Characterization of the Cannabis Terpene Myrcene in Joint Pain and Inflammation in a
Rat Model of Rheumatoid Arthritis

by

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For my father, Chuck

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Abstract

Rheumatoid arthritis is a progressive inflammatory condition resulting in joint damage and debilitating pain. Current treatments present with variable efficacy and negative side effects, leaving pain management a priority. The endocannabinoid system has been targeted to relieve both inflammation and pain in disease conditions. Specifically, the non-euphoric cannabis terpene myrcene offers analgesic actions in acute pain models, and anti-inflammatory potential *in vitro*. We investigated the effect of both acute and chronic administration of myrcene alone, and in combination with cannabidiol, on pain and inflammation in the Freund's complete adjuvant (FCA) rat model of rheumatoid arthritis. The involvement of the endocannabinoid system was also explored. Evoked and spontaneous pain behaviour were analyzed using von Frey hair algesiometry, dynamic weight bearing, and locomotor activity. Inflammatory parameters assessed were oedema, leukocyte trafficking, blood flow to the injured area, cytokine levels, and joint histopathology.

Our study found that both acute and chronic administration of myrcene attenuated evoked mechanical allodynia, but did not improve FCA-induced weight bearing deficits, and did not alter locomotor activity. Acute administration of myrcene reduced leukocyte trafficking. Chronic administration reduced leukocyte trafficking and blood flow, yet had no effect on cytokine levels or disease progression. These anti-inflammatory effects were mediated via the endocannabinoid system; however, endocannabinoid-dependent analgesia was not confirmed.

Excluding blood flow, the analgesic and anti-inflammatory effects of myrcene were comparable to diclofenac. The combination of myrcene with a suboptimal dose of cannabidiol did not enhance any of the above parameters.

Compared to other analgesic treatments, myrcene may be a safer therapeutic to use in conjunction with disease-modifying treatments to adequately manage pain and inflammation in rheumatoid arthritis.

List of Abbreviations Used

2-AG	2-arachidonoylglycerol
AA	Antigen arthritis
AAC	Arthritis Alliance of Canada
ACEA	Arachidonyl-2'-chloroethylamide
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AEA	Arachidonylethanolamine
AIA	Adjuvant-induced arthritis
ANOVA	Analysis of variance
APC	Antigen presenting cell
bDMARD	Biological disease modifying anti-rheumatic drug
BL	Baseline
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CB_1R	Cannabinoid receptor-1
CB_2R	Cannabinoid receptor-2
CBD	Cannabidiol
CCAC	Canadian Council of Animal Care
CCD	Charged-coupled device
CD	Cluster of differentiation
cDMARD	Conventional disease modifying anti-rheumatic drug
CGRP	Calcitonin gene-related peptide
CIA	Collagen-induced arthritis
COX	Cyclooxygenase
Crem	Cremophor
CRP	C-reactive protein
CYP	Cytochrome
D7	Day seven
DAG	Diacylglycerol

DAS	Disease activity score
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DWB	Dynamic weight bearing
EDTA	Ethylenediaminetetraacetic acid,
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatology
FAAH	Fatty acid amide hydrolase
FCA	Freund's complete adjuvant
GPCR	G-protein coupled receptor
GPR55	G-protein receptor 55
GTP	Guanosine-5'triphosphate
Hr	Hour
i.artic.	Intraarticular
i.p.	Intraperitoneal
IA	Inflammatory arthritis
ICAM-1	Intercellular adhesion molecule 1
ICAM-2	Intercellular adhesion molecule 2
IFN-γ	Interferon-gamma
IL	Interleukin
IVM	Intravital microscopy
JAM	Junctional adhesion molecule
JIA	Juvenile idiopathic arthritis
JNK	c-Jun N-terminal kinase
kg	Kilogram
L	Litre
LASCA	Laser speckle contrast analysis
LFA	Leukocyte function-associated antigen
Mac-1	Macrophage-1
MAGL	Monoacylglycerol lipase

MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCP	Metacarpophalangeal
MHC	Major histocompatibility complex
MIA	Monoiodoacetate
mg	Milligram
Min	Minutes
MMP	Matrix metalloproteinases
ml	Millilitre
MTP	Metatarsophalangeal
NADA	N-arachidonoyldopamine
NEA	N-oleoylethanolamine
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NIH	National Institute of Health
NK	Natural killer
NKA	Neurokinin-A
NMDA	N-Methyl-D-aspartate
NS	Not significant
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PAMP	Pathogen associated molecular pattern
PEA	Palmitoylethanolamine
PECAM	Platelet endothelial cell adhesion molecule
pg	Picogram
PG	Prostaglandin
PIP	Proximal interphalangeal
PIP_2	Phosphatidylinositol 4,5-biphosphate
PLC	Phospholipase-C
PLD	Phospholipase-D
PRR	Pattern recognition receptor
PU	Perfusion units

RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid Factor
s.c.	Subcutaneous
Sal	Saline
SAPE	Streptavidin-PE
SF	Synovial fluid
SP	Substance P
T0	Time zero
Th	Helper T-cell
THC	Tetrahydrocannabinol
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid-1
UCLA	University Committee on Laboratory Animals
VCAM-1	Vascular cell adhesion molecule 1
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

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Chapter 1: Introduction

1.1   Overview of arthritis in Canada

Arthritis is an umbrella term for over 100 conditions affecting the musculoskeletal system (Krustev et al. 2015). Derived from the Greek 'arthro' meaning joint and 'itis' meaning inflammation, arthritis targets the joints: the synovial membrane, joint capsule, cartilage, tendons, ligaments and surrounding tissues (Fu et al. 2018). It is one of the most prevalent chronic diseases and can impact all persons; however, both prevalence and incidence of these conditions is higher in females and older age individuals (Public Health Agency of Canada 2017).

Patients with arthritic conditions often present with joint stiffness, redness, swelling and pain (Lee and Weinblatt 2001). Commonly affected joints include, but are not limited to the hip, the knee joint, the spine, and the hands (Lagacé et al. 2010). Persistent symptoms and resulting damage can cause physical disabilities, reduced mental well-being, and lessening of one's overall quality of life (Lagacé et al. 2010).

As the leading cause of disability in North America, arthritis also asserts a substantial economic burden and strain on the healthcare system and on a jurisdiction's economy (Lagacé et al. 2010). Combined direct and indirect costs in Canada associated with arthritis are estimated to be 6.4 billion dollars in the year 2000 alone (Lagacé et al. 2010).

Unfortunately, at this time there is no cure for arthritis (Bombardier et al. 2011). Therefore, the treatment has focused on managing pain and sustaining joint function with the goal of preventing overall disability (Bombardier et al. 2011).

1.2   Knee joint anatomy

It is important to outline the anatomical structure, vasculature and innervation of the knee joint to have a better understanding of how physiological pain transmission and inflammation propagation can influence the musculoskeletal system under arthritic conditions.

1.2.1   Structure of the human knee joint

The knee joint, as illustrated in Figure 1.1, is a crucial synovial joint with a modified hinge capability allowing for both flexion and extension (Welsh 1980). Vital for providing stability during load bearing activities (Abulhasan and Grey 2017), the knee joint consists of the conjoining femoral condyle and tibial plateaux (Welsh 1980). The patella, better known as the knee-cap, is another bony component of the knee joint (Welsh 1980). Secured to the tibial tuberosity and the quadriceps muscles by the patellar ligament, the patella glides over the femur and tibia with help from articular cartilage (Abulhasan and Grey 2017). As a classical synovial joint, the knee joint contains a fluid filled capsule with a membranous synovial layer lining the inner surface (Welsh 1980).
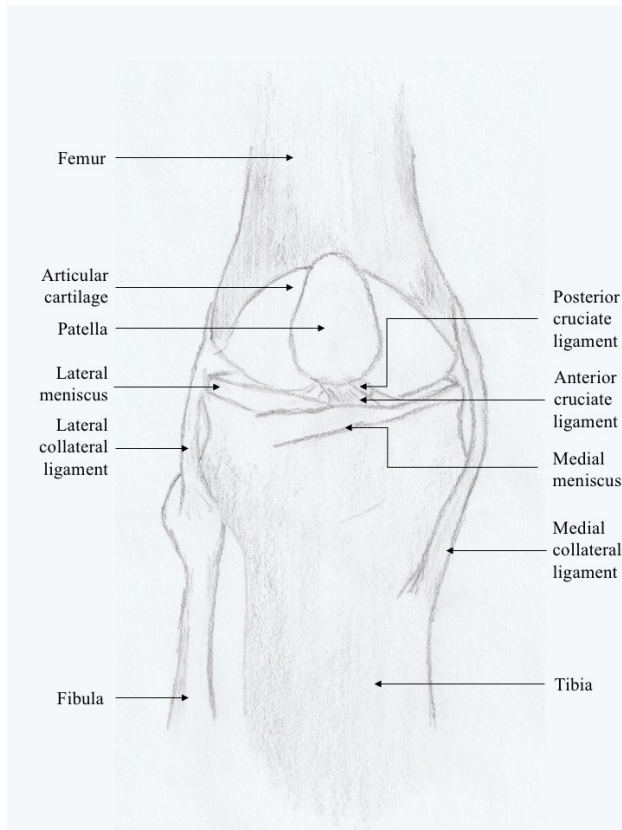
Figure 1.1 Anatomy of the right knee joint. Author's drawing based on: Centeno and Clinuic et al. (2015).

Fibrous strands of tissue known as ligaments, ensure the stability of the joint itself (Welsh 1980). Two groups of ligaments exist to support the knee joint: collateral

ligaments and cruciate ligaments (Abulhasan and Grey 2017). The medial collateral ligament connects the femur and tibia and reinforces against valgus movement (Abulhasan and Grey 2017). The lateral collateral ligament attaches from the femur to the fibular head to reinforce against varus movement (Abulhasan and Grey 2017). The anterior cruciate ligament is the primary support, maintaining rotational stability, meanwhile the posterior cruciate ligament resists posterior displacement of the bones in the joint (Abulhasan and Grey 2017).

Unique to the knee joint, a cartilaginous tissue known as the meniscus lies between the femur and tibia (Welsh 1980). Located both laterally and medially, the menisci are responsible for shock absorption and reduction of friction from bones that would otherwise rub together (Abulhasan and Grey 2017). Unlike other tissues in the knee joint, the inner portions of the menisci and the entire cartilage are avascular (Welsh 1980).

1.2.2   Vasculature of the knee joint

The knee is also made up of an extensive vascular network (Figure 1.2) (Scapinelli 1968). The primary feeding arterial supplies are the supreme genicular artery (highest genicular artery), the medial and lateral superior genicular arteries, the medial and lateral inferior genicular arteries, the middle genicular artery, and the anterior or posterior tibial recurrent artery (Scapinelli 1968). Also pictured, the descending branch of the lateral femoral circumflex recurrent fibular artery also provides blood supply to the knee joint (Scapinelli 1968).
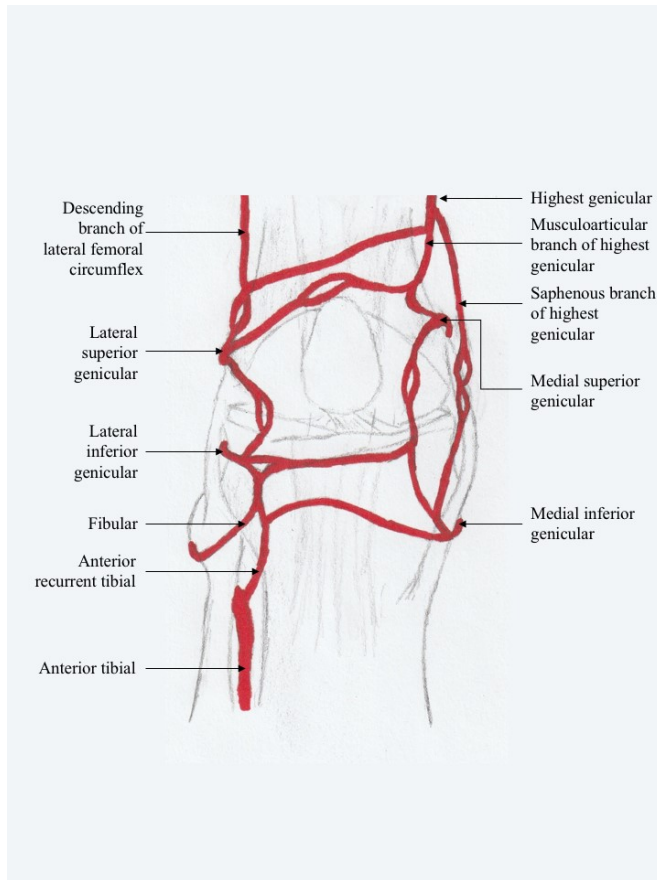
Figure 1.2 Arterial vasculature of the right knee joint. Author's drawing based on: Gray (1918).

Innervation of the knee joint

In addition to the intricate vascular network, the knee joint is highly innervated by afferent and efferent fibers which make it possible to communicate from the periphery to the central nervous system (Freeman and Wyke 1967; Kennedy et al. 1982). Kennedy et al. (1982) has described a posterior and anterior innervation patterns within the human knee joint. The posterior group consists of the posterior articular nerve and the obturator nerve (Freeman and Wyke 1967). These nerves innervate the posterior portion and medial aspect of the joint capsule respectively (Freeman and Wyke 1967). The anterior group includes articular branches from the femoral, common peroneal, and saphenous nerve (Kennedy et al. 1982). Branches from the femoral nerve play a distinct role in innervating the quadriceps muscles and they can also innervate the anteromedial aspect of the joint capsule (Kennedy et al. 1982). The lateral articular and recurrent peroneal

branches from the common peroneal nerve innervate the lateral portion of the joint capsule, meanwhile the infrapatellar branch deriving from the saphenous nerve innervates the inferior capsule area (Kennedy et al. 1982; Vas et al. 2014). These anatomical distinctions are common to human, rat, and cat species (Freeman and Wyke 1967; Kennedy et al. 1982; Hildebrand et al. 1991).

Fiber type (Table 1.1) has also been investigated (Kennedy et al. 1982). Type I, II, III and IV were each found to innervate the joint (Kennedy et al. 1982; Ferrell et al. 1992). Type I afferents included myelinated Aα fibers responsible for proprioception (Ferrell et al. 1992). Slightly smaller type II myelinated Aβ afferents were found to transmit proprioception (Ferrell et al. 1992). Myelinated Aδ type III fibers together with unmyelinated type IV, C-fibers are responsible for transmitting noxious signals (Ferrell et al. 1992). Small, non-myelinated efferent fibers originating from the posterior and medial articular nerves were also determined to innervate the joint (Hildebrand et al. 1991). These fibers make up two thirds of the unmyelinated fibers (Hildebrand et al. 1991).

Table 1. 1 Descriptive characteristics of primary afferent fibers. Adapted from Krustev et al. (2015), with additional info from Ferrell (1992) and Urch (2007).

Fiber Type	Diameter (μm)	Myelin	Conductance (m/s)	Function/ Activation	Area of innervation	Pain Sensation
Afferent						
Aα (I)	10-18	Thick	60-100	Proprioception	Ligaments	N/A
Aβ (II)	5-12	Thick	20-70	Proprioception	Fibrous layer	N/A
Aδ (III)	1-5	Thin	2.5-20	Nociception (Thermal, mechanical)	Fibrous layer, synovium	Pricking, short-acting
C (IV)	<1	N/A	< 2.5	Nociception (Thermal, chemical, mechanical)	Fibrous layer, synovium	Dull, aching, non-localized
Efferent						
Symp-athetic	0.4-2.4	N/A	1	Sympathetic/ vasomotor tone	Posterior/ Medial region of joint capsule	N/A

1.3   Physiological basis of pain

The International Association for the Study of Pain defines pain as the culmination of sensory and emotional experiences from damaging stimuli (Loeser and Treede 2008). Nociception, distinct from pain, is the objective outcome of a noxious stimulus that is transmitted by afferents to the central nervous system (Loeser and Treede 2008).

1.3.1   Types of pain

Pain is characterized into three main components: nociceptive, inflammatory and neuropathic pain (Loeser and Treede 2008). Nociceptive pain occurs somatically or viscerally through activation of nociceptors on peripheral afferents (Loeser and Treede 2008; Su et al. 2014). Evolutionarily important as a warning defense mechanism that alerts the body to danger, nociceptors may be activated by chemical mediators, thermal disturbances, or inflammatory mediators from tissue injury (Prescott and Ratte 2012). Activation of nociceptors prompts a centrally mediated withdrawal response along with redness, swelling, and pain sensations which vary from sharp-stabbing to dull-aching (Prescott and Ratte 2012). These sensations remain while the stimulus is present (Su et al. 2014).

Inflammatory pain originates as nociceptive response by chemical, mechanical, or thermal activation of nociceptors in the periphery (Kidd and Urban 2001; Xu and Yaksh 2011). Unlike nociceptive pain however, inflammatory pain can occur secondarily without the noxious stimuli actually being present (Xu and Yaksh 2011). Inflammatory mediators released in response to tissue injury or other damage are able to alter the sensitization of nociceptors on neuronal afferents making them more responsive, known as hyperalgesia (Prescott and Ratte 2012). This can also result in allodynia, where a typically a non-noxious stimulus will induce pain sensations (Loeser and Treede 2008). Pro-inflammatory mediators responsible for inflammatory pain include prostaglandins, bradykinins, cytokines/interleukins, serotonin, glutamate, histamine, or hydrogen or potassium ions (Prescott and Ratte 2012).

Neuropathic pain, on the other hand, arises through injury or disease to the somatosensory nervous system (Loeser and Treede 2008). This can occur centrally by

lesions to the brain or spinal cord, or peripherally by damage to the Aβ, Aδ or C-fibers (Colloca et al. 2017). Neuropathic pain can occur in a number of neurogenerative disorders, diabetes, or even arthritis and usually presents as tingling sensations (Klooster et al. 2007; Colloca et al. 2017; Philpott et al. 2017).

Pain is often characterized by duration (Treede et al. 2015). In humans, the term chronic pain is used to describe pain that persists for more than three-months, whereas acute pain encompasses the time beforehand (Treede et al. 2015).

1.3.2   Pain transmission

The overall mechanics of pain can be described as three primary components: transduction, transmission, and modulation (Osterweis et al. 1987). The transduction phase begins in the periphery, where nociceptive fibers can detect the products of a noxious event (Yam et al. 2018). The interaction of an excitatory, pro-inflammatory molecule with its respective receptor on the nociceptive fiber initiates a chemical depolarization of the afferent's terminal (Osterweis et al. 1987). If the intensity of the stimuli are high, the threshold potential is surpassed, and an electrical impulse known as an action potential is directed towards the neuron's cell body in the dorsal root ganglion and, subsequently, to the spinal cord (Osterweis et al. 1987; Yam et al. 2018). Small diameter fibers capable of transmitting noxious signals from the periphery to the central nervous system (Table 1.1) have been identified as thinly myelinated Aδ fibers and non-myelinated C-fibers (Kitahata 1994; Yam et al. 2018).

Nociceptors synapse onto second order neurones in the dorsal horn of the spinal cord in laminae I and II. Signals are then propagated from the spinal cord to the thalamus (Osterweis et al. 1987) and then relayed to specific areas of the somatosensory cortex and limbic system where they are processed into the physiological and emotional components of pain (Osterweis et al. 1987).

The magnitude of nociception and/or pain can be worsened by modulation of transmission at various sites within the periphery or central nervous system (Osterweis et al. 1987). Peripheral sensitization occurs when there is persistent activation of the nociceptors by inflammatory mediators (Yam et al. 2018). As a consequence, the firing threshold is reduced and a lower number of nociceptive mediators are required to activate

the fiber (Yam et al. 2018). Like peripheral sensitization, central sensitization results from reduced firing potential (Bennett 2000). Due to these persistent signals from the periphery, an influx of SP and glutamate are released within the dorsal horn synapse (Bennett 2000; Yam et al. 2018). Cohesive binding of these abundant neuromodulators remove magnesium from the N-methyl-D-aspartate (NMDA) channel (Bennett 2000). This allows for increased glutamate binding and ultimately increased excitability of the second order neuron (Bennett 2000). Secondary allodynia, a painful response to a non-noxious stimulus (Loeser and Treede 2008), may also occur as a result of central sensitization (Yam et al. 2018). Nociceptive fibers, along with peripheral and central sensitization, is also implicated in neurogenic inflammation (Heppelmann and Pawlak 1997).

1.3.3   Neurogenic inflammation

The concept of neurogenic inflammation was first described by Bayliss et al. (1901). Experimental evidence of vasodilation in a dog's hind limb by stimulation of the sensory afferents implicated the peripheral nervous system in this type of inflammatory response (Bayliss 1901; McGillis and Fernandez, 1999) The origin of the neurogenic inflammatory response has since been elucidated to peripheral antidromic impulses which bifurcate into neighbouring branches of the activated C fiber afferent (Lewis and Zotterman 1927; Jancsó et al. 1967). This branching potential, known as the axon reflex (Lewis and Grant 1924), prompts the release of vesicle-stored neuropeptides (Brain 1997). SP, CGRP, neurokinin-A (NKA), and VIP are each implicated in the neurogenic inflammatory response (Brain 1997). Of these, SP and CGRP are heavily researched and associated with neurogenic inflammation (Brain 1997).

Release of pro-inflammatory and algogenic neuropeptides into the periphery can result in increased activation and afferent firing (Schaible et al. 2009). These peptides have also been implicated in the sensitization of peripheral fibers by reducing the nociceptive firing threshold (Schaible et al. 2009). Aa a result, neurogenic inflammation can contribute to a damaging inflammatory and nociceptive feedback loop, facilitating the chronicity of some inflammatory pathologies (Prescott and Ratte 2012).

1.4   The human immune system and acute inflammation

1.4.1   Innate immunity

The innate immune system is the body's non-specific, first line of defense against invading pathogens or disease (Alberts et al. 2002). The virtually immediate response instigates the cardinal signs associated with inflammation: redness, warmth, swelling and pain (Bennett et al. 2018). The human body is equipped to detect foreign substances that have escaped the physical barriers put in place by the innate immune system (Alberts et al. 2002). Pattern recognition receptors (PRRs) are able to discern between self and foreign through the recognition of pathogen-associated molecular patterns (PAMPs); sequences uniquely exhibited by pathogens (Bennett et al. 2018).

Pattern recognition occurs with the help of circulating white blood cells known as leukocytes (Molnair and Gair 2013). Leukocytes are a broad category of immune cells tasked with cleaning up and eliminating foreign or damaged material (Molnair and Gair 2013). Monocytes, macrophages, mast cells and dendritic cells are all examples of leukocytes (Molnair and Gair 2013). The transmigration of circulating leukocytes to the site of inflammation is described in detail later in this section. Recognition of a pathogen precipitates phagocytosis of the substance and recruitment of more leukocytes to the scene (Bennett et al. 2018). Macrophages, dendritic cells, and neutrophils are known for their phagocytic behaviours (Alberts et al. 2002; Bennett et al. 2018). However, neutrophils are the most abundant leukocyte and are often the preliminary leukocyte in the removal process (Molnair and Gair 2013). Natural killer (NK) cells lyse pathogens rather than dealing with them through phagocytosis (Bennett et al. 2018).

Detection and elimination of foreign material also promotes the secretion of cytokines (Zhang and An 2007); proteins responsible for communicating the inflammatory message (Zhang and An 2007). Production of cytokines by the innate immune response can occur mainly by macrophages and neutrophils (Zhang and An 2007). Interleukins (IL) are leukocyte-secreted cytokines known to exhibit both pro-inflammatory and anti-inflammatory actions (Zhang and An 2007). These cytokines can stimulate nearby leukocytes, resulting in activation and production of more immune mediators (Bennett et al. 2018). They also interact with lymphocytes belonging to the adaptive immune response (Bennett et al. 2018). Tumor necrosis factor alpha (TNF-α),

IL-1β, and IL-6 are integral pro-inflammatory cytokines whereas IL-4 and IL-10 are common anti-inflammatory cytokines (Bennett et al. 2018).

The innate system is also responsible for activating the adaptive immune response (Alberts et al. 2002; Bennett et al. 2018). Phagocytic leukocytes, like macrophages and dendritic cells, are characterized as antigen presenting cells (APCs) (Bennett et al. 2018). Fragments of the engulfed foreign pathogen reappear on the external surface of the APC and are delivered to the lymphatic organs and the lymphocyte populations (Alberts et al. 2002).

1.4.2 Other acute immune mediators

Prostaglandins are also known to contribute to the acute inflammatory response (reviewed by Morteau 2000; Ricciotti and FitzGerald 2011). During an inflammatory event, arachidonic acid is freed from the phospholipid membrane by phospholipase A (Ricciotti and FitzGerald 2011). Arachidonic acid is then converted to prostaglandin H_2 by prostaglandin H_2 synthases known as cyclooxygenase enzymes (COX1 or COX2) (Morteau 2000). From there, various prostaglandins are produced including: prostaglandin E_2 (PGE_2), prostaglandin D_2, prostaglandin I_2 and prostaglandin F_2 (Morteau 2000; Ricciotti and FitzGerald 2011). Thromboxane A_2 is also produced from prostaglandin H_2 (Ricciotti and FitzGerald 2011).. PGE_2 is commonly known for its pyretic and inflammatory actions which occur by increasing vascular permeability and inducing oedema (Morteau 2000). PGE_2 has also been implicated in algogenic processes by binding afferent terminals (Osterweis et al. 1987). Although prostaglandins can act as pro-inflammatory mediators, they also play a role in homeostatic functions by exerting protective mechanisms (Morteau 2000). For example, prostaglandin synthesis in the gastrointestinal system aids gastric motility, promotes gastric secretions and protects against injury (Morteau 2000). COX-1 has been linked to helpful homeostatic prostaglandin synthesis, whereas COX-2 enzymes are often implicated in the inflammatory response (Ricciotti and FitzGerald 2011).

Several other mediators within the inflammatory mixture play a critical role in the production of inflammation (Basbaum et al. 2010). Histamine for example, can be released from degranulated mast cells, resulting in oedema (Rosa and Fantozzi 2013).

Leukotrienes are also upregulated during an inflammatory response and contribute to the inflammatory response (Osterweis et al. 1987). Bradykinins can also activate nociceptors, sensitize peripheral afferents and increase vascular permeability (Osterweis et al. 1987).

1.4.3   Leukocyte trafficking

When the body suffers an injury, infection, or inflammatory event, immune mediators such as leukocytes are required to help fight and repair the damage (Nourshargh and Alon 2014). To do this, they must transmigrate across the vascular endothelium of post-capillary venules in order to access the inflamed area (Nourshargh and Alon 2014). As pictured in Figure 1.3, this process is called leukocyte extravasation or diapedesis (Muller 2011). Under normal homeostatic conditions, leukocytes are free-flowing and are carried along by the laminar blood flow (Muller 2011). During an inflammatory event, post-capillary venules create signals that increase the likelihood that leukocytes will interact with the endothelium (Muller 2011). This protective process can become pathogenic if the process is unregulated, executed excessively, or directed against self-tissues (Nourshargh and Alon 2014).

Initiating the extravasation process is known as capturing (Ley 1996). At this point, leukocytes make weak but rapid associations with endothelial selectins, such as L-selectin and P-selectin, and capture them from mainstream blood flow (Ley 1996; Muller 2011). Continuous, rapid, on and off binding with P-selectin and newly activated E-selectin promote greater endothelium-leukocyte interactions, known as the rolling state of the extravasation process (Muller 2011). Visually, leukocytes 'roll' along the endothelial wall (Ley 1996). Activated cells can then produce chemoattractant molecules such as IL-8, C5a, and leukotriene B4 responsible for activating integrins (Ley 1996). Activated integrins promote endothelium-based adhesion ligands such as intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-2 (ICAM-2), and vascular cell adhesion molecule-1 (VCAM-1) (Muller 2011). This leads to the adhesion of leukocytes to the endothelium (Muller 2011). Transmigration of the adherent leukocytes typically occurs at the tight junctions between endothelial cells in the vascular wall (Muller 2011). Leukocytes can then extravasate by squeezing through the junction into the interstitial

space to perform their immunoregulatory effects (Ley 1996; Muller 2011; Nourshargh and Alon 2014).
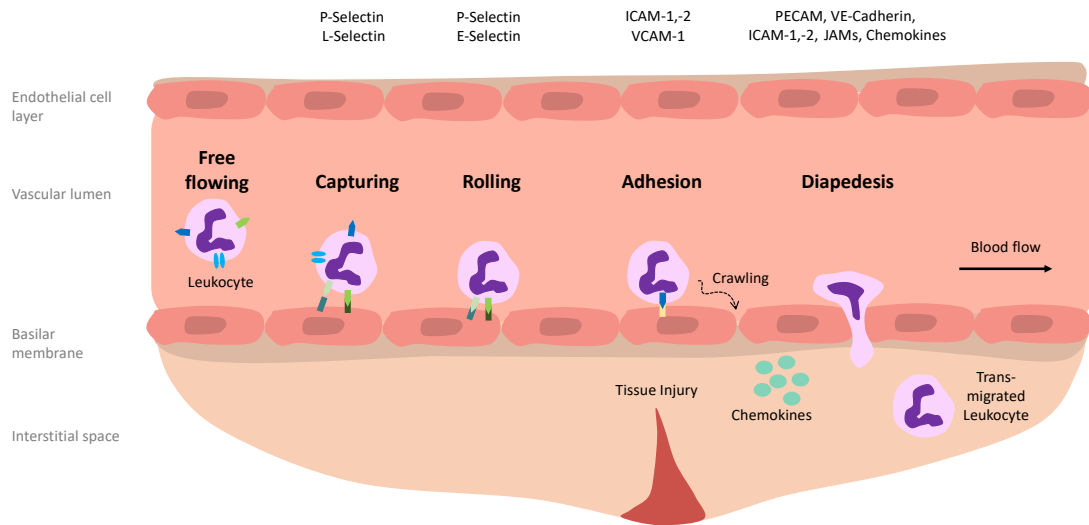
Figure 1.3 Integrin and adhesion molecule involvement in the leukocyte extravasation process. Adapted from Nourshargh et al. (2014). ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; VE-cadherin, vascular endothelial cadherin.

1.5   Inflammatory arthritis

Inflammatory arthritis (IA) is a subcategory of arthritis characterized by inflammatory flares within the joints and surrounding tissues that can develop acutely or over time (Ledingham et al. 2017). IA conditions often involve autoimmune disorders, where the body's innate immune system mounts a self-tissue immune response instead of invading foreign substances (Ledingham et al. 2017). The systemic nature of these conditions is yet another distinguishing attribute, where several joints may be simultaneously impacted (Ledingham et al. 2017). Commonly known conditions of IA are rheumatoid arthritis (RA), psoriatic arthritis, ankylosing spondylitis, juvenile idiopathic arthritis, and systemic lupus (Ledingham et al. 2017).

1.5.1 Rheumatoid arthritis

Of all of the inflammatory conditions, RA is the most prevalent (Ledingham et al. 2017). This polyarthritic condition is a chronic progressive arthritis that typically localizes in a few joints, before spreading bilaterally and producing systemic effects in other organs (Lee and Weinblatt 2001).

RA is estimated to affect over 300 thousand Canadians, roughly one percent of the nation's population (Public Health Agency of Canada 2017). The prevalence of RA is expected to rise to 1.3 percent over the next 30 years (Bombardier et al. 2011). The autoimmune condition is reported to be 2-3 times more prevalent in women (Smolen and Steiner 2003). The onset of RA can commence at any age (Smolen and Steiner 2003). However, the majority of newly diagnosed cases occur after the age of 40 and the condition is reported to be more prevalent in the aging population (Bombardier et al. 2011; Public Health Agency of Canada 2017).

According to the Lee and Weinblatt (2001), pre-disease symptoms are often non-specific. They frequently include a low-grade fever, body soreness, mild fatigue and a general "unwell" feeling (Lee and Weinblatt 2001). Patients may also present with symptoms involving their musculoskeletal system including soreness, morning stiffness, and tenderness in one or more joint areas and surrounding tissues (Aletaha and Smolen 2018). Due to the inflammatory component of this arthritis, one or more joint areas may also experience redness, warmth, and swelling (Lee and Weinblatt 2001). These symptoms can affect larger diarthrodial joints such as the knee joint or hip joints (Lee and Weinblatt 2001; Aletaha and Smolen 2018). More frequently, smaller joints such as the metacarpophalangeal (MCP) joints and proximal interphalangeal (PIP) joints in the hands and metatarsophalangeal (MTP) joints in the feet are also involved by themselves or in association with larger joints (Lee and Weinblatt 2001). As the disease progresses, bilateral and/or symmetric symptoms manifest (Aletaha et al. 2010). Patients may experience restricted mobility or loss of function in the affected joints (Lee and Weinblatt 2001). Severe cases of long-lasting fatigue, loss of appetite and weight loss may also accompany the previously described symptoms (Lee and Weinblatt 2001).

RA patients may present with radiological evidence of articular damage (Aletaha and Smolen 2018). Radiological abnormalities include bone erosion, joint space

narrowing, cartilage damage and ankylosis (Lee and Weinblatt 2001; Aletaha and Smolen 2018). The joints in the hand may also experience subluxation (Lee and Weinblatt 2001; Aletaha and Smolen 2018).

Synovitis, an inflamed synovial lining, is one of the defining histological features of RA (Aletaha et al. 2010). Perpetuated by an infiltration of immune cells into the synovial layer (McInnes and Schett 2011), cellular hyperplasia of the synovium occurs and can extend from 3 cells thick to up to 20 cells thick (Smolen and Steiner 2003). Additionally, increased vascularity known as angiogenesis (Gerritsen 2008) also transpires as a support mechanism as the enlarged synovial tissue requires an augmented blood-supply (Lee and Weinblatt 2001).

The physically debilitating symptoms of RA, such as extreme persistent pain and loss of musculoskeletal mobility, can impose a significant burden on the personal, psychological, and social well-being of the patient (Lütze and Archenholtz 2007). Personal burdens can include difficulty caring for one's self and the inability to perform daily personal care tasks (Lagacé et al. 2010). The Arthritis Alliance of Canada (AAC) reports that more than 50 thousand Canadian RA patients find personal hygiene tasks, grooming tasks, going to bathroom, and getting dressed difficult (Bombardier et al. 2011). These patients often rely on, or become dependent on, the sustained care from friends and family to perform essential tasks (Lütze and Archenholtz 2007). Socially, RA patients report the need to modify basic life activities such as leisure and recreation based on energy levels, disease progression and severity of disease symptoms (Lütze and Archenholtz 2007). Persistent symptoms, loss of dependence or social participation, and self-insecurities can create negative impacts on a patient's psychological well-being which may lead to depression or anxiety (Dickens et al. 2002; van Dyke et al. 2004).

Rheumatoid arthritis can also play a significant role in workplace disability (Gunnarsson et al. 2015). According to the Arthritis Alliance of Canada, 0.75% of the labor force has diagnosed early to late stage RA, a number that is estimated to double in the next thirty years (Bombardier et al. 2011). Missed work and productivity losses from RA patients equated to financial losses of 252-million dollars annually within the U.S. (Gunnarsson et al. 2015). In addition to increased long-term disability leave, a separate

study reported that RA patients were unlikely to return to work post-disability leave (Hansen et al. 2017).

1.5.1.1   *Risk factors and pathophysiology of rheumatoid arthritis*

Although the exact cause of RA is unknown, several genetic and environmental factors are known to influence one's chance of getting the disease and exacerbate its effects (Deane et al. 2017). Environmental factors such as heavy/long-duration cigarette use (Hutchinson et al. 2001; Costenbader et al. 2006), air pollutants and dust exposure are associated with a higher relative risk of RA (Deane et al. 2017). Bacteria in the oral cavity and specific flora such as *Prevotella copri* in the gut microbiome have also been connected with elevated incidences of RA (Scher et al. 2012; Scher et al. 2013).

Unmodifiable risk factors acknowledged by Deane et al. (2017) include sex, genetic susceptibility, and shared inheritance. As mentioned previously, RA is 2-3x more likely to occur in females (Public Health Agency of Canada 2017). The exact basis for this is unknown; however, hormonal involvement is thought to be a factor whereas pregnancy and breast feeding have been associated with fewer cases of disease onset and disease symptom remission (Silman et al. 1992; Karlson et al. 2004). Similarly, post-partum periods have been associated with exacerbation of the condition (Silman et al. 1992).

Heritability and genetic interplay have also been linked to RA (Koumantaki et al. 1997; Frisell et al. 2016). Occurrence of RA is higher in populations that have a close family relative with the disease (Koumantaki et al. 1997; Frisell et al. 2016). Additionally, incidence rates are higher in monozygotic twins relative to dizygotic twins suggesting some genetic link (MacGregor et al. 2000). Compellingly, studies report that genetics may account for roughly 60 percent of disease susceptibility in certain cohorts (MacGregor et al. 2000).

Genetic modification can also alter susceptibility to the disease (Firestein 2003). One identified genetic epitope that confers susceptibility is a variable region in the human leukocyte antigen (HLA) locus within the major histocompatibility complex (MHC) (Gregersen et al. 1987; Firestein 2003). Deemed the 'shared epitope' by Gregersen and colleagues (1987), this region is thought to modify antigen presentation of arthritogenic

peptides by the MHC complex, thus influencing onset and pathogenesis of the disease (Gregersen et al. 1987; Firestein 2003; Mateen et al. 2016).

The self-reactivity of RA is in part due to autoantibodies and their loss of tolerance to innate antigens (Guo et al. 2018). Rheumatoid factor (RF), discovered by Waaler in 1940, is an antibody that binds to the Fc region of immunoglobulin G (IgG) (Waaler 1940; Franklin et al. 1957). Presence of the autoantibody can be indicative of an increased disease severity (Agrawal et al. 2007). Produced by B-cells or T-cells in response to presentation of an antigen (Ingegnoli et al. 2013), RF is thought to contribute to RA pathogenesis through creation of immune complexes by binding with IgG and subsequent displacement in the synovium (Zvaifler 1973; Ingegnoli et al. 2013). This facilitates recruitment of immune cells equipped to perform phagocytosis (Ingegnoli et al. 2013).

Yet another important autoantibody involved in the pathogenesis of RA is the anti-citrullinated protein antibody (ACPA) (Kurowska et al. 2017). Citrullination occurs as a post-translational deamination of an arginine residue by peptidylarginine, altering the peptide so that it is considered foreign and may be recognized by antigen presenting cells (Derksen et al. 2017). Peptides affected by this modification include fibrin, fibrinogen and vimetin (Kurowska et al. 2017). These cells present antigens to B and T cell populations, where ACPAs are created and propagate the autoimmune component of the disease (Choy 2012). ACPAs, once generated, form immune complexes with citrullinated proteins and promote activation of immune cells such as macrophages (Sokolove et al. 2011). Seropositive in 60-80 percent of RA patients (Agrawal et al. 2007; Aggarwal et al. 2009), this antibody is considered an established indicator of the disease and an accurate future predictor of disease onset (reviewed by Kurowska et al. 2017).

Inflammatory mediators, constituting the innate and adaptive immune system, play a critical role in driving the pathogenesis of RA as illustrated in Figure 1.4 (Hussein et al. 2008; Mateen et al. 2016). Dendritic cells, macrophages, monocytes, and natural killer cells are all involved in establishing the inflammatory pathogenesis of RA through detection, phagocytosis, presentation to the adaptive immune cells, and production of inflammatory mediators such as cytokines (Choy 2012). Commonly elevated cytokines are described in Table 1.2.

Table 1. 2 Major cytokines found in the serum and synovial fluid of RA patients.

Cytokine	Serum	Synovial Fluid	Reference
TNF-alpha	+	+	(Tetta et al. 1990) (Hamed et al. 2007)
IL-1B	+	+	(Hamed et al. 2007)
IL-6	+	+	(Hamed et al. 2007) (Houssiau et al. 1988)
IL-12	+	+	(Kim et al. 2000)
IL-17[c]	+	+	(Metawi et al. 2011)
IL-4[a]	+	/	(Rivas et al. 1995)
IL-10[a]	+	+	(Cush et al. 1995)

*Synovial fluid (SF) is not relative to healthy controls, rather is elevated relative to osteoarthritis (OA) SF
[a] Ant-inflammatory cytokines
[b] Relative to SF from healthy controls
[c] Positively correlated with SF levels

TNF-α and IL-1β are critical cytokines that exert very similar pathophysiological actions (Choy and Panayi 2001). Both cytokines are mainly secreted by monocytes, macrophages, as well as B and T cell populations (Choy and Panayi 2001). Primarily, they act to propagate inflammation in the affected area by stimulating other immune cells to further produce cytokines (Choy and Panayi 2001). Increased levels of matrix metalloproteinases (MMPs) from fibroblasts and chondrocytes are also a direct result of IL-1β and TNF-α stimulation, resulting in connective tissue and cartilage damage (Choy 2012). Adding to their inflammatory influence, TNF-α and IL-1β have demonstrated the ability to up-regulate adhesion molecule expression on fibroblasts and endothelial cells (Choy and Panayi 2001). The proliferation of adhesion molecule expression imparted by these cytokines can aid in the recruitment and transmigration of leukocytes to the inflamed area (Choy 2012). Specifically, TNF-α may play a role in the recruitment of neutrophils to the joint, where their secretory proteases can trigger cartilage damage (Choy and Panayi 2001). IL-1β also mediates bone erosion and structural joint damage through the activation of osteoclasts (Mateen et al. 2016).

Similar to TNF-α and IL-1β, IL-6 is also produced by monocytes, macrophages, and T-cells; but can also be produced by chondrocytes and endothelial cells (Choy and Panayi 2001). IL-6 can stimulate and cause proliferation of T-cells, B-cells, and fibroblasts (Choy and Panayi 2001). Analogous to the previous cytokines (Choy and Panayi 2001), IL-6 also up-regulates adhesion molecules, recruits and stimulates neutrophil production of cartilage degrading enzymes, and can activate osteoclasts contributing to significant bone damage (Choy 2012; Mateen et al. 2016). Uniquely, IL-6 can aid in the production of vascular endothelial growth factor (VEGF), a growth factor deemed responsible for the proliferation of pannus (Mateen et al. 2016).

Produced by monocytes and dendritic cells, IL-12 contributes to inflammation by stimulating natural killer cells to produce interferon-gamma (IFN-γ) (Mateen et al. 2016). In a positive feedback loop, IFN-γ reverts back and acts on these first-line immune cells to produce more IL-12 (Choy and Panayi 2001). This cytokine is also known to act on helper T-cells and create a shift to a Th-1 profile (Mateen et al. 2016).

IL-17 has recently been identified as an arthritogenic cytokine (Hussein et al. 2008). Produced by CD4+ T-cells and natural killer cells, IL-17 mimics other cytokines and increases production of TNF-α, IL-1β, IL-6, IL-8 and MMPs from fibroblasts (Mateen et al. 2016). This interleukin can also contribute to osteoclast-induced bone damage, MMP-induced cartilage damage, and recruitment of immune cells to the affected area through production of a chemotactic gradient (Choy 2012).

Anti-inflammatory mediators such as IL-4 and IL-10 are also elevated in RA patient samples (Cush et al. 1995; Rivas et al. 1995; Verhoef et al. 2001). These mediators contribute to anti-inflammatory effects by inhibiting further production of pathogenic molecules like TNF-α, IL-1β, IL-6, IL-8, and IL-12 (Isomäki et al. 1997; Mateen et al. 2016). Inhibition of MMPs has also been observed with these anti-inflammatory cytokines and this could prevent further damage of connective tissues (Mateen et al. 2016). Patients with mild forms of the disease tend to have upregulations of IL-10 suggesting that this cytokine has the potential to mitigate disease severity (Verhoef et al. 2001). Despite their notable up-regulation and anti-inflammatory effects, the actions of these cytokines may not be enough to suppress the disease completely (Choy and Panayi 2001).
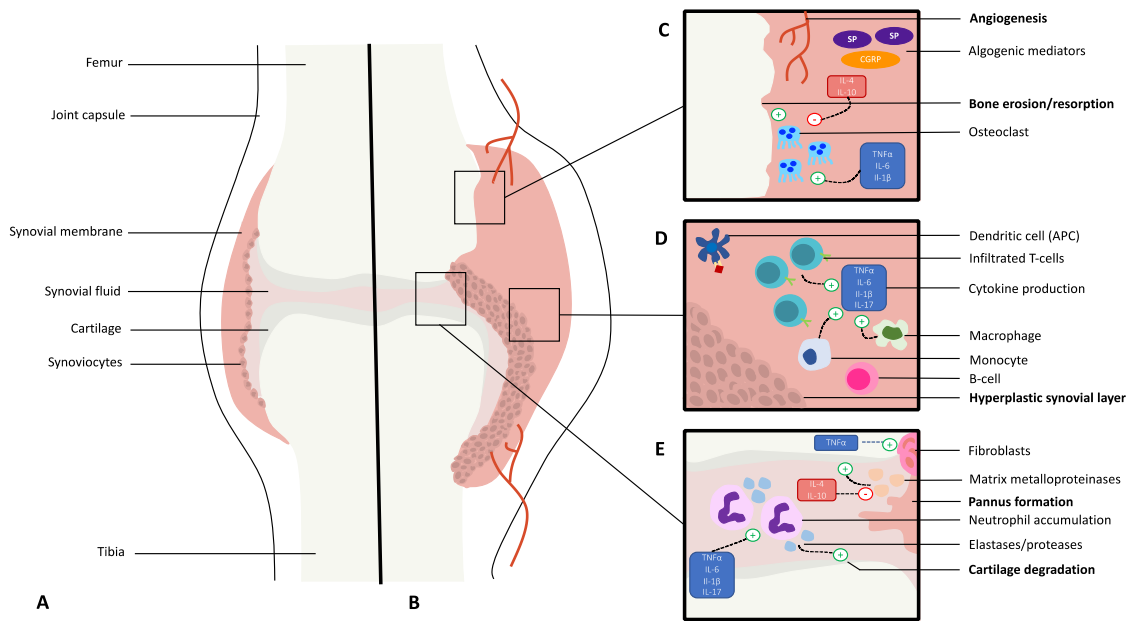
Figure 1.4 Healthy control knee joint (A) compared to a knee joint affected by rheumatoid arthritis (B). Bone erosion (C), synovial hyperplasia with immune cell infiltration (D), and cartilage degradation (E) are depicted. Clinical signs of rheumatoid arthritis are bolded. Adapted from Smolen and Steiner (2003). APC, antigen presenting cell; TNFα, tumor necrosis factor-alpha; IL, interleukin; (+) stimulation or (-) inhibition of a pathological process.

Inflammatory mediators not only contribute to tissue damage, but also play a central role in the pain experienced by RA patients (Schaible et al. 2002). Joint-innervating sensory fibers express cytokine receptors, pattern recognizing receptors (toll-like receptors; TLR), transient receptor potential receptors (TRP), among others (Osterweis et al. 1987). Inflammatory mediators are able to activate their respective receptors on nociceptors leading to the generation of action potentials which are then transmitted centrally (Yam et al. 2018). Furthermore, RA patients exhibit increased levels of SP in both plasma and synovial fluid compared to controls and OA patients (Marshall et al. 1990; Hernanz et al. 1993). These aspects contribute to increased excitability known as peripheral sensitization (Schaible 1996). CGRP and VIP were also upregulated in synovial fluid in RA patients compared to OA patients (Hernanz et al. 1993), contributing

to the vasodilatory neurogenic inflammatory response, as well as transmission of pain (Russell et al. 2014).

The upregulation of algogenic neuropeptides is also a feature of central sensitization in RA patients (Schaible 1996; Dessein et al. 2000; Schaible et al. 2002). Peripheral and central sensitization may be responsible for the thermal and mechanical hyperalgesia observed in RA patients compared to healthy individuals (Schaible et al. 2002; Edwards et al. 2009).

1.5.1.2   Diagnostics

The 2010 College of Rheumatology/European League Against Rheumatism guidelines (Aletaha et al. 2010), adapted from the 1987 version (Arnett et al. 1988), remains one of the most widely used classification criteria for definitive diagnosis of RA. For the scale to be employed, the patient must present with at least one joint exhibiting clinical synovitis (Aletaha et al. 2010). The classification criteria then utilizes a cumulative score-based algorithm in the categories of joint involvement, serology, acute phase reactants and duration to confirm a diagnosis (Table 1.3; Aletaha et al. 2010). A score above or equal to six, accompanied by one seropositive test and one positive acute phase reactant is indicative of the definitive presence of RA (Aletaha et al. 2010).

Table 1. 3 American college of Rheumatology (ACR)/European League Against Rheumatism (EULAR) Classification Criteria for the diagnosis of rheumatoid arthritis. Adapted from Aleteha et al. (2010).

2010 American college of Rheumatology/European League Against Rheumatism Classification Criteria	
Joint Involvement	
1 large joint	0
2-10 large joint involvement	1
1-3 small joint involvement (with or without large joint involvement)	2
4-10 small joint involvement (with or without large joint involvement)	3
>10 small joint involvement (at least 1 small joint must be involved)	5
Serology	
Negative RF and Negative ACPA	0
Low positive RF or low positive ACPA	2
High positive RF or high positive ACPA	3
Acute Phase Reactants	
Normal CRP or normal ESR	0
Abnormal CRP or abnormal ESR	1
Symptom Duration	
Less than or equal to six weeks	0
More than six weeks	1

ACPA, Anti-citrullinated protein antibody; CRP, C-reactive protein; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate.

1.5.1.3 Current rheumatoid arthritis treatments

Research into the pathophysiology of RA has paved the way for the development of clinical therapeutics to address disease progression and symptom management (Singh et al. 2015). Early diagnosis and treatment has been identified as paramount to the successful management of this chronic condition (Singh et al. 2015). Early treatment targeting inflammatory-induced damage may lend to reducing disease severity, pain severity, and can aid in improving overall functional outcomes and quality of life (Lard et al. 2001; Nell et al. 2004).

Over time, the treatment paradigm of RA has transformed to prioritize the attenuation of the damage and pathogenesis associated with the disease (Singh et al. 2015). This clinical objective produced the discovery of conventional disease modifying anti-rheumatic agents (cDMARD) and biological disease modifying anti-rheumatic

agents (bDMARD) (Nurmohamed and Dijkmans 2005). Clinically available and commonly used cDMARDs include methotrexate, leflunomide, sulfasalazine, minocycline, and hydrochloroquine (Nurmohamed and Dijkmans 2005). cDMARD therapeutics predominantly target pathological steps in the inflammatory pathway responsible for progressive destruction (Benjamin and Lappin 2019). For example, methotrexate, the gold standard for cDMARD prescription (Nurmohamed and Dijkmans 2005), functions to inhibit dihydrofolate reductase resulting in altered protein synthesis and increased adenosine, which can ultimately inhibit neutrophil and cytokine synthesis (Nurmohamed and Dijkmans 2005; Friedman and Cronstein 2019). Various studies have reported the efficacy of methotrexate in reducing disease scores and radiographic damage compared to placebo (Kremer and Phelps 1992; Strand et al. 1999). Similar to methotrexate, leflunomide can also alter protein synthesis, reduce production of pro-inflammatory cytokines and adhesion molecules, but may also inhibit NF-κB (Nurmohamed and Dijkmans 2005; Guo et al. 2018).

Despite efficacy, both methotrexate and leflunomide can inflict adverse side effects that may limit treatment compliance (Kremer and Phelps 1992; Strand et al. 1999). Common adverse effects shared by both treatments were gastrointestinal irritations (nausea, diarrhea, abdominal pain), centrally mediated headaches and dizziness, skin disturbances, alopecia, and infections (Kremer and Phelps 1992; Strand et al. 1999; Cannon et al. 2004; Nurmohamed and Dijkmans 2005). Methotrexate may also be responsible for respiratory infections like pneumonitis, mouth sores, malignancies and leukopenia (Nurmohamed and Dijkmans 2005).

Biologic DMARD treatments distinctly target specific inflammatory mediators liable for the structural damage associated with RA (Feely 2010). This group of therapeutics is often used in combination with a cDMARD rather than as a monotherapy (Singh et al. 2012; Singh et al. 2015). Biologics largely consist of TNF-alpha inhibitors such as infliximab, adalimumab, etanercept, golimumab, and certolizumab pegol (Feely 2010; Singh et al. 2012; Guo et al. 2018). Through various mechanisms, inhibition can reduce inflammation by preventing further production of inflammatory mediators and chemokines and by limiting immune cell migration and infiltration, thus reducing inflammation (Feely 2010; Guo et al. 2018). Moderate adverse effects include

gastrointestinal disturbances, urinary tract infections, respiratory infections, and cutaneous reactions at the injection site (Moreland et al. 1997). More serious effects include increased risk of hypertension and heart failure (Moreland et al. 1997; Nurmohamed and Dijkmans 2005).

Other pro-inflammatory cytokines targeted by bDMARDs include IL-1β (anakinra, canakinumab, rilonacept), IL-17 (secukinumab) and IL-6 (tocilizumab) (Guo et al. 2018). Biologicals can also target immune cell populations such as B-cells (rituximab, ofaturmumab, belimumab, atacicept, and tabalumab), T-cells (abatacept and belatacept), and kinases within inflammatory pathways (tofacitinib, baricitinib, and filgotinib) (Guo et al. 2018).

Although attenuating disease progression is one treatment strategy, adequate pain management is of utmost importance to the patient population (Klooster et al. 2007). Opioids are effective at relieving acute joint pain but their long-term use is problematic (Crofford 2010). Despite the rising use of opioids in RA patients, their analgesic efficacy is questionable (Whittle et al. 2013). A review conducted by the Cochrane Library concluded limited (30%) analgesic efficacy and a lack of functional improvement across several North American and European trials using a comparative placebo (Whittle et al. 2013). Limited analgesic efficacy of opioids in treating chronic RA-associated pain is likely attributed to tolerance (Benyamin et al. 2008; Crofford 2010), down-regulation of opioid receptors in affected tissues (Li et al. 2005), and desensitization of opioid receptors (Ueda and Ueda 2009).

The most commonly noted adverse effects associated with opioid use are gastrointestinal including nausea, vomiting (Cepeda et al. 2003), and constipation (Cherny et al. 2001; Swegle and Logemann 2006). Cognitive adverse events such as drowsiness, euphoria, hallucinations, sedation, and confusion are notable side effects (Cherny et al. 2001; Benyamin et al. 2008; Crofford 2010). Central cardiovascular (orthostatic hypertension and bradycardia) and respiratory adverse events (respiratory depression and bronchoconstriction) have also been observed at higher doses (Cepeda et al. 2003; Crofford 2010; Solomon et al. 2010). Addiction, dependence, tolerance, abuse and overdose potential are harmful disadvantages associated with chronic opioid use (reviewed by Benyamin et al. 2008).

