# One cell, two cell, female cell, male cell: Sex Differences in Immune Cell Activation

by

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# Abstract

The overarching idea of this thesis stemmed from previous work in our lab, where it was noticed that females and males exhibited different disease trajectories in a model of Multiple Sclerosis. Combined with our observation of different outcome metrics for peripheral nociception suggested that something interesting was happening in the female peripheral nervous that may not be happening in males, or to the same extent. This idea is also relevant to clinical MS and other autoimmune diseases, where women not only have different incidence rates than men but also exhibit subtleties in disability and chronic pain. It is well established that there are sex differences in immune cell activation or pathways in immune activation, so we aimed to investigate if these differences were present specifically in the peripheral nervous system structures responsible for pain signal generation.

To begin my investigation, I took a general approach and aimed to look from an overhead view by RNA sequencing male and female DRGs to identify major differences. This encompassed most of Chapter 2, where I analyzed bulk RNA and miR sequencing data from male and female DRGs. I discovered that the female DRGs had an incredible amount of both gene and miR dysregulation, focusing on genes related to the innate immune system and phagocytic roles. I discovered that miR-21a-5p, a microRNA previously known to be upregulated in CNS tissues in MS and EAE, was also upregulated in the DRG of both sexes. I identified a candidate gene for follow-up investigation (TLR7) as it was highly upregulated in the female DRGs and is well described in phagocytic innate immune cells.

Chapter 3 took a reductionist approach by focusing on the innate immune cells indicated in the first chapter. I stimulated male and female cells with TNF-alpha, one of the cytokines

responsible for activating immune cells, as it was more prevalent in the female disease course in EAE and interesting in this system. I did not find anything that contradicted the previously described M1-like phenotype, but there were some interesting subtleties. Females had a much higher mitotic capability, as well as higher motility and sensitivity for activation. I found some differences in cytokine profiles, particularly in the levels of CCL12, CCL22, and IL-16 as standout candidates for sex differential effects, which may have knock-on effects on inflammatory pain. This suggests that this cytokine profile could potentially help resolve inflammatory pain.

In Chapter 4, I continued the reductionist approach and shifted the *in vitro* system from bone marrow-derived macrophages to dorsal ganglion neurons. By culturing neurons in different types of inflammatory conditioned media, we investigated the plastic properties that these medias could impart on the neurons. Interestingly, I found that the conditions exhibiting high amounts of growth also contained neurons with highly spontaneous electrical activity immediately after plating, which then became quiescent over time. I further explored the link between excitability and plasticity by modulating excitability using pharmacokinetic tools and discovered that exogenous silencing of neurons prevented later outgrowth.

This thesis aimed to investigate sex differences in the innate immune system and their potential implications in peripheral pain signal generation in Multiple Sclerosis. Through a combination of bioinformatics, *in vitro* cell culture, and pharmacological approaches, I discovered unique effects of the innate immune system on each sex of neurons. My findings suggest that understanding the molecular mechanisms of these pathways may provide valuable information to guide pharmacological therapeutics that could benefit one or both sexes. Furthermore, my work highlights the importance of considering sex differences in autoimmune diseases such as

Multiple Sclerosis, as they not only have different incidence rates between the sexes but also exhibit subtleties in disability and chronic pain. Ultimately, my research provides insight into the role of the innate immune system in sex-specific pain signaling and identifies potential targets for future therapeutic interventions.

# Preface

This thesis is an original work by Timothy N. Friedman. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Assessing sensory function in EAE", AUP00000274, Approved Fall 2007.

Parts of the introduction chapter were written as part of my thesis proposal, *One cell, two cell, female cell, male cell: Sex differences in Immune Cell Activation*, (October 2019).

Chapter 2 of this thesis has been published as:

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I was responsible for concept formation, data collection, analysis, and manuscript composition. MSY, AC, and MD collected data. CAJ and AEF were involved with editing the manuscript. BJK was the supervisory author involved with concept formation and manuscript edits.

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I was responsible for concept formation, data collection, analysis, and manuscript composition. OC and CZ collected data and analyzed samples. KL and JM assisted with sample preparation and data collection. JM, TB, JRP, and AMWT were involved with editing the manuscript. BJK was the supervisory author involved with concept formation and manuscript edits.

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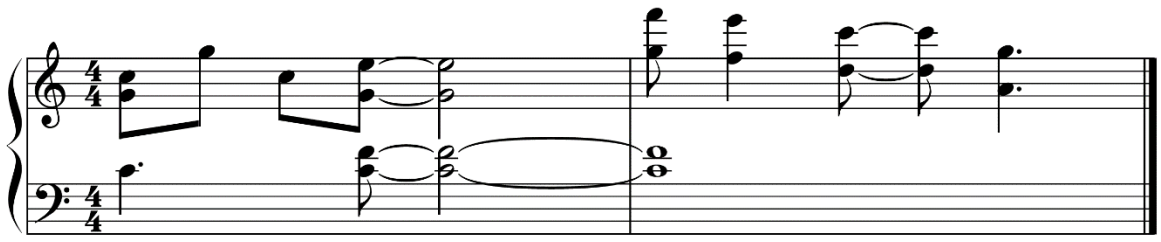
I was responsible for concept formation, data collection, analysis, and manuscript composition. SML, and TH collected data. SML, HTK, and JRP were involved with editing the manuscript. BJK was the supervisory author involved with concept formation and manuscript edits.

# Dedication

To Sharon:

I distinctly remember a conversation where we decided that our career plan was to graduate with doctorates and run a lab together. It wasn't a very well thought out plan but we were barely adults and plans at that age rarely are. I am sorry that you aren't here to see it but I want you to know that part of this belongs to you.

We did it. Rest in peace.



# Acknowledgements

I've heard the phrase that pursuing a PhD is a marathon, not a sprint. Well, I've always been a sprinter so learning to slow down, pace myself, and enjoy the journey has been a (frustratingly) wonderful experience.

Thank you:

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To my family who have been with me since the beginning,

and to my best friends who have been with me for almost as long.

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# Abbreviations and Symbols

| | |
|---|---|
| **IASP** | International Association for the Study of Pain |
| **DRG** | Dorsal Root Ganglion |
| **CNS** | Central Nervous System |
| **PNS** | Peripheral Nervous System |
| **CIP** | Congenital Insensitivity to Pain |
| **TNFα** | Tumor Necrosis Factor Alpha |
| **CGRP** | Calcitonin Gene Related Peptide |
| **NFκB** | Nuclear Factor Kappa-light-chain-enhancer of activated B |
| **MAPK** | Mitogen-Activated Protein Kinase |
| **IL** | Interleukin |
| **PAMP** | Pathogen Associated Molecular Pattern |
| **DAMP** | Damage Associated Molecular Pattern |
| **TLR** | Toll-Like Receptor |
| **ROS** | Reactive Oxygen Species |
| **COX** | Cyclooxygenase |
| **NSAID** | Non-Steroidal Anti-Inflammatory Drug |
| **MS** | Multiple Sclerosis |
| **EAE** | Experimental Autoimmune Encephalomyelitis |
| **MOG35-55** | Myelin Oligodendrocyte Glyocoprotein (35-55) |
| **CFA** | Complete Freund's Adjuvant |
| **DPI** | Days Post Immunization |
| **RA** | Rheumatoid Arthritis |
| **SLE** | Systemic Lupus Erythematosus |
| **NMO** | Neuromyelitis Optic |
| **E2** | Estradiol |
| **NGS** | Next Generation Sequencing |
| **miR/miRNA** | microRNA |
| **DEG/DEmiR** | Differentially Expressed Gene/microRNA |
| **BMDM** | Bone Marrow Derived Macrophage |
| **CM** | Conditioned Media |
| **MCM** | Macrophage Conditioned Media |
| **T-MCM** | TNFα-stimulated Macrophage Conditioned Media |
| **IL-4-MCM** | IL-4-stimulated Macrophage Conditioned Media |
| **PFA** | Paraformaldehyde |
| **NDS** | Normal Donkey Serum |
| **(D)PBS** | (Dulbecco's) Phosphate Buffered Saline |
| **HBSS** | Hank's Buffered Salt Solution |
| **ANOVA** | Analysis of Variance |
| **SEM** | Standard Error of Mean |
| **RTG** | Retigabine |
| **KV** | Voltage-gated Potassium channel |
| **ROI** | Region of Interest |
| **AUC** | Area Under the Curve |

# Chapter 1: Introduction

## 1.1 The Burden of Pain:

Pain is a complex and multidimensional phenomenon that affects millions of people worldwide, leading to a significant burden on individuals, healthcare systems, and the economy[1]. Although difficult to estimate, the cost of lost work productivity due to pain is staggering and conservative estimates for European countries range in the billions for annual costs[2, 3] amount. Adults in the US lose approximately 5 hours of effective work each week to chronic pain, costing hundreds of billions of dollars each year[4, 5]. Additionally, the impact of pain on an individual's quality of life cannot be overlooked, with a significant decrease in overall well-being and functioning[6, 7, 8, 9, 10, 11]. Chronic pain syndrome and pain symptoms are prevalent in many populations, affecting approximately 20%[12, 13] or higher of adults worldwide. Furthermore, pain is often accompanied by comorbidities, such as depression and anxiety, as well as other types of diseases, such as cancer and arthritis, making it a challenging condition to manage. Therefore, it is crucial to understand the nuances of pain and its associated conditions to develop effective strategies for alleviating this burden.

### 1.1.1 What is pain?

To fully comprehend the magnitude of the pain problem, it is essential to understand what pain is and how it is defined. Pain is a complex sensory and emotional experience associated with actual or potential tissue damage. The International Association for the Study of Pain (IASP) defined pain in 1979 as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."[14] In 2020, IASP updated its definition to include the emphasis that pain is always a personal experience that is influenced to varying degrees by biological, psychological, and social factors[15].

Pain is a complex process that involves the activation of specialized sensory receptors called nociceptors, which detect potentially harmful stimuli, such as heat, cold, or mechanical pressure, in peripheral tissues. The term 'nociceptor' was coined in 1906 by Charles Scott Sherrington[16] from the Latin word for meaning to harm, 'nocere'. This definition was profoundly impactful on pain research by providing a clear direction of the need to understand the special sensation of harm.

It is worth noting that despite involving the detection of harm, pain is primarily a beneficial experience because it alerts individuals that something is wrong and requires attention. This adaptive mechanism allows individuals to respond to potentially harmful stimuli and protect themselves from further injury. Therefore, pain is not something we should aim to eliminate entirely. However, when pain becomes chronic or unmanageable, it can lead to significant physical, emotional, and economic burdens.

### 1.1.2 Nociception

It is essential to differentiate between the sensory experience of nociception and the perceptual construct of pain. While nociception refers to the detection and transmission of potentially harmful stimuli by specialized sensory receptors, pain is a complex perceptual experience that arises from the integration of sensory, emotional, and cognitive inputs.

In terms of anatomy, nociception involves the activation of specialized sensory neurons called nociceptors, whose cell bodies are located in the dorsal root ganglia (DRG) of the peripheral nervous system and innervate all peripheral tissues[17]. Although these neurons are also able to sense cool and warm temperature, the primary function of these neurons is to carry specific sensory information related to noxious stimuli, such as mechanical pressure[18], extreme temperatures[19, 20], and chemical irritants[21]. The nociceptor population of DRG neurons are the small-diameter, lightly

myelinated A-delta fibers, and the unmyelinated C fibers. These neurons are molecularly distinct[22, 23] from other sensory neurons, with unique receptor proteins that enable their nociceptive capabilities[24]. In contrast, other sensory neurons, such as A-beta fibers, primarily carry non-noxious sensory information, such as temperature and pressure. However, these sensory modalities can overlap with noxious stimuli, such as detecting the difference between cool[25] and noxious cold[26].

The dorsal root ganglia serve as a critical site for the integration of nociceptive information. The DRGs are a series of small structures located just outside the spinal column, where sensory nerves from the periphery converge and transmit information to the spinal cord and undergo signal modulation in the central nervous system[27, 28, 29]. Additionally, the dorsal root ganglia contain other cell types, such as glia and immune cells, that can influence pain signaling[30, 31, 32, 33, 34].

Therefore, understanding the physiology of nociception is critical to understanding the complex perceptual experience of pain, as sensory inputs are integrated into a conscious experience of pain.

## 1.2 Why does pain arise?

As previously mentioned, pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. The key phrase in the definition is "actual or potential," meaning that pain is not limited to only actual tissue damage but also includes the potential for damage.

Pain serves as a protective mechanism that provides critical information to individuals to prevent further harm and promote healing. The perception of pain can prompt individuals to take action to prevent injury, such as moving away from a noxious stimulus, or to seek medical attention or rest. Furthermore, pain can also teach us which stimuli are potentially harmful, allowing us to learn from our experiences to avoid pain in the future.

Interestingly, there's also natural variation among humans in their sensitivity to nociceptive stimuli. For example, some people may be extremely sensitive to chemical irritants such as capsaicin, the chemical responsible for the spiciness of chili peppers[35], while others have a much higher tolerance and may find the experience pleasant[36]. In addition, women report greater pain severity in addition to greater tolerances, suggesting that adaptive pain may vary by sex[37, 38].

While pain is a necessary function for survival, there are some individuals who do not experience pain or have varying degrees of pain insensitivity[39, 40, 41]. Some individuals can feel the sensation of pain but do not experience the unpleasantness associated with it[42, 43], while others are completely insensitive to all nociceptive stimuli[44].

Pain insensitivity can arise from genetic mutations or other neurological conditions that affect the sensory or emotional processing of nociceptive information[45, 46]. While the absence of pain may seem like a desirable condition, individuals with congenital insensitivity to pain (CIP) face significant challenges and health risks[47, 48]. Individuals with CIP are often prone to injuries, such as fractures, burns, and cuts, that go unnoticed due to the absence of pain signals. These injuries can accumulate over time, leading to long-term health problems and reduced lifespan[49]. Therefore, it is essential to understand the complex nature of pain and its adaptive properties in promoting health and survival. While pain may be an unpleasant experience, it plays a critical role in preventing further harm and promoting healing.

### 1.2.1 Pain after Injury

Pain arises from tissue damage, ranging from minor cuts to significant injuries. In this scenario, pain signals the real hazards posed to the individual. The physical act of injury triggers the release of various factors, such as cytokines, chemokines, inflammatory factors, reactive oxygen species, prostaglandins, and cellular debris into the site of injury[50]. These factors can influence the function

of the peripheral nervous system, specifically the peripheral neurons that directly innervate the site of injury[51, 52, 53, 54].

The detection of these factors by specialized sensory receptors, the nociceptors, provides critical information on the type, extent, and localization of tissue damage to the central nervous system. These inputs are integrated into a perceptual model of pain, leading to a conscious awareness of pain[55, 56, 57].

Furthermore, the response of the body to injury involves mechanisms of inflammation, identified by Aulus Cornelius Celsus (c. 25 BCE – 50 CE) with the four signs of redness, swelling, heat, and pain[58, 59]. Both the direct tissue injury and the subsequent inflammatory response provide adaptive information to the central nervous system to prioritize protection of the injured site until regeneration is complete.

For example, stubbing your toe results in a high amount of pain associated with that foot. The injured foot is protected from further injury by shifting reliance during walking to the other foot or by promoting immobile behavior entirely. When the toe has healed and the inflammation and swelling subside, the pain resolves and function can continue as normal.

### 1.2.2 Pain after Infection

Perceived damage to the biological system may also include infections caused by viral and bacterial pathogens. While not all infections result in tissue damage[60], they have the potential to do so and, therefore, are ideally prevented from establishing before they can cause harm. Since infections pose a risk to an individual's health, the immune system has evolved as a crucial line of defense against potential pathogens[61].

Upon detection of an infection, the innate immune system triggers a cascade of signaling mechanisms under a general ‘systemic inflammatory response to sepsis’[62, 63, 64]. This cascade

involves the release of cytokines, chemokines, inflammatory factors, reactive oxygen species, and prostaglandins, which overlap with those triggered by the factors released in response to real tissue damage[65, 66, 67, 68, 69]. This is distinctly apparent in the usage of attenuated vaccines, which use mimicry to engage the immune response without the risk of infection. The inflammatory response triggered by vaccination is identical to that of natural infections, leading to pain and other symptoms associated with this immune response[70]. Thus, both actual and perceived threats to the system are processed in a similar manner and result in the sensation of pain[71]. However, the lack of clearly defined regions of infection can sometimes make it difficult to accurately localize the perception of pain in space[72].

Like injury, pain associated with infection generally resolves after the infection is cleared. However, infectious agents have evolved mechanisms to modulate the inflammatory response to prevent destruction through suppressing cytokine production and immune cell function[73, 74]. These persistent infections present an unresolving source of inflammation and may be responsible for pain persisting after the initial phase of infection[75, 76, 77]. As the immune response and associated inflammation can vary with different types or extents of infection, understanding the immune system and how it is activated and interacts with the peripheral nervous system is essential in understanding the inflammatory consequences of pain.

### 1.2.3 Pain from Inflammation

As pain is associated with inflammation resulting from either injury or infection, it is important to understand the inflammatory process and how it can trigger nociception. Inflammation is a complex biological response to harmful stimuli, such as tissue damage or infection, which involves a range of cells and molecular signaling pathways.

One of the key players in the inflammatory process is tumor necrosis factor-alpha (TNFα), a cytokine that is produced by a range of cells, including macrophages, T cells, and NK cells[78, 79]. TNFα is a pleiotropic cytokine that can activate a variety of signaling pathways and induce a range of cellular responses, including cell survival, proliferation, and apoptosis, depending on the context of its expression and the cell type involved[80, 81].

In the context of pain, TNFα can sensitize nociceptors by inducing the expression and activation of various ion channels, including voltage-gated sodium channels, that are involved in nociceptive transmission[82, 83, 84, 85, 86, 87]. TNFα release can also modulate the actions of neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), that are involved in pain transmission and contribute to the development of chronic pain[69, 88].

TNFα exerts its effects through binding to its receptor, TNFR1, which is expressed on a range of cell types, including nociceptors[89]. The binding of TNFα to TNFR1 can trigger a cascade of intracellular signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway[90] and the mitogen-activated protein kinase (MAPK) pathway[69], which promote inflammation and pain.

Specifically, the activation of the NF-κB pathway by TNFα can lead to the upregulation of various inflammatory genes, such as interleukin-1β (IL-1β)[91] and interleukin-6 (IL-6)[92], that can contribute to the development of pain. The activation of the MAPK pathway by TNFα can lead to the phosphorylation and activation of various ion channels, such as Nav1.8, that are involved in nociceptive transmission[93].

However, TNFα can also signal through a second receptor, TNFR2, which can have anti-inflammatory effects[94, 95]. Activation of TNFR2 can lead to the production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and can promote tissue repair and regeneration[96, 97]. This

dual role of TNFα, as both a pro-inflammatory and anti-inflammatory cytokine, highlights the complexity of the inflammatory response and its role in pain.

In addition to the role of TNFα, there are several other major inflammatory mechanisms that are involved in pain. Toll-like receptors (TLRs) are a family of pattern recognition receptors that are expressed on a range of cells, including immune cells[67] and nociceptors[98]. The activation of TLRs can lead to the production of pro-inflammatory cytokines, such as TNFα and interleukin-1β (IL-1β)[99, 100, 101]. TLRs can detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and can initiate an inflammatory response[102, 103]. PAMPs and DAMPs are danger signals that are released during infection or tissue damage. PAMPs are typically associated with pathogens, such as bacteria or viruses, while DAMPs are associated with cellular damage. Detection of these danger signals can lead to an inflammatory response and the production of pro-inflammatory cytokines that can contribute to the development of pain[104, 105, 106].

The NLRP3 inflammasome is a multi-protein complex that can sense a variety of danger signals, including PAMPs and DAMPs. The activation of the NLRP3 inflammasome can lead to the production of the pro-inflammatory cytokine IL-1β and the subsequent recruitment of immune cells and other inflammatory mediators to the site of injury or infection[107, 108]. The activation of the NLRP3 inflammasome has been implicated in a range of chronic inflammatory diseases, including chronic pain[109].

Reactive oxygen species (ROS) are also known to play a critical role in the development of pain associated with inflammation. During inflammation, immune cells such as macrophages and neutrophils release ROS as part of the innate immune response[110] and can directly activate nociceptors as well as being directly produced by peripheral neurons themselves[111]. ROS can also

promote the production of pro-inflammatory cytokines and chemokines that further contribute to inflammation and pain[112]. In addition, ROS can activate intracellular signaling pathways that promote neuronal hyperexcitability and sensitization, leading to increased pain perception[113].

Prostaglandins and bradykinins are inflammatory mediators that are produced in response to injury or inflammation. Prostaglandins are produced by the cyclooxygenase (COX) enzymes, while bradykinin is generated from a plasma protein called kininogen by the action of kallikrein. Prostaglandins[114, 115] and bradykinin[116, 117] can sensitize nociceptors and enhance the transmission of pain signals to the central nervous system[118].

The inflammatory response is a complex and multifaceted process that involves a range of cells and molecular pathways. Understanding the specific mechanisms involved in inflammation-induced pain is critical to developing effective treatments for chronic pain. By targeting specific molecular cascades, such as those activated by TNFα, clinicians develop therapies that address the underlying causes of pain and promote healing.

## 1.3 Chronic Pain Syndromes: converting ‘good’ pain to ‘bad’ pain

While pain is generally avoided because it is unpleasant, we need to keep in mind that pain is an adaptive system that serves a beneficial purpose. This type of pain, which we might call ‘good’ pain[119], is useful and therefore an overall positive experience.

However, following this reasoning there is also pain that might be considered "bad": maladaptive pain. This type of pain is no longer serving a beneficial purpose but is still extremely unpleasant, an overall negative experience[120]. Maladaptive pain can result from a range of factors, including changes in the way that the central nervous system processes nociceptive signals, unresolving inflammation, and nerve damage. Chronic pain is an example of maladaptive pain, and it can have a significant impact on quality of life and overall health.

An ideal intervention would prevent maladaptive pain without impacting the adaptive pain system, but this has turned out to be a challenging goal. Finding the correct balance between preventing maladaptive pain and preserving the beneficial aspects of pain is an ongoing area of research requiring further investigation into the mechanisms responsible for the initiation of maladaptive pain.

### 1.3.1 Central Sensitization

Understanding the mechanisms underlying the generation of pain is crucial to identify the factors that can cause the system to malfunction, leading to the development of chronic pain. One example of this is central sensitization, which is the process by which the central nervous system becomes hypersensitive to receiving inputs from the periphery in response to extreme and persistent pain[121, 122] or through widespread inflammation[123, 124]. This process can result in the central nervous system anticipating pain and becoming responsive to signals that would normally not elicit pain[125], leading to the development of both allodynia[126] and hypersensitivity[127].

Research into these mechanisms has shown that additional ion channels are inserted into the membranes of spinal neurons[128, 129] and synapses are strengthened[130, 131], resulting in amplified signal transmission into the central nervous system similar to turning up the gain on a guitar amp[132]. However, like a guitar amp that has been turned up too high, this amplified signal transmission can also lead to low threshold signals being transmitted and an output generated from the background noise, resulting in the development of chronic pain.

### 1.3.2 Failure of Pain Resolution

The adaptive pain system can also fail due to a breakdown in the pain resolution processes. Recent studies have shown that for inflammatory-mediated pain to subside after the noxious stimuli is removed, the system needs to receive signals from mediators called resolvins[133, 134, 135,

[136]. These resolvins help the system return to its baseline state and demarcate the point at which the pain should subside[137, 138].

In the context of injury and infection mediated by inflammation, it is essential not only to diminish pro-inflammatory mechanisms but also to establish pro-resolving mechanisms at the end[139, 140, 141]. Factors that can influence pain resolution include the type and severity of injury or infection, as well as underlying factors of the individual such as sex and predisposition to inflammatory processes[142, 143, 144]. Impaired pain resolution can lead to chronic pain syndromes, which can be debilitating and have a significant impact on an individual's quality of life. Recent lines of investigation have shown that modulating pro- and anti-inflammatory activity without promoting resolving activity can result in a state of chronic and persistent pain[145, 146].

Thus, investigating the processes of pain resolution is as critical as understanding the mechanisms of pain activation. Identifying factors that contribute to impaired pain resolution and developing targeted therapies that promote resolution can lead to more effective pain management strategies and improved quality of life for individuals living with chronic pain.

### 1.3.3 Neuropathic Pain

Neuropathic pain is a complex and debilitating type of pain that can result from a variety of conditions, such as diabetes[147], nerve injury[148], infection[149], or inflammation[150]. It is often described as burning, shooting, or stabbing and can be accompanied by tingling or numbness in the affected area[151]. While the underlying mechanisms of neuropathic pain are not yet fully understood, recent studies suggest that it may be caused by structural plasticity in the sensory input pathways. This plasticity can result in a rewiring of the nervous system that distorts normal pain processing[151], leading to the encoding of non-painful stimuli as painful and vice versa. Treatment of neuropathic pain is a significant clinical challenge, as traditional pain-relief strategies may be

ineffective[152]. As such, it is crucial to identify the biological factors involved in the induction of structural plasticity in the nociceptive nervous system, including sex differences, to develop effective tools and inform strategies for the prevention and treatment of neuropathic pain.

## 1.4 Insufficient Treatments for Chronic Pain

### 1.4.1 Opioids

Opioids are powerful pain-relieving medications that are often used to treat acute pain, such as pain after surgery, pain from accidental injury, or pain due to a medical condition. Due to their potency, opioids are commonly prescribed for moderate to severe acute pain[153]. They are often used in postoperative settings, as well as in emergency departments for pain relief from injuries or other acute conditions[154]. Opioids can be given orally, intravenously, or by injection, and the dose is typically adjusted based on the severity of the pain and the patient's response to the medication. Opioids work by binding to specific receptors in the brain and spinal cord, which reduces the perception of pain and increases pain tolerance. Many types of opioids are used clinically including: potent opioids such as morphine and fentanyl, semi-synthetic opioids like oxycodone and hydrocodone, weak opioids like codeine, and synthetic opioids like tramadol. For a review of current opioid practices and pharmacology, see Pathan & Williams (2012)[155] as well as Volkow & Blanco (2021)[156].

Although they are useful for the treatment of acute pain, long-term consumption of opioids can lead to addiction and tolerance to the pain-relieving effects[153, 157]. The usage of opioids in the treatment of chronic pain is contentious, with mixed evidence regarding their efficacy and risk profiles for long-term pain management[158]. Opioid use is associated with impacts on nerve function[159], gut health[160], and other organ systems[161, 162], contributing to the opioid epidemic and

its associated consequences. Further, opioids are not consistently effective in all types of chronic pain. Clinical trials on opioids usage for treating autoimmune-related chronic pain demonstrated a high proportion of non-responders[163]. As side effects and risks are high with chronic opioid usage, sufficient pain relief is expected to offset these negatives. Thus, alternative pain management strategies are needed that have fewer side effects and can be used for long-term management of chronic pain.

### 1.4.2 Anti-Inflammatories

Given that pain can largely be driven by inflammatory activity, it follows that preventing inflammation would prevent the related pain. This can be accomplished using anti-inflammatories such as non-steroidal anti-inflammatory drugs (NSAIDs) which are commonly used to relieve pain and reduce inflammation. NSAIDs function by inhibiting the production of prostaglandins, which produced by the enzyme isoforms COX-1 and COX-2[164]. COX-1 is constitutively expressed in many tissues and is responsible for the production of prostaglandins that help maintain normal physiological functions, such as protecting the stomach lining and regulating blood flow to the kidneys. In contrast, COX-2 is an inducible isoform that is primarily expressed in inflammatory cells and is responsible for producing prostaglandins that cause pain and inflammation[165]. NSAIDs can inhibit both COX-1 and COX-2 enzymes, thereby reducing the production of prostaglandins and leading to a decrease in pain and inflammation[165]. However, NSAID usage can also lead to side effects such as increased cardiovascular risk[166], gastrointestinal ulcers and bleeding[167, 168], as well as impaired kidney function[169, 170]. As a result, some NSAIDs have been developed to selectively inhibit COX-2 while sparing COX-1, with the aim of reducing side effects[171, 172]. However, the concept of preventing pain by preventing inflammation is flawed as inflammation is not inherently a bad thing and serves a crucial role in the processes of regeneration after injury and

immune activity in response to infection. In fact, interfering with the pain signal could also interfere with the resolution of pain, leading to an increased risk of chronic pain by blocking the beneficial effects of inflammation[173]. Thus, the use of anti-inflammatory medications should be judiciously employed in the management of pain.

