific differences in MMP activity or the expression of common algogenic molecules, administering the signaling peptide, TIMP-1 (C), in T1KO mice attenuated hypersensitivity. Therefore, during acute inflammation TIMP-1 reduces hypersensitivity, in part, through a novel receptor-mediated mechanism which may protect against the emergence of pathological pain. Although I focused on the role of TIMP-1 during cutaneous inflammation, these findings may provide insight to how TIMP-1 attenuates nerve injury pain and other conditions that have an inflammatory component.

I. TIMP-1 and inflammatory hyperalgesia

Clinical interest in the role of TIMP-1 during disease progression has provided evidence that TIMP-1 may be neuroprotective during inflammation (141). In order to determine how TIMP-
I may be protective and affect nociception, I first needed to know how inflammation alters the expression of TIMP-1 along the peripheral nociceptive circuit. It is well established that CFA injected into tissue produces a localized inflammatory response (72). This was verified in my experimental paradigm to which cutaneous administration of CFA significantly upregulated cutaneous TIMP-1 protein levels for 7-days post-injection (Refer to Figure 3.1C). Within 1-day post-injection, I found that TIMP-1 increased 8.3-fold and then increased another 0.42-fold at 3-day post-injection (Figure 4.1). When I assessed the progression of mechanical hyperalgesia to the temporal expression of cutaneous TIMP-1, I observed that although absolute protein expression was greatest at day 3, when hypersensitivity emerged in WT mice, the largest increase in TIMP-1 expression occurred before the onset of hyperalgesia. This change in TIMP-1 expression, between baseline and 1 day, suggests that the relative change in expression, rather than absolute levels of TIMP-1, may determine the functional effect of TIMP-1.

In experimental inflammatory models, TIMP-1 is often described as an immediate protein that is induced during the acute phase of inflammation (180, 181, 215). In human ulcers, such as surgical wounds or UVB-induced photodamage, TIMP-1 is upregulated by keratinocytes to provide protection during the wound healing response, either by suppressing apoptosis or attenuating the synthesis of TNFα (173, 228, 229). I also found that during the acute phase of CFA-induced inflammation that TIMP-1 was upregulated in keratinocytes. Therefore, the induction of TIMP-1 may be neuroprotective by attenuating the expression of algogenic molecules, such as TNFα, resulting in antinociception (141, 181, 191-193). To examine this further, future studies should interrogate cell-specific expression of TIMP-1, either through murine conditional gene knockout or knockdown methods, to assess whether keratinocyte-derived TIMP-1 reduces nociception during inflammatory conditions.
In comparison to skin, TIMP-1 expression in the SC and DRG remained unchanged over 7-days. Although, I did not detect changes in TIMP-1 protein levels in these tissues this result may be indicative of milder tissue damage produced by diluted CFA (1:1). A similar result was reported in the gene array conducted by Parkitna and colleagues (168), rats that received subcutaneous injection of diluted CFA failed to show differential TIMP-1 mRNA expression in DRG or SC compared to rats that underwent CCI. In chapter 2, I suggested that the differential upregulation of TIMP-1 between CCI and CFA may be indicative of the roles TIMP-1 plays in varying levels of tissue damage. This is clinically relevant because patients that seek medical care have varying levels of inflammation as well as self-reported pain. Therefore, to determine whether TIMP-1 levels are differentially altered by injury severity and higher pain levels, I conducted a preliminary experiment whereby I assessed TIMP-1 protein levels following undiluted CFA (5uL; s.c.), a more severe inflammatory stimulus, administered in WT mice. Following injection, I assessed mechanical allodynia for 15-days and collected skin from an additional cohort of mice at 1-day post-CFA for TIMP-1 ELISA (230). In comparison to my previous experiment that assessed TIMP-1 levels following mild inflammation (refer to Figure 3.1C), I found that severe inflammation caused by non-diluted CFA resulted in a 50% reduction in TIMP-1 protein expression (Figure 4.2 A) that corresponded with an increase in mechanical hypersensitivity relative to naïve mice (Figure 4.2 B). These data suggest that varying levels inflammation affect the expression of TIMP-1, which may predict the development of pain-like behaviors. Specifically, increased injury severity may be associated with increased pain resulting from a reduction in TIMP-1 expression. If injury severity is positively associated with transition to a chronic state then, administering recombinant TIMP-1 might alleviate nociception and potentially, the transition to chronic pain. While my research suggests that there is a therapeutic benefit for the management of
inflammatory pain using TIMP-1, there is some indication that TIMP-1 may slow the rate of cutaneous wound healing (141, 174, 231, 232). If this is true, then future work should focus on identifying the optimal dosing for TIMP-1 therapeutic TIMP-1 administration that prevents adverse downstream effects.