Non-steroidal anti-inflammatory drugs (NSAIDs) are an over the counter or prescribed class of drug whose mechanism involves the inhibition of prostaglandin synthesis from arachidonic acid (Crofford 2013). Two main enzymes are responsible for the hydrolysis of arachidonic acid into an intermediate prostaglandin called prostaglandin H_2 (Morteau 2000). These enzymes are cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Crofford 2013). Typical NSAIDs are non-selective, meaning they inhibit both the homeostatic COX-1 enzyme and the inducible COX-2 enzyme, which is typically activated during inflammatory events (Crofford 2013). Examples of non-selective NSAIDs include: acetylsalicylic acid, diclofenac, naproxen, ibuprofen, nabumetone, piroxicam, and indomethacin (Crofford 2013).

Across studies examining NSAID use, treatment withdrawal was typically determined by lack of efficacy and adverse effect profiles (Wasner et al. 1981; Shi et al. 2004). Gastrointestinal side effects are considered to be the most common complaint (Crofford 2013). These may include dyspepsia, abdominal pain, diarrhea, constipation, nausea or vomiting (Wasner et al. 1981; Silverstein et al. 2000), but also may include development of gastrointestinal ulcers, bleeding, intestinal obstructions or perforations, esophagitis, or colitis (Crofford 2013). Incidence of renal complications and cardiac events are also serious adverse events associated with NSAIDs (Silverstein et al. 2000; Crofford 2013). Crofford (2013) mentions that NSAID use may lead to kidney or heart failure, hypertension, myocardial infarction, and stroke in the general population. However, risk of adverse cardiac events appears unaltered in an inflammatory polyarthritis population (Goodson et al. 2009).

Attribution of side effect profiles to the non-selectivity of typical NSAIDs prompted the discovery and employment of COX-2 selective inhibitors (Simon et al. 1998; Chen et al. 2008). Common COX-2 selective inhibitors include celecoxib, rofecoxib, etoricoxib, valdecoxib, lumiracoxib, meloxicam, and etodolac (Chen et al. 2008). Selective inhibition of prostaglandin synthesis in inflammatory or pathological states was able to reduce the occurrence of gastrointestinal adverse effects, while maintaining similar efficacy (Collantes et al. 2002; Shi et al. 2004). However, renal adverse events were not altered, and the incidence of cardiac adverse events was increased in selective COX-2 inhibitors compared to non-selective NSAIDs (reviewed by

Fine 2013). Significant cost of these selective inhibitors may also deter sustained use (Fine 2013). Acetaminophen may act as an analgesic through inhibition of prostaglandin formation through inhibition of the COX-2 enzyme (Graham and Scott 2005), or by preventing reuptake of endocannabinoids (Hama and Sagen 2010; Klinger-gratz et al. 2018). Tolerability of this treatment is high, however over 60 percent of RA patients found that it lacked efficacy (Wolfe et al. 2000).

1.5.1.4 *Experimental rheumatoid arthritis*

Many experimental rodent models of RA are used to replicate the human disease in an *in vivo* setting (Brand 2005; Roy and Ghosh 2013; Choudhary et al. 2018). Popular experimental modes include collagen-induced arthritis (CIA), antigen-induced experimental arthritis (AA), and adjuvant-induced arthritis (AIA) (Brand 2005; Roy and Ghosh 2013; Choudhary et al. 2018).

Freund's complete adjuvant (FCA) is widely used to induce a chronic adjuvant polyarthritis in rodents. Initially described by Pearson (1956), dried, heat-killed *Mycobacterium tuberculosis* suspended in mineral oil is injected intradermally proximal to lymphatic drainage areas (Taurog et al. 1988). Exposure to this foreign bacteria and oil mixture induces the production of cytokines and chemokines such as TNF-alpha, IL-12, and IL-6 (Billiau and Matthys 2001).

Systemic inoculation with the FCA emulsion and resulting immune activation produces a robust acute inflammatory response which gives way to a chronic inflammation within 9-14 days (Taurog et al. 1988; Bendele 2001). Increased joint temperature, increased joint circumference/paw volume (oedema), functional weight bearing limitations and joint/tendon tenderness are observed compared to control groups (Pearson 1956; Bendele 2001; Snekhalatha et al. 2013). Radiographic changes, as observed in RA, such as joint space narrowing and bone damage are evident within 21 days (Taurog et al. 1988; Butler et al. 1992; Bendele 2001; Snekhalatha et al. 2013). Histological changes including synovitis, enlarged synovial layer, immune-cell infiltration, granuloma formation and pannus formation are also characteristic of this model (Bendele 2001).

Systemic delivery of FCA has since been modified to a local injection proximal to the joint (Grubb et al. 1991; Donaldson et al. 1993) or intra-articularly (Butler et al. 1992) to address concerns regarding systemic effects in the model (Butler et al. 1992; Chillingworth and Donaldson 2003). Tibiotarsal or knee joints are frequently used to investigate FCA-induced monoarthritis (Donaldson et al. 1993; Mcdougall et al. 1995). FCA has also been associated with ipsilateral joint destruction, pannus formation and increased production of algogenic mediators such as SP and CGRP in the dorsal root ganglia (Butler et al. 1992; Donaldson et al. 1993; Schaible et al. 2002) and in the spinal cord (Hammell et al. 2016). Although less severe than systemic polyarthritis, these pathologies allow for comparisons between treated and non-treated joints and investigation of clinically relevant disease manifestations in an ethical manner (Chillingworth and Donaldson 2003).

1.6   Overview of cannabis

1.6.1   Plant origin and historic medical cannabis use

Evidence of *Cannabis sativa* pollen has been traced back to over 19.6 million years ago (McPartland et al. 2019). While cannabis cultivation is now widespread (Small 2015), cannabis originated in central Asia near the Tibetan Plateau in the Himalaya mountains at Ningxia in China (McPartland et al. 2019). Considered one of the oldest cash crops (Clarke and Watson 2007), *Cannabis sativa* provides a plethora of important products including fiber in clothing, human and livestock food consumption, recreational narcotics and medical remedies (Clarke and Watson 2007). South-Asian cultivated plants are typically high in psychoactive compounds and sought after for recreational purposes, whereas the European based cannabis chemovars are characteristically used for textile purposes (Clarke and Watson 2007).

Sexually dioecious, female plants are known to produce resin-based compounds from their glandular trichomes (Small 2015). Male varieties lack glandular trichomes but contribute to cultivation through pollination (Small 2015). As of 2014, over 545 chemical constituents have been identified within the cannabis plant (El Sohly and Gul 2014).

The earliest known descriptions of medical use occurred in 2700 BC in the Pents'au ching in China (Zuardi 2006). During this time, cannabis was indicated for

rheumatic conditions, gastrointestinal disturbances, and reproductive disorders (reviewed by Zuardi 2006). The spread to India led to cannabis-related medical use as an analgesic, anti-convulsant, anaesthetic, among other indications (Mikuriya 1969; Zuardi 2006). Introduction into Western societies wasn't documented until the 19th century (Hand et al. 2016). Experiments by William O'Shaughnessy demonstrated efficacy in treating both rheumatic and respiratory illnesses (Hand et al. 2016). Despite its therapeutic promise, cannabis was made illegal by the Canadian government in 1923 (Act to Prohibit the Improper use of Opium and Other Drugs 1923). In 2001, access to the plant for clinical use was granted under the 'Access to Cannabis for Medical Purposes' regulations (Controlled Substances Act 2001). These regulations were repealed when recreational use of cannabis was legalized in 2018 under the Cannabis Act (Bill C-45 2018).

1.6.2   Cannabis use in arthritis

The Canadian Arthritis Society (Arthritis Society 2019) reports that 65% of prescribed medical cannabis users in Canada are, in fact, arthritis patients. Medical cannabis use was also noted in a UK arthritis patient cohort, which made up 21 percent of medical use (Ware et al. 2005). Relief of chronic pain is typically the predominant reason for the use of medical cannabis (Ware et al. 2005). Anecdotally, arthritis patients experience improved joint function, sleep and reduced joint pain when using cannabis (Blake et al. 2006). Ware et al. (2005) report that 68 percent of patients they interviewed reported that cannabis ameliorated their symptoms. Studies report that medical cannabis can act as a substitute for other therapeutics; one study reported a 64 percent reduction in opioid use while NSAIDs consumption also dramatically declined (Boehnke et al. 2016; Baron et al. 2018).

Although cannabis shows efficacy in alleviating the pain of arthritic conditions (Baron et al. 2018), adverse side-effects are also observed (Volkow et al. 2014). For example, cannabis users self-reported experiencing panic attacks, instances of psychosis, and adverse physical symptoms (Thomas 1996). Acutely, cannabis can impair reasoning (Morrison et al. 2009), affect recall memory and reduce cognitive performance in non-chronic users (Morrison et al. 2009). Hart et al. (2001) noted increased decision making time. Euphoria is yet another effect reported by cannabis users, accompanied by an

increased heart rate (Hart et al. 2001). Long-term effects also occur and may include bronchitis and lung-infections (Volkow et al. 2014). Cannabis-induced neuronal damage in memory predominant brain areas may be associated with adverse memory effects of long-term use (Zalesky et al. 2012). Patton el al. (2002) also described a two-fold increase in the development of depression and anxiety with moderate cannabis use. Impaired learning, retention or recall of information were also reported (Hart et al. 2001). Dependence and addiction are also a significant concern in long-term cannabis users although further research is required in this area (Thomas 1996).

1.7   Endocannabinoid system

The endocannabinoid system is an innate, widespread physiological system in the human body, and other vertebrate species (Elphick and Egertová 2001), involved in the maintenance of homeostasis and physical or mental well-being (Battista et al. 2012). Labelled as the "eat, sleep, forget and protect" system by Di Marzo et al. (1998), the endocannabinoid system has a profound role in mood, sleeping, appetite, temperature control, cognition, immune function, motor coordination, and reproduction as reviewed by Clarke and Watson (Clarke and Watson 2007). Three integral components of the endocannabinoid system include the cannabinoid receptors, endogenous cannabinoid molecules, and enzymes responsible for the synthesis and degradation of these compounds (Battista et al. 2012; Lu and Mackie 2016).

1.7.1   Receptors and signaling transduction

Cannabinoid receptors are necessary for ligands to exert their influence and to enable the widespread physiological functions of the endocannabinoid system (Lu and Mackie 2016). Matsuda et al. (1990) identified the first cannabinoid receptor (CB_1R) in 1990. The current literature suggests that CB_1R is primarily located in the central nervous system (Herkenham et al. 1990). Discovery of the receptor in peripheral areas, including sensory afferents and immune cells, has more recently been reported (Bouaboula et al. 1993; Richardson et al. 1998). Unlike the CB_1R, the cannabinoid-2 (CB_2R) discovered shortly afterwards by Munro et al. (1993) is acknowledged as a peripherally-located receptor (Howlett 2002). CB_2R is largely localized on the cell membranes of immune

cells or immunogenic organs (Howlett et al. 2002) but can also be found on afferent nerve terminals (Griffin et al. 1997; Schuelert and McDougall 2008) and in the central nervous system (Gong et al. 2006). Both of these receptors are G-protein coupled receptors (GPCR) consisting of seven transmembrane spanning domains (Howlett 2002). They also exhibit pertussin toxin sensitivity, which infers $G_{i/o}$ protein coupling (Howlett et al. 1986). Heightened expression of CB_1R and CB_2Rs has been detected in the synovium of RA patients compared to OA patients suggesting a role of the endocannabinoid system in inflammatory joint disease (Richardson et al. 2008). A third associated orphan receptor, GPR55, has been identified (Sawzdargo et al. 1999), but its role as a cannabinoid receptor is still controversial.

Activation of the CB_1R and CB_2R with agonist ligands initiates an inhibitory cascade by inhibition of adenylyl cyclase to attenuate cyclic adenosine monophosphate (cAMP) production thereby preventing the phosphorylation of protein kinase A (Matsuda et al. 1990). This agonism leads to the inhibition of voltage-gated calcium channels, preventing the positively charged influx of calcium (Guo 2004). Activation of distal cannabinoid receptors and pre-junctional receptors on afferent nerve terminals (Howlett 2002) results in hyperpolarization of the pre-synaptic terminal, preventing the release of neurotransmitters capable of transmitting neural signals (Matsuda et al. 1990; Howlett 2002). This can also lead to the activation of mitogen-activated protein kinase (MAPK) pathways resulting in the down-regulation of genes and reduced production of cytokines, adhesion molecules and other pro-inflammatory mediators (Wartmann et al. 1995; Kobayashi et al. 2001).

Coined the ionotropic cannabinoid receptors (Akopian et al. 2008), the transient receptor potential family of cation channels has a significant contribution to endocannabinoid-mediated processes (Caterina et al. 1997). Found predominantly in the periphery and specifically on afferent nerve terminals (Cho and Valtschanoff 2008), these ligand-gated cation channels are known for their pro-inflammatory, nociceptive and temperature sensing actions (Caterina et al. 1997). Specifically, cannabinoids have been shown to bind to the transient receptor potential vanillioid-1 cation channel (TRPV1) (Ahluwalia et al. 2003). TRPV1 overstimulation can also produce anti-nociceptive effects through receptor desensitization and algogenic neuropeptide depletion (Ahluwalia et al.

2003; Ruparel et al. 2011). Engel et al. (2011) demonstrated that TRPV1 channels were prevalent in the synovial fibroblasts of RA patients and pre-clinical evidence indicates the presence of TRPV1-positive nerve fibres in the knee joint of mice (Cho and Valtschanoff 2008).

1.7.2 *Endocannabinoids; Metabolic pathways of the endocannabinoids*

The existence of humanized cannabinoid receptors inferred the evolutionary production of endogenous ligands (Zou and Kumar 2018). Several lipid-based molecules possess the ability to interact with conventional and atypical cannabinoid receptors and have been termed endocannabinoids (Lu and Mackie 2016). Seven endocannabinoids have so far been identified both peripherally and centrally: arachidonylethanolamine (AEA or anandamide), 2-arachidonoylglycerol (2-AG), virodhamine, noladin ether, N-arachidonoyldopamine (NADA), palmitoylethanolamine (PEA) and N-oleoylethanolamine (NEA) as described by Battista et al. (2012). Of these, AEA, discovered by Devane et al. (1992) and 2-AG, identified by Mechoulam et al. (1995) are the principle endocannabinoids that have been researched the most (Battista et al. 2012).

An important feature of endocannabinoids is their characteristic on-demand synthesis (Figure 1.5), rather than vesicle storage (Di Marzo et al. 1994). Activation of a membrane bound receptor, prompts the conversion of phospholipid precursors into specific endocannabinoids (Lu and Mackie 2016). Described by Devane et al. (1992), the synthesis of anandamide involves the conversion of phosphatidylethanolamine to N-arachidonoyl phosphatidylethanolamine by N-acyltransferase followed by conversion with N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) (Di Marzo et al. 1994; Di Marzo 2008). Anandamide is degraded by the fatty acid amide hydrolase (Di Marzo 2008). Mechoulam et al. (1995) subsequently discovered 2-AG in 1995. The intermediate molecule diacylglycerol (DAG) is synthesized by converting phosphatidylinositol 4,5-biphosphate (PIP_2) with phospholipase C (PLC) (Di Marzo et al. 1994; Di Marzo 2008). 2-AG is formed when diacylglyerollipase (DAGL) acts on DAG to reach its final endocannabinoid state (Di Marzo 2008). Monoacylglycerollipase (MAGL) is responsible for the inherent breakdown of 2-AG (Di Marzo 2008). COX2 enzymes are yet another degradative mechanism (Di Marzo 2008). Investigation

regarding the transport and reuptake of these molecules is unclear and research to identify these mechanisms is on-going (Zou and Kumar 2018).
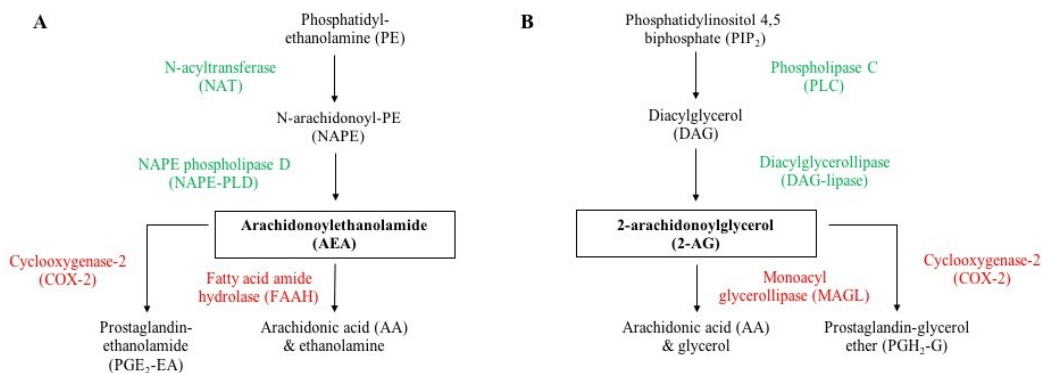
Figure 1.5 Arachidonoylethanolamide or anandamide (A) and 2-arachidonoylglycerol (B) biosynthesis and degradation pathways. Enzymes responsible for synthesis are listed in green; while degradative enzymes are in red. Adapted from: Di Marzo (2008).

1.8   Cannabis compounds

1.8.1   Synthetic compounds

Due to the promising research being conducted with phytocannabinoids, synthetic cannabinoids have emerged as a new stream of research (Zou and Kumar 2018). These compounds are created to structurally mimic the phytocannabinoids or to possess improved cannabinoid receptor binding affinity (Zou and Kumar 2018). Selective CB_1R agents with high binding affinity include R-(+)-methandamide, arachidonoyl-2'-chlorethylamine (ACEA), and arachidonylcyclopropylamide (Lin et al. 1998; Hillard et al. 1999). In addition, CB_2R selective compounds also exist and include JWH 133, JWH 015 and HU308 (Hanus et al. 1999; Huffman et al. 2003). Non-selective synthetic derivatives are also available. Most commonly investigated have been CP 55 940, HU210 (synthetic delta-9 tetrahydrocannabinol ligand) and WIN-55-212 (Mechoulam et al. 1988). These compounds have the ability to interact with both of the cannabinoid receptors, but may show preferred affinity to one (Mechoulam et al. 1988). Although created with pharmacological therapeutic intent, those with CB_1R binding affinity have

been used illegally under the names 'K2', 'spice' or 'black mamba' (reviewed by Hourani and Alexander 2018).

Synthetic inhibitors of cannabinoid receptors have also been designed. Pertwee et al. (1995) was the first to demonstrate that AM630 (6-iodopravadoline) could be used as an antagonist for the CB_2R. In the study, AM630 competitively antagonized non-selective cannabinoid compounds like CP 55-940 and WIN-55-212 in the mouse vas deferens (Pertwee et al. 1995). Using CB_2 transfected cells, further studies demonstrated that AM630 could prevent CP 55-940 activation and $[^{35}S]$-GTPγS binding to the CB_2R and was determined to be an inverse agonist of the CB_2R (Ross et al. 1999). N-(morpholin-4-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM281) was developed as an improvement to the CB_1R antagonist $[^{123}I]$-(pperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) (Lan et al. 1999). The binding affinity K_i value for AM281 binding to CB_1R was 12nM (Lan et al. 1999) and effectively inhibited the acetylcholine releasing actions of WIN-55-212 in the rat hippocampus (Gifford et al. 1997).

1.8.2   Phytocannabinoids

Phytocannabinoids are naturally-occurring cannabis-derived molecules synthesized in the glandular trichomes of the cannabis plant (Clarke and Watson 2007; Russo 2011). Contributing to 30 percent of the dried weight of the flower tops, phytocannabinoids originate from the compound geraniol (Clarke and Watson 2007). The most recent tally speculates that over 120 phytocannabinoids have been identified (Morales et al. 2017). These compounds are hydrocarbon based (C_{21} structured) and combined with oxygen molecules (Morales et al. 2017). While they are known to interact with the endogenous cannabinoid receptors (Gertsch et al. 2010), their binding affinity is not limited to the endocannabinoid system (Russo 2011).

The most abundant and perhaps the most well-known phytocannabinoid is delta-9 tetrahydrocannabinol (THC) which is notorious for its psychoactive properties (Russo 2011). Other abundant phytocannabinoids include: delta-8 tetrahydrocannabinol, cannabinol, cannabigerol, cannabichromene, cannabidiol, tetrahydrocannabivarin, cannabivarin and cannabidivarin (Morales et al. 2017). Beyond recreational use for

purely euphoric experiences, these compounds are now contributing to novel medical therapies (Morales et al. 2017; Russo and Marcu 2017).

1.8.2.1 *Cannabidiol*

Cannabidiol (CBD), chemically identified as 2-[1R-3-methyl-6R-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol, is a naturally occurring phytocannabinoid (Russo 2011). Discovered by Mechoulam and Shvo (1963), CBD has a chemical structure of $C_{21}H_{30}O_2$ and a molecular weight of 314.469g/mol (World Health Organization 2017). CBD is naturally produced in the glandular trichomes of the cannabis plant (Clarke and Watson 2007) and can be found in many chemovars of cannabis (Russo and Marcu 2017). Production of CBD originates from cannabigerolic-acid, which is transformed to cannabidiolic-acid by cannabidiolic-acid synthase (Taura et al. 2007). Cannabidiolic-acid produces the final product of CBD when exposed to heat or light through decarboxylation (Taura et al. 2007). The crystalline solid is insoluble in water but can be dissolved in dimethyl sulfoxide (DMSO) and ethanol (World Health Organization 2017).

One of the most appealing attributes of CBD is its inherent non-euphoric properties (Consroe et al. 1979). Consroe et al. (1979) showed that cohorts receiving just oral CBD had no psychomotor deficits and were timely in a production based timed test. These participants also reported feeling 'sober', 'not drugged' and 'alert' compared to control (Consroe et al. 1979). Supporting this claim, participants receiving CBD cigarettes reported feeling no "high" sensations (Haney et al. 2016). The non-euphoric attributes of CBD relative to THC may be in part due to receptor mechanisms. For example, CBD is reported to have little to no affinity for the orthosteric site of CB_1Rs, those responsible for centrally-mediated psychoactive effects (reviewed Pertwee 2008). Other studies have demonstrated that CBD acts as an allosteric modulator of the CB_1R, thus diminishing the effects of CB_1R agonists like THC and 2-AG (Laprairie et al. 2015). CB_2R affinity is also relatively low (reviewed Pertwee 2008). However, CBD has been demonstrated to act as an agonist at TRPV1 channels (Bisogno et al. 2001; Costa et al. 2004; Philpott et al. 2017), which may lend to its anti-inflammatory properties explained

later. Russo et al. (2005) also outlined that CBD may activate 5-HT serotonergic receptors, or may activate GPR55 (Ryberg et al. 2007).

Metabolism of CBD occurs mainly in the liver by human liver microsomes (Jiang et al. 2011). Although eight mono-hydroxylated metabolites have been identified (6α-OH-, 6β-OH-, 7-OH-, 1"-OH-, 2"-OH-, 3"-OH-, 4"-OH- and 5"-OH-), hydroxylation to 6α-OH-, 6β-OH-, and 7-OH-CBDs are most prominent (Jiang et al. 2011). Jiang et al. (2011) demonstrated that cytochromes, CYP3A4 and CYP2C19 were the enzymes responsible for the metabolism of CBD into the commonly described metabolites.

1.8.2.1.1 CBD in pain and inflammation

Pre-clinical literature has demonstrated the efficacy of CBD in treating neuropathic pain (De Gregorio et al. 2019) and cancer pain (Ward et al. 2014). These actions are thought to be mediated by TRPV1 receptors and 5-HT$_A$ receptors respectively (Ward et al. 2014; De Gregorio et al. 2019). Clinically, CBD has shown efficacy in reducing peripheral neuropathic pain (Serpell et al. 2014) and a THC:CBD combination reduced pain associated with multiple sclerosis (Russo and Guy 2006). As a result, Sativex® (1:1 THC-CBD ratio), indicated for multiple sclerosis pain, is a federally approved therapeutic with CBD (Pertwee 2009). The effect of CBD on inflammation in preclinical models has also been thoroughly reviewed and is suggested to be mediated by CB$_2$R, TRPV1 channels or adenosine receptors (Burstein 2015).

1.8.2.1.2 CBD in arthritis (pre-clinical/clinical)

Pre-clinically, CBD has been effective in alleviating symptoms associated with arthritic conditions (Malfait et al. 2000; Costa et al. 2004; Costa et al. 2007; Hammell et al. 2016; Philpott et al. 2017). In the monoiodoacetate (MIA) model of OA in rats, CBD (300μg) administered locally over the arthritic joint was able to reduce mechanical allodynia (acutely and prophylactically) and improve weight bearing on the injured limb (Philpott et al. 2017). CBD was also able to reduce model-induced neuropathy and dose-dependently reduced noxious firing in afferent fibers (Philpott et al. 2017). CBD also reduced inflammation by lowering both rolling and adherent leukocytes, but not blood flow to the affected area (Philpott et al. 2017). TRPV1 antagonists attenuated the

improvements in mechanical allodynia and partially inhibited the reductions of leukocyte trafficking. AM630, the CB_2R antagonist, also prevented leukocyte trafficking inhibition (Philpott et al. 2017).

CBD has also been employed in various experimental RA models (Malfait et al. 2000; Costa et al. 2004; Costa et al. 2007; Hammell et al. 2016). In the mouse CIA model, oral and intraperitoneal administration of CBD was able to improve clinical scoring of joint involvement and reduce levels of IFN-γ and Il-12 at optimal doses of 25mg/kg and 5mg/kg respectively (Malfait et al. 2000). Interestingly, Malfait et al. (2000) demonstrated a bell-shaped dose-response curve. Oral administration of CBD in a carrageenan-induced model of synovitis showed reductions in oedema, attenuation of thermal hyperalgesia, reductions in PGE_2, as well as reduced levels of nitric oxide and COX enzymes in the affected tissue (Costa et al. 2004).

The effects of CBD have been investigated in the FCA model of RA with both transdermal application and oral administration (Costa et al. 2007; Hammell et al. 2016). Orally delivered and transdermal application of CBD were both able to increase latency to paw licking thus improving thermal hyperalgesia (Costa et al. 2007; Hammell et al. 2016). Oral administration also attenuated mechanical hyperalgesia in rats (Costa et al. 2007). Improvements in hyperalgesia were antagonized by TRPV1 receptor antagonists (Costa et al. 2007) coinciding with results by Philpott et al. (2017). In these models, CBD was also able to modulate pro-inflammatory targets. PGE_2 and nitric oxide were reduced by oral administration (Costa et al. 2007). Additionally, CBD prevented synovial enlargement, reduced immune cell infiltration into the synovium, and reduced expression of CGRP in the spinal cord (Hammell et al. 2016). These results are indicative of CBD being an effective analgesic and anti-inflammatory agent in RA, especially in the FCA model(Costa et al. 2007; Hammell et al. 2016).

Clinically approved use of CBD is limited to date, but is gaining acceptance as a viable therapeutic option (Blake et al. 2006). A randomized controlled trial showed that Sativex (1:1 ratio of CBD and THC) could improve disease progression in RA as measured by the Disease Activity Scale (DAS) and McGill pain questionnaire compared to the placebo cohort (Blake et al. 2006). Additionally, a trial investigating the effects of pure CBD in psoriasis and OA is currently ongoing (National Institute of Health 2010).

1.8.3 Terpenes

The terpenes are comprised of basic hydrocarbon units (Singh and Sharma 2015). Following a minimalistic formula of C_5H_8 terpenes are categorized by how many isoprene units they contain (Clarke 2008). A monoterpene consists of two isoprene units ($C_{10}H_{16}$), a sesquiterpene comprises of three ($C_{15}H_{24}$), and a diterpene is made up of four ($C_{20}H_{32}$). Any terpenes thereafter are referred to as poly-terpenes (Clarke 2008). Terpenes can also be classified as aliphatic or cyclic (Clarke 2008). Generally, terpenes are colorless liquid compounds that are insoluble in water (Fichan et al. 1999). They are highly volatile and easily oxidizable (Clarke 2008).

Originally, terpenes were known primarily for their purpose as an ingestible toxin to predators, or a sticky defense mechanism in plants and insect vertebrate species (Gershenzon and Dudareva 2007; Singh and Sharma 2015). They are also found in essential oils and are used cosmetically, as remedies, in fragrances, and as flavoring agents (Singh and Sharma 2015). In cannabis, the terpenes are synthesized in the glandular trichomes of the plant and are responsible for the pungent, yet unique odors of the various chemovars (Singh and Sharma 2015). Over 200 terpenes have been identified to date within the cannabis plant (Russo and Marcu 2017). The cannabis-derived monoterpenes include: limonene, alpha or beta-pinene, myrcene and linalool (Russo and Marcu 2017). Commonly referenced sesquiterpenes within the cannabis plant are beta-caryophyllene, nerolidol, and humulene (Russo and Marcu 2017). In addition to their aromatic benefit (Singh and Sharma 2015), Russo et al. (2017) reviews that they have several medical advantages and may be helpful in treating cancer, neurological disorders, chronic inflammatory conditions and chronic pain.

1.8.3.1 Myrcene

Myrcene, or more specifically 7-Methyl-3-methylene-1,6-octadiene, is an acyclic monoterpene ($C_{10}H_{16}$) with a molecular weight of 136.24g/mol (National Institute of Health 2010). This colourless liquid is highly volatile and has a high boiling point of 167 ºC (Behr and Johnen 2009). Myrcene has two isotypes: beta-myrcene and alpha-myrcene as illustrated in Figure 1.6 (Behr and Johnen 2009). Ruzicka and Stoll discovered in 1924 that beta-myrcene was the naturally occurring isotype (myrcene will henceforward be

used to refer to the naturally occurring beta-myrcene isotype) (Behr and Johnen 2009). Myrcene can be extracted or steam distilled from essential oils (Behr and Johnen 2009) but can also be synthesized directly by pyrolyzing the monoterpene beta-pinene (from turpentine) at temperatures exceeding 450 ºC (Behr and Johnen 2009). Through various chemical modifications, myrcene can be converted to other terpene products and pheromones as described by Behr & Johnen (2009).

Limited information was available concerning myrcene's metabolic and pharmacological properties. A study by the National Institute of Health in 2010 described that myrcene was preferentially metabolized into 10-hydroxylinalool (Madyastha and Srivatsan 1987). Elevated levels of CYP2B suggest that myrcene is broken down by this inducible liver enzyme (Freitas et al. 1993; NIH, 2010). Further investigation showed 10-hydroxylinalool, 7-methyl-3-methyele-oct-6-ene-1,2-diol, 1-hydroxymethyl-4-isopropenyl cyclohexanol, 10-carboxylinalool and 2-hydrxy-7-methyl-3-methyelene-oct-6-enoic acid metabolites in the urine of rats treated with myrcene (National Institute of Health 2010). The elimination half-life of myrcene has been reported as 285 minutes (roughly 4.75 hours) in one study investigating post-natal effects of myrcene (Delgado et al. 1993). As with most terpenes, myrcene is insoluble in water (Weidenhamer et al. 1993; Fichan et al. 1999), but soluble in alcohol or ethers (Behr and Johnen 2009).

Myrcene generates a woody, turpentine, musty odor with hints of fruits (Behr and Johnen 2009). It can be found in many essential oils derived from natural products like lemongrass, hops, bay leaves, and thyme (Behr and Johnen 2009). Its natural derivation and aromatic characteristics lend itself to the common use in fragrances, cosmetics, flavoring, and essential oil remedies (Behr and Johnen 2009). Myrcene is also the most abundant monoterpene found within cannabis (Ross and Elsohly 1996; Booth et al. 2017), and is present in many chemovars (Baron et al. 2018). Of the three most prevalent cannabis chemovars in an arthritic patient cohort (OG Shark, Cannatonic, and Sweet Skunk CBD), myrcene was the most abundant monoterpene (Baron et al. 2018).

The mechanism of action of myrcene is controversial and has long been debated. An initial study by Da Silva et al. (1991) demonstrated that exploratory behaviour was not hindered by myrcene, nor did it contribute to or cause anxiolytic properties (Da Silva et al. 1991). However, a separate study found that intraperitoneal administration of

myrcene (100-200mg/kg) decreased locomotor behaviours such as the numbers of quadrant crosses, number of rearing events and incidence of grooming (Do Vale et al. 2002). The same doses also reduced time spent on the rotarod which measures neuromuscular coordination and showed increased sleeping time in a pentobarbital-induced sleeping test (Do Vale et al. 2002). These sedative effects were not noted at the lower dose of 50mg/kg (Do Vale et al. 2002). At 25mg/kg, entries into the open arms in the elevated plus maze, which tests exploratory/anxiolytic behaviour, were reduced compared to controls, but time spent in the open arms was not altered (Do Vale et al. 2002). The sedative properties of myrcene, as demonstrated by this study, determined it was responsible for the sedative, or more colloquially defined, 'couch lock' properties (Russo and Marcu 2017). These varying results could be attributed to either the differences in doses or the route of administration.

Although having been deemed safe by the American Food and Drug Administration, the European Council, and the Flavor Extract Manufacturers' Association in 1965 (Behr and Johnen 2009), myrcene may have toxicological effects. Several studies proposed that myrcene may be linked to nephrosis in the kidneys and increased incidence of renal tumors in male rats (National Institute of Health 2010; Cesta et al. 2013). In mice, hepatotoxicity was demonstrated and was accompanied with increased incidences of adenomas and carcinomas (National Institute of Health 2010). These events occurred significantly less in females of both species (National Institute of Health 2010). Lethal oral doses were noted at 1g/kg in the 2 year repeated administration study and 2mg/kg in the 3 month repeated administration study (National Institute of Health 2010). The lethal oral dose for a single exposure was 11.39g/kg in rats and 5.06g/kg in mice (Paumgartten et al. 1990). A single exposure lethal dose of 5g/kg in rats and 2.25g/kg when injected into the peritoneal cavity in mice was demonstrated (Paumgartten et al. 1990). Single exposure toxicity symptoms associated with higher doses included piloerection, rapid breathing, nasal bleeding, hypoactivity, loss of righting, ataxia, and hypertonus of the stomach muscles (Paumgartten et al. 1990). Paumgartten et al. (1998) also reported that myrcene did not affect incidences of female mating patterns or female pregnancy. Despite slight elevations in kidney and liver weights, no other maternal or fetal toxicities were noted (Paumgartten et al. 1998). No

genotoxicity was noted from myrcene administration (National Institute of Health 2010). Critiques of these studies suggest that these doses are not proportionate to the relative exposure humans would encounter in naturally derived products (Tisserand and Yong 2014).

1.8.3.1.1 The analgesic properties of myrcene

Essential oils containing a moderate amount of myrcene have demonstrated analgesic effects. The essential oils of *Eremanthus erythropappus* (10.03% myrcene) and *Bougatnvella glabra* (4.4% myrcene), administered orally, were both able to increase reaction time to a noxious thermal stimulus in mice and rats (Sousa et al. 2008; Ogunwande et al. 2019). *Bougatnvella glabra* was more effective than the standard control aspirin at 100mg/kg (Ogunwande et al. 2019). *Eremanthus erythropappus* also demonstrated the ability to reduce paw licking in mice in the formalin test and dose-dependently inhibited writhing by 10-27 percent in the acetic acid chemonociception test (Sousa et al. 2008). Essential oil derived from *Teucrium stocksianum* (8.64% myrcene), administered into the peritoneal cavity, displayed similar writhing inhibition in mice (Shah et al. 2012). Additionally, the essential oil derived from *Chamaecypris obtusa* (26.4% myrcene), administered intra-articularly 4.5 hours after induction of carrageenan, an experimental model of inflammatory arthritis, was able to improve weight bearing on the ipsilateral limb (Suh et al. 2016). These improvements to weight bearing were comparable with the NSAID indomethacin (Suh et al. 2016). The promising analgesic effects exhibited by treatment with these essential oils cannot solely be attributed to myrcene, as it is only one of the many terpene components within the essential oils that may be individually or synergistically contributing to the exhibited analgesia.

Few studies have examined the analgesic effects of isolated pure myrcene. The first, performed by Rao et al. in (1990), investigated the anti-nociceptive effects of myrcene isolated from *Cymbopogon citratus* in mice. Myrcene dose dependently (10mg/kg or 20mg/kg; i.p.) increased reaction time to paw licking in the hot plate test (Rao et al. 1990). The antinociceptive effects in this test were observed for 2 hours and were found to be less efficacious compared to the standard opioid analgesic morphine (Rao et al. 1990). When administered subcutaneously (20mg/kg or 40mg/kg), myrcene

treated animals had fewer instances of writhing in the acetic acid test (Rao et al. 1990). The authors demonstrated that both naloxone, a non-selective opioid receptor antagonist, and yohimbine, an alpha-2 adrenergic receptor antagonist, partially inhibited both the improvement in reaction time and reduction of acetic-acid induced writhes (Rao et al. 1990). Based on these findings, Rao et al. (1990) concluded that myrcene may be exerting its analgesic effects by inhibiting the pre-synaptic alpha-2 adrenergic receptor, and subsequently inhibiting endogenous opioid release as described by Pettibone and Mueller (1981).

Similarly, Lorenzetti et al. (1991) investigated the peripheral acting mechanism of myrcene isolated from *Cymbopogon citratus*. Oral administration of lemon grass oil (15-120mg/kg) and isolated myrcene (5-405mg/kg) to rat cohorts was able to attenuate hyperalgesia induced by carrageenan and PGE_2, but not dibutyryl-cAMP (Lorenzetti et al. 1991). Both preparations were also able to reduce the number of writhes induced by peritoneal injection of acetic acid (Lorenzetti et al. 1991). Unlike the previous study, oral administration of myrcene did not significantly improve reaction time in mice using the hot-plate test (Lorenzetti et al. 1991). Interestingly, Lorenzetti et al. (1991) demonstrate that the same dose of myrcene administered for five consecutive days was able to maintain the same level of analgesia whereas consecutive administration of morphine did not. No mechanistic investigations were reported. In summary, this study showed that myrcene shows similar analgesic patterns to its derivative essential oil and that its analgesic effects occurred without tolerance (Lorenzetti et al. 1991).

A more recent study confirmed the previous antinociceptive findings of myrcene in mice using myrcene isolated from *Ocimum gratissimum* (Paula-Freire et al. 2013). Myrcene (5,10mg/kg; administered orally) was able to increase the time it took for paw-licking to occur in the hot plate test and this was sustained until the last testing point at four hours (Paula-Freire et al. 2013). Similar to Rao et al. (1990), naloxone was able to attenuate the myrcene-induced improvement in reaction time suggesting mu-opioid receptor involvement (Paula-Freire et al. 2013). Licking latency was also increased in both the first and second phase of the formalin test at the highest dose of myrcene (10mg/kg) (Paula-Freire et al. 2013). Morphine demonstrated enhanced analgesic effects in the hotplate test, except at the four hour time point where the analgesic effects had

subsided and the effects were less pronounced in the first phase of the formalin test (Paula-Freire et al. 2013). Uniquely, Paula-Freire et al. (2013) found no effects of a higher dose of myrcene (80mg/kg) on motor coordination with the rotarod test, but noted an increase in locomotion and climbing. Slight reductions in ambulation and defecation were noted in toxicology studies at a dose of 100mg/kg (Paula-Freire et al. 2013). In conclusion, these findings suggest that myrcene is an effective analgesic in acute pain models without substantial toxicology or impairments to locomotion (Paula-Freire et al. 2013).

1.8.3.1.2   The anti-inflammatory properties of myrcene

Plant-derived essential oil products containing myrcene have also demonstrated anti-inflammatory properties. *Eremanthus erythropappus* (10.03% myrcene) and *Bougainvillea glabra* (4.4% myrcene) both reduced carrageenan-induced paw oedema in rats when administered orally (Sousa et al. 2008; Ogunwande et al. 2019). Despite improving paw oedema, the preparation from *Bougatnvella glabra* was inferior to a high-dose of the NSAID diclofenac at 100mg/kg (Ogunwande et al. 2019). Leukocyte trafficking was also altered by essential oils derived from *Eremanthus erythropappus* and *Zingiber officinale* Roscoe (14% myrcene). Sousa et al. (2008) showed that oil from *Eremanthus erythropappus* could reduce the number of leukocytes present in a pleural exudate in a carrageenan-induced model of pleurisy. Ginger oil extracts (*Zingiber officinale*) showed reductions in multiple aspects of leukocyte kinetics (Nogueira De Melo et al. 2011). Rolling, adherent, and transmigrated leukocytes in the vasculature and surrounding scrotal chamber were all reduced when given the essential oil preparation 4 hours after carrageenan-induced inflammation (Nogueira De Melo et al. 2011).

Porophyllum ruderale, a myrcene-based essential oil from the Asteraceae species, also prevented leukocyte migration and was also able to inhibit RA-relevant inflammatory mediators (Souza et al. 2003). Oral treatment of oil from *Porophyllum ruderale* ultimately inhibited production of IFN-γ, but also inhibited the anti-inflammatory cytokine IL-4 in a lipopolysaccharide pleurisy model (Souza et al. 2003). Similarly, synovial tissue levels of IL-1β, IL-6, TNFα and COX-2 were decreased as early as 2 hours post-treatment with *Chamaecypans obtusa* in rats with carrageenan-

induced inflammation (Suh et al. 2016). Reductions in the same inflammatory mediators (except TNFα) were observed in meniscal tissue (Suh et al. 2016). Definitive conclusions surrounding anti-inflammatory properties of myrcene are difficult to ascertain due to the chemical diversity of the essential oils and the unreported concentration of myrcene.

Only one study has examined the anti-inflammatory effects of pure myrcene. Using both human chondrocytes from donors undergoing knee arthroplasty and a human chondrocyte cell line (C-28/I2), the authors demonstrated that myrcene could inhibit IL-1β-induced nitric oxide production and inducible nitric oxide synthase mRNA levels (Rufino et al. 2015). Additionally, myrcene attenuated NF-κB activation by inhibiting kinase phosphorylation (Rufino et al. 2015). Rufino et al. (2015) also showed that myrcene could inhibit extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, p38 phosphorylation and c-Jun N-terminal kinase (JNK) phosphorylation suggesting the inhibition of all three MAPK inflammatory pathways. Reduced levels of MMPs and increased levels of their tissue inhibitors (TIMPs) were observed (Rufino et al. 2015), further substantiating the anti-inflammatory and potentially chondroprotective properties of myrcene. These findings suggest that myrcene may be a valuable component in preventing inflammation by attenuating activation of arthritic inflammatory pathways and by inhibition of cartilage degrading mediators (Rufino et al. 2015).

1.9   The entourage effect

Cannabis sativa has a complex chemical profile (El Sohly and Gul 2014). Identification and isolation of specific compounds has enabled research of their individual therapeutic properties, referred to as monotherapy (Bonn-Miller et al. 2018). However, studies demonstrating better efficacy with whole plant extracts, relative to their isolated properties, suggest a synergistic interaction amongst the array of chemical constituents (Ryan et al. 2006; Russo 2019). Synergy is observed when the combination of compounds demonstrates an effect greater than that of each of the compounds individually (Berenbaum 1989). Interestingly, Ryan et al. (2006) showed a heightened calcium response to a 1:1 THC:CBD extract mix compared to the individual compounds alone (Ryan et al. 2006). Ben-Shabat et al. (1998) showed that inactive compounds on their own (2-lino-glycerol and 2-palm-glycerol) could enhance binding of 2-AG, thus

potentiating adenylyl cyclase inhibition. They coined the synergistic effects of the cannabis compounds the 'entourage effect' (Ben-Shabat et al. 1998).

The inter-relationship between THC and CBD has been summarized in the literature (Russo and Guy 2006). CBD was able to attenuate THC-induced adverse events including intoxication, sedation, and other centrally-mediated effects (reviewed by Russo and Guy 2006). Relevant to this study, CBD enhanced the analgesic properties of THC, a known ligand of CB_1R and CB_2R by increasing latency to thermal reactions in the hotplate test (Karniol and Carlini 1973). However, CBD did not potentiate analgesia associated with abdominal contractions in the formalin test (Welburn et al. 1976). Additionally, inactive doses of phytocannabinoids like THC were able to enhance anti-nociception when combined with an active dose of a mu-opioid (Smith et al. 1998). These findings suggest that the combination of more than one cannabis-related compound may demonstrate synergistic interactions or qualitative 'pleiotropic actions' thus increasing therapeutic efficacy (Williamson 2001).

1.10 Measuring pain and inflammation in arthritic rodents

1.10.1 Nociceptive and pain behaviour measurements in rodents

1.10.1.1 Dynamic weight bearing

RA patients use affected joints less frequently due to pain (Lee and Weinblatt 2001). Functional deficits include reduced load bearing of affected joints/limbs and impaired gait (Lee and Weinblatt 2001) Dynamic weight bearing (DWB) measures spontaneous pain, where no stimulus is applied, by allowing for the quantification of weight distribution between a rodent's four limbs (Deuis et al. 2017). The animal is placed in a closed area and allowed unrestricted movement, while pressure sensors on the floor of the enclosure and video recordings allow for the capture of load bearing data (Piel et al. 2014). This method allows for the examination of unilateral nociception as it quantifies weight bearing deficits between ipsilateral and contralateral paws (Quadros et al. 2015; Deuis et al. 2017).

Studies show that inflammatory models posit weight bearing deficits (Griffioen et al. 2015; Quadros et al. 2015). Specifically, FCA-injected mice demonstrated reduced weight bearing on the FCA-injected paw. Similar findings were noted in a CIA and

antigen induced model of RA (Griffioen et al. 2015). Improvement in weight bearing deficits have been demonstrated when using analgesic substances (Quadros et al. 2015). Recovery of the ipsilateral weight bearing deficit was observed when NSAIDs, biologics, and DMARDs were administered (Quadros et al. 2015). These findings suggest that dynamic weight bearing analysis is an effective tool to interpret nociception and pain (Griffioen et al. 2015; Quadros et al. 2015).

1.10.1.2 Locomotor activity

Arthritis patients can experience pain when performing routine daily activities like walking short distances or climbing the stairs (Bombardier et al. 2011). To avoid pain, these activities are curtailed (Lagacé et al. 2010). As a result, locomotor activity has been used as a non-invasive indicator of spontaneous pain in rodent models (Deuis et al. 2017). Locomotor analysis can be performed using an open field setup where the animal is permitted unrestricted movement (Piel et al. 2014). These movements are captured by a camera and/or sensors/lasers placed around the arena providing an unbiased inference of pain (Piel et al. 2014).

Although acute noxious stimuli may increase locomotion due to innate escape reflexes (Chuang and Lin 1994), inflammatory arthritis and its associated disease conditions can reduce locomotor behaviour (Matson et al. 2006). Research shows that inflammatory arthritic conditions reduced general movements (horizontal locomotion) and vertical rearing (Matson et al. 2006). The attenuation of these limitations with the administration of morphine corroborates the use of this methodology as an inferred measurement of pain (Matson et al. 2006). Other locomotor tests in the literature include time spent immobile, distance travelled, grooming, and feeding behaviours (Deuis et al. 2017).

1.10.1.3 Von Frey hair algesiometry

Evoked pain behaviour is another method used to investigate nociceptive pain responses in animal models (Deuis et al. 2017). Unlike the two previously noted spontaneous measures, this technique involves applying a non-noxious stimulus to elicit a withdrawal response, representative of mechanical allodynia (Gregory et al. 2013; Deuis

et al. 2017). One of the most commonly used evoked pain test is von Frey hair algesiometry, invented by Maximilian von Frey (Deuis et al. 2017). While automatic and manual versions exist, manual applications of von Frey hairs are standard (Deuis et al. 2017). Filaments of various bending force apply non-noxious tactile stimuli to the ipsilateral hind paw in a sequential manner (Chaplan et al. 1994). This process is explained further in the methods section. The force required for production of a paw withdrawal in fifty percent of animals is then calculated (Dixon 1980). This test is used to examine the inflammatory referred pain, possibly attributed to central sensitization (Muley et al. 2016).

Von Frey hair algesiometry has been used to demonstrate mechanical allodynia in FCA-injected rats (Liu et al. 2009; Li et al. 2014). Withdrawal threshold was drastically altered in FCA cohorts compared to control (Liu et al. 2009; Li et al. 2014). Analgesics, including morphine, were able to improve withdrawal thresholds in these animals (Li et al. 2014). Von Frey algesiometry has also been used in many neuropathic pain models (Li et al. 2014) and osteoarthritis models (Philpott et al. 2017). This widely used test is efficacious for inferring pain in inflammatory models (Liu et al. 2009; Li et al. 2014; Philpott et al. 2017).

1.10.2 Inflammatory measurements in rodents

1.10.2.1 Intravital microscopy and leukocyte trafficking

Intravital microscopy (IVM) is a form of *in-vivo* microscope imaging that permits the visualization of cellular and biological events like leukocyte trafficking in real time (Weigert et al. 2010; Herr et al. 2015). It is most commonly used for imaging of brain microcirculation, tumor pathology, and the immunology of vasculature within tissue or organs (Masedunskas et al. 2012).

The area of interest is displayed on a fluorescent microscope in an anesthetized animal (Weigert et al. 2010). Efforts to maintain the moist environment and pH of tissues are made, however fully recapitulating biologic conditions is difficult to maintain, this being one disadvantage of the technique (Weigert et al. 2010). Using a single-photon, or a two/three-photon microscope, cellular interactions between leukocytes and the endothelium can be visualized using fluorescent light (Weigert et al. 2010) when labeled

with an exogenous fluorescent probe – rhodamine 6G. With the help of a high resolution camera, these interactions can be video recorded for later analysis (Masedunskas et al. 2012). Typically, one-minute videos are recorded at several (3-4) vessels for each time in question enabling quantification of rolling and adherent leukocytes (Atherton et al. 1972; Herr et al. 2015).

Leukocyte trafficking using intravital microscopy has been used in previous joint arthritis models (Krustev et al. 2017; Philpott et al. 2017) and joint inflammation models (Krustev et al. 2014; Krustev et al. 2015). These studies demonstrated elevated leukocyte-endothelial interactions that could be modified by prospective anti-inflammatory agents (Krustev et al. 2014; Krustev et al. 2015; Muley et al. 2016; Krustev et al. 2017; Philpott et al. 2017). As a result, IVM has therefore been shown to be an efficacious technique for *in vivo* investigation of joint inflammation.

1.10.2.2 Laser speckle contrast analysis

Increased blood flow to an injured/inflamed area is typical during an inflammatory response (Bennett et al. 2018). Laser speckle contrast analysis (LASCA), developed by Briers and Webster in (Briers and Webster 1996), is a technique that extrapolates the velocity of particles moving within a medium (Briers and Webster 1996). With a maximum penetrating depth of 1mm, LASCA can be used to investigate blood flow in the capillary microcirculation networks (Briers 2001). When the laser hits the surface of an object, the light scatters producing an interference pattern or speckle pattern (Briers and Webster 1996; Briers 2001). If the object in question is moving, this speckle pattern constantly fluctuates. During blood flow analysis, light can scatter off exposed capillary walls and red blood corpuscles (Briers and Webster 1996). Using a CCD camera and a frame grabber, images of the varying speckle patterns are captured and transformed into a contrasting false colour map (Briers and Webster 1996). The contrast in speckle pattern intensities relates to the speed of the objects in the illuminated area, allowing for the inference of cellular velocity (Briers 2001).

Using the PeriCam system, blood flow velocity is assigned arbitrary perfusion units for quantification. At a frame capture rate of 25 images per second and a computation time of 0.02 seconds, LASCA is effectively a real time method for

measuring blood flow to an area of interest (Briers 2007). Clinically, LASCA has been used to analyze cutaneous burn injuries, microcirculation injuries, ophthalmological pathologies, and much more (Briers et al. 2013). Pre-clinically, differences in speckle patterns and inferred blood flow have been utilized in the paws of FCA-injected mice suggesting hyperemia at the arthritic site (Son et al. 2014).

1.10.2.3 Joint oedema

As discussed previously, oedema is a cardinal sign of inflammation (Bennett et al. 2018). As such, this parameter is a reproducible, quantifiable occurrence investigated in many inflammatory conditions including OA and RA models (Adães et al. 2014; Tian et al. 2015). Several methodologies are used to asses oedema in rodent models, which depend on the site of inflammation (Sharma et al. 2004). For example, oedema of the paw can be inferred by measuring the total paw volume using a plethysmometer (Sharma et al. 2004). Joint oedema, however, is more practically quantified by measuring articular diameter (Tian et al. 2015). These measurements are typically made with digital calipers (Tian et al. 2015). FCA produces significant increases in both ankle joint and knee joint diameters compared to controls (Liu et al. 2009; Tian et al. 2015; Hammell et al. 2016). Joint diameter or circumference is also a targeted parameter for the testing of anti-inflammatories (Tian et al. 2015; Hammell et al. 2016).

1.10.2.4 Cytokine multiplex analysis

Collection of serum or plasma is a relatively non-invasive way to infer systemic inflammation by measuring pro-inflammatory or anti-inflammatory cytokines (Zhou et al. 2010). Multiplex technology permits analysis of multiple cytokines within a single sample using antibody coated beads (Leng et al. 2008). Cytokines will bind to their respective antibody creating a complex, which binds to a fluorescently-labelled secondary antibody (Leng et al. 2008).

Plasma, a whole-blood sample including fibrinogen and other proteins, is acquired using ethylenediamine tetra acetic acid (EDTA) to prevent clotting (Zhou et al. 2010). Some studies argue that serum detection of cytokines is more sensitive (Gruen et al. 2016), yet other studies argue that sample clotting may alter cytokine levels and that

plasma samples exhibit higher cytokine stability (Thavasu et al. 1992; Scott et al. 1996; Zhou et al. 2010). Cytokine levels were typically constant when comparing plasma versus serum samples (Aziz et al. 1999; Gruen et al. 2016).

In the FCA model of RA in rats, IL-1β, IL-12, IL-2 and TNFα serum cytokines are elevated 19 days post intraarticular FCA-induction (Liu et al. 2009). IL-10, however, showed marked decreases compared to control animals (Liu et al. 2009). Kim et al. (2016) showed significant increases in serum levels of TNF-α, IL-1β and IL-6 ten days post intradermal paw FCA-induction.