### 1.4.3 Nerve Blocks

Nerve blocks or analgesics are another pharmacological tool that is commonly used to assist with pain management. Anesthetics, which can be given locally for nerve blocks or systemically for diffuse pain, work by binding to and blocking ion channels in the nerve cell membrane. These sodium channels are responsible for generating and propagating the electrical impulses that transmit pain signals along the nerve fibers to the brain. By blocking these channels, local anesthetics prevent the electrical impulses from traveling along the nerve fibers and reaching the brain, which reduces or eliminates the sensation of pain. This approach is particularly useful for managing pain in contexts where it cannot be avoided, such as post-surgical pain[174, 175].

Other classes of drugs may be used to modulate nerve function less directly, such as anti-depressants and anti-epileptics. Tricyclic antidepressants, such as amitriptyline and nortriptyline, can be effective in managing chronic pain conditions such as neuropathic pain and migraine headaches[176, 177]. The exact mechanism by which tricyclic antidepressants reduce pain is unknown, but it is thought to involve the modulation of TLR signaling in the brain and spinal cord[178, 179].

Anticonvulsants, such as gabapentin and pregabalin, are typically used to treat epilepsy but can also be effective in managing neuropathic pain conditions[180, 181]. The mechanism by which anticonvulsants reduce pain is not fully understood, but it is thought to involve the modulation of nerve impulses and the release of certain neurotransmitters in the CNS[182, 183]. Anticonvulsants may

also have some sedative effects, which can help with sleep disturbances that are often associated with chronic pain[184]. As all classes of nerve-modulating drugs carry their own side effects with mixed efficacies, alternative pain management strategies that are effective for long-term management with fewer side effects are necessary[185].

### 1.4.4 Diet and Exercise

Emerging evidence indicates that diet and exercise may have significant effects on the molecular mechanisms that control the inflammation involved in the development of chronic pain. Diets rich in anti-inflammatory foods, such as whole grains, fruits, vegetables, and lean protein sources can reduce the production of pro-inflammatory molecules such as cytokines and chemokines that contribute to chronic pain[186, 187, 188]. In particular, the Mediterranean diet, which is high in omega-3 fatty acids, has been shown to have potent pro-resolving inflammatory effects[189].

Similarly, regular exercise has been shown to attenuate pain behaviour in both rodents[190, 191] and humans[192, 193]. Exercise can also stimulate the release of anti-inflammatory molecules, such as interleukin-10 and transforming growth factor-beta, which can help to mitigate chronic pain[194]. Importantly, regular exercise supports a greater capacity for the neuroplasticity involved in descending modulation of pain[195] and sensory fiber sprouting[196], suggesting that the pain-modulating effects are due to structural changes in neurons or circuits.

## 1.5 Factors that Influence Treatment of Chronic Pain

### 1.5.1 Age

Age is a significant factor in chronic pain, as older individuals accumulate injuries and medical conditions that can contribute to chronic pain, but also for adolescents with developing brain circuits vulnerable to pain[197]. In addition, age-related declines in the musculoskeletal and nervous

systems can result in reduced physical function, exacerbating chronic pain. Differing sensitivities in nerve activity of aging nociceptors[45, 198] can also affect pain processing in the brain, leading to increased pain sensitivity and reduced pain tolerance[199]. Furthermore, older adults may be taking multiple medications that can interact with each other and contribute to chronic pain or exacerbate existing pain[200, 201, 202]. Finally, social isolation, which is more common among older adults, can exacerbate psychological factors such as depression and anxiety, which can worsen chronic pain[203, 204, 205].

### 1.5.2 Genetics

Additionally, there are genetic factors that can contribute to chronic pain[197, 206]. Studies have identified various genetic polymorphisms[207] that are associated with an increased risk of chronic pain, including genes directly involved in pain perception[208, 209]. For example, mutations in the SCN9A gene, which encodes for a sodium channel involved in pain signaling, have been associated with increased sensitivity to pain[210, 211]. Similarly, variations in genes involved in the regulation of inflammation, such as IL-1[212] and TNFα[213], have been linked to chronic pain conditions such as low back pain and irritable bowel syndrome respectively. Furthermore, genetic variations in stress response pathways, such as the hypothalamic-pituitary-adrenal axis, may contribute to chronic pain by increasing the production of stress hormones that can exacerbate pain perception[214].

### 1.5.3 Sex

An essential aspect of chronic pain is the influence of sex as a biological variable, which forms a significant focus of the thesis. Notably, chronic pain syndromes exhibit a higher prevalence rate among females, along with a greater extent of burden and pain intensity. While socio-economic factors may also play a role, it is noteworthy that women experience a significant proportion of

chronic pain globally. Therefore, the study of sex differences in pain processing and pain perception is critical to identify the underlying mechanisms that contribute to the development and persistence of chronic pain.

Chronic pain syndromes are more common in women[215, 216]. This is reflected in animal models where female rodents are more sensitive to pain[215]. Even accounting for pain syndromes of accompanying reproductive system changes such as endometriosis, syndromes like trigeminal neuralgia still present with female biases[217]. Many mechanisms for these biases have been proposed, including sex hormones, genetics, and environment. Despite documentation on this phenomenon dating back decades, it is unfortunate that few studies account for the variable of sex when studying pain. The studies that do often don't measure sex hormones which have been shown to modulate nociception. For example, estrogen can be both pro- and anti-nociceptive, while testosterone is more unilaterally anti-nociceptive[217]. However, the mechanism of sex-hormones on the pain axis requires further elucidation.

Sex differences in pain have been well-documented, with women being more greatly affected by chronic pain syndromes in both pre-clinical and clinical studies[215]. The historically predominant use of male animals and exclusion of female participants in clinical trials has led to a lack of understanding of sex differences in pain, and a need for greater consideration of sex as a variable in pain research. This may be especially important in the development of pain management therapies. Generalizing pain therapies to both sexes may not be effective, as the pain processing pathways and mechanisms can differ between males and females. Although males and females share many genetic and developmental similarities, there are important differences in their chromosomal and hormonal makeup that can predispose females to different types or amounts of

pain. For example, females have been shown to have differently operating immune and inflammatory mechanisms that contribute to pain sensitivity.

The omission of females from preclinical research using the excuse that females are more difficult to work with because of their reproductive variables or menstrual cycles is past its date of expiration. The investigation of sex differences in pain is a critical area of research that requires careful consideration of potential variables that may affect pain processing pathways and mechanisms. Although there may be no differences observed between males and females in some studies, it is important to acknowledge that these differences may exist and require investigation.

## 1.6 Pain in Autoimmunity

Pain is a common symptom of autoimmune diseases such as multiple sclerosis (MS)[218, 219]. In autoimmune diseases, the body's immune system mistakenly attacks healthy tissues and organs, leading to inflammation and tissue damage. This can result in pain, stiffness, and swelling in the affected areas. Additionally, autoimmune responses can damage nerve tissue[220, 221], likely resulting in neuropathic pain.

Unlike other types of pain, autoimmune-related pain may lack an external trigger for an adaptive response. Pain in autoimmune diseases can persist even in the absence of overt injury and treating it can be challenging due to the stochastic nature of its induction. However, there are similarities between autoimmune pain and other types of pain, such as inflammation and tissue damage.

Managing pain in autoimmune diseases often involves targeting inflammation and immune activation[222] but it is important to understand the underlying mechanisms that lead to pain in these syndromes. Investigating pain in autoimmunity and identifying targeted therapies that address its underlying mechanisms, including sex-specific differences, can lead to more effective pain

management strategies and improved quality of life for individuals living with autoimmune diseases.

### 1.6.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease that primarily affects the central nervous system[223]. In MS, the immune system mistakenly attacks and damages the protective covering of nerve fibers, leading to inflammation and damage to the nerve fibers themselves. This can result in a wide range of symptoms, including pain, numbness, tingling, muscle weakness, and difficulty with coordination and balance. MS has different types, including relapsing-remitting MS, secondary-progressive MS, and primary-progressive MS, each with its own unique course and symptoms[224, 225, 226]. Treatment for MS often involves medications that target the underlying immune dysfunction, such as disease-modifying therapies[227, 228, 229] or immunosuppressive drugs[230, 231]. These medications can help reduce inflammation and slow down the progression of the disease, which can help to prevent or delay disability. Pain is a common symptom in MS[232, 233] but is often viewed as a by-product[234] of the disease rather than a distinct entity that is actively managed.

### 1.6.2 EAE

Experimental autoimmune encephalomyelitis (EAE) is an animal model that is commonly used to study the pathogenesis of multiple sclerosis (MS) and to test potential treatments[235, 236]. In this model, animals are immunized with myelin antigens to induce an autoimmune response, resulting in an inflammatory reaction in the central nervous system (CNS).

EAE can be induced in various animal species, including mice, rats, and non-human primates, and can produce a range of neurological symptoms similar to those seen in MS, such as muscle weakness, paralysis, and pain[237, 238]. By inducing EAE, researchers can study the

underlying mechanisms of MS, such as the role of immune cells in the development of CNS inflammation, the effect of specific genetic or environmental factors on disease progression, and the potential for new treatments.

EAE has been used to test various therapeutic strategies for MS, including immunomodulatory drugs and cell-based therapies[239, 240, 241]. Results from EAE studies can provide valuable insights into the efficacy and safety of these treatments, as well as their potential mechanisms of action. It is important to note that while EAE shares many similarities with MS, it is not a perfect model and may not fully recapitulate all aspects of the disease. Therefore, findings from EAE studies should be interpreted with caution and validated in human clinical trials.

### 1.6.3 Sex Differences in MS/EAE Pain

It is well established that the incidence rates of MS are female biased. This bias has increased and is now estimated at over 3:1[242, 243, 244]. Female biases extend into many other autoimmune diseases, including rheumatoid arthritis (RA), Systemic Lupus Erythematosus (SLE), and myasthenia gravis[245]. The recurrent female bias in autoimmunity suggests some sex-related factor responsible for this gap but has remained elusive.

Hormones have been pursued as one of these factors. During pregnancy, the hormone levels of women are dramatically different than that of baseline. This shift in hormones is accompanied by a lessening of autoimmune severity in RA and MS, but a worsening of lupus, suggesting a complicated landscape of hormonal and autoimmune interaction[242]. One of these fluctuating hormones, estradiol (E2), was successful in ameliorating the clinical signs of EAE in non-pregnant mice[246]. Similarly, testosterone has also been shown to display protective effect in EAE, complicating the story further[247].

Sex differences are also extremely evident in animal models, including the EAE model of MS[248, 249]. However, comparing disease severity, the direction of bias is mouse-strain specific. For example, Papenfuss and colleagues showed that in the SJL strain, the females presented with higher disease severity. However, the B10.PL and PL/J strains showed a male bias. Interestingly, the C57/BL6 and NOD strains were observed to have no bias[250]! The complication of obvious sex differences but a lack of established causal mechanisms is a persistent confound on current research.

### 1.6.4 Looking Outwards to the Peripheral Nervous System

Chronic pain is a complex and multifactorial condition that is often poorly understood. While much research has focused on the central nervous system (CNS) and its role in chronic pain, recent evidence suggests that the PNS may also play a critical role. The PNS includes all nerves outside of the brain and spinal cord, including sensory nerves that transmit information about pain and touch to the CNS.

There are several factors that suggest the need to look to the PNS for a better understanding of chronic pain. First, the immune system plays a critical role in the development and maintenance of chronic pain, and immune cells are present in the peripheral tissues where nociceptors are located. Thus, factors related to immunity in the periphery may be important in the development of chronic pain.

In addition, recent evidence has found that the PNS is impacted in multiple sclerosis/ experimental autoimmune encephalomyelitis (MS/EAE)[251, 252], a disease that primarily affects the CNS. This suggests that the PNS may be involved in the development of chronic pain, even in diseases that primarily affect the CNS.

Further, there are major sex differences in the prevalence and severity of chronic pain[216], and recent evidence suggests that these differences may be related to differences in the PNS. For example, studies have found sex differences in immunity and signaling pathways from the peripheral tissues that contribute to pain perception[253, 254, 255].

Taken together, these findings highlight the need to focus on the PNS for a better understanding of chronic pain. Further research is needed to fully elucidate the role of the PNS in chronic pain, particularly with respect to immune function, disease-specific impacts, and sex differences. Such research could lead to the development of novel therapeutic approaches that target the PNS for the management of chronic pain.

# Summary and Purpose

Pain is a ‘good’ thing; an adaptive mechanism that promotes survival and health. Unfortunately, this dynamic process is susceptible to damage and dysregulation, transforming ‘good’ pain into ‘bad’ chronic pain. It is well described that females are more susceptible to maladaptive chronic pain with evidence implicating the peripheral nociceptive signaling pathways over the central pain pathways. These factors are evident in autoimmune diseases like multiple sclerosis which we can model in animals with EAE to investigate the underlying mechanisms that these sex differences confer. This dissertation aims to implicate fundamental sex differences in the interaction of the peripheral immune and nervous systems as a critical mediator of chronic pain induction. By elucidating the fundamental sex differences in chronic pain, this research may lead to the development of novel therapeutic approaches that target the peripheral nervous and immune systems and improve pain management for all individuals.

# Chapter 2:

# Profiling the microRNA signature of the peripheral sensory ganglia in experimental autoimmune encephalomyelitis (EAE)

The following chapter has been published as:

TNF (Friedman, T.N.), MSY (Yousuf, M. S.), AC (Catuneanu, A.), MD (Desai, M.), CAJ (Juźwik, C. A.), AEF (Fournier, A. E.), BJK (Kerr, B. J.) (2019), Profiling the microRNA signature of the peripheral sensory ganglia in experimental autoimmune encephalomyelitis (EAE). *J. Neuroinflammation* 16.

I was responsible for concept formation, data collection, analysis, and manuscript composition. MSY, AC, and MD collected data. CAJ and AEF were involved with editing the manuscript. BJK was the supervisory author involved with concept formation and manuscript edits.

## Motivation

My introduction to this project came at a time when the lab was just beginning to move into the world of bioinformatics. A current student at the time has identified a curious phenomenon in EAE; male EAE animals that developed pain would always develop bilateral pain, whereas female EAE animals could present with unilateral or bilateral pain. A central mechanism of pain was the prevailing idea in EAE – which certainly seemed to be the case for males – but anecdotal evidence suggested a peripheral involvement in the females. A series of papers provided evidence to support this, including the following chapter. As a first foray into bioinformatics, this analysis was incredibly fruitful. The complete dichotomy of male and female transcriptomic dysregulation sold me on the idea that male and female EAE are not the same. By extension, I began to question the idea that any disease can be assumed to have a consistent mechanism between the sexes, and it solidified my ideals of investigating sex differences in all future experiments. Despite the weight of literally doubling the workload by including both sexes all the time, I believe it is an incredibly important 'control' for all early investigations and the future of pre-clinical research as a field.

## 2.0 Abstract

Background

Multiple Sclerosis is an autoimmune disease with a distinct female bias, as well as a high prevalence of neuropathic pain in both sexes. The dorsal root ganglia (DRG) contains the primary sensory neurons that give rise to pain, and damage to these neurons may lead to neuropathic pain. Here, we investigate the sex differences of the DRG transcriptome in a mouse model of MS.

Methods

Next Generation Sequencing was used to establish RNA and microRNA profiles from the DRG of mice with MOG35-55 induced EAE, a model of CNS inflammation that mimics aspects of MS. Differential expression and multiple meta-analytic approaches were used to compare expression profiles in immunized female and male mice. Differential expression of relevant genes and microRNAs were confirmed by qPCR.

Results

3520 genes and 29 microRNAs were differentially expressed in the DRG of female mice with MOG35-55-EAE, while only 189 genes and 3 microRNAs were differentially expressed in males with MOG35-55-EAE. Genes related to the immune system were uniquely regulated in immunized female mice. Direct comparison of sex within disease indicates significant differences in interferon and phagosomal pathways between the sexes. miR-21a-5p is the primary dysregulated microRNA in both sexes, with females having additional dysregulated microRNAs, including miR-122-5p.

Conclusions

This study provides evidence that females are uniquely affected by MOG35-55-EAE and that this difference may result from additional signalling not present in the male. The altered transcriptome of females correlates with other studies finding hyperactivity of pain-sensing neurons and suggests underlying sex-specific pathways for neuropathic pain.

Keywords: micro-RNA, pain, inflammation, Multiple Sclerosis, DRG

## 2.1 Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system, characterized by demyelination, disturbances in neuronal function and progressive neurodegeneration[1]. Experimental autoimmune encephalomyelitis (EAE) is a mouse model used to study the pathophysiology of MS, where an autoimmune response is artificially induced against the myelin of the central nervous system (CNS). The model produces immune-related demyelination of the CNS[2,3,4]. People with MS experience altered motor function but recent estimates report that 63% of people living with MS are additionally negatively impacted by disturbances to sensation such as chronic, neuropathic pain[5]. Neuropathic pain arises from damage to the neurons involved in transmitting the 'pain' signal to the brain. This damage can be localized to any part comprising the pain system, from the pain-sensing periphery to the pain-perceiving central nervous system[6].

The dorsal root ganglia (DRG) residing in the peripheral nervous system (PNS) contain primary sensory neurons including pain-sensing neurons. The precise contribution of DRG neurons and the mechanisms mediating neuropathic pain in MS and EAE remain elusive. While transcriptome dysregulation has been studied in the CNS and immune cell populations in the context of EAE[7,8,9], less research has been done on the PNS, a potential source of neuropathic pain in the disease. Recently, our laboratory has described differences in the development of pain states between female and male mice in the EAE model. These differences include differences in immune and neurodegenerative histochemical and oxidative stress profiles[2,3,4]. These sex differences extend beyond the CNS, affecting the DRG[3]. Knowing that sex differences exist in EAE and that there are functional alterations correlated to pain, we sought to describe the transcriptional profile of the DRG in EAE from female and male mice with the disease.

MicroRNAs (miRNAs) are ~22 nucleotide long, single-stranded RNA molecules that repress translation of RNA species with complementary sequences[10]. miRNAs have risen to the forefront of research into the regulation of the transcriptome in pathophysiological states. It is estimated that the majority of all transcriptionally related pathways are under miRNA regulation[11]. Increasing amounts of evidence suggest that 'miRNA-signatures' in various tissues can be correlated with specific disease states. As miRNAs can function as one-to-many vectors by regulating the expression of multiple genes, we were also interested to see if a 'miRNA-signature' existed at the level of the DRG in addition to a functional analysis of the 'transcriptome signature'. This information could yield insights into the mechanisms of pain in EAE and MS more generally.

Here, we identify EAE-related miRNAs that are differentially expressed in both male and female DRGs. We compare the two sex-specific signatures at the transcriptional level for mice with EAE. We describe the analysis of RNASeq and miRSeq datasets, and functional clustering of the differentially expressed genes and miRNAs into disease relevant signatures. Our findings highlight extensive sex differences that must be considered with animal models of disease and identify potential targets for pain modifying therapies in the disease.

## 2.2 Methods

MOG35-55-EAE Generation

Female and male 6-8 week old C57BL/6 mice were acquired from Charles River Canada, and habituated for two weeks in the housing facility with baseline handling and behavioural testing. After this period, MOG35-55-EAE, (hereon referred to as 'EAE') was induced by 50µg subcutaneous injections of myelin oligodendrocyte glyocoprotein (MOG35-55; Peptide Synthesis Facility, University of Calgary) emulsified in Complete Freund's Adjuvant (CFA) at a

concentration of 1.5mg/ml. An additional intraperitoneal injection of 300ng of pertussis toxin in 0.2ml saline was given on the day of induction as well as 48 hours later. Control (termed CFA hereon after) mice received identical injection protocols except for the lack of emulsified MOG. CFA is known to produce transient alterations in immune activity[12] but is still an effective tool for EAE induction and an appropriate control. Mice were assessed daily for clinical progression of EAE following a four-point scale: grade 0, normal mouse; grade 1, flaccid tail (disease onset); grade 2, mild hindlimb weakness with quick righting reflex; grade 3, severe hindlimb weakness with slow righting reflex; grade 4, hindlimb paralysis in one hindlimb or both. Tissue was extracted on the first day a mouse presented with clinical signs (see below) that is denoted as 'onset'.

Tissue Collection and Total RNA Extraction

Animals were euthanized by Euthanyl® (sodium pentobarbital) overdose injected intrapertioneally. After injection, animals were monitored for level of consciousness and dissections did not proceed until no response to toe pinch or corneal contact was observed. Cardiac punctures were performed to confirm euthanization and animals were perfused with 10mL of saline. Whole DRGs were immediately extracted and suspended in Qiazol (Qiagen Biosystems) and stored at -80°C for later processing. Upon collection of the full tissue set, total RNA was extracted using miRNeasy® Mini kits (Qiagen Biosystems).

Next Generation Sequencing

Total RNA was supplied to ArrayStar Inc. for sequencing. Total RNA was enriched by oligoDT magnetic beads and library preparation was completed using KAPA Stranded RNA-Seq Library Prep Kit. Transcriptome data was acquired by sequencing on an Illumina HiSeq 4000 machine according to manufacturer's instructions. MicroRNA data was acquired by: 3'-adapter ligation

with T4 RNA ligase 2 (truncated), 5'-adapter ligation with T4 RNA ligase, cDNA synthesis with RT primer, PCR amplification, and extraction and purification of ~130-150 bp PCR amplified fragments and sequencing on an Illumina HiSeq 2000 machine according to manufacturer's instructions.

Analysis of Differential Expression

Both RNASeq and miRSeq differential expression was investigated through the R[13] package DESeq2. Briefly, raw transcript sequences from NGS were aligned to the UCSC 'mm10' database of known genes using the Genomic Features package function 'exonsBy'. miR read counts were directly uploaded into R. The resulting data structures were processed through the DESeq2 package following the author's instructions found in their published vignette[14,15,16]. The results of each contrasting analysis (eg. M EAE vs. M CFA) were published in tabular format for usage in subsequent analyses. Genes were considered significantly differentially expressed using the threshold of $q < 0.1$ after Benjamini-Hochberg correction. Clustergram and PCA analyses were performed using MATLAB (MATLAB and Statistics Toolbox Release 2017b, The MathWorks, Inc., Natick, Massachusetts, United States).

Ontological Analysis with GOrilla/REViGo

Single gene lists ranked by p-adjusted values were analyzed with the GOrilla[17] web software, using a p-value threshold of 10E-9. Significant GO terms from each category (Process, Function, Component) were then processed through REViGO[18] to screen for redundant terminology.

Functional Analysis with Ingenuity Pathway Analysis

Activation patterns of canonical pathways were analyzed through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis)[19]. Briefly, gene

lists ranked by p-adjusted values were analyzed using the 'Core Analysis' feature using default parameters and a p-adjusted cutoff of 0.1.

qPCR Validation

For validation of DEmiRs, 50ng of Total RNA was reverse transcribed using the miScript II RT Kit (Qiagen Biosystems). miRs were detected with the Mm_miR-21_2 and Mm_miR-122a_1 miScript Primer Assays (Qiagen Biosystems) using the miScript SYBR Green PCR Kit (Qiagen Biosystems). Values detected were standardized to sno72. For validation of DEGs, 100ng of Total RNA was processed with DNase I (Invitrogen) followed by reverse transcription using Superscript III (Invitrogen). Transcripts were detected using the following primers: Ppia: RT² qPCR Primer Assay for Mouse Ppia (Qiagen Biosystems), C3: Forward, GAGGCACATTGTCGGTGGTG, Reverse, CCAGGATGGACATAGTGGCG, C5ar1: Forward, ATGGACCCCATAGATAACAGCA, Reverse, GAGTAGATGATAAGGGCTGCAAC, Stat1: RT² qPCR Primer Assay for Mouse Stat1 (Qiagen Biosystems) RT² qPCR Primer Assay for Mouse Tlr7 (Qiagen Biosystems), RT² qPCR Primer Assay for Mouse Tlr8 (Qiagen Biosystems), RT² qPCR Primer Assay for Mouse Trem2 (Qiagen Biosystems). Transcripts detected were standardized to Ppia values. All qPCR reactions were 20uL volumes utilizing a StepOnePlus machine (Applied Biosystems).

qPCR Statistical Analysis

Statistical analysis was performed using Graphpad Prism 6.01. qPCR analysis utilized two-way ANOVA testing with Sidak's correction for multiple testing. qPCR statistics were performed on untransformed values but plotted as linearized values for ease of visualization. Alpha was set to 0.05 for all testing.

## 2.3 Results

RNASeq

To detect a transcriptional signature within the DRG of EAE mice, we performed Next Generation Sequencing (NGS) of DRGs taken from mice at the onset of the disease when pain hypersensitivity is evident[2,3,20]. Roughly 20,000 genes were detected by sequencing. We observed gross differential expression of genes (DEGs) in mice subjected to EAE both in female (3520 DEGs) and male (189 DEGs) mice relative to their sex-specific CFA controls (Fig. 2.1a, Supplementary Fig. 2.1, 2.2) (Wald test; $q < 0.1$). To visualize variation in animal profiles and expression patterns, we performed a Principal Component Analysis of all animals by all genes. This unbiased approach projects numerous complex variables into simpler dimensions, allowing for appreciation of relatedness between samples based on their proximity. The first two components captured almost half of the total variation within the entire sequencing set, with 29 and 19 percent respectively (Fig. 2.1b). Cluster analysis (MATLAB clustergram) of all animals by the sum of female and male DEGs showed high specificity for EAE over CFA control, but minimal specificity for sex (Fig. 2.1c).

Detection of EAE-related sex-specific signatures

To contextualize the significant genes into functional groups from known ontologies, we enlisted the DAVID[21], REACTOME[22], GOrilla[17], REViGO[18], and PANTHER[23,24,25] algorithms. Analysis of DEGs generated by DESeq analysis for significantly enriched GOslim (Gene Ontology) terms for Cell Compartment (Fig. 2.2a) showed a similar pattern of gene regulation for females and males. The Cell Compartment analysis references genes known to localize in specific regions of the cell. While many terms were identical in enrichment (i.e. ‘membrane’) further analysis of GO

terms revealed some unique variations in Cell Compartment, as males included enrichment in mitochondrial terms whereas females were enriched in vesicular related terms. (Table 2.1 and 2.2) Remarkably, analysis of DEGs by REACTOME pathways to cluster molecular events, revealed a striking sex-specific signature (Fig. 2.2b). The majority of pathways enriched in females such as Immune system related pathways were completely absent in males. Male EAE mice contained significant enrichment of a single unique term: "SLC-mediated transport", with all others sharing overlap with females.

Validation of RNASeq

To confirm the differential expression of transcripts indicated by RNASeq analysis and of special interest through subsequent meta-analyses, we performed qPCR on relevant target genes of interest (Fig. 2.3). Validation targets were chosen based on criteria of differential expression between EAE and CFA condition, differential expression between female and male EAE conditions, and inclusion in significant canonical pathways of Ingenuity Pathway Analysis. (Supplementary Fig. 2.1,2.2,2.5,2.6) In females, we confirmed the upregulation of C3, C5ar1, Stat1, Trem2, Tlr7, and Tlr8, in concordance with RNASeq predications. Interestingly, we also detected significant downregulation of C3, Stat1, Trem2, and Tlr8 in male EAE compared to their control (Table 2.3).

miRSeq

In addition to sequencing for gene coding fragments, we acquired NGS data for almost 2,000 microRNAs (miRNAs). Differential expression was determined using an identical methodology to the RNASeq data above. We detected 29 differentially expressed miRNAs (DEmiRs) in the DRG of EAE females relative to CFA female controls, but only 3 DEmiRs in EAE males (Fig. 2.4a, Supplementary Fig. 2.3,2.4) (Wald test; $q < 0.1$). All DEmiRs detected in males (miR-21a-

5p, miR-21c, and miR-142a-5p) were similarly detected in females and displayed similar patterns of upregulation. Multidimensional variation was visualized by PCA analysis of all expressed miRNAs (Fig. 2.4b) and cluster analysis of DEmiR expression values was carried out in a similar manner to the RNAseq data set (Fig. 2.4c). We did not however, identify a separation by disease by miRSeq variation unlike the RNASeq suggesting that any disease-related alterations may be more subtle.

Differences in transcriptional signatures by sex within EAE

We next wanted to directly compare females and males with EAE to determine the effect of sex on the transcriptional profiles in the DRG. To accomplish this, we first compared the groups of 'Female EAE' and 'Male EAE' to find ~6000 DEGs. To account for genes differentially expressed as a result of sex (such as Y-linked genes), we removed DEGs from an 'all Female' vs. 'all Male' comparison, leaving 869 DEGs (Supplementary Fig. 2.5). We were interested in the functional significance of the DEGs and relied upon Ingenuity Pathway Analysis to investigate hypothetical activation of known pathways. Direct comparison yielded terminology related to metabolism (i.e. LXR/RXR Activation), phagocytosis (i.e. Acute Phase Response) and interferon signaling (Table 2.4, Supplementary Fig. 2.6). Negative log(p-values) correspond to increasing levels of significance ($-\log(\leq 0.05) \geq 1.3$); ratios represent the proportion of DEGs / # of genes in canonical pathway; and z-scores represent the predicted activation state of the pathway, with positive and negative being activated and inhibited, respectively. The heightened activation state of these pathways suggests an increased amount of immune-related inflammation in the DRGs of females compared to males with EAE.