II. Endogenous TIMP-1 expression attenuates pain-like behaviors

To address whether the upregulation of endogenous TIMP-1 is important for delaying the progression of hyperalgesia I compared the effect of cutaneous inflammation in global TIMP-1 KO mouse strain to responses in WT mice that share the C57BL/6 background. In response to mild inflammation, I found that KO mice exhibited increased mechanical and thermal sensitivity following inflammation relative to WT mice (refer to Figure 3.4B and D). Which begged the question, in general, are T1KO mice more hypersensitive? Although I did not detect any baseline differences to mechanical or thermal stimulation, I did observe that T1KO mice had increased responsiveness to mechanical stimulation following physiological saline that was not recapitulated in WT mice. These data imply that endogenous TIMP-1 expression is protective for suppressing hypersensitivity associated with even slight tissue damage generated by needle insertion and possibly, mechanical-stress induced by tissue distention.

During the acute phase of injury persistent activation of peripheral nociceptors may contribute to the development of central pain (233-235). In clinical cases of spinal cord injury and post-stroke pain, “early sensory hypersensitivity” that occurred immediately following injury increased the likelihood for patients to develop central pain (236-238). Similarly, in a study of patients that recently underwent surgery, patients that reported hyperaesthesia25 within 1 year of surgery were more at risk for developing persistent pain (239, 240). Therefore, to determine

25 Hyperaesthesia is excessive physical sensitivity, particularly of the skin.
whether the presence of TIMP-1 is protective for the development of pathological-like pain behaviors, I assessed mechanical allodynia and contralateral hypersensitivity in T1KO and WT mice. I found that mice lacking TIMP-1 developed rampant mechanical allodynia in non-inflamed skin ipsilateral and contralateral to CFA-administration. While WT mice showed brief development of mechanical alldynia, T1KO mice developed persistent alldynia. Moreover, the replacement of full-length recombinant TIMP-1 at the site of CFA-injection prevented these behaviors from developing in T1KO mice. These results suggest that in the absence of TIMP-1, prolonged cutaneous hypersensitivity may engage peripheral, and potentially central, mechanisms that ultimately lead to pathological pain-like behaviors but can be prevented by increasing the bioavailability of TIMP-1 at the site of injury. Therefore, it is possible that TIMP-1 released in inflamed tissue attenuates peripheral sensitization and prevents subsequent central changes that contribute to allodynia and mirror image pain. The question now becomes how does TIMP-1 affect persistent hyperalgesia and the development of alldynia?