1.11 Experimental hypothesis and objectives

As described above, previous studies have shown that myrcene is an effective analgesic in rodent acute pain models by working through cannabinoid, opioid and adrenergic pathways (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013). In an *in vitro* study, myrcene demonstrated anti-inflammatory properties by reducing arthritogenic pro-inflammatory mediator production (Rufino et al. 2015). Additionally, CBD has exhibited analgesic, anti-inflammatory, and disease modifying properties in the FCA and CIA models of RA (Malfait et al. 2000; Costa et al. 2007; Hammell et al. 2016).

The analgesic, anti-inflammatory, and disease-modifying properties of myrcene have not yet been investigated in an *in vivo* model of RA. Nor has its interaction with the endocannabinoid system been tested. Additionally, the synergism of myrcene and CBD has not yet been evaluated in acute or disease models. Based on these understandings, the purpose of this project was to investigate the analgesic, anti-inflammatory and disease modifying potential of acute or chronic administration of myrcene in the FCA model of RA. The involvement of the endocannabinoid system and the synergism of myrcene with CBD were also investigated.

Our hypothesis was that **acute and chronic administration of myrcene would reduce pain and inflammation via the endocannabinoid system in FCA-injected rats.** Secondarily, we hypothesized that a sub-clinical dose of the phytocannabinoid CBD would enhance the analgesic and anti-inflammatory effect of myrcene in FCA-injected rats. To evaluate our hypothesis, we had three main objectives:

Objective 1: to assess the analgesic and anti-inflammatory effect of two acute doses of myrcene administered in FCA-injected rats.

Objective 2: to investigate the effect of repeated doses of myrcene on pain, inflammation and disease progression over a 21-day period in the model.

Objective 3: to characterize the synergistic effects of myrcene and CBD when administered acutely in the FCA rodent model of RA.

Chapter 2: Methods and Materials

2.1 Animals

Male Wistar rats (236-432g pre-induction) from Charles River Laboratories, Senneville, Quebec, Canada were dually housed in ventilated racks at a temperature of 22 ± 2ºC with a 12 hour light:12 hour dark cycle. (lights on 7:00 - lights off 19:00). Cages were lined with sterile wood chips, enviro-dry shavings, and environmental enrichments. Animals had unrestricted access to standard lab chow and water. The animals were allowed to acclimate to the facility for one week prior to experimentation. All animal ethics protocols (15-117, 17-114, 16-077, 18-089) were compliant with Dalhousie's University Committee on Laboratory Animals (UCLA) and adhered to the Canadian Council of Animal Care (CCAC) guidelines.

2.2 Experimental rheumatoid arthritis

Freund's complete adjuvant (Sigma F5881) was used to chemically induce rheumatoid monoarthritis in the right knee joint. Animals were deeply anesthetized (2% isoflurane, 100% O_2; 1L/min) until the flexor-withdrawal reflex was absent. Prior to induction, the knee-joint was shaved, swabbed with sterile alcohol (70% isopropyl alcohol; Fisher Scientific Company, Ottawa, Ontario, Canada). With the knee-joint gently flexed, a 30-guage needle attached to a 1ml syringe was inserted anteriorly between the femoral head and tibial plateau into the joint space. Twenty-five microliters of Freund's complete adjuvant was injected into the posterior region of the joint before the needle was then withdrawn slightly until resting in the anterior chamber, at which time another 25μl was administered. The knee-joint was then flexed and extended for ten seconds to distribute the Freund's complete adjuvant throughout the joint space.

2.3 Pain measurements

2.3.1 Von Frey hair algesiometry

Mechanical allodynia was assessed using von Frey hair algesiometry (adapted from Chaplan et al. 1994). Animals were acclimated to a quiet testing room for at least

one hour prior to assessment. Animals were transferred to the testing apparatus 10 minutes before testing commenced. The testing apparatus consisted of an elevated (61cm), six-chambered, Plexiglass structure (31cm x 9.5cm x 25cm/chamber) with a wire mesh flooring (Figure 2.1A). This allowed for unrestricted access to the plantar surfaces of ipsilateral and contralateral hind paws of each animal. Von Frey hairs (4.31-5.18g) were used for tactile sensitivity assessment (North Coast Medical, Gilroy, CA, USA; Figure 2.1B).

Beginning with the lowest bending force (4.31g), von Frey filaments were applied perpendicular to the plantar surface of the hind paw until a slight bend was achieved; and held for two seconds. Withdrawal responses, or lack thereof, were recorded during the application or removal of the filament. No visible behavioural response was deemed a negative response and prompted the next thicker hair in the graded series to be applied. If the animal exhibited a positive withdrawal response, as indicated by the retraction or flick of the paw and/or licking of the paw area, the preceding filament (less bending force) was then employed. If the response was ambiguous the current stimulus was repeated. Testing continued until a cut off force (5.18g hair) was achieved or four additional responses were recorded after the initial positive response (Figure 2.1C). Five seconds were allotted in between each filament application.

The 50% withdrawal threshold was calculated using the following equation:

$$50\% \, Threshold = \frac{(10^{[Xf + \kappa\delta]})}{10{,}000}$$

Where Xf is the bending force of the last von Frey filament used (in log units), κ is the tabular value for the pattern of the last six responses and δ is the mean difference (in log units) between stimuli.

2.3.2 Dynamic incapacitance

Dynamic weight bearing measurements were assessed using a Dynamic Incapacitance chamber (Bioseb, Boulogne, France). Animals were brought to the testing room to acclimate to the local environment for at least one hour prior to testing. During

testing, each animal was placed into the Bioseb Perspex testing chamber (24.5cm x 24.5cm x 33.0cm), which contains a touch/pressure sensitive floor (Figure 2.2A). The animals were observed for a period of three minutes during which both video and weight bearing patterns were recorded using a DFK22AUC03 camera. Hind-limb weight bearing and hind-limb weight bearing surface area were analyzed (Figure 2.2B). Weight placed on the ipsilateral paw was represented as a percentage of the combined hind-limb weight and ipsilateral hind paw surface area was calculated as percent contralateral surface area. Von Frey hair algesiometry and dynamic incapactiance measurements were taken consecutively.

2.3.3 Activity measures

Activity was used as a proxy to measure pain behaviour and levels of animal sedation. The three-minute videos generated by the Bioseb dynamic incapacitance software were analyzed to measure three types of activity: quadrant crossings, quantity of rears, and time spent rearing.

To quantify quadrant crossings, the square-shaped arena was divided equally into four quadrants. The animal was considered to cross quadrants if either the fore-paws or hind-paws, in addition to over fifty percent of the animal's body, entered a new quadrant. Entry, followed by reversal back into the previous quadrant was deemed two crosses. Crossing could occur into adjoining quadrants or diagonally, which in either case was considered as only one crossing. Validated crosses were quantified for the entirety of the three-minute video.

Rearing was analyzed by quantifying the number of times the animal stood on its hindlimbs, in addition to the time spent rearing. A rearing event was counted if the animal raised its front paws, bearing weight solely on the hind limbs. A new rear was quantified every time the animal returned its forelimbs to ground position and subsequently elevated. Time spent rearing was measured by manually timing the period where the animal raised its front limbs and remained upright. Timing was stopped when the forelimbs returned to the floor. Total time spent rearing was calculated as the sum of all rears within the three-minute period.

2.4   Inflammatory measures

2.4.1   *Joint oedema*

Joint oedema was assessed by measuring joint diameter using digital callipers (Traceable Products, Webster, Texas, USA). Three measurements were recorded along the joint line in a medio-lateral plane and averaged. Baseline measurements were taken immediately before induction of arthritis and throughout acute (0-180 minutes) and chronic (0-21 days) time courses.

2.4.2   *Intravital microscopy*

2.4.2.1   *Surgical preparation*

Surgical preparation was performed for all acute inflammatory time courses (Figure 2.3). Animals were anesthetized with 25% urethane in saline (2ml loading dose; 0.5-0.7ml top-ups every 20 minutes) until ocular blink and pedal withdrawal reflexes were absent. Internal temperature (37 ± 1ºC) was recorded using a rectal thermometer and kept stable with a thermostatically controlled heating pad during surgical preparations and throughout the entirety of the experiments (TC-1000 Rat probe/monitor; CWE Inc., Ardmore, Pennsylvania, USA).

Once a stable, deep plane of anesthesia was achieved, the animal was placed on a surgical board and the ipsilateral joint was stabilized by placing a leg stage underneath the knee prior to securing the hind paw to the board with Transpore tape. A longitudinal midline incision was made down the midline of the animal from below the chin to the sternum. Next, the surrounding fat and skin layers were blunt dissected to expose the sternohyoid muscle. Blunt dissection was continued through the sternohyoid muscle, and the trachea was isolated. A transverse incision was made on a cartilaginous section of trachea using micro-surgical scissors and a tracheal cannula (PE-205; 5cm) was inserted and secured with two sutures (4-0 surgical silk thread). Once inserted, any liquid or mucous in the trachea was extracted to ensure a clear, unobstructed airway.

The carotid artery was then isolated from the surrounding tissue and vagus nerve using blunt dissection techniques. Once isolated, the distal end of the artery was tied off with two surgical knots to halt blood flow. A surgical clamp was inserted between two untied sutures at the proximal end of the carotid artery. A small incision was made in the

artery using micro-surgical scissors and a fine cannula (PE-50; 25cm) containing warmed 1% heparinized saline was inserted into the carotid and secured with the two sutures. The cannula was attached to a 3-way stopcock and a 5ml syringe. The carotid artery cannulation was performed in order to record mean arterial pressure (MAP) measurements via a BLPR2 pressure transducer connected to a calibrated, bridged amplifier (World Precision Instruments, Sarasota, Florida, USA).

The jugular vein was then isolated, and cannulated (PE-40; 25cm) with tubing flushed with warmed 1% heparinized saline. Rhodamine 6G (0.1ml; 5mg/10ml; Sigma Aldrich, St. Louis, Missouri, USA), a fluorescent dye, was injected to label leukocytes within the systemic circulation.

The knee joint was subsequently exposed by removing a small section of skin directly above the joint. Connective tissue and fascia were removed to expose the underlying microvasculature of the knee joint capsule.

Endpoint measurements for the chronic series of experiments did not require tracheal, carotid or jugular cannulation as only one time point was recorded. Instead, rhodamine 6G (0.1ml; 5mg/10ml) was administered by tail vein injection (1ml syringe; 30g needle) to avoid unnecessary invasive surgery.

2.4.2.2   *Leukocyte trafficking assessment*

Intravital microscopy was used to assess leukocyte trafficking within the microvasculature of the ipsilateral knee joint (Figure 2.4). Rhodamine 6G-labelled leukocytes were visualized with fluorescent light (530nm absorbance; 556nm fluorescence; Brackmann 2000) using a Leica DM2500 microscope, with a HCX APOL 20X objective and HC Plan 10X eyepiece. Straight, unbranched post capillary venules (15-50μm) located in the knee joint were selected and three, one-minute videos were recorded at a final magnification of 200x using a Leica DFC 3000 camera (Leica Microsystems Canada Inc, Richmond Hill, Ontario, Canada). Additional camera recording parameters are detailed in Table 2.1.

The videos were then analyzed offline in a blinded manner, where rolling and adherent leukocytes were quantified (Figure 2.5). Rolling leukocytes were defined as cells moving slower than blood flowing past an arbitrary line perpendicular to the vessel

of interest. The number of rolling leukocytes per minute was quantified. Leukocytes were considered adherent if they were stuck to the endothelium and were immobile for more than 30 seconds within a 100µm span of the postcapillary venule (leukocytes/100µm). The number of adherent leukocytes was quantified for each of the one-minute recordings.

2.4.3   Laser speckle contrast analysis

Laser speckle contrast analysis, using the PeriCam PSI System (Perimed Inc, Ardmore, PA) was used to assess blood perfusion to the knee joint of interest (Figure 2.6). A one-minute recording was taken at a working distance of 10cm and a frame capture rate of 25 images/second using PIMSoft software (Version 1.5.4.8078). LASCA recordings were performed consecutively with IVM recordings throughout the inflammatory time course. Post euthanasia, a "dead scan" recording was taken of the joint and subtracted from experimental perfusion values as a biological zero to mitigate any Brownian motion or tissue optical interference. The speckle pattern was subsequently analyzed offline in a blinded manner and recorded in arbitrary perfusion units for each time-specific recording. Blood perfusion to a specific region of interest which corresponded to the joint capsule was recorded (Figure 2.7).

2.4.4   Cytokine analysis

2.4.4.1   Blood collection and plasma separation

Plasma samples were collected to assess systemic cytokine levels in treated versus non-treated cohorts at endpoint of the chronic administration study. Collection was performed on the same cohorts of animals used for IVM and LASCA recordings.

Whole blood samples were collected by intracardiac puncture using a 5ml syringe at endpoint on day 21. The sample was transferred immediately into a 6ml EDTA coated tube and placed on ice. Samples were centrifuged at 1000g at 4 ºC for 10 minutes within 30 minutes of collection. Plasma supernatant was transferred into a labelled Eppendorf tube, and stored at -80 ºC until use.

2.4.4.2 Multiplex

A ProcartaPlex™ Multiplex immunoassay was custom created and purchased from Thermo Fisher (ThermoFisher, Burlington, Ontario, Canada). The Rat Custom ProcartaPlex 5-plex (Lot#196356000; Cat# PPX-05-MXTZ9MK) included detection beads for IL-1β, IL-10, IL-17A, IL-6, and TNF-alpha (Table 2.2).

Frozen aliquots were thawed on ice and centrifuged at 10,000 x g for 5-10 minutes and then diluted with 1X universal assay buffer (1:2), provided with the kit. Antigen standards and a 4-fold serial dilution of the reconstituted standard were prepared as per the ProcartaPlex™ Mutliplex Immunoassay for Convenience and Mix&Match Panels User Guide (Figure 2.8; ThermoFisher Scientific 2017). The magnetic beads were vortexed for 30 seconds and 40μl of the beads was added to each well, before subsequently removing the liquid and 150μl of wash buffer. Standards and pre-diluted plasma samples (50μl) were added to the bead-coated wells. Blank wells were filled with 50μl of 1X universal assay buffer. The plate was subsequently sealed, covered with the blackened plate cover, and incubated at room temperature on a microplate shaker at 500rpm (VWR; Friendswood, Texas, USA) for 2 hours. Post-incubation, the plate was washed a total of three times. Twenty microliters of the detection antibody mixture (1X) was added to each well and incubation took place at room temperature while shaking for 30 minutes (500rpm), followed by three washes. Next, 40μl of Streptavidin-PE (SAPE) was added to each well and incubated at room temperature while shaking (500rpm) for 30 minutes. The plate was then prepared for analysis by adding 120μl of Reading Buffer into each well and incubating for five minutes (ThermoFisher Scientific 2017).

The prepared plate was then read using a Bio-Plex 200 system (Bio-Rad, Hercules, California, USA) and analyzed in a blinded manner using Bio-Plex manager 6.0 (Bio-Rad, Hercules, California, USA).

2.5   Joint damage measures

2.5.1 Joint harvest

At endpoint on day 21 after FCA injection, a select cohort of animals (n=8) was used for joint histology. Animals were deeply anesthetized with 2% isoflurane until the

flexor-withdrawal reflex was absent and then were trans-cardially perfused with 60 ml of saline, followed by 60ml of 4% paraformaldehyde.

The surrounding skin was removed from the ipsilateral joint using a large pair of surgical scissors. The joint was removed by cutting the femur, tibia and fibula bones using Rongeurs. Excess fat and tissue were excised from the joint before immersion in a 50ml Falcon tube with 4% paraformaldehyde for 24 hours. At 24 hours, the isolated knee was washed with sterile water and transferred to 70% ethanol for storage.

2.5.2   *Joint histopathology*

Joints from the myrcene treated (n=4) and vehicle treated (n=4) cohort were sent to Bolder BioPATH Inc. (Boulder, CO, USA) for joint histopathological analysis. Joint histopathology and analysis were conducted by Alison M. Bendele; statistical analysis was performed by George Bendele and histologic tissue preparation was conducted by HistoTox Labs, Inc (Boulder, CO, USA).

As per HistoTox Labs, Inc and Bolder BioPATH protocols, the knee-joints were decalcified for 4-5 days in 5% formic acid (Bendele 2019). After trimming, the joints were halved in the frontal plane. Both halves were embedded in a paraffin block (Bendele 2019). The blocks were subsequently sliced (8μm thick) at 200μm intervals throughout the block and one section was chosen for toluidine blue staining (0.04%; prepared with acetate buffer working solution) (Gerwin et al. 2010). The joint section was then viewed microscopically and scored for four parameters: inflammation (Table 2.3), cartilage damage (Table 2.4), bone resorption (Table 2.5), and a total score of all three parameters (Bendele 2019). Joint preparation, staining, and scoring protocols are described by Gerwin et al. (2010).

2.6   Materials

2.6.1   *Drugs and reagents*

Solutions of myrcene (1 & 5mg/kg) diluted in soybean oil, were made up fresh on the morning of the day of administration. CBD (200μg), AM281 (75μg), AM630 (75μg), and diclofenac (1mg/kg) were dissolved in a mixture of DMSO, cremophor, and saline (1:1:8) and were also prepared on the day of administration from stock solutions (10x

concentration in 100% DMSO stored at -20 ºC). Respective vehicle preparations were a 50μl bolus of 100% soybean oil or DMSO, cremophor, saline mixture (1:1:8) without drug. A detailed description of the drugs used can be found in Table 2.6.

Standard solutions of 0.9% sodium chloride USP, isoflurane, Euthansol, and Freund's complete adjuvant (stored at 4ºC) were not altered.

Solutions of rhodamine-6G (5mg/10ml), paraformaldehyde (4%; 40g/1L) and heparinized saline (1%; 0.5ml/50ml) were prepared using a 0.9% sodium chloride saline solution and stored at 4ºC. Rhodamine-6G was stored in the dark. Urethane (25%; 12.5g/50ml) was also prepared using a 0.9% sodium chloride saline solution and stored at room temperature (21 ºC). Ethyl alcohol (95%) was diluted to a 70% concentration with distilled water and stored at room temperature.

A more detailed description of the drugs and reagents used in this project can be found in Table 2.6-2.7.

2.6.2   Equipment

Equipment, as previously described, is also summarized in Table 2.8.

2.7   Statistical analyses

All data were presented as an average percent baseline ± SEM. Normality was assessed using the Kolmogorov Smirnov normality test. Normally distributed data was analyzed using a parametric one-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* test, two-way ANOVA with Bonferroni's *post-hoc* test, or the unpaired Student's t-test. One-way data distributions that did not pass normality, and scored data (joint histopathology) used the non-parametric equivalent. Two-factored data that did not pass normality used the parametric test in the absence of a non-parametric equivalent. A P-value less than 0.05 was considered significant.

2.8 Tables

Table 2. 1 Leica DFC 3000 camera recording parameters in the Leica Application Suite (LAS v.4.5.0 software).

Parameter	Setting
Brightness	20%
Gamma	0.96
Capture format	1296x966; Full frame HQ
Live format	432x322, 3x3, HQ binning
Automatic	HDR/Averaging

Table 2. 2 Cytokine targets and respective identifiers provided with the ProcartaPlex™ Multiplex immunoassay – ThermoFisher, Burlington, Ontario, Canada (ThermoFisher Scientific 2017).

Target Name	Bead Number	Std1 Concentration pg/ml	Standard
IL-1beta	19	53100	Standard Mix A
IL-10	15	24600	Standard Mix A
IL-17A	53	8550	Standard Mix A
IL-6	18	8950	Standard Mix A
TNF-alpha	45	11800	Standard Mix A

Table 2. 3 Rat knee inflammation scoring criteria as described by Bolder BioPATH, Inc – Boulder, Colorado, USA (Bendele 2019).

Score	Intensity Label	Description
0	Normal	
0.5	Minimal	Generally focal infiltration of inflammatory cells in synovium
1	Minimal	Diffuse infiltration of inflammatory cells in synovium
2	Mild	Diffuse infiltration of inflammatory cells in synovium
3	Moderate	Diffuse infiltration with moderate edema in synovium and extending into periarticular tissues
4	Marked	Diffuse infiltration with marked edema in synovium and extending into periarticular tissues
5	Severe	Diffuse infiltration with severe edema in synovium and extending into periarticular tissues

Table 2. 4 Rat cartilage damage scoring criteria as described by Bolder BioPATH, Inc – Boulder, Colorado, USA (Bendele 2019).

Score	Intensity Label	Description
0	Normal	
0.5	Very Minimal	Focal or only a few marginal zones affected
1	Minimal	Minimal to mild loss of toluidine blue staining with no obvious chondrocyte loss or collagen disruption, lesions mainly in marginal zones
2	Mild	Mild loss of toluidine blue staining with focal mild (superficial chondrocyte loss and/or collagen disruption mainly in marginal zones may have 1-2 small areas of more severe degeneration)
3	Moderate	Moderate loss of toluidine blue staining with multifocal moderate (depth to middle zone) chondrocyte loss and/or collagen disruption on at least one surface but still mainly in marginal zones, may have 1-2 small areas of more severe degeneration
4	Marked	Marked loss of toluidine blue staining with multifocal marked (depth to deep zone or tidemark) chondrocyte loss and/or collagen disruption on at least one surface
5	Severe	Severe diffuse loss of toluidine blue staining with multifocal severe (depth to tidemark) chondrocyte loss and/or collagen disruption affecting more than 2 cartilage surfaces

Table 2. 5 Rat knee bone resorption scoring criteria as described by Bolder BioPATH, Inc – Boulder, Colorado, USA (Bendele 2019).

Score	Intensity Label	Description
0	Normal	
0.5	Very minimal	Normal on low magnification but with the earliest hint of small focal areas of resorption in a marginal zone
1	Minimal	Small definite areas of resorption in marginal zones only
2		Small areas of resorption in marginal zones and extending up the distance across the affected surface
3	Moderate	Areas of resorption extend beyond marginal zones > ¼ but less than ½ of subchondral surface
4	Marked	Areas of resorption extend beyond marginal zones > ¼ but less than ¾ of the subchondral surface
5	Severe	Areas of resorption extend beyond marginal zones > ¾ of the subchondral surface

Table 2. 6 List of drugs used for acute and chronic delivery experiments.

Drug	Description	Dose/Administration route	Source
AM281	Cannabinoid-1 receptor antagonist GPR55 antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboximide	75μg (s.c. or topical over exposed area)	Cayman Chemicals; Ann Arbor, Michigan, USA
AM630	Cannabinoid-2 receptor antagonist 6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-4-methoxyphenyl)menthanone	75μg (s.c. or topical over ipsilateral knee-joint)	Cayman Chemicals; Ann Arbor, Michigan, USA
Cannabidiol	Natural phytocannabinoid Cyclohexene (2-[(1R-6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3 benzenediol)	200μg (s.c. or topical over ipsilateral knee-joint)	Tocris Bioscience, Bio Techne; Abingdon, UK
Diclofenac	Non-steroidal anti-inflammatory drug	1mg/kg ~ 0.35mg (s.c. or topical over ipsilateral knee-joint)	Sigma Aldrich; St. Louis, Missouri, USA
Euthansol	Pentobarbital sodium with phenytoin sodium. Short-acting barbituric acid. Used as an euthanasia solution	100mg/kg; intracardiac	CDMV; Dartmouth, Nova Scotia, Canada
Isoflurane	Fluorinated ether. Used as a general anesthetic.	2% isoflurane, 100% O_2; 1L/min	CDMV; Dartmouth, Nova Scotia, Canada
Myrcene	Acyclic monoterpene Organic hydrocarbon 7-Methyl-3-methylene-1,6-octadiene	1mg/kg ~ 0.49μl/50μl 5mg/kg ~ 2.45μl/50μl (s.c. or topical over ipsilateral knee-joint)	Toronto Research Chemicals; Toronto, Ontario, Canada
Urethane	Organic ethyl carbamate. Carbamic acid ester. Used as an irreversible anesthetic	25% dissolved in saline 1.2-1.5mg/kg i.p.	Sigma Aldrich; St. Louis, Missouri, USA

Table 2. 7 List of reagents used to complete the study's experimental objectives.

Reagent	Description	Source
Cremophor EL	Non-ionic emulsifying solvent. Used as vehicle for reagents unable to dissolve in water	Sigma Aldrich; St. Louis, Missouri, USA
Dimethyl sulfoxide	Organosulfur solvent used to dissolve polar and non-polar compounds.	Sigma Aldrich; St. Louis, Missouri, USA
Ethyl alcohol 95%	Alcohol based solvent	Commercial Alcohols, Brampton, Ontario, Canada (purchased from central stores at Dalhousie University)
Ethyl alcohol anhydrous	Alcohol based solvent	Commercial Alcohols, Brampton, ON, Canada (purchased from central stores at Dalhousie University)
Freund's Complete adjuvant	Mineral oil emulsion with heat killed *Mycobacterium tuberculosis* RA-inducing agent	Sigma Aldrich; St. Louis, Missouri, USA
Heparin	Anionic sulfated glycosaminoglycan. Used as a blood anticoagulant.	CDMV; Dartmouth, Nova Scotia, Canada
Mineral Oil	Inert liquid paraffin oil Used to retain moisture	Lawtons; Halifax, NS, Canada
Paraformaldehyde	Polymer of formaldehyde. Used as a tissue/joint fixative	Fisher Scientific Company; Ottawa, Ontario, Canada
Rhodamine 6G	Leukocyte-labelling fluorophore	Sigma Aldrich; St. Louis, Missouri, USA
Saline	0.9% Sodium chloride injection USP (1000ml)	B. Braun Medical Inc., Scarborough, Ontario, Canada (purchased from central stores)
Soy bean oil (#1)	Solvent; oil extracted from soy bean flakes	Superstore; Halifax, NS, Canada
Soy bean oil (#2)	Solvent; oil extracted from soy bean flakes	Sigma Aldrich; St. Louis, Missouri, USA

Table 2. 8 List of equipment used to complete the study's experimental objectives.

Equipment	Model	Manufacturer
Analytical balance	AL-54	Mettler Toledo; Mississauga, Ontario, Canada
Blood perfusion analysis software	PIMSoft V.1.5.4.8078	Perimed Inc., Ardmore, Pennsylvania, USA
Blood perfusion machine	PeriCam PSI *Normal Resolution* System	Perimed Inc., Ardmore, Pennsylvania, USA
Blood pressure monitor	Pressure Monitor BP-1	World Precision Instruments, Sarasota, Florida, USA
Blood pressure transducer	BLPR2	World Precision Instruments, Sarasota, Florida, USA
Caliper	Electronic caliper with digital display.	Traceable Products, Webster, Texas, USA.
Cytokine plate analysis software	Bio-Plex manager 6.0	Bio-Rad, Hercules, California, USA
Cytokine plate reader	Bio-Plex 200	Bio-Rad, Hercules, California, USA
Cytokine plate washer	Bio-Plex Pro™ II	Bio-Rad, Hercules, California, USA
Dynamic weight bearing instrument	BIO-DWB-AUTO-R; DFK22AUC03 camera	Bioseb, Boulogne, France
Intravital microscope	Leica DM2500 microscope; HCX APOL 20X objective; HC Plan 10X eyepiece; Leica DFC 3000 camera	Leica Microsystems Canada Inc., Richmond Hill, Ontario, Canada
Intravital microscopy software	Leica Application Suite (LAS v.4.5.0)	Leica Microsystems Canada Inc., Richmond Hill, Ontario, Canada
Micropipettes	2-1000μl VWR or Eppendorf Research	VWR; Friendswood, Texas, USA Fisher Scientific Company; Ottawa, Ontario, Canada
Microplate Shaker	Microplate Shaker	VWR; Friendswood, Texas, USA

Table 2.8 cont'd

Equipment	Model	Manufacturer
Scale #1,2 ,3	#1 AR5120 #2 CS Series: CS2000 #3 CP2202 S	#1-2: Ohaus, Parsippany, New Jersey, USA. #3: Sartorius Mechanotronics, Göttigen, Germany.
Temperature control monitor	TC-1000 temperature controller	CWE Inc., Ardmore, Pennsylvania, USA
Temperature controlled heating pad with probe	TC-1000 Rat	CWE Inc., Ardmore, Pennsylvania, USA
Von Frey chamber	Custom	Concept Plastic, Dartmouth, Nova Scotia, Canada
Von Frey hairs	Touch Test 20 Piece Full Kit; NC12775	Touch Test Sensory Evaulator, North Coast Medical, Gilroy, California, USA
Water bath	Microprocessor Controlled 280 Series	World Precision Instruments, Sarasota, Florida, USA

2.9   Figures

A

B

C

3.61
3.84
4.08
4.17
4.31
4.56
4.74
4.93
5.07
5.18

3.61
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5.07
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3.61
3.84
4.08
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4.31
4.56
4.74
4.93
5.07
5.18

x – positive withdrawal
o – no observable withdrawal
Xf = 5.18; 'oxooox' – XO sequence
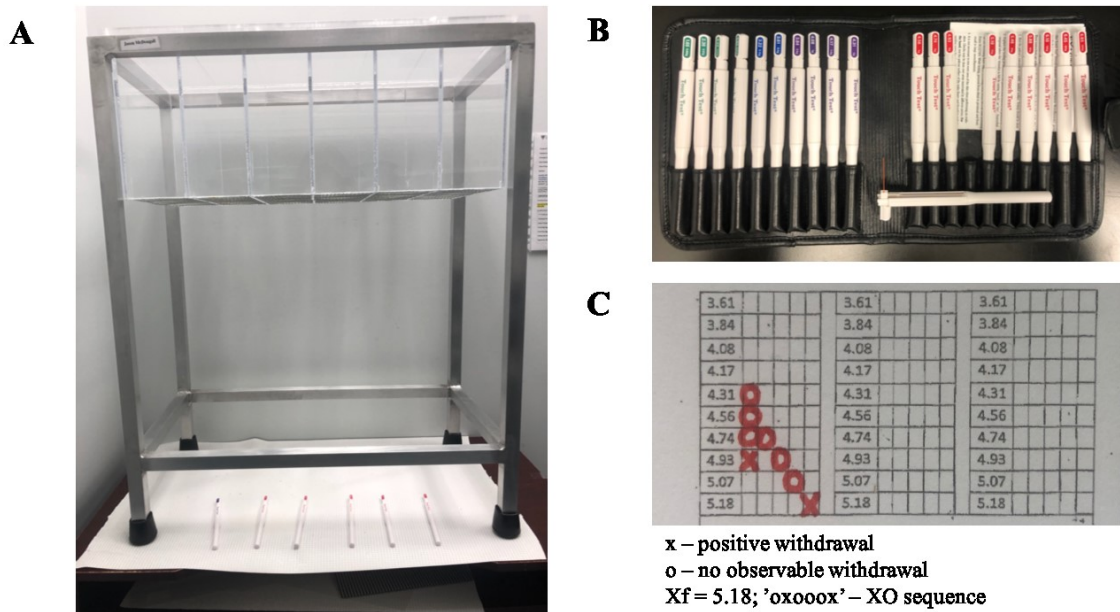
Figure 2.1 Von Frey hair algesiometry experimental set up using the elevated six-chambered von Frey apparatus with mesh flooring (A). Mechanical allodynia is investigated using von Frey filaments (B) and (C) recorded/analyzed using Dixon's up down method (Dixon 1980) adapted from Chaplan et al. (1994).
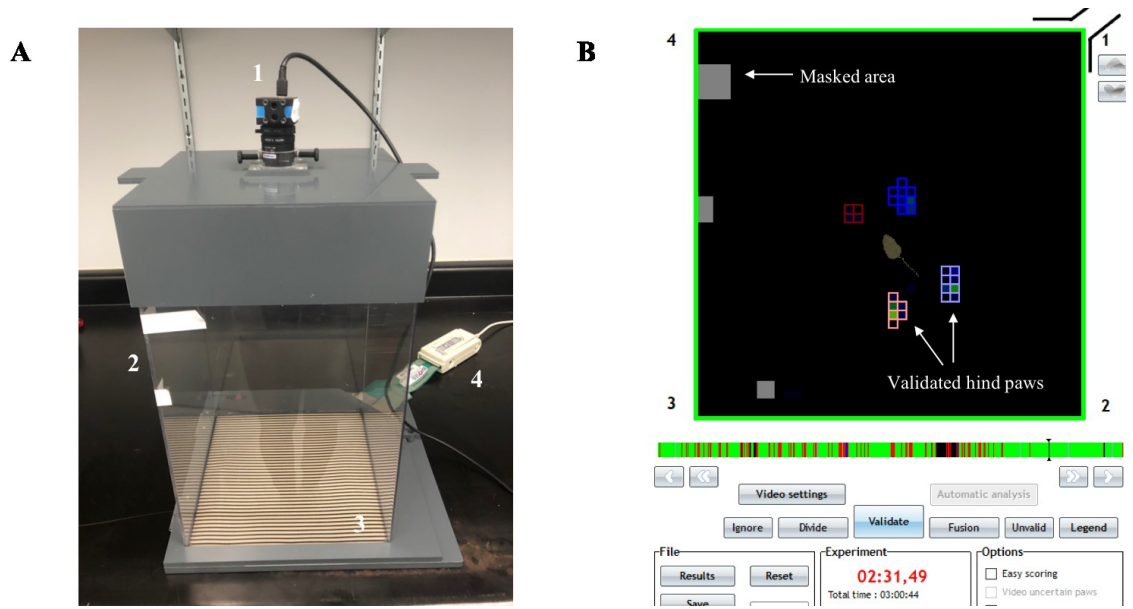
Figure 2.2 Dynamic weight bearing incapactiance experimental set up (A) and respective offline video/sensor pattern analysis (B). Unvalidatable masked areas and correctly validated left and right hind paws are labelled.

Important experimental components include:

      1: DFK22AUC03 camera

      2: Bioseb Perspex testing chamber

      3: Touch/pressure sensor pad

      4: Sensor pad connection handle
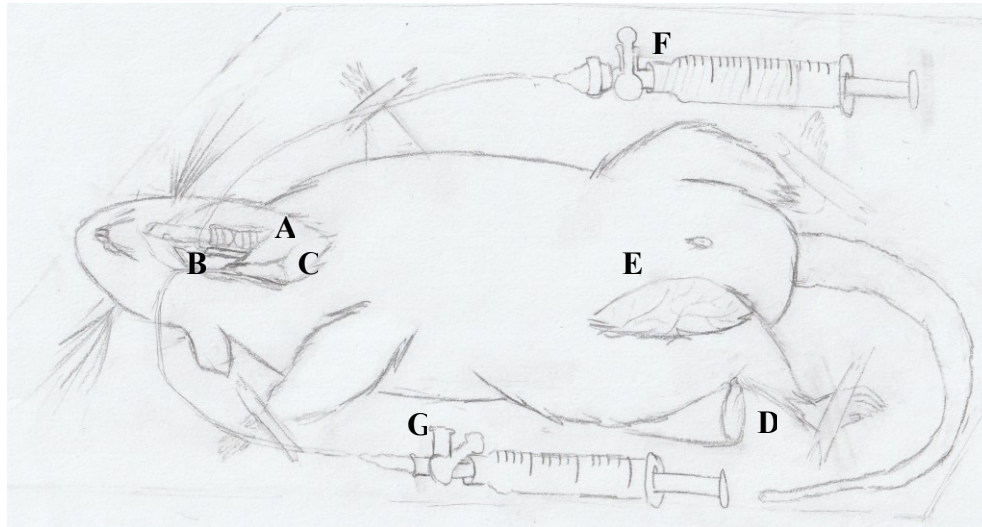
Figure 2.3 Acute surgical preparation visualization. (A): Tracheal cannulation. (B): Carotid artery cannulation. (C): Jugular vein cannulation. (D): Leg brace made from an Eppendorf tube to secure joint. (E): Exposed microvasculature of the knee-joint. (F): Stop-cock controlled cannula filled with 1% heparinized saline; connected to blood pressure recorder. (G): Stop-cock controlled cannula filled with 1% heparinized saline; used to administer rhodamine-6G.
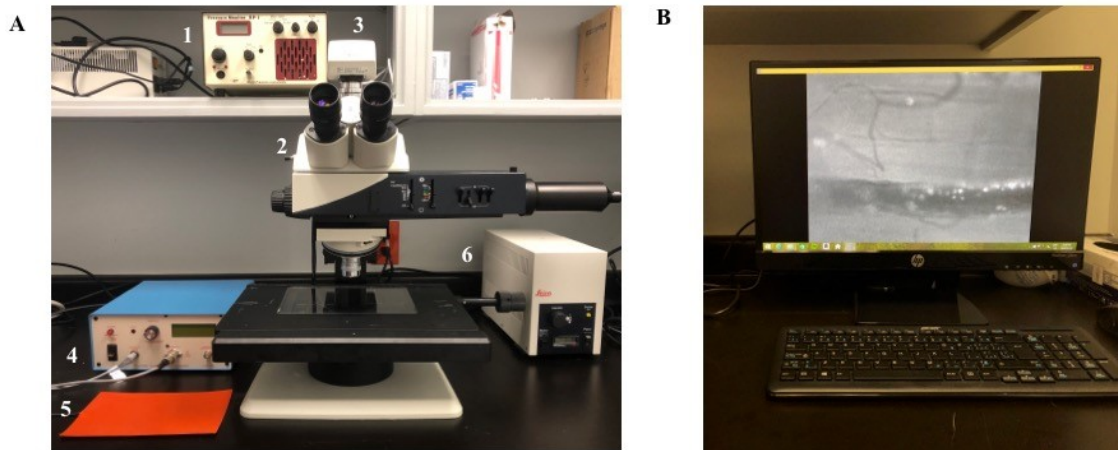
Figure 2.4 Intravital microscopy experimental set up (A) and recording of the video for offline analysis (B).

Important experimental components include:

    1: BP-1 pressure Monitor

    2: Leica DM2500 microscope (HCX APOL 20X objective and HC Plan 10X eyepiece)

    3: Leica DFC 3000 camera

    4: TC-1000 temperature controller

    5: TC-1000 rat heating pad and probe

    6: External light source for Leica DM2500 light microscope
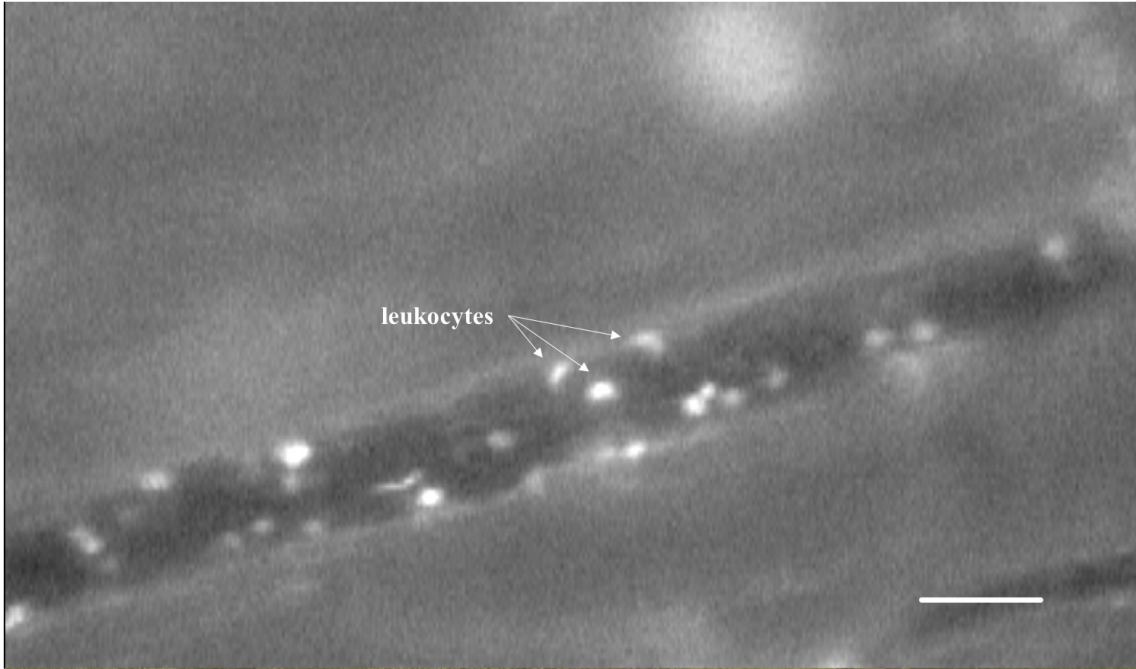
Figure 2.5 Post-capillary venule in the knee joint containing Rhodamine 6G labelled leukocytes. Scale bar equals 50μm.
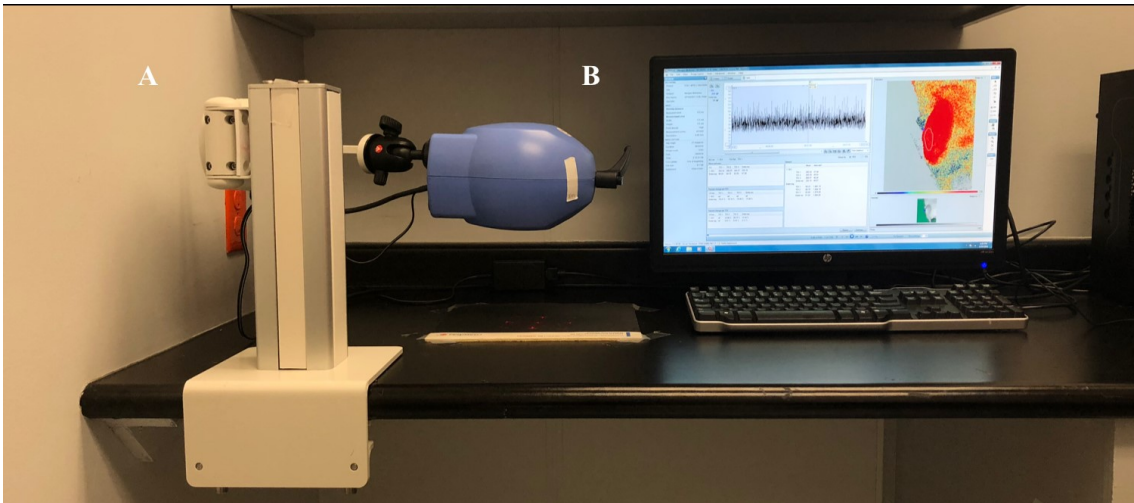
Figure 2.6 Laser speckle contrast analysis (LASCA) experimental setup; the PeriCam PSI System (A) used to capture the blood perfusion to the knee joint recording, which is then analyzed by the PIMSoft software (B).
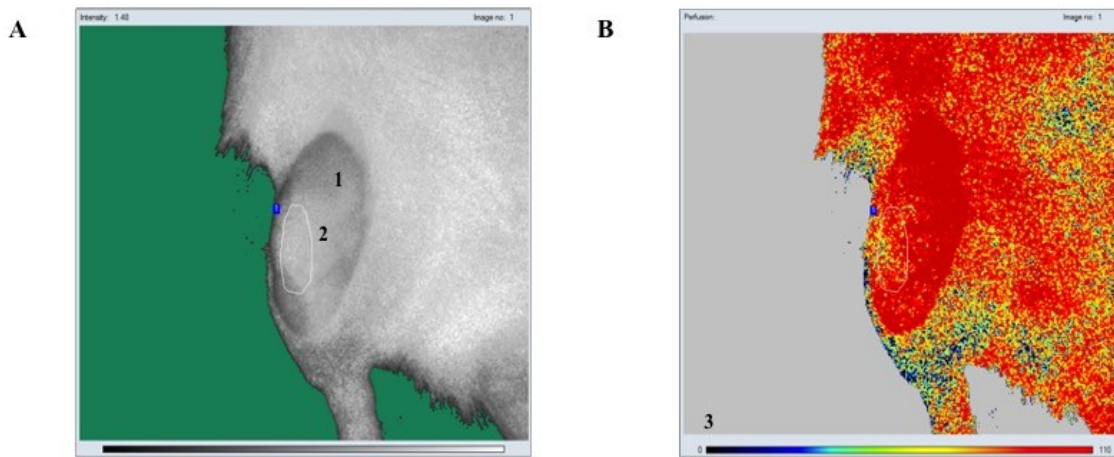
Figure 2.7 Laser speckle contrast (LASCA) analysis via PIMSoft (V.1.5.4.8078) software, including the intensity image (A) and perfusion view (B).

Important components include:

1. Dissected joint area
2. Region of interest over the knee joint
3. Perfusion intensity scale bar (blue – lowest; red – highest)
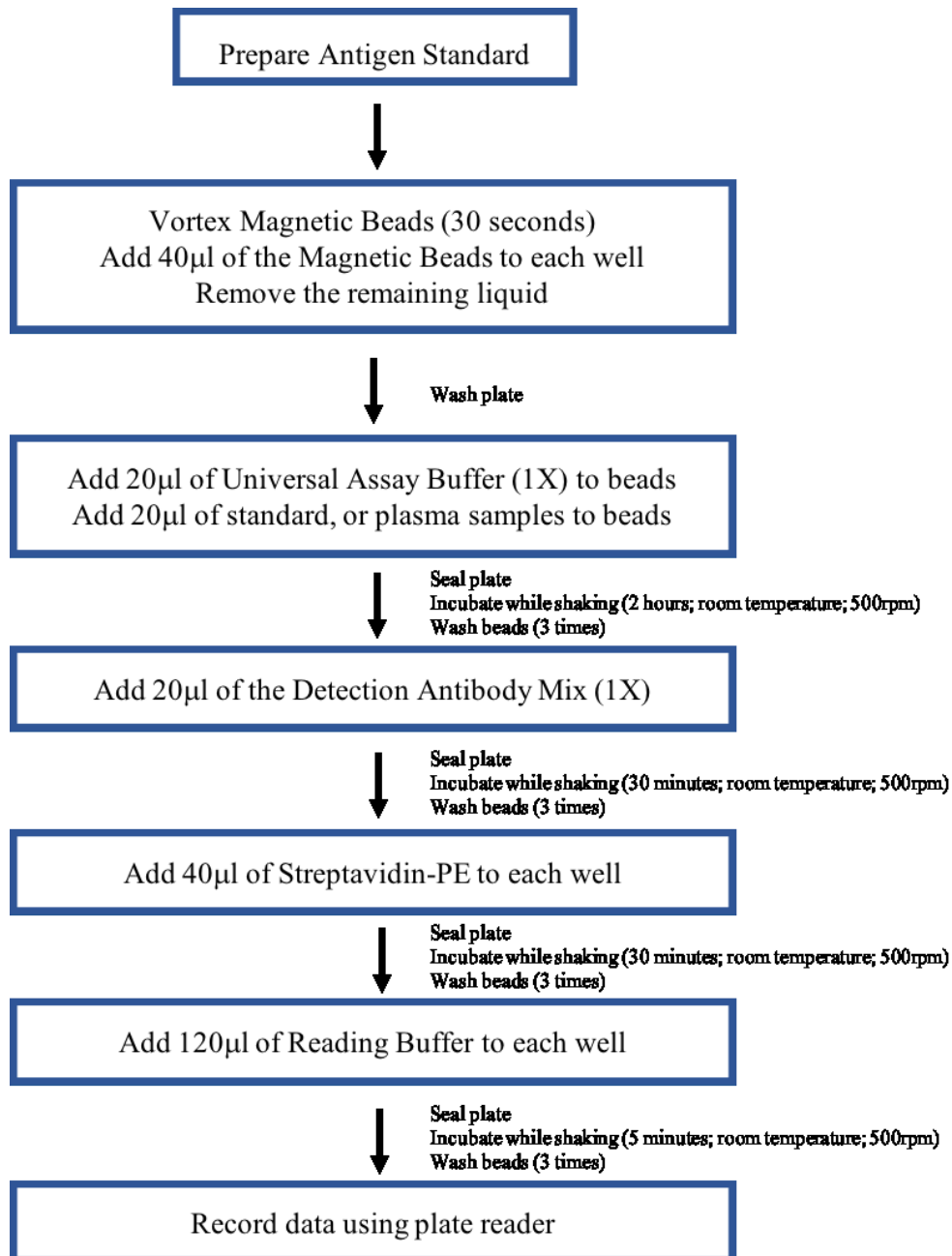
Figure 2.8 ProcartaPlex™ Mutliplex Immunoassay preparation protocol (Thermo Fisher, Burlington, Ontario, Canada). Adapted from the ProcartaPlex™ Mutliplex Immunoassay for Convenience and Mix&Match Panels User Guide (ThermoFisher Scientific 2017).

Chapter 3: Effect of Acute Treatment of Myrcene on Pain and Inflammation Induced by Freund's Complete Adjuvant

3.1 Background and hypotheses

Treatment strategies for RA focus on targeting disease modification (Singh et al. 2015); however, adequate pain management remains a predominant concern of patients (Klooster et al. 2007). Conventional and biologic DMARDs have shown some efficacy in preventing inflammation and associated joint damage (Nurmohamed and Dijkmans 2005); however, discontinuation due to lack of efficacy or intolerable adverse effects is common (Nurmohamed and Dijkmans 2005). Supplemental treatments such as opioids and NSAIDs also remain ineffective in treating the chronicity of the disease and are riddled with their own adverse effects (Crofford 2010; Crofford 2013). Therefore, the search for an effective therapeutic presenting with limited adverse effects is ongoing.

A significant number of arthritis patients use medical cannabis for symptom relief (Ware et al. 2005), reporting reduced pain and improved joint function (Blake et al. 2006). The efficacy of medical cannabis as an analgesic therapeutic in RA patients is logical due to the involvement of the endocannabinoid system in the inhibition of pain transmission (Russo and Hohmann 2013) and the presence of cannabinoid receptors within the joints (Schuelert and McDougall 2008).

Cannabis harbours a diverse chemical profile with over 545 identified compounds, including over 200 terpenes (El Sohly and Gul 2014). Terpenes are hydrocarbons responsible for the pungent odor of the cannabis plant (Singh and Sharma 2015). Their analgesic and anti-inflammatory potential has also been brought to light recently (reviewed by Russo and Marcu 2017).

Myrcene is the most abundant monoterpene in the cannabis plant and tends to occur in strains preferred by arthritis and chronic pain patients (Baron et al. 2018). Although most commonly studied as a fraction of an essential oil, isolated myrcene has demonstrated analgesic properties in acute pain models (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013). Oral and subcutaneous administration of myrcene were able to attenuate writhing in the noxious acetic acid test (Rao et al. 1990; Lorenzetti et al.

1991; Paula-Freire et al. 2013). Latency to paw licking during the hot plate test was also improved by oral and intraperitoneal administration of myrcene (Rao et al. 1990; Paula-Freire et al. 2013). Inhibition of these analgesic effects was prevented when naloxone or yohimbine were administered, implying the involvement of opioid and alpha-2 adrenergic receptors in the analgesic mechanism (Rao et al. 1990; Paula-Freire et al. 2013).

To date, no research has investigated the effect of acutely administered myrcene on pain and inflammation in RA. The involvement of the endocannabinoid system in the actions of myrcene has not yet been examined *in vivo*. Therefore, we investigated the analgesic and anti-inflammatory potential of acutely delivered myrcene in both naïve and FCA-injected rats. We also explored the mechanistic actions of myrcene and the involvement of cannabinoid receptors.

The hypotheses tested in this study were:

I. **Acute administration of myrcene will reduce spontaneous pain behaviour and mechanical allodynia in FCA-injected rats via the endocannabinoid system.**

II. **Acute treatment of myrcene will reduce knee joint inflammation in the FCA model via the endocannabinoid system.**

3.2 The effects of myrcene treatment in a naïve cohort

3.2.1 Pain behaviour methods and experimental design

Preliminary experiments were carried out to test the effect of myrcene in naïve animals. A dose was chosen based on a systemic dose used by Rao et al. (1990).

Joint diameter, von Frey hair algesiometry and dynamic weight bearing measurements were recorded prior to commencing the three hour pain behaviour time course. All animals received the following treatments:

I.   Vehicle – soy bean oil (50μl; subcutaneously over the knee joint)

II.   Myrcene (1mg/kg; 50μl; subcutaneously over the knee joint)

Following treatment, pain behaviour was assessed over a three hour time course (protocol schematic shown below). Animals that had received vehicle treatment were then re-used to assess the effect of myrcene treatment after a 2-day wash out period.
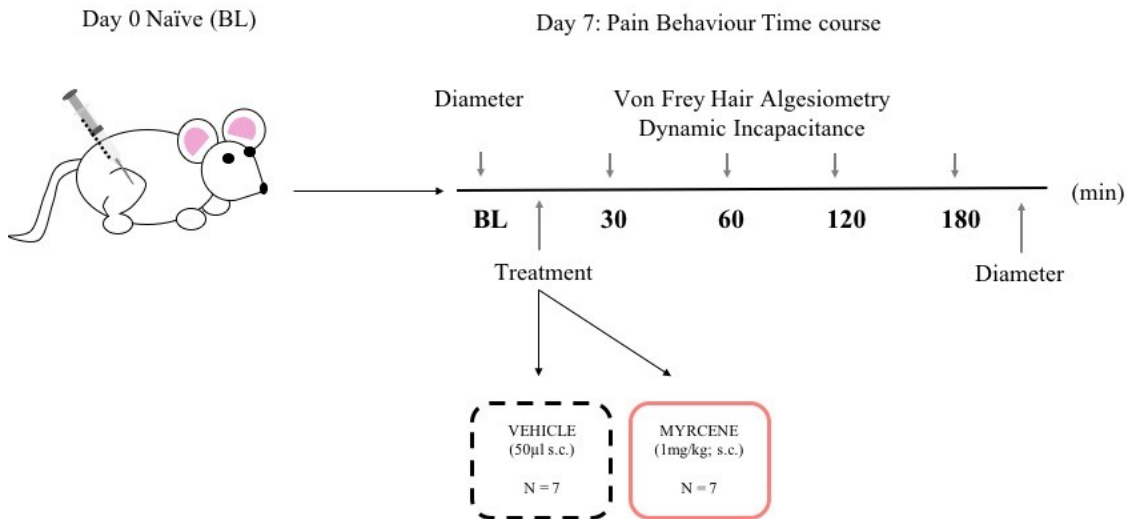
3.2.2 Pain behaviour results

The data for the time course did not pass normality, but were analyzed using a two-way RM ANOVA in the absence of a non-parametric equivalent. Withdrawal thresholds between myrcene and vehicle-treated groups were not significantly different (Figure 3.1A, $P > 0.05$, n = 7 per group). Treatment with myrcene did not significantly change the weight borne on the ipsilateral hind paw (Figure 3.1B, $P > 0.05$, n = 7 per time point) or surface area used to weight bear Figure 5.1C, $P > 0.05$, n = 7 per time point) compared to vehicle-treatment.