Validation of miRSeq

We next wanted to validate the differential expression of miRNAs in both females and males with EAE. We identified miR-21a-5p as the most significantly dysregulated miRNA in both female and male EAE relative to respective CFA control; as well as miR-122-5p as being significantly dysregulated in female EAE alone. We validated that miR-21a-5p (hereon referred to as miR-21) was increased in both female and male EAE mice relative to CFA controls by 3.78 and 4.76-fold, respectively (Fig. 2.5a, b). There was no significant difference in the level of miR-21 upregulation between the sexes (2-way ANOVA, disease main effect $F_{1,34} = 79.21$ , $P < 0.0001$, followed by Sidak's multiple comparison test for disease within females, $t = 6.794$, $q < 0.0001$, and males, $t = 6.021$, $q < 0.0001$). miR-122-5p (hereon referred to as miR-122) was confirmed to be significantly upregulated in females only relative to CFA female controls by 4.36-fold, with a significant difference between the sexes (2-way ANOVA, sex main effect $F_{1,32} = 9.803$, $P = 0.0037$, followed by Sidak's multiple comparison test for disease within females, $t = 2.675$, $q = 0.0232$, and males, $t = 1.940$, $q = 0.1188$) (Fig. 2.5c, d).

## 2.4 Discussion

EAE is a complex disease that primarily targets the CNS. Less is known about how the PNS is affected in EAE. We have shown that a disease-related transcriptional signature is detectable in the DRG of EAE mice, and that sex is an important factor in the characterization of this signature. To accomplish this, we utilized Next Generation Sequencing for mRNA and miRNAs from the DRG at the clinical onset of EAE and profiled the differentially expressed genes and miRNAs to acquire a functional signature for each sex. The identification of functional signatures for complex disease states or comorbidities such as pain is relevant for prompting strategic experimental design and stratifying future therapies.

## Detecting the Signature

We first wanted to describe the transcriptome of our animals, considering sex as a factor. Sex differences have been previously described in immunity[26,27,28] and inflammatory responses[29,30], and both of these processes play a key role in our disease model. Indeed, we found extensive differential expression of the transcriptome in female EAE mice but only small amounts of differentially expressed genes (DEGs) in male EAE mice. Large changes in gene expression have been reported in the DRG after peripheral nerve injury and these changes have been associated with neuropathic pain behaviours[31,32]. This supports previous evidence from our laboratory, indicating that female but not male DRG neurons are hyper-responsive at the onset of EAE[3]. In addition to these differences in the quantity of DEGs, we also found major differences in the functional signature of this altered transcriptional profile. Our data indicates that there are extensive immune and cell-signalling related processes disturbed in the female DRGs, a potential by-product of immune cells transiently localized there[33].

## Sex-specific Disease Effects

Comparing the sexes directly, we noted the appearance of phagosomal activation pathways, complement, and interferon signalling as being differentially affected in female EAE versus their CFA controls (Table 2.3). The phagosome related terms contained references to DEGs such as Trem2, Csf1r, Tlr7 and most of the complement system. Taken together, this data indicates sex-specific dysfunction of inflammatory activity in phagocytic cells. Laffont et. al have reported increased interferon responses of estrogen activated human plasma dendritic cells to TLR7 ligands, suggesting that this mechanism may be sex-hormone linked[34]. Tlr7 is an X-linked gene and this may account for the sex-bias we observed, as X chromosome genes may escape silencing.

Interferon signalling is an approved target for MS therapy, as beta-interferons have been utilized in relapsing-remitting MS since the late 1990s[35]. Although the effects of interferon inhibitors are modest, all clinical trials conducted to date have been with mixed female and male populations. The possibility of female specific interferon dysregulation raises the possibility of higher efficacy in a subset of the clinical population. We validated the female-EAE-specific increased expression of Stat1 – a downstream effector of Types I and II interferons – suggesting that interferon signalling is activated in these animals. In male EAE mice however, Stat1 levels were significantly reduced suggestive of a silencing of interferon signalling. As beta-interferon therapy would activate these pathways, it follows that their effect may have differential effects based on sex.

Activation of interferon signalling through Stat1 typically results in increased inflammation and enhanced immune activity[36]. While these pathways do seem to be activated in the diseased group of females, it is apparent that it is not the case for the males. The high activation of interferon-signalling and Stat1 in females is complemented by a similar increase in transcripts associated with inflammatory macrophages, including most members of the complement system. Complement proteins are involved with the recruitment and phagocytic capacity of innate immune cells[37,38]. Based on our transcriptomic data, females may be poised to specifically respond to interferon stimulation, resulting in heightened phagocytic activity in the DRG in response to EAE.

Accounting for the Signature

To account for the transcriptomic changes observed in EAE, we also sequenced the miR-transcriptome. We observed a small amount of overlap between the detected DEmiRs in each sex, corresponding to miRNAs previously linked to EAE and the MS literature: miRs-21[39,40] and -142[41]. The most significant miRNA for both sexes, miR-21, was both highly expressed and confirmed to be significantly upregulated in both female and male EAE DRGs. MS brain lesions

have been shown to be enriched with miR-21 in patients with MS but not neuromyelitis optica (NMO)[42]. NMO is an autoimmune disease with similar symptomatology, potentially indicating that this signature is specific to MS/EAE pathology. Therefore, miR-21 may be suitable as a marker of EAE. miR-21 has been well studied in the context of cancer, giving rise to its label of an 'oncomiR'[43,44,45]. Interest in miR-21 as a regenerative marker is also emerging with evidence that it is pro-regenerative[46] and immunomodulatory[47]. Macrophages uptake miR-21 laden exosomes after peripheral nerve injuries[48] where it can act as a direct ligand for Toll-like Receptors 7 and 8[49,50]. Interestingly, miR-21 is affected by sex-hormones, containing a direct response element for androgens[51] and directly affected by estradiol signalling[52,53]. As miR-21 was similarly upregulated in both sexes, we additionally screened for miRNAs unique to a sex. miR-122 was only upregulated in the diseased female DRG. miR-122 is primarily described for its expression in the liver, however it is known to increase in the circulation after injury[54,55] and may interact with Type I interferon signalling[56]. Together with the transcriptomic signatures, our data indicates that the female DRGs may be uniquely affected by the epigenetic regulation of miRs-21 and -122. Although EAE is canonically not thought of as a PNS disease, the presence of the typical 'MS-miR-signature' indicates that female DRGs may be affected concordantly with CNS lesions.

## 2.5 Conclusion

To our knowledge, this is the first presentation of transcriptome and miR-transcriptome dysregulation in DRGs using the EAE mouse model. We have shown that the miR-signature is primarily defined by miR-21, bearing similarity to previously described findings in the spinal cord and serum of mice with EAE, and MS lesions. We present evidence of a female biased dysregulation of both 'omic' analyses, the presence of distinct functional transcriptomic signatures

for each sex, and that interferon signalling and phagosome function may be indicated for future analysis. Acknowledging fundamental differences between the sexes is not only important for experimental design, but for the development of potential sex-specific therapies. Our study provides a platform to study sex-specific changes in the DRG of EAE and the role that miRNAs may have on their transcriptome.

## 2.6 Appendix

Abbreviations: Multiple Sclerosis (MS); Experimental autoimmune encephalomyelitis (EAE); central nervous system (CNS); dorsal root ganglia (DRG); peripheral nervous system (PNS); MicroRNAs (miRNAs); myelin oligodendrocyte glyocoproteinn (MOG); Complete Freund's Adjuvant (CFA); Next Generation Sequencing (NGS); differential expression of genes (DEGs); Gene Ontology (GO); RNA sequencing (RNAseq); microRNA sequencing (miRseq)

Declarations: Ethics approval and consent to participate: All animal studies complied with the Canadian Council on Animal Care Guidelines and Policies and were approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta.

Consent for Publication: Not applicable.

Availability of Data and Materials: All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: AC conducted the EAE experiment. TNF, MSY, and MD performed tissue extraction and qPCR validation. TNF analyzed and interpreted the sequencing data. TNF, CAJ, AEF and BJK were involved in experimental design and manuscript preparation. All authors read and approved the final manuscript.

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## 2.7 Figures

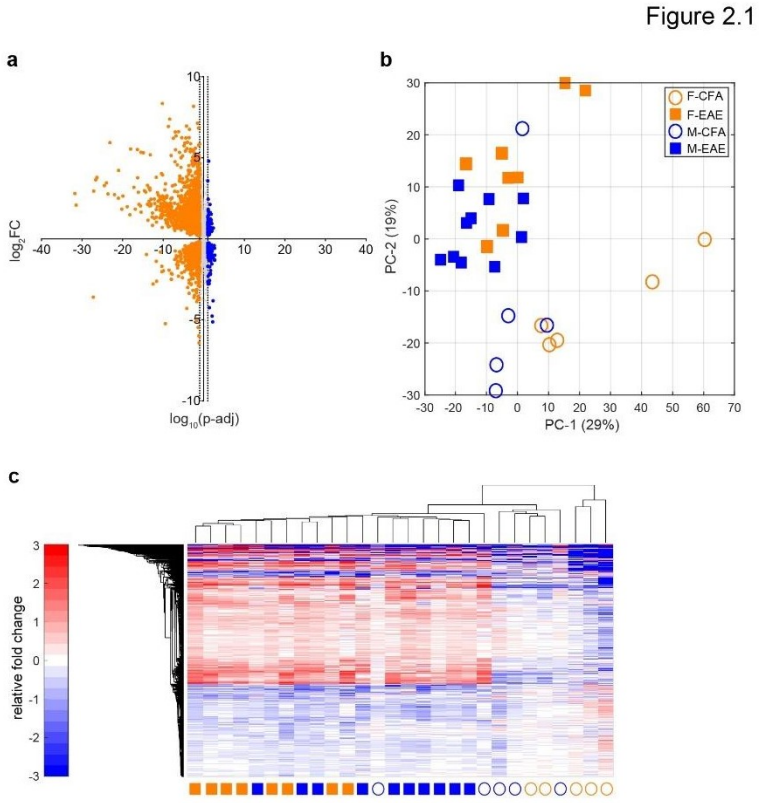


Figure 2.1

EAE primarily affects female DRGs over males at the transcriptional level. (a) Joined volcano plots of DEGs; female DEGs = 3520, male DEGs = 189. Vertical dotted lines represent log10(q = 0.1) as cutoff threshold for significance. (b) Principal Component Analysis suggests separation of the EAE and CFA DRGs when analyzed by all mRNA sequencing data. Top two components are plotted. (c) Heatmaps of all DEGs in EAE further visualize the clustering of EAE. Values are Reads / Totals Reads normalized to the average of the CFA condition. DEG = Differentially expressed gene.

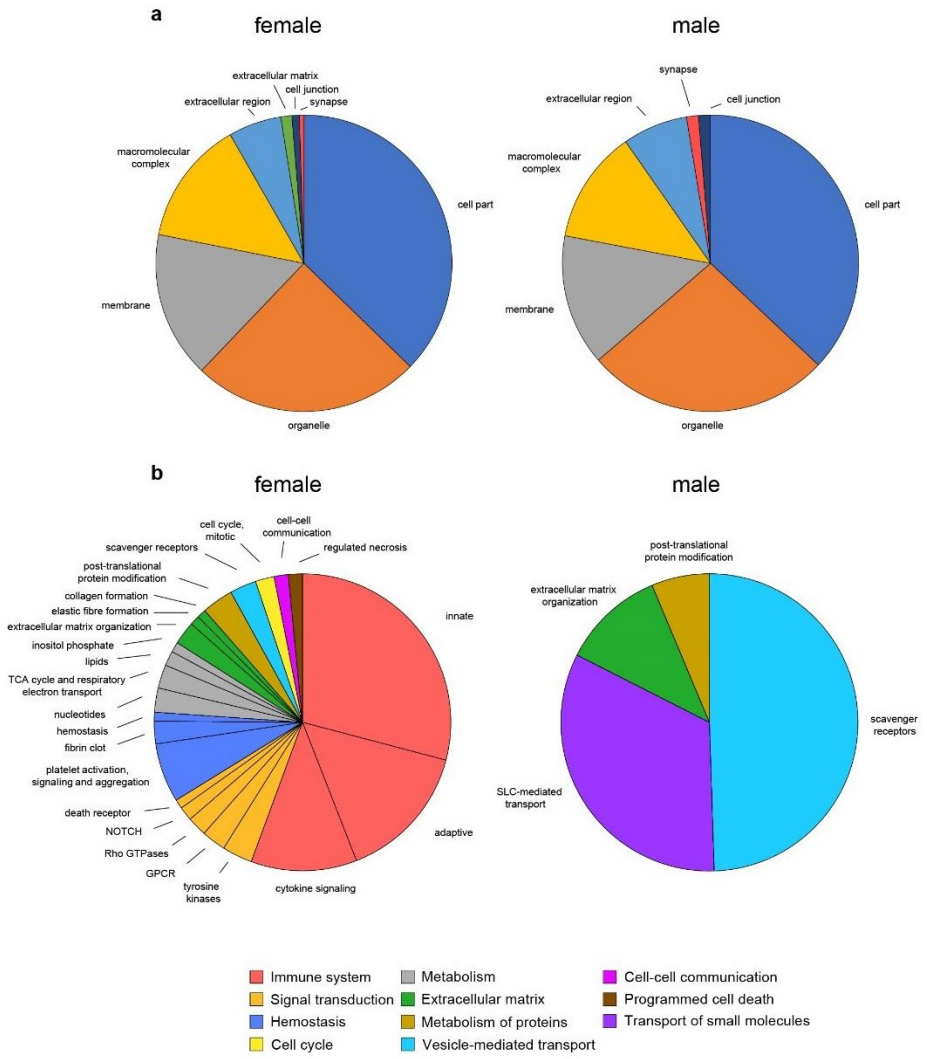


Figure 2.2

Female and male DRGs exhibit unique functional RNA signatures. (a) Both sexes show similar PANTHER Pathways GO cell compartment terms. (b) However, REACTOME pathways of female and male DEGs show a distinct profile, suggesting that the function of each set of DEGs may be different. Colour legend represent grouped terms under the REACTOME hierarchy. Wedges are scaled to # DEGs in pathway / # genes defined in the respective pathway.

Figure 2.3

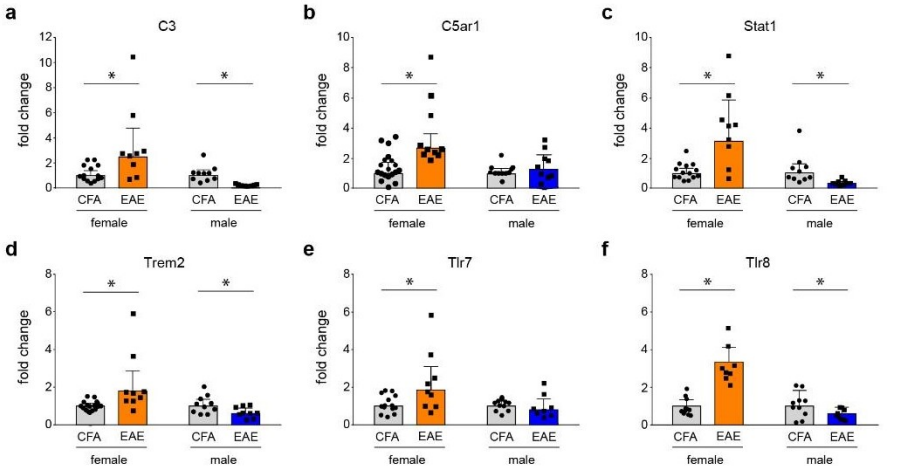


Figure 2.3

Validation of DEGs by qPCR. Bar graphs of (a) C3 (b) C5ar1 (c) Stat1 (d) Trem2 (e) Tlr7 and (f) Tlr8. * $p < 0.05$, 2-way ANOVAs with Sidak's multiple comparison test. Bars indicate geometric mean with 95% confidence interval.

Figure 2.4

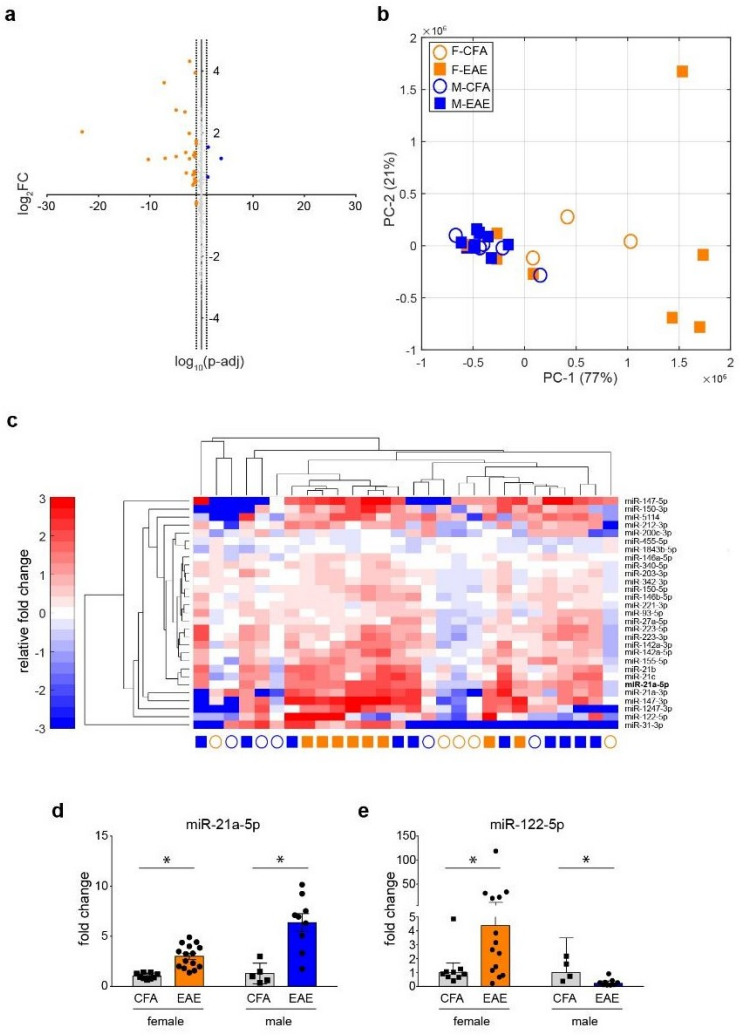


Figure 2.4

miR-21a-5p is the candidate miR for EAE in females and males. (a) Joined volcano plots of DEmiRs; female DEmiRs = 29, male DEmiRs = 3. Vertical dotted lines represent log10(q = 0.1) as cutoff threshold for significance. (b) Principal Component Analysis shows minimal separation of EAE and CFA animals by all miR sequencing data. Top two components are plotted. (d) qPCR validation of miR-21a-5p in female and male DRGs. (e) qPCR validation of miR-122-5p in female and male DRGs. * $p < 0.05$, 2-way ANOVAs with Sidak's multiple comparison test. Bars indicate geometric mean with 95% confidence interval. DEmiR = differentially expressed miRs

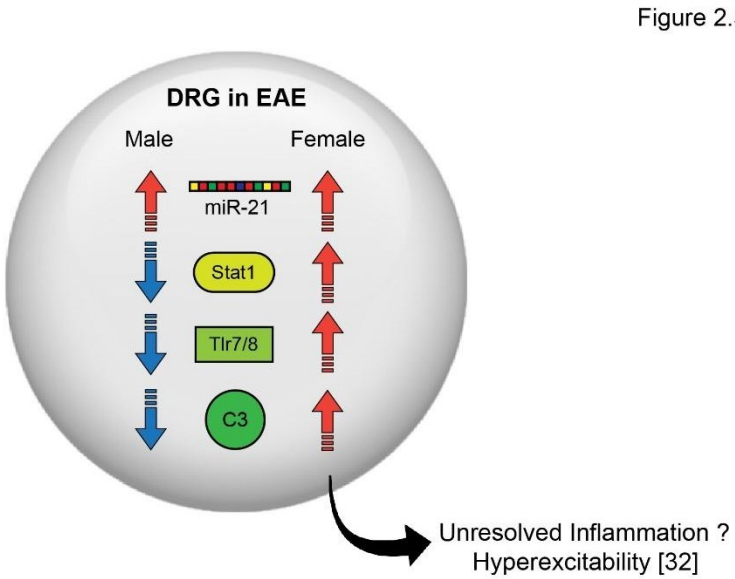


Figure 2.5

Schematic of a hypothetical DRG in EAE. Although we observe increased miR-21 in both sexes, only females display the increased inflammatory signalling (see reference).

Table 2.1

Table 2.1: GOrilla/REViGO analysis of **differentially expressed genes in female EAE**. Top 10 non-dispensable terms are reported.

| REViGO Term | -log(p-value) | Dispensability | Frequency |
|---|---|---|---|
| extracellular region | 23.8069 | 0 | 2.38% |
| cell surface | 19.1549 | 0 | 0.24% |
| membrane | 27.0114 | 0 | 61.59% |
| vesicle | 9.279 | 0 | 1.36% |
| extracellular region part | 30.5186 | 0 | 1.31% |
| side of membrane | 20.0101 | 0 | 0.21% |
| membrane region | 12.4225 | 0.037 | 0.12% |
| external side of plasma membrane | 19.3224 | 0.046 | 0.06% |
| cell part | 20.251 | 0.087 | 52.39% |
| cytoplasmic part | 14.284 | 0.214 | 12.66% |

Table 2.2

Table 2.2: GOrilla/REViGO analysis of **differentially expressed genes in male EAE**. Top 10 non-dispensable terms are reported.

| REViGO Term | -log(p-value) | Dispensability | Frequency |
|---|---|---|---|
| membrane | 19.9136 | 0 | 61.59% |
| NADH dehydrogenase complex | 12.2565 | 0 | 0.04% |
| organelle | 9.8665 | 0 | 20.79% |
| extracellular region part | 11.5017 | 0 | 1.31% |
| respiratory chain | 9.8239 | 0.035 | 0.30% |
| cell surface | 11.9872 | 0.045 | 0.24% |
| mitochondrial membrane part | 11.4377 | 0.053 | 0.38% |
| cell part | 17.4437 | 0.093 | 52.39% |
| receptor complex | 9.9747 | 0.233 | 0.12% |
| cytoplasmic part | 16.0414 | 0.261 | 12.66% |

Table 2.3

Table 2.3: qPCR Statistics for DEGs and DEmiRs

| DEG/DEmiR | Female | | Male | | Main Effect | p-value | 2° effect | p-value |
|---|---|---|---|---|---|---|---|---|
| | Fold Change | Adjusted p-value | Fold Change | Adjusted p-value | | | | |
| *C3* | 2.48 | 0.0029 | 0.17 | < 0.0001 | Sex | < 0.0001 | Disease | 0.0384 |
| *C5ar1* | 2.69 | 0.0079 | 1.23 | 0.8121 | Disease | 0.0170 | ~ | ~ |
| *Stat1* | 3.13 | < 0.0001 | 0.33 | 0.0005 | Sex | < 0.0001 | ~ | ~ |
| *Trem2* | 1.80 | 0.0072 | 0.61 | 0.0375 | Sex | 0.0004 | ~ | ~ |
| *Tlr7* | 1.85 | 0.0182 | 0.80 | 0.6256 | Sex | 0.0174 | ~ | ~ |
| *Tlr8* | 2.82 | < 0.0001 | 0.60 | 0.0478 | Sex | < 0.0001 | ~ | ~ |
| *miR-21* | 3.78 | < 0.0001 | 4.76 | < 0.0001 | Disease | < 0.0001 | ~ | ~ |
| *miR-122* | 4.36 | 0.0232 | 0.22 | 0.1188 | Sex | 0.0037 | ~ | ~ |

Table 2.4

Table 2.4: Canonical pathways **differentially expressed between female vs. male EAE**.

| Ingenuity Canonical Pathways | -log(p-value) | Ratio | z-score |
|---|---|---|---|
| LXR/RXR Activation | 5.06 | 0.145 | 2.673 |
| Acute Phase Response Signaling | 4.44 | 0.116 | 1.155 |
| Neuroinflammation Signaling Pathway | 3.57 | 0.0871 | 0.229 |
| Complement System | 3.54 | 0.212 | 1.342 |
| FXR/RXR Activation | 3.12 | 0.113 | N/A |
| Phagosome Formation | 2.88 | 0.107 | N/A |
| Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells | 2.39 | 0.185 | 1 |
| Hepatic Fibrosis / Hepatic Stellate Cell Activation | 2.21 | 0.0838 | N/A |
| GP6 Signaling Pathway | 2.14 | 0.0909 | 0.302 |
| Interferon Signaling | 2.12 | 0.161 | 2.236 |

## 2.8 Supplemental Materials

### Supplementary Figure 2.1

Spreadsheet of RNASeq differential expression analysis between EAE and CFA condition in females.

### Supplementary Figure 2.2

Spreadsheet of RNASeq differential expression analysis between EAE and CFA condition in males.

### Supplementary Figure 2.3

Spreadsheet of miRSeq differential expression analysis between EAE and CFA condition in females.

### Supplementary Figure 2.4

Spreadsheet of miRSeq differential expression analysis between EAE and CFA condition in males.

### Supplementary Figure 2.5

Spreadsheet of RNASeq differential expression analysis between EAE conditions of females and males.

### Supplementary Figure 2.6

Spreadsheet of Ingenuity Pathway Analysis of differentially expressed genes between female and male EAE conditions.

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# Chapter 3:

# Sex Differences in Peripheral Immune Cell Activation:

# Neuro-Immune Interactions with Consequences for Pain

The following chapter has been accepted at *Brain, Behaviour, and Immunity* as:

TNF (Friedman, T.N.), OC (La Caprara, O.), CZ (Zhang, C.), KL (Lee, K.), JM (May, J.), CAF (Faig, C. A.), TB (Baldwin, T.), JRP (Plemel, J. R.), AMWT (Taylor, A. M. W.), BJK (Kerr, B. J.) (2023), Sex Differences in Peripheral Immune Cell Activation: Neuro-Immune Interactions with Consequences for Pain. *Brain, Behaviour, and Immunity.*

I was responsible for concept formation, data collection, analysis, and manuscript composition. OC and CZ collected data and analyzed samples. KL and JM assisted with sample preparation and data collection. JM, TB, JRP, and AMWT were involved with editing the manuscript. BJK was the supervisory author involved with concept formation and manuscript edits.

## Motivation

The major takeaway of Chapter 2 was the massive difference between the male and female EAE transcriptome. Despite both sexes displaying equivalent levels of pain and physical disability, the dysregulation of the male DRG was almost insignificant compared to the female DRG. Hypotheses ranged from a female-specific signaling mechanism to a resilient male system. Despite previous studies in our lab identifying similar quantities of immune cell influx into the DRG of both male and female mice with EAE, the most parsimonious explanation for the extent of female dysregulation was an increased presence of the phagocytic innate immune cells predicted by the gene ontology analysis.

Building off that idea, I considered the idea that the relationship between innate immune cells (i.e. macrophages) and the DRG cells (i.e. DRG neurons) fundamentally differed between the sexes. Either 1) female macrophages are more robustly recruited under inflammatory conditions, or 2) female DRG neurons are more capable of robustly recruiting macrophages under inflammatory conditions. As EAE is a difficult model to consistently replicate as well as issues with throughput and cost, I decided to strip down this system into its base components and test fundamental differences *in vitro*. I established a protocol for primary cell culture of bone-marrow derived macrophages (BMDMs) and began characterizing male and female inflammatory activation parameters.

Understanding that macrophage activity must interact with neuronal activity *in vivo* due to proximity and the appearance of neuronally driven symptomology (i.e. pain), I intentionally designed experiments that would allow for transfer of experimental variables between experiments. This led to the generation of BMDM conditioned media and the investigation of its contents under the assumption that factors in the conditioned media would be prime candidates for

signaling onto nearby neurons in the proposed scenario where female DRGs are increased in activated macrophages.