III. TIMP-1 and the local inflammatory response

The resolution of inflammation is important for tissue homeostasis. Ideally, acute inflammation only persists while wound healing occurs however, the lack of resolution can lead to chronic pathological conditions (241). The resolution process was believed to be passive until the recent discovery that pro-resolving molecules actively orchestrate the inflammatory ceasefire (242-245). Of the few pro-resolving mediators previously identified, resolvin E1 (RvE1) and resolvin D1 (RvD1), which are biosynthesized from omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively, have remarkable efficacy for treating inflammation as well inflammatory pain induced by CFA as well as nerve-injury (246-248).
In my model, mice lacking TIMP-1 exhibited persistent inflammatory hyperalgesia and allodynia compared to their WT controls which suggested that TIMP-1 may recruit molecules involved in the resolution of inflammation. To determine whether TIMP-1 regulates the expression these molecules, I used ELISAs to assay total expression of RvD1 and RvE1 in hairy skin for 7 days following inflammation. When I examined RvD1 expression, I found T1KO mice had overall, significantly decreased RvD1 expression over 7 days post-CFA (Figures 4.3B). RvD1 has been shown to produce antinociception through the modulation of temperature-sensitive transient receptor potential ion channels, or TRP channels, expressed by nociceptors (e.g., TRPA1, TRPV3, and TRPV4). It is possible that in the absence of TIMP-1, and the reduction of RvD1, prolongs the activity of certain TRP channels on peripheral nociceptors leading to persistent hyperalgesia. In comparison, RvE1 expression was significant increased at baseline and 7 days post-CFA in T1KO mice (Figure 4.3C). RvE1 is known to suppress neutrophil infiltration, paw edema, and proinflammatory cytokine expression as well as attenuate mechanical allodynia and thermal hyperalgesia caused by TNFα and microglial activation in the spinal cord (246, 247). Therefore, in mice lacking TIMP-1, elevated cutaneous RvE1 expression may compensate for the loss of TIMP-1 during the inflammatory process. Given the remarkable potency of resolvins and well-known side effects of opioids and COX inhibitors, future studies that address the relationship between TIMP-1 and resolvins may further explain their antinociceptive affects.

IV. TIMP-1 receptor attenuates hyperalgesia

The extracellular environment consists of a dynamic network of proteins and cells that are connected by cell adhesion receptors. Cell adhesion receptors, such as integrin receptors, tether cells by attaching their intercellular intermediate filaments to the basement membrane of tissues (249). As a result, changes in the extracellular environment, such as ECM remodeling during tissue
damage, are communicated through integrin receptors. The bidirectional communication between integrin receptors and ECM allows cells to sense the changing environment during mechanical or osmotic stress by activating downstream intracellular pathways that can affect cellular functions important for maintaining tissue homeostasis. During nerve injury and inflammation, degraded ECM proteins bind to integrin receptors on nociceptors and increase their excitability through the MAPK/ERK pathway \((224)\). Supporting this, our data also show that administration of TIMP-1(C), which binds directly to CD63 \((161)\), is effective at reducing inflammatory hypersensitivity. Given that TIMP-1 is cleared in minutes to hours following administration \((250, 251)\), and that the effects of exogenous TIMP-1 administration can last days following a single injection of rmTIMP-1, I hypothesize that TIMP-1 may signal to maintain a balance between antinociception or hypersensitivity following tissue damage.

Because it was not possible for me to directly manipulate CD63 (due to the lack of availability of knockout mouse strains or functional-grade antibodies), I manipulated \(\beta_1\) integrin activity with a function-blocking antibody, Ha2/5, administered at the time of inflammation. I found that Ha2/5 administration \((10\text{ng/\muL; s.c.})\) alleviated mechanical hypersensitivity compared to inflamed T1KO mice that did not receive Ha2/5 (Figure 4.5 A, B). This result indicates that in the absence of TIMP-1 the activation of \(\beta_1\) integrin contributes to the emergence of hypersensitivity during inflammation. I propose that cutaneous inflammation upregulates keratinocyte-derived TIMP-1 to delay the onset of hypersensitivity through the activation of CD63 and that in the absence of TIMP-1, stimulation of \(\beta_1\) integrin receptor by the inflammatory milieu, causes the emergence of inflammatory hypersensitivity. It is possible that independent activation of CD63 or \(\beta_1\) integrin results in antinociception or hypersensitivity, respectively.
It has been proposed that CD63 and β1 integrin may work in a competitive fashion to facilitate cellular functions (140, 158, 252). Specifically, TIMP-1 activation of CD63 and β1-integrin was shown to activate β-catenin to facilitate oligodendrocyte maturation but competed with the canonical Wnt pathway that also activated β-catenin to promote cell migration (158). Perhaps, in a similar light, TIMP-1 mediated CD63 signaling and β1 integrin activation by ECM molecules compete to activate downstream effects that either result in antinociception or hypersensitivity. Perhaps, the induction of TIMP-1 functions as a physiological brake pedal to reduce the onset of nociception during tissue damage and protect against the development of long-term changes within the nervous system. Evidence for this occurred when mice lacking TIMP-1 were injected with physiological saline, the tissue distension or disruption of skin integrity of the injection may have stimulated β1 integrin on cutaneous nociceptors and caused rampant hyperalgesia that was not recapitulated in WT mice because TIMP-1 was available to reduce β1 integrin-induced hyperalgesia. Future directions include studying the downstream pathways of CD63 and β1 integrin to determine if they converge on similar effector proteins involved in antinociception that may become counteracted by the activation of β1 integrin (158). Another competing hypothesis is the involvement of Piezo family of protein receptors of which Piezo2 is a major transducer of mechanical stimulation (253). Sensory neuron expression of Piezo2 has been shown to modulate rapid adapting currents (254). Moreover, specific stimulation of Piezo2 by bradykinin, a potent inflammatory mediator, can sensitize sensory neurons and contribute to the development of mechanical allodynia (255). Therefore, it is possible that TIMP-1 receptor-mediated affects may counteract other known signaling cascades involved in mechanical allodynia and nociception as a whole. Future directions that aim to address how TIMP-1 signaling facilitates antinociception may provide insight to this receptor-mediated mechanism.
V. TIMP-1 and glial cells