3.2.3 Inflammation methods and experimental design

Joint diameter, leukocyte trafficking and blood perfusion data were recorded in naïve male Wistar rats following one of the following treatments:

I.   Vehicle – soy bean oil (50μl; subcutaneously over the knee joint)

II.   Myrcene (1mg/kg; 50μl; subcutaneously over the knee joint)

As described in full detail in Chapter 2 (section 2.4.2-2.4.3), animals were examined using intravital microscopy to investigate leukocyte trafficking over a one hour time course. LASCA measurements were also recorded consecutively during the same time course (outlined below).
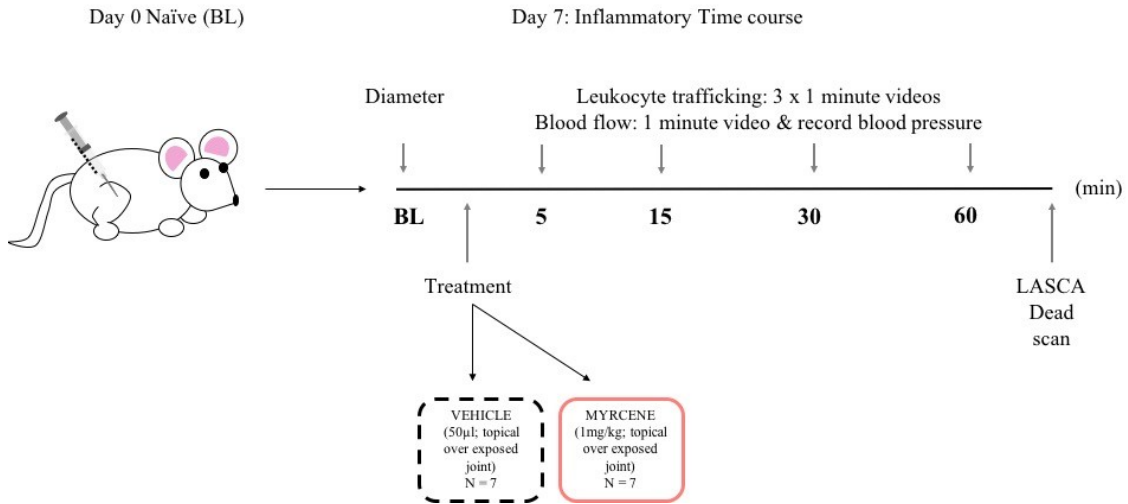
3.2.4   Inflammation results

Joint diameter was not significantly different between treatment groups at the conclusion of the three hour time course (Figure 3.2A, $P > 0.05$, n = 7 per group). Additionally, rolling leukocytes were not significantly different between the myrcene and vehicle-treated cohorts (Figure 3.2B, $P > 0.05$, n = 5-6 per time point). Adherent leukocytes increased over time in both groups; reaching $481.00 \pm 58.1\%$ baseline in the vehicle-treated group compared to $313.30 \pm 66.79\%$ baseline in the myrcene-treated cohort. Adherent leukocytes in the myrcene-treated group were significantly different than vehicle-treated animals (Figure 3.2C, $P < 0.0001$, n = 5-6 per time point). Blood perfusion to the affected area was also not significantly different between treatments in naïve animals (Figure 3.2D, $P > 0.05$, n = 5-6 per time point).

3.3   The effects of acute myrcene treatment on pain behaviour in the FCA model

3.3.1   Methods and experimental design

Myrcene has demonstrated analgesic qualities in the acute hot plate, formalin and acetic acid tests (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013), but has

not been tested in any disease models. We wanted to characterize the effect of acute administration of myrcene on pain in the FCA model of RA.

A 50μl bolus of FCA was injected intraarticularly into the right knee joint of deeply anaesthetised male Wistar rats (see section 2.2 for further details). Prior to arthritis induction, the animals were subjected to baseline pain behaviour measurements (von Frey hair algesiometry, dynamic weight bearing). A secondary baseline was recorded on day seven after FCA injection (0 minutes) prior to starting the inflammatory assessment time course. Separate cohorts received one of the following treatments:

I.   Myrcene vehicle – soy bean oil (50μl; subcutaneously over the knee joint)

II.   Myrcene (1mg/kg; 50μl; subcutaneously over the knee joint)

III.   Myrcene (5mg/kg; 50μl; subcutaneously over the knee joint)

IV.   Diclofenac vehicle – DMSO/cremophor/saline (1:1:8; 50μl; subcutaneously over the knee joint)

V.   Diclofenac (1mg/kg; 50μl; subcutaneously over the knee joint)

Diclofenac and its respective vehicle were tested in this study as a positive control to FCA-induced pain and inflammation. The dose used was based on the systemic dose used by Hasani et al., (2011). Following treatment, von Frey hair algesiometry and dynamic weight bearing recordings were measured at 30, 60, 120 and 180 minutes (see section 2.3.1-2.3.2 for more details).

Joint diameter was also measured using these same cohorts on day zero, day seven at 0 minutes and 180 minutes (section 2.4.1).

Day 0: FCA induction (50μl i.artic)   Day 7: Pain Behaviour Time course
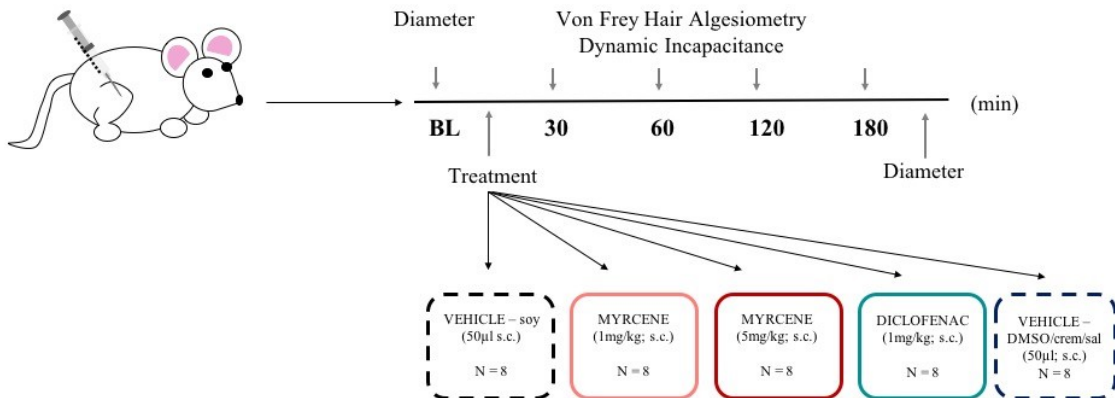
3.3.2 Results

3.3.2.1 FCA-injected animals exhibit secondary allodynia and weight bearing deficits

Withdrawal threshold was significantly decreased (from 14.36 ± 0.18g to 7.76 ± 0.36g) seven days after FCA-induction compared to pre-induction baseline measurements (Figure 3.3A, $P < 0.0001$, N = 80 per group). At baseline, animals placed on average 49.80 ± 0.34 percent of their weight on their ipsilateral paw. This was significantly reduced to 29.41 ± 1.30 percent seven days post FCA-induction (Figure 3.3B, $P < 0.0001$, n = 76-78 per group). Baseline measurements indicated that the surface area animals placed on their ipsilateral limb was on average 99.69 ± 0.93 percent of the surface area used in their contralateral limb. This was significantly reduced to 76.52 ± 11.20 percent seven days post FCA-induction (Figure 3.3C, $P < 0.0001$, n = 74-76 per group).

3.3.2.2 Diclofenac attenuates evoked and spontaneous pain behaviour in the FCA model

The effect of diclofenac on evoked and spontaneous pain behaviour was investigated to act as a positive control. Withdrawal threshold time course data (Figure 3.4A) did not pass normality, however these data were tested using the parametric two-way RM ANOVA as a non-parametric equivalent was lacking. Diclofenac significantly improved withdrawal threshold compared to vehicle (Figure 3.4A, $P < 0.05$, n = 8 per time point). Percent weight borne on the ipsilateral limb was also improved by diclofenac treatment (Figure 3.4B, $P < 0.0001$, n = 7-8 per time point). However, diclofenac-treated

animals did not use a larger surface area while weight bearing was similar to vehicle controls (Figure 3.4C, P > 0.05, n = 7-8 per time point).

3.3.2.3 *Myrcene improved secondary allodynia but did not improve weight bearing deficits*

Withdrawal threshold was significantly different amongst myrcene-treated and vehicle treated cohorts (Figure 3.5A, P < 0.05, n = 8 per time point). The highest improvement in withdrawal threshold occurred at 120 minutes post treatment where the 1mg/kg dose was 211± 17.93% and the 5mg/kg myrcene dose was 269.30 ± 63.27% higher than the time zero baseline measurement.

Myrcene-treated cohorts did not exhibit significant improvements in the percent weight borne on the ipsilateral hind paw when compared to the vehicle-treated cohort (Figure 3.5B, P > 0.05, n = 8 per time point). Percent contralateral surface area borne on the ipsilateral hind paw was also not significantly different between vehicle and myrcene treated animals (Figure 3.5C, P > 0.05, n = 8 per time point).

3.4   Involvement of the endocannabinoid system in myrcene-induced analgesia

3.4.1   *Methods and experimental design*

Previous studies demonstrated that myrcene may be acting through mu-opioid receptors and α2-adrenoreceptors (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013). No *in vivo* studies have looked at endocannabinoid system involvement in myrcene responses. To do so, we administered either the synthetic CB_1R antagonist AM281 or the CB_2R antagonist AM630 ten minutes prior to myrcene treatment as per the protocol used by Philpott et al. (2017). The animal cohorts tested in this series of experiments were:

I.   Antagonist vehicle – DMSO/cremophor/saline (1:1:8; 50µl; subcutaneously over the knee joint)

     + Myrcene (1mg/kg; 50µl; subcutaneously over the knee joint)

II.   AM630 (75µg; 50µl; subcutaneously over the knee joint) +

Myrcene (1mg/kg; 50μl; subcutaneously over the knee joint)

III.　　AM281(75μg; 50μl; subcutaneously over the knee joint) +

　　　　Myrcene (1mg/kg; 50μl; subcutaneously over the knee joint)

IV.　　AM630 (75μg; 50μl; subcutaneously over the knee joint) +

　　　　myrcene vehicle – soy bean oil (50μl; subcutaneously over the knee joint)

V.　　AM281 (75μg; 50μl; subcutaneously over the knee joint) +

　　　　myrcene vehicle – soy bean oil (50μl; subcutaneously over the knee joint)

Previously completed data used for comparison:

i.　　Myrcene vehicle – soy bean oil (50μl; subcutaneously over the knee joint)

ii.　　Myrcene (1mg/kg; 50μl; subcutaneously over the knee joint)
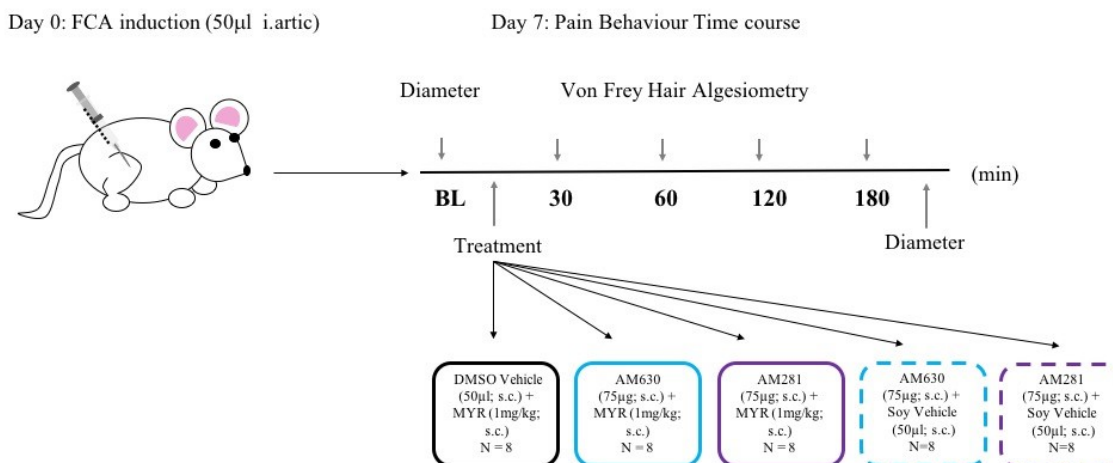
3.4.2　Results

3.4.2.1　CB$_1$ and CB$_2$ receptor antagonists did not attenuate myrcene-induced analgesia

Overall, withdrawal threshold varied across treatment cohorts in the CB$_1$R antagonist (AM281) 3 hour time course (Figure 3.6A, $P < 0.0001$, n = 8 per time point). AM281 administered prior to myrcene significantly reduced withdrawal threshold compared to myrcene-treatment at 60min ($P < 0.01$) and 120 minutes ($P < 0.0001$). However, vehicle (DMSO/cremophor/saline) with myrcene also showed significant reductions of withdrawal threshold compared to myrcene at those time points ($P < 0.05$

and P < 0.001). AM281 with myrcene, or vehicle (soy bean oil) were not significantly different from each other.

Significant variation in withdrawal threshold also occurred in the 3 hour time course with a CB_2 R antagonist (Figure 3.6B, $P < 0.01$, n = 8 per time point). Treatment with AM630 prior to myrcene, significantly attenuated myrcene-induced withdrawal threshold improvement at 60 minutes ($P < 0.001$) and 120 minute ($P < 0.0001$) in *post-hoc* comparisons. However, there was also a significant *post-hoc* reduction of withdrawal threshold between myrcene and vehicle (DMSO/cremophor/saline), and AM630 120 minutes after treatment ($P < 0.01$). In addition, AM630 with myrcene versus AM630 with vehicle (soy bean oil) did not differ in *post-hoc* analysis ($P > 0.05$).

The antagonistic effect of the CB receptor antagonists was compared at myrcene's most efficacious time point, 120 minutes. Overall, withdrawal threshold varied significantly between treatments 120 minutes after treatment (Figure 3.6C, $P < 0.0001$, n = 8 per group). Both AM630 and AM281 attenuated the myrcene-induced improvement in withdrawal threshold ($P < 0.0001$ and $P < 0.001$). However, vehicle (DMSO/cremophor/saline) with myrcene also attenuated withdrawal improvement ($P < 0.01$) and did not differ from either antagonist treatment ($P > 0.05$).

3.5 Comparing the analgesic effects of myrcene to a positive control at peak effect time

3.5.1 Methods and experimental design

From the data previously compiled within this study, the peak effect of myrcene in pain behavioural testing was determined to be 120 minutes post-treatment, while the peak effect for diclofenac was determined to be 30 minutes post-treatment. The top dose for myrcene was used for comparison. Treatments were compared at their respective peak effect time points.

3.5.2   Results

3.5.2.1   The analgesic effect of myrcene was comparable to the positive control diclofenac

At peak response, the effect of myrcene on withdrawal threshold was not significantly different from the analgesic effect of diclofenac (Figure 3.7A, $P > 0.05$, n = 8 per group). Comparisons of the percent weight borne on the ipsilateral hind paw and percent surface area of the contralateral paw also showed no significant differences between myrcene-treatment and the positive control (Figure 3.7B and Figure 3.7C, $P > 0.05$, n = 8 per group).

3.6   The effects of myrcene on inflammation in the FCA model

3.6.1   Methods and experimental design

In these experiments, we explored the effects of acute administration of myrcene on FCA-induced joint inflammation. Baseline joint diameter was measured prior to FCA induction and again on day seven to confirm inflammation (section 2.4.1). Animals were then anesthetized with 25% urethane in saline (2ml loading dose; 0.5-0.7ml top-ups every 20 minutes) and surgical preparations were performed as described in section 2.4.2.1. These included tracheal, carotid and jugular cannulations, administration of the fluorescent nuclear stain rhodamine 6G, and exposure of the knee joint.

One-minute blood perfusion LASCA recordings and three one-minute recordings using intravital microscopy were taken at baseline and periodically throughout the one hour time course. Mean arterial pressure was also recorded at each of the time points. Separate cohorts of animals received one of the following treatments:

I.   Myrcene vehicle – soy bean oil (50μl; topically over the exposed knee joint)

II.   Myrcene (1mg/kg; 50μl; topically over the exposed knee joint)

III.   Myrcene (5mg/kg; 50μl; topically over the exposed knee joint)

IV.   Diclofenac vehicle – DMSO/cremophor/saline (1:1:8; 50μl; topically over the exposed knee joint)

V.   Diclofenac (1mg/kg; 50μl; topically over the exposed knee joint)

At the end of the time course, a biological zero LASCA recording was taken post-euthanasia and subtracted from all perfusion values to account for Brownian motion and tissue reflection.
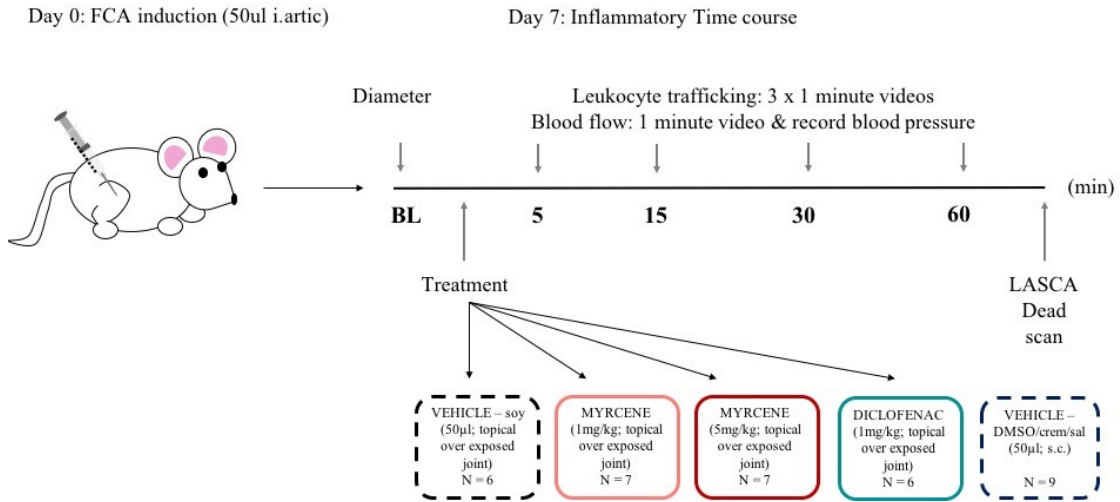
3.6.2 Results

3.6.2.1 FCA-injected animals exhibited joint oedema, increased leukocyte trafficking and hyperaemia

Average joint diameter was significantly elevated from 9.91 ± 0.04mm baseline measurements to an average of 12.12 ± 0.09mm when measured seven days post FCA-induction (Figure 3.8A, $P < 0.001$ n = 80 per group).

Rolling leukocytes were significantly increased at baseline on day seven in FCA-injected animals (86.75 ± 2.84 leukocytes) compared to naïve (49.58 ± 5.36 leukocytes) (Figure 3.8B, $P < 0.0001$, n = 11-64). Adherent leukocytes were also slightly increased from 1.06 ± 0.13 leukocytes in naïve animals to 1.94 ± 0.26 leukocytes in day seven FCA-injected animals (Figure 3.8C, $P < 0.001$, n = 11-64 per group). FCA animals exhibited higher mean perfusion, 234.0 ± 10.81PU, compared to that of naïve animals, 155.7 ± 13.91 PU (Figure 3.7D, $P < 0.05$, n = 11-64).

3.6.2.2 Diclofenac improved leukocyte trafficking and blood flow but joint oedema was unchanged

Joint diameter, relative to day seven baseline, was unchanged by diclofenac treatment (Figure 3.9A, $P > 0.05$, $n = 8$ per group). However, diclofenac significantly attenuated rolling leukocytes within the one-hour time course (Figure 3.9B, $P < 0.01$, $n = 6$-9 per time point). The greatest reduction ($66.04 \pm 6.23\%$ baseline) occurred 30 minutes after treatment. Time course adherent leukocyte data were not normally distributed, but were analyzed using a two-way RM ANOVA in the absence of a non-parametric equivalent. There was no change in adherent leukocytes between diclofenac and vehicle-treated animals (Figure 3.9C, $P > 0.05$, $n = 6$-9 per group).

Blood flow to the knee joint was also reduced with diclofenac treatment (Figure 3.9D, $P < 0.01$, $n = 6$-9 per time point).

3.6.2.3 Myrcene did not improve joint diameter compared to vehicle

There was an overall significant variation of joint diameter relative to baseline between myrcene and vehicle-treated cohorts (Figure 3.10A, $P < 0.01$, $n = 8$ per group). *Post-hoc* testing showed that both the 1mg/kg and 5mg/kg dose of myrcene did not differ from vehicle measures ($P > 0.05$). The joint diameter, relative to day seven baseline, was significantly increased in the 5mg/kg group compared to the 1mg/kg group ($P < 0.01$).

3.6.2.4 Myrcene reduced leukocyte rolling but not adherent leukocytes or blood flow to the knee

Rolling leukocytes were significantly reduced in the myrcene-treated cohorts compared to vehicle (Figure 3.10B, $P < 0.0001$, $n = 6$-7 per time point). The largest reduction of leukocytes for 1mg/kg of myrcene was $52.22 \pm 6.18\%$ of baseline rolling leukocytes at 30 minutes and $48.76 \pm 5.35\%$ of baseline rolling leukocytes at 60 minutes for the 5mg/kg myrcene dose. There was no significant difference between the two myrcene doses ($P > 0.05$).

Adherent leukocytes showed a gradual increase over the time course. Percent baseline values for vehicle, 1mg/kg and 5mg/kg of myrcene were 383.4 ± 148.5, 195.7 ± 31.1, and $324,8 \pm 44.28\%$ respectively. There was no significant different between the

myrcene-treated and vehicle-treated cohorts (Figure 3.10C, $P > 0.05$, n = 6-7 per time point). Mean perfusion was also not significantly different between myrcene and vehicle treated groups (Figure 3.10D, $P > 0.05$, n = 6-7 per time point).

3.7 Involvement of the endocannabinoid system in inflammation

### 3.7.1 Methods and experimental design

We examined cannabinoid receptor involvement by administering AM281, AM630 or vehicle ten minutes prior to applying vehicle or myrcene treatment. More specifically, separate cohorts received one of the following treatment combinations:

I. Antagonist vehicle – DMSO/cremophor/saline (1:1:8; 50µl; topically over the exposed knee joint) + Myrcene (1mg/kg; 50µl; topically over the exposed knee joint)

II. AM281 (75µg; 50µl; topically over the exposed knee joint) + Myrcene (1mg/kg; 50µl; topically over the exposed knee joint)

III. AM630 (75µg; 50µl; topically over the exposed knee joint) + Myrcene (1mg/kg; 50µl; topically over the exposed knee joint)

IV. AM630 (75µg; 50µl; topically over the exposed knee joint) + myrcene vehicle – soy bean oil (50µl; topically over the exposed knee joint)

V. AM281 (75µg; 50µl; topically over the exposed knee joint) + myrcene vehicle – soy bean oil (50µl; topically over the exposed knee joint)

The antagonist data were compared to data reported in section 3.4.2. Those treatments included:

i. Myrcene vehicle – soy bean oil (50µl; topically over the exposed knee joint)

ii. Myrcene (1mg/kg; 50µl; subcutaneously over the knee joint)

The same arthritis induction, surgical procedures, and inflammatory measurements were followed as in section 3.4.1. LASCA dead scans were performed at the end of the one hour time course post-euthanasia to obtain a biological zero value.
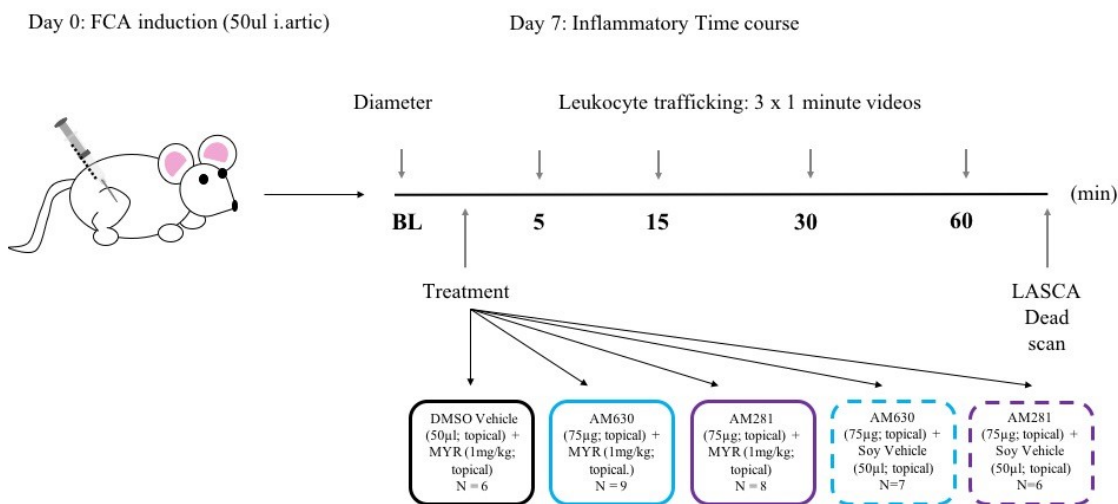
3.7.2 Results

3.7.2.1 CB_1 and CB_2 receptor antagonists inhibited leukocyte rolling over a one hour period

The mechanism by which myrcene mediates leukocyte rolling was investigated using the cannabinoid receptor antagonists AM630 (CB_2) and AM281 (CB_1). AM281 significantly attenuated the myrcene-induced reductions in leukocyte rolling over a one hour period (Figure 3.11A, $P < 0.0001$, $n = 6\text{-}9$ per time point). Antagonist vehicle (DMSO/cremophor/saline) with myrcene was not different from myrcene alone ($P > 0.05$). AM281 with myrcene was also not significantly different from AM281 with the myrcene vehicle ($P > 0.05$).

AM630 administered prior to myrcene also significantly attenuated the myrcene-induced reduction of rolling leukocytes throughout the time course (Figure 3.11B, $P < 0.0001$, $n = 6\text{-}9$ per time point). Vehicle (DMSO/cremophor/saline) with myrcene did not significantly attenuate rolling leukocytes ($P > 0.05$) Additionally, there was no difference between AM630 with myrcene or AM630 with myrcene's vehicle ($P > 0.05$).

3.7.2.2 *The anti-inflammatory effect of myrcene is attenuated by both CB_1 and CB_2 receptor antagonists at 30 minutes*

The effects of CB_1R and CB_2R antagonists were examined at 30 minutes when co-administered with myrcene. There was a significant variation overall amongst myrcene-treated, individual antagonists administered prior to myrcene, and respective vehicles (Figure 3.11C, $P < 0.0001$, n = 6-9 per group). *Post-hoc* analysis confirmed that myrcene reduced leukocyte rolling ($P < 0.01$). The CB_2R antagonist AM630 significantly attenuated the myrcene-induced reduction of rolling leukocytes ($P < 0.05$), as did the CB_1R antagonist ($P < 0.001$). Both antagonists administered prior to myrcene were significantly different than their respective vehicle in the presence of myrcene ($P < 0.01$, $P < 0.0001$). The *post-hoc* comparison of the myrcene-treated and vehicle (DMSO/cremophor/saline) with myrcene groups was not significantly different ($P > 0.05$).

3.8 Comparing the effects of myrcene to diclofenac at peak effect time

3.8.1 *Methods and experimental design*

Based on data previously collected within this chapter, the confirmed anti-inflammatory effect of myrcene was compared to that of the positive control diclofenac at their respective peak effect times. The peak effect for diclofenac was 30 minutes; the peak effect of myrcene was also at 30 minutes.

3.8.2 *Results*

3.8.2.1 *The anti-inflammatory effects of myrcene were not different from diclofenac*

Modulation of joint diameter was not different between myrcene and diclofenac-treated animals (Figure 3.12A, $P > 0.05$, n = 8 per group). Myrcene-induced reductions in leukocyte trafficking were not significantly different from reductions observed when using the positive control (Figure 3.12B, $P > 0.05$, n = 6-7 per group). The effect on adherent leukocytes was also not significantly different between diclofenac and myrcene-treated animals (Figure 3.12C, $P > 0.05$, n = 6-7 per group). Diclofenac lowered blood flow to the knee joint significantly more than myrcene treatment (Figure 3.12D, $P < 0.01$, n = 6-7 per group).

3.9   Chapter summary

FCA-injected animals exhibited mechanical secondary allodynia and weight bearing deficits seven days after arthritis induction. These animals also experienced increases in joint oedema, leukocyte trafficking and blood flow to the knee joint area. All of the parameters except surface area and adherent leukocytes were modulated with the use of the positive control, diclofenac.

By itself, myrcene did not induce pain or inflammation in pilot naïve animal studies. Acutely, both of the myrcene doses reduced secondary mechanical allodynia, but did not improve FCA-induced weight bearing deficits. However, we could not confirm that the analgesic effects were occurring via the endocannabinoid system. Myrcene also attenuated rolling leukocytes via endocannabinoid-dependent mechanisms, but had no significant impacts on joint oedema, leukocyte adhesion, or blood flow. With the exception of blood flow, the analgesic and anti-inflammatory effects of myrcene were comparable to those of diclofenac.

3.10 Tables

Table 3. 1 Myrcene treatment result summary.

Parameter	Myrcene 1mg/kg	Myrcene 5mg/kg
Pain behaviour summary	Myrcene improved evoked but not spontaneous pain behaviour	
Withdrawal threshold	Improved withdrawal threshold	Improved withdrawal threshold
Ipsilateral weight bearing	No improvement	No improvement
Ipsilateral surface area	No improvement	No improvement
Inflammation summary	Myrcene improved leukocyte trafficking	
Joint oedema	No improvement	No improvement
Rolling leukocytes	Reduced leukocyte rolling	Reduce leukocyte rolling
Adherent leukocytes	No improvement	No improvement
Blood perfusion to knee	No improvement	No improvement

Table 3. 2 Diclofenac treatment result summary.

Parameter	Result
Pain behaviour summary	Diclofenac reduced evoked and spontaneous pain behaviour
Withdrawal threshold	Improved withdrawal threshold
Ipsilateral weight bearing	Improved weight bearing
Ipsilateral surface area	No improvement
Inflammation summary	Diclofenac reduced leukocyte trafficking and blood flow
Joint oedema	No improvement
Rolling leukocytes	Reduced leukocyte rolling
Adherent leukocytes	No improvement
Blood perfusion to knee	Reduced blood flow

Table 3. 3 Comparison of myrcene treatment with the positive control diclofenac treatment.

| Parameter	Myrcene (5mg/kg) vs. Diclofenac (1mg/kg)
Pain behaviour summary	Analgesia between myrcene and diclofenac did not differ
Withdrawal threshold	No difference
Ipsilateral weight bearing	No difference
Ipsilateral surface area	No difference
Inflammation summary	The anti-inflammatory actions of myrcene were not superior to diclofenac
Joint oedema	No difference
Rolling leukocytes	No difference
Adherent leukocytes	No difference
Blood perfusion to knee	Diclofenac reduced blood flow

3.11 Figures
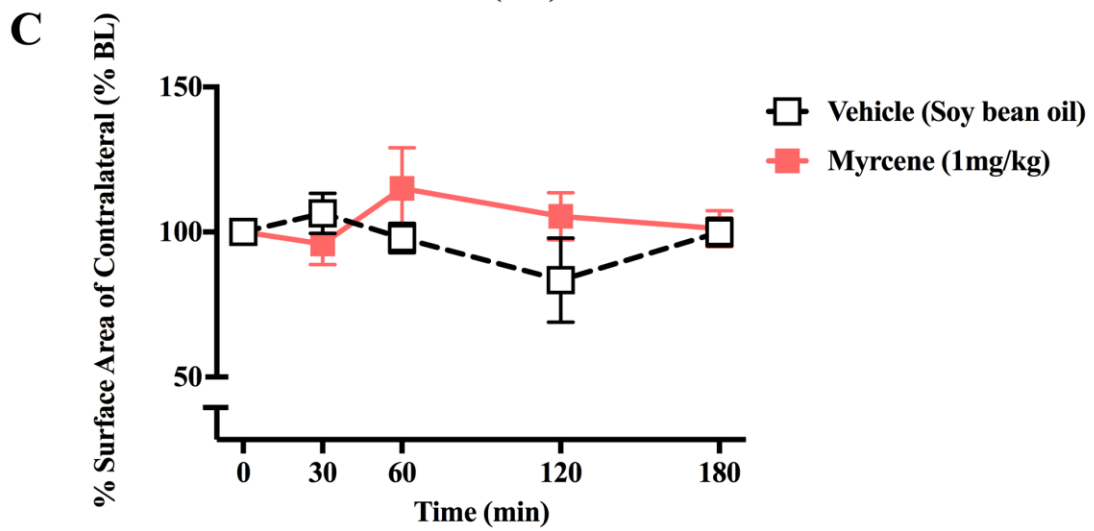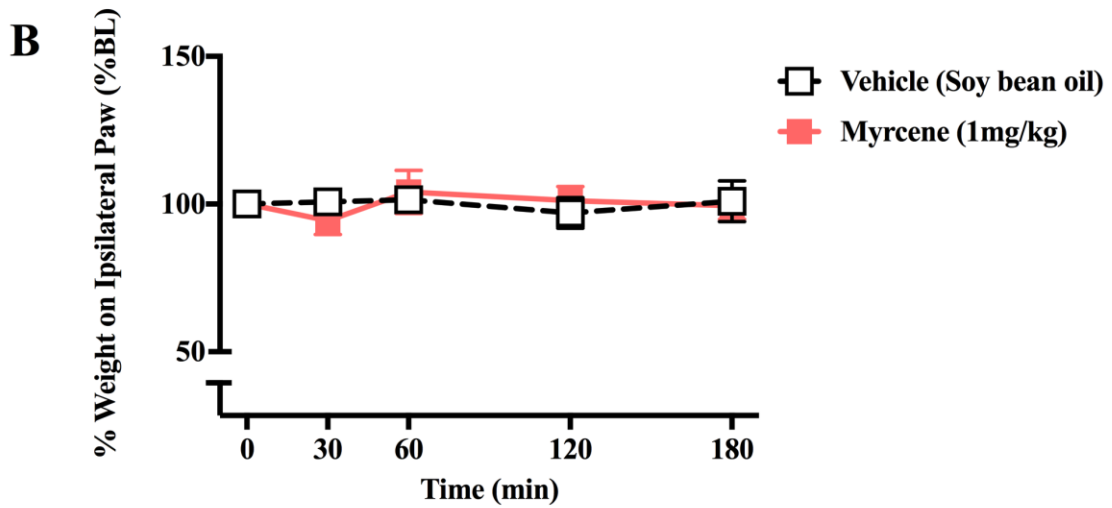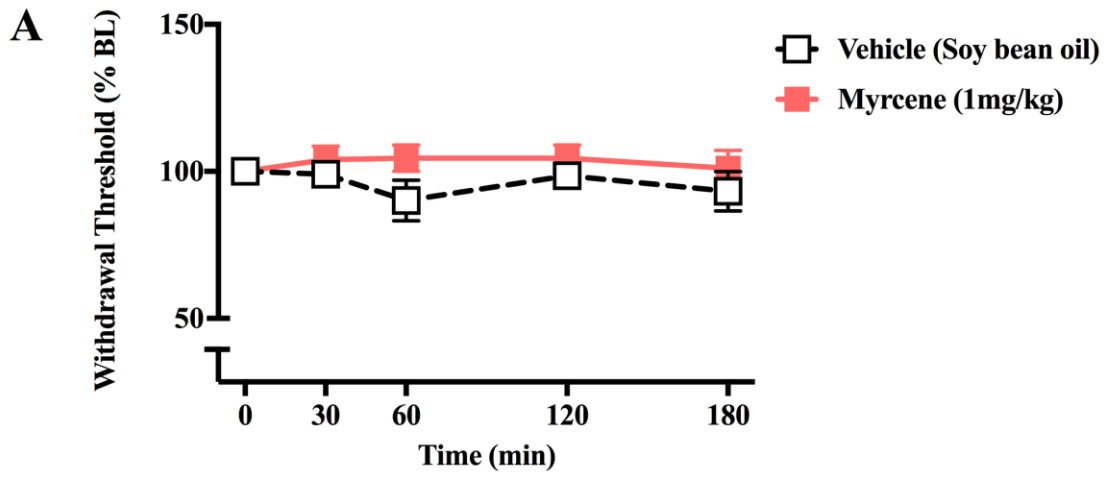
Figure 3.1 Effect of myrcene on evoked and spontaneous pain behaviour in naïve animals.

(A) There was no significant difference in tactile sensitivity between the myrcene-treated cohort and vehicle-treated cohort ($P > 0.05$, two-way RM ANOVA, n = 7 per time point). **(B)** Weight borne on the ipsilateral paw ($P > 0.05$, two-way RM ANOVA, n = 7 per time point) and **(C)** surface area borne on the ipsilateral paw ($P > 0.05$, two-way RM ANOVA, n = 7 per group) relative to baseline, were not significantly different between naïve animals that received vehicle or myrcene. Data are standardized to baseline and presented as mean ± SEM. ANOVA, analysis of variance; BL, baseline, RM, repeated measures.

A

Joint Diameter (% t0 BL)

NS

Vehicle (Soy bean oil)
Myrcene 1mg/kg

B

Rolling Leukocytes (% BL)

Vehicle (Soy bean oil)
Myrcene (1mg/kg)

Time (min)

C

Adherent Leukocytes (% BL)

Vehicle (Soy bean oil)
Myrcene (1mg/kg)

Time (min)
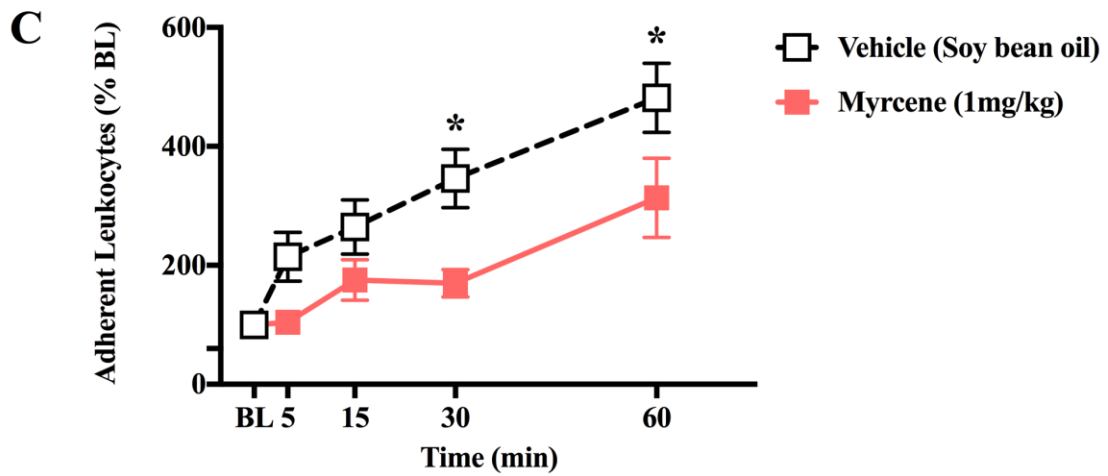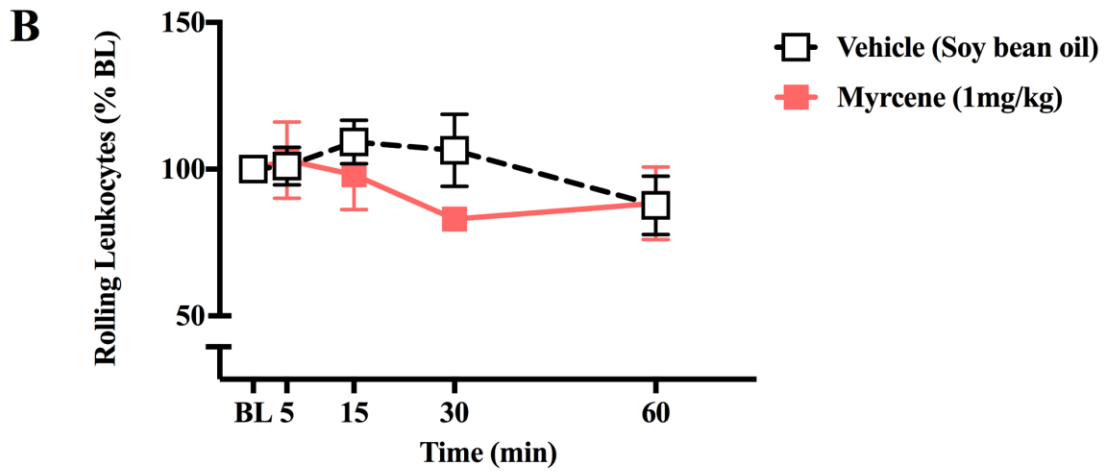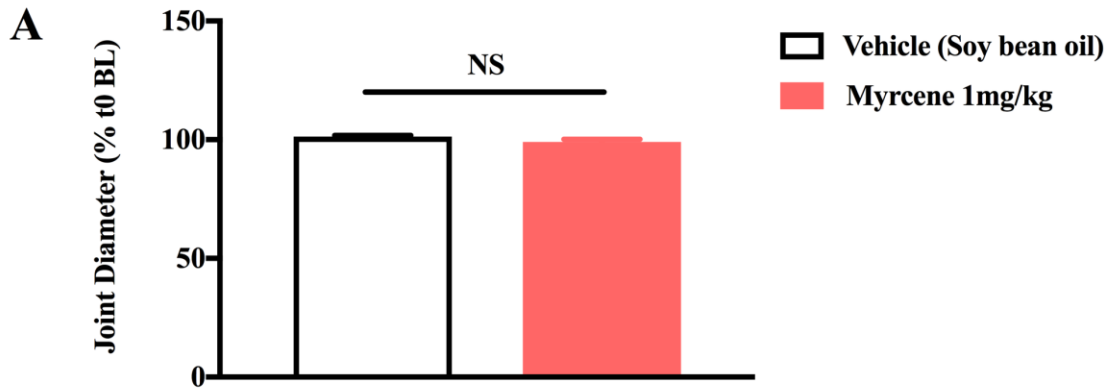
Figure 3.2 Effect of myrcene on leukocyte trafficking and blood perfusion in the microvasculature in the knee joint.

(A) Joint diameter relative to the baseline on the day of testing (t0) was not significantly different between the vehicle-treated or myrcene-treated cohorts ($P > 0.05$, Mann-Whitney U test, n = 7 per group). **(B)** Rolling leukocytes were not significantly different between vehicle or myrcene-treated naïve cohorts ($P > 0.05$, two-way RM ANOVA, N = 5-6 per time point). **(C)** Myrcene-treated animals had significantly reduced adherent leukocytes compared to vehicle controls ($P < 0.001$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 5-6 per time point). *Post-hoc* significance was demonstrated at 30 and 60 minutes post-treatment (*$P < 0.05$). **(D)** Mean perfusion was not significantly different between treatment groups throughout the one hour time course ($P > 0.05$, two-way RM ANOVA, n = 5-6 per time point). Data are standardized to baseline and presented as mean ± SEM. ANOVA, analysis of variance; BL, baseline; NS, not significant; RM, repeated measures.
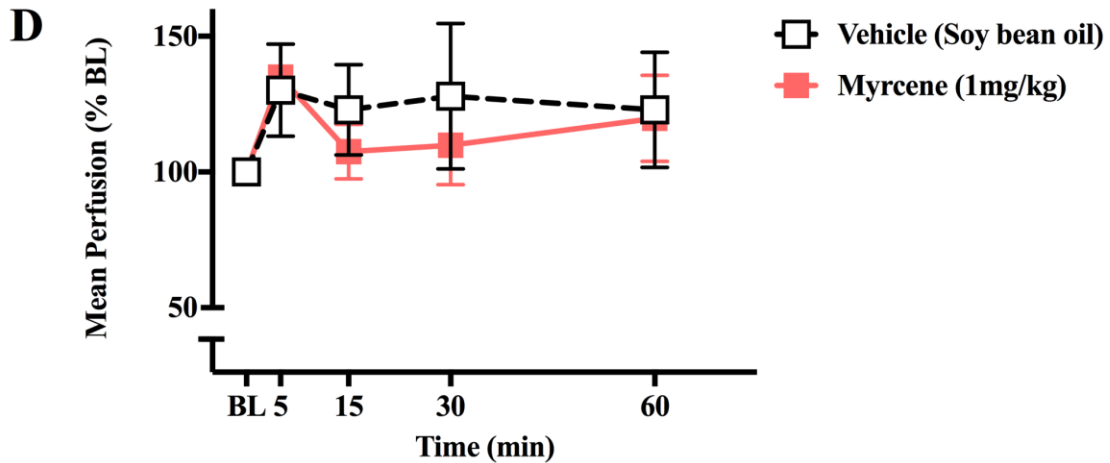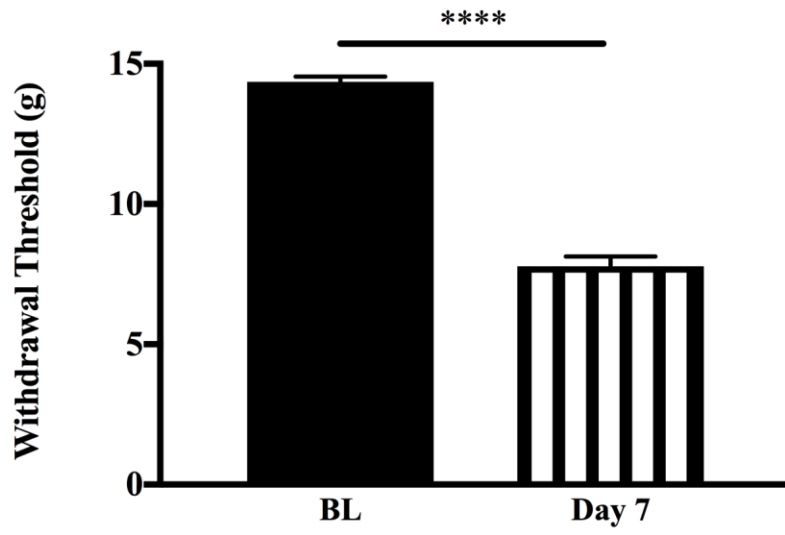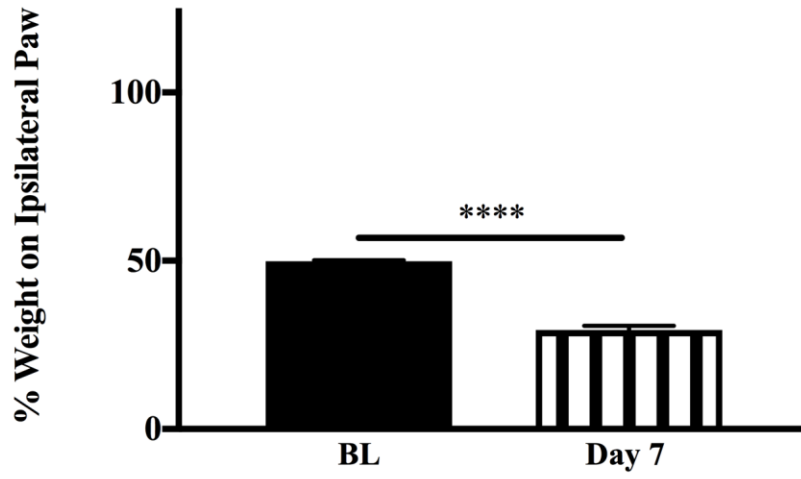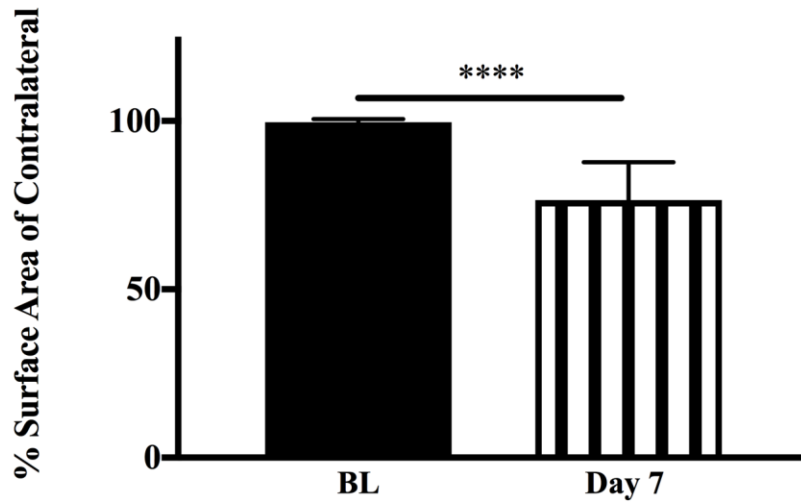
Figure 3.3 Confirmation of mechanical allodynia and weight bearing deficits with FCA.

(A) Day 7 withdrawal threshold in FCA-injected rats was significantly reduced from baseline measurements taken prior to FCA induction ($P < 0.0001$, paired Wilcoxon test, n = 80 per group). **(B)** Percent weight borne on the ipsilateral hind paw ($P < 0.0001$, paired Student's t-test, n = 75-77 per group) and **(C)** percent surface area of the contralateral paw ($P < 0.0001$, paired Wilcoxon, n = 74-76 per group) in FCA-injected rats on day seven was significantly different compared to their pre-induction baseline measurements. Data is presented as mean ± SEM. BL, baseline; FCA, Freund's complete adjuvant.

A

B

C
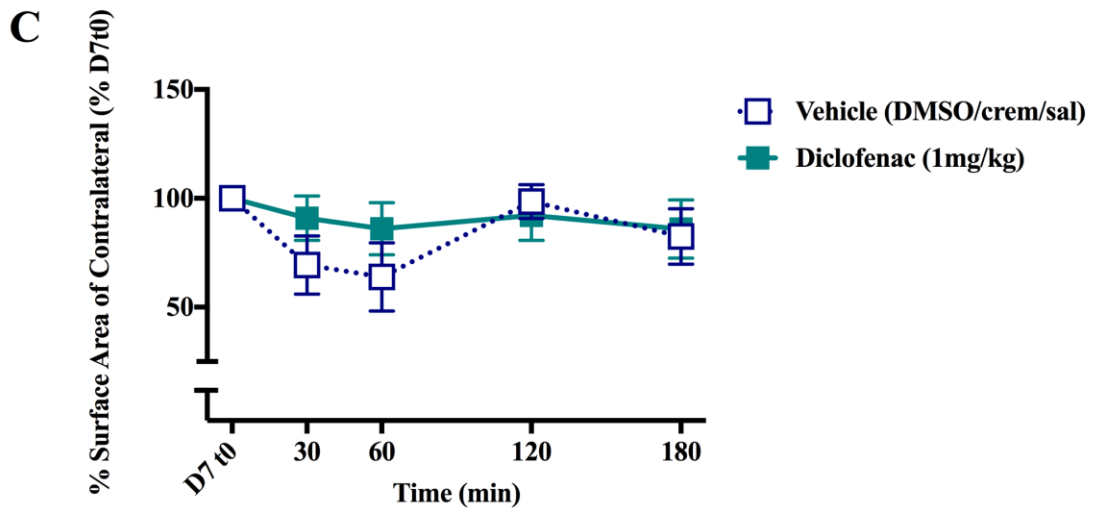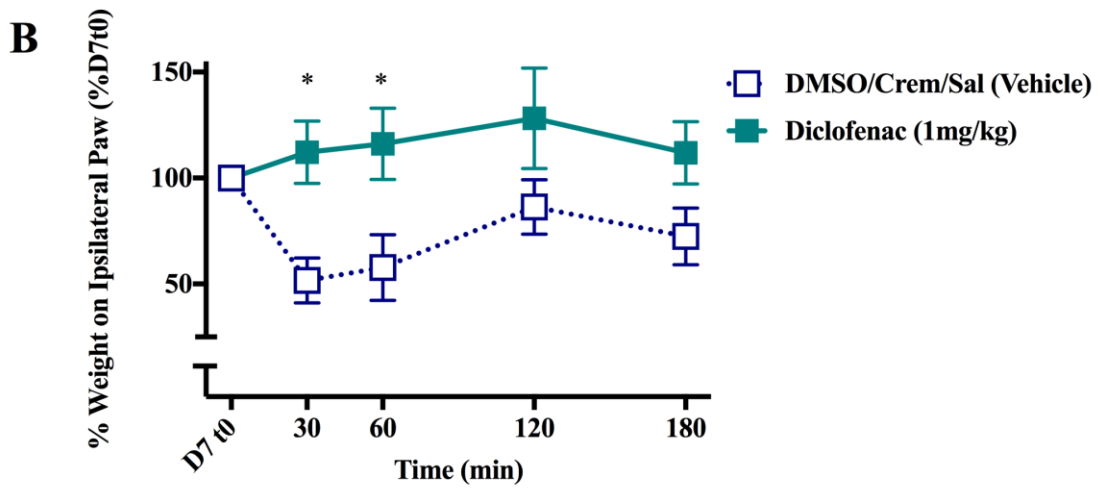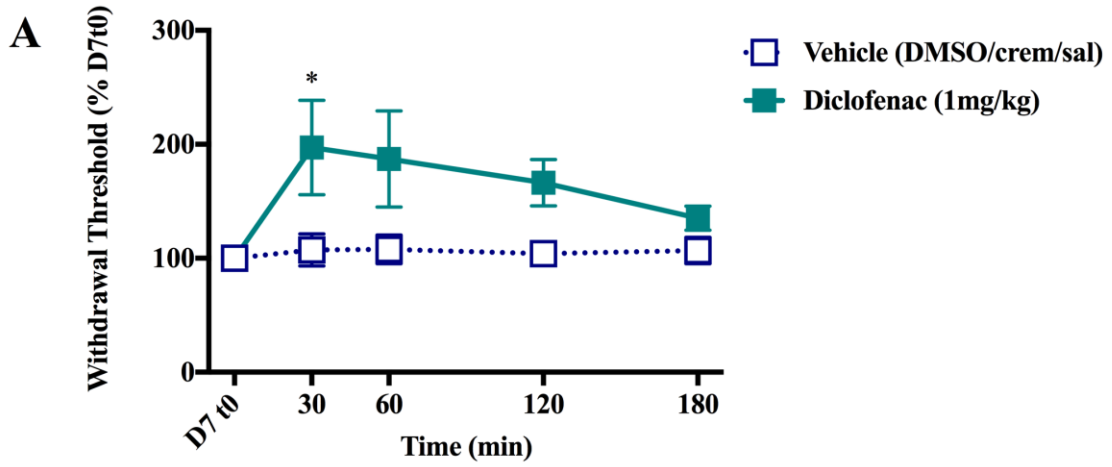
Figure 3.4 Effect of diclofenac on evoked and spontaneous pain behaviour.