## 3.0 Abstract

Decades of research into chronic pain has deepened our understanding of the cellular mechanisms behind this process. However, a failure to consider the biological variable of sex has limited the application of these breakthroughs into clinical application. In the present study, we investigate fundamental differences in chronic pain between male and female mice resulting from inflammatory activation of the innate immune system. We provide evidence that female mice are more sensitive to the effects of macrophages. Injecting small volumes of media conditioned by either unstimulated macrophages or macrophages stimulated by the inflammatory molecule TNFα lead to increased pain sensitivity only in females. Interestingly, we find that TNFα conditioned media leads to a more rapid resolution of mechanical hypersensitivity and altered immune cell recruitment to sites of injury. Furthermore, male and female macrophages exhibit differential polarization characteristics and motility after TNFα stimulation, as well as a different profile of cytokine secretions. Finally, we find that the X-linked gene *Tlr7* is critical in the proper resolution of pain in models of acute, chronic, and systemic autoimmune inflammation in both sexes. Altogether, these findings suggest that although the cellular mechanisms of pain resolution may differ between the sexes, the study of these differences may yield more targeted approaches with clinical applications.

Keywords: BMDM, macrophage, pain, inflammation, pain resolution, TNFα, sex differences, DRG, TLR7, innate immune activation.

## 3.1 Introduction

Women suffer from chronic pain syndromes more than men[1, 2, 3]. Differing levels of sex hormones and the complement of sex chromosomes influencing sexually dimorphic development could explain this phenomenon. Effective management of chronic pain, however, represents an unmet clinical need. This is in part due to the fact that research toward developing new therapeutic strategies for pain have often failed to consider the underlying sex differences in the mechanism mediating pain.

It is now well established that the immune system interacts with pain processing differently between the sexes[4, 5]. Depletion of specific populations of immune cells in animals, such as microglia, alters pain responses in a sex-specific manner[6]. Furthermore, recent evidence has described a female-bias of peripheral immune cell involvement in chronic pain[7, 8], suggesting an incomplete understanding of fundamental differences of sex-differences in peripheral immunity. Recently, our group has identified *Tlr7*, an X-linked gene, as being differentially expressed between male and female peripheral nervous tissue in animals with an inflammatory auto-immune condition (EAE) that is associated with heightened pain sensitivity[9]. The encoded protein TLR7 is canonically defined as a pathogen-associated molecular pattern (PAMP) receptor and is involved in the activation of innate immune cells[10]. As such, we sought to explore its involvement in the activation of the innate immune system in males and females and to determine if any differences uncovered could account for differential pain responses between the sexes.

Bone marrow derived macrophages (BMDMs) are classical innate immune cells responsible for the constant surveillance of their niches, free to encounter stimuli both foreign and endogenous[11]. Upon encountering an ‘inflammatory’ stimulus present after injury or

infection, these macrophages undergo a response that is beneficial for clearing up debris from tissue damage and conveying protection from invading bacteria and viruses[12]. In vitro, this phenotype can be reproduced by incubation with inflammatory molecules like tumour necrosis factor alpha (TNFα)[13, 14, 15]. In addition to their roles in acute inflammation, macrophages may also assist in wound healing and metabolic support which is thought to direct the recovery phase after injury or infection[16]. This phenotype can be achieved by incubation with the cytokine IL-4 which produces an enlarged, foamy appearance with reduced mobility[12, 15]. More recently however, data from single cell sequencing experiments has revealed that macrophages can adopt many different phenotypes with much more complexity than the oversimplified, dichotomous M1 or M2 phenotypes[17, 18, 19].

Knowing that differential sex characteristics modulate both pain and immunity, we have now conducted a series of experiments that explore the variable of sex in the context of innate immune cell activation and the impact that this has on inflammatory pain. We aimed to better define the phenotype and functional roles of male and female BMDMs in inflammatory pain and its resolution. We have used the pro-inflammatory cytokine TNFα to stimulate BMDMs and find that secreted factors from TNFα-stimulated female BMDMs are unique in their ability to promote the resolution of pain. Female BMDMs also display unique cytokine and histochemical profiles under inflammatory stimulation, and we provide evidence that the X-linked gene *Tlr7* plays a role in this sex-specific phenomenon. Understanding the interplay between pain and immunity and how this interplay is affected by biological sex will better inform future clinical practices to account for sex differences in the genesis of pain.

## 3.2 Materials and Methods

### 3.2.1 Animals

#### Wildtype Animals

Unless otherwise stated, all mice are wild-type C57/BL6, purchased from Charles River and included in experiments at 8-10 weeks of age (C57BL/6NCrl, Strain Code: 027, https://www.criver.com/products-services/find-model/c57bl6-mouse?region=24#). All mice were given access to food and water ad libitum and maintained in the University of Alberta facility under 12-hour light/dark cycles.

#### TLR7 KO Animals

*Tlr7* -/- breeders were purchased from The Jackson Laboratory (B6.129S1-Tlr7tm1Flv/J, Strain Code: 008380, https://www.jax.org/strain/008380). The colony was maintained in the University of Alberta's facilities and cross-bred with littermates to ensure continued depletion of the gene from the colony. Confirmation of gene absence was achieved by PCR from ear-notch samples, performed by Transnetyx (https://www.transnetyx.com/).

### 3.2.2 Bone Marrow Derived Macrophage (BMDM) Cultures

#### L929 Cells and Media

ATCC: NCTC clone 929; Areolar Fibroblast (Mus Musculus) cryopreserved cells were obtained as a generous donation from Dr. Christopher Power at the University of Alberta and stored at -80 °C until use. To generate L929 media, cells were thawed, expanded, and passaged until confluence. Media was fully changed and collected after one week, replaced with fresh media and collected after the second week. This "Week 1" and "Week 2" media was added to the base macrophage media (DMEM -/- including 10% Fetal Bovine Serum, and 1% of each: Penicillin/Streptomycin,

sodium pyruvate, and Glutamax) as 1:10 dilutions and used to generate BMDMs from naïve bone marrow stem cells.

Naïve Cells

Female and male 6 to 8 week old C57BL/6 mice were acquired from Charles River Canada, and habituated for approximately 1 week in the housing facility. After this period, animals were euthanized by Euthanyl® (sodium pentobarbital) injected intraperitoneally. After injection, animals were monitored for level of consciousness and dissections did not proceed until no response to toe pinch or corneal contact was observed. To confirm euthanasia, animals were intra-cardially perfused with 10 mL of cold saline. Femurs were bilaterally dislocated and kept on ice for further processing. Excess fascia and muscle tissue was removed by scraping with a scalpel blade (FEATHER, #22) and sterilized by a 5 minute incubation in 70% EtOH. Bone marrow was exposed by clipping the bony process from both ends of the femur and flushed with warm L929 BMDM media (see above) using a 20G, 5/8 in. needle. Flushed cells were collected in 50 mL conical tubes and pelleted at 1200 RPM. Cell pellets were resuspended by pipetting and the resulting single-cell suspensions were passed through a 70 um mesh filter to remove excess debris. Samples were approximated for viable density using Trypan Blue and a hemocytometer. Samples were adjusted to a final concentration of 1 million cells per milliliter. 10 mL of samples were plated on sterile 10 cm petri dishes and allowed to differentiate for 8 to 10 days. Media was changed on days 5 (half-change) and 7 (full-change). On day 9, media was removed and cells were washed once with room temperature sterile PBS before adding cold PBS. Cells were scraped from the petri dish using a Cell Scraper (Falcon, 1.8 cm blade, 353085) and collected in a 50 mL conical tube. The scraped cells were pelleted at 1200 RPM and resuspended in warmed L929 media. A small volume of cells was stained with Trypan Blue and viable cells were counted using a

hemocytometer. Total cell counts were extrapolated from the counted value and diluted to a final concentration of 300,000 cells/mL by adding an appropriate volume of L929 media. 100 uL of the cell suspension was seeded onto poly-L-lysine coated plates allowed to rest for 48 hours to reattach. Immediately prior to stimulation, media was fully changed to 'blank media' (DMEM -/- including 1% of each: Fetal Bovine Serum, Penicillin/Streptomycin, sodium pyruvate, and Glutamax).

### 3.2.3 BMDM Stimulation and Macrophage Conditioned Media

Seeded plates were stimulated with recombinant TNFα (R&D Biosciences, 410-MT) at concentrations of 200, 1000, or 5000 pg/mL. A cursory literature search revealed that TNFα is standardly used at much higher doses (greater than 20 ng/mL) but previous analysis of physiological levels of TNFα in inflammatory pain models suggested a lower dose would be more relevant[20]. Early optimization experiments found that the presented range provided sufficient activation without extensive cell death. Cells were incubated with the stimulus for 24 hours after which cells were fixed in 4% paraformaldehyde (PFA) in preparation for immunocytochemistry. Conditioned media (CM) from macrophages was generated by replacing stimulation media with fresh blank media and incubated for an additional 6 hours. Media collected from control cells was designated as Macrophage Conditioned Media (MCM) while media collected from TNFα-stimulated cells was designated as TNFα-stimulated Macrophage Conditioned Media (T-MCM). Additional blank media from conditioned media experiments was stored for usage as 'vehicle control'. Media was stored at -80 °C until cytokine quantification or behavioural usage, avoiding freeze-thaws.

### 3.2.4 Animal Behaviour

For all experiments involving measurements of animal behaviour, animals were randomly assigned to groups and experimenters were blinded in respect to animal treatment groups. At the conclusion of the experiment, the data was unblinded and analyzed.

Assays - Von Frey

Evoked mechanical paw withdrawal thresholds were measured with von Frey filaments (0.01g-2g) using the up-down method[21]. Filaments of increasing force, beginning with the 0.4 gram filament, were applied to the paw for a maximum of 5 seconds unless a pain response was observed. Once a pain response was observed, an additional four filaments were tested using increasing or decreasing force depending on whether each elicited a pain response. A lesser force followed a painful stimulus, while a greater force followed a non-painful stimulus. These responses were used to calculate the 50% positive response paw withdrawal threshold (PWT), which is the force required to elicit a pain response to 50% of stimulus exposures as described in Dixon[22]. For testing, mice were habituated to individual, clear plexiglass chambers on an elevated wire mesh screen for 30 minutes per day for 3 consecutive days, followed by a baseline assessment and subsequent experimental behaviour testing.

Assays - Formalin

Using a 29G needle (BD, 1/2 in., 324703), mice were placed in a clear plexiglass container after a 30 uL injection of 1% formalin (formaldehyde solution in sterile saline) into the left hind paw and observed for nocifensive responses binned in 5 minute increments over a total period of 60 minutes. Nocifensive responses were defined as any scratching, biting, licking, or guarding behaviours directed at the injected paw and all behavioural testing was performed by a single trained experimenter.

Hind paw Injections - Conditioned Media

Using a 29G needle (BD, 1/2 in., 324703), mice were injected with 30 uL of blank media (vehicle), macrophage conditioned media (MCM), or TNFα-macrophage conditioned media (T-MCM) into the left hind paw. Baseline Von Frey measurements were taken 24 hours prior to injection and reassessed at 24 and 48 hours post-injection.

Hind paw Injections - CFA

Using a 29G needle (BD, 1/2 in., 324703), mice were injected with 100 uL of Complete Freud's Adjuvant (CFA, 1 mg/ml, Sigma) into the left hind paw. Baseline Von Frey measurements were taken 24 hours prior to injection and reassessed at 3, 7, 10, 14, 16, 21, and 28 days post-injection. This modified CFA protocol is preferred over protocols with smaller volumes due to its ability to induce a more robust and prolonged pain syndrome without diminishing locomotive capacity, causing excessive inflammation, or leading to self-mutilation (unpublished observations). The modified protocol was developed and approved by on-site veterinary staff for usage and produces pain lasting greater than 4 weeks, allowing for the study of chronic pain adaptations.

Hind paw Injections – CFA with Imiquimod

Using a 29G needle (BD, 1/2 in., 324703), mice were injected 100 uL of imiquimod solution (Imiq, 500 uM in sterile saline) or vehicle (VEH, 0.21% DMSO) into the left hind paw at 3, 6, 8, 10, and 12 days post-injection of CFA on day 0 as described above. Imiquimod solutions were prepared by preparing stock solutions in DMSO and further dilution in sterile saline. Baseline Von Frey measurements were taken 24 hours prior to injection and reassessed at 3, 7, 14, 21, 28, 42, 47, 49, and 54 days post-injection.

### 3.2.5 Hind paw Immunohistochemistry

Hind paw glaborous skin was collected after completion of in vivo behavioural experiments. Animals were euthanized and intra-cardially perfused with ice-cold 0.9% saline followed by ice-cold 4% PFA. 2mm biopsy samples of the injected/tested area were collected utilizing a 2 mm biopsy punch, using contralateral hind paws as controls. Samples were post-fixed in 4% PFA for an additional day, followed by cryopreservation for 48 hours in a 30% sucrose solution in 0.1 M phosphate buffer. Cryopreserved samples were frozen in Optimal Cutting Temperature (OCT Tissue-Tek, Sakura, 4583) and sectioned into 12 um slices on a Cryostat, collected on Superfrost Plus (Fisherbrand, 2-0370246) slides.

For IHC analysis, slides underwent a previously described standard IHC protocol[23, 24]. Briefly, slides were washed in 1x phosphate buffered saline (PBS) for 5 minutes, repeated three times. Tissue sectioned were incubated with 10% NDS in PBS containing 0.2% Triton X for one hour at room temperature to prevent unspecific antibody binding. Blocking media was then removed and replaced with antibody solution with corresponding dilutions of antibody (see Supplemental Table 1) overnight at room temperature. The following day, slides were triple washed in 1x PBS and incubated with antibody solution containing corresponding fluorescent conjugated secondary antibodies (see **Supplemental Table 3.1**) for one hour at room temperature. Slides were washed a final three times and mounted with coverslips using FluoromountG (Invitrogen, 00-4958-02). Following staining, slides were imaged on a Zeiss AxioObserver.Z1 at 20x magnification. Two sections with one widefield of view each were imaged per sample. No additional processing was performed. Images were saved as .tif files for analysis using ImageJ. Cell counts were obtained by manual quantification of positively labeled cells by a blinded experimenter.

### 3.2.6 Cytokine Luminex Analysis

Media from conditioned media BMDM experiments was collected at the 6 hour post change time-point. 100-200 uL of media was collected and immediately frozen and stored at -80 °C. Samples were shipped to Eve Technologies in Calgary, Alberta (https://www.evetechnologies.com/) on dry-ice for analysis. The multiplexing analysis was performed using the Luminex™ 200 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Forty-five markers were simultaneously measured in the samples using Eve Technologies' Mouse Cytokine 45-Plex Discovery Assay® which consists of two separate kits: one 32-plex and one 13-plex (MilliporeSigma, Burlington, Massachusetts, USA). The assay was run according to the manufacturer's protocol. Assay sensitivities of these markers range from 0.3 – 30.6 pg/mL for the 45-plex. Individual analyte sensitivity values are available in the MilliporeSigma MILLIPLEX® MAP protocol. Observed analyte concentrations were reported after fitting to a cubic spline regression against a standard curve, under default settings of Bio-Plex Manager™ software (Bio-Rad Laboratories, Hercules, California, USA). Values reported as out of range for extrapolation were substituted for true zeroes. All samples were analyzed for cytokine concentrations for: 6Ckine, EPO, Fractalkine, IFNB-1, IL-11, IL-16, IL-20, MCP-5, MDC, MIP-3A, MIP-3B, TARC, TIMP-1; Medias from Figure 3.5 were additionally analyzed for cytokine concentrations for: Eotaxin, G-CSF, GM-CSF, IFN-y, IL-1a, IL-1B, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1a, MIP-1b, MIP-2, RANTES, TNF-a, VEGF.

### 3.2.7 Immunocytochemistry

At experimental endpoints, an equivalent volume of 8% PFA was added to the media of 96 well glass-bottom plates (Falcon, 353219) containing the adherent cells. Samples were incubated at room temperature in the diluted fixative for 15 minutes and then washed 3 times in Dulbecco's

Phosphate Buffered Saline (DPBS). Permeabilization and non-specific IgG binding was blocked by one hour incubation with 10% Normal Donkey Serum (NDS) in DPBS containing 0.2% Triton X at room temperature, followed by an overnight incubation with targeted antibodies to the protein of interest diluted in DPBS (**Supplemental Table 3.1**). The following day, cells were washed 3 times in DPS, incubated with DAPI (Invitrogen, D1306) and secondary antibodies for one hour at room temperature before three final washes and imaging. Cells strongly labeled with the associated fluorophore after background level adjustment were considered as positive phenotypes. For cell viability assays, a 10x stock solution of 5% Calcein AM (Invitrogen, C3099), 5% Propidium Iodide (Invitrogen, P3566) and 10% NucBlue (Invitrogen, R34605) was prepared. The stock solution was incubated with the live cells after 1:10 dilution for 15 minutes at 37 °C before imaging. Cells strongly labeled by Calcein AM/Propidium Iodide were considered as viable/non-viable respectively, with minimal overlap of signals detected. Imaging was performed on an ImageXpress Micro system and analyzed using MetaXpress 6.

### 3.2.8 qPCR quantitation and analysis

100 ng of total RNA was processed with DNase I (Invitrogen) followed by reverse transcription using Superscript III (Invitrogen). Transcripts were detected using the following primers: RT2 qPCR Primer Assay for Mouse Ppia, Tnfa, Il6, Tgfb, and Il10 (Qiagen Biosystems). Transcripts detected were standardized to Ppia values. All qPCR reactions were 20 uL volumes utilizing a StepOnePlus machine (Applied Biosystems). Statistical analysis was performed using Graphpad Prism 6.01. qPCR analysis utilized two-way ANOVA testing with Sidak's correction for multiple testing. qPCR statistics were performed on untransformed values but plotted as linearized values for ease of visualization. Alpha was set to 0.05 for all testing.

### 3.2.9 Migration Assay

BMDM seeded plates were prepared as described above. Immediately prior to TNFα stimulation, the monolayer of cells was disturbed by dragging a P200 pipette tip across the surface. The 'scratched' wells underwent a full media change to remove any dislodged cells and stimulated with TNFα or control media. The plate was immediately transferred into the environmental chamber of the ImageXpress Micro system and imaged under brightfield illumination every 15 minutes for 24 hours at 37 °C with supplemental $CO_2$. Images were exported and stacked into a time series using ImageJ. Images were thresholded to a background value and the area defining the scratch was manually defined at t = 0. The percentage of this area occupied by thresholded cells was repeated for each frame and subtracted by the initial value.

### 3.2.10 Trans-well Assay and Flow Cytometry

A single cell suspension of splenocytes was generated by mechanical dissociation of a whole spleen from a wildtype animal. Using a hemocytometer, the suspension was adjusted in concentration to 166 million cells/mL and 150 uL of this suspension (2e6 cells) was plated in the top chamber of a transwell plate (Corning, Transwell® Permeable Supports, 6.5 mm - 5 um, 3421). The bottom chamber was filled with blank solution, solution containing the positive control CCL19 at a concentration of 40 ng/mL (R&D Biosciences, 440-M3) or media from the BMDM CM experiments. The complete apparatus was incubated at 37 °C and 10% $CO_2$ for 5 hours before the flow-through (containing the migrated cells) was collected. These cells were pelleted, washed, and stained for the following targets: CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, CD45.2, F4/80, and the viability marker Zombie Aqua. The full details of antibodies and stains used in flow cytometry can be found in **Supplemental Table 3.2**. Stained samples were analyzed on a BD LSRFortessa X-20 (BD Biosciences) using single-stains to calibrate spectral profiles. The input sample was used as a reference of cell sub-populations to determine the proportion of cells that

successfully migrated through the transwell. Complete gating strategy can be found in **Supplemental Figure 3.2**.

## 3.3 Results

### 3.3.1 MCM/T-MCM and Animal Behaviour

To explore the link between pain and neuro-immune interactions, we first wanted to determine if macrophages secrete factors that could lead to pain hypersensitivity. Hind paw injection of media conditioned by sex-matched BMDMs allowed for testing of uni-directional macrophage signalling without feedback from the presence of the cells themselves (**Figure 3.1A, A'**). Injection of control or conditioned media (CM) into the hind paws of mice from all treatments and sexes resulted in reduced pain thresholds during the acute phase (< 12 hours). All male conditions returned to baseline values after 24 hours and onwards (2-way ANOVA, time effect $F_{5,95} = 8.190$, $p < 0.05$, **Figure 3.1B**). Interestingly, both the MCM and the conditioned media from TNFα stimulated macrophages (T-MCM) from females triggered a more persistent reduction in mechanical thresholds compared to the full resolution of pain in the control media group after 24 hours. However, the female mice that received T-MCM recovered to baseline sensitivity after 24 hours and became hyposensitive at 48 hours while tactile hypersensitivity in female mice receiving MCM remained unresolved at 24 hours and only recovered to baseline sensitivity at 48 hours (2-way ANOVA, treatment secondary effect $F_{2,17} = 4.557$, $p < 0.05$; 24 hr F Blank vs. 24 hr F MCM, $p < 0.05$, **Figure 3.1C**).

After assessing mechanical sensitivity following MCM and T-MCM treatment, we performed histochemical staining of the injection site within the skin at 48 hours post injection (**Figure 3.2**). All male conditions contained a consistently low quantity of CD45+ cells.

Concurrent with the lasting hypersensitivity, the tissue from females treated with MCM contained significantly increased numbers of CD45+ cells compared to media-only injected controls. By contrast, females treated with T-MCM with resolving hypersensitivity had levels of CD45+ cells that were similar to media only controls at the experimental endpoint (2-way ANOVA, interaction effect $F_{2,24}= 7.607$, $p = 0.0028$; F Media vs. F MCM, $p = 0.0151$, **Figure 3.2G**). Therefore, accumulation of leukocytes into the skin correlates with the degree of pain hypersensitivity.

### 3.3.2 BMDM CM as a regulator of chemotaxis

Although CM injection in females was able to elicit heightened pain responses and the greatest pain response correlated to tissues with a heightened presence of immune cells, these independent results could not indicate if the influx of immune cells was directly related to the injection of the conditioned media. Therefore, we next tested whether the CM from BMDMs could be directly chemotactic for immune cells (naïve splenocytes). Using a transwell system, we assessed the migration of splenocytes in response to blank media, MCM and T-MCM from female BMDMs by flow cytometry. Most migrating splenocytes were of B/T cell lineages, with a smaller percentage of myeloid origin. Among leukocytes induced to migrate by female T-MCM, we observed an enrichment in the CD3+ cells as well as the CD4+ and CD8+ subsets (two-tailed t-test, CD3: $t = 2.687$, $p < 0.05$; CD4: $t = 2.440$, $p < 0.05$; CD8: $t = 2.758$, $p < 0.05$; **Supplemental Figure 3.1**) suggesting that the treatment of female BMDMs with TNFα can affect the production of chemotactic secreted factors for T lymphocytes. No change was detected in the rate of myeloid cell migration (data not shown).

### 3.3.3 Macrophage Conditioned Media

After establishing that macrophage conditioned media (MCM and T-MCM) can elicit pain and exploring the plausibility of chemotactic factors altering immune cell migration, we analyzed the cytokine content of the media by Luminex®. A total of 45 separate cytokines were analyzed including standard markers consistent with inflammatory macrophage activation (CXCL1/2/10, CCL2/4/5) as well as secondary activation markers (RANTES, MIP1a, IL-16 etc.)

When examining the levels of these cytokines in MCM and T-MCM, we found that cytokines exhibiting the most significant changes between MCM and T-MCM grouped into three categories: no sex differences (CXCL1, CXCl2, CXCL10, CCL2, CCL4, CCL5, 2-way ANOVA, TNFα effect $F_{1,12} > 40$, $p < 0.05$, **Figures 3 A-F**), sex-dependent differences (CCL12: 2-way ANOVA, sex effect $F_{1,12} = 85.32$, $p < 0.05$; CCL22: 2-way ANOVA, interaction effect $F_{1,12} = 64.66$, $p < 0.05$, **Figures 3.3G, H**), and sex-specific differences (IL-16, 2-way ANOVA, interaction secondary effect $F_{1,12} = 7.906$, $p < 0.05$, **Figure 3I**). Most detected cytokines differed in media conditioned by TNFα-treated cells in both sexes, displaying an expected activation profile of primary markers. Secondary markers revealed a more subtle distinction between the sexes: CCL12 levels increased in both sexes but is more pronounced in males while CCL22 was decreased in both sexes but this effect was more pronounced in females. Of all the cytokines analyzed, only IL-16 displayed a sex-specific effect. Levels of IL-16 were reduced to approximately 75% of baseline in the female T-MCM condition, making it a promising target for follow-up analysis. A full summary of cytokine quantification with fold changes can be found in **Supplemental Table 3.3**.

### 3.3.4 BMDMs and TNFα Stimulation

To assess any overt differences in BMDM function or viability between the sexes, naïve BMDMs from male and female animals were exposed to a range of TNFα doses in the same order of magnitude that has been detected physiologically during inflammation. No significant effect was seen in the proportion of healthy cells. Females showed a significantly higher proportion of dead or dying cells after accounting for the TNFα effect, measured by PI+/CalceinAM- staining (2-way ANOVA, sex secondary effect $F_{1,40} = 6.238$, $p < 0.05$, **Figure 3.4A**). Concurrent with the increased rate of death, female BMDMs displayed a greater proliferative capacity over males even in the absence of stimulation. This proliferative capacity was significantly lessened by the maximal concentration of TNFα (5000 pg/mL) (2-way ANOVA, sex main effect $F_{1,40} = 52.89$, $p < 0.05$, **Figure 3.4B**). Further characterization of these cells using qPCR revealed a similar transcriptomic profile of activation in both sexes (**Supplemental Figure 3.3**) but using the classical markers for inflammatory (iNOS) and anti-inflammatory (Arg1) cells revealed dose-dependent increases in both markers with a more pronounced effect in the iNOS labeling of female BMDMs (iNOS: 2-way ANOVA, sex secondary effect $F_{1,40} = 20.36$, $p < 0.05$; Arg1: sex secondary effect $F_{1,40} = 3.659$, $p = 0.0629$, **Figures 3.4C, D**).

We continued our assessment of BMDM activity by subjecting confluent cultures of BMDMs to a motility assay with/without the moderate dose of TNFα (1000 pg/ml). Male macrophages did not change motile capacity with TNFα stimulation (**Figure 3.5 A-C**). Interestingly, female macrophages were more motile in the absence of any stimulation but after TNFα stimulation, this motility was reduced. The total recovered scratch area was determined for males (RM 2-way ANOVA, TNFα effect $F_{1,10} = 0.04$, $p = 0.8499$, **Figure 3.5C**) and females (RM 2-way ANOVA, TNFα effect $F_{1,10} = 12.94$, $p < 0.05$, **Figure 3.5F**).

### 3.3.5 *Tlr7* regulates Inflammatory Cytokine Levels

Previous work had established TLR7 as a potential mediator of sex specific differences in inflammatory pain[9]. TLR7 is an X-linked gene that is selectively and highly expressed on macrophage and dendritic cells in the periphery and is a mediator of macrophage activity. Additionally, signaling through TLR7 has been shown to negatively regulate the expression of many cytokines[25, 26, 27]. Therefore, we explored the impact of TLR7 on the cytokine profile of female BMDMs from *Tlr7* -/- mice by measuring cytokine production in the basal state and after TNFα stimulation. We found that female *Tlr7* -/- media varies drastically from its wild-type counterpart in cytokine content (**Figure 3.6**). Specifically, the increased levels of CCL12, CCL17, and CCL22 following TNFα stimulation (blank vs. + TNFα) was absent in the *Tlr7* -/- conditions.

To determine the active role of TLR7 in the TNFα-driven cytokine response, the TLR7 agonist imiquimod (Imiq) was layered as a co-stimulator with TNFα in wildtype and *Tlr7* -/- BMDMs. This demonstrated that TNFα sensitivity can be heightened by TLR7 activation (+ TNFα vs. + TNFα + Imiq) (2-way ANOVA, genotype tertiary effect $F_{1,24} > 10$, $p < 0.05$, **Figures 3.6 A-C**). Treatment of female *Tlr7* -/- BMDMs with TNFα lead to a decrease in IL-16 to levels seen in wildtype cells, however the absolute levels of IL-16 remain higher than baseline in all conditions (2-way ANOVA, genotype secondary effect $F_{1,24} = 267.7$, $p < 0.05$, **Figure 3.6D**). Additionally, the levels of IL-16 are dramatically increased even under unstimulated *Tlr7* -/- conditions, illustrating the requirements of TLR7 for the regulation of the expression proinflammatory cytokines like IL-16 under basal and inflammatory conditions.