Although neurons are required for the transduction of noxious stimuli, many studies suggest that the interactions between nervous system and nonneuronal cells can contribute to pain processing (132-134, 247, 256, 257). In the central nervous system astrocytes comprise approximately 50% of all cells (258, 259). As a whole, astrocytes are important for the developing nervous system because they provide metabolic and trophic support during neurogenesis (260, 261). During inflammation caused by experimental autoimmune encephalitis, excitotoxic injury, and kainite-induced seizures (185, 262, 263), astrocytes upregulate TIMP-1 around areas of immune infiltration and demyelination. One hypothesis for the function of astrocyte-derived TIMP-1 is to restrict neuroinflammation by decreasing immune cell extravasation into the spinal cord, cytokine release, and MMP-mediated myelin destruction (262). Other studies have shown that during inflammatory conditions astrocyte-specific TIMP-1 affects their phenotypic response to inflammatory modulators and their function during wound healing (252). Previous reports have shown in rats, that subcutaneous administration of CFA activates astrocytes in the spinal cord contribute to inflammation hyperalgesia (82, 264). Other studies using various methods of inflammation have shown that activated astrocytes facilitate the mechanical allodynia (265).

To determine whether cutaneous CFA-injection affected spinal astrocytes and if the absence of TIMP-1 altered their phenotype, I isolated lumbar L2/L3 spinal cord segments from wildtype and T1KO mice and examined expression of GFAP using IHC. Interestingly, I found that the GFAP expression was enhanced in T1KO mice relative to WT controls (Figure 4.4A), suggesting that acute inflammation results in reactive astrogliosis throughout the spinal cord (266, 267). Perhaps in the absence of TIMP-1, similar to what has been reported in nerve injury, spinal astrocytes become reactive and less efficient at regulating extracellular glutamate or they release
neuroactive molecules leading to neuronal excitability (268-270). Therefore, in WT mice, astrocytic-derived TIMP-1 may serve a protective function to provide metabolic support for activated neurons and/or restrict neuroinflammation within the spinal cord thus reducing hyperresponsiveness to noxious stimulation.