(A) Diclofenac significantly improved withdrawal threshold compared to vehicle (DMSO/crem/sal) ($P < 0.05$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 8 per time point). Post-hoc analysis revealed a significant improvement in withdrawal threshold in diclofenac-treated animals at 30 minutes (*$P < 0.05$). **(B)** Diclofenac significantly improved percent weight borne on the ipsilateral paw throughout the three hour time course ($P < 0.0001$, two-way ANOVA with Bonferroni's *post-hoc* test, n = 7-8 per time point). During *post-hoc* analysis, diclofenac improved ipsilateral weight bearing at 30 minutes (*$P < 0.05$) and 60 minutes (*$P < 0.001$). **(C)** Percent contralateral surface area was not improved in diclofenac versus vehicle-treated cohorts ($P > 0.05$, two-way ANOVA, n = 7-8 per time point). Data are standardized as percent day seven baseline and presented as mean ($\pm$ SEM). ANOVA, analysis of variance; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; RM, repeated measures; Sal, saline; t0, time zero.

A

B

C
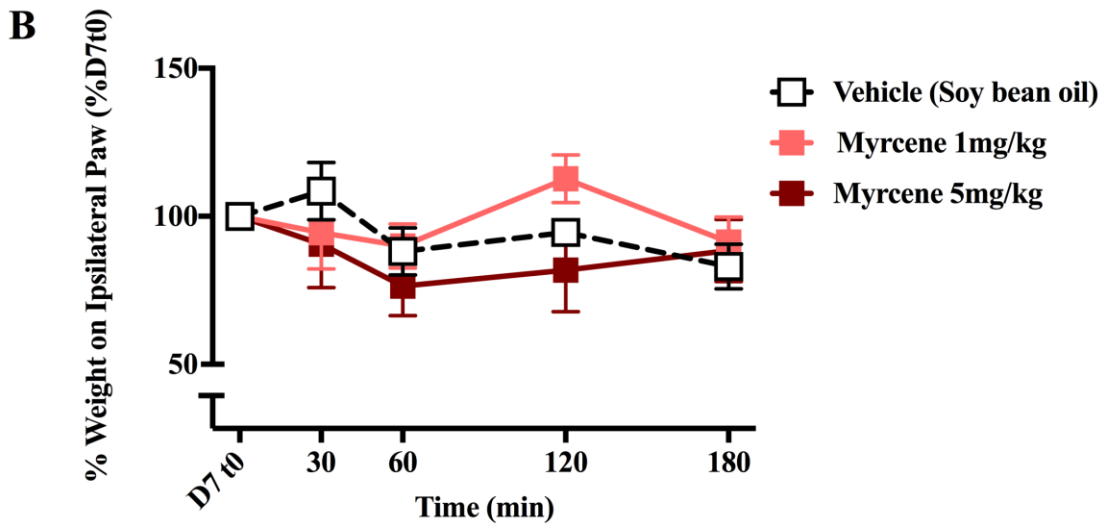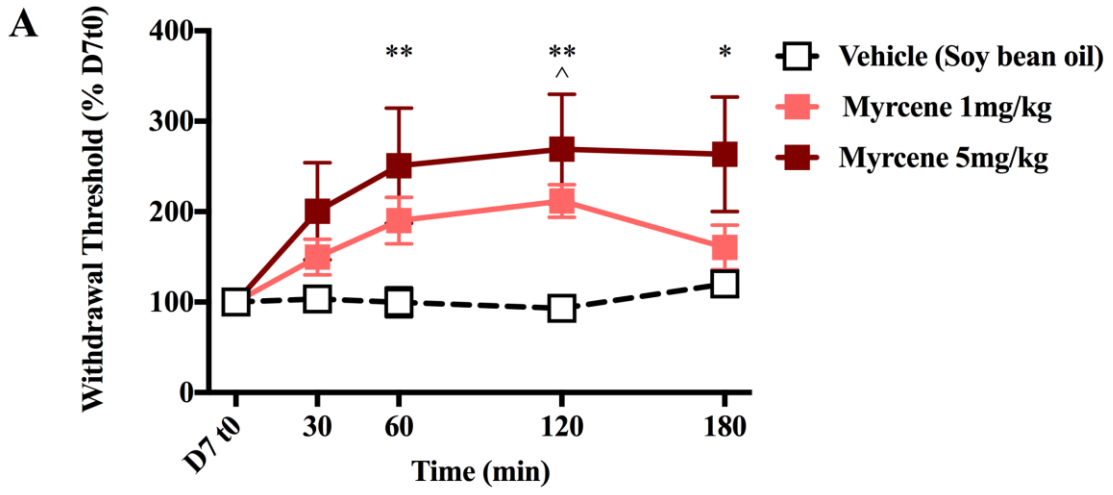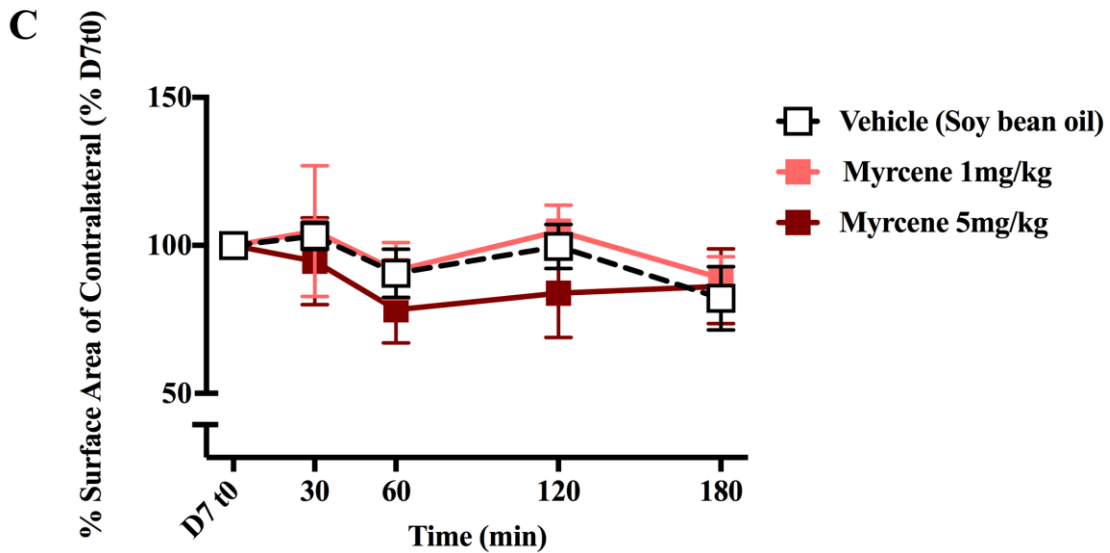
Figure 3.5 Effect of myrcene treatment on evoked and spontaneous behaviour in FCA-injected animals.

(A) There was a significant difference in withdrawal threshold, relative to baseline, between myrcene-treated cohorts and vehicle-treated animals ($P < 0.05$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 8 per time point). *Post-hoc* testing revealed that myrcene (5mg/kg) significantly increased withdrawal threshold at 60 minutes (**$P < 0.01$), 120 minutes (**$P < 0.01$) and 180 minutes (*$P < 0.05$) compared to vehicle. Myrcene (1mg/kg) significantly improved withdrawal threshold at 120 minutes (^$P < 0.05$) in *post-hoc* comparison to vehicle. There were no significant differences between 1mg/kg of myrcene and 5mg/kg of myrcene. **(B)** Weight borne on the ipsilateral limb relative to baseline ($P > 0.05$, two-way RM ANOVA, n = 8 per time point) and **(C)** surface area placed on the ipsilateral paw ($P > 0.05$, two-way RM ANOVA, n = 8 per time point) were not significantly different amongst treatments. Data are standardized to day seven baseline and presented as mean ± SEM. ANOVA, analysis of variance; RM, repeated measures.
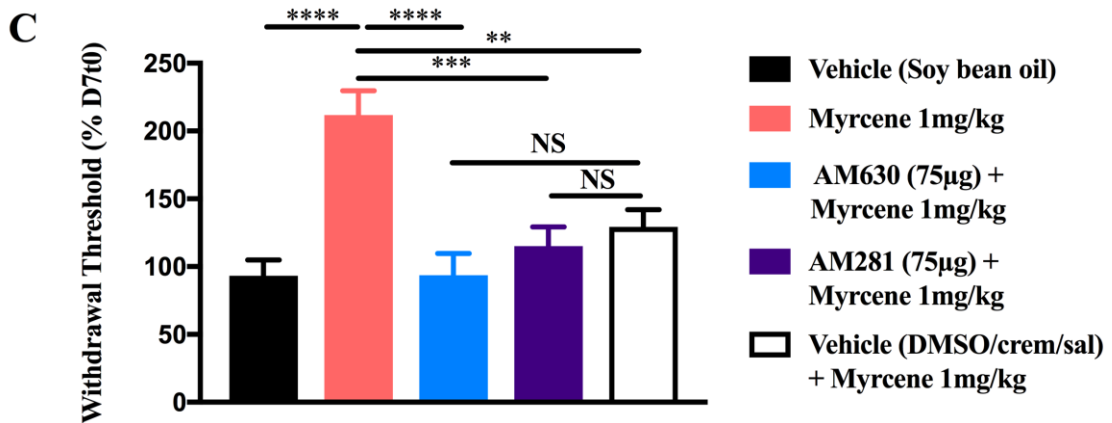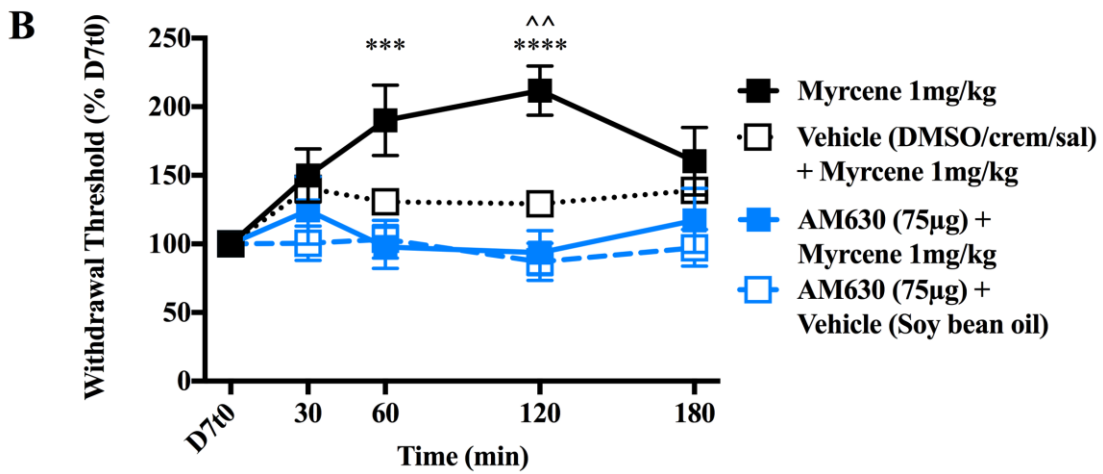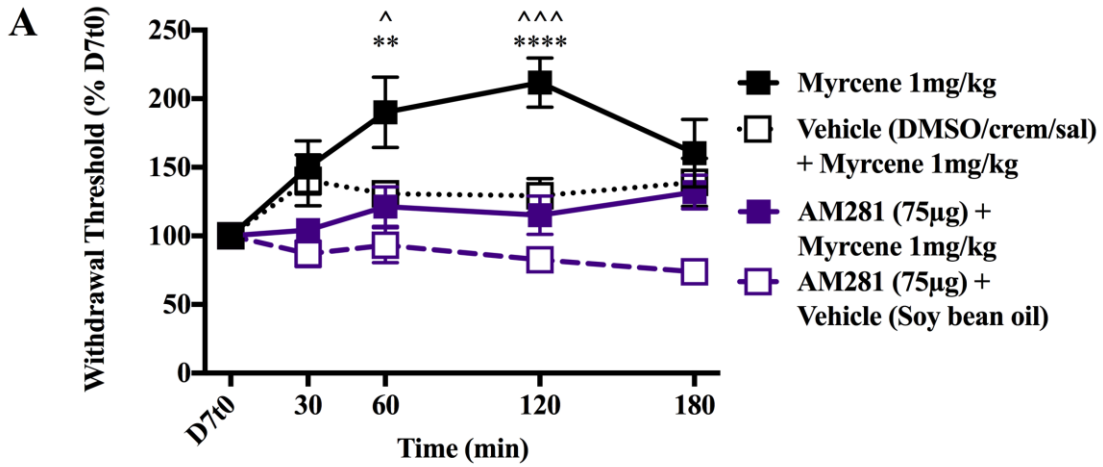
Figure 3.6 The effect of cannabinoid receptor antagonists on myrcene-induced analgesia.

(A) Withdrawal threshold varied significantly between myrcene treatment, antagonist treatments and respective controls in a 3 hour time course ($P < 0.0001$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 8 per time course). *Post-hoc* comparison revealed that the withdrawal threshold in the group receiving AM281 with myrcene compared to myrcene was significantly reduced at 60 minutes (**$P < 0.01$) and 120 (****$P < 0.0001$) minutes. At 60 and 120 minutes, vehicle (DMSO/crem/sal) was significantly reduced compared to the myrcene-treated group (^$P < 0.05$; ^^^$P < 0.001$). AM281 with myrcene and AM281 with vehicle (soy bean oil) comparisons did not show *post-hoc* significance ($P > 0.05$). **(B)** There was a significant difference of withdrawal threshold between myrcene, myrcene plus CB$_2$R antagonist AM630, vehicle (DMSO/crem/sal) with myrcene, and AM630 with vehicle (soy bean oil)-treated groups ($P < 0.01$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 8 per time point). AM630 with myrcene attenuated the improvement in withdrawal threshold observed in the myrcene-treated group at 60 (***$P < 0.001$) and 120 (****$P < 0.0001$) minutes. There was also *post-hoc* significance between myrcene, and vehicle (DMSO/crem/sal) with myrcene at 120 minutes (^^$P < 0.01$). *Post-hoc* comparisons between myrcene plus AM630 and vehicle (soybean oil) with AM630 were not significant ($P > 0.05$). **(C)** Withdrawal threshold amongst treatments, antagonists and appropriate vehicles varied significantly at 120 minutes ($P < 0.0001$, one-way ANOVA with Bonferroni's *post-hoc* test, n = 8 per group). The withdrawal threshold was increased significantly in the myrcene-treated group compared to its vehicle (****$P < 0.0001$). AM630 (****$P < 0.0001$) and AM281 (***$P < 0.001$) with myrcene exhibited significantly reduced withdrawal thresholds *post-hoc* compared to myrcene at 120 minutes. Vehicle (DMSO/crem/sal) plus myrcene showed no post-hoc significance between either CB receptor antagonists ($P > 0.05$), but was significantly reduced compared to myrcene-treatment (**$P < 0.01$). Data are standardized to day seven baseline and presented as mean ± SEM. ANOVA, analysis of variance; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; NS, not significant; RM, repeated measures; Sal, saline; t0, time zero.
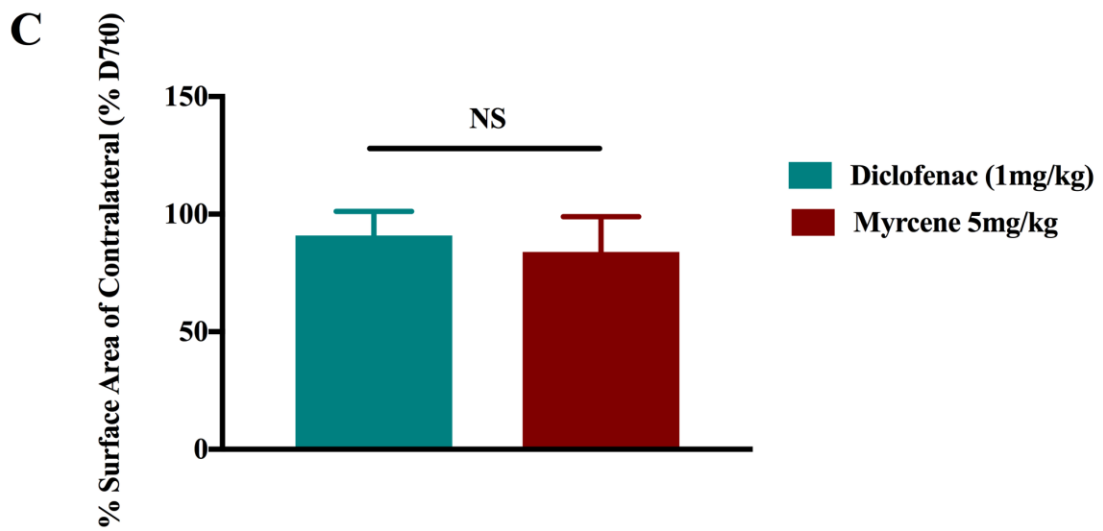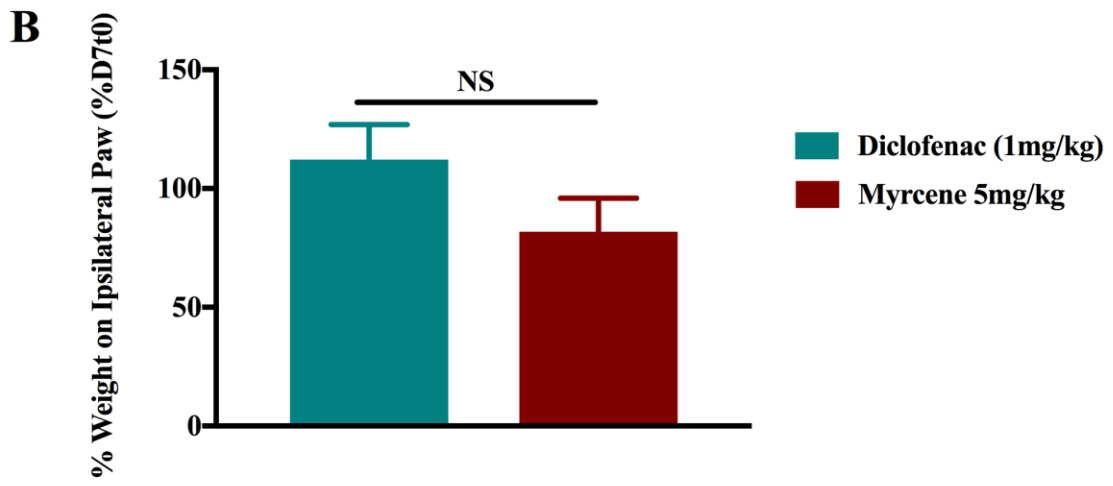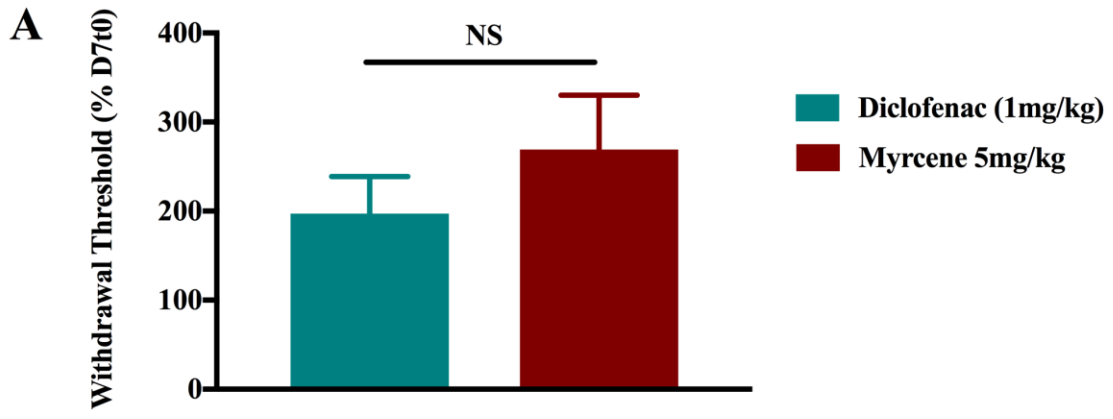
Figure 3.7 Effect of myrcene on pain behaviour compared to the positive control diclofenac at peak effect time.

(A) Improvements in withdrawal threshold were not significantly different between myrcene and diclofenac-treated animals ($P > 0.05$, Mann-Whitney U test, $n = 8$ per group). **(B)** Weight borne on the ipsilateral paw and **(C)** surface area used to weight bear were not significantly different between myrcene and diclofenac-treated cohorts ($P > 0.05$, unpaired Student's t-test, $n = 8$ per group). For these comparisons, the peak effect time was 30 minutes for diclofenac and 120 minutes for myrcene. Data are standardized to baseline on day seven and presented as mean $\pm$ SEM. D7, day seven, DMSO, dimethyl sulfoxide; Crem, cremophor; NS, not significant; Sal, saline; t0, time zero.
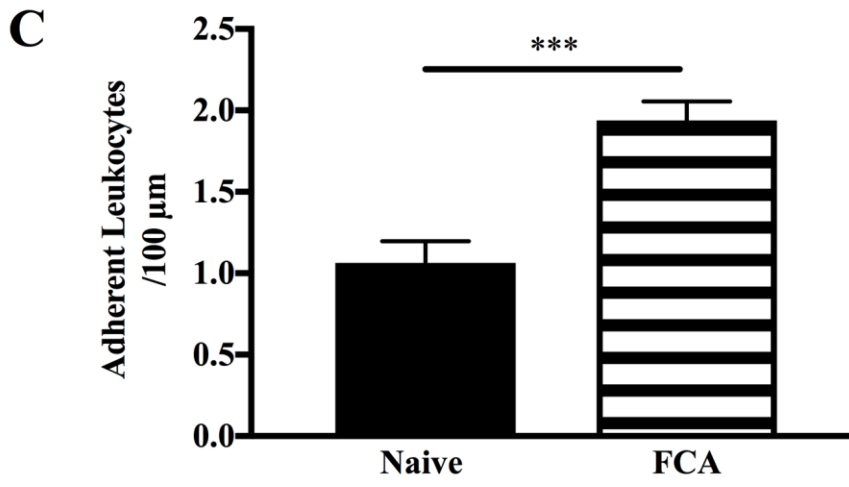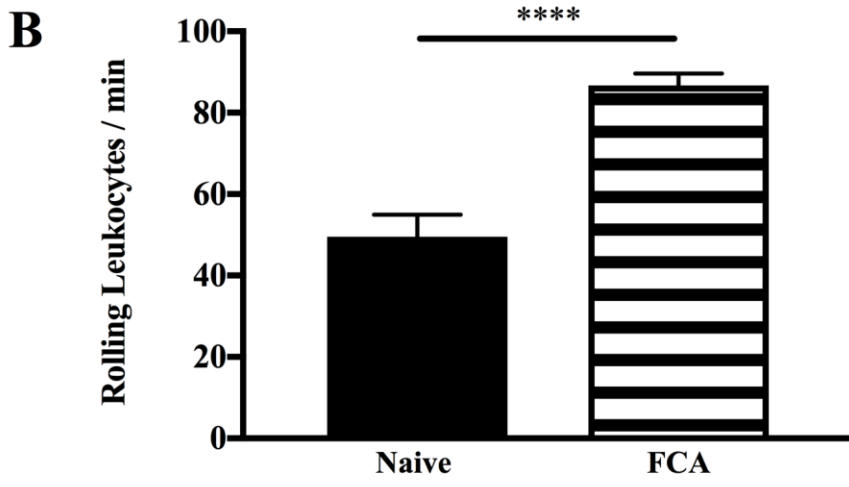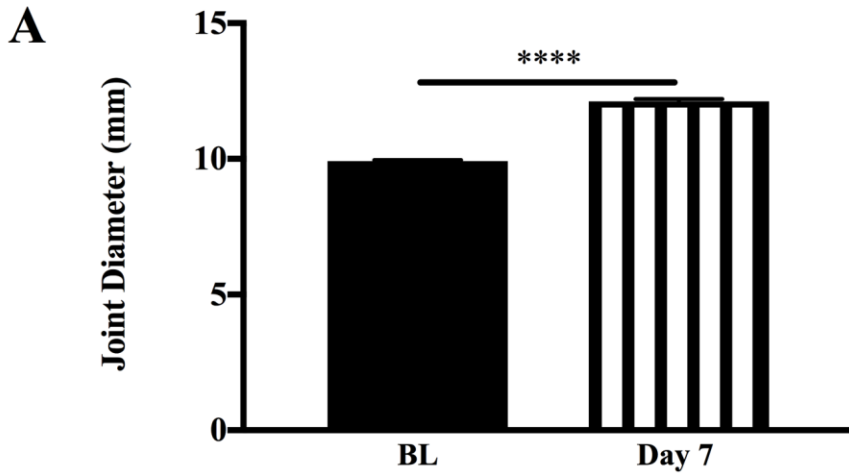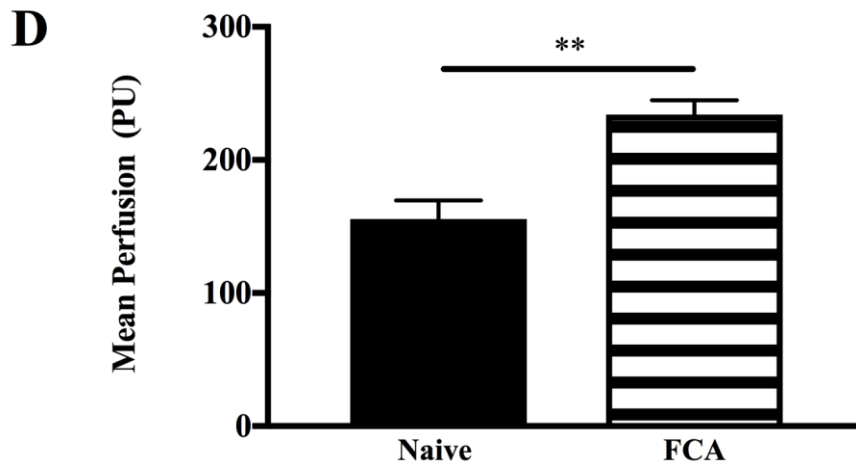
Figure 3.8 Confirmation of inflammation in FCA-injected rats.

(A) Ipsilateral joint diameter was significantly increased in FCA-injected rats on day seven compared to baseline pre-induction measurements (****P < 0.0001, paired Student's t-test, n = 24 per group). **(B)** The FCA cohort had a significantly higher percent change from baseline joint diameters compared to naïve animals (****P < 0.0001, Mann-Whitney U test, n = 11-20 per group). **(C)** The number of rolling leukocytes (****P < 0.0001, unpaired Student's t-test, n = 11-64 per group) and **(D)** adherent leukocytes (****P < 0.0001, unpaired Student's t-test, n = 11-64 per group) were significantly increased in FCA-injected rats on day seven compared to a naïve cohort. **(E)** Mean perfusion in FCA-injected rats on day seven was also significantly increased compared to naïve animals (**P < 0.01, unpaired Student's t-test, n = 11 – 64 per group). Data are presented as mean ± SEM. BL, baseline; FCA, Freund's complete adjuvant; PU, perfusion units.

A

Joint Diameter (% D7 t0)

NS

Vehicle (DMSO/crem/sal)
Diclofenac (1mg/kg)

B

Rolling Leukocytes (% BL)

*** *

Time (min)

□ Vehicle (DMSO/crem/sal)
■ Diclofenac (1mg/kg)

C

Adherent Leukocytes (% BL)

Time (min)

□ DMSO/Crem/Sal (Vehicle)
■ Diclofenac (1mg/kg)
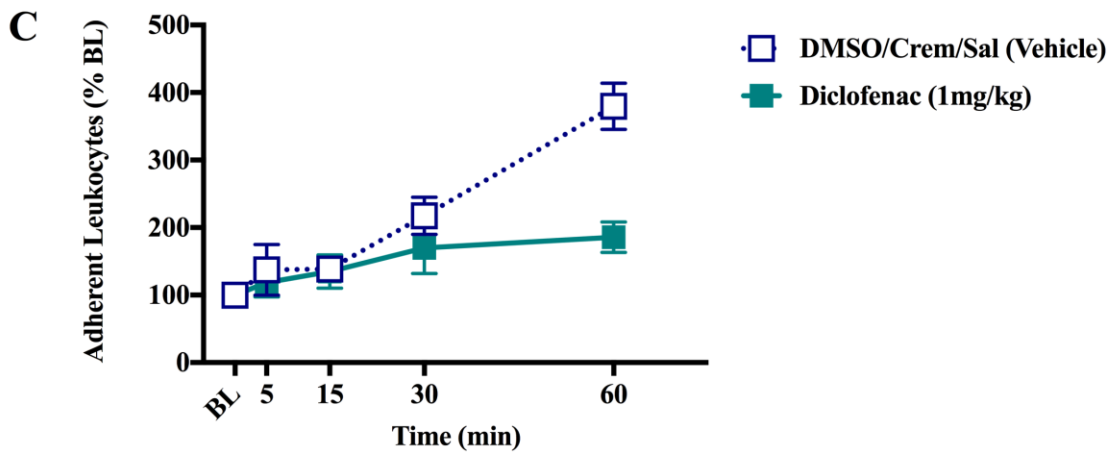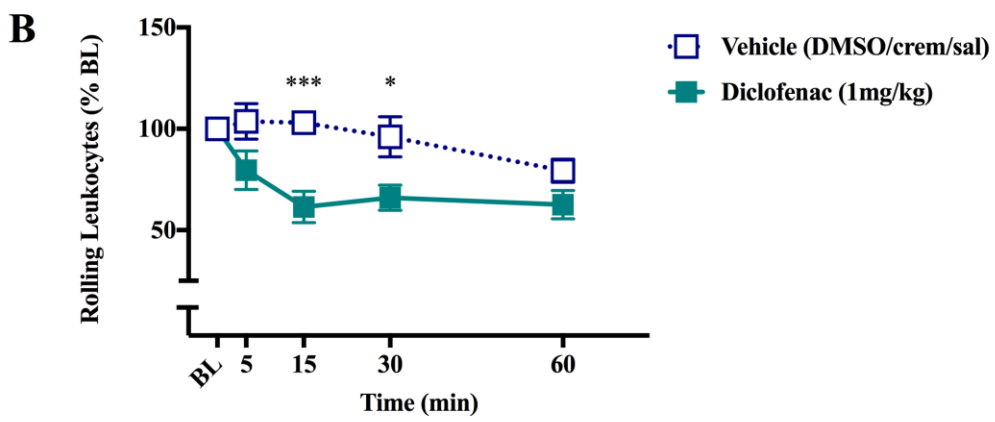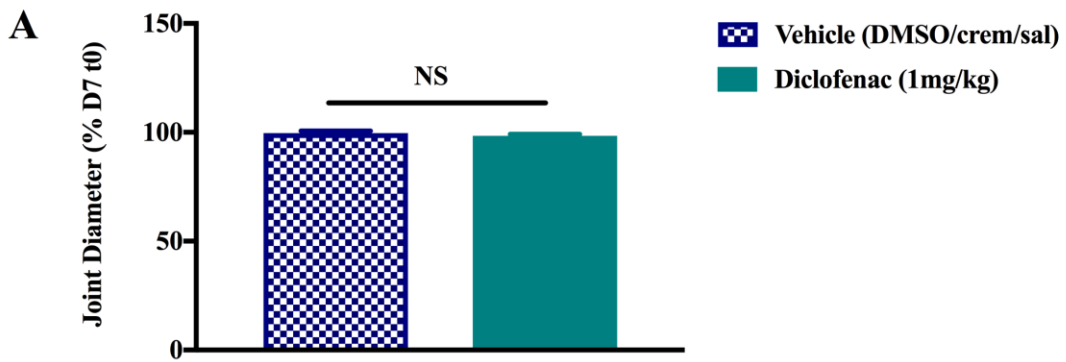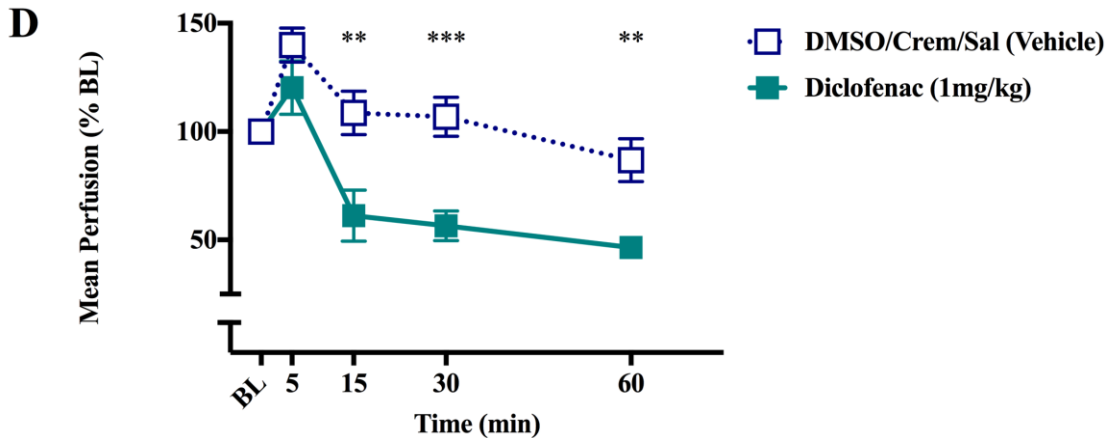
Figure 3.9 Effect of diclofenac compared to vehicle on local inflammation.

(A) Joint diameter, relative to baseline, was not significantly different between diclofenac and vehicle-treated animals ($P < 0.05$, unpaired Student's t-test, n = 8 per group). **(B)** Diclofenac significantly reduced leukocyte rolling compared to vehicle ($P < 0.01$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 6-9 per time point). *Post-hoc* testing showed that diclofenac reduced leukocyte rolling at 15 (***$P < 0.001$) and 30 minutes (*$P < 0.05$). **(C)** There was no significant difference in adherent leukocytes between diclofenac and vehicle-treated animals ($P > 0.05$, two-way RM ANOVA, n = 6-9 per time point) **(E)** Diclofenac also significantly reduced blood flow to the knee joint ($P < 0.01$, two-way ANOVA with Bonferroni's *post-hoc* test, n = 6-9 per time point). *Post-hoc* comparisons showed a significant reduction at 15 minutes (**$P < 0.01$), 30 minutes (***$P < 0.001$) and 60 minutes (**$P < 0.01$). Data are standardized as percent baseline, or percent cumulative change and are presented as mean ($\pm$ SEM). ANOVA, analysis of variance; Crem, cremophor; DMSO, dimethyl sulfoxide; NS, not significant; RM, repeated measures; Sal, saline.
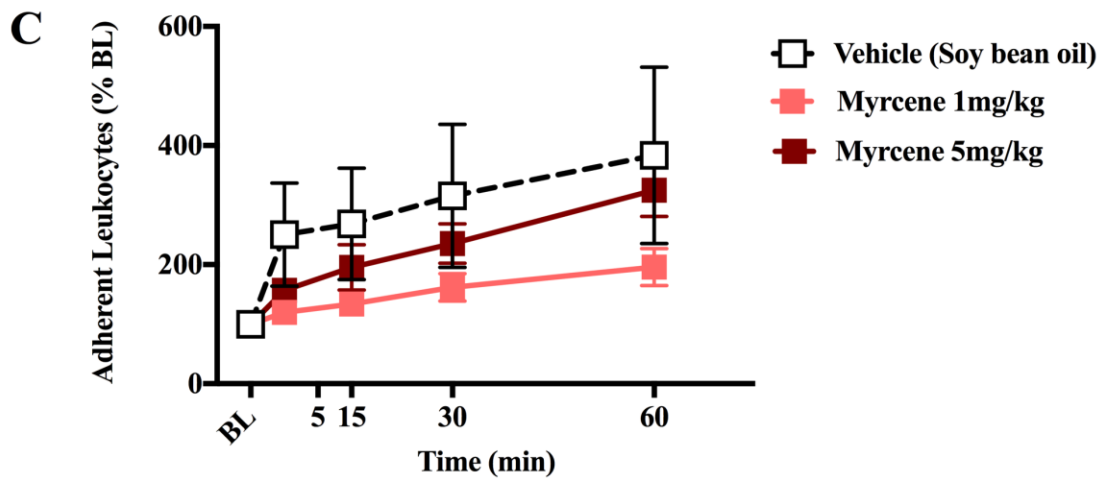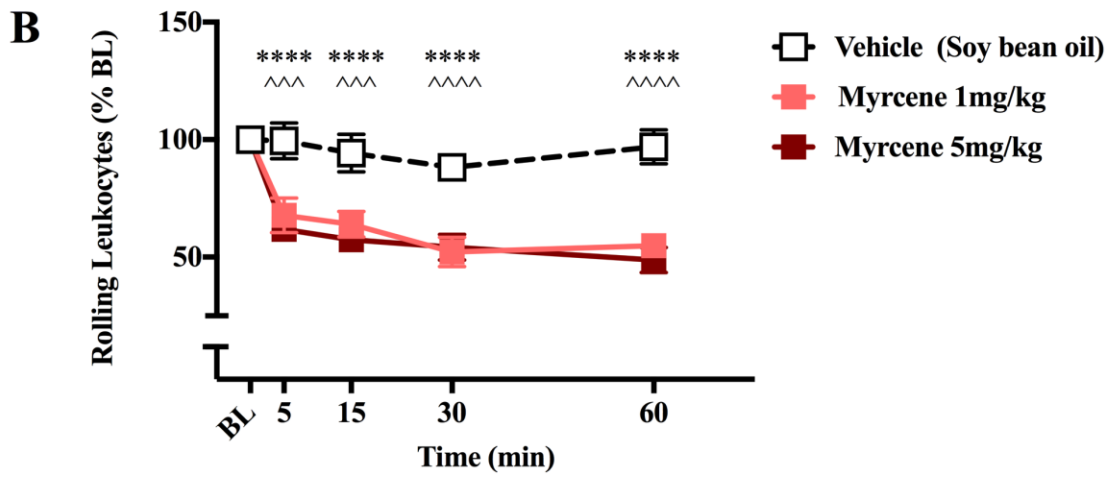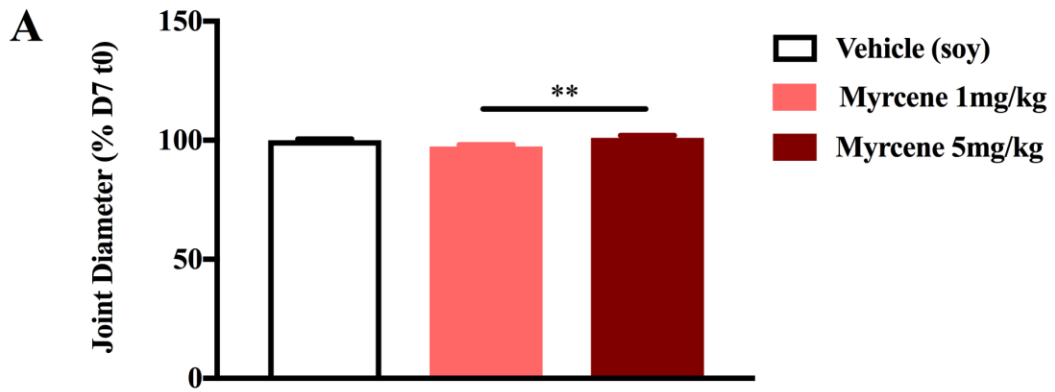
Figure 3.10 The effect of myrcene treatment on leukocyte trafficking and blood perfusion in FCA-inflamed knee joints.

(A) There was significant variation of joint diameter between treatments ($P < 0.01$, one-way ANOVA with Bonferroni's *post-hoc* test, n = 8 per group). Neither myrcene dose varied from vehicle ($P > 0.05$), but there was a significant difference between myrcene doses (**$P < 0.01$). **(B)** Rolling leukocytes, relative to baseline, were significantly different amongst treatments ($P < 0.0001$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 6-7 per time point). Treatment with 1mg/kg and 5mg/kg of myrcene significantly reduced rolling leukocytes compared to vehicle-treatment at 5, 15 (^^^$P < 0.001$; ***$P < 0.001$), 30 and 60 minutes (^^^^$P < 0.0001$; ****$P < 0.0001$) in *post-hoc* analysis. There was no *post-hoc* differences between the myrcene-treated groups ($P > 0.05$). **(C)** Adherent leukocytes relative to baseline were not significantly different between treatment groups ($P > 0.05$, two-way RM ANOVA, n = 6-7 per group). **(D)** Mean perfusion to the affected area did not significantly differ between myrcene-treated and vehicle-treated cohorts ($P > 0.05$, two-way RM ANOVA, n = 6-7 per group). Data are standardized to baseline and presented as mean ± SEM. ANOVA, analysis of variance; BL, baseline; RM, repeated measures.

A

Legend:
- Myrcene 1mg/kg
- Vehicle (DMSO/crem/sal) + Myrcene 1mg/kg
- AM281 (75µg) + Myrcene 1mg/kg
- AM281 (75µg) + Vehicle (Soy bean oil)

Significance markers (top): ** *** **** *

Y-axis: Rolling Leukocytes (% BL), 0 to 150
X-axis: Time (min); BL 5, 15, 30, 60

B

Legend:
- Myrcene 1mg/kg
- Vehicle (DMSO/crem/sal) + Myrcene 1mg/kg
- AM630 (75µg) + Myrcene 1mg/kg
- AM630 (75µg) + Vehicle (Soy bean oil)

Significance markers (top): * ** **

Y-axis: Rolling Leukocytes (% BL), 0 to 150
X-axis: Time (min); BL 5, 15, 30, 60

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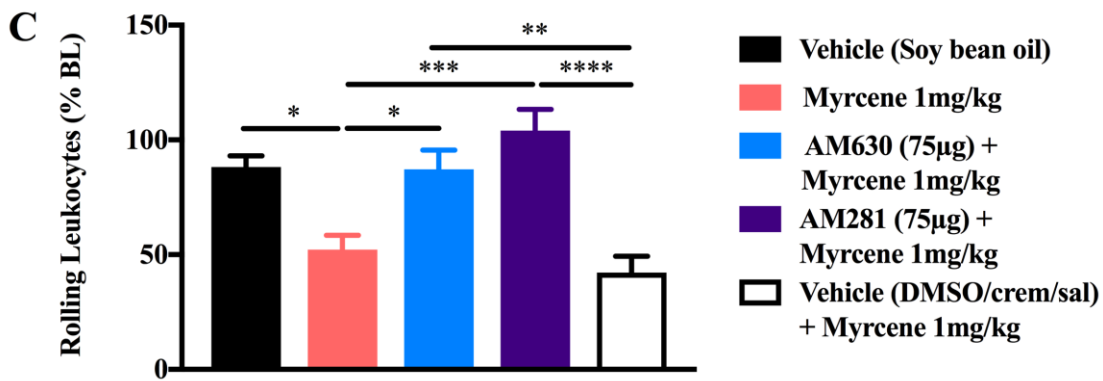
Figure 3.11 The effect of cannabinoid receptor antagonists on myrcene-induced rolling leukocyte reductions.

(A) Leukocyte rolling was significantly different between myrcene, myrcene plus CB_1R antagonist AM281, vehicle (DMSO/crem/sal) with myrcene, and AM281 with vehicle (soy bean oil)-treated groups. ($P < 0.0001$, two-way ANOVA with Bonferroni's *post-hoc* test, n = 5-9 per time point). AM281 with myrcene significantly increased leukocyte rolling at 5 (**$P < 0.01$), 15 (***$P < 0.001$), 30 (****$P < 0.0001$) and 60 (*$P < 0.05$) minutes post-treatment in *post-hoc* testing. Post-hoc comparisons between myrcene and vehicle (DMSO/crem/sal) were not significant ($P < 0.05$) at any time point. Myrcene plus AM281 and vehicle (soy bean oil) *post-hoc* comparisons were also not significant ($P < 0.05$). **(B)** There was a significant difference in rolling leukocytes between groups receiving myrcene, myrcene and the CB_2R antagonist AM630, myrcene and AM630's vehicle, and AM630 plus myrcene's vehicle ($P < 0.0001$, two-way ANOVA with Bonferroni's *post-hoc* test, n = 5-9 per time point). *Post-hoc* analysis showed that AM630 increased leukocyte rolling at 15 (*$P < 0.05$), 30 (**$P < 0.01$) and 60 (**$P < 0.01$) minutes compared to myrcene-treated animals. There was no significant *post-hoc* comparisons between myrcene and myrcene plus antagonist vehicle ($P > 0.05$) during. *Post-hoc* comparison between myrcene and AM630, and AM630 with vehicle (soy bean oil) was not significantly different ($P > 0.05$). Other *post-hoc* comparisons were not relevant to the research objective. **(C)** At 30 minutes, there was a significant overall variation between myrcene, the CB receptor antagonists and respective vehicles ($P < 0.0001$, one-way ANOVA with Bonferroni's *post-hoc* test, n = 5-9 per group). *Post-hoc*

comparisons showed that myrcene reduced leukocyte rolling compared to its vehicle (*P < 0.05). AM630 (*P < 0.05) and AM281 (***P < 0.001) significantly increased rolling leukocytes. Both AM630 and AM281 were significantly different than their vehicle (**P < 0.01/***P < 0.001) in *post-hoc* comparisons. Other *post-hoc* comparisons were not directly pertinent to the research question. Data are standardized to baseline and presented as mean ± SEM. ANOVA, analysis of variance; BL, baseline; Crem, cremophor; DMSO, dimethyl sulfoxide; RM, repeated measures; Sal, saline.
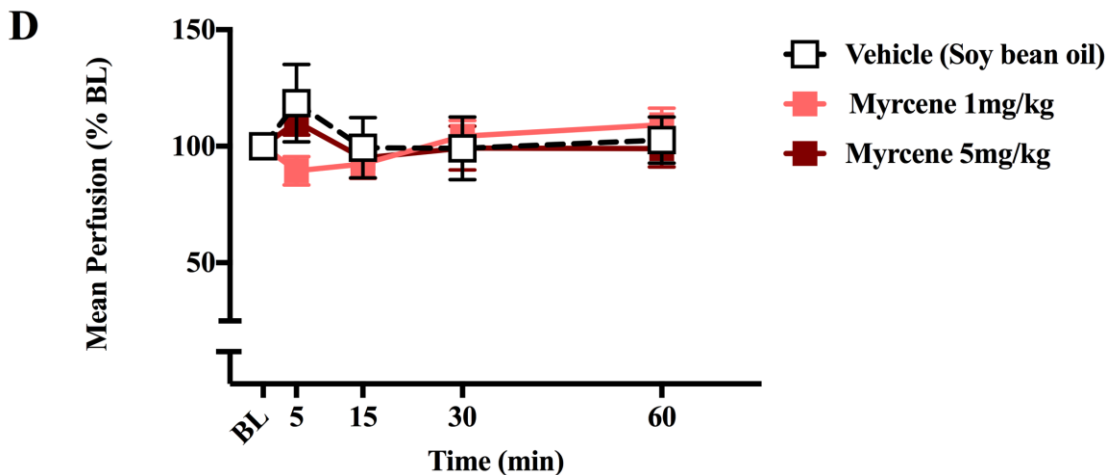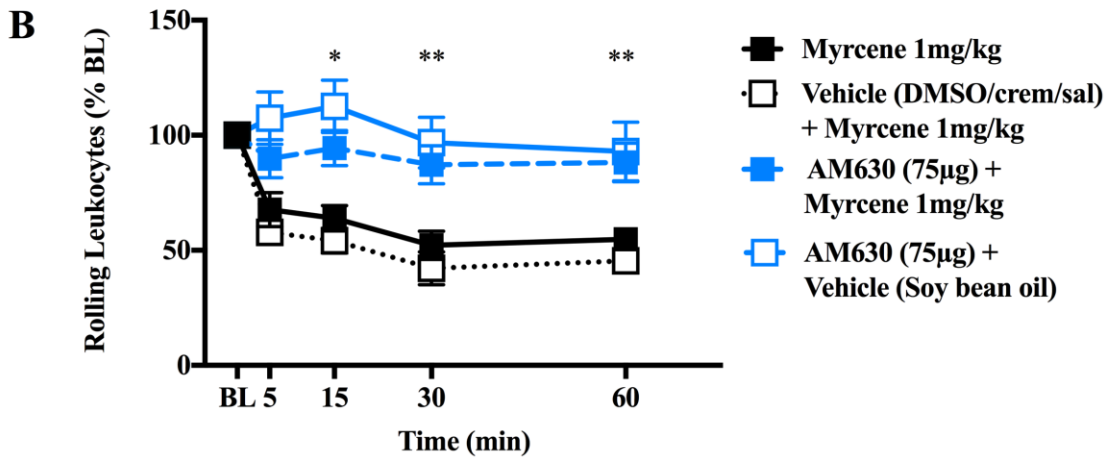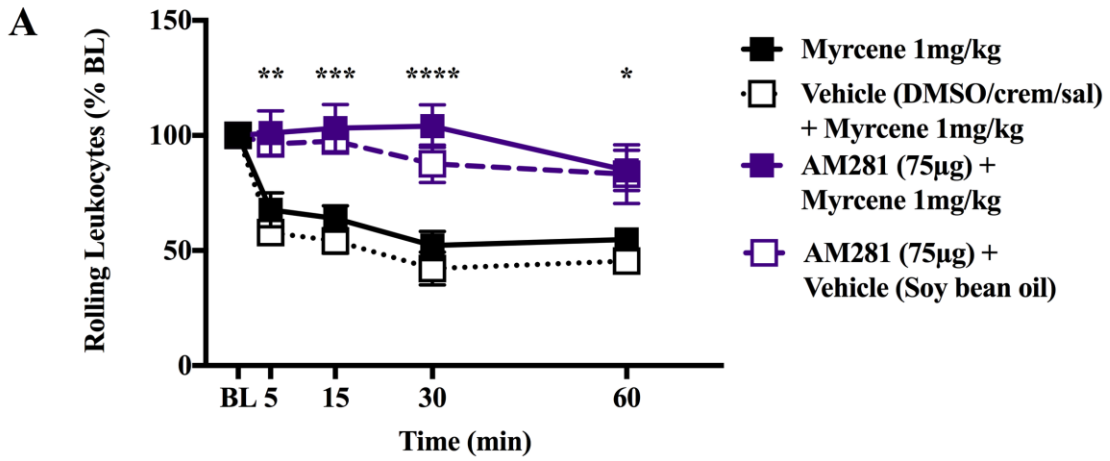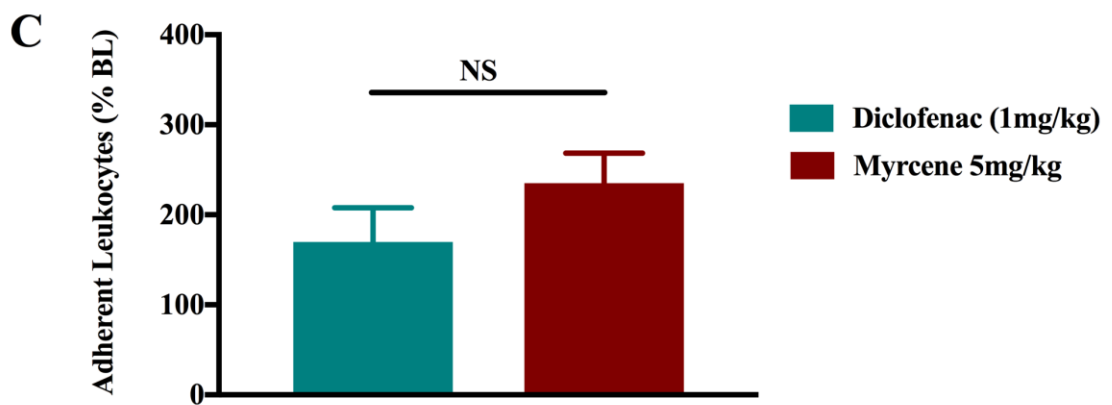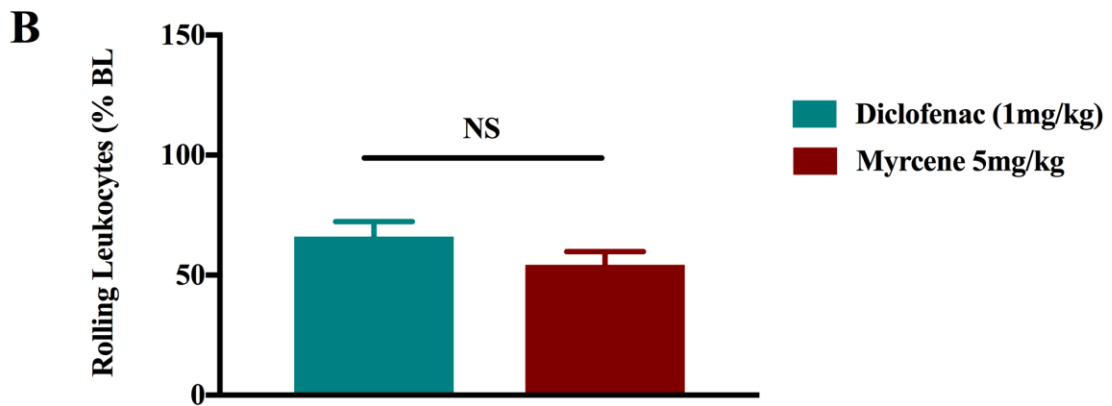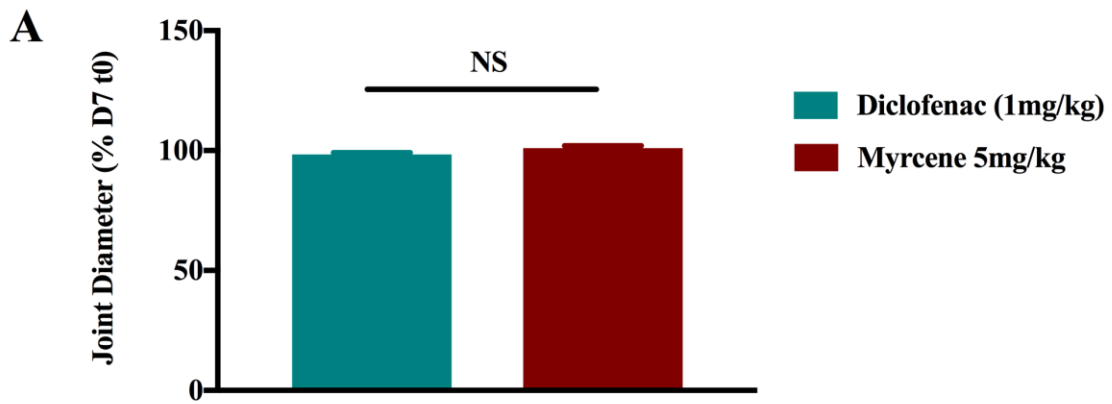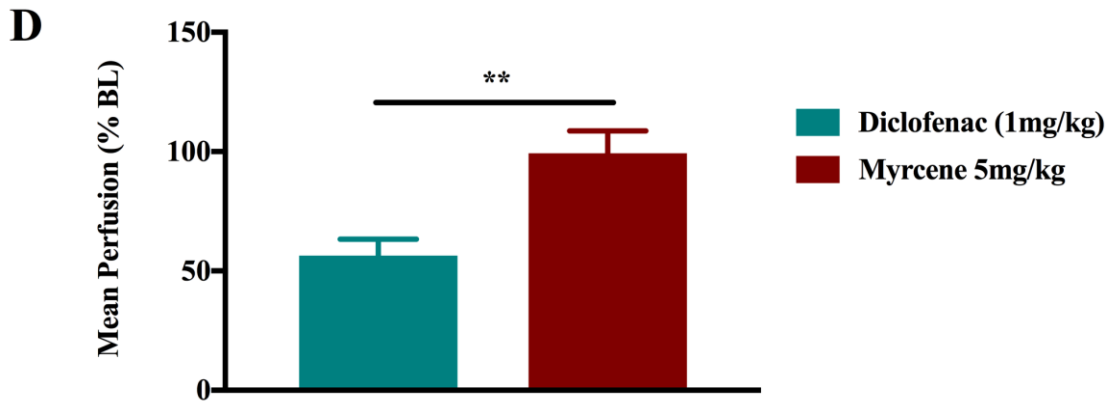
Figure 3.12 Effect of myrcene on inflammation compared to diclofenac.