### 3.3.6 Hind paw CFA & Formalin Injections

After establishing that CM from BMDMs differ by sex in both cytokine content and pain induction as well as differing in content when female BMDMs lack *Tlr7*, we sought to investigate the reciprocal involvement of TLR7 in various in vivo models of pain. Utilizing the *Tlr7* -/- animal line, animals lacking *Tlr7* displayed heightened and persistent nociceptive hypersensitivity in both the formalin model (**Figures 3.7 A, B**) and the CFA model of chronic inflammatory pain (**Figures 3.7 C, D**). In the formalin model, male *Tlr7* -/- animals displayed increased nocifensive behaviour at all measured timepoints including baseline, but this increase was not significant (2-way ANOVA, genotype secondary effect $F_{1,24} = 4.255$, $p = 0.0692$, **Figure 3.7A**). Female *Tlr7* -/- animals were identical to their wildtype counterparts in the acute phase of the formalin response but exhibited a more prolonged, persistent second phase compared to wild type controls (2-way ANOVA, genotype secondary effect $F_{1,10} = 6.503$, $p = 0.0289$, **Figure 3.7B**).

Additionally, both sexes of *Tlr7* -/- animals exhibited more prolonged pain hypersensitivity in response to CFA. Although the absolute level of tactile allodynia in response to CFA was not different between wild type and *Tlr7* -/- mice, the *Tlr7* -/- animals exhibited an impaired pain resolution in response to CFA. At 28 days post-injection, male and female *Tlr7* -/- animals had recovered to approximately 40 and 20% of baseline threshold respectively compared to the fully recovered wildtype animals (RM 2-way ANOVA, interaction effect $F_{5,75} > 3$, $p < 0.05$, **Figures 3.7C, D**).

### 3.3.7 *Tlr7* -/- CFA + Imiquimod

After establishing that mice lacking *Tlr7* show increased pain levels and delayed pain resolution in three separate in vivo models, we next tested whether direct agonism of TLR7 could affect pain hypersensitivity or enhance the rate of recovery in the CFA model. We chose to conduct this experiment in female mice because our evidence indicated that females were more dependent on

this TLR7 system for pain resolution. Direct agonism of the TLR7 receptor using the selective agonist imiquimod did not appear to be immediately analgesic, however, imiquimod treatment did promote a faster resolution of pain after CFA injection compared to vehicle treated controls (2-way ANOVA, treatment secondary effect $F_{1,10} = 10.93$, $p < 0.05$, **Figure 3.8A**). The AUC of pain scoring was significantly greater in the imiquimod treated mice reflecting the more rapid resolution to baseline mechanical withdrawal thresholds following CFA injection (Kolmogorov-Smirnov test, $p < 0.05$, **Figure 3.8B**).

## 3.4 Discussion

### 3.4.1 BMDM CM as a vector for pain

As the first line of immune defense in peripheral tissues, macrophages are responsible for mounting appropriate responses to threats to the individual. A hallmark of the appropriate macrophage response is the initiation of a rapid influx of other immune cells into the site of injury or infection. Secretions from these cells (cytokines, exosomes etc.)[28, 29, 30] promote inflammatory responses, including the production of the cytokines TNFα, IL-1β, and Type I interferon cascades. Activity of these 'first-responders' initiates a coordinated response that ensures a coordinated adaptive immune response to infection, proper debris clearance after trauma, and promoting regeneration of tissues after injury. These responses can also affect neuronal signalling, manifesting as alterations in sensation and motor control but most commonly as pain. While acute pain is beneficial to ensure proper health of an individual, pain lasting beyond this helpful period represents a large economic and psychological burden on modern societies. Pain as a disability results in an high amount of work days lost per year per worker[31], and is an economic burden[32, 33, 34]. Chronic pain unequally affects men and women, so combined with ours and others' evidence

for sex-biased innate immune activation, we sought to investigate the involvement of sex-specific macrophage activity on pain outcomes.

Using a model of indirect macrophage transfer using the cell-free conditioned media from male and female macrophages, we found that injections of the CM from BMDMs elicited pain responses that varied depending on the activation profile of the BMDMs in a sex-specific manner. Although both sexes exhibited pain responses in the acute phase, we found that females had very distinct pain responses that depended on the activation profile of the CM. Despite lacking an exogenous stimulus, macrophages at rest also generated conditioned media with robust cytokine content but did not differ between males and females. Interestingly, while 'unstimulated' MCM produced prolonged pain hypersensitivity, the CM from BMDMs stimulated with TNFα (T-MCM) generated a resolving pain phenotype in female mice. As the cytokine profiles of the CM from the different sexes were almost identical except for a few factors, it is unlikely that differential cytokine/chemokine production alone is the cause for these different behavioural profiles. One possible explanation is that females have higher levels or enhanced sensitivity of the corresponding receptors and are therefore more sensitive to the CM. This hypothesis is congruent with literature supporting a female-bias toward macrophages in peripherally mediated pain. Macrophage-targeted inhibition of COX-2 has been shown to inhibit inflammation in the CFA model and confer greater anti-nociceptive properties in male over female mice, highlighting the beneficial effect of inflammation in female biology[35]. Furthermore, mechanical hypersensitivity can be induced by a female-specific axis of IL-23 and IL-17 through macrophages and is dependent on estrogen[36]. Our data supports the notion of a female-specific mechanism of innate immune driven nociception following cutaneous tissue injury but requires further study to investigate the underlying mechanisms.

### 3.4.2 Sex as a biological variable in macrophage activation

The polarization of macrophages (as well their CNS counterparts, microglia) under inflammatory stimuli remains a contentious topic to this day[11, 37]. More recent sequencing studies have shown that macrophages exist as a diverse group with many subpopulations, and transition into many different clusters based on the exact paradigm. Representing this complex situation as existing in the linear space is an oversimplification but is still useful for basic contexts and may be sufficient to understand functional roles[12]. As many of the original studies typically only used male mice, sex as a biological variable is another dimension that must now be considered. Many auto-immune diseases present with sex biases in incidence rates as well as disease trajectories suggesting that inherent differences between the sexes may contribute to these reports. It is apparent that 'homeostatic' macrophages are not inactive; unstimulated macrophages of each sex may be uniquely primed with activation responses. If these activation responses are different, do these macrophage responses also vary by sex? Our data demonstrates that the answer to this question is in the affirmative. Using a classic inflammatory molecule (TNFα)[14, 38], we show that female BMDMs are more sensitive to activation. Although the trajectory of change is similar between the sexes, there are subtle differences in multiple metrics such as proliferation, motility, and cytokine profiles that impact on functional outcomes like the resolution of pain hypersensitivity. Our findings align with existing literature that supports the notion of sex-specific differences in macrophage activation. For instance, subtle sex differences have been reported in cytokine levels of CCL22 in IL-4 stimulated BMDMs, with females displaying a bias towards increased expression after IL-4 stimulation[39]. In contrast, our usage of TNFα as an opposing pro-inflammatory mechanism revealed an inverted effect, with females trending towards greater regulation. These results suggest that CCL22 may possess sex-specific regulatory mechanisms

linked to inflammation. A separate study investigating LPS and TNFα as stimulation factors found that male macrophages displayed heightened activation[40]. Although not explored in this study, it is worth noting that LPS stimulation is well known to favor the activation of male macrophages, which is consistent with the higher membrane presentation of associated Toll-like receptors (TLRs) in male cells[41]. The authors also indicated that TNFα stimulation favors males, although the dosing of TNFα used in that study was 20 times higher. This discrepancy may suggest that sex differences in BMDM activation are even more nuanced, with our data suggesting that females may exhibit heightened sensitivity to lower grades of inflammation, while males may possess a higher capacity for activation responses to higher grades of inflammation. These findings underscore the importance of considering sex as a biological variable in macrophage activation studies, as it can contribute to a more comprehensive understanding of the complex mechanisms underlying immune responses.

### 3.4.3 TNFα stimulation drives resolving pain – a female specific phenomenon

Inflammation is well-characterized in its ability to induce acute pain. Once the tissue is healed, however, the pain typically resolves. Preventing the development of chronic pain, or pain that does not properly resolve, is a clinically relevant goal. Interestingly, we found that while both sexes of our CM injected mice displayed acute pain responses, female mice displayed a delay in resolution that was significantly diminished in the group receiving CM from macrophages stimulated with the pro-inflammatory cytokine TNFα (T-MCM). This suggests that at least in females, inflammation may have both pro- and anti-nociceptive effects. The pro-nociceptive effects are well established under classical inflammatory pathways and can be seen as an ‘accelerator pedal’ for driving the acute pain response. However, the anti-nociceptive effect is less well understood but may represent the inverse: a self-limiting ‘brake pedal’ for the adaptive resolution of pain. Recent

meta-analyses of clinical trials involving pain outcomes corroborate this hypothesis; usage of nonsteroidal anti-inflammatory drugs (NSAIDs) correlates with an increase in chronic pain reports suggesting that preventing early inflammatory responses may be detrimental in terms of pain resolution[42]. This may have significant negative impacts on health with chronic usage[43]. As T-MCM was ineffective in male mice, an interesting future direction could investigate the source of the discrepancy by repeating experiments with male and female mice receiving media generated from the opposite sex's macrophage. Any translatable effect of female T-MCM into males would provide evidence of the female macrophage being critical and not the sex of the receptive nociceptive system.

### 3.4.4 TLR7 as a regulator for the resolution of pain. Indication of sex-specificity?

We know from previous work that the female DRG is more affected in the EAE model of neuroinflammation than males, even with equivalent pain and clinical scores[9, 20]. When investigating this phenomenon, we became interested in TLR7 as it was one identified gene that exhibited a sex-specific upregulation in the DRGs of female mice. TLR7 is part of the Toll-like receptor family, a group of receptors highly specialized to sense 'pathogen-associated molecular patterns' or PAMPs which are indicative of viral infection and also happens to be an X-linked gene. TLR7 is abundantly expressed in innate immune cells including macrophages. The choice of the formalin and CFA models for testing pain was driven by the need to simulate distinct pain scenarios. The formalin model was employed as an acute, rapidly resolving model to replicate adaptive pain commonly experienced in everyday life, with effects lasting for approximately an hour. In contrast, the CFA model was utilized to investigate chronic pain by inducing a pain syndrome that persist and resolve over multiple weeks. This longer duration enables the study of chronic pain mechanisms and the exploration of maladaptive pain, which may emerge following

significant injuries or infections. By incorporating both the formalin and CFA models, we aimed to comprehensively investigate different dimensions of pain experiences, encompassing acute adaptive pain and chronic maladaptive pain. Interestingly, using a *Tlr7* -/- mouse line, we found that both males and females lacking *Tlr7* had more prolonged pain responses in the CFA model of cutaneous tissue inflammation but only modulated the late phase of formalin induced pain in female mice lacking *Tlr7*. This suggests that TLR7 engages female-biased signaling pathways that may be necessary to limit inflammation and facilitate the resolution of pain without modulating steady-state or acute pain. Indeed, we find that administration of the TLR7 selective activator, imiquimod, promoted a more rapid resolution of pain in CFA treated mice, suggesting that engagement of TLR7 may be a useful strategy to promote a more rapid resolution of pain. Interestingly, despite confirmation of TLR7's absence, imiquimod was still able to produce a response in *Tlr7* -/- BMDMs suggesting off target effects. The most likely candidate for this is TLR8, a homolog with similar but reduced profiles of expression compared to TLR7[44, 45, 46]. Further complicated by differences between the mouse and human orthologs, the mechanistic differences between TLR7 and 8 activation by imiquimod remains an open question and limit our interpretation of a TLR7-only effect. Although our knockout studies confirmed that both sexes utilize TLR7 as a regulator of the resolution of pain and immune-related inflammation, this mechanism may be less critical in males due to the absence of prolonged pain under our CM injection paradigm. The attenuated impact observed in *Tlr7* -/- males is corroborated by prior research examining male pain within the formalin model[47]. However, it is worth noting that no investigation regarding potential sex-related differences was conducted, as female mice were not included in the study. Other works investigating pain in *Tlr7* -/- have reported no sex differences[48], highlighting the possibility of mouse strain and pain modality specific effects. Investigation of the

involvement of TLR7 in the mechanisms behind the failure to resolve pain in females may provide more targeted strategies for therapeutics.

### 3.4.5 Interplay of TNFα, IL-16, and *Tlr7*. Implications for clinical pain modulation potential?

The observed faster resolving phenotype in female mice upon T-MCM injection, combined with the analysis of the CM suggests a potential involvement of IL-16 as it was the only cytokine to exhibit a female-specific effect. Given that IL-16 is a known chemokine for immune cells expressing CD4[49, 50], future directions could focus on exploring the specific role of IL-16 in mediating this response and its potential implications in pain resolution. In addition, it is worth noting that the levels of IL-16 were found to be modulated by the presence of *Tlr7*. This observation further strengthens the potential significance of investigating the interplay between TNFα, IL-16, and *Tlr7*. Such investigations hold promising implications in terms of clinical relevance, particularly considering that the *Tlr7* agonist imiquimod is already approved for usage in humans[51, 52]. Exploring the intricate relationship between TNFα, IL-16, and *Tlr7* may open up new avenues of research with the potential to contribute to our understanding of pain modulation and offer insights for future therapeutic interventions.

## 3.5 Conclusion

To our knowledge, this is the first demonstration of sex as a biological variable in classical BMDM activation. We show that female and male cells exhibit similar but distinct activation profiles *in vitro*. Injection of CM from these cells into mouse hind paws promotes similar but distinct pain phenotypes, depending on the sex of the recipient mouse. Together, this data shows the importance of sex as a biological variable in consideration of immune mediated inflammation as well as providing a framework to dissect the molecular mechanisms further. Based on our data, we

postulate that *Tlr7*, an X-linked gene and seemingly biased towards higher levels of expression in females, may play a critical role in facilitating the adaptive resolution of pain linked to tissue injury and inflammation.

## 3.6 Appendix

Abbreviations

Experimental autoimmune encephalomyelitis (EAE); Central nervous system (CNS); Dorsal root ganglia (DRG); Peripheral nervous system (PNS); Complete Freund's Adjuvant (CFA); Tumor necrosis factor alpha (TNFα); Toll-like receptor 7 (TLR7); Pathogen-associated molecular pattern (PAMP); Bone marrow derived macrophage (BMDM); Macrophage conditioned media (MCM); Imiquimod (Imiq); Paw Withdrawal Threshold (PWT); Paraformaldehyde (PFA); Phosphate buffered saline (PBS); Normal donkey serum (NDS);

Declarations

*Ethics approval and consent to participate:* All animal studies complied with the Canadian Council on Animal Care Guidelines and Policies and were approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta.

*Consent for Publication:* Not applicable.

*Availability of Data and Materials:* All data generated or analysed during this study are included in this published article and its supplemental information files.

*Competing interests:* The authors declare that they have no competing interests.

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*Authors' contributions:* TNF performed cell culture and in vitro imaging experiments. OC performed behavioural analyses with assistance from CZ. CZ performed histochemical quantitation. TNF, OC and BJK were involved in experimental design and manuscript preparation. All authors read and approved the final manuscript.

*Facilities:* Experiments were performed at the University of Alberta Faculty of Medicine & Dentistry Cell Imaging Core, RRID:SCR_019200, the University of Alberta Faculty of Medicine & Dentistry Flow Cytometry Facility, RRID:SCR_019195, and the University of Alberta Faculty of Medicine & Dentistry High Content Analysis Core, RRID:SCR_019182; These facilities receive financial support from the Faculty of Medicine & Dentistry, the Department of Medical Microbiology and Immunology, the Li Ka Shing Institute of Virology, the University Hospital Foundation, and Canada Foundation for Innovation (CFI) awards to contributing investigators.

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## 3.7 Figures

Figure 3.1.

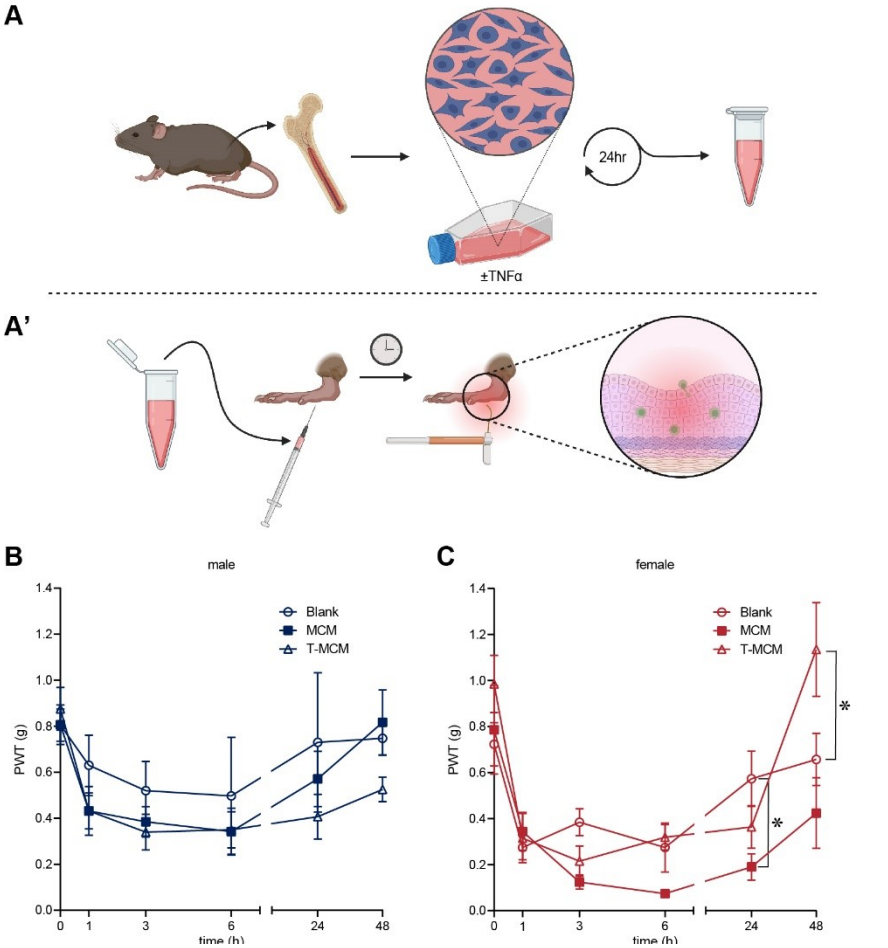



Figure 3.1

Female mice exhibit prolonged pain after hindpaw injection and TNFα dependant pain resolution. (A) Schematic of BMDM CM generation. (A'') Schematic of CM injection paradigm. (B, C) Changes in withdrawal threshold to mechanical stimuli applied to hindpaws following hindpaw CM injection in male (B) and female (C) mice over a 48 hour post-injection period. n = 5 per group; * $p < 0.05$. Bars and symbols with error bars represent mean ± SEM. Data is analyzed by repeated-measures two-way ANOVA and Sidak's multiple comparison test. PWT = Paw Withdrawal Threshold.

**Figure 3.2.**

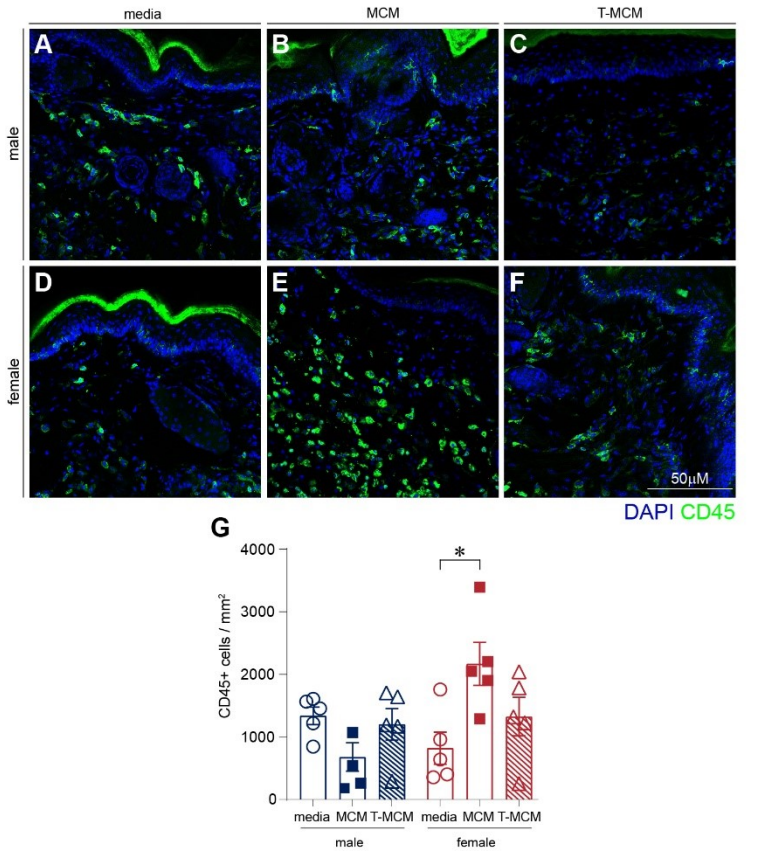



Figure 3.2

Injected hindpaws of female mice with unresolving pain contain greater quantities of CD45+ cells 48 hours post-injection of BMDM CM. (A-F) Representative images of injected mouse hindpaws, stained for CD45 (green) cells and DAPI (blue). Only the female mice receiving MCM injection showed increases in CD45+ cell counts. (G) Quantification of CD45+ cells Scale bar = 50µm; n = 5 per group; * $p < 0.05$. Bars with error bars represent mean ± SEM. Data is analyzed by two-way ANOVA with Sidak's post hoc test.

**Figure 3.3.**

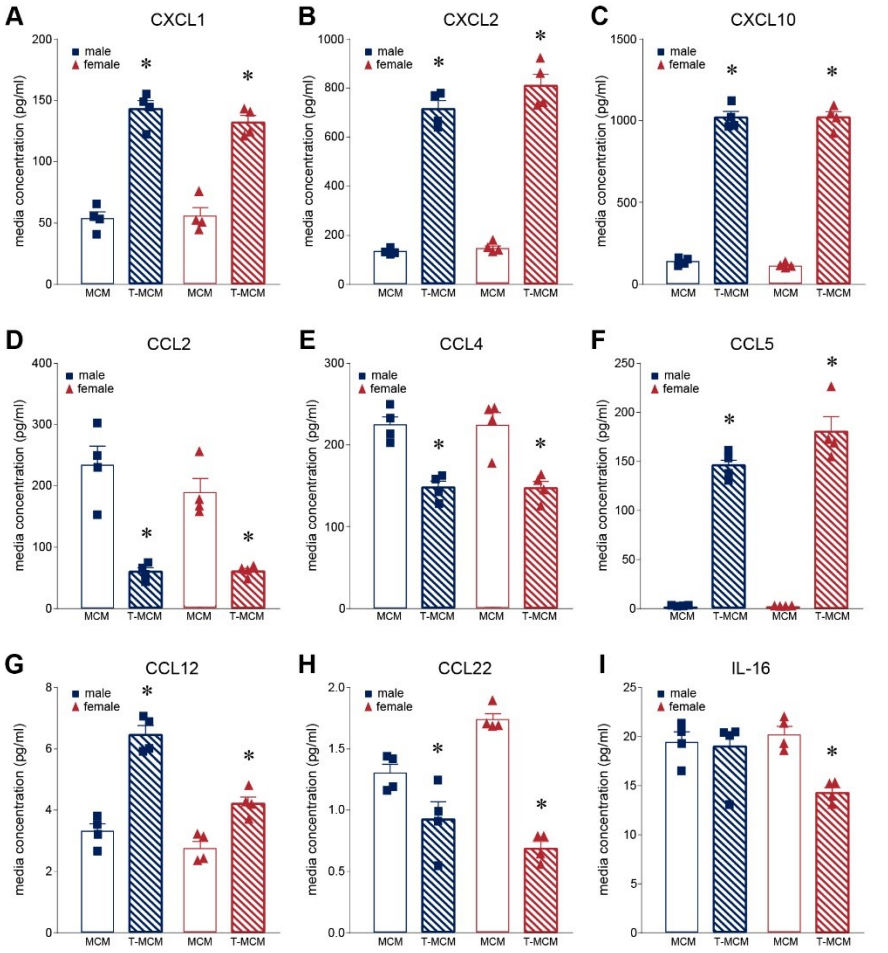



Figure 3.3

BMDM-conditioned media (CM) analysis of cytokine content reveals both sex dependent and independent effects. BMDMs were stimulated for 24 hours with or without TNFα and then allowed to condition fresh blank media for an additional 6 hours before collection. A total of 45 cytokines were profiled in the CM. Example cytokines with TNFα-mediated effects include CXCL1, 2, 10, CCL2, 4, 5 with no sex differences (A-F) as well as CCL12 and CCL22 that vary by sex (G, H). Only IL-16 was sex-specific for a TNFα effect (I). n = 4 per group, each n represents a replicate well of BMDM stimulation and conditioned media; * $p < 0.05$. Bars with error bars represent mean ± SEM. All comparisons are made to sex-matched control. Data is analyzed by two-way ANOVA with Sidak's post hoc test.

**Figure 3.4.**

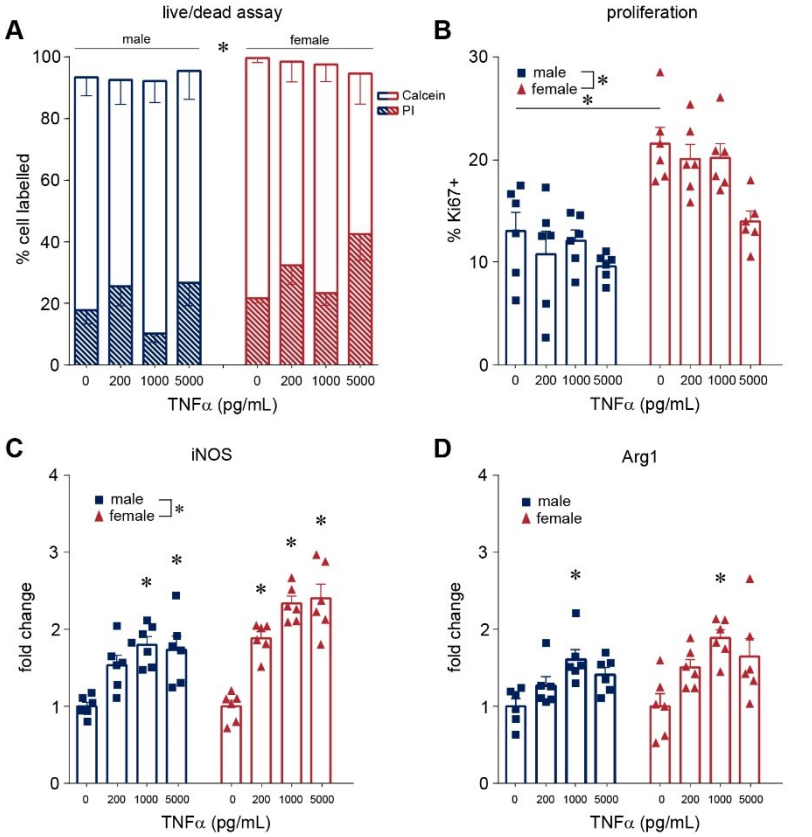



Figure 3.4

Sex differences in macrophage markers of proliferation and polarization. (A) Proportions of BMDMs stained with the live cell marker CalceinAM (open bars) or the dead cell marker propidium iodide (PI, hashed bars). (B) Absolute proportions of BMDMs with nuclear labeling of the proliferative marker Ki67. Both males and female showed reduced Ki67 labeling with escalating TNFα dosage with females additionally showing higher staining without TNFα stimulation. (C, D) Normalized changes in proportions of BMDMs labeled with the classical M1 marker iNos (C) or the classical M2 marker Arg1 (D) Data are normalized and compared to the blank conditions of the respective sex unless otherwise stated. n = 6 per group; * $p < 0.05$. Bars with error bars represent mean ± SEM. Unless otherwise stated, all comparisons are made to sex-matched control. Data is analyzed by two-way ANOVA with Sidak's post hoc test.

Figure 3.5.