Previously, I showed that global TIMP-1KO mice displayed robust threshold changes to thermal and mechanical stimulation. However, I could not test whether TIMP-1 released from specific cell types contribute to the progression of pain following inflammation. Together, based on the enhanced GFAP expression in T1KO mice and what is known about reactive astrocytes in chronic pain, I postulated that astrocytic-derived TIMP-1 may reduce hypersensitivity associated with central mechanisms. To examine whether astrocyte-derived TIMP-1 regulates the development of mechanical allodynia, I subcutaneously administered CFA into transgenic mice with TIMP-1 deleted from GFAP+ cells (Crocker et al., unpublished) and assessed mechanical responding for 7 days. I found that compared to WT mice, deletion of TIMP-1 from GFAP+ cells resulted in persistent mechanical allodynia that did not resolve by 7 days (Figure 4.4B). Combined with my hypothesis that TIMP-1 is protective, what is understood about sex hormones, and that TIMP-1 expression in females in polymorphic, mosaic expression of TIMP-1 in females, may alter noxious sensation across different tissues (53, 148, 271). Understanding the role of TIMP-1 in pain sensitivity may provide implications that may improve sex-specific pain management.

Despite what is known about astrocytes, TIMP-1, and pain, I cannot rule out the contribution of TIMP-1 from other GFAP+ cells. In the peripheral nervous system, satellite glial cells (SGCs) appear similar to grey matter astrocytes, also called protoplasmic astrocytes, that exist in close proximity to neuronal cells bodies within the central nervous system (272). Both SGC and astrocytes express a variety of ion channels and transporters for neurotransmitters that can regulate
the extracellular space (273, 274). SGCs are commonly characterized by proteins commonly expressed by astrocytes, including gap junction proteins, Connexin 43, GFAP, the calcium sensing protein S100, as well as the inward rectifying potassium channel Kir4.1 (275-277). SGCs surround the cell bodies of individual primary afferents that reside in the dorsal root ganglia (DRG) and even form gap junctions with other SGCs in between neuronal cell bodies (Figure 4.6)(272, 278-281). Bidirectional communication between the cell bodies of sensory neurons and SGCs is important for metabolic support, survival, as well as functional support during pathological conditions (272). Neurons release glutamate, ATP, nitric oxide, CGRP and substance P, which can bind to receptors on SGCs and induce calcium signaling (256, 276, 277, 280, 281). Previous experiments that have assessed these interactions suggest that both SGCs and their companion neurons upregulate TIMP-1 mRNA following sciatic nerve transection (144). In my model of cutaneous inflammation, I did not report any changes in TIMP-1 expression in whole DRG protein lysates. However, IHC revealed a difference in TIMP-1 expression in cells surrounding the cell bodies of sensory neurons 1-day post-inflammation. Therefore, within the DRG, SGCs may upregulate TIMP-1 during tissue damage to attenuate nociceptive excitability.

A common method used to manage inflammatory pain is through the use of NSAIDs. NSAIDs inhibit COX activity and decrease the production of pro-inflammatory mediators. Specifically, COX-2 aids in the synthesis and release of PGE2. The release of PGE2 can stimulate E prostanoid receptors on nociceptors, which eventually causes peripheral sensitization through the transactivation of noxious heat transducer TRPV1 (282, 283) and the voltage-gated sodium channel, NaV1.8 (284-286). The inhibition of COX-2 decreases inflammation, neuronal excitability, and pain behaviors (98, 287, 288). Interestingly, the activation of nociceptors and surrounding SGCs has been both shown to increase expression of COX-2 (109). Then LPS-
induced inflammation was shown to activate toll-like receptor-4 (TLR4) signaling on SGCs which resulted in the activation of COX-2, as well as the upregulation of TNFα mRNA (289). In another study, the administration of a potent TLR4 receptor antagonist, called LPS-RSU, attenuated neuropathic pain in rats caused by CCI (290). They showed that antagonism of TLR4 altered the protein expression of pronociceptive molecules such as, IL-1β and IL-6, while also increasing the ratio of TIMP-1 to MMP-9 in both spinal cord and DRG. The authors suggest that antagonism of TLR4 may cause analgesia through the production of antinociceptive factors such as TIMP-1. Therefore, in the context of the GFAP conditional TIMP-1 KO mice, the absence of TIMP-1 expressed by SGCs in the DRG may contribute to the progression of hyperalgesia. To tease apart the functional contribution of TIMP-1 from astrocytes and SGCs in the development of hyperalgesia, future studies should administer TIMP-1 protein constructs directly into the DRG or spinal cord or examine inflammatory responses in new lines of transgenic mice targeting deletion of TIMP-1 under SGC-specific promoters. One such promoter could be foxd3, which is a marker of neural crest cells and peripheral glia (291). These data suggest that depending on the tissue, more proximal expression of TIMP-1 in innervated, inflamed tissues may be important for gating the onset of hyperalgesia or in more distal tissues, TIMP-1 may attenuate neuronal excitability associated with the development of allodynia and mirror image pain.