(A) Joint diameter was not altered between diclofenac and myrcene-treated cohorts (P > 0.05, unpaired Student's t-test, n = 8 per group). There were also no significant changes in **(B)** rolling leukocytes or **(C)** adherent leukocytes between diclofenac and myrcene-treated animals (P > 0.05, unpaired Student's t-test, n = 6-7 per group). (D) Diclofenac treatment significantly reduced blood flow to the knee joint compared to myrcene treatment (**P < 0.01, Mann-Whitney U test, n = 6-7 per group). Data are standardized to percent baseline and presented as mean ± SEM. BL, baseline; NS, not significant.

Chapter 4: The Effects of Repeated Administration of Myrcene on Pain, Inflammation and Joint Damage

Disclosures: The joint histopathology preparation and scoring were conducted and reported by Bolder BioPATH Incorporated.

4.1   Background and hypotheses

Having established the acute effects of myrcene (previous chapter), we wanted to investigate the effect of repeated administration of myrcene on pain behaviour, inflammation and disease progression as many RA treatments are routinely taken throughout the disease to manage chronic symptoms (Nurmohamed and Dijkmans 2005).

One study assessed repeated administration of myrcene on prostaglandin-E_2 induced hyperalgesia (Lorenzetti et al. 1991). The authors found that myrcene provided a consistent level of analgesia over five days of oral treatment, suggesting that animals did not develop tolerance to the chosen dose (Lorenzetti et al. 1991). Myrcene has also been deemed a sedative terpene as it can reduce exploratory behaviour and locomotor coordination, while increasing time spent sleeping at high doses (Do Vale et al. 2002). However, several studies show opposing findings on the effect of myrcene on locomotor behaviour (Da Silva et al. 1991; Do Vale et al. 2002; Paula-Freire et al. 2013). Locomotion is often used as an indicator of nociception in disease models but can also provide insight into motor control and alertness (Deuis et al. 2017). Currently, no literature has examined the effects of repeated administration of myrcene on pain or locomotor activity in the FCA model of RA.

Discovering a therapeutic with the ability to mitigate the inflammatory flares responsible for disease progression could be a valuable advancement (Singh et al. 2015). The anti-inflammatory potential of myrcene has been investigated in a human chondrocyte cell model (Rufino et al. 2015). Researchers showed that myrcene prevented nitric oxide production and also prevented the phosphorylation of integral proteins in the NF-κB, JNK, ERK ½ and p38 pathways (Rufino et al. 2015). Additionally, myrcene reduced MMPs responsible for cartilage damage and increased their respective TIMP

inhibitors (Rufino et al. 2015). These findings suggest that myrcene may be an effective anti-inflammatory mediator and could alter disease progression (Rufino et al. 2015). No studies to our knowledge examined the effect of myrcene on RA chronic disease progression.

We investigated the effect of repeated subcutaneous administration of myrcene on pain behaviour, activity, inflammation and disease progression in FCA monoarthritic rats over the course of 21 days.

Hypotheses tested in this study are as follows:

I. **Repeated administration of myrcene will improve withdrawal threshold, improve dynamic weight bearing, and increase locomotor activity.**

II. **Repeated doses of myrcene will reduce joint oedema, prevent leukocyte trafficking, and reduce pro-inflammatory cytokines.**

III. **Repeated delivery of myrcene will reduce overall joint damage.**

4.2 Methods and experimental design

We chose to repeatedly administer the higher 5mg/kg dose in order to maximize analgesic, anti-inflammatory, and disease modifying potential of the treatment regimen. Baseline (BL) ipsilateral joint diameter, dynamic weight bearing (DWB), activity and von Frey hair measurements were recorded. FCA (50μl) was injected intraarticularly in anesthetized (2% isoflurane at 100% O_2,1L/min) male Wistar rats on day zero (section 2.2). Twenty-four hours post FCA induction (day 1) von Frey, DWB, activity and ipsilateral joint diameter were assessed again before starting the treatment regimen (section 2.3). Cohorts received one of the following treatments:

I. Vehicle (100% soy bean oil; 50μl; subcutaneously over the knee joint)

II. Myrcene (5mg/kg; 50μl in vehicle; subcutaneously over the knee joint)

The identical treatment was administered on days 1, 2, 3, 7, 10, 14, 17 and 21. Treatment days were chosen to model intense early treatment, followed by sustained

maintenance treatments. Pain behaviour measurements (von Frey, DWB and activity) were assessed prior to receiving treatment on each of the listed days and 2 hours post treatment. Joint oedema was assessed only prior to receiving treatment on the given days (section 2.4.1). IVM and LASCA recordings, plasma samples and joints were collected from the same animal cohorts at the end of the study (day 21-22; see section 2.4).

Day 0 (BL): FCA induction
(50µl i.artic)

Von Frey Hair Algesiometry[a], Dynamic Incapacitance[a], Activity[a], Joint Diameter[b]

IVM & LASCA (n=7-8)

(day)

1   2   3   7   10   14   17   21

Treatment

Plasma (n=7-8) & joint collection (n=4)

VEHICLE – soy
(50µl; s.c.)

N = 8-12

MYRCENE
(5mg/kg; s.c.)

N = 8-12

[a]Measurements were performed prior to receiving treatment and 2 hours post treatment on the given day
[b]Measurements were recorded only prior to receiving treatment

4.3   Results

4.3.1   *Repeated administration of myrcene attenuates mechanical allodynia but not spontaneous pain behaviour*

The effect of myrcene on withdrawal threshold throughout the time course is shown in Figures 4.1A and 4.1B. These data did not pass normality, but were tested using a parametric two-way ANOVA in the absence of a non-parametric equivalent. Withdrawal threshold before receiving treatment each day was significantly increased in the myrcene-treated cohort compared to vehicle (Figure 4.1A, $P<0.0001$, n = 12 per time point). *Post-hoc* testing revealed that myrcene improved withdrawal threshold on days 3 ($P < 0.01$), 7 ($P < 0.001$), 10 ($P < 0.05$), 14 ($P < 0.01$), 17 ($P < 0.001$) and 21 ($P < 0.05$). There was also a significant improvement of withdrawal threshold in the myrcene-treated cohort compared to vehicle controls 2 hours after treatment (Figure 4.1B, $P < 0.0001$, n =

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11-12 per time point). Myrcene-treated animals exhibited an improved withdrawal threshold on days 1 ($P < 0.01$), 2 ($P < 0.001$), 3 ($P < 0.01$), 7 ($P < 0.0001$), 10 ($P < 0.01$), 14 ($P < 0.05$), 17 ($P < 0.01$) and 21 ($P < 0.0001$).

There was no significant change in the amount of weight borne by the ipsilateral hind paw during pre-treatment analysis (Figure 4.2A1, $P > 0.05$, n = 8 per time point) or post treatment (Figure 4.2A2, $P < 0.05$, n = 7-8 per time point) between myrcene-treated or vehicle-treated animals. Pre-treatment (Figure 4.2B1, $P > 0.05$, n = 8 per time point) and post-treatment (Figure 4.2B2, $P > 0.05$, n = 7-8 per time point) analysis of ipsilateral paw surface area used to bear weight showed no significant difference between treatment cohorts.

4.3.2   *Locomotor activity is not altered by repeated treatment with myrcene*

Repeated treatment with myrcene did not significantly increase quadrant crossing during pre-treatment (Figure 4.3A1, $P > 0.05$, n = 8 per time point) or post-treatment (Figure 4.3A2, $P > 0.05$, n = 8 per time point) analysis when compared to vehicle controls. Myrcene-treated animals exhibited no difference in the number of rears during pre-treatment (Figure 4.3B1, $P > 0.05$, n = 8 per time point). No significant difference occurred throughout the 2 hour time course (Figure 4.3B2, $P > 0.05$, n = 8 per time point). Time spent rearing was also not significantly different in myrcene-treated and vehicle-treated animals in either pre-treatment investigations (Figure 4.3C1, $P > 0.05$, n = 8 per time point) or during post-treatment analysis (Figure 4.3C2, $P > 0.05$, n = 8 per time point).

4.3.3   *Myrcene treatment blood flow and leukocyte trafficking but not chronic oedema*

In the 21 day time course, joint oedema measurements were significantly different in the myrcene treated cohort compared to their vehicle counterparts (Figure 4.4, $P > 0.05$, n = 12 per time point). Specific time points were not significantly different between the groups in *post-hoc* testing. At the conclusion of the time course, myrcene-treated animals had fewer rolling leukocytes (Figure 4.5A, $P < 0.01$, n = 7-8 per group) and fewer adherent leukocytes (Figure 4.5B, $P < 0.05$, n = 7-8 per group) compared to control treated animals. Blood perfusion to the affected knee joint was also significantly reduced

by repeated myrcene treatment compared to vehicle treated controls at the conclusion of the time course (Figure 4.5C, $P < 0.01$, n = 7-8).

4.3.4   *Myrcene does not alter any systemic cytokine levels after repeated treatment*

Analysis of systemic inflammation showed that repeated treatment with myrcene did not significantly alter levels of IL-1β (Figure 4.6A, $P > 0.05$, n = 7 per group), IL-17A (Figure 4.6B, $P > 0.05$, n = 7 per group), IL-6 (Figure 4.6C, $P > 0.05$, n = 7 per group), or TNF-α (Figure 4.6D, $P > 0.05$, n = 7 per group). Levels of IL-10 were also unchanged by myrcene treatment (Figure 4.6E, $P > 0.05$, n = 7 per group). Levels of these cytokines were lower than detectable limits in naïve animals.

4.3.5   *Myrcene is unable to reverse joint damage after repeated treatment*

Repeated treatment with myrcene did not reverse overall summed joint damage (Figure 4.7C, $P < 0.05$, n = 4 per group). Recurrent treatment also had no significant effect on joint inflammation (Figure 4.8A, $P < 0.05$, n = 4 per group), cartilage destruction (Figure 4.XB, $P < 0.05$, n = 4 per group), pannus formation (Figure 4.8C, $P < 0.05$, n = 4 per group) or bone resorption (Figure 4.8D, $P < 0.05$, n = 4 per group) in histologically analyzed knee joints.

4.4   Chapter summary

Repeated administration of myrcene over the 21 day chronic time course improved withdrawal threshold, an evoked measure of pain, but did not improve spontaneous pain parameters such as ipsilateral weight bearing or hindpaw surface area utilized throughout the time course. Locomotor activity was unchanged during pre-treatment and post-treatment analysis (Table 4.3). Overall, repeated treatment with myrcene improved local inflammation as evidenced by reducing leukocyte trafficking and blood perfusion to the affected area. Myrcene had no significant effect on oedema, systemic inflammation or joint damage compared to vehicle controls (Table 4.4).

4.5   Tables

Table 4. 1 Histopathological breakdown of inflammation, pannus, cartilage and bone resorption scores in knee joints from rat cohorts receiving either vehicle or myrcene treatment over a 21 day time course. Scoring and histological preparations were performed/reported by Bolder BioPath, Boulder, Colorado, USA (Bendele 2019).

Group			Histopathology Scores (0-5)		
Vehicle treated	**Inflammation**	**Pannus**	**Cartilage**	**Bone Resorption**	**Summed Score**
CV1	3	0.5	0.5	0.5	4.5
CV2	3	0.5	0.5	0.5	4.5
CV3	2	0.5	0.5	0	3
CV4	1	0	0	0	1
Myrcene treated	**Inflammation**	**Pannus**	**Cartilage**	**Bone Resorption**	**Summed Score**
CM1	3	0.5	0.5	0.5	4.5
CM2	4	0.5	0.5	0.5	5.5
CM3	3	0.5	0.5	0.5	4.5
CM4	2	0.5	0.5	0	3

CV, chronic vehicle; CM, chronic myrcene.

Table 4. 2 Histopathological observations of knee joints in rat cohorts receiving either repeated vehicle or myrcene treatment over a 21 day time course. Observations, made by Bolder BioPath (Colorado, USA), were provided with the internal report (Bendele 2019).

Group	Histopathological Observation
Vehicle Treated	
CV1	Moderate synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, cartilage damage and bone resorption. Microspheres approximately 30µm diameter in infiltrate.
CV2	Moderate synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, cartilage damage and bone resorption. Microspheres approximately 30µm diameter in infiltrate.
CV3	Mild synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, and cartilage damage. Focal iatrogenic injury to lateral tibia (needle stick). Microspheres approximately 30µm diameter in infiltrate.
CV4	Minimal synovial/periarticular infiltrate of macrophages mainly, focal iatrogenic injury to lateral tibia (needle stick). Microspheres approximately 30µm diameter in infiltrate.
Myrcene Treated	
CM1	Moderate synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, cartilage damage and bone resorption. Microspheres approximately 30µm diameter in infiltrate.
CM2	Marked synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, cartilage damage and bone resorption. Microspheres approximately 30µm diameter in infiltrate.
CM3	Moderate synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, cartilage damage and bone resorption. Microspheres approximately 30µm diameter in infiltrate.
CM4	Mild synovial/periarticular infiltrate of macrophages mainly, fewer neutrophils, marginal zone pannus, and cartilage damage. Focal iatrogenic injury to lateral tibia (needle stick). Microspheres approximately 30µm diameter in infiltrate.

CV, chronic vehicle; CM, chronic myrcene.

Table 4. 3 Pain behaviour result summary.

Parameter	Pre-treatment	Post-treatment
Pain behaviour summary	Myrcene improved evoked but not spontaneous pain	
Von Frey: withdrawal threshold	Improved withdrawal threshold	Improved withdrawal threshold
DWB: ipsilateral weight bearing	No improvement	No improvement
DWB: ipsilateral surface area	No improvement	No improvement
Activity summary	Myrcene largely did not enhance locomotor activity	
Quadrant crossing	No improvement	No improvement
Quantity of rears	No improvement	No improvement
Time spent rearing	No improvement	No improvement

DWB, dynamic weight bearing.

Table 4. 4 Inflammation and joint damage result summary.

Parameter	Result summary
Inflammation summary	Myrcene reduced local but not systemic inflammation
Joint oedema	No improvement in joint oedema
Leukocyte trafficking: rolling	Reduced rolling leukocytes
Leukocyte trafficking: adherent	Reduced adherent leukocytes
Blood perfusion to affected area	Reduced blood perfusion
Cytokine analysis	No improvement in cytokine levels
Joint damage summary	Myrcene did not alter joint damage
Inflammation infiltration	No improvement
Pannus formation	No improvement
Cartilage destruction	No improvement
Bone resorption	No improvement

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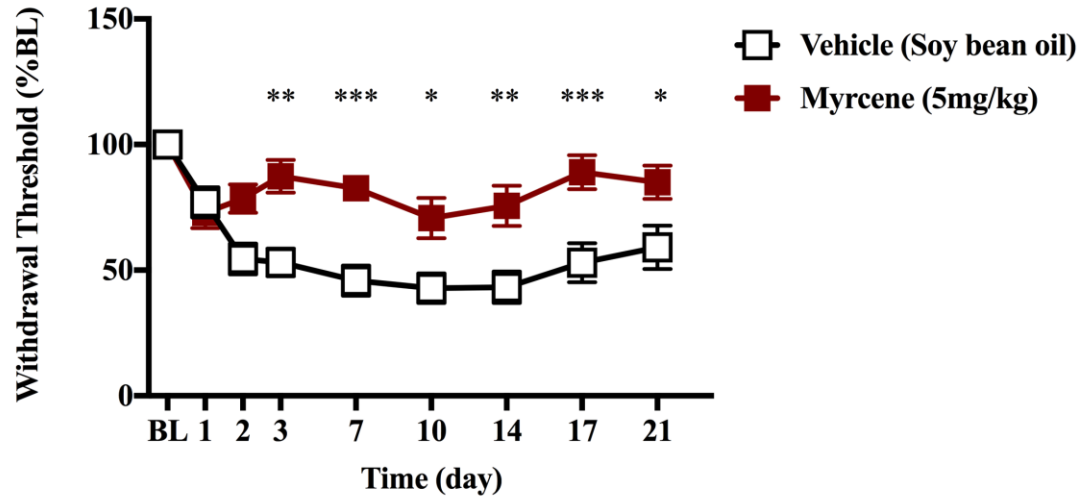
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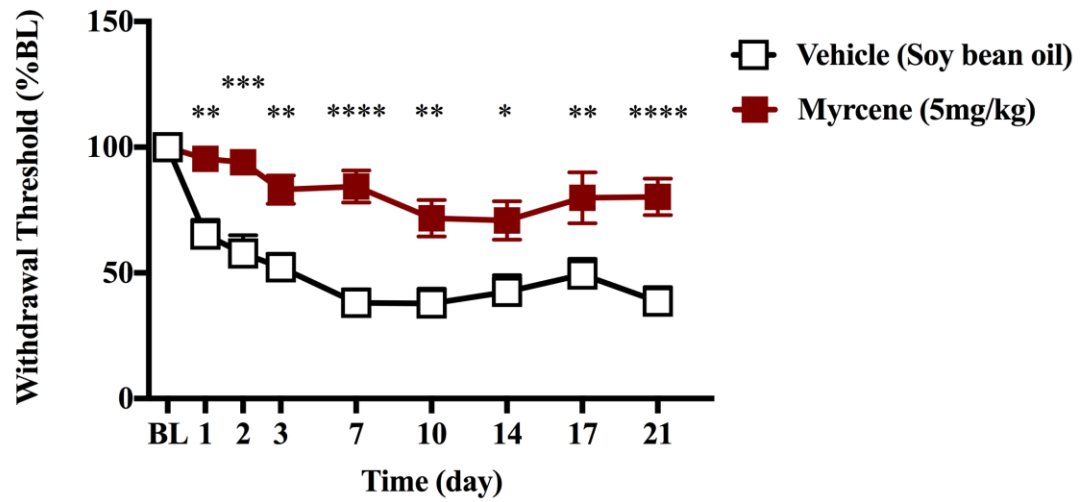
Figure 4.1 The effect of repeated myrcene treatment on evoked pain behaviour.

(A) Withdrawal threshold was significantly higher in the myrcene versus vehicle-treated cohort (P < 0.0001, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 12 per time point) when tested prior to receiving treatment each day. *Post-hoc* analysis revealed that myrcene treatment improved withdrawal threshold on days 3 (**P < 0.01), 7 (***P < 0.001), 10 (*P < 0.05), 14 (**P < 0.01), 17 (***P < 0.001) and 21 (*P < 0.05). **(B)** Withdrawal threshold was also significantly higher two hours post-treatment in the myrcene-treated cohort versus vehicle controls (P < 0.0001, two-way ANOVA with Bonferroni's *post-hoc* test, n = 11-12 per time point). *Post-hoc* comparisons were significant on days 1 (**P < 0.01), 2 (***P < 0.001), 3 (**P < 0.01), 7 (****P < 0.0001), 10 (**P < 0.01), 14 (*P < 0.05), 17 (**P < 0.01) and 21 (****P < 0.0001). Withdrawal threshold data over the 21-day time course did not pass normality, but was analyzed using a parametric two-way ANOVA in the absence of a non-parametric equivalent. An ordinary two-way ANOVA was used due to a difference in data measurements at one of the time points. Data are standardized to percent baseline and presented as mean ± SEM. ANOVA, analysis of variance; BL, baseline; RM, repeated measures.

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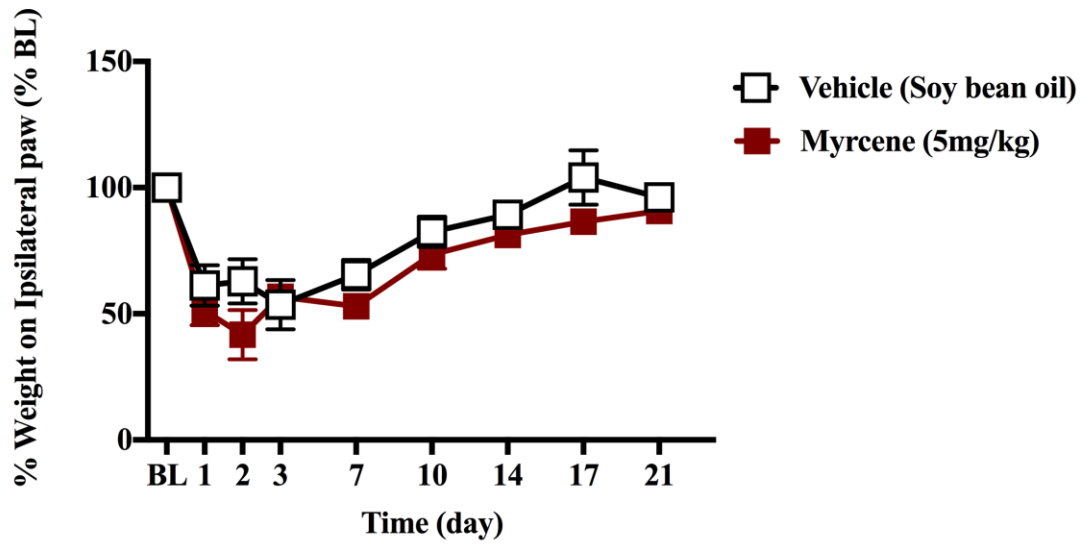
A2
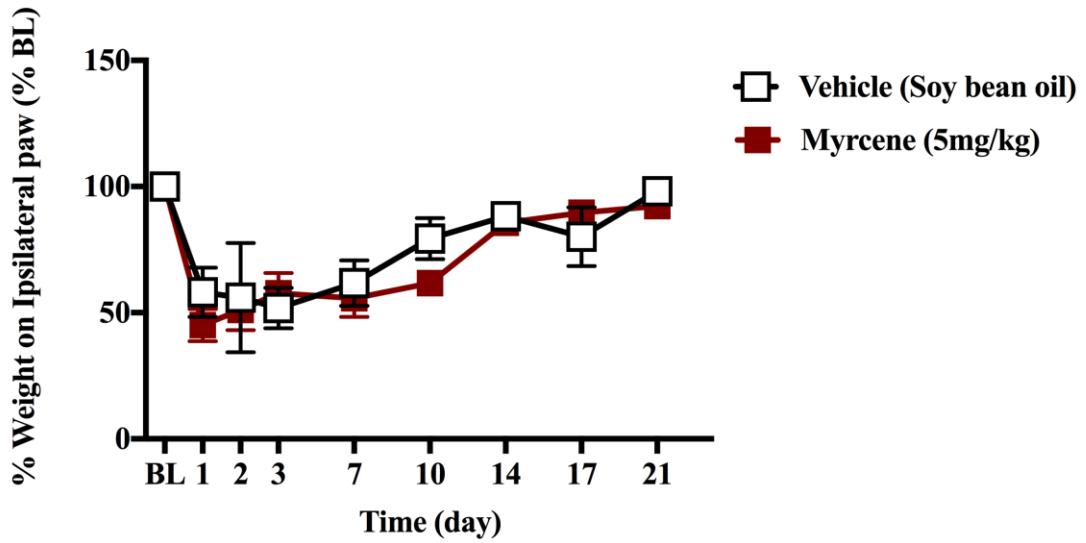
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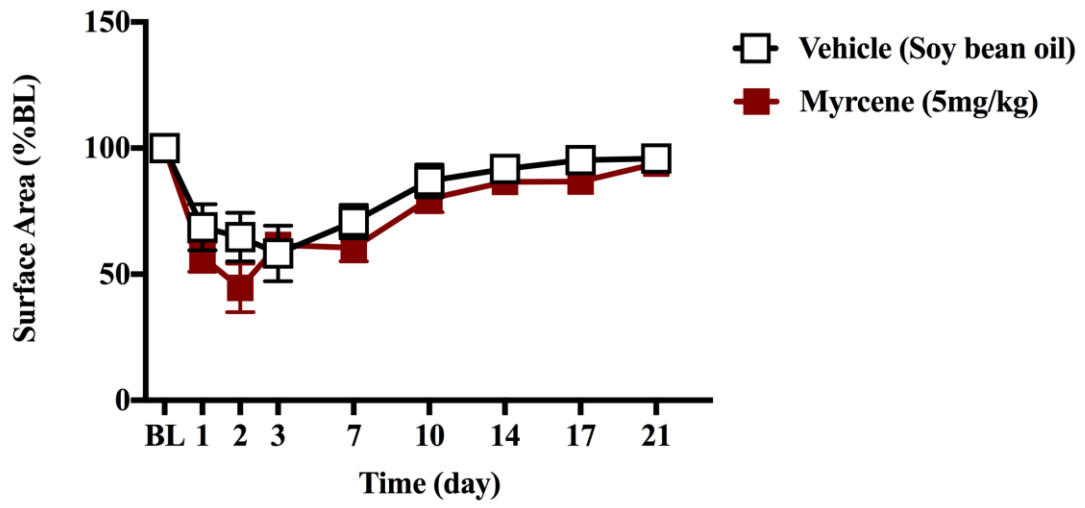
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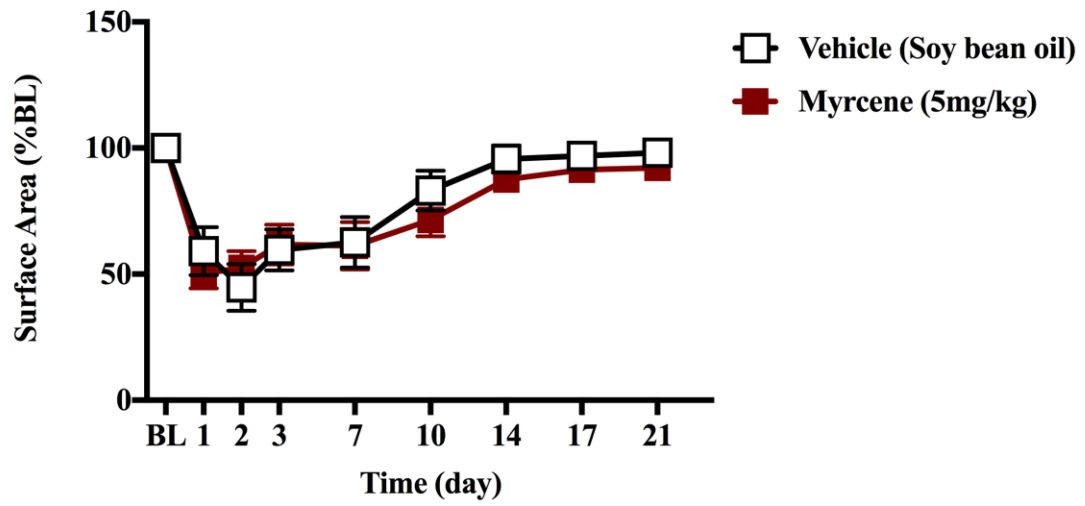
Figure 4.2 Effect of repeated myrcene treatment on spontaneous pain behaviour.

Dynamic weight bearing on the ipsilateral paw was not significantly different in the myrcene-treated cohort compared to vehicles when investigated (**A1)** prior to treatment (P > 0.05, two-way RM ANOVA, n = 8 per group) or **(A2)** 2 hours post-treatment (P > 0.05, two-way RM ANOVA, n = 8 per time point). Surface area of the ipsilateral paw was not significantly different in both **(B1)** pre-treatment (P > 0.05, two-way ANOVA, n = 7-8 per time point) and **(B2)** post-treatment analysis (P > 0.05, two-way ANOVA, n = 7-8 per time point). Post-treatment testing used an ordinary two-way ANOVA due to a missing data point. Data are standardized to baseline and presented as mean ± SEM. ANOVA, analysis of variance; BL, baseline; RM, repeated measures.

B1

B2

C1

C2
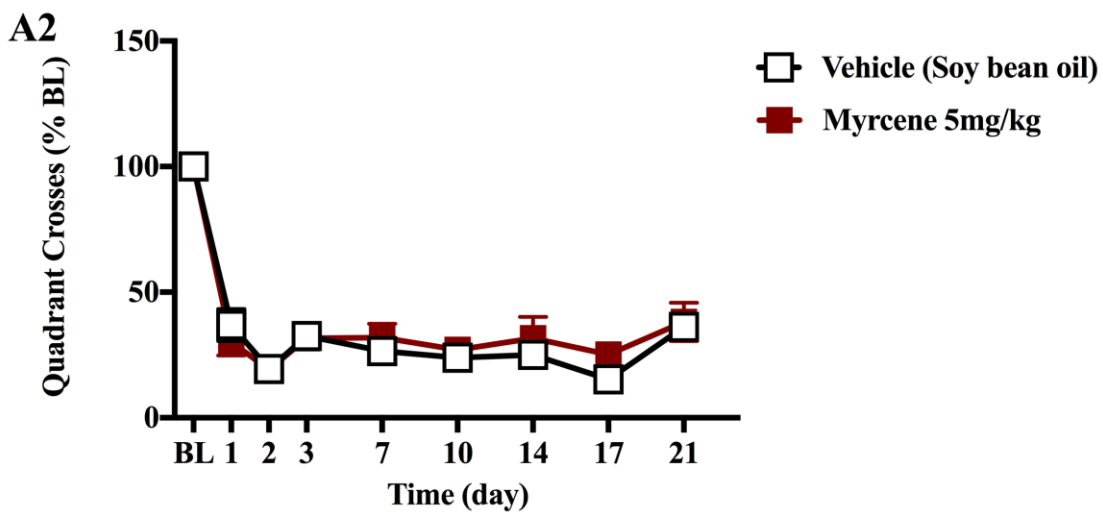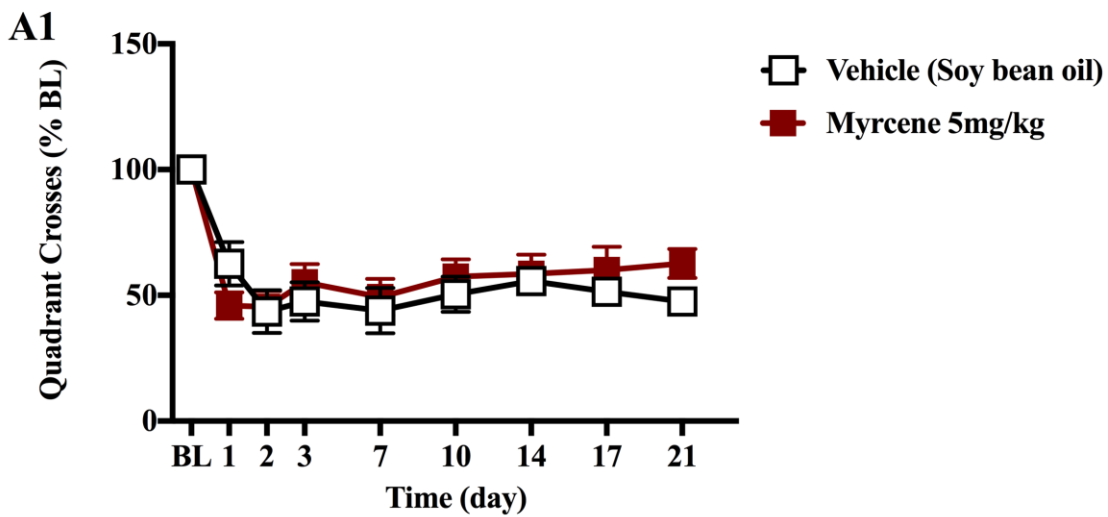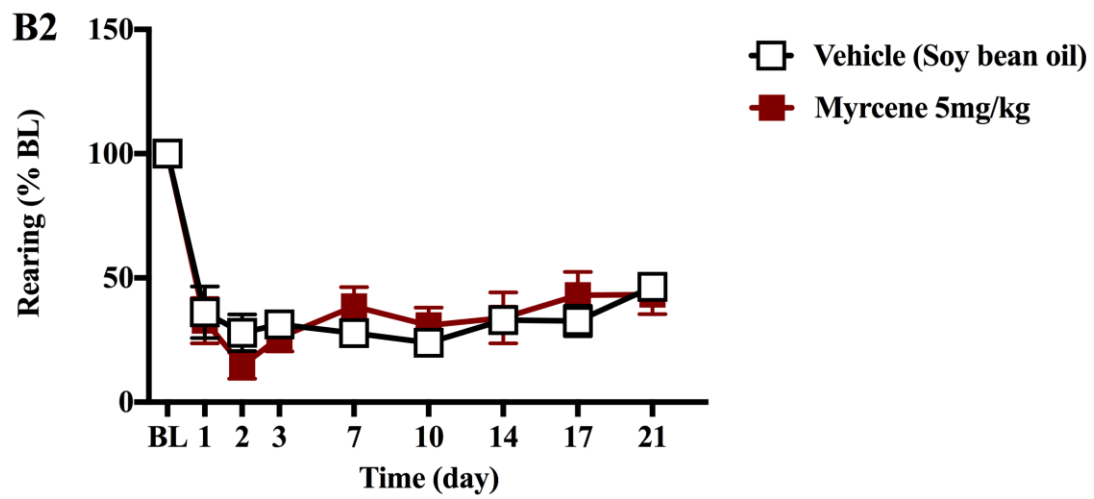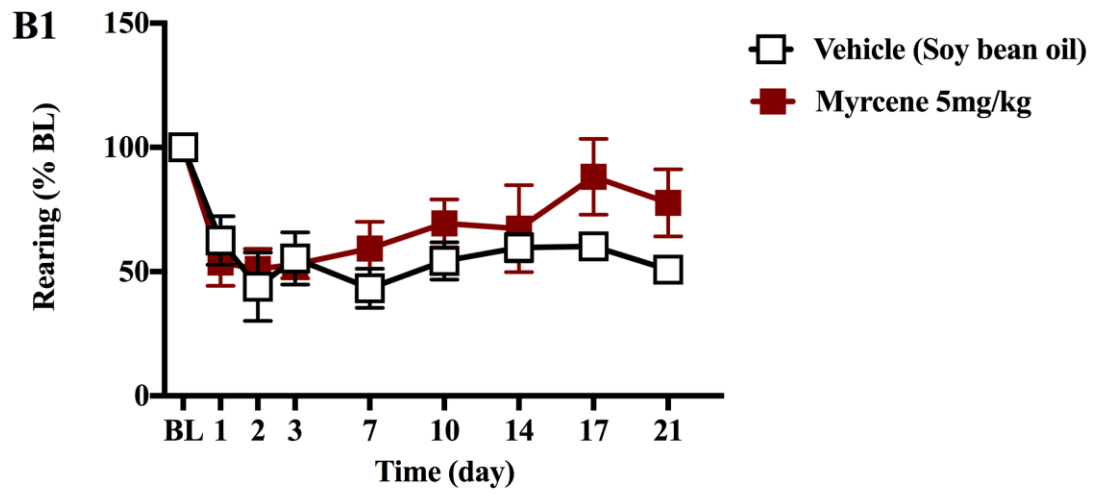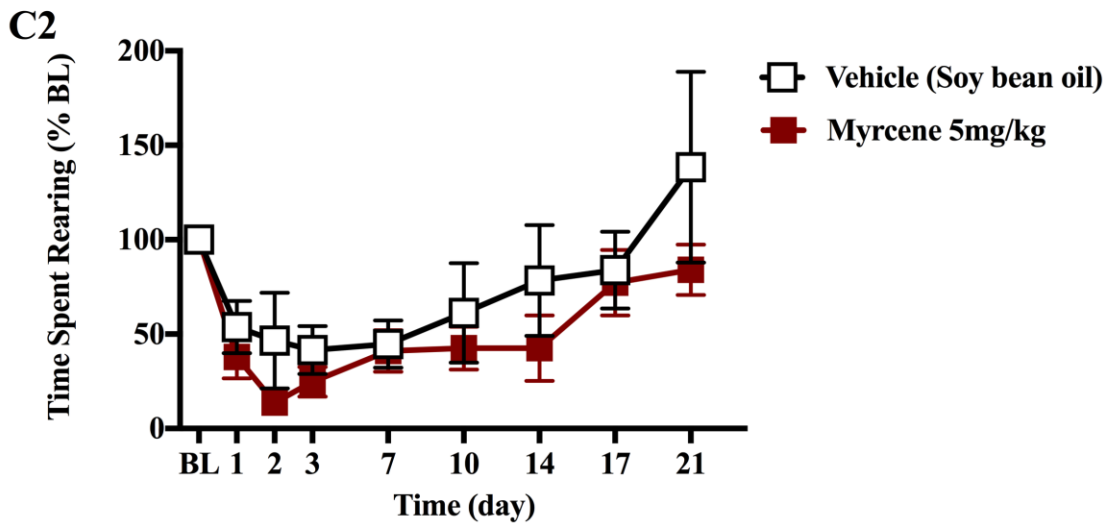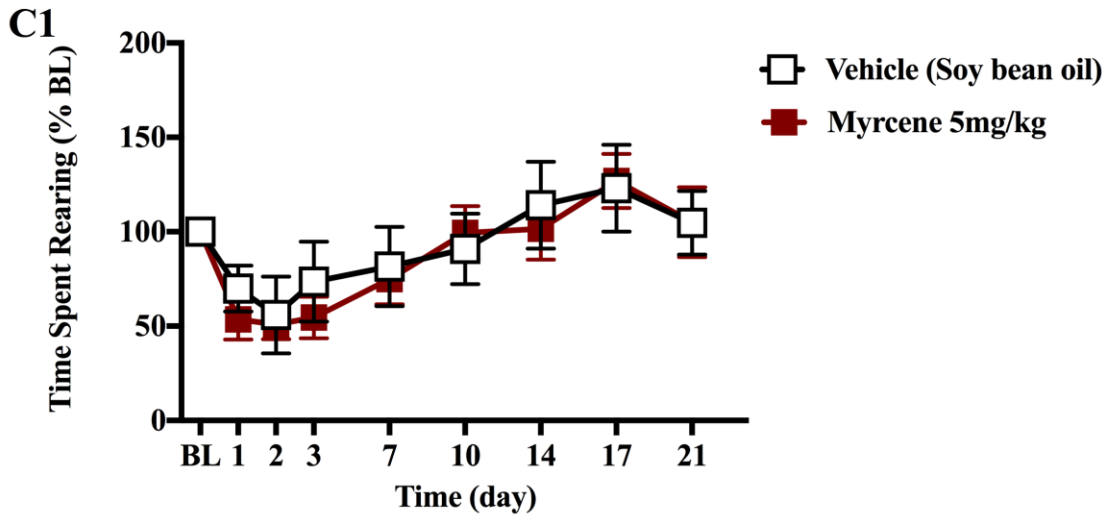
Figure 4.3 Pre- and post-treatment analysis of locomotive activity.

Pre-treatment **(A1)** and post-treatment **(A2)** analysis of quadrant crossing was not significantly different from controls (P > 0.05, two-way RM ANOVA, n = 8 per time point). **(B1)** Repeated myrcene administration significantly increased the number of rearings during pre-treatment analysis (P < 0.05, two-way RM ANOVA with Bonferroni's *post-hoc*, n = 8 per time point), but had no effect during post-treatment **(B2)** analysis (P > 0.05, two-way RM ANOVA, n = 8 per time point). **(C1)** Pre-treatment and **(C2)** post-treatment analysis of time spent rearing was not significantly different between myrcene treated animals and vehicle controls (P > 0.05, two-way RM ANOVA, n = 8 per time point). Data are presented as mean (± SEM). ANOVA, analysis of variance; BL, baseline; RM, repeated measures.
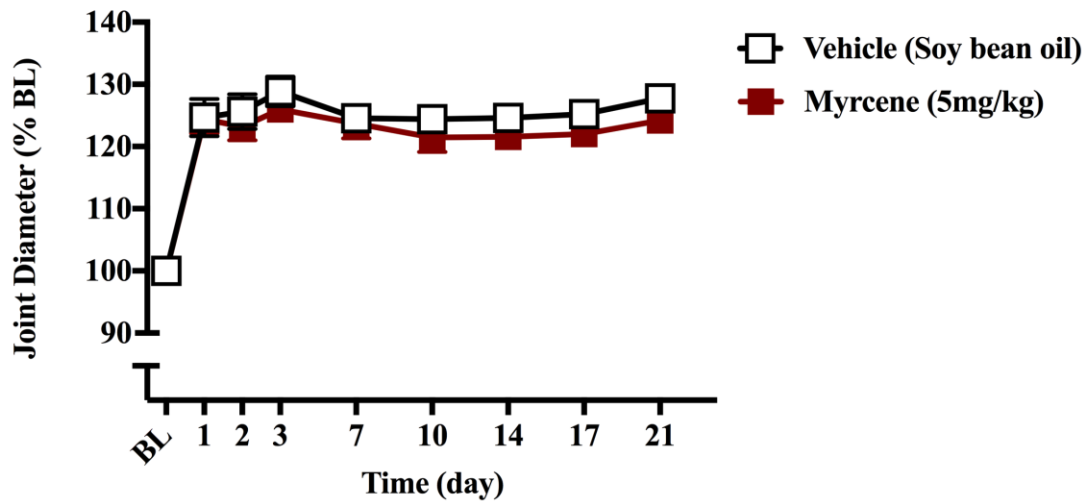
Figure 4.4 The effect of recurrent myrcene treatment on FCA-induced joint oedema over a 21 day chronic time course.

Myrcene did not significantly improve joint oedema ($P > 0.05$, two-way RM ANOVA, n = 12 per time point) over the 21 day period. There was no *post-hoc* significance to report. Data are presented as mean (± SEM) for each time point. ANOVA, analysis of variance; BL, baseline; RM, repeated measures.
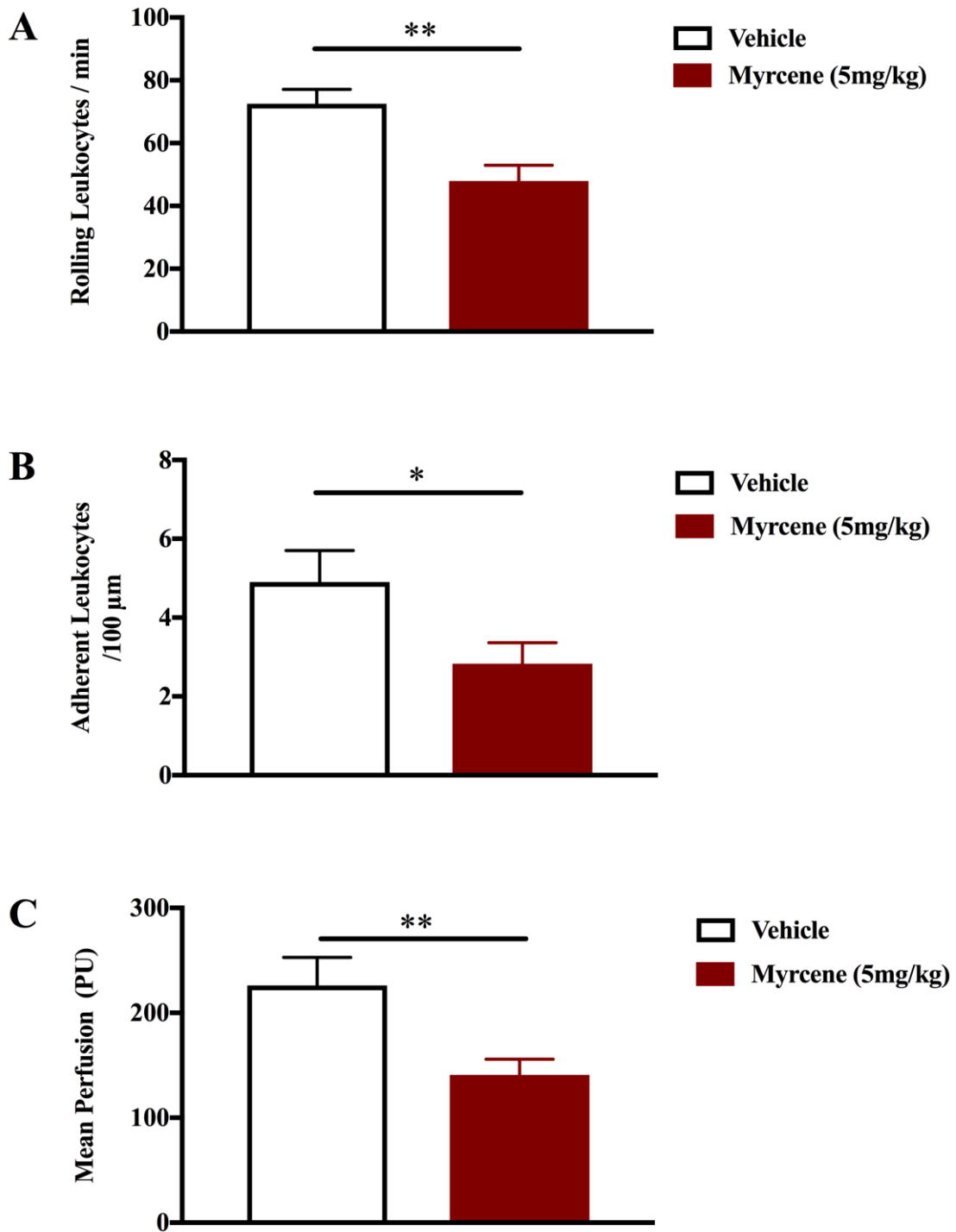
Figure 4.5 Myrcene exhibits anti-inflammatory properties after repeated administration.

Repeated administration of myrcene significantly reduced the number of **(A)** rolling leukocytes (**$P < 0.01$, unpaired student's t-test, n = 7-8) and **(B)** adherent leukocytes (*$P < 0.05$, unpaired student's t-test, n = 7-8) compared to vehicle controls on day 21-22. **(C)** Mean perfusion to the affected knee joint was also significantly decreased in myrcene-treated animals compared to vehicle controls on days 21-22 (**$P < 0.01$, Mann-Whitney U test, n = 7-8). Data are presented as mean ($\pm$ SEM). BL, baseline; PU, perfusion units.
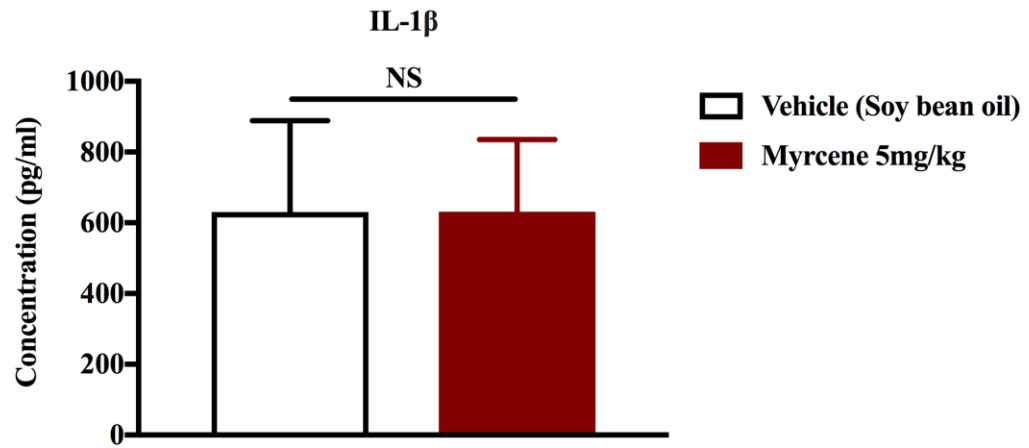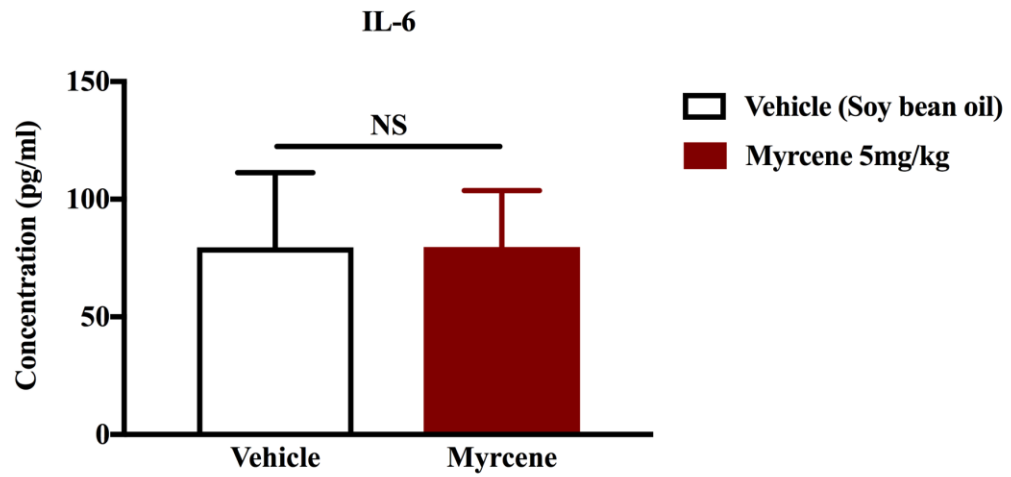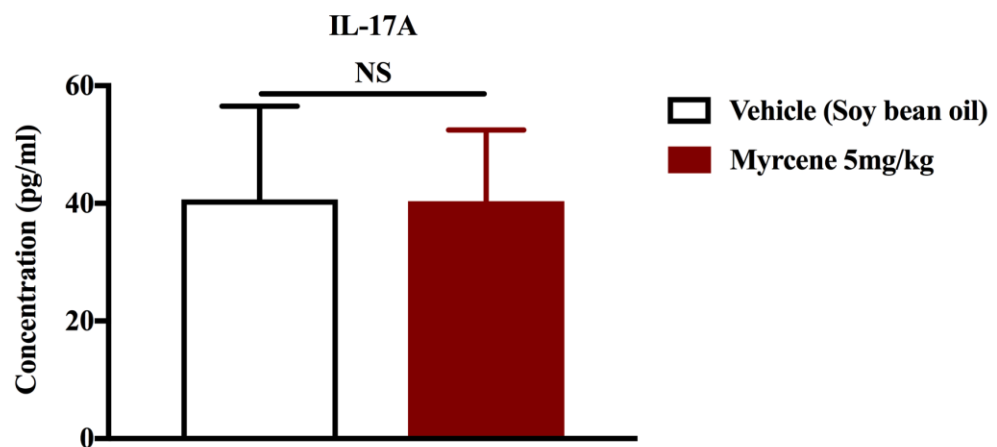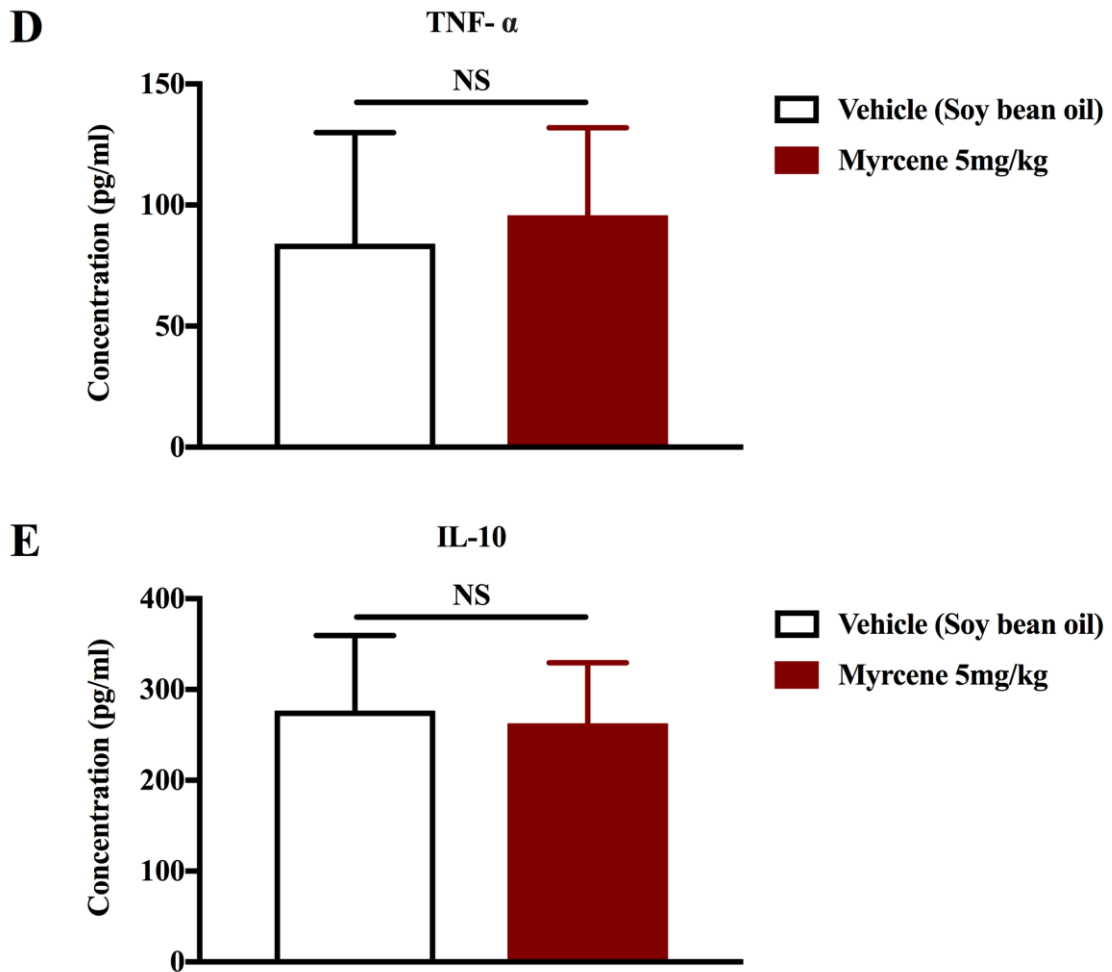
Figure 4.6 Comparison of plasma cytokine concentrations (pg/ml) at end point between myrcene treated and vehicle treated cohorts.