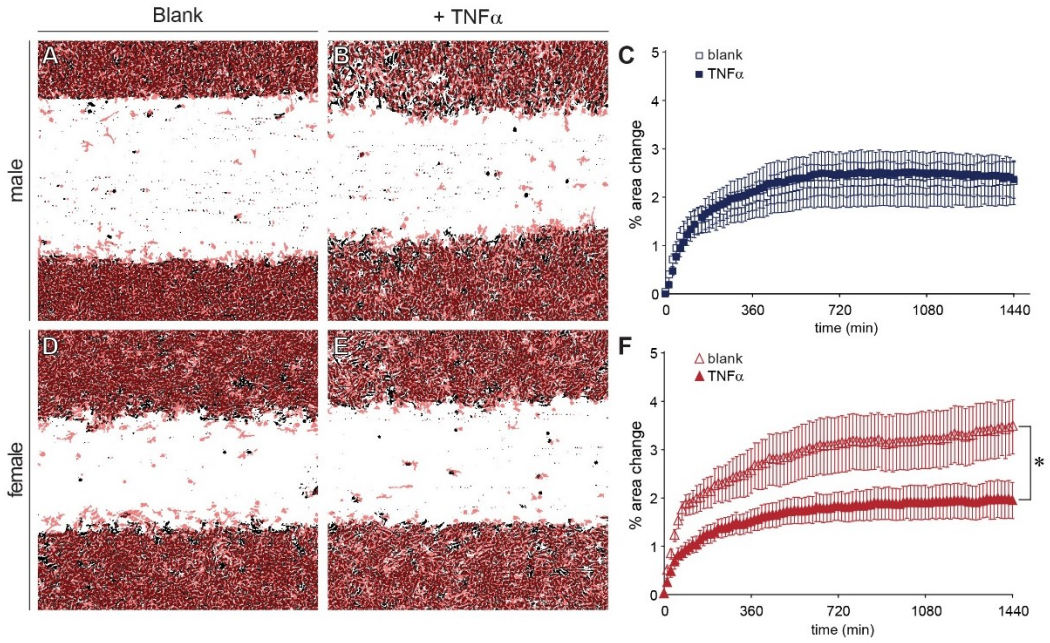



Figure 3.5

Female BMDMs exhibit enhanced motility which can be ameliorated by TNFα stimulation. A confluent layer of BMDMs was manually scratched using a P200 pipette tip and imaged at 15 minute intervals for 24 hours. (A, B, D, E) Representative stacks of male (A, B) and female (D, E) BMDM scratch assay ± TNFα, with the overlay (red) depicting thresholded cells at the final timepoint compared to the initial timepoint (black). (C, F) Change in the male (C) and female (F) cell footprint within the defined scratch area at t = 0. Each timepoint is normalized to the initial area. n = 6 per group; * $p < 0.05$. Symbols with error bars represent mean ± SEM. Data is analyzed by repeated-measures two-way ANOVA.

**Figure 3.6.**

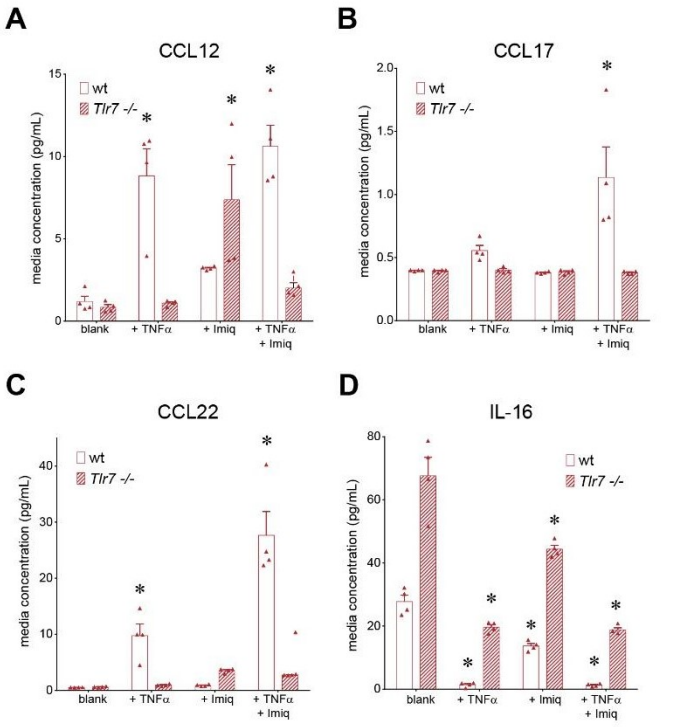



Figure 3.6

TNFα regulated cytokines are additionally regulated by TLR7 and the TLR7 agonist Imiquimod. Female BMDMs from wt and Tlr7 -/- animals were stimulated for 24 hours with TNFα, Imiquimod, or both and then allowed to condition fresh blank media for an additional 6 hours before collection. (A-C) The TNFα effect on CCL12, CCL17, and CCL22 is ameliorated with the loss of *Tlr7*. (D) The female-specific cytokine IL-16 levels in CM from wt and *Tlr7* -/- female BMDMs. n = 4 per group, each n represents a replicate well of BMDM stimulation and conditioned media; * $p < 0.05$. Bars with error bars represent mean ± SEM. All comparisons are made to treatment-matched control. Data is analyzed by two-way ANOVA with Sidak's post hoc test.

**Figure 3.7.**

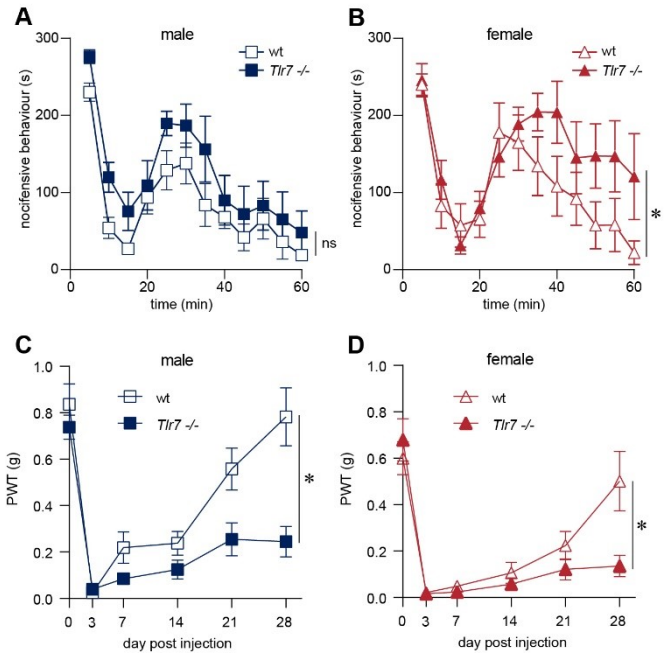



Figure 3.7

Animals lacking *Tlr7* display increased and unresolving pain phenotypes. Male (A) and female (B) nocifensive behavioural quantitation following hindpaw formalin injection in wt and *Tlr7* -/- mice. Hindpaw injection of formalin causes acute pain lasting approximately 15 minutes, followed by an extended period of pain lasting approximately an hour. Animals were video recorded for 60 minutes post-injection and the playback was quantitated for periods of nocifensive behaviour by a blinded experimenter. Male (C) and female (D) withdrawal thresholds to mechanical stimuli following hindpaw CFA injection in wt and *Tlr7* -/- mice, with day 0 representing baseline measurements. Hindpaw injection of CFA causes prolonged sensitivity to mechanical stimuli that resolved over a period of 4 to 6 weeks. n = 5-10 per group; * $p < 0.05$. Bars with error bars represent mean ± SEM. Data is analyzed by repeated-measures two-way ANOVA with Sidak's multiple comparison test. CFA = Complete Freund's Adjuvant. PWT = Paw Withdrawal Threshold.

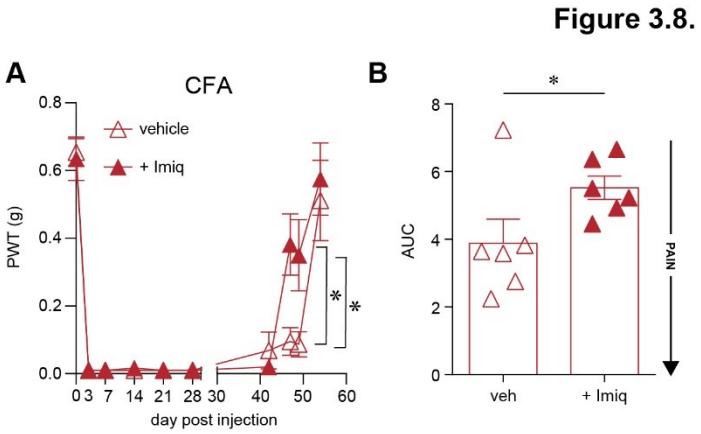
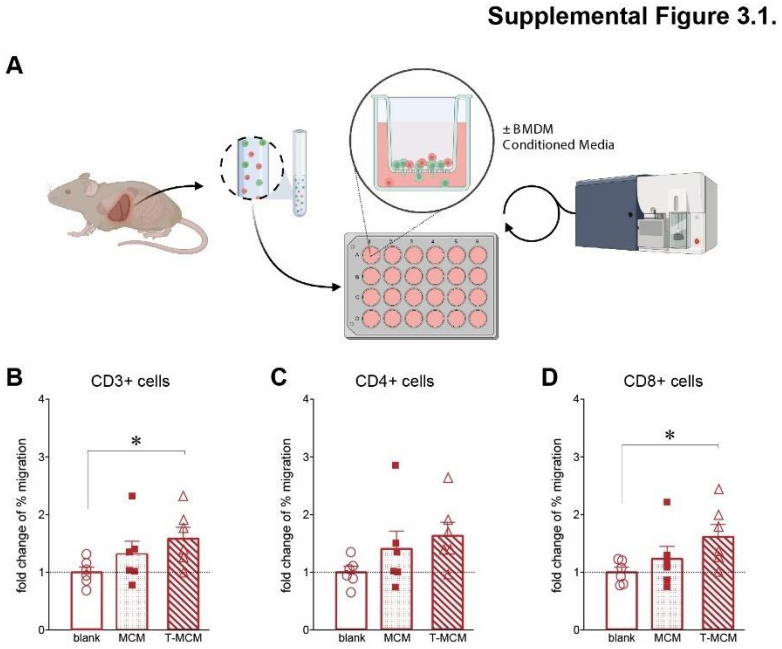



Figure 3.8

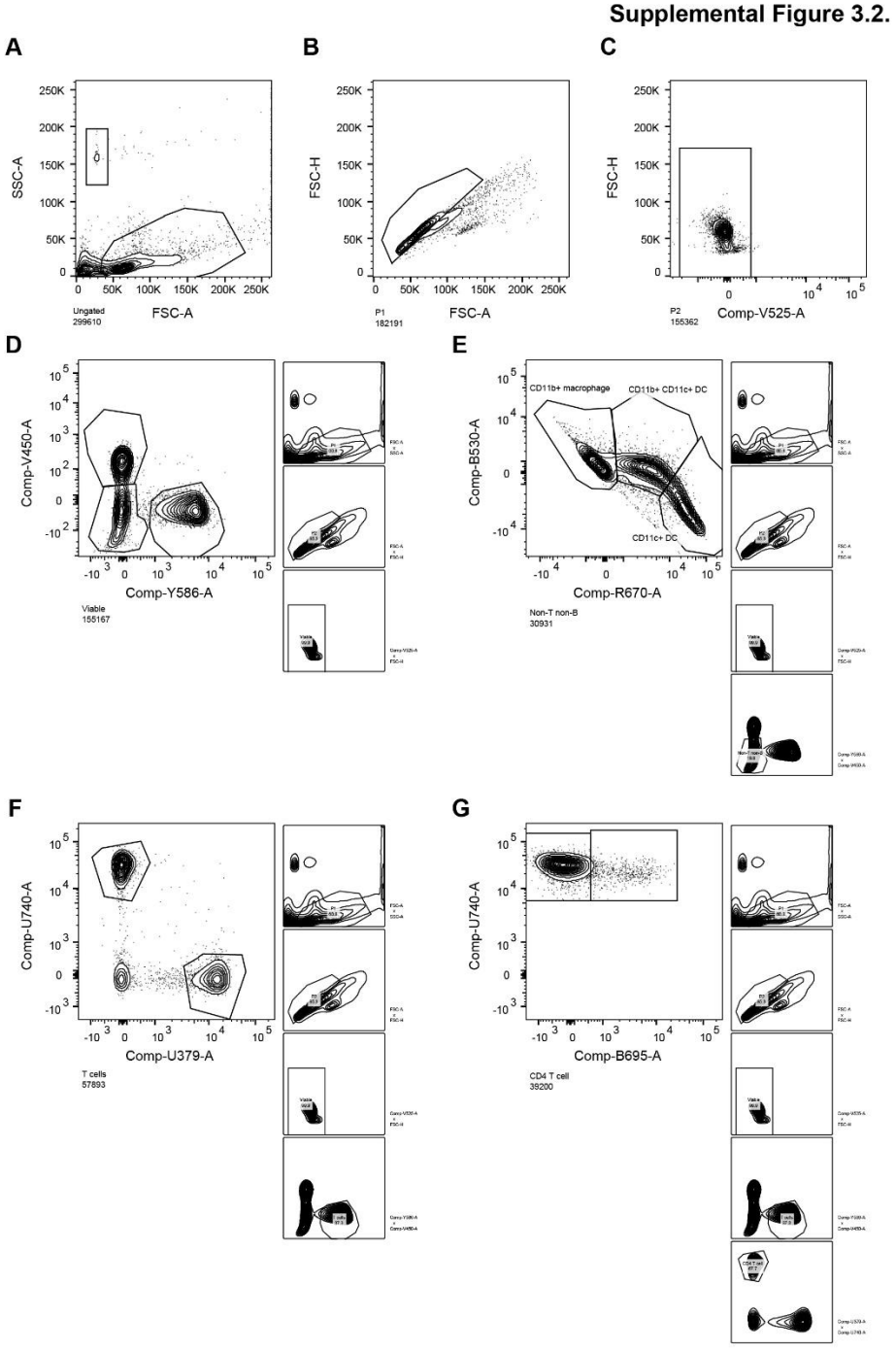
The TLR7 agonist imiquimod promotes speedier resolution of pain after hindpaw injection of CFA. Female mice received hindpaw co-injections of CFA and imiquimod with additional imiquimod hindpaw injections on days 3, 6, 8, 10, and 12. Control mice received vehicle injections replacing imiquimod. (A) Withdrawal thresholds to mechanical stimuli following hindpaw CFA injection in wt and *Tlr7* -/- mice were assessed until day 54. Mice receiving imiquimod treatment partially recovered approximately one week before the vehicle group. (B) AUC transformation of data from Panel A summarizing the overall pain response. n = 6 per group; * $p < 0.05$. Bars and symbols with error bars represent mean ± SEM. Data is analyzed by repeated-measures two-way ANOVA with Panel B including a Kolmogorov-Smirnov test. PWT = Paw Withdrawal Threshold. AUC = Area Under the Curve.

## 3.8 Supplemental Materials



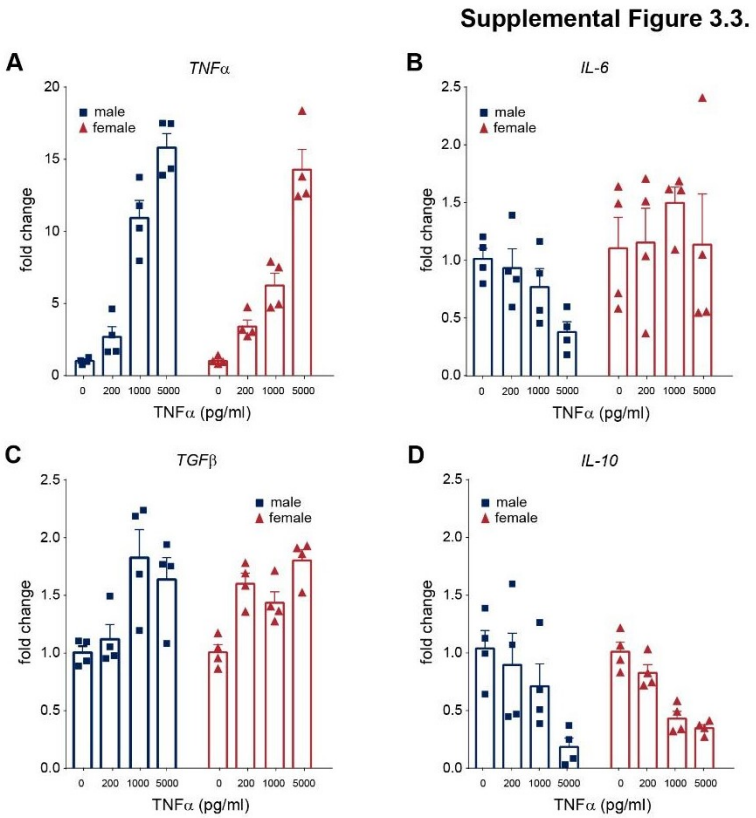

Supplementary Figure 3.1

Female BMDM conditioned media (CM) promotes increased chemotaxis of mixed splenocytes in a transwell system. Splenocytes from naïve female mice were isolated and incubated in the top chamber of a transwell allowing movement into the lower chamber. (A) Schematic of splenocyte isolation and transwell assay. The proportions of total available cells labeling for CD3 (B), CD4 (C), and CD8 (D) were quantified by FACS, normalizing to the input counts of the top chamber and the passive flow in the blank control. n = 6 per group, each n represents a replicate well of three combined BMDM CM transwell incubation experiments; * $p < 0.05$. Bars with error bars represent mean ± SEM. Data is analyzed by two-way ANOVA with Sidak's post hoc test.




Supplementary Figure 3.2

Gating strategy for flow cytometry of chemotactic splenocyte assay. Cells were isolated from standardizing counting beads (A), before single cell (B) and viability (C) gates were applied. B and T cells were isolated (D) while non-B, non-T cells were further characterized by CD11b/c staining (E). CD4+ and CD8+ T-cells were further characterized (F) and CD4+ T-reg cells were additionally gated (G).




Supplementary Figure 3.3

BMDMs display TNFα dose-dependant profiles of common macrophage polarizing transcripts. The transcripts for TNFα (A), IL-6 (B), TGFβ (C), and IL-10 (D) were quantified by qPCR. n = 4 per group; * $p < 0.05$. Bars with error bars represent mean ± SEM. Data is analyzed by two-way ANOVA with Sidak's post hoc test.

## Supplementary Table 3.1

Summary of antibodies and reagents used in immuno-histo/cyto-chemistry experiments.

**Supplementary Table 3.1.**

| | Target | Species | Dilution | Supplier | Product Code |
|---|---|---|---|---|---|
| Primary Antibodies | Ki67 | ms anti-ms | 1:500 | BD Biosciences | 550609 |
| | iNOS | rb anti-ms | 1:500 | Sigma | ABN26 |
| | Arg1 | gt anti-rt | 1:500 | Novus | NB100-59740 |
| | CD45 | rt anti-ms | 1:200 | BD Biosciences | 550539 |
| | CD3 | rt anti-hu | 1:200 | Bio-Rad | MCA1477 |
| | CD4 | rt anti-ms | 1:200 | Bio-Rad | MCA2691 |
| | CD8 | rt anti-ms | 1:200 | Bio-Rad | MCA1768 |
| Secondary Antibodies | 488 | dk anti-ms | 1:500 | Jackson ImmunoResearch | 715-546-151 |
| | 488 | dk anti-rt | 1:500 | Jackson ImmunoResearch | 712-546-153 |
| | 594 | dk anti-rb | 1:500 | Jackson ImmunoResearch | 711-586-152 |
| | 647 | dk anti-gt | 1:500 | Jackson ImmunoResearch | 705-606-147 |
| | DAPI | N/A | 1:1000 | Invitrogen | D1306 |
| | | | | | |
| Other | Normal Donkey Serum | | | Sigma | 566460 |
| | Fluoromount G Mounting Medium | | | Thermofisher | 00-4958-02 |
| | Tissue-Tek O.C.T Compound | | | Sakura | 4583 |
| | Superfrost Plus Microscope Slides | | | Fisherbrand | 22-0370246 |
| | 96 well, Tissue Culture Treated Plate | | | Falcon | 353219 |
| | Poly-L-lysine | | | Sigma | P8920 |
| | Sterile Disposable Biopsy Punch | | | Integra | 33-31 |

## Supplementary Table 3.2

Summary of antibodies and reagents used in fluorescence-activated cell sorting experiments.

**Supplemental Table 3.2.**

| | Supplier | Product Code | Clone | Fluorophore |
|---|---|---|---|---|
| CD3 | Thermofisher | 12-0031-82 | 145-2C11 | Y586 |
| CD4 | BD Biosciences | 612843 | RM4-5 | U740 |
| CD8 | BD Biosciences | 562786 | 53-6.7 | U379 |
| CD11b | Miltenyi Biotec | 130-113-802 | REA592 | APC |
| CD11c | Invitrogen | 50-0114-82 | N418 | R670 |
| CD19 | Invitrogen | 48-0193-82 | eBio1D3 | V450 |
| CD25 | Invitrogen | 45-0251-82 | PC61.5 | B710 |
| CD45.2 | Biolegend | 109824 | 104 | R780 |
| F4/80 | Biolegend | 123113 | BM8 | PE-Cy7 |
| Zombie Aqua | Biolegend | 423102 | N/A | V525 |

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# Chapter 4:

# Plasticity of Mouse Dorsal Root Ganglion Neurons by Innate Immune Activation is Influenced by Electrophysiological Activity

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I was responsible for concept formation, data collection, analysis, and manuscript composition. SML, and TH collected data. SML, HTK, and JRP were involved with editing the manuscript. BJK was the supervisory author involved with concept formation and manuscript edits.

## Motivation

The conclusions of Chapter 3 suggested that the female innate immune system is indeed more sensitive to inflammation than the male. In addition to increased sensitivity, I found that there may be some subtle mechanics in the female system that was absent in the male. For example, finding that TNFα stimulation of macrophages regulated only female IL-16, I began thinking that inflammation in females may be unique enough to account for some of the discrepancies that appear in animal behaviour and human disease prevalence.

Similar to the ideas that led me to investigate BMDMs *in vitro*, I wanted to investigate how these differences could extrapolate onto neuronal mechanisms. To proceed with this, I developed a protocol for primary cell culture of DRG neurons, aiming to investigate parameters of plasticity with a single cell resolution.

The decision to investigate plasticity was prompted by conversations around mechanisms of the induction of chronic pain and central sensitization. Several new lines of investigation around the concept of 'maladaptive plasticity' led me to question the role of inflammation in this context: could female sensory neurons be inherently primed to engage in this phenomenon? By considering females as more likely to acquire this phenotype despite similar mechanics of inflammation to males, we may be able to reconcile the differences in incidence of chronic pain in the general population. Furthermore, by additionally querying the role of excitability in this paradigm, I wanted to consider the role that these three variables together could have on pain. Translating the findings from Chapter 3 into the neuronal system of Chapter 4 was an organic progression of these goals and sets the stage for future experiments to complete this transformation back into the whole animal system.

## 4.0 Abstract

The intricate interplay between inflammation, excitability, and plasticity in dorsal root ganglion (DRG) neurons is a relatively unexplored area of research. In this study, we aimed to investigate the role of excitability in the early plastic response of DRG neurons. Our findings revealed that the early phase of excitability is critical for the engagement of plastic processes, and that neuronal excitability profiles are linked through time and to the structural phenotype of individual neurons. Specifically, we observed that neuronal excitability induced by inflammation may be involved in the plastic response of DRG neurons. These results suggest that targeting the excitability of DRG neurons may provide a novel therapeutic avenue to manipulate their plastic response to inflammatory stimuli. Further studies are needed to elucidate the underlying mechanisms of this relationship, which could ultimately lead to the development of new treatments for neuropathic pain and other disorders involving sensory nerve dysfunction.

## 4.1 Introduction

Pain is a prevalent symptom in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, and its management remains a challenge. Based on the current understanding of their pathophysiological mechanisms, these diseases involve the dysregulated activation of the innate and adaptive immune system, leading to high levels of inflammation that damage tissue in the periphery and central nervous system along with the sensitization of primary sensory neurons leading to pain and disability[1, 2, 3, 4, 5].

It should be noted however, that inflammation is not always detrimental. For instance, after injury, immune-mediated inflammation can trigger regenerative mechanisms, assisting in debris clearance or promoting regeneration of lost cells or structures[6, 7, 8]. Inflammation is a dynamic process that can be initiated in response to various stimuli and then resolved when no longer required[9, 10, 11]. In individuals with multiple sclerosis (MS), an inflammatory autoimmune disease characterized by periods of attacks and remissions, these inflammatory processes can coincide with periods of disability. Although inflammation can resolve in MS, it is not always the case, and persistent low-grade inflammation may lead to neurodegeneration, emphasizing the importance of the resolution process[12, 13]. Chronic inflammation can lead to disability and may also play a role in the induction of chronic pain. As inflammation is adaptive and designed to resolve, its persistence may indicate an impairment in the resolution process.

Dorsal root ganglion (DRG) neurons have an innate capacity for growth after injury to their distal axons[14, 15]. This plasticity can be demonstrated in cell culture. Dissociated DRG neurons can readily establish and send out neurites in short periods of time, a process that can be encouraged by the priming of these cells with ‘conditioning lesions’[16, 17, 18] or discouraged by inhibition of

retrograde transport along the injured axon[19, 20]. Furthermore, the structural plasticity of neurons can be modulated by pharmacological manipulation of ion channels. For example, inhibition of neuronal activity with gabapentin (an inhibitor of $Ca^{2+}$ channel trafficking to the membrane[21]) can modulate synaptogenesis and neural circuitry rewiring[22, 23]. This effect is also observed in vivo as gabapentinoids can be used to increase functional regeneration of axons after spinal cord injury[24, 25, 26]. The growth state of sensory neurons can also be modulated by inflammation[27, 28, 29, 30]. Not only can the inflammatory response to injury, even potentially sterile injury, have effects on outgrowth, but inflammation triggered by adaptive or innate immunity can also affect outgrowth. Therefore, inflammation can be seen as a critical driver of structural plasticity and the growth status of sensory neurons[31].

It is also widely recognized that inflammation can alter the excitability of neurons, and traditional mechanisms of inflammation can result in increased action potential frequencies and lowered thresholds for action potentials (i.e., sensitization). Inflammatory mediators like TNFα and IL-1β can regulate neuronal expression of voltage-gated sodium and potassium channels, increasing neuronal excitability[32, 33, 34, 35, 36]. This phenomenon has been observed in both electrophysiological recordings of single cells[37] and nerve conduction studies[38, 39]. Heightened excitability and sensitization of peripheral sensory neurons results in an increased perception of pain, although this phenomenon may resolve as the inflammation subsides.

The relationship between inflammation, excitability, and structural plasticity of DRG neurons is complex. While inflammation can independently affect both the excitability and growth status of neurons, increasing excitability through electrical stimulation can promote axonal growth, indicating that there may be a connection between the two processes[40, 41, 42]. Additionally, some researchers have proposed that the inflammation-induced changes in excitability may be directly

involved in changes to neuronal growth status[43, 44, 45]. Increasing neuronal excitability using optogenetics enhanced axonal growth in the presence of an inflammatory stimulus[46, 47]. This suggests that the increase in neuronal excitability may be one of the mechanisms by which inflammation promotes structural plasticity.

Overall, the relationship between inflammation, excitability, and the capacity for structural plasticity of DRG neurons is complex and likely involves multiple signaling cascades. To investigate this relationship, we conducted a series of experiments examining the effect of inflammation on neuronal excitability and neurite outgrowth. Our findings indicate that an early phase of increased neuronal excitability plays a critical role in initiating the change in growth status of primary sensory neurons, and that the neuron's excitability is intricately linked to its structural phenotype.

## 4.2 Materials and Methods

### 4.2.1 Animals

Unless otherwise stated, all mice are wild-type C57/BL6, purchased from Charles River and included in experiments at 8-10 weeks of age (C57BL/6NCrl, Strain Code: 027, https://www.criver.com/products-services/find-model/c57bl6-mouse?region=24#). All mice were given access to food and water ad libitum and maintained in the University of Alberta facility under 12-hour light/dark cycles.

### 4.2.2 Bone Marrow Derived Macrophage (BMDM) Cultures and Conditioned Media

Bone marrow derived macrophage cultures were generated under a previously described protocol (Friedman, et. al., unpublished). Briefly, female and male 8-10 week old C57BL/6 mice were

acquired from Charles River Canada, and habituated for approximately 1 week in the housing facility. After this period, animals were euthanized by Euthanyl® (sodium pentobarbital) injected intraperitoneally. After injection, animals were monitored for level of consciousness and dissections did not proceed until no response to toe pinch or corneal contact was observed. Bone marrow cells were isolated from femurs and resuspended by pipetting to single-cell suspensions and filtered to remove excess debris. Bone marrow cells were allowed to differentiate into macrophages (BMDMs) for 8-10 days in L929 media.

### 4.2.3 BMDM Conditioned Media

Upon completed differentiation, bone marrow derived macrophages were harvested and resuspended into freshly prepared DMEM containing 1% each of sodium pyruvate, Glutamax, Pen/Strep, and FBS. This media is hereon referred to as 'low-serum media'. Cells were suspended at a concentration of 300,000 cells per mL and cultured in T75 flasks by addition of 23.7mL of suspended cells. This concentration was chosen for consistency of cell density in relation to surface area from the previously established protocol. BMDM flasks were allowed to rest for 48 hours after which a full change of media was performed. A small volume of concentrated TNFα, IL-4, or vehicle (low-serum media) was added and allowed to incubate for 24 hours. After incubation, the stimulation media was fully replaced with a final volume of low-serum media and the stimulated BMDMs were allowed to rest for 6 hours. This '6 hour CM' was captured at the experimental endpoint, aliquoted, and stored at -80°C until further usage avoiding freeze-thaw cycles.