VI. TIMP-1 and clinical data

Often in human diseases MMPs and TIMPs have been used as biomarkers to assess neural disease progression (i.e. MS, diabetes, HIV-induced dementia, Parkinson’s disease, Alzheimer’s disease) (166, 292). One example of this is in diabetes-induced peripheral neuropathy (foot ulcerations), which is a common cause of amputations. Specifically, elevated levels of MMPs compared to TIMP-1 have been important physiological indicators of oxidative stress which may
cause foot ulcerations through oxidative stress and mitochondrial dysfunction in neurons (293, 294). Moreover, high glucose levels in fibroblasts increased MMP production and decreased TIMP-1 expression indicating a protective function of TIMP-1 during neuropathy, presumably through MMP mediated affects. In a paper published in 2016, revealed that diabetic patient derived fibroblasts treated with Myricetin26 increased TIMP-1 levels (by 72%) and decreased MMP-9 expression (by 68%) compared to fibroblasts that were untreated (295).

However, there are discrepancies throughout the literature regarding the relationship between TIMP-1 expression and disease severity (169, 292). For instance, serum levels of TIMP-1 from multiple sclerosis patients are similar or reduced compared to healthy controls. It is not clear why elevated MMP:TIMP ratios are inconsistent across different disease conditions, but this could be the result of alternatively spliced TIMP-1 mRNA (296). The gene that codes for TIMP-1 is located on the X-chromosome and is comprised of 6 exons (263). In humans, several splice variants have been detected however the role of each variant in regulating inflammatory or neuropathic pain has yet to be defined (296, 297). Using cancer cell lines, Usher and colleagues identified that TIMP-1 mRNA variants which lack either, exon 2 or exon 5 in various cancer cell lines (297). The former is commonly referred to as TIMP1-v2 and is unique because it lacks the signal peptide sequence or C terminus domain (297). Suggesting that in humans, TIMP-1 may have different protein isoforms which may affect its functioning or contribute to its role in pathology. Moreover, systematic characterization of each variant across human disease conditions or research using preclinical mouse models has not been fully conducted thus, leaving many questions about the function of each variant in pain. However, one could postulate that in chronic pain patients that have elevated levels of systemic TIMP-1, these individuals may express TIMP1-

---

26 Bioflavonoid found in tea, berries, fruits, and vegetables is known for its anti-inflammatory effects
v2 whereby, the antinociceptive effect of TIMP-1 is absent because the C terminus is missing. In addition, since TIMP-1 is X-linked perhaps mosaic expression in females contributes to underlying pain severity. Addressing the expression of TIMP-1 isoforms across disease conditions and different human populations may resolve some of the discrepancies between the protective effects and the disease-promoting effects of TIMP-1.