Repeated myrcene did not significantly alter the levels of the pro-inflammatory cytokines Il-1β **(A)**, Il-6 **(B)**, IL-17A **(C)**, TNF-α **(D)** or the anti-inflammatory cytokine Il-10 **(E)**. Data are presented as mean ± SEM. P > 0.05, compared with vehicle control, Mann-Whitney U test (all panels), n = 7-8 per group. IL, interleukin; NS, not significant; pg, picogram; TNF-α, tumor necrosis factor-alpha.
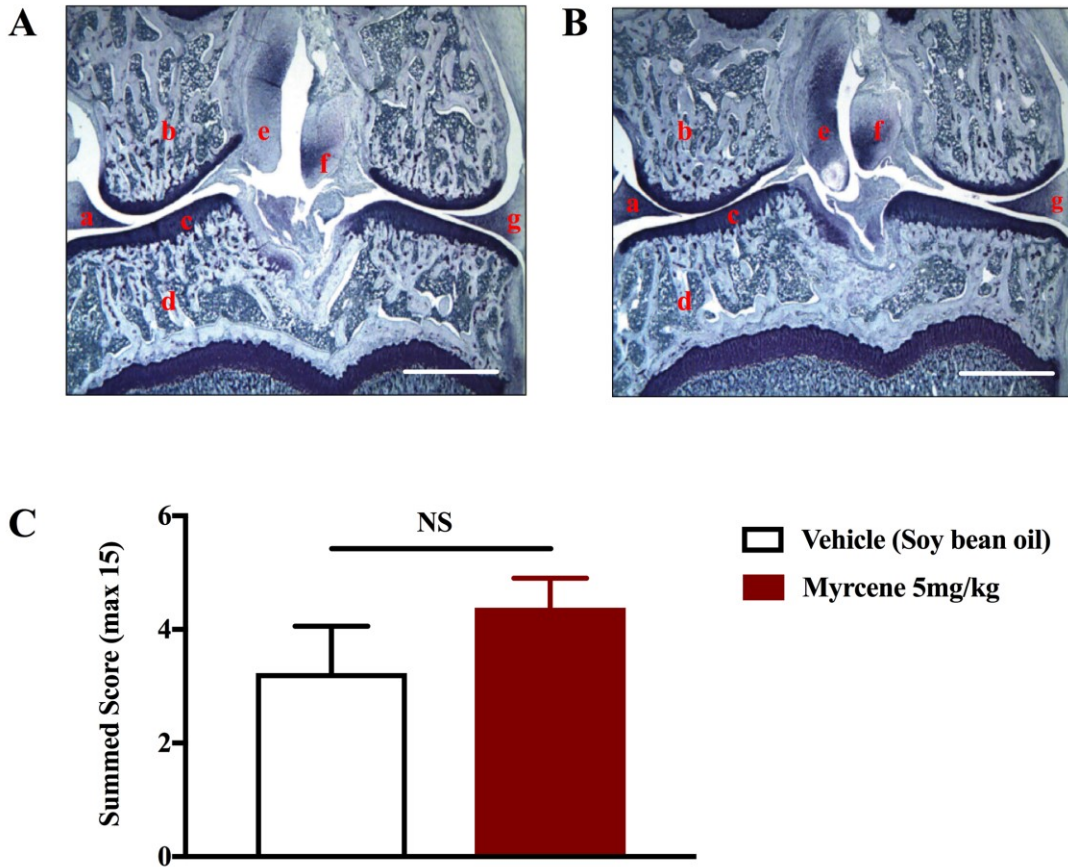
Figure 4.7 Effect of repeated administration of myrcene on joint damage.

Representative frontal plane section of myrcene treated and vehicle treated knee joints stained with toluidine blue are presented in the upper panel. Pictures are viewed at a magnification of 16X. **(A)** The representative section from the vehicle treated cohort shows mild inflammation, minimal cartilage damage and minimal pannus formation. **(B)** The knee joint pictured from the myrcene-treated group shows moderate inflammation, minimal cartilage damage and minimal pannus formation. Pictures, scoring and histological preparations were performed by Bolder BioPath (Colorado, USA). **(C)** Myrcene had no effect on overall joint damage, the summed score from the histopathological scoring analysis of inflammation, cartilage damage, pannus formation and bone resorption. $P < 0.05$, Mann-Whitney U test, n = 4 per group. Data are presented as mean $\pm$ SEM. a: lateral meniscus, b: femur, c: cartilage, d: tibia, e: anterior cruciate ligament, f: posterior cruciate ligament; g: medial meniscus. NS, not significant. Scale bar equals 2.5mm.
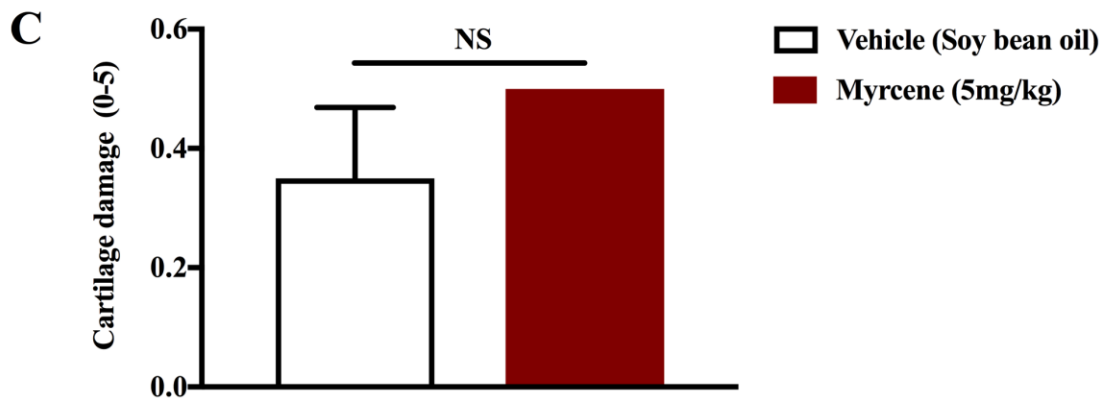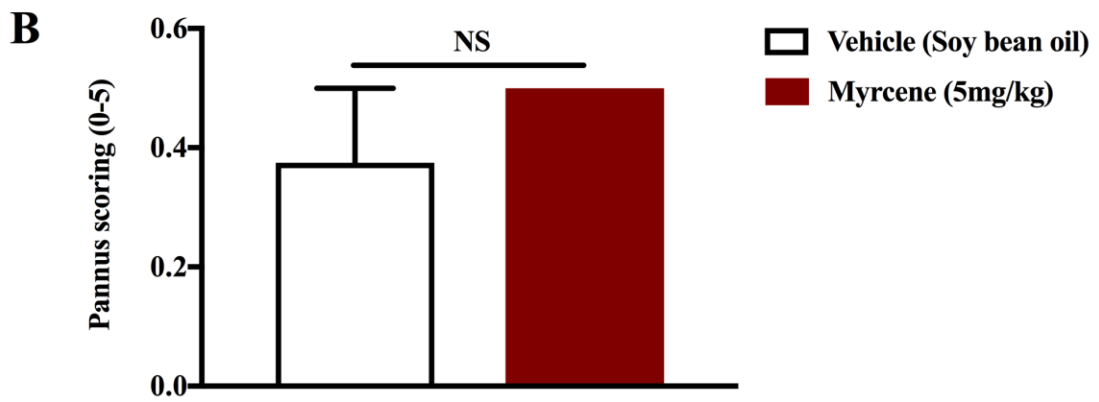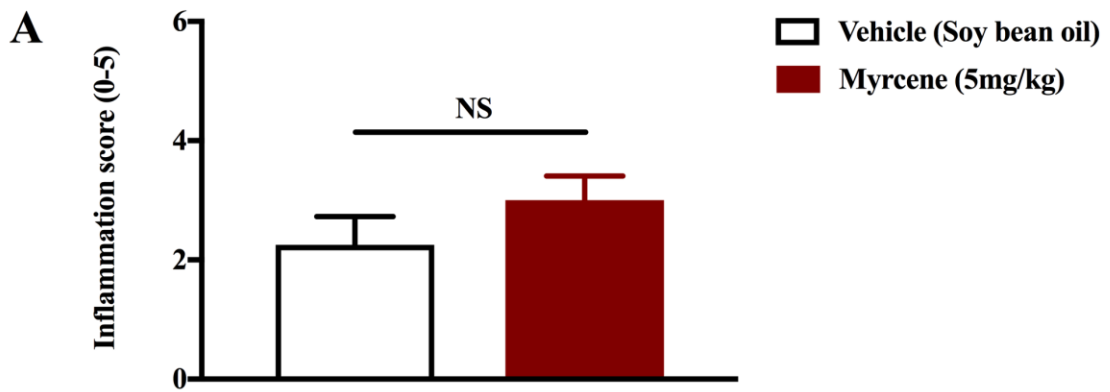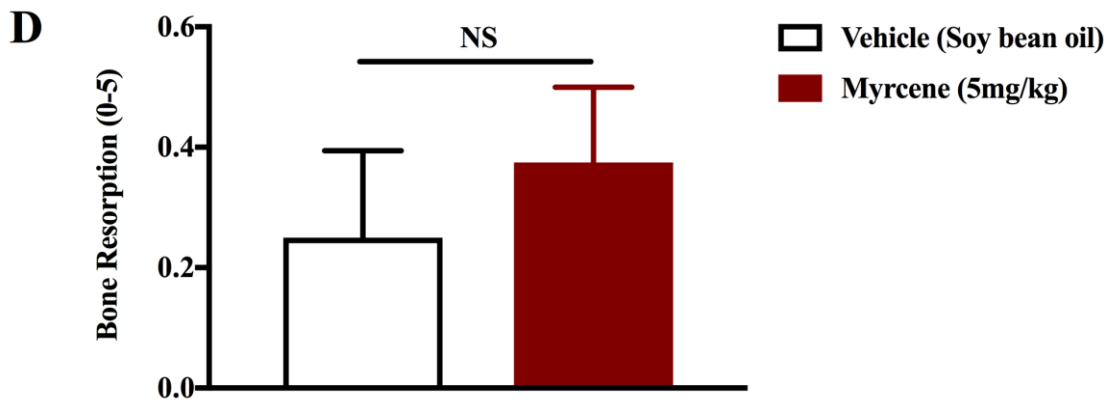
Figure 4.8 Knee joint histopathology comparison between myrcene treated and vehicle treated cohorts.

Histopathological scoring (0-5) showed that myrcene did not decrease pannus formation **(A)**, inflammation infiltration **(B)**, cartilage degradation **(C)** or bone resorption **(D)** compared to soy bean vehicle controls. Scoring and histological preparations were performed by Bolder BioPath (Colorado, USA). Data are presented as mean ($\pm$ SEM). P > 0.05, compared with vehicle control, Mann-Whitney U test (all panels), n = 4 per group). NS, not significant.

Chapter 5: Combination Effects of Cannabidiol and Myrcene on Inflammatory Joint Pain and Inflammation

5.1 Background and hypotheses

Phytocannabinoids are a diverse array of chemicals in the cannabis plant which make up the bulk of the dried flower composition (Clarke and Watson 2007). A variety of phytocannabinoid and terpene profiles in medical cannabis have been used to treat the symptoms associated with arthritis (Ware et al. 2005; Baron et al. 2018). A great deal of pharmacological and medical interest has been devoted to the individual cannabinoids (Russo and Marcu 2017); however, less attention has been afforded to the terpenes.

The non-euphoric phytocannabinoid CBD has recently been tested as an analgesic and anti-inflammatory agent in animal models of OA (Philpott et al. 2017) and RA (Malfait et al. 2000; Costa et al. 2007; Hammell et al. 2016). Transdermal application of CBD protected against thermal hyperalgesia (Hammell et al. 2016), while oral administration attenuated both thermal and mechanical hyperalgesia (Costa et al. 2007). The anti-nociceptive actions of CBD were reversed when using a TRPV1 antagonist suggesting a desensitization-dependent analgesic effect (Costa et al. 2007; Hammell et al. 2016). Systemic CBD was able to decrease FCA-induced inflammation, with associated reductions in nitric oxide and PGE_2 (Costa et al. 2007). Transdermal CBD also attenuated joint oedema and decreased CGRP expression at the level of the spinal cord (Hammell et al. 2016).

Cannabinoid monotherapy and synthetic therapeutics matching natural cannabinoid content ratios have shown various levels of efficacy when compared to that of the natural plant (Ryan et al. 2006). This has sparked a debate about the possible synergy of the chemical constituents of the cannabis plant (Ryan et al. 2006). Coined the 'entourage effect', Ben-Shabat and Mechoulam (1998) demonstrated that two inactive cannabis-related compounds could work together to produce a positive response. The combination of two compounds can work synergistically to elicit a greater response than the effects of individual compounds on their own (Berenbaum 1989). Studies have demonstrated that CBD has the ability to augment the analgesic potential of THC

(Karniol and Carlini 1973), while mitigating the adverse central side-effects involved with CB_1R binding (Russo and Guy 2006). Similar effects were observed when CBD was combined with morphine (Smith et al. 1998).

Both Lorenzetti et al., (1991) and Paula-Freire et al. (2013) compared the effects of myrcene by itself to the effects of its respective essential oil extract and showed that they both exhibited similar analgesic properties. These extracts, however, were terpene-based and did not contain phytocannabinoids (Lorenzetti et al. 1991; Paula-Freire et al. 2013).

Few studies have investigated the synergistic effects of myrcene with other cannabis compounds. We chose to combine myrcene with CBD due to the demonstrated efficacy of CBD in the FCA model (Costa et al. 2007; Hammell et al. 2016) and its reported synergism with THC (Karniol and Carlini 1973; Russo and Guy 2006). As such, we investigated the effects of CBD alone, and CBD in combination with myrcene, on pain and inflammation in FCA-injected rats.

Hypotheses that were investigated within this study include:

I.   **Low dose CBD will have no effect on pain or inflammation in FCA-injected rats.**

II.   **The combination of CBD and myrcene will have greater anti-inflammatory and analgesic actions than myrcene or low dose CBD alone in the FCA model.**

5.2   Effect of acute CBD administration on pain behaviour

5.2.1   *Methods and experimental design*

In this series of experiments, the same FCA induction, acute pain behaviour and joint oedema protocols were used as described in section 3.2.1. The cannabidiol versus vehicle experiments were performed originally to determine a suboptimal dose for use in the combination therapy. The dose of CBD was chosen based on previously published work demonstrating that 200µg of CBD was ineffective at reducing pain and inflammation using similar tests in a rat model of OA (Philpott et al. 2017). The lowest

dose of myrcene was chosen to use in combination to see if a synergistic effect could be achieved in combination. Cohorts received one of the following treatments:

I.   CBD (200µg; 50µl; subcutaneously over the knee joint)

II.   CBD (200µg) + Myrcene (1mg/kg); 50µl; subcutaneously over the knee joint

Day 0: FCA induction (50µl i.artic)   Day 7: Pain Behaviour Time course
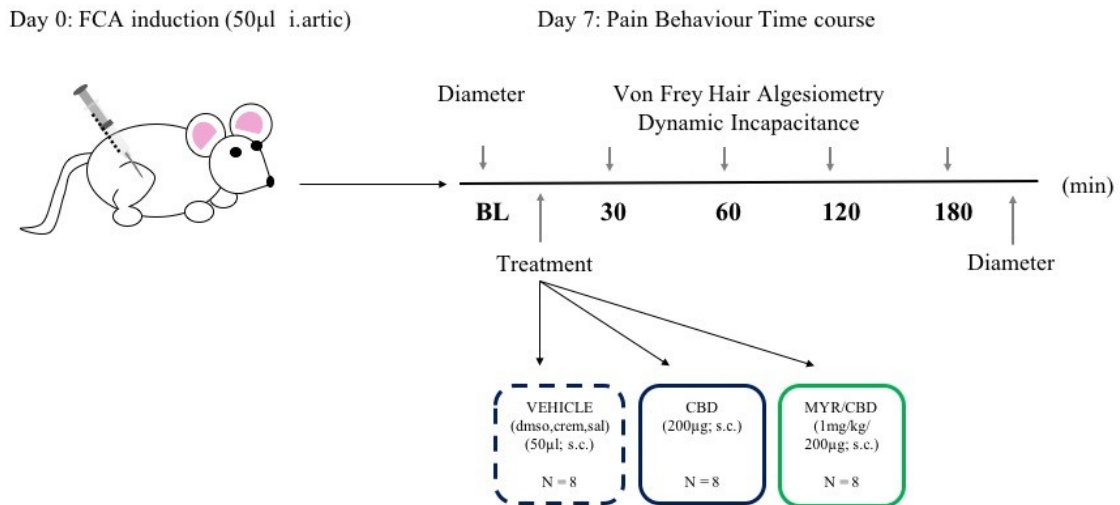
Previously completed data from Chapter 3 was used as a comparison to the combination data. These data included:

i.   Myrcene Vehicle – soy bean oil (50µl; subcutaneously over the knee joint)

ii.   Myrcene (1mg/kg; 50µl; subcutaneously over the knee joint)

iii.   CBD Vehicle – DMSO/cremophor/saline (1:1:8; 50µl; subcutaneously over the knee joint)

5.2.2   Results

5.2.2.1   FCA-injected animals exhibited mechanical allodynia and weight bearing deficits

Evoked pain behaviour and weight bearing analysis was performed seven days post FCA-induction. Average withdrawal threshold was significantly reduced from 14.61

± 0.23g in naïve pre-induced animals to 8.176 ± 0.74g in day seven FCA-injected rats (Figure 5.1A, P < 0.0001, n = 24 per group). Percent weight borne by the ipsilateral hind paw was also significantly reduced in day seven FCA-injected animals (27.57 ± 2.13%) compared to their pre-induction baseline (49.51 ± 0.65%) measurement (Figure 5.1B, P <0.0001, n = 24 per group). The weight bearing deficit was also accompanied by a significant reduction of surface area relative to the contralateral limb (Figure 5.1C, P < 0.0001, n = 24 per group), decreasing from equal surface area between contralateral and ipsilateral (100.1 ± 1.63%) to using 62.98 ± 4.12% surface area compared to the contralateral hind paw in day seven FCA-injected rats.

5.2.2.2 *Low dose CBD did not reduce mechanical allodynia or spontaneous pain behaviour*

Subcutaneous administration of low dose CBD did not significantly improve withdrawal threshold over a three hour period when compared to vehicle controls (Figure 5.2A, P > 0.05, n = 8 per time point). Neither weight borne on the ipsilateral limb (Figure 5.2B, P > 0.05, n = 8 per time point) nor percent contralateral surface area used to weight bear (Figure 5.2C, P > 0.05, n = 8 per time point) in CBD-treated animals was statistically different from vehicle controls over the time course.

5.2.2.3 *Combination therapy improved mechanical allodynia but did not improve weight bearing deficits compared to vehicle*

The time course data for withdrawal threshold did not pass normality, but was tested using a two-way RM ANOVA in the absence of a non-parametric equivalent. Investigation of withdrawal threshold showed that combination therapy significantly improved mechanical allodynia compared to vehicle (Figure 5.3A, P < 0.05, n = 8 per time point). *Post-hoc* significance was observed 30 minutes post-treatment (P < 0.05). In addition, percent weight borne on the ipsilateral paw was not significantly different (Figure 5.3B, P > 0.05, n = 8 per time point), nor did percent contralateral surface area significantly vary in the combination-treated group compared to the vehicle group (Figure 5.3C, P > 0.05, n = 8 per time point).

5.2.2.4   Lack of synergistic analgesia with combination treatment compared to drugs alone

Overall, there were significant changes in withdrawal threshold between the five treatment cohorts (Figure 5.4A, $P < 0.0001$, n = 8 per group). *Post-hoc* analysis revealed that myrcene treatment significantly improved withdrawal threshold compared to its respective vehicle ($P < 0.001$). Low dose CBD alone was not significantly different from its respective vehicle ($P > 0.05$). The combination of myrcene + CBD did not produce an enhanced anti-allodynic effect compared to vehicles or the individual drugs alone ($P > 0.05$).

Percent weight placed on the ipsilateral paw and percent surface area of the contralateral paw relative to baseline were also compared two hours post-treatment between myrcene, CBD, the combination therapy, and their respective vehicles. Both weight placed on the ipsilateral paw (Figure 5.4B, $P > 0.05$, n = 8 per group) and surface area placed on the ipsilateral paw (Figure 5.4C, $P > 0.05$, n = 8 per group) showed no significant difference between each group.

5.3   Effect of acute CBD treatment on local inflammation

5.3.1   Methods and experimental design

The same FCA induction and acute inflammatory time course methods were used as outlined in Section 3.4.1. The acute effect of CBD versus vehicle was examined first to confirm a suboptimal dose of CBD which could be compared to the combination therapy. Individual cohorts received one of the following treatments:

I.   CBD (200µg; 50µl; topically over the exposed knee joint)

II.   CBD (200µg) + Myrcene (1mg/kg); 50µl; topically over the exposed knee joint)

Day 0: FCA induction (50ul i.artic)
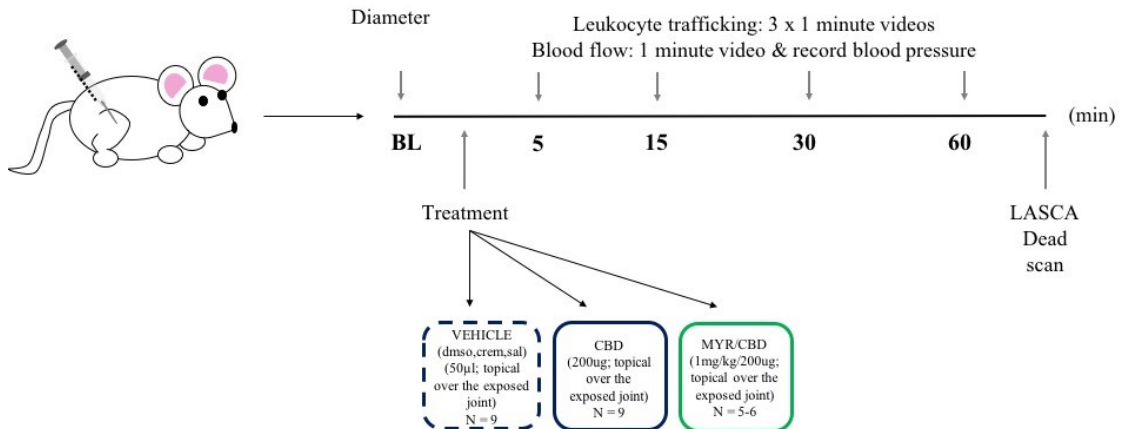
Previously completed data were used to compare with the combination therapy. Therapeutic treatments that are re-represented in this section include:

i.   Myrcene vehicle – soy bean oil (50µl; subcutaneously over the knee joint)

ii.   Myrcene (1mg/kg; 50µl; subcutaneously over the knee joint)

iii. CBD vehicle – DMSO/cremophor/saline (1:1:8; 50µl; subcutaneously over the knee joint)

Combination treatment was ultimately compared with individual treatments and their respective vehicles one hour post-treatment as this was the time point that myrcene exhibited the greatest anti-inflammatory effect in the acute study from Chapter 3.

5.3.2   Results

5.3.2.1   FCA-induced local joint inflammation

Inflammatory time course parameters were assessed on day seven in FCA-injected animals. Joint diameter had significantly increased from 9.90 ± 0.08mm to 12.73 ± 0.18mm seven days after FCA injection (Figure 5.5A, $P < 0.0001$, n = 24 per group). Day seven FCA-injected animals had a significantly higher average rolling leukocyte count of 78 ± 3.42 compared to 49.58 ± 5.36 in naïve animals (Figure 5.5B, $P < 0.0001$, n

= 11-24) per group. Adherent leukocyte counts were not significantly different in day seven FCA-injected animals compared to naïve animals (Figure 5.5C, P > 0.05, n = 11-24 per group). Mean perfusion was increased from 155.7 ± 13.91 PU in naïve animals to 203.6 in FCA-injected animals (Figure 5.5D, P < 0.05, n = 11-24 per group)

5.3.2.2 *Low dose CBD had no effect on joint oedema, leukocyte trafficking or blood flow in inflamed knee microvasculature*

Administration of CBD had no effect on joint diameter at the conclusion of the time course compared to vehicle (Figure 5.6A, P > 0.05, n = 8 per group). Administration of CBD subcutaneously over the knee joint did not significantly reduce rolling leukocytes (Figure 5.6B, P > 0.05, n = 9 per time point) in the hour-long time course compared to vehicle controls. Time course data for adherent leukocytes did not pass normality, but were analyzed using a two-way RM ANOVA in the absence of an equivalent non-parametric test. There was no significant effect of CBD administration on adherent leukocytes compared to vehicle (Figure 5.6C, P > 0.05, n = 8 per time point). In addition, treatment with CBD had no significant effect on mean perfusion to the joint (Figure 5.6D, P > 0.05, n = 8 per time point.

5.3.2.3 *Combination treatment reduced leukocyte trafficking, but had no effect on blood flow or oedema*

Joint diameter was not significantly altered three hours after combination treatment (Figure 5.7A, P > 0.05, n = 8 per group). Combination therapy significantly reduced leukocyte rolling compared to vehicle (Figure 5.7B, P < 0.0001, n = 5-9 per time point). Adherent leukocyte data did not pass normality, however were analyzed using a two-way RM ANOVA in the absence of a non-parametric equivalent. Adherent leukocyte levels were not significantly different between the combination-treated group versus vehicle-treated rats (Figure 5.7C, P > 0.05, n = 5-9 per time point). Finally, blood flow to the knee joint was not altered in the combination-treated group when compared to vehicle (Figure 5.7D, P > 0.05, n = 5-9 per time point).

5.3.2.4   Combination treatment did not improve oedema, vascular inflammation or blood flow compared to myrcene

Vascular inflammation was compared one hour post-treatment between myrcene, CBD, combination therapy, and their respective vehicles at 30 minutes. Joint diameter relative to day seven baseline did not significantly vary across all treatments (Figure 5.8A, P > 0.05, n = 8 per group). Overall, the percent decrease in rolling leukocytes varied significantly amongst treatments (Figure 5.8B, P < 0.001, n = 6-9 per group). Significant reductions in rolling leukocytes were noted between the myrcene treated group and its vehicle (P < 0.05) during *post-hoc* analysis. *Post-hoc* analysis revealed that CBD did not alter the percent reduction of rolling leukocytes compared to vehicle treatment or combination treatment (P > 0.05). Combination treatment was also not significantly different from its vehicle, or when compared to the myrcene alone group (P > 0.05).

Adherent leukocytes relative to baseline did not significantly vary amongst groups when compared one hour post-treatment (Figure 5.8C, P > 0.05, n = 6-9 per group). Vascular perfusion to the knee joint did not significantly vary (Figure 5.8D, P > 0.05, n = 6-9 per group) amongst individual treatments, combination treatment and their respective vehicles at one hour post-treatment.

5.4   Chapter summary

Acute treatment with low dose CBD did not reduce FCA-induced mechanical allodynia in evoked pain behaviour tests and did not improve weight bearing as a measure of spontaneous pain behaviour. Over the three hour time course, combination therapy improved withdrawal threshold, but not ipsilateral weight bearing or hind paw surface area. Administration of myrcene + CBD did not improve withdrawal threshold or dynamic weight bearing/surface area when compared to vehicle, CBD alone or myrcene by itself.

Treatment with low dose CBD alone was also unable to attenuate leukocyte trafficking and hyperemia to the knee joint. Combination therapy reduced rolling leukocytes but not adherent leukocytes or blood flow to the knee joint area. Although combination therapy reduced rolling leukocytes compared to its vehicle, the anti-

inflammatory effect was not different from the individual treatment groups 30 minutes post-treatment. No differences were observed in leukocyte adherence or perfusion to the knee joint 30 minutes post-treatment.

5.5 Tables

Table 5. 1 CBD treatment result summary.

Parameter	Result
Pain behaviour summary	CBD did not reduce pain behaviour
Withdrawal threshold	No improvement
Ipsilateral weight bearing	No improvement
Ipsilateral surface area	No improvement
Inflammation summary	CBD did not decrease inflammation
Joint oedema	No improvement
Rolling leukocytes	No improvement
Adherent leukocytes	No improvement
Blood perfusion	No improvement

Table 5. 2 Combination treatment result summary.

Parameter	Result
Pain behaviour summary – at 120 minutes	Combination did not enhance analgesia
Withdrawal threshold	Improved withdrawal threshold
Ipsilateral weight bearing	No improvement
Ipsilateral surface area	No improvement
Inflammation summary – at 60 minutes	Combination did not enhance anti-inflammatory effects
Joint oedema	No improvement
Rolling leukocytes	Reduced leukocyte rolling
Adherent leukocytes	No improvement
Blood perfusion	No improvement

Table 5. 3 Combination treatment result summary.

Parameter	Versus Vehicle	Versus CBD	Versus Myrcene
Pain behaviour summary	Combination therapy was not different from monotherapy		
Withdrawal threshold	No difference	No difference	No difference
Ipsilateral weight bearing	No difference	No difference	No difference
Ipsilateral surface area	No difference	No difference	No difference
Inflammation summary	Inflammation was generally not improved by combination therapy		
Joint oedema	No difference	No difference	No difference
Rolling leukocytes	*Reduced rolling leukocytes**	No difference	No difference
Adherent leukocytes	No difference	No difference	No difference
Blood perfusion	No difference	No difference	No difference

*Significance was determined by *post-hoc* analysis

5.6   Figures

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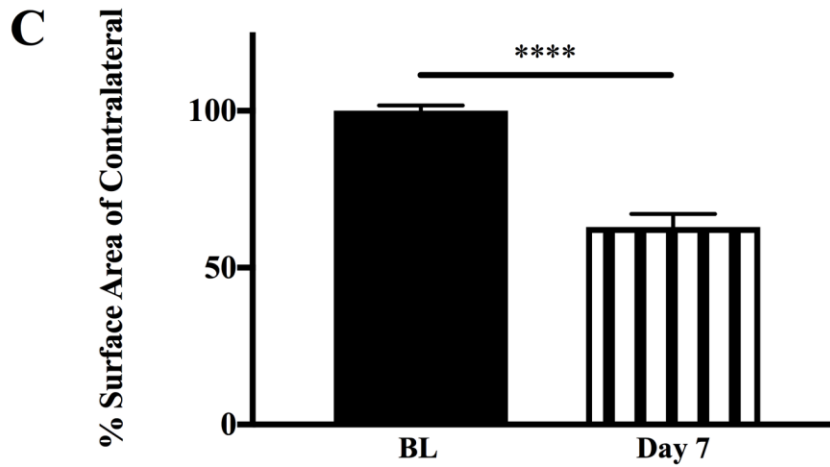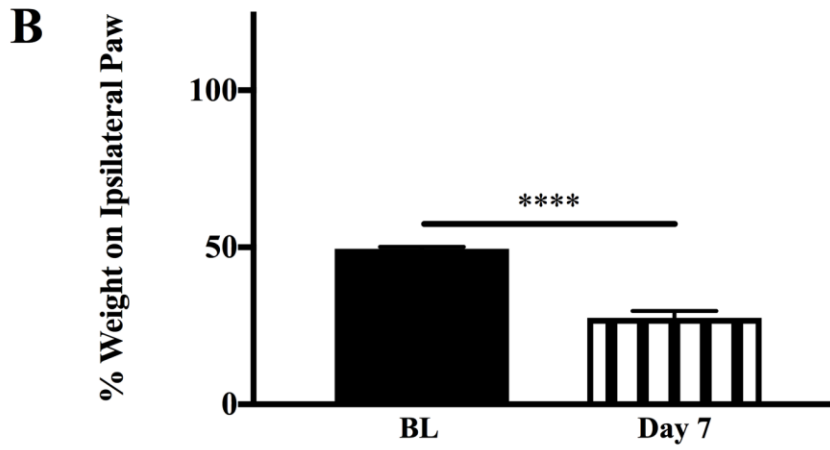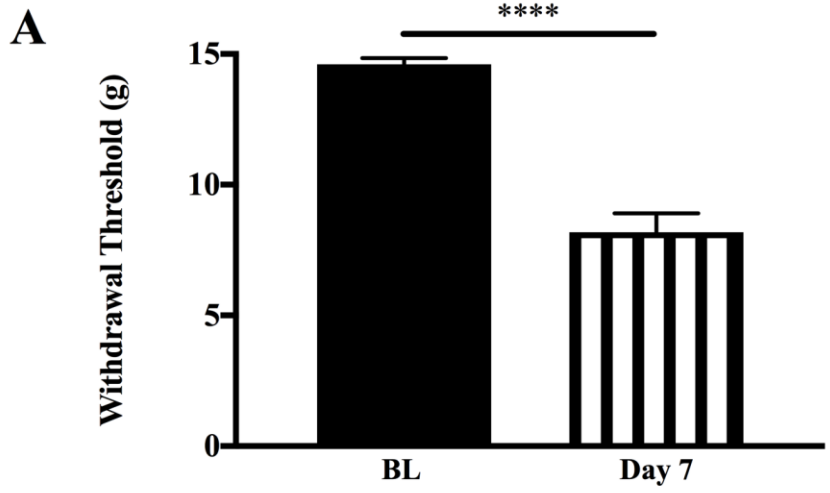
Figure 5.1 Confirmation of mechanical allodynia and weight bearing deficits in day seven FCA-injected rats.

(A) Withdrawal threshold was significantly reduced on day seven in FCA-injected rats compared to pre-induction baseline withdrawal threshold (****$P < 0.0001$, paired Wilcoxon test, n = 24). **(B)** Percent weight borne by the ipsilateral paw relative to baseline (****$P < 0.0001$, paired-student's t-test, n = 8-24) and **(C)** percent surface area of the contralateral paw placed by the ipsilateral paw relative to the baseline measurements (****$P < 0.0001$, paired-Student's t-test, n = 8-24) was significantly reduced in day seven FCA-injected animals. Data are presented as mean ± SEM. BL, baseline; FCA, Freund's complete adjuvant.

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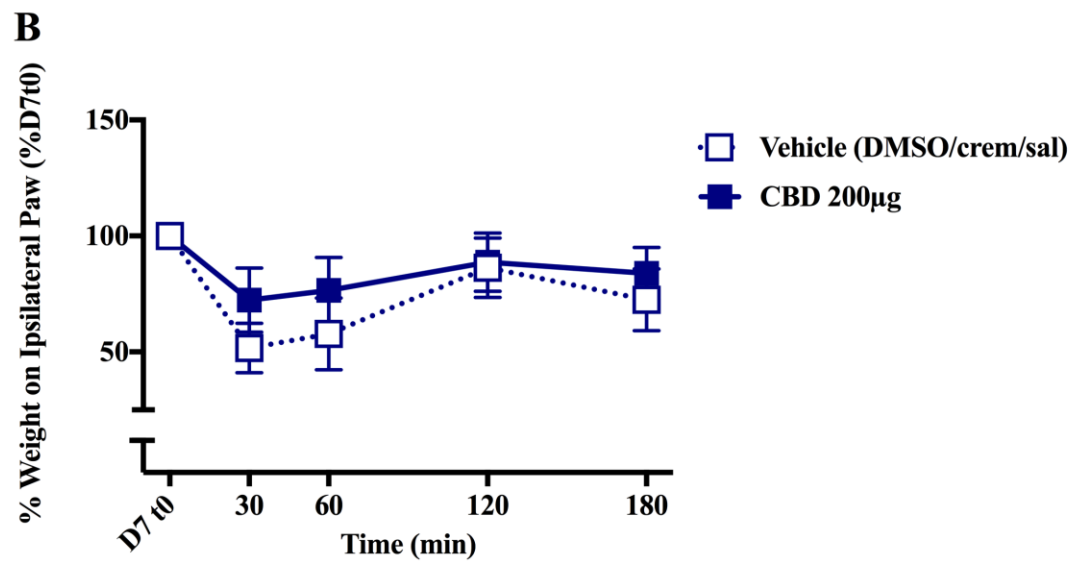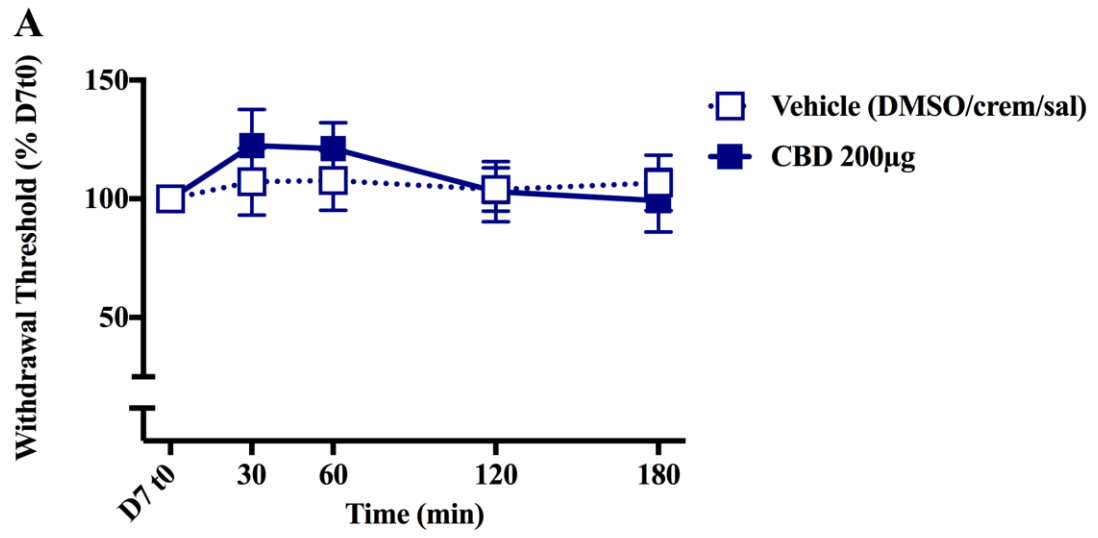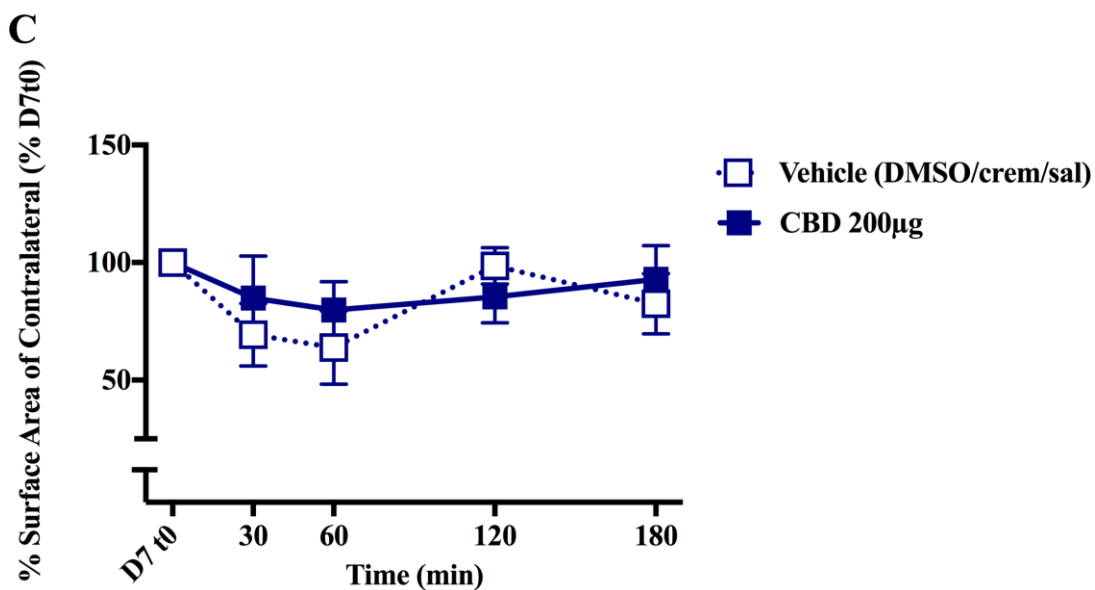
Figure 5.2 Effect of CBD on evoked and spontaneous pain behaviour.

Acute administration of low dose CBD did not significantly improve **(A)** withdrawal threshold in the 3 hour time course ($P > 0.05$, two-way RM ANOVA, n = 8 per time point). **(B)** Administration of CBD also did not significantly improve percent weight borne on the ipsilateral paw ($P > 0.05$, two-way RM ANOVA, n = 8 per time point) or the **(C)** percent contralateral surface area placed on the ipsilateral hind paw ($P > 0.05$. two-way RM ANOVA, n = 8 per time point). Data are standardized as percent day seven baseline and are presented as mean ± SEM. ANOVA, analysis of variance; CBD, cannabidiol; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; RM, repeated measures; Sal, saline; t0, time zero.

A

B

C

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Figure 5.3 Effect of combination therapy on evoked and spontaneous pain behaviour.

(A) Withdrawal threshold was significantly increased in animals treated with the combination therapy compared to vehicle ($P < 0.05$, two-way RM ANOVA with Bonferroni's *post-hoc* test, n = 8 per time point) **(B)** Percent weight borne on the ipsilateral paw was not significantly greater than vehicle over the 3 hour time course ($P > 0.05$, two-way RM ANOVA, n = 8 per time point). **(C)** Percent contralateral surface area was not significantly different between combination therapy and vehicle throughout the time course ($P > 0.05$, two-way RM ANOVA, n = 8 per time point). Data are standardized as percent day seven baseline and are presented as mean ($\pm$ SEM). ANOVA, analysis of variance; CBD, cannabidiol; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; RM, repeated measures; Sal, saline; t0, time zero.

A

Withdrawal Threshold (% D7t0)

***   NS
       NS
       NS
NS

- ☐ Vehicle (Soy bean oil)
- 🟥 Myrcene 1mg/kg
- ▦ Vehicle (DMSO/crem/sal)
- 🟦 CBD 200μg
- 🟩 Myrcene 1mg/kg + CBD 200μg

B

% Weight on Ipsilateral Paw (%D7 BL)

NS

- ☐ Vehicle (Soy bean oil)
- 🟥 Myrcene 1mg/kg
- ▦ Vehicle (DMSO/crem/sal)
- 🟦 CBD 200μg
- 🟩 Myrcene 1mg/kg + CBD 200μg

C

% Surface Area of Contralateral (% D7t0)

NS

- ☐ Vehicle (Soy bean oil)
- 🟥 Myrcene 1mg/kg
- ▦ Vehicle (DMSO/crem/sal)
- 🟦 CBD 200μg
- 🟩 Myrcene 1mg/kg + CBD 200μg

Figure 5.4 Effects of combined myrcene and CBD on pain behaviour compared to individual treatment 120 minutes after treatment.

Overall, there was a statistical significance in withdrawal threshold at 120 minutes **(A)** across all treatments ($P < 0.0001$, one-way ANOVA with Bonferroni's *post-hoc* test, n = 8 per group). *Post-hoc* comparisons showed that the myrcene-treated cohort was statistically significant from its vehicle (***$P > 0.001$), but CBD treatment alone was not significant from its vehicle ($P > 0.05$). The myrcene and CBD combination was not significantly different from vehicle ($P > 0.05$) or CBD monotherapy ($P > 0.05$). **(B)** There was no significant difference between treatments in percent weight borne on the ipsilateral paw ($P > 0.05$, one-way ANOVA, n = 8 per group) or **(C)** percent contralateral surface area used to weight bear on the ipsilateral limb ($P > 0.05$, one-way ANOVA, n = 8 per group) at two hours post-treatment. Data are standardized as percent day seven baseline and is presented as mean ($\pm$ SEM). ANOVA, analysis of variance; CBD, cannabidiol; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; NS, not significant; Sal, saline; t0, time zero.

A

Joint Diameter (mm)

Baseline — Day 7

****

B

Rolling Leukocytes / min

Naive — FCA

****

C

Adherent Leukocytes /100 µm

Naive — FCA

P = 0.0519
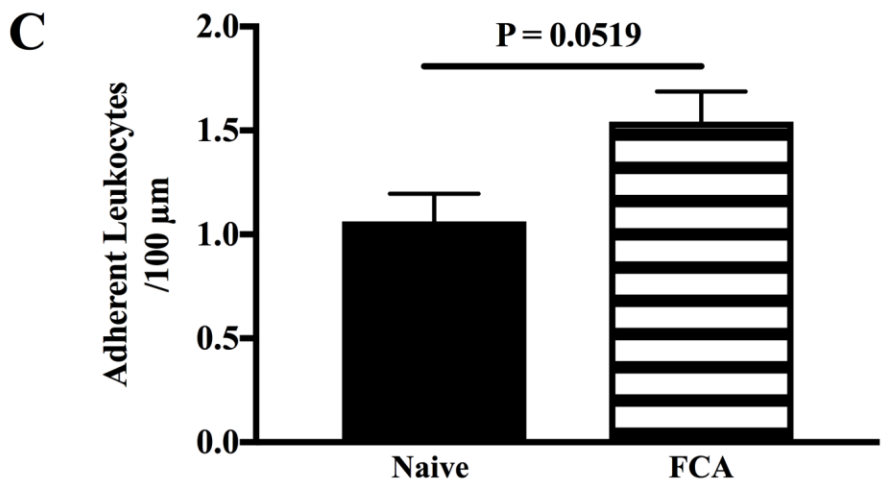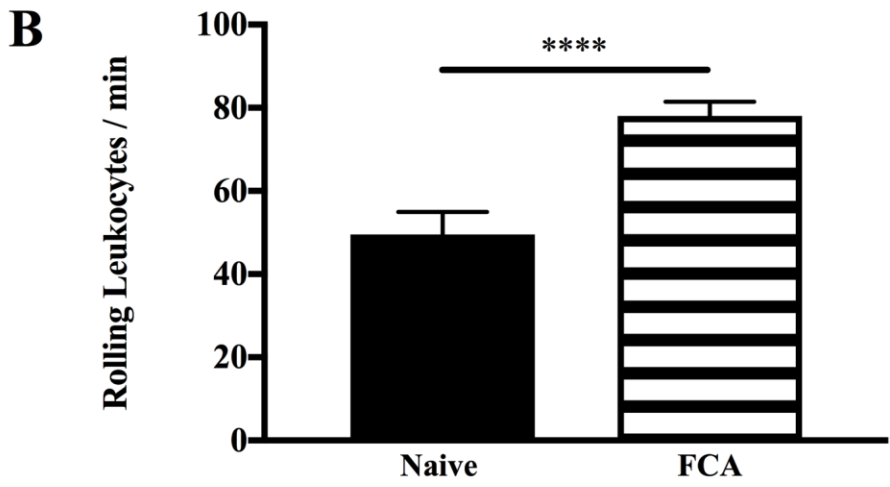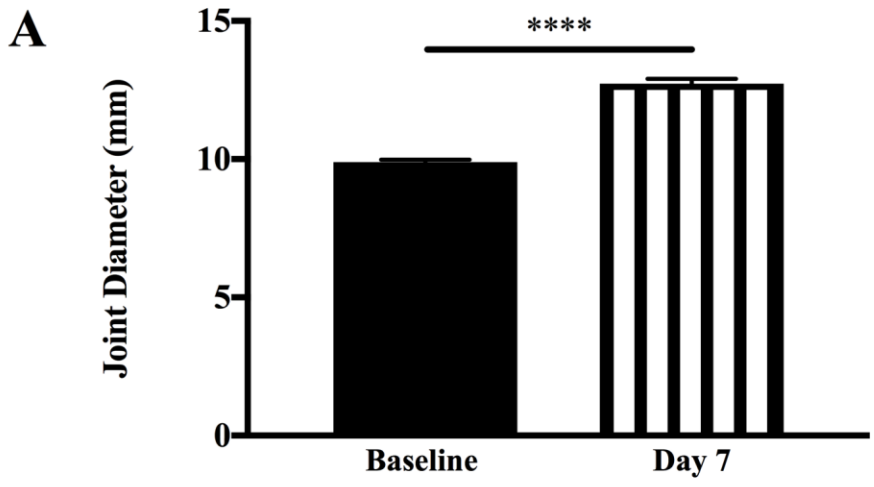
Figure 5.5 Confirmation of FCA-induced inflammation and hyperaemia on day seven.

(A) Joint diameter had significantly increased on day seven in FCA-injected rats compared to pre-induction baseline joint diameter (****$P < 0.0001$, unpaired Student's t-test, n = 24 per group). **(B)** Rolling leukocytes were significantly reduced in FCA-injected animals on day seven compared to that of naïve animals (****$P < 0.0001$, unpaired Student's t-test, n = 11-24). **(C)** There was no difference in adherent leukocytes between day seven FCA-injected animals and naïve animals ($P > 0.05$, unpaired Student's t-test, n = 11-24). **(D)** Mean perfusion was significantly elevated in day seven FCA animals compared to naïve animals ($P > 0.05$, unpaired Student's t-test, n = 11-24). Data are presented as mean ± SEM. BL, baseline; FCA, Freund's complete adjuvant; PU, perfusion units.

A Joint Diameter (% D7 t0)

NS

Vehicle (DMSO/crem/sal)
CBD 200μg

B Rolling Leukocytes (% BL)

Vehicle (DMSO/crem/sal)
CBD 200μg

Time (min)
BL 5   15   30   60

C Adherent Leukocytes (% BL)

Vehicle (DMSO/crem/sal)
CBD 200μg

Time (min)
BL 5   15   30   60
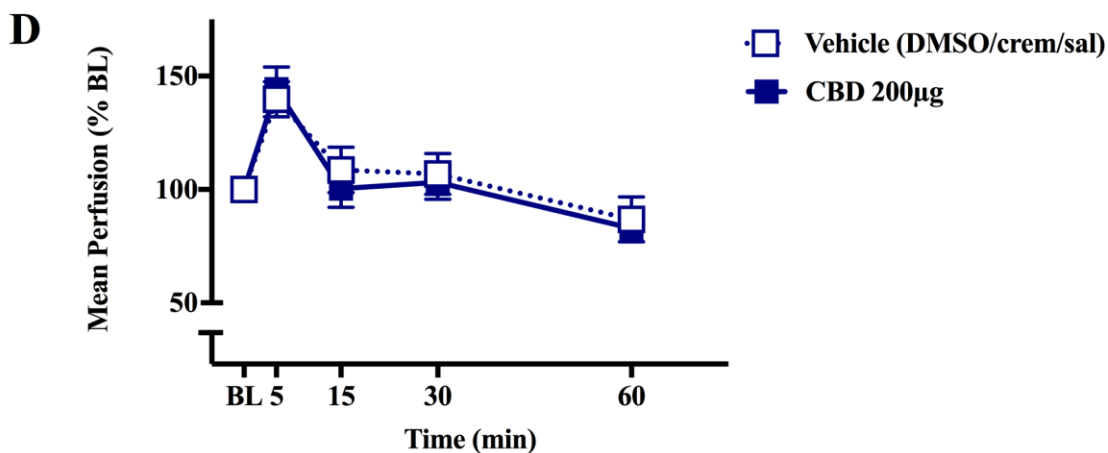
Figure 5.6 Effect of CBD on inflammation and blood flow in the knee joint microvasculature.