### 4.2.4 Dorsal Root Ganglia Neuron Cultures

Dorsal root ganglion (DRG) neurons were acquired from male and female mice, using a modified protocol from previously described work[48]. Briefly, animals were euthanized by Euthanyl® (sodium pentobarbital) injected intraperitoneally. After injection, animals were monitored for level of consciousness and dissections did not proceed until no response to toe pinch or corneal contact was observed. Cardiac punctures were performed to confirm euthanization and animals were perfused with 10mL of ice-cold saline. Perfused animals underwent spinal laminectomies and gross dissection of the spinal cord to expose the DRGs. DRGs were micro-dissected from the spinal column, taking care to remove as much residual nerve as possible while avoiding damage to the DRG. Isolated DRGs were placed in ice-cold HBSS -/- until dissections were completed. To acquire single-cell suspensions of DRGN, the HBSS -/- was replaced with a warmed dilution of Stemxyme I (Worthington, LS004106) and DNase (Worthington, LS002007) in HBSS -/- and incubated in a 37 °C water bath for approximately 45 minutes. Following digestion, enzyme activity was quenched with equal volumes of low ovomucoid (Worthington, LS003086) and mechanically titrated with a P1000 pipette until tissue was fully dissociated. The cell suspension was filtered through a 70 um mesh filter (Biologix, 15-1070) and gently layered on top of a 20% BSA solution. This layered gradient was spun at 300G for 10 minutes at room temperature to pellet neuronal cells and remove cellular debris (mainly myelin). Debris was gently removed, and the cell pellet was resuspended in a small volume of 0.5% BSA in HBSS -/- and quantified for neuronal yield with a hemocytometer. The cell suspension was adjusted to 1000 cells / 100uL and 100uL of this suspension was added to equilibrated media in a 24 well (CellVis, P24-1.5H-N), poly-D-lysine coated plate (Sigma, P6407). For electrophysiological recordings, cells were plated onto glass coverslips (Fisher, 1254583) identically coated in poly-D-lysine and transferred into the

electrophysiological recording setup at experimental timepoints. Unless otherwise stated, plated neurons were incubated at 37 °C with 10% $CO_2$ for 48 hours. A full list of reagents used in cell culture experiments can be found in **Supplementary Table 4.1**.

### 4.2.5 Pharmacology

For experiments involving retigabine (RTG), a master stock of 50mM was prepared by reconstituting 10ng of lyophilized RTG (Tocris, 6233) in DMSO, aliquoted, and stored at -20 °C. On experimental timepoints, a 10x concentration (200 uM) was prepared by dilutions of thawed aliquots in DMEM. The final concentration of RTG was adjusted by direct dilution into plate wells.

### 4.2.6 Immunocytochemistry

At experimental endpoints, an equivalent volume of 8% PFA was added to the media of culture plates containing the adherent cells. Samples were incubated at room temperature in the diluted fixative for 15 minutes and then washed 3 times in DPBS. Permeablization and non-specific IgG binding was blocked by one hour incubation with 10% NDS in DPBSTx 0.2% at room temperature, followed by an overnight incubation with rabbit anti-BIII Tubulin (Sigma, T2200) diluted in DPBS. The following day, cells were washed 3 times in DPS, incubated with DAPI (Invitrogen, D1306) and 594-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch, 711-586-152) for one hour at room temperature before a final three washes. Imaging was performed on an ImageXpress Micro system and analyzed using MetaXpress 6.

### 4.2.7 Neurite Extension Well Average analysis

Imaging was performed on an ImageXpress Micro system, using a single protocol for all experiments. Fluorescence images were collected with a 10x objective, corresponding to a field-of-view of 1406um2 per image, tiled in a 6-by-6 grid with 10% overlap. Basic neurite extension

as performed using the 'Neurite Extension' plugin in the MetaXpress 6 software. Data was aggregated by well, averaging outgrowth of Total # of Neuronal Cell Bodies / Total # of Neurites. Experiments were replicated with internal controls for normalizing, where one replicate corresponds to a single well's average for that condition. Additional metrics were retrieved from the 'Neurite Extension' analysis including: # of Neuronal Cells, Total Outgrowth, # of Branches per Cell, # of Processes per Cell, and % Significant Outgrowth.

### 4.2.8 Whole-cell Current Clamp Electrophysiology

For generation of action potentials from mouse Dorsal Root Ganglion Neurons (DRGNs), recordings were acquired from current-clamp mode using an Axopatch 200B amplifier (Molecular Devices), Digidata 1440 digitizer and Clampex10 software (Molecular Devices). Whole-cell configuration was obtained in voltage-clamp mode before manually switching to current-clamp recording mode. Recordings were filtered at 5 kHz and sampled at 10 kHz. Patch pipettes were manufactured from soda lime capillary glass (Thermo Fisher Scientific) using a Sutter P-97 (Sutter Instrument) puller. Electrodes had a tip resistance of 2-4 MΩ when filled with an internal (pipette) solution. Pipette solution was comprised of; 130 mM K-gluconate, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM EGTA, 2 mM CaCl2, and 10 mM HEPES (adjusted to pH 7.2 with KOH). The bath was perfused with an external solution containing: 135 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 10 mM HEPES (adjusted to pH 7.3 with NaOH). Patch clamp experiments were performed at room temperature (22 ± 1°C). DRG neuron dimensions (cell size) were estimated using a microscope eyepiece reticle (27 mm, 10 mm scale). For identification of IB4+ cells, DRG neurons were labelled with isolectin GS-B4 Alexa Fluor™ 488-conjugated antibody (Thermo Fisher, I21411) at least 15 minutes prior to transferring cells to the recording chamber. Images of DRG neurons in the recording chamber were acquired using a high resolution USB2.0 CMOS,

1280 x 1024, Camera (Thorlabs, DCC1645C) and ThorCam™ software. The resting membrane potential was determined immediately following whole-cell break-in at I = 0 pA. Threshold (Rheobase) was established by the first action potential to be elicited by a series of 3s stepwise current injections that increased from 0 pA in 10 pA increments. Action potential frequencies were calculated by the number of spikes during the 3 second stepwise current injections from 0 pA in 10 pA increments. Frequency of action potentials during acute retigabine application (RTG) was analyzed using the event detection, Threshold search feature of Clampfit 10.7. The frequency of action potentials was calculated by the number of spikes over the time exposed to a specific condition (control, RTG application, washout). The baseline was set at the resting membrane potential at the beginning of the recording. The threshold level for the inclusion criteria of an action potential was set at 0 mV. Patch clamp recordings and analysis were performed independent from DRG extraction and culture; as such, the electrophysiologist was blinded to all experimental groups.

## 4.3 Results

### 4.3.1 DRG neuronal plasticity after BMDM CM stimulation

Previous experiments from our group have shown that the conditioned media from male and female macrophages after pro or anti-inflammatory stimulation elicit different pain phenotypes when injected into the hind paw of adult mice (Friedman, et. al., unpublished). In the current study, we sought to establish the relationship between the growth status of sensory neurons and the conditioned media that elicited these different pain phenotypes. We first cultured DRG neurons in the base media used in macrophage conditioned media experiments to confirm if neurons would readily survive and extend neurites in a non-traditional media. Optimization experiments

confirmed that DRG neurons attached and begin neurite outgrowth within 24 hours post-plating in base 'low-serum' media and survive at least 96 hours in culture (data not shown). We next investigated the effect on outgrowth of neurons in the presence of conditioned media from unstimulated male or female bone marrow derived macrophages (macrophage conditioned media, MCM), or with the conditioned media from sex-matched macrophages after undergoing stimulation with either tumor necrosis factor alpha (TNFα macrophage conditioned media, T-MCM) or interleukin-4 (IL-4-MCM). Neurons were cultured for 48 hours in the presence of these different conditioned media and structural plasticity was assessed by βIII tubulin staining (**Figure 4.1B-I**). Mean outgrowth of neurites from neurons was quantified at the experimental endpoint for both male (**Figure 4.1J**) and female (**Figure 4.1K**) treated neurons. Both male and female neurons incubated with the standard, unstimulated macrophage condition medium (MCM) had no change in outgrowth compared to regular media treated controls. Female DRG neurons however, specifically demonstrated an increase in neurite outgrowth after incubation with female T-MCM (2-way ANOVA, treatment effect $F_{3,76} = 48.60$, $p < 0.05$, F Blank vs. F T-MCM, $p < 0.05$, **Figure 4.1K)**. Male neurons incubated with the male equivalent T-MCM did not exhibit a statistically significant increase in neurite outgrowth, although the growth pattern of the male neurons did suggest a similar trend (2-way ANOVA, M Blank vs. M T-MCM, $p = 0.09$, **Figure 4.1J)**. Both male and female neurons exhibited significantly enhanced neurite outgrowth after incubation with IL-4-MCM media. To assess whether the sex of the neuron or the sex of the macrophage generating the conditioned media was critical, we performed a sex swap experiment where female neurons were incubated with the conditioned media generated from male macrophages as well as the reversed conditions. Interestingly, male neurons did not exhibit a statistically significant increase in neurite outgrowth when incubated with female T-MCM (which was effective on female-

matched neurons). Conversely, female neurons did not exhibit increased growth in the presence of male T-MCM. Both sexes showed an increase in outgrowth after treatment with IL-4-MCM regardless of its source, demonstrating that this anti-inflammatory media is more generalizable than its inflammatory counterpart (2-way ANOVA, treatment effect $F_{3,76} = 39.11$, $p < 0.05$, **Supplementary Figure 4.1**).

4.3.2 Electrophysiological assessment of DRG neurons early after culturing

The function of neurons is dependent on both their structural and excitability profiles. We next assessed the excitability profiles of neurons treated with the different macrophage conditioned medias using whole-cell current clamp recordings, incubated under identical conditions to previous experiments. Although both sexes show changes in structural plasticity by treatment, we decided to focus on female DRG neurons due to previous work indicating a specific mechanism of pain in our auto-immune inflammatory mouse model[49, 50, 51] as well as the female bias of autoimmunity and chronic pain in the human population[52]. We analyzed general metrics of neuronal excitability including the minimum electric current required to elicit an action potential (rheobase), the resting membrane potential, and the action potential frequency after 6 hours of incubation in the different conditioned medias. We found that all conditions displayed varying degrees of spontaneous activity and quiescence, with the T-MCM and IL-4-MCM treated neurons exhibiting the most spontaneous activity (**Figure 4.2C**). Interestingly, neurons treated with T-MCM and IL-4-MCM also exhibited the lowest rheobase and had reduced resting membrane potential (RMP) relative to media only treated neurons (**Figures 4.2D-E**). The action potential frequency of the T-MCM condition was similar to the MCM and blank media controls, while the IL-4-MCM condition displayed a higher action potential frequency with identical current injections (**Figure 4.2F**). Interestingly, the neurons with the greatest excitability profiles

(spontaneous activity, lowered rheobase and resting membrane potential) at 6 hours correlated with the neurons exhibiting the greatest structural outgrowth at 48 hours described in **Figure 4.1**. These findings suggest a potential positive relationship between increased early excitability of the sensory neuron and later increased structural plasticity.

4.3.3 Electrophysiological assessment of DRG neurons in established cultures

To evaluate neurons at later time points in culture, we conducted an identical electrophysiological assessment at the 48-hour time point. Notably, we observed changes in the excitability profiles in all treatment conditions. Specifically, the MCM condition exhibited no spontaneous activity at this later timepoint, also exhibiting the highest rheobase and the most negative resting membrane potential. In contrast, the T-MCM and IL-4-MCM treated neurons still displayed some spontaneous activity along with a moderate rheobase and a shift in resting membrane potential (**Figures 3C-F**).

4.3.4 Growth status and electrophysiological characteristics of DRG neurons

As we were interested in relating electrical excitability to structural plasticity, we noted that neurons adopt distinct morphologies at the 48-hour time point in culture. At this time point, neurons were consistently observed to fall into three categories: a population of neurons yet to extend any neurites a population that display a highly complex, ‘arborizing’ phenotype characterized by numerous branches from the primary outgrowth, and a population that extend ‘elongating’ neurites characterized by low amounts of branching and a high displacement of primary growth (**Figures 4.4A-C**). Identifying and subdividing these morphological populations by visual inspection at the time of patch clamp recording, we found that the spontaneous activity seen in the T-MCM and IL-4-MCM conditions were restricted to neurons with no outgrowth. This

restriction of spontaneous activity to neurons in the 'no outgrowth' category was true in the blank media control condition as well. Furthermore, neurons in the 'arborizing' and 'elongating' categories were primarily quiescent regardless of treatment condition (**Figures 4.4Bi, Ci**). Neurons adopting the arborizing phenotype were less likely to be excitable in the MCM and T-MCM conditions but were more excitable in the IL-4-MCM condition. Conversely, neurons that adopted an 'elongating' phenotype were more varied based on the conditioned media treatment they received. Overall, these data suggest that incubation of neurons with different types of inflammatory conditioned media convey unique excitability profiles and unique structural plasticity profiles.

### 4.3.5 Structural plasticity is modulated by Kv7 channel activity

To assess the direct impact that excitability has on structural plasticity we turned to pharmacological manipulation of these neurons in our in vitro system. Retigabine (RTG) is an anticonvulsant drug that was initially developed as a treatment for epilepsy. It acts as a positive allosteric modulator of voltage-gated potassium channels (Kv7), which stabilizes the resting membrane potential and reduces neuronal excitability[53, 54, 55]. We repurposed this drug to act as a general inhibitor of neuronal activity to mimic the quiescent phenotype we identified in vitro. The effect of RTG is near instantaneous as repetitively evocable neurons exhibit a complete loss of electrical activity upon wash in of RTG during recording. Repetitive firing of neurons is then restored when RTG is washed off (1-way ANOVA, $F_{2,14} = 4.40$, $p < 0.05$, Control vs. RTG, $p = 0.0584$, RTG vs. Washout, $p < 0.05$, **Figure 4.5B**). The basal neurite outgrowth of male and female neurons cultured in the presence of retigabine is significantly reduced after 48 hours in culture (2-way ANOVA, treatment effect $F_{1,92} = 15.54$, $p < 0.05$, **Figure 4.5H**). Although the quiescent phenotype was primarily shown in the later stages of outgrowth, we additionally tested the addition

of RTG at a later timepoint (24 hours post plating), but this had an identical effect to the early addition (data not shown). These data suggest that neurons are dependent on increased excitability and high levels of spontaneous activity for later outgrowth.

### 4.3.6 Plasticity driven by BMDM CM is dependent on excitability

To assess whether the growth promoting effects on neurite outgrowth from the different macrophage conditioned medias are reliant on changes to the excitability profile of the neurons, we treated DRG neurons for 48 hours with MCM, T-MCM or IL-4-MCM in the presence or absence of RTG. In all media conditions, RTG diminished neurite outgrowth for both sexes (2-way ANOVA, RTG effect $F_{3,36} > 14.36$, $p < 0.05$, **Figure 4.6Q-R**). Interestingly, the effect of RTG on outgrowth was not able to fully prevent the positive effect of CM treatment, suggesting that additional mechanisms distinct from the excitability profile can mediate aspects of plasticity in DRG neurons.

## 4.4 Discussion

### 4.4.1 BMDM Conditioned Media conveys plastic potentials to DRG neurons

Building upon previous research indicating sex differences in innate immune inflammatory activity, we have investigated the sex specific changes in DRG sensory neuron structural plasticity and excitability when exposed to inflammatory mediators from innate immune cells (BMDMs). Here, we demonstrate that incubating peripheral neurons with sex-matched inflammatory conditioned media from innate immune cells can impact both the structural plasticity and excitability profiles of these neurons. We find that excitability parameters of neurons that exhibit high amounts of structural outgrowth correspond to the greatest shift in excitability, becoming

quiescent after an initial period of high spontaneous activity. Furthermore, we demonstrate that a pharmacological intervention limiting neuronal activation through RTG's effect on Kv7 can prevent this capacity for structural plasticity in the inflammatory conditions.

## 4.2 DRG neurons are highly plastic cells

In early neuronal development, the maturation of neurons involves complex signaling cascades that are crucial for the transcriptional profile and development of the functional component of these cells[56, 57, 58]. Maintenance of neuronal circuitry in adult organisms is tightly regulated and these plastic processes can result in adaptive or maladaptive consequences depending on the context. For instance, heightened pain sensitivity during inflammation may trigger acute plasticity that is adaptive for preventing further injury, but intense or prolonged inflammation also has the capacity to promote signalling within sensory neurons that leads to long term changes and chronic pain syndromes[59, 60, 61].

Pain management is a critical aspect of first-line treatments for nervous system injury. However, strategies that directly address pain without considering the structural consequences have proven to be ineffective in preventing chronic pain. Thus, it may be worthwhile to consider both the functional and structural aspects of neural plasticity when developing pain management strategies. This involves understanding the interplay between activity-dependent phenomena and regenerative outgrowth and investigating the mechanisms that link neuronal excitability and structural plasticity. Such approaches could lead to more effective treatments for chronic pain syndromes.

## 4.3 DRG neuronal plasticity involves alterations in excitability

Recent work has explored a related line of inquiry and demonstrated that in vitro, neurons initially dedifferentiate to an "embryonic" state before undergoing transcriptional maturation concomitant with associated structural plasticity[62, 63, 64]. Moreover, transcriptional re-maturation of regenerating axons involves genes related to excitability and signalling, with pharmacogenetic targeting of these genes significantly modulating outgrowth[65]. Together with the findings presented here, these results suggest that inflammation-induced molecular cascades that promote neurite outgrowth are inexorably linked with changes in neural excitability/activity.

Activity-dependent phenomena are well-documented in regenerative contexts, and an excellent example of this is rehabilitation therapy that remains one of the most effective treatments for spinal cord injury, restoring function and preventing maladaptive pain syndromes. Inactivity has direct consequences on nervous system activity, similar to muscle tone and strength, which operate in a "use it or lose it" manner and require constant input for maintenance. Although it is rare for a neuron to lose all input in vivo, as it does in a dissociative culture system, evidence indicates that peripheral neurons may atrophy or even die following long-term sensory deprivation[66, 67, 68]. In this context, it is possible that pain after injury plays a crucial role in promoting the proper re-maturation of healthy neuronal circuitry. Therefore, the excessive use of analgesics in clinical settings may potentially hinder regenerative outgrowth[69, 70]. The experience of pain may be a necessary part of the healing process; acknowledging and pursuing this hypothesis may lead to better outcomes.

While the precise mechanism linking excitability and structural plasticity remains unclear, several plausible hypotheses can be tested. One such hypothesis is that ionic gradients along neuronal membranes allow for calcium influx during action potentials. Calcium is a well-known second

messenger and cofactor for many different molecular cascades that promote both the growth of axons but also neuronal sensitization and pain hypersensitivity[64, 71].

4.4 DRG neuronal plasticity: the relationship between excitability and pain

Plasticity, excitability, and pain are interconnected processes in the nervous system. The maturation and maintenance of neuronal circuits involves signaling cascades that regulate the functional component and structural generation and pruning of synapses, membrane potentials, and sensory receptor insertion. These processes involve different cell types, including glia[72] and immune cells[73], and are activity driven[74, 75]. The consequences of these plastic processes can be adaptive or maladaptive, such as sickness behavior and chronic pain. Pain is a crucial factor in injury and inflammation, and current treatments for nervous system injury often involve analgesics. However, addressing pain function without considering structural consequences has proven ineffective. Additionally, environmental factors play a crucial role in the interplay between plasticity, excitability, and pain.

## 4.5 Conclusions

The relationship between neuronal excitability and structural plasticity is complex and dynamic, with evidence suggesting that they may be inextricably linked. Both *in vitro* and *in vivo* studies have demonstrated the importance of activity-dependent phenomena in promoting regenerative processes and maintaining nervous system function. The role of pain in this process is an intriguing area of investigation, with some evidence suggesting that it may be necessary for proper re-maturation of healthy circuitry. The mechanisms underlying the link between excitability and plasticity remain unclear, but the role of $Ca^{2+}$ as a second messenger and cofactor for molecular cascades presents a plausible hypothesis that warrants further investigation. Overall, a deeper

understanding of the relationship between neuronal excitability and structural plasticity has important implications for the development of new strategies to promote nerve regeneration and functional recovery after injury or disease.

## 4.6 Appendix

Abbreviations

Multiple Sclerosis (MS); Experimental autoimmune encephalomyelitis (EAE); central nervous system (CNS); dorsal root ganglia (DRG); peripheral nervous system (PNS); Complete Freund's Adjuvant (CFA)

Declarations

Ethics approval and consent to participate: All animal studies complied with the Canadian Council on Animal Care Guidelines and Policies and were approved by the Animal Care and Use Committee: Health Sciences for the University of Alberta.

Consent for Publication: Not applicable.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: TNF and TH performed cell culture and *in vitro* imaging experiments. SML performed and analyzed electrophysiological recordings. TNF, SML, JRP, HTK, and BJK were involved in experimental design and manuscript preparation. All authors read and approved the final manuscript.

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## 4.7 Figures

Figure 4.1.

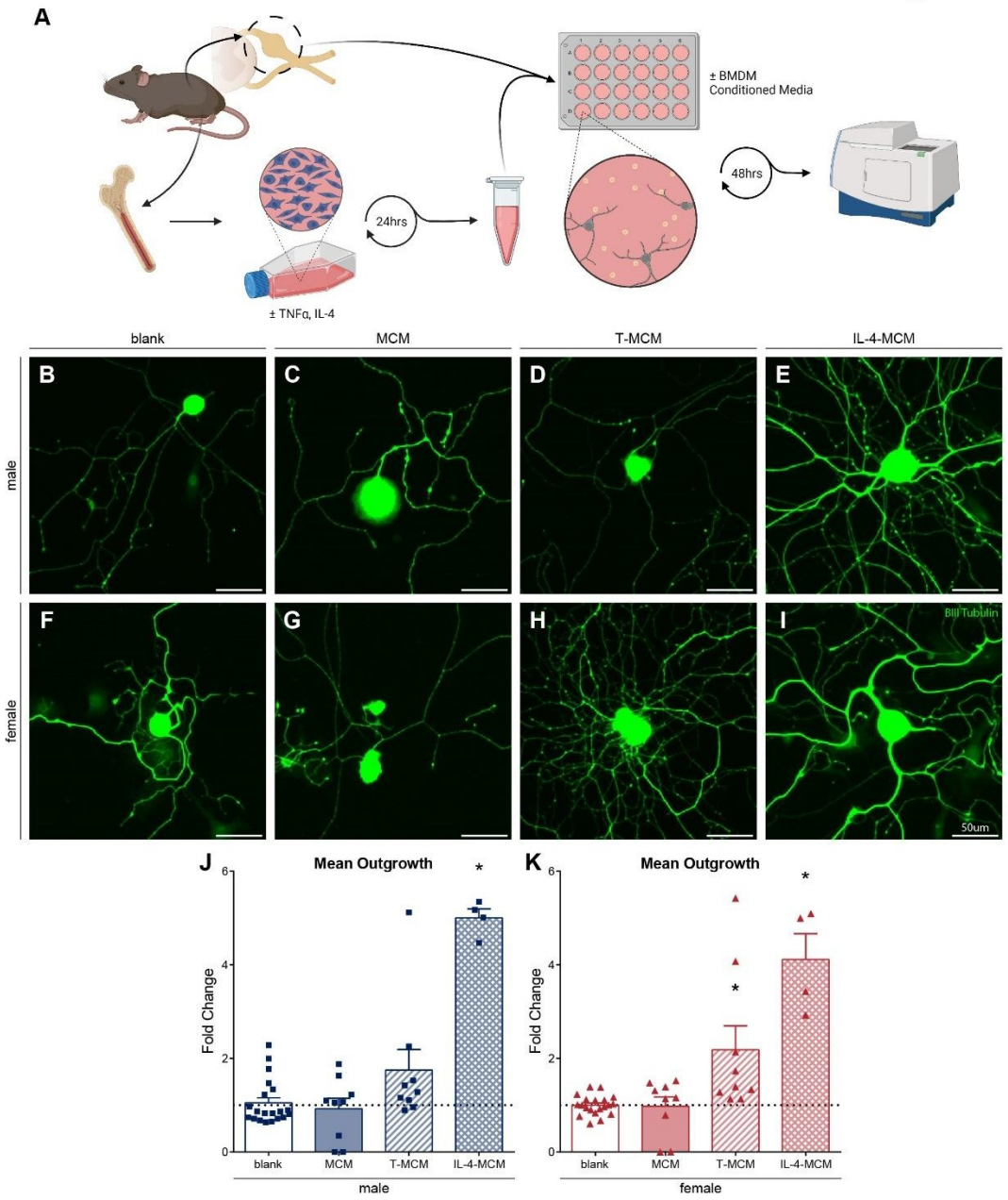



Figure 4.1

Male and female DRG neurons display increased neurite outgrowth in anti-inflammatory and female-specific pro-inflammatory BMDM-conditioned media. (A) Schematic of DRG neuron cultures and CM stimulation. (B-I) Representative images of neurites stained with BIII tubulin at experimental endpoint. Quantification of male (J) and female (K) neurites with sex-matched condition media treatments, normalized to the extent of outgrowth measured in the control

(blank) conditions. Scale bar = 50μm; * $p < 0.05$. Data is analyzed by two-way ANOVA including Sidak's multiple comparison test. Bar graphs represent mean +- SEM.

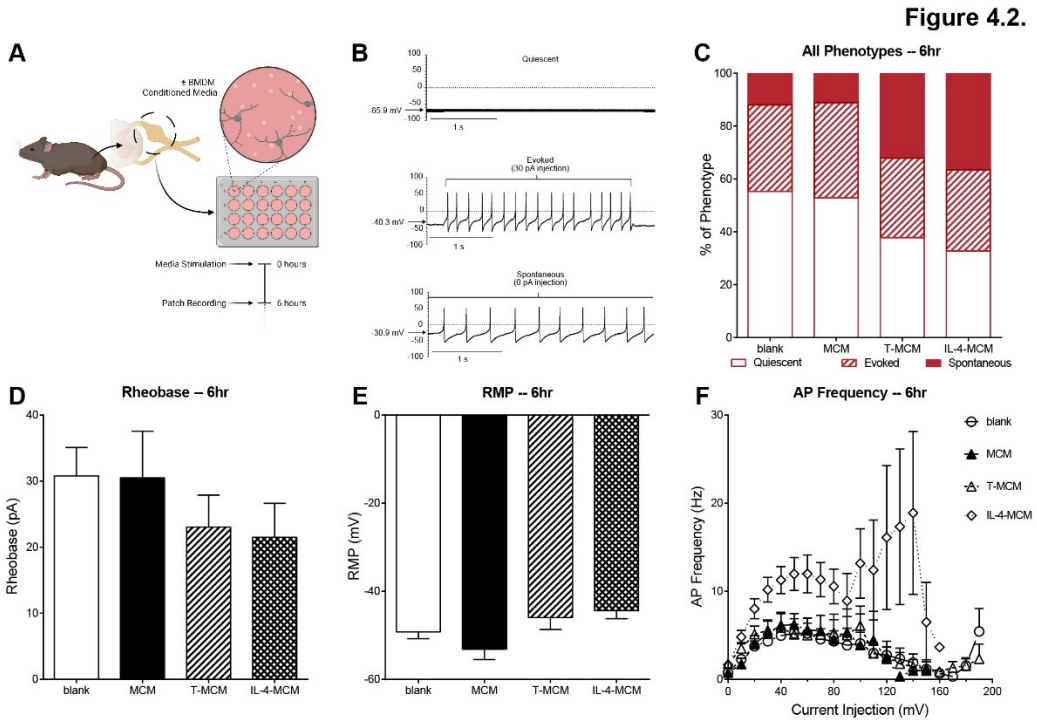



Figure 4.2

Whole-cell patch clamp acute recordings of female DRG neurons incubated in BMDM-conditioned media (CM) reveals distinct firing patterns and electrophysiological profiles. (A) Schematic of experimental workflow; neurons were recorded 6 hours post-plating and CM stimulation. (B) Example traces of the three characteristic firing patterns: Quiescent, Evoked, and Spontaneous. (C) Quantification of categorical labels for stereotypic firing patterns across CM treatments. Quantification of (D) rheobase, (E) resting membrane potential (RMP), and (F) action potential (AP) frequency across CM treatments. n = 4 per group; * $p < 0.05$. Data is analyzed by two-way ANOVA with Sidak's post hoc test. Bar graphs represent mean +- SEM. Categorical data is represented by parts-of-whole transformation and analyzed by Fisher's exact test on untransformed values.