Others have proposed an alternative view of TIMP-1 function, that the balance of free, unbound TIMP-1 and the proteolytic response to tissue damage can differentially affect the functional outcome of TIMP-1 (141). For example, in conditions where MMP activity is elevated, and TIMP-1 is primarily bound to MMPs, TIMP-1 may interact with different receptors that result in signaling (141). In contrast, overexpression of TIMP-1 can impede MMP driven proteolysis of ECM proteins (298) and mediate fibrosis, by binding CD63–Integrin β1 receptor complex expressed by endothelial cells (159). Moreover, Kawasaki and colleagues showed that mice lacking MMP-9 had an attenuated pain phenotype following peripheral nerve injury [Kawasaki, 2008 #71]. This could be a direct consequence of the absence MMP-9 or the result of increased MMP-free unbound TIMP-1 which is available to bind cell-receptors, such as CD63 on primary afferents, resulting in antinociception. I showed using a dose-response curve that pain was only attenuated in mice that were administered 10ng/μL of rmTIMP-1 but did with 100ng/μL concentration (Figure 4.7). This suggests that at higher concentrations, TIMP-1 may saturate potential binding partners or competitively inhibit both analgesia and nociceptive mechanisms that ultimately, contribute to disease. Therefore, the amount of TIMP-1 that can bind to cell receptors may determine functional outcomes. Combined with the knowledge that in humans TIMP-1 is alternately spliced, administering effective doses of TIMP-1 (C) terminus peptide may provide an novel solution to attenuating pathological pain by directly affecting the nociceptive circuit.
VII. Concluding Statement

My thesis research on the role of TIMP-1 in the development of hyperalgesia provides insight to the diversity functions TIMP-1 may play in the pathology of pain. In addition, these findings suggest that assessing the concentration of TIMP-1 at the site of hypersensitivity may indicate pain severity following tissue damage and that the administration of recombinant TIMP-1 may limit peripheral hypersensitivity as well as the central processes involved in pathological pain. Further research is required to determine if this mechanism directly affects nociceptors through the activation of CD63 or another signaling receptor to attenuate hypersensitivity. Compared to an indirect consequence of TIMP-1 binding to nonneuronal cell populations, such as keratinocytes or glial cells, to prevent the release of algogenic molecules. These experiments will be important for determining the mechanism of TIMP-1-induced antinociception or analgesia. I strongly believe that understanding the receptor-mediated functions of TIMP-1 in the context of pain conditions may provide an innovative, non-opioid based strategy for attenuating pain associated with both nociceptive and non-nociceptive mechanisms of chronic pain.
Figure 4-1: Calculation of delta TIMP-1 protein expression. Change in TIMP-1 expression, or delta, was calculated between consecutive days following CFA-injection (adapted from Figure 3-1C). The greatest change occurred between baseline and 1-day following CFA compared to the difference between 1 and 3-days post-injection, when absolute levels of TIMP-1 were the highest. p<0.05, and error bars depict SEM.
Figure 4-2: Assessment of TIMP-1 in varying levels of inflammation. A) Increasing CFA concentration decreases levels of cutaneous TIMP-1 expression and B) and severity of mechanical hypersensitivity in WT mice. * = indicates statistical significance relative to naïve controls, and # indicates statistical significance relative to CFA conditions, \( p<0.05 \), and error bars depict SEM.
A

Bar graph showing the levels of RvD1 in WT and T1KO genotypes over 7 days post inflammation. The x-axis represents days post inflammation (naive, 1, 3, 5, 7), and the y-axis represents pg/mL. The graph illustrates a significant increase in RvD1 levels over time for both genotypes.

B

Bar graph showing the levels of RvD1 in WT and T1KO genotypes. The x-axis represents genotypes (WT, T1KO), and the y-axis represents pg/mL. The graph indicates a statistically significant difference (*).