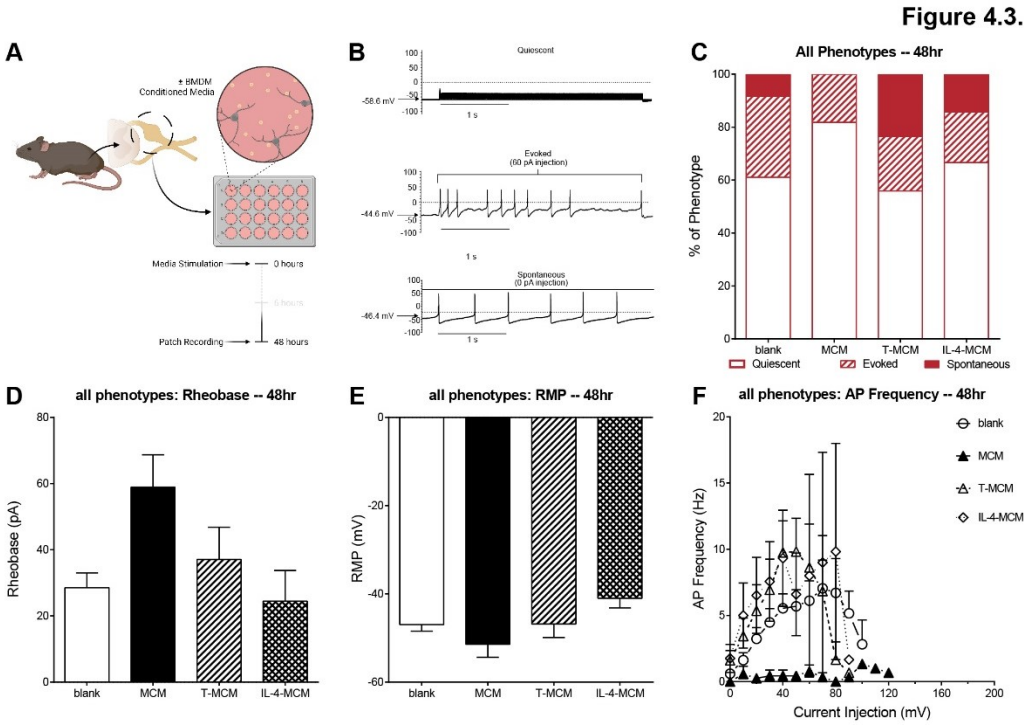



Figure 4.3

Whole-cell patch clamp recordings of established female DRG neurons incubated in BMDM-conditioned media (CM) reveals distinct firing patterns and electrophysiological profiles. (A) Schematic of experimental workflow; neurons were recorded 48 hours post-plating and CM stimulation. (B) Example traces of the three characteristic firing patterns: Quiescent, Evoked, and Spontaneous. (C) Quantification of categorical labels for stereotypic firing patterns across CM treatments. Quantification of (D) rheobase, (E) resting membrane potential (RMP), and (F) action potential (AP) frequency across CM treatments. n = 4 per group; * $p < 0.05$. Data is analyzed by two-way ANOVA with Sidak's post hoc test. Bar graphs represent mean +- SEM. Categorical data is represented by parts-of-whole transformation and analyzed by Fisher's exact test on untransformed values.

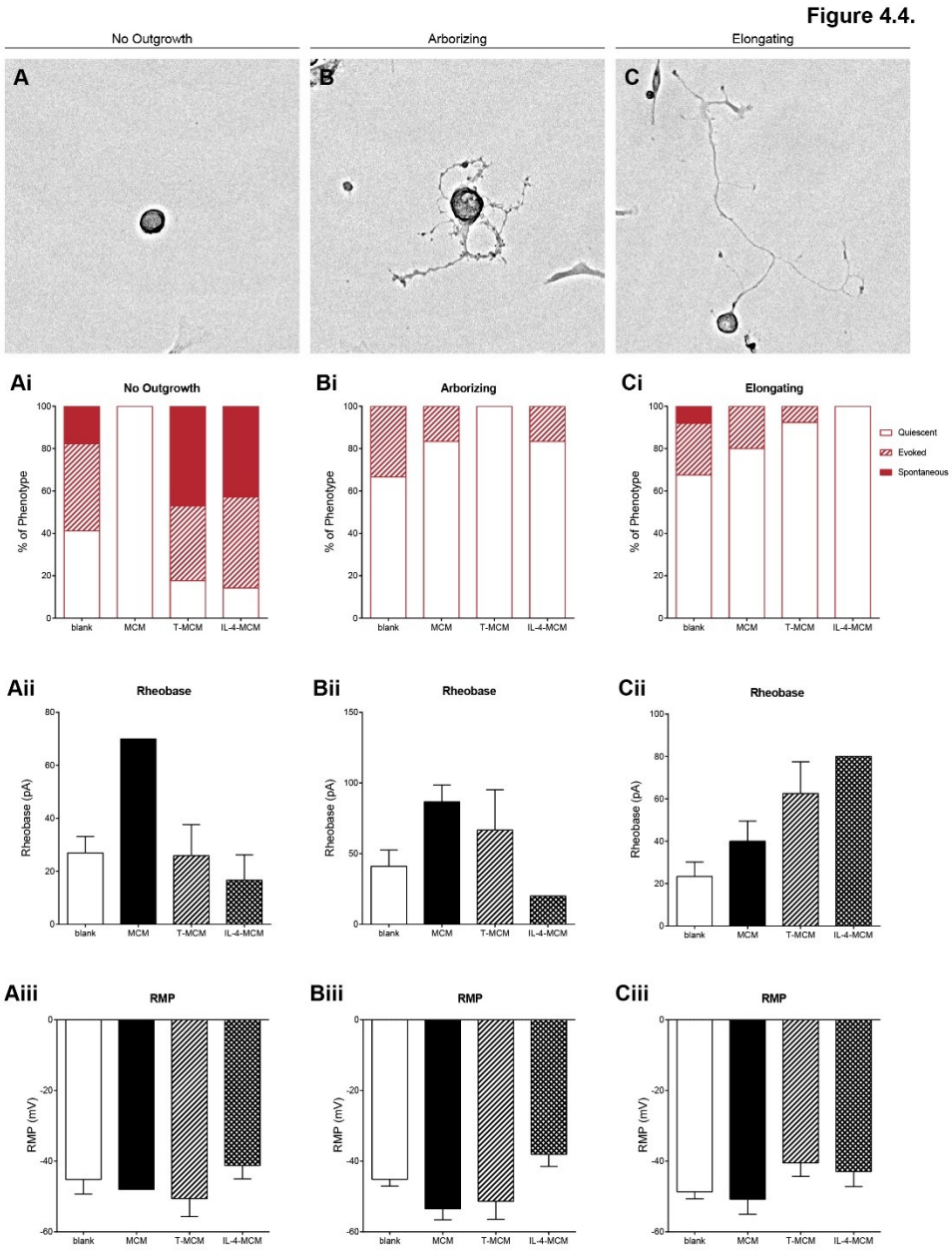



Figure 4.4

Whole-cell patch clamp recordings of established female DRG neurons incubated in BMDM-conditioned media (CM) reveals distinct firing patterns and electrophysiological profiles based on structural phenotype. (A-C) Representative images at 48 hours post plating of (A) neurons with no outgrowth, (B) arborizing outgrowths, and (C) elongating outgrowths. (A'-C') Summary of the three characteristic firing patterns in each structural phenotype by CM treatment. (A''-C'') Summary of rheobase in each structural phenotype by CM treatment. (A'''-C''') Summary of the resting membrane potential (RMP) in each structural phenotype by CM treatment. * $p < 0.05$. Data is analyzed by one-way ANOVA with Sidak's post hoc test. Bar graphs represent mean +- SEM.

Categorical data is represented by parts-of-whole transformation and analyzed by Chi-squared test on untransformed values.

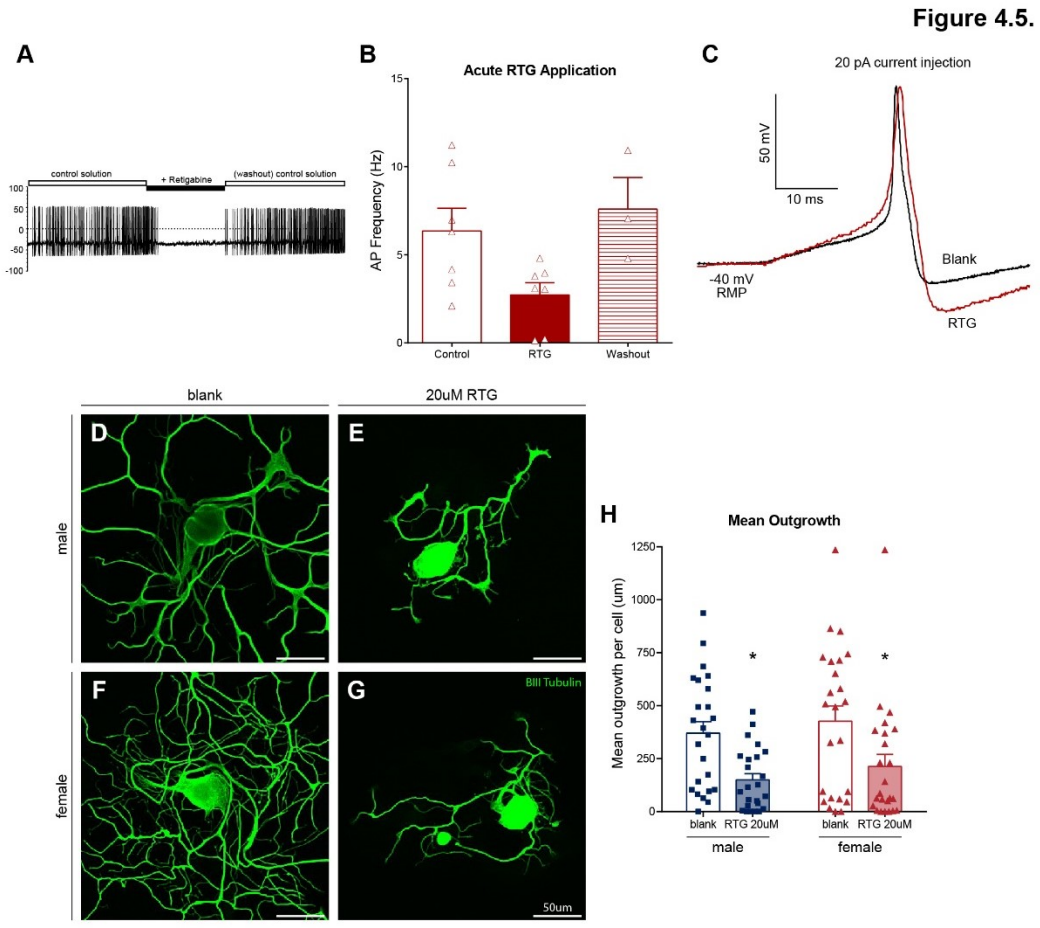



Figure 4.5

Neurite outgrowth of DRG neurons is diminished by retigabine (RTG) treatment. (A) Example traces of spontaneously firing DRG neurons which become quiescent under RTG treatment. (B) Quantification of action potential (AP) frequency in binned time domains centred around the period of RTG stimulation. (C) Example traces of the three characteristic firing patterns: Quiescent, Evoked, and Spontaneous. (D-G) Representative images of neurites stained with BIII tubulin at experimental endpoint. (H) Quantification of male and female neurites after treatment with RTG or control (blank). Scale bar = 50μm; * $p < 0.05$. Data is analyzed by two-way ANOVA including Sidak's multiple comparison test. Bar graphs represent mean +- SEM..

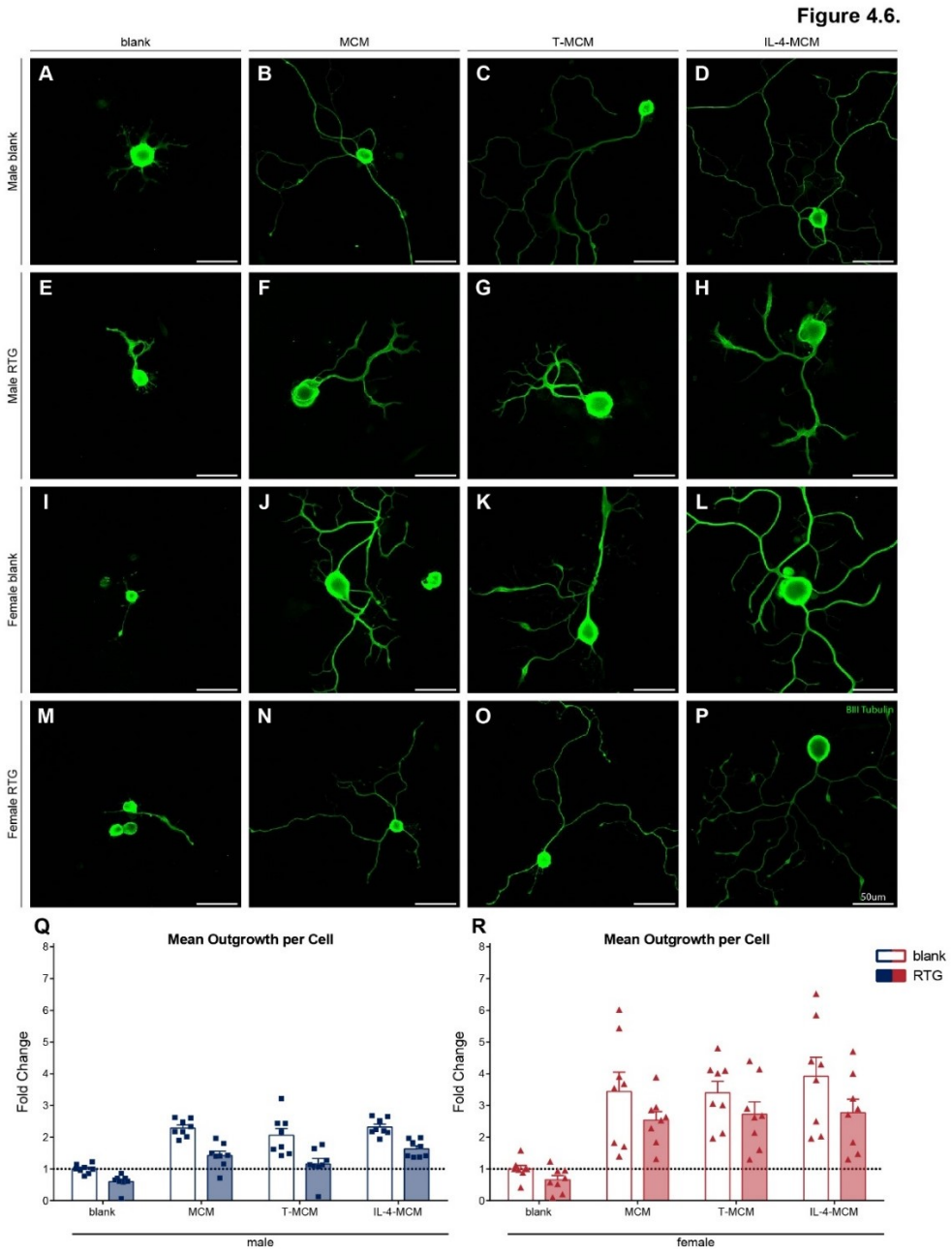
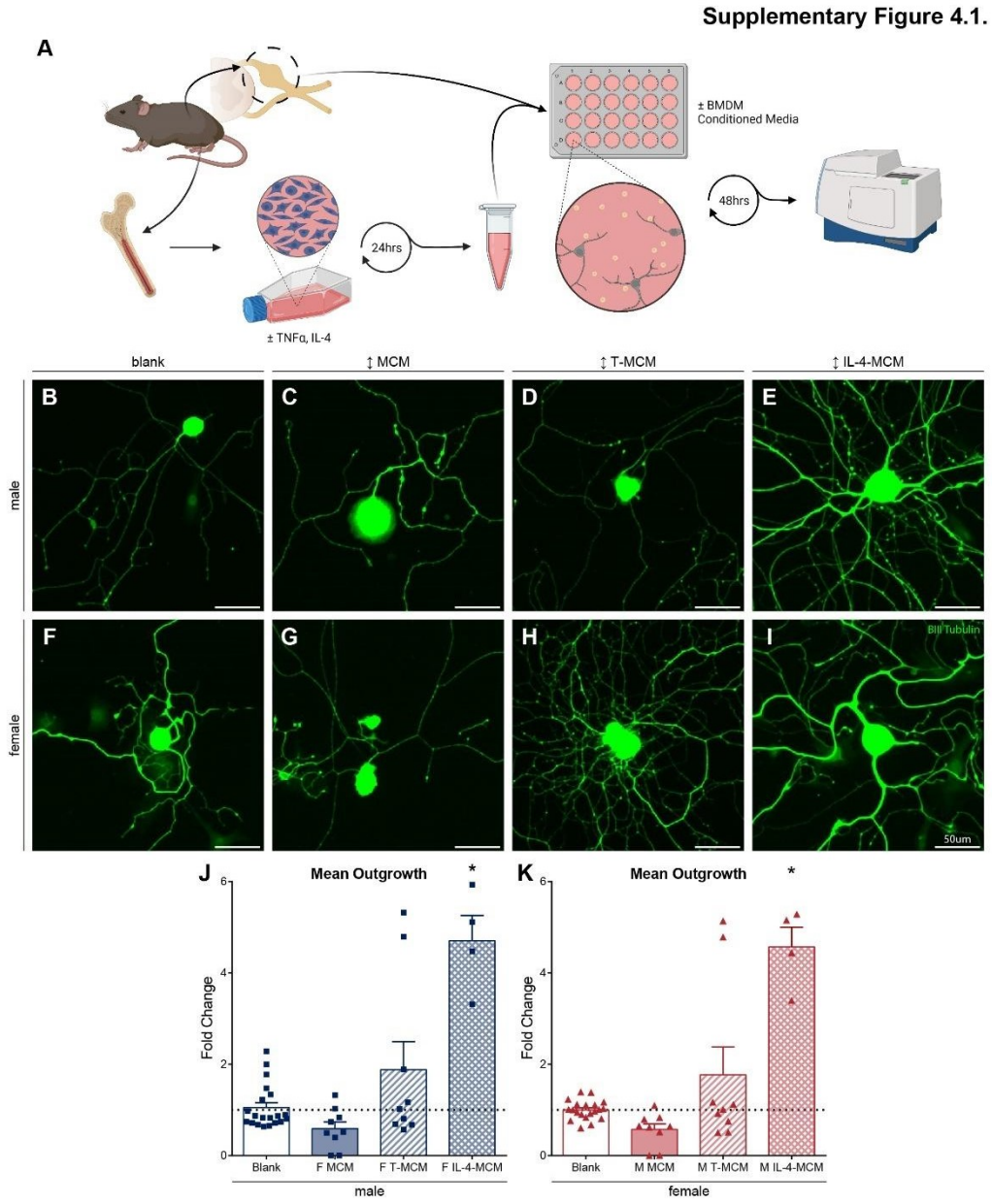



Figure 4.6

Retigabine treatment of male and female DRG neurons with BMDM-conditioned media reduces outgrowth. (A-P) Representative images of neurites stained with BIII tubulin at experimental endpoint. Quantification of male (Q) and female (R) neurites with sex-matched condition media stimulation and RTG treatment or control (blank), normalized to the extent of outgrowth measured in the master control (blank + blank) condition. Scale bar = 50µm; * $p < 0.05$. Data is analyzed by two-way ANOVA including Sidak's multiple comparison test. Bar graphs represent mean +- SEM.

## 4.8 Supplemental Materials




Supplementary Figure 4.1

Male and female DRG neurons display increased neurite outgrowth in sex-swapped anti-inflammatory and female-specific pro-inflammatory BMDM-conditioned media. (A) Schematic of DRG neuron cultures and CM stimulation. (B-I) Representative images of neurites stained with BIII tubulin at experimental endpoint. Quantification of male (J) and female (K) neurites with sex-swapped condition media treatments, normalized to the extent of outgrowth measured in the control (blank) conditions. Scale bar = 50μm; * $p < 0.05$. Data is analyzed by two-way ANOVA including Sidak's multiple comparison test. Bar graphs represent mean +- SEM.

## Supplementary Table 4.1

Summary of antibodies and reagents used in immunocytochemistry experiments.

**Supplementary Table 4.1.**

| | Target | Species | Dilution | Supplier | Product Code |
|---|---|---|---|---|---|
| Primary Antibodies | BIII Tubulin | rb anti-hu | 1:1000 | Sigma | T2200 |
| | | | | | |
| Secondary Antibodies | 488-IB4 | N/A | 1:100 | Fisher | I21411 |
| | 594 | dk anti-rb | 1:500 | Jackson ImmunoResearch | 711-586-152 |
| | DAPI | N/A | 1:1000 | Invitrogen | D1306 |
| | | | | | |
| | | | | | |
| Other | 24 well, Tissue Culture Treated Plate | | | CellVis | P24-1.5H-N |
| | 24 well, Tissue Culture Treated Plate | | | Falcon | 353047 |
| | 15mm Glass Coverslips | | | Fisher | 1254583 |
| | Poly-D-lysine | | | Sigma | P6407 |
| | Normal Donkey Serum | | | Sigma | 566460 |
| | Retigabine (RTG) | | | Tocris | 6233 |
| | | | | | |
| Equipment | Axopatch 200B amplifier | | | Molecular Devices | |
| | Digidata 1440 digitizer | | | Molecular Devices | |
| | Clampex10 | | | Molecular Devices | |
| | ImageJ | | | NIH | |
| | MATLAB | | | Mathworks | |
| | Clampfit 10.7 | | | Molecular Devices | |
| | | | | | |
| Plugins | Stitching | | | FIJI | [Ref 34] |
| | NeuriteTracer | | | FIJI | [Ref 35] |
| | Simple Neurite Tracer (SNT) | | | FIJI | [Ref 36, 37] |

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# Chapter 5: Conclusions

## 5.1 Summary of Findings

This thesis used both a general and reductionist approach to identify potential mechanisms underlying sex differences in pain processing. The analysis of bulk RNA and miRNA sequencing data from male and female DRGs revealed significant dysregulation in genes related to the innate immune system and phagocytic roles, including the upregulation of miR-21a-5p in both sexes. Chapter 2 focused on this general approach and discovered that female DRGs had an incredible amount of both gene and miRNA dysregulation, with candidate gene TLR7 highly upregulated in the female DRGs.

The reductionist approach of Chapter 3 focused on the innate immune cells and revealed higher mitotic capability, motility, and sensitivity for activation in female immune cells compared to males, as well as differences in cytokine profiles. In particular, levels of CCL12, CCL22, and IL-16 stood out as potential candidates for sex differential effects that could have knock-on effects on inflammatory pain.

Chapter 4 continued the reductionist approach by investigating the plastic properties of neurons cultured in different types of inflammatory conditioned media. Interestingly, the conditions exhibiting high amounts of growth also contained neurons with highly spontaneous electrical activity immediately after plating, which then became quiescent over time. The study further explored the link between excitability and plasticity by modulating excitability using pharmacokinetic tools and discovered that exogenous silencing of neurons prevented later outgrowth.

## 5.2 Overall Significance of Findings

The investigation presented in this thesis provides important insights into the mechanisms underlying autoimmune-induced pain and highlights the importance of considering sex as a biological variable in pain research. The findings suggest that females may be more heavily dysregulated compared to males in this context, which could have important implications for the development of more effective pain management strategies.

One major issue in the current treatment of autoimmune pain is the failure to consider sex as a biological variable. Men and women are often grouped together in clinical trials, despite evidence that they may respond differently to treatment. The discovery of a potential female-specific mechanism in autoimmune-induced pain suggests that treatments that fail to consider this variable may be greatly confounded and may only work in one sex and not the other. The study of the innate immune system in Chapter 3 also showed that male and female BMDMs were distinctly sensitive to inflammation, which further emphasizes the importance of considering sex as a biological variable in the development of disease-modifying therapies that target this system.

The investigation also sheds light on the plastic properties of DRG neurons in response to inflammatory stimulation. The findings indicate that DRG neurons have different capacities for growth, depending on the type of inflammatory conditioned media they are exposed to. This highlights the importance of promoting the right type of growth, as endless promotion of growth can lead to side effects like pain without further increasing functional recovery. These findings have important implications for the development of novel pain management strategies that take into account the plastic properties of DRG neurons and the potential sex differences in pain processing.

## 5.3 Nociplastic Pain

The more we learn about pain and nociception, the more it seems to defy being placed in a neat little box. Terminology for chronic pain conditions seem limited, suggesting the need for expansion of our pain vocabulary[256, 257]. Nociplastic pain is a relatively new concept in pain research that refers to pain that arises from altered nociceptive processing in the absence of ongoing peripheral nociceptive input. This type of pain is thought to result from changes in the central nervous system's processing of pain signals and may occur in the context of chronic pain conditions like fibromyalgia, chronic headache, and irritable bowel syndrome[257, 258]. The underlying mechanisms of nociplastic pain are not well understood, but recent research has suggested that it may involve changes in central sensitization, alterations in descending pain modulatory pathways, and changes in central input of nociceptive signals[258, 259].

One important consideration in the study of nociplastic pain is the potential for plasticity in the circuitry involved in pain processing. While plasticity in the nervous system is often associated with maladaptive changes that contribute to chronic pain, it is also possible that this plasticity could be modulated in a way that is utilized by the system. By taking advantage of the flexibility of cellular mechanisms, it may be possible to develop therapeutics that assist with pain management in a way that avoids maladaptive changes in the nervous system[259]. Developing the proper vocabulary to discuss the problem is a critical first step in avoiding another opioid epidemic[260].

This thesis investigates the impact of sex on the innate plastic properties of DRG neurons under inflammation. As nociplastic pain is more common with comorbidities of autoimmune diseases[261], it is important to investigate these properties further to develop precision medical therapies for its treatment.

## 5.4 Future Directions

Moving forward, there are several important directions for future research in the area of nociplastic pain. One important area of investigation is the dynamics of the nociplastic system, including the cellular and molecular events that underlie changes in pain processing. Live imaging techniques could be used to capture dynamic cellular events and provide insights into the mechanisms underlying nociplastic pain. Additionally, techniques such as topological reconstructions could be used to quantitate changes in the plasticity of DRG neurons involved in pain processing. As structure in neurons directly relates to function, this investigation may uncover further evidence of sex differences and hint at the mechanism behind it.

Another important direction for future research is the integration of single-cell sequencing and cell culture work to focus on nociceptors. By identifying whether plasticity is a trait of all DRG neurons or something special about nociceptors, researchers could gain a better understanding of the cellular mechanisms underlying nociplastic pain. This could also help to identify new targets for the development of targeted therapies with greater precision.

Overall, the investigation of nociplastic pain is an exciting area of research with important implications for pain management. By continuing to investigate the underlying mechanisms of this type of pain and developing targeted therapies that take into account the dynamics of the nociplastic system, it may be possible to improve pain management and quality of life for individuals with chronic pain conditions.

## 5.5 Concluding Statements

This thesis aimed to investigate sex differences in the innate immune system and their implications in peripheral pain signal generation in Multiple Sclerosis. The first chapter of this

work analyzed bulk RNA and miRNA sequencing data from male and female dorsal root ganglia (DRG) to identify major sex differences. It was found that the female DRGs had an incredible amount of gene and miRNA dysregulation, focusing on genes related to the innate immune system and phagocytic roles. In Chapter 3, the innate immune cells indicated in Chapter 2 were focused on and it was found that female cells had higher mitotic capability, as well as higher motility and sensitivity for activation. Chapter 4 concluded the reductionist approach and shifted the *in vitro* system from bone marrow-derived macrophages to dorsal ganglion neurons. By culturing neurons in different types of inflammatory conditioned media, the plastic properties that these medias could impart on the neurons were investigated.

The findings of this research suggest that understanding the molecular mechanisms of these pathways may provide valuable information to guide pharmacological therapeutics that could benefit one or both sexes. Furthermore, this work highlights the importance of considering sex differences in autoimmune diseases such as Multiple Sclerosis, as they not only have different incidence rates between the sexes but also exhibit subtleties in disability and chronic pain. Ultimately, this research provides insight into the role of the innate immune system in sex-specific pain signaling and identifies potential targets for future therapeutic interventions.

In conclusion, this thesis presents a comprehensive investigation of sex differences in the innate immune system and their potential impact on peripheral nociception in Multiple Sclerosis. By utilizing a combination of bioinformatics, *in vitro* cell culture, and pharmacological approaches, the work highlights unique sex effects of the innate immune system on DRG neurons. Overall, the research findings suggest the importance of considering sex differences in autoimmune diseases and their implications for future therapeutic interventions.

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