C

Bar graph showing the levels of RvE1 in WT and T1KO genotypes over 7 days post inflammation. The x-axis represents days post inflammation (naive, 1, 3, 5, 7), and the y-axis represents pg/mL. The graph illustrates a significant increase in RvE1 levels over time for both genotypes.
Figure 4-3: Mice lacking TIMP-1 have altered expression of proresolving mediators. A) Analysis of time course of cutaneous RvD1 expression in WT and T1KO mice using ELISA. B) Average RvD1 expression over 7 days is significantly decreased in T1KO mice. C) Analysis of time course of cutaneous RvE1 expression in WT and T1KO mice using ELISA. T1KO mice have elevated expression of RvE1 at baseline and 7 days post-CFA administration. n=4/condition, \( p<0.05 \), and error bars depict SEM.
Figure 4-4: Astrocyte-derived TIMP-1 attenuates persistent inflammatory pain. Lumbar spinal cord staining for GFAP expression at 1-day post-CFA. Lumbar L2L3 spinal cord segments were isolated from 4% PFA perfused wildtype and T1KO mice, embedded in OCT, and cut at 20 um sections. Slices were stained for Glial fibrillary acidic protein (GFAP) conjugated to cy3 (red) to image astrocytes and DAPI (blue) to image cell nuclei. Compliments of S. Crocker lab. B) WT and conditional knockout mice that have inactivated TIMP-1 in cells that express GFAP (T1cKO; Crocker et al., unpublished) received subcutaneous CFA injections (10 μL, s.c.) and mechanical allodynia was assessed for 7 days. Compared to WT mice, GFAP -T1cKO mice remain hypersensitive at day 7. n=6, p<0.05, and error bars depict SEM.
Figure 4-5: Blocking β1 integrin activity attenuates hypersensitivity in T1KO mice. T1KO mice at the time of CFA injection (10 µL, s.c.) received injections of monoclonal anti-rat CD29 Ha2/5 (10 ng/µL, 10 µL, s.c.; BD Pharmingen, Franklin Lakes, NJ.) to antagonize β1 integrin signaling. (A) Blocking activity of β1 integrin with Ha2/5 prevents inflammation-induced hypersensitivity following CFA administration. Mechanical thresholds were assessed for 7 days following inflammation and are presented as change from baseline. (B) Averaged PWT across time. Inflamed T1KO mice that received Ha2/5 exhibit significantly higher mechanical thresholds overall compared to T1KO mice. T1KO mice administered Ha2/5 at the time of inflammation exhibit mechanical thresholds that are not different from naïve T1KO mice (n=8/condition). Mechanical thresholds were assessed for 7 days following inflammation and are presented as change from baseline (statistics). *p<0.05, error bars depict SEM, *significant compared to naïve control, # significant compared to experimental control.
Figure 4-6: 3D surface rendering of SGCs around neurons from naive DRG using CLARITY. PFA perfused naïve lumbar DRG from a mouse line expressing tdTomato under the control of the GFAP-gene were stained for NeuN. We used a modified version of the method presented in Yang et al. (299). Images were taken on Zeiss LSM 780 confocal microscope at 30X, 2μm step. Bitplane IMARIS x64 version 8.2.1. was used for image processing. (top) Z-stack images of naive DRG from GFAP-tdTomato using CLARITY. Neurons were immunostained for NeuN+ (green) and GFAP+ SGCs express tdTomato and fluoresce red. 9x magnification, 2μm step. (bottom) 3D surface rendering of SGCs around neurons 30X magnification, 2μm step. In collaboration with Stephen Yeung.
Mechanical Allodynia

Change from BL (g)

Vehicle
1 ng/uL
10 ng/uL
100 ng/uL

Days Post Inflammation

1 3 5 7
Figure 4-7: Assessment of mechanical allodynia during dose response curve of rmTIMP-1 administration. T1KO mice that received 10ng/uL rmTIMP-1 (N) at the time of CFA-injection showed attenuated mechanical allodynia 24 hours post-injection. Other doses of TIMP-1, at 1ng/uL or 100ng/uL were not significantly different from inflamed T1KO mice at any timepoint following injection.

C. Brifault, H. Kwon, W. M. Campana, S. L. Gonias, LRP1 deficiency in microglia blocks neuro-inflammation in the spinal dorsal horn and neuropathic pain processing. *Glia* **0**.


119


B. Wu *et al.*, NMR Structure of Tissue Inhibitor of Metalloproteinases-1 Implicates Localized Induced Fit in Recognition of Matrix Metalloproteinases. (2000).


185. S. Rivera et al., Tissue inhibitor of metalloproteinases-1 (TIMP-1) is differentially induced in neurons and astrocytes after seizures: evidence for developmental, immediate early gene, and lesion response. *Journal of Neuroscience* 17, 4223-4235 (1997).
206. M. Lee et al., Tissue inhibitor of metalloproteinase 1 regulates resistance to infection. *Infection and Immunity* 73, 4 (2005).