(A) There was no significant difference in joint diameter between the CBD-treated and vehicle-treated cohorts at 180 minutes (P > 0.05, unpaired Student's t-test, n = 8 per group). **(B)** The time course data for adherent leukocytes did not pass normality, but was tested using a two-way RM ANOVA in the absence of a non-parametric equivalent. There was no significant difference in the number of rolling leukocytes amongst the groups (P > 0.05, two-way RM ANOVA, n = 9 per time point). **(C)** There was no significant difference in the adherent leukocytes between CBD and vehicle treated animals (P > 0.05; two-way RM ANOVA (n = 9 per time point). **(D)** Mean perfusion was also not significantly changed with acute CBD administration (P > 0.05, two-way RM ANOVA, n = 9 per time point). Data was standardized as percent baseline or percent cumulative change and is presented as mean (± SEM). ANOVA, analysis of variance; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; NS, not significant; RM, repeated measures; Sal, saline; t0, time zero.
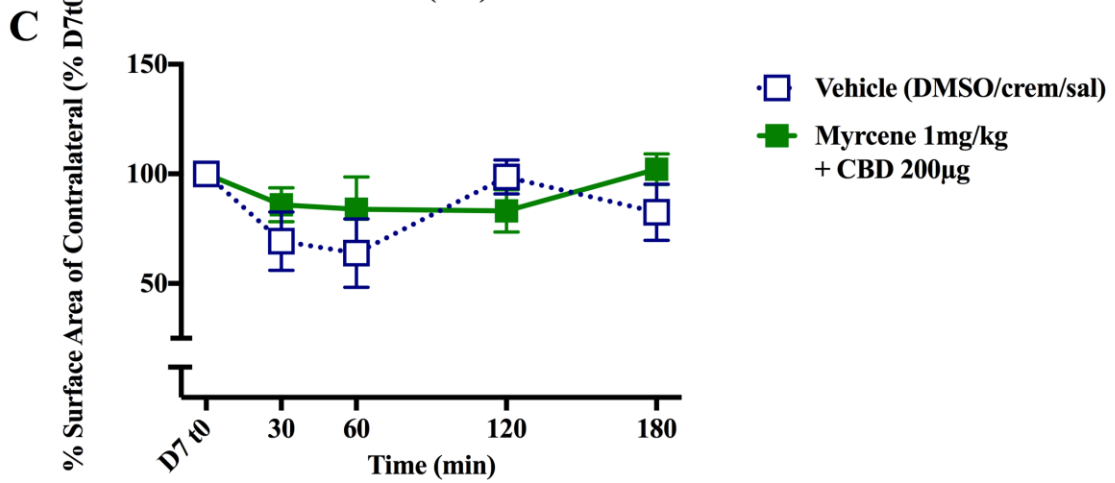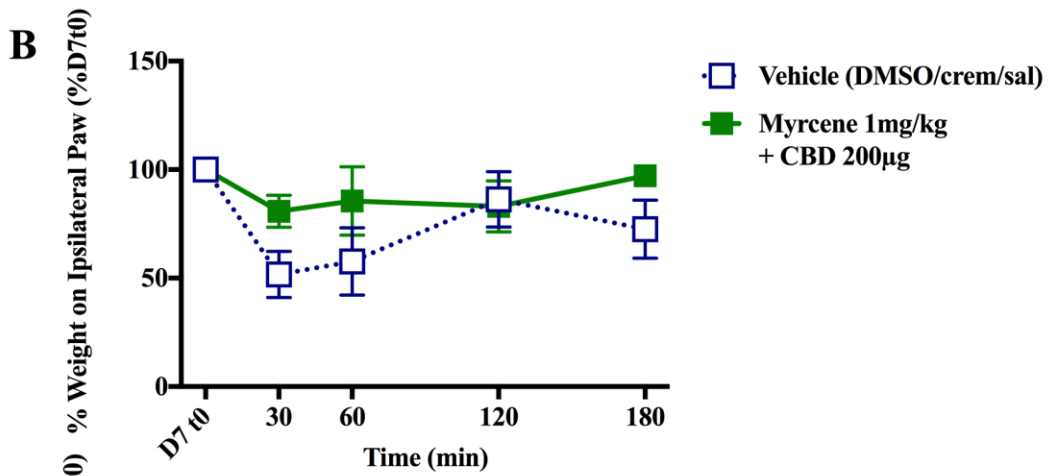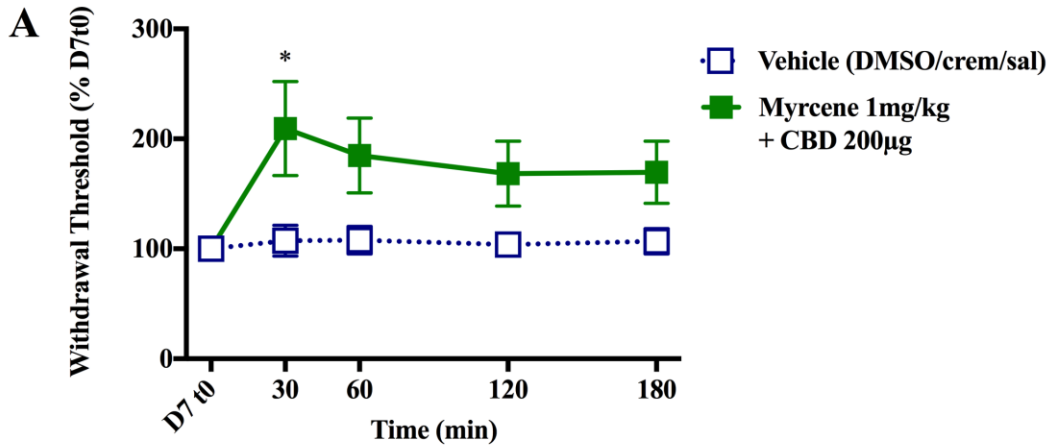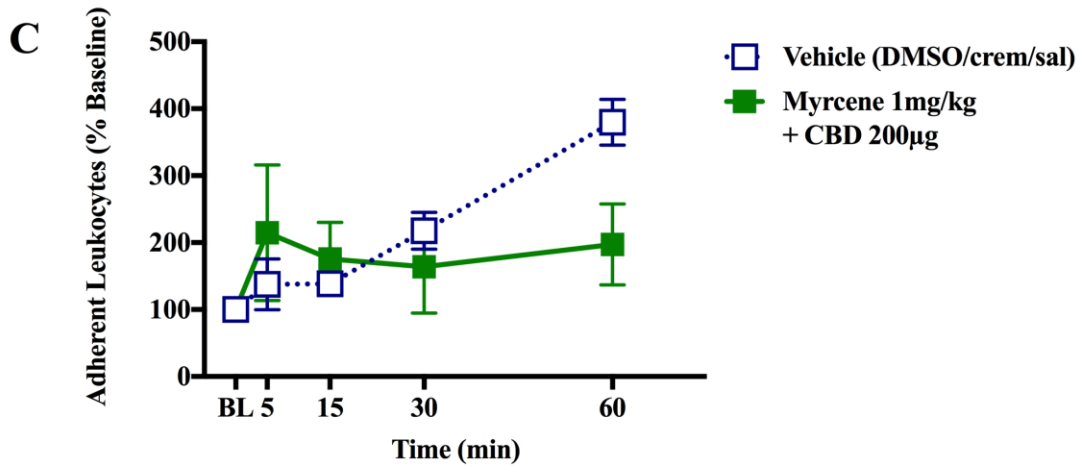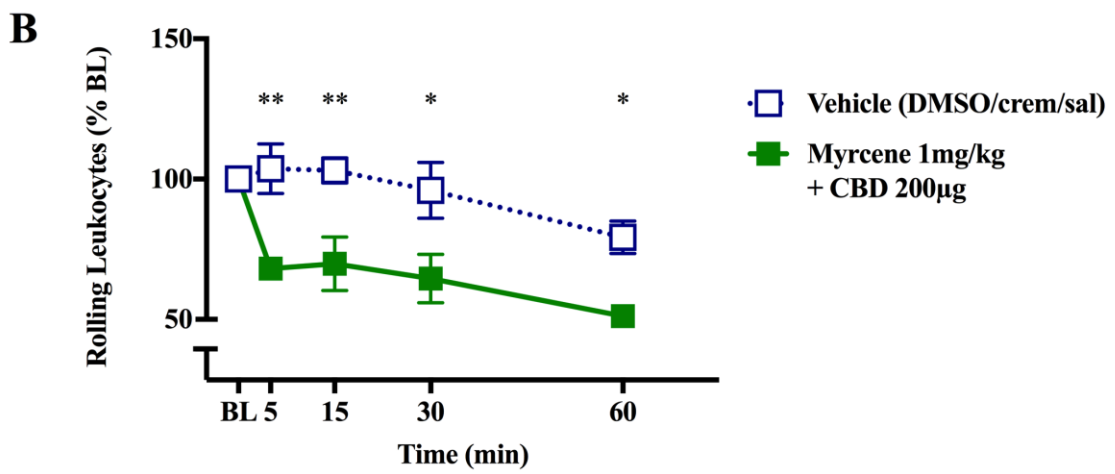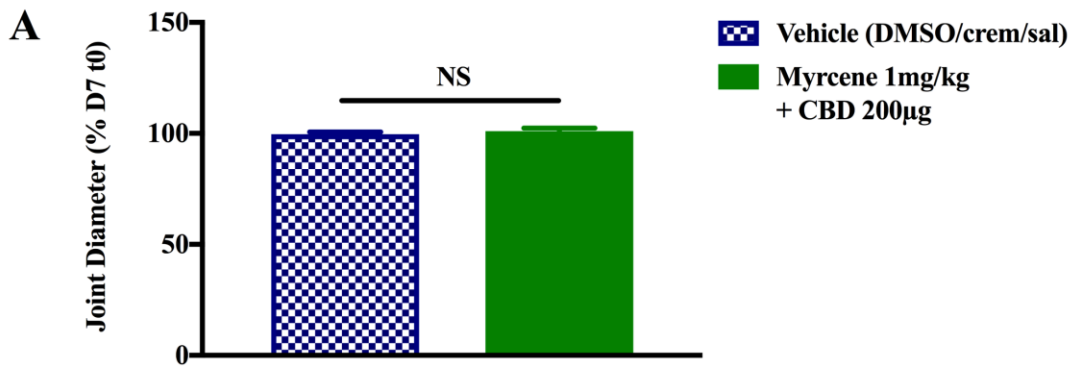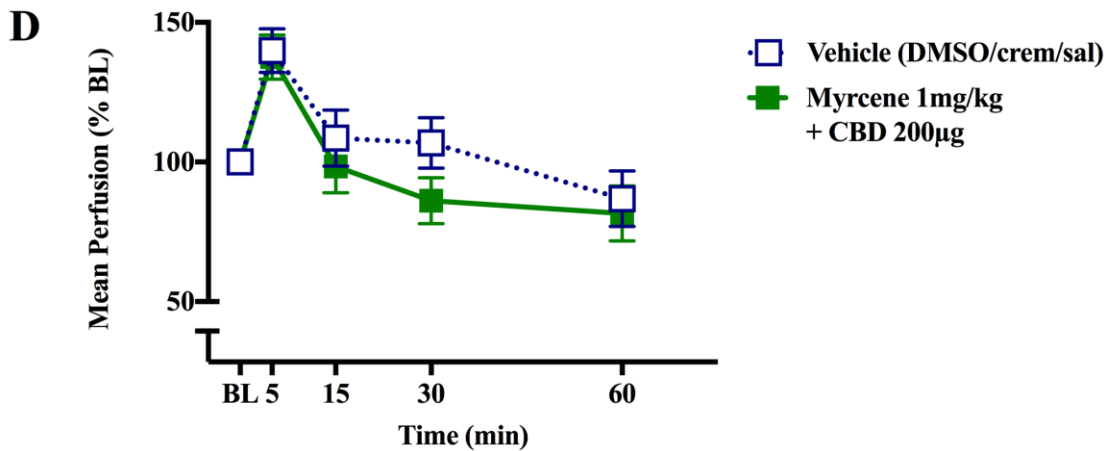
Figure 5.7 The effect of combination therapy versus vehicle on local inflammation.

(A) Joint diameter was not significantly different 3 hours after treatment between combination and vehicle-treated groups (P > 0.05, unpaired Student's t-test, n = 8 per group). **(B)** There was a significant difference in leukocyte rolling between the combination and vehicle-treated cohorts (P < 0.0001, two-way ANOVA, n = 5-9 per time point). *Post-hoc* analysis showed that combination significantly reduced leukocyte rolling at 5 (**P < 0.01), 15 (**P < 0.01), 60 (*P < 0.05) and 60 minutes (*P < 0.05). **(C)** The progression of adherent leukocytes over one hour is did not pass normality, but was analyzed with a two-way ANOVA, in the absence of a non-parametric equivalent. Adherent leukocytes were not significantly altered between combination and vehicle-treated groups (P > 0.05, two-way ANOVA, n = 5-9 per time point). **(D)** There was no significant difference in blood flow to the joint between combination and vehicle treated groups (P > 0.05, two-way ANOVA, n = 5-9 per time point). Ordinary two-way ANOVAs were performed due to a missing time point. Data was standardized as percent baseline, or percent cumulative change and is presented as mean (± SEM). ANOVA, analysis of variance; CBD, cannabidiol; Crem, cremophor; DMSO, dimethyl sulfoxide; NS, not significant; Sal, saline.

A

Joint Diameter (% D7 t0)

NS

- ☐ Vehicle (Soy bean oil)
- ☐ Myrcene 1mg/kg
- ☒ Vehicle (DMSO/crem/sal)
- ■ CBD 200µg
- ■ Myrcene 1mg/kg + CBD 200µg

B

Rolling Leukocytes (% BL)

NS

NS

NS NS

*

- ☐ Vehicle (Soy bean oil)
- ☐ Myrcene 1mg/kg
- ☒ Vehicle (DMSO/crem/sal)
- ■ CBD 200µg
- ■ Myrcene 1mg/kg + CBD 200µg

C

Adherent Leukocytes (% BL)

NS

- ☐ Vehicle (Soy bean oil)
- ☐ Myrcene 1mg/kg
- ☒ Vehicle (DMSO/crem/sal)
- ■ CBD 200µg
- ■ Myrcene 1mg/kg + CBD 200µg

167
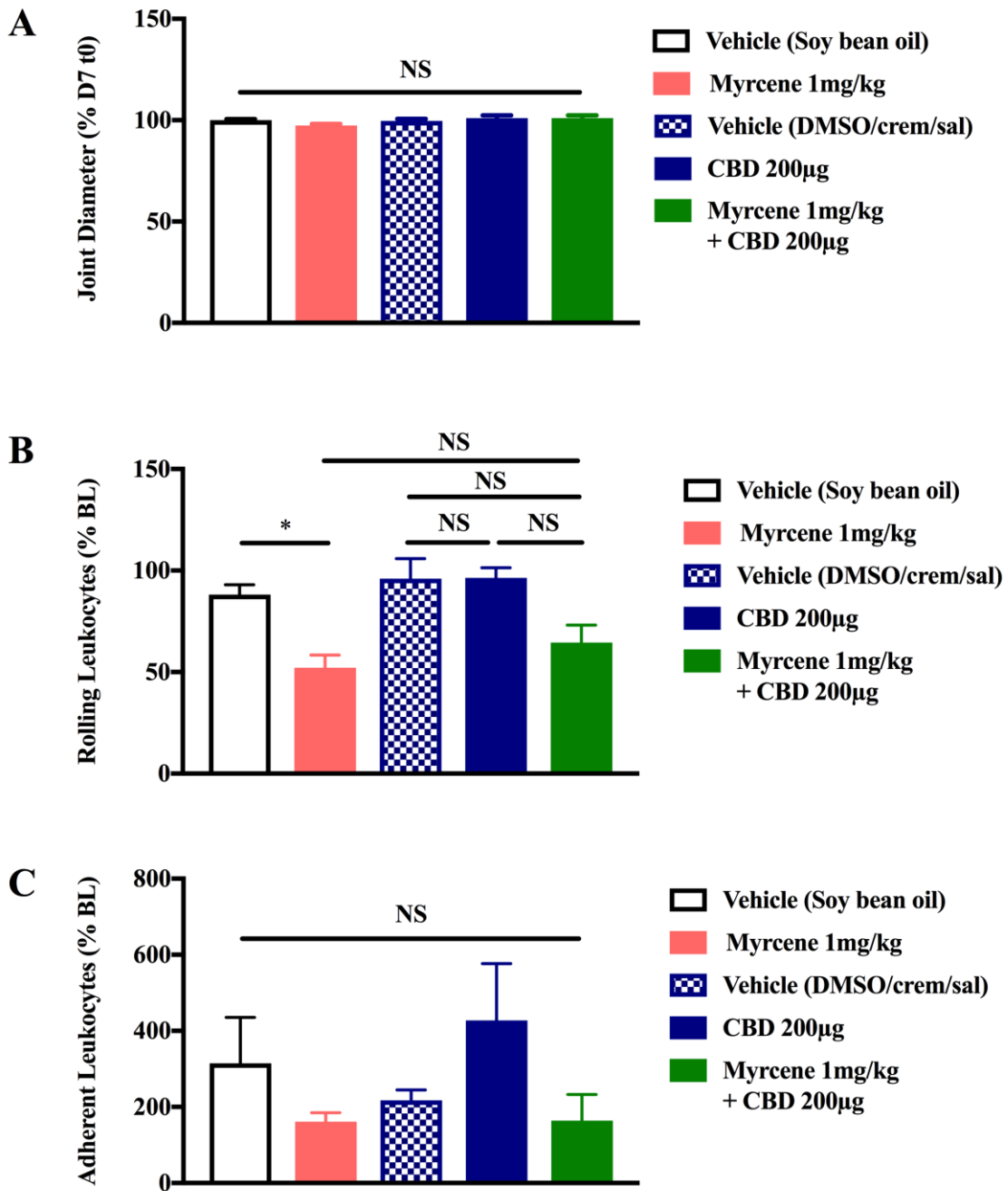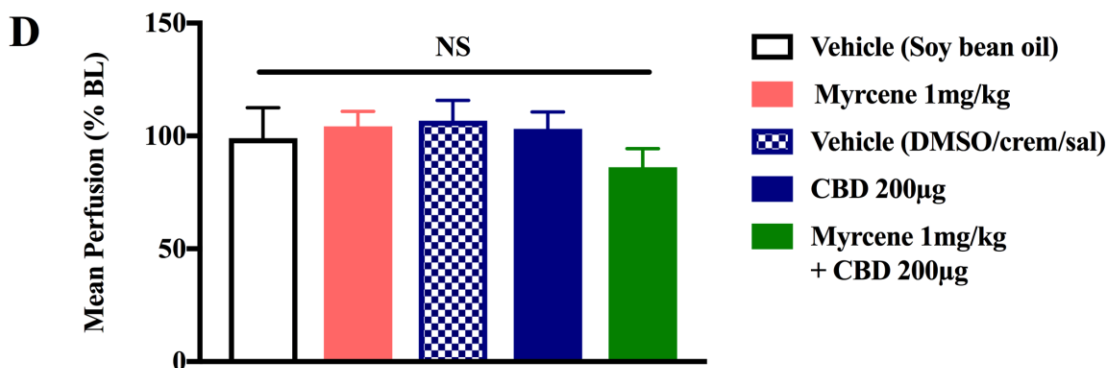
Figure 5.8 Effect of myrcene and CBD combination compared to individual therapies on joint oedema, leukocyte trafficking and blood flow in the inflamed knee joint microvasculature at 30 minutes.

(A) Joint diameter represented as percent day seven baseline did not vary significantly across treatments ($P > 0.05$, one-way ANOVA, n = 8 per group). **(B)** Overall, there was a significant difference in rolling leukocytes amongst treatments ($P < 0.001$, one-way ANOVA with Bonferroni's *post-hoc* test, n = 6-9 per group). *Post-hoc* comparisons indicate that rolling leukocytes were significantly reduced between myrcene and its vehicle (*$P < 0.05$). There was no *post-hoc* difference between myrcene or CBD when compared to the combination treatment, or between combination and its vehicle ($P > 0.05$). **(C)** There was no significant difference in leukocyte adherence amongst treatments ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparisons test, n = 6-9 per group). **(D)** There was no significant difference in mean perfusion to the knee joint between treatments ($P > 0.05$, one-way ANOVA, n = 6-9 per group). Data are standardized as percent day seven baseline, or percent cumulative change and is presented as mean ± SEM. ANOVA, analysis of variance; CBD, cannabidiol; Crem, cremophor; D7, day seven; DMSO, dimethyl sulfoxide; NS, not significant; Sal, saline; t0, time zero.
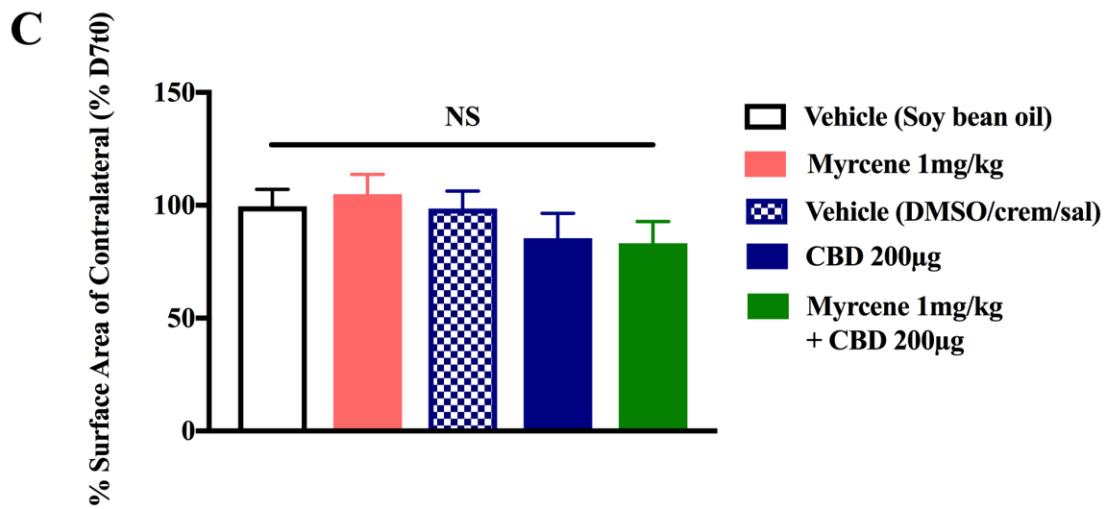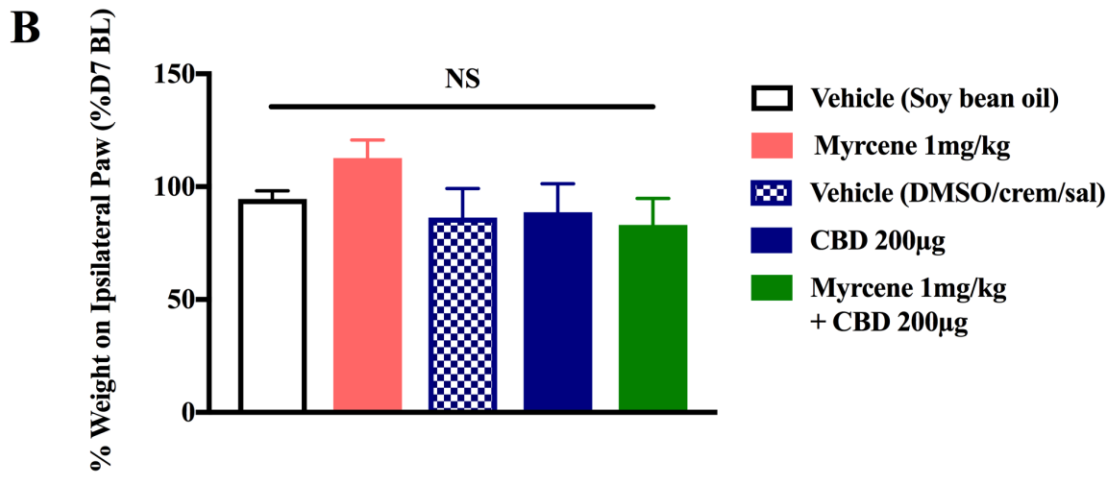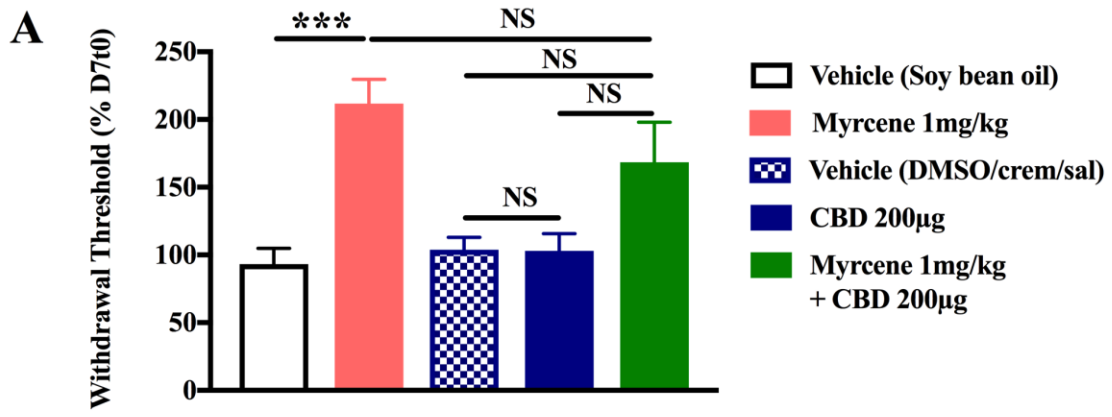
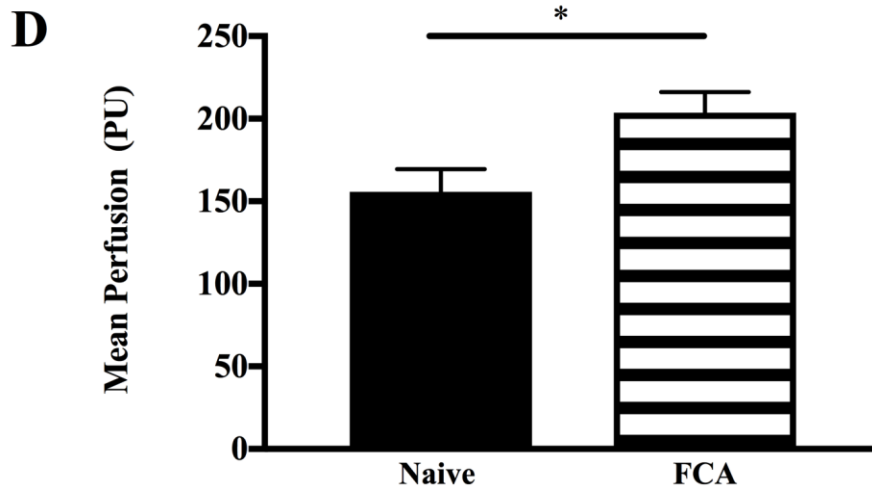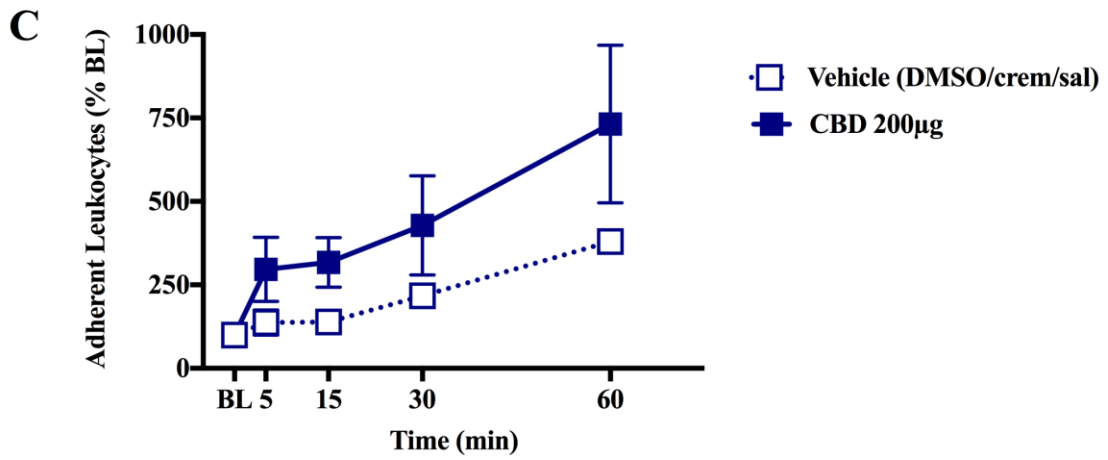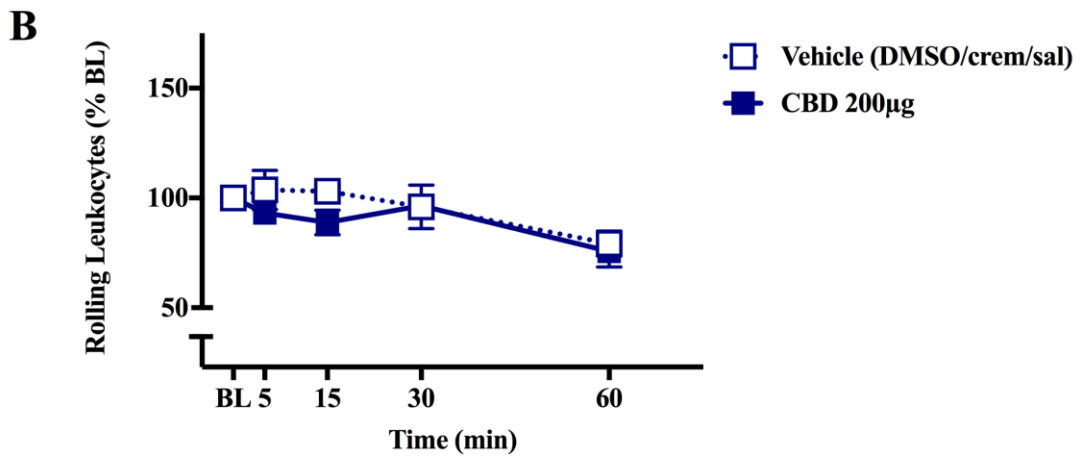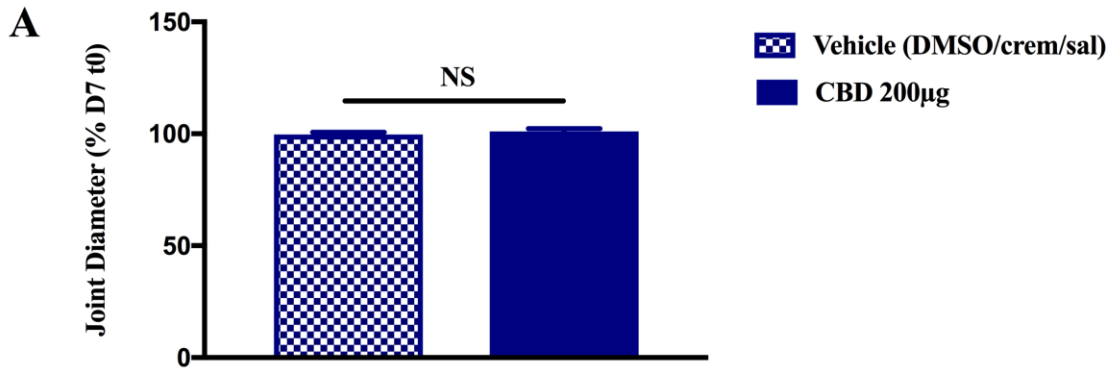# Chapter 6: Discussion and Conclusions

Rheumatoid arthritis is an aggressive and debilitating inflammatory condition that often leaves patients with tissue damage, joint destruction and chronic pain (Lee and Weinblatt 2001). Despite access to moderately effective disease modifying agents, side effect profiles and adequate pain management remain as significant concerns (Nurmohamed and Dijkmans 2005). The body's endocannabinoid system can be used to counteract both pain and inflammation in diseases including RA (Lu and Mackie 2016). Agonism of the cannabinoid receptors in affected joint areas (Schuelert and McDougall 2008), on immune cells (Howlett et al. 2002), and throughout the nociceptive pathway (Richardson et al. 1998) may help modulate joint pain and inflammation (Ethan B Russo and Hohmann 2013). As a result, medical cannabis has been successfully used as an analgesic to manage joint pain (Ware et al. 2005; Blake et al. 2006), notwithstanding its adverse euphoric and cardiovascular effects (Volkow et al. 2014). Isolation and investigation of non-euphoric cannabinoids and terpenes aims to address these concerns while maintaining safe, therapeutic efficacy (Russo and Marcu 2017).

This study adds to pre-existing literature by showing for the first time that the cannabis terpene myrcene can exert anti-allodynic effects and modulate leukocyte trafficking via the endocannabinoid system when administered acutely and chronically in the FCA model of RA.

While combination therapy with low dose CBD did not enhance the therapeutic efficacy, we propose that myrcene may be a safer approach as its therapeutic benefit was equal to that of diclofenac.

6.1   Effect of acute and chronic myrcene treatment on pain

In our preliminary studies, acute treatment with myrcene did not induce secondary allodynia or create weight bearing deficits in naïve animals. Thus, in normal animals myrcene has no direct effect on mechanosensation at the doses tested in our study.

As shown in previous studies, day seven FCA animals exhibited lower withdrawal thresholds (Liu et al. 2009; Li et al. 2014) which is indicative of secondary allodynia in the arthritis model (Loeser and Treede 2008). The demonstration of mechanical allodynia

in FCA animals may be the result of central sensitization as it measures a referred type of pain (Gregory et al. 2013), where the threshold for activating nociceptive afferents is reduced (Yam et al. 2018). Day seven FCA animals also exhibited weight bearing deficits, which has previously been documented in the literature (Griffioen et al. 2015). The unwillingness of the animals to place weight on the affected limb suggests they are feeling spontaneous pain as no evoked stimulus is being applied (Griffioen et al. 2015; Deuis et al. 2017). These results confirm that FCA was producing evoked and spontaneous pain in the treated rats.

After confirming that our model induced pain, we then sought to investigate if the observed pain behaviour could be modulated with a positive control. Diclofenac was chosen as it is a commonly used NSAID to treat inflammatory pain (Crofford 2013). Diclofenac was able to attenuate both mechanical allodynia and improve weight bearing deficits in adjuvant monoarthritic animals. Previous studies have shown that diclofenac is effective in reducing mechanical allodynia in the FCA polyarthritis model (Boyce-rustay et al. 2010; Macedo et al. 2016). Our work corroborates previous studies suggesting that diclofenac improves weight bearing deficits in day seven FCA animals (Huntjens et al. 2009). These findings ultimately demonstrate that the test parameters are sensitive enough to detect analgesia in the FCA monoarthritic model (Li et al. 2014; Philpott et al. 2017). As such, we initially sought to investigate the analgesic potential of the cannabis terpene myrcene in this model.

Acute treatment with 1 and 5mg/kg of myrcene subcutaneously over the knee joint improved mechanical allodynia induced by adjuvant monoarthritis but had no beneficial effect on weight bearing deficits. From this, we can infer that both doses of myrcene were able to reduce evoked pain, but neither dose was sufficient to reduce spontaneous pain behaviour over the three hour testing period. The attenuation of mechanical allodynia may be a result of myrcene modulating nociceptive firing, thus limiting the reflexive withdrawal response to a non-noxious tactile stimulus (Yam et al. 2018). This is consistent with findings by Lorenzetti et al. (1991) who demonstrated that myrcene could inhibit prostaglandin and carrageenan-induced hyperalgesia in response to noxious heat. Other studies have demonstrated that oral, intraperitoneal and subcutaneous administrations of myrcene could reduce acetic acid-induced writhing and increase the

latency to lick in formalin and hot-plate tests (Rao et al. 1990; Paula-Freire et al. 2013). The lack of analgesia observed in the spontaneous pain behaviour tests contrasts with previous analgesic evidence reported in the literature (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013). These differences may be attributed to investigation of both evoked and spontaneous pain behaviours, which were not performed in the previous studies. In addition to nociceptive pain, our model was a chronic inflammatory model involving an inflammatory pain resulting from progressive tissue damage where the other studies tested an acute nociceptive type of pain (Kidd and Urban 2001; Loeser and Treede 2008).

Another difference is that our study, by design, gave myrcene locally to try and mitigate any unwanted systemic/central side effects, whereas previous studies administered myrcene systemically (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013). Chronic disease inflammation and a confined local dose of myrcene may not have been sufficient to alter spontaneous pain behaviour in our study.

There is an ongoing need for multifaceted pain assays when testing novel analgesics in complex animal models (Mogil 2009; Bryden et al. 2015). The lack of improvement in weight bearing, despite significant improvements in secondary allodynia, may be partially explained by the absence of a cognitive aspect of pain in reflexive, evoked pain assays (Mogil 2009; Cobos and Portillo-Salido 2013). Therefore, modulating the behavioural change may be more complex than simply modulating sensory nerve firing (Cobos and Portillo-Salido 2013). Peripheral administration of myrcene may also support this theory as it would not access supra-spinal regions of the nervous system to alter the conscious perception of pain. Additionally, a substantial change in peripheral input and sensitization may be required for the animal to consciously decide to weight bear on the injured limb rather than distributing weight through the non-injured limbs (Cobos and Portillo-Salido 2013). A study by Huntjens et al. (2009) also demonstrated a discrepancy between therapeutic modulation of spontaneous and evoked pain behaviour in the FCA model. Although this study reports efficacy of NSAIDS in improving weight bearing rather than allodynia, it shows that the attenuation of both behaviours may not be simultaneously achieved (Huntjens et al. 2009).

Having characterised the analgesic potential of myrcene in the FCA model, our experiment then explored if these effects of myrcene were mediated through the endocannabinoid system. Our findings were inconclusive in determining the analgesic mechanism of action of myrcene. Both the CB_1R and CB_2R antagonists attenuated the myrcene-induced improvement in mechanical allodynia suggesting endocannabinoid involvement. However, the antagonist vehicle also showed similar attenuations, which confounds the conclusion that the attenuation was due to antagonism of the CB_1R or CB_2R. A possible explanation for this could be a later observation that myrcene precipitates out of solution when initially dissolved in high concentrations of DMSO; although this was not evident in the acute inflammatory experiments. Because the antagonists and myrcene were not pre-mixed together and were not administered subcutaneously as a single bolus, the possible precipitation of myrcene may have hindered its absorption by the tissue and the analgesic potential via this route that was not observed elsewhere. Therefore, we could not conclude that the analgesic actions of myrcene were mediated via the endocannabinoid system.

While frequently used as an antagonist to confer CB_1R mediated effects, AM281 has also been shown to act on GPR55 receptors at micromolar concentrations (Ryberg et al. 2007; Henstridge et al. 2010). Although these studies demonstrate that AM281 may not be selectively inhibiting CB_1Rs (Ryberg et al. 2007; Henstridge et al. 2010), the doses required to activate GPR55 are higher than the concentrations used within this study. This leads us to believe that the attenuation of myrcene-induced analgesia by AM281 would be occurring through CB_1R inhibition rather than GPR55.

This study is the first to report on the interaction of myrcene with the endocannabinoid system. It is possible that the analgesic effects of myrcene are working through a separate analgesic pathway, such as the endogenous opioid system (Rao et al. 1990; Paula-Freire et al. 2013). Two out of the three previously published studies investigating the analgesic effects of myrcene *in vivo* have demonstrated that its effects can be inhibited with administration of naloxone, a non-selective opioid receptor antagonist (Rao et al. 1990; Paula-Freire et al. 2013). Rao et al. (1990) also explored the possibility of alpha-adrenoreceptor potentiation of endogenous opioid release as an analgesic mechanism since yohimbine also reversed myrcene's analgesic effect. As these

receptors are also on pre-junctional afferent terminals, their agonism may also prevent nociceptive firing (Khasar et al. 1995). The mechanism in which myrcene orchestrates analgesia in the FCA model of inflammatory arthritis warrants further investigation. A future study could investigate the effect of antagonizing opioid and adrenergic receptors as suggested by the results of Rao et al. (1990).

Opioid receptors have been shown to act peripherally to reduce chronic inflammatory pain (Labuz et al. 2006). Opioid receptors, like cannabinoid receptors, are found in the knee joint (Li et al. 2005). Opioid receptor agonism on pre-junctional afferent terminals and along the axon, can attenuate nociceptive firing by preventing the afferent from reaching its firing threshold (Sehgal et al. 2011). This mechanism can prevent nociceptive transmission from reaching the dorsal horn and may also prevent the release of neurogenic peptides responsible for peripheral sensitization (Sehgal et al. 2011). Activation of opioid receptors on peripheral immune cells may also promote analgesia by subsequent release of endogenous opioid peptides from macrophages and mast cells (Labuz et al. 2006; Iwaszkiewicz et al. 2013). As we did not confirm experimentally that myrcene was acting solely in the periphery, it is possible that myrcene is acting to attenuate central sensitization in the dorsal horn of the spinal cord (Sehgal et al. 2011; Iwaszkiewicz et al. 2013). Opioid agonism in the dorsal horn may prevent the release of algogenic sensitizing mediators, known as presynaptic inhibition (Sehgal et al. 2011; Iwaszkiewicz et al. 2013). Hyperpolarization of the post-synaptic terminal of the second order neuron would also inhibit spinal transmission of the nociceptive signal (Sehgal et al. 2011).

A direct comparison with a positive control showed that the analgesic effect of myrcene was equal to that of diclofenac. Although diclofenac seemingly alters more parameters in the pain behaviour assay, the magnitude of the effect is not different when compared to that of myrcene. Since similar analgesic profiles were observed, myrcene may be a safer alternative to diclofenac based on the toxic side-effects of long-term NSAID use (Crofford 2013).

Having established the acute effects of myrcene on joint mechanosensitivity, the effects of chronic repeated treatment was tested as this approach may be more relevant to long-term arthritis sufferers. Myrcene was able to improve withdrawal thresholds, but not

weight bearing deficits, before receiving treatment and 2 hours after receiving treatment each day. Once again, these findings suggest that repeated doses are maintaining reductions in the reflex-driven pain response and continue to modulate peripherally driven central sensitization and referred pain. Spontaneous behaviour remained unaltered despite repeated doses, reaffirming that myrcene may not be modulating the cognitive aspect of the pain experience, or that the effect on peripheral nociception is not substantial enough to translate into behavioural modifications (Cobos and Portillo-Salido 2013).

These findings are consistent with research investigating the effect of repeated administration of other terpenes on allodynia (Guénette et al. 2007; Paula-Freire et al. 2014). Beta-caryophyllene and eugenol have demonstrated sustained analgesia with chronic administration (Guénette et al. 2007; Paula-Freire et al. 2014). For example, oral beta-caryophyllene sustained analgesia after being treated for 14 consecutive days in a model of chronic constriction of the sciatic nerve (Paula-Freire et al. 2014). Orally administered eugenol did not exhibit analgesic effects until day 5 of treatment, also in a nerve constriction model (Guénette et al. 2007). Our work supports these assertions that cumulative administration of a terpene can sustain or enhance analgesia over a protracted period of time (Guénette et al. 2007; Paula-Freire et al. 2014).

Throughout the chronic administration study we also investigated the effect of myrcene on locomotor activity. This study revealed that myrcene did not alter quadrant crosses, number of rears, or time spent rearing. The lack of effect on activity was observed in both pre-treatment and post-treatment testing. This suggests that cumulative doses of myrcene did not affect spontaneous activity and was therefore not acting centrally to alter motor circuits or cause any sedative effects. Our outcomes agree with previous literature from Da Silva et al. (1991) and Paula-Freire et al. (2013), who demonstrated that orally administered myrcene did not affect exploratory behaviour. Research by Do Vale et al. (2002) inferred that myrcene could hinder locomotion but locomotor deficits dissipated at lower doses. This supports observations in our study that the low doses of myrcene used here were not acting at higher centres outside of the periphery.

6.2 Effect of acute and chronic myrcene treatment on inflammation

Preliminary studies revealed that acute treatment with myrcene did not produce inflammation in naïve animals. Again, these findings corroborate previous literature that support the use of myrcene in therapeutic testing (Rao et al. 1990; Lorenzetti et al. 1991; Paula-Freire et al. 2013). As a result, we wanted to investigate the anti-inflammatory potential of myrcene in an inflammatory model of joint disease.

Day seven FCA animals exhibited significant increases in oedema, leukocyte trafficking, and blood flow, confirming a robust inflammatory reaction in the joints of FCA-injected animals as previously described (Johnston and Kubes 1999; Liu et al. 2009; Son et al. 2014; Tian et al. 2015; Hammell et al. 2016). Testing of a positive control confirmed that diclofenac was able to reduce leukocyte rolling and blood flow to arthritic knee joints. However, diclofenac had no effect on joint oedema, and did not significantly reduce adherence of leukocytes, despite data trending towards that conclusion. The dose of diclofenac, the short dosing regimen of diclofenac and the time that these parameters were observed may contribute to these findings. The modulations of leukocyte trafficking are consistent with previous literature (Macedo et al. 2016; Akramas et al. 2017). It has been suggested that the anti-inflammatory actions of NSAIDs on leukocytes are mediated by the down regulation of adhesion molecules such as ICAM and P-selectin (Rodrigues et al. 2008). Despite not modulating knee joint oedema, diclofenac has been shown to reduce paw oedema in the FCA model (Macedo et al. 2016). This could be due to the systemic administration in these experiments or the use of higher doses of the drug in that study (Macedo et al. 2016). These findings ultimately show that inflammation in the monoarthritic FCA model can be modulated by exogenously applied drugs.

Myrcene applied topically over the exposed knee joint reduced rolling leukocytes, but had no effect on leukocyte adherence, joint hyperaemia or joint oedema at either dose tested. The anti-rolling effect of myrcene may be due to the action of myrcene on specific adhesion molecules. Rolling initiation is regulated by P-selectin and E-selectin (Ley 1996; Muller 2011). Slow rolling as well as adhesion of leukocytes to the endothelium involves ICAM and VCAM molecules (Muller 2011). Therefore, the differential effect of myrcene on rolling, but not adhesion may be due to preferential regulation of selectins rather than adhesion molecules (Ley 1996); this theory warrants further testing. Previous

work has shown that terpenes such as alpha-pinene, beta-pinene, limonene and beta-caryophyllene are able to down regulate these extravasation-dependent selectins (Johard et al. 1993; d'Alessio et al. 2014) and adhesion molecules (Zhang et al. 2017).

The anti-inflammatory effect of pure myrcene *in vivo* had not yet been tested. The reduction of rolling leukocytes by acutely administered myrcene in the FCA model coincides with previous findings that myrcene exhibited anti-inflammatory properties (Rufino et al. 2015). However, only broad comparisons could be inferred as the only previous study investigated nitric oxide production and MAPK pathway activation *in vitro* using human chondrocytes (Rufino et al. 2015). Despite the *in vitro* nature of the study, MAPK activation has been shown to downregulate adhesion molecules in the presence of inflammation (Guo 2004), which could explain our findings.

Investigation of endocannabinoid system involvement in leukocyte trafficking revealed that CB_1R and CB_2R antagonism attenuated the myrcene-induced reductions in leukocyte rolling. Although AM281 is not entirely selective for the antagonism of CB_1Rs, higher concentrations than used in this study are necessary to promote activation of GPR55 as previously stated (Ryberg et al. 2007; Henstridge et al. 2010). Therefore, it is unlikely that the attenuating effects of AM281 are occurring through GPR55 in our experiments.

It is known that cannabinoid receptors, especially CB_2R are upregulated in inflammatory environments (Pertwee 2009; Miller and Devi 2011). An increase in the quantity of receptors may promote or enhance binding of myrcene to CB_2R, thus providing an anti-inflammatory mechanism for myrcene that was not observed under normal conditions (Duncan et al. 2008).

Despite no previous literature linking myrcene and the endocannabinoid modulation of peripheral inflammation, the involvement of the endocannabinoid system in the regulation of myrcene-induced leukocyte-endothelial interactions is not a novel concept (Turcotte et al. 2016). Agonism of the CB_2 receptor by synthetic cannabinoids, phytocannabinoids and terpenes has demonstrated reductions in leukocytes (Zhao et al. 2010; Lehmann et al. 2012; Turcotte et al. 2016; Philpott et al. 2017; Zhang et al. 2017). CB_2R-induced down regulations of selectin and adhesion molecules responsible for leukocyte rolling and adhesion are thought to be the reason for the attenuation of

leukocytes undergoing the extravasation (Zhao et al. 2010; Lehmann et al. 2012; Zhang et al. 2017). The CB_2R is coupled to a $G_{i/o}$ protein which can inhibit phosphorylation of kinases responsible for gene expression and can down regulate expression and transcription of adhesion molecules and pro-inflammatory cytokines (Howlett et al. 2002). Similarly, CB_1R agonism can inhibit the pre-junctional terminal from firing thus preventing antidromic release of pro-inflammatory peptides which would result in neurogenic inflammation (Richardson et al. 1998).

The novel finding that myrcene exerts anti-inflammatory effects through the endocannabinoid system adds to the growing body of literature showing that terpenes have therapeutic value (as reviewed by Russo and Marcu 2017). While comparing to diclofenac, a clinically used NSAID, myrcene showed equiefficacious anti-inflammatory actions against rolling leukocytes, whereas neither treatment modulated adherence. Diclofenac, however, was able to modulate blood perfusion to the affected knee joint area. Although CB_1R and CB_2R agonism has been implicated in the control of vasodilation and haemodynamic changes (Richardson et al. 1998), the chosen dose or time frame that we looked at may not have been favorable to capture myrcene's full impact on this parameter. Neither diclofenac nor myrcene were able to alter joint oedema. This may be owing to reductions of blood flow preceding changes in oedema, the dose of diclofenac used, the short-term that diclofenac was administered or the sensitivity of the calipers.

Unlike an acute inflammatory event, RA presents with persistent and chronic inflammation (Aletaha et al. 2010). As a result, repeated treatment with a therapeutic may be necessary to take effect on the insurmountable inflammation and sustain anti-inflammatory effects throughout the progression of chronic inflammation (Singh and Sharma 2015). In our research, repeated treatment with myrcene over a 21 day time period ultimately reduced local inflammation by attenuating leukocyte trafficking (both rolling and adherent) and reducing blood flow to the knee joint compared to vehicle cohorts. Although joint oedema was not altered, our findings suggest that myrcene exhibited enhanced anti-inflammatory actions after repeated administration. Thus, chronic myrcene treatment appears to be better at reducing inflammation compared to an acute administration.

To our knowledge, no studies have investigated the effect of repeated administration of myrcene on chronic inflammation. Additionally, very few terpene studies touch on the anti-inflammatory actions of terpenes after repeated administration. One study by Vijayalaxmi et al. (2015) investigated the anti-inflammatory effects of repeated administration of beta-caryophyllene in the FCA model over 21 days. Their work showed that repeated administration of beta-caryophyllene was able to attenuate both joint oedema and clinical inflammation scores, while also showing reductions in nitrite levels and radical oxygen species at the end of the experiment (Vijayalaxmi et al. 2015). Despite measurement and terpene differences, these two compounds show comparable CB_2 and opioid-like mechanisms (Katsuyama et al. 2013). This corroborates our findings that repeated administration may mitigate chronic inflammation.

The more encompassing anti-inflammatory effect of myrcene during repeated treatment could be attributed to the up-regulation of CB_1Rs and CB_2Rs in chronic pain models (Siegling et al. 2001; Pertwee 2009) and inflammatory conditions (Miller and Devi 2011; Concannon et al. 2015; Jean-Gilles et al. 2015). Increased expression of these receptors, as reviewed by Pertwee (2009) and Miller et al. (2011), can increase efficacy of the agonist. Duncan et al. (2008) show that in an LPS-induced inflammatory state, a CB_2R agonist can improve contractility of the intestines, which is not observed in basal conditions. Similar improvements in inflammation were observed with up-regulations of CB_1Rs in colitis (Kimball et al. 2006). Reductions in these parameters for the chronic study, but not in the acute treatment, could also be attributed to the timeframe in which the parameters were investigated. During acute studies, the inflammatory time course was limited to one hour; while in the chronic study, joint exposure and inflammatory measures were performed after 21 days of inflammation. Due to behavioural testing that was also performed on day 21, the inflammatory parameters were recorded approximately six hours after treatment, compared to the acute analysis performed throughout one hour. As a result, the treatment had been on board longer and we may have captured a more efficacious time point.

Despite local anti-inflammatory effects, repeated administration of myrcene did not alter systemic cytokine levels compared to vehicle controls. Essential oils with myrcene, as well as with other terpenes, have shown the ability to reduce systemic

cytokines (Souza et al. 2003; Suh et al. 2016). This suggests that myrcene may be exerting its anti-inflammatory actions independently of cytokine regulation. Examples of this may include reductions of neurogenic mediators or other inflammatory mediators (Richardson et al. 1998). It is possible that the local nature of myrcene administration, by design, may have prevented it from acting systemically. The previous studies had all used systemic administration routes (Souza et al. 2003; Suh et al. 2016). Additionally, FCA was induced as a monoarthritic local model rather than as a systemic disease. The local nature of our model may have limited the more profound systemic inflammatory response that is observed in some other models (Taurog et al. 1988; Bendele 2001). Assessing levels of these molecules in synovial fluid may be more appropriate than plasma analysis since the cytokines may have been confined to the joint in the monoarthritic model rather than in the general circulation (Barton et al. 2007).

Repeated administration of myrcene also did not have any effect on joint damage parameters including inflammatory cell infiltration, pannus formation, bone resorption and cartilage damage. These findings suggest that myrcene was not influencing disease progression in this model. *In vitro* studies demonstrated that myrcene reduces levels of MMPs, which are known to induce joint damage (Choy 2012), and increase levels of their endogenous inhibitors (Rufino et al. 2015). This work contradicts our findings as it suggests that myrcene should have the potential to alleviate joint damage (Rufino et al. 2015); however, the dosing or exposure time may not have been adequate enough to for inhibit or repair damage in this model. Elsewhere, repeated systemic administration of the sesquiterpene beta-caryophyllene, which exhibits similar mechanisms of action to myrcene, attenuated joint damage in day 21 FCA animals (Vijayalaxmi et al. 2015), suggesting that other terpenes can influence damage in this model. The systemic nature of this study, and the systemic dosing of terpenes may contribute to the differential results. For example, systemic FCA may create a more robust onset of damage (Bendele 2001) compared to the monoarthritic model, leaving more room for modulation to occur.

6.3 Effect of low-dose CBD alone, and in combination with myrcene on pain and inflammation

Considering the vast chemical profile of cannabis and the entourage phenomenon of these compounds (Russo 2011), we wanted to see if combining myrcene with a phytocannabinoid could enhance the analgesic potential of the individual compounds (Do Vale et al. 2002). We chose to investigate CBD since this cannabinoid has been shown to be efficacious in the FCA model (Costa et al. 2007; Hammell et al. 2016), and because of the distinct receptor mechanism by which CBD exerts its effects (Laprairie et al. 2015; Hammell et al. 2016; Philpott et al. 2017). Targeting multiple receptor pathways may enhance the therapeutic benefits of myrcene and CBD when given together. We chose a low dose of CBD to test for any synergistic effects with myrcene. The dose of CBD we chose did not improve mechanical allodynia or weight bearing in day seven FCA-injected rats. At this given dose, our findings support that of Philpott and colleagues, who also showed no improvements in pain behaviour in the MIA model of osteoarthritis confirming that we had chosen a subclinical dose of CBD (Philpott et al. 2017). The low dose of CBD also had no anti-inflammatory effects in the model as evidenced by a lack of change in joint oedema, leukocyte trafficking or blood perfusion. After confirming that low dose CBD did not induce analgesia or reduce inflammation, we combined the 200μg dose with 1mg/kg of myrcene to explore the synergistic potential of the combination. Compared to the acute myrcene studies, the combination improved mechanical allodynia but had no effect on weight bearing deficits. Similarly, the combination attenuated leukocyte rolling, but had no effect on oedema, leukocyte adhesion or perfusion. However, the combination therapy was not significantly different from treatment with myrcene alone. These findings suggest that myrcene and CBD are not acting synergistically to enhance anti-nociception or to reduce inflammation. The absence of any enhancement in analgesia and anti-inflammation infers that myrcene was the primary active compound in the combination therapy.

The lack of synergism may be attributed to the compounds used, or the doses used within our experimental design. As observed in our acute studies, the 1mg/kg and 5mg/kg doses were not dose-dependent, and a higher dose was not tested because of the sedative effects that occur at higher concentrations. It is possible that the doses of myrcene we

used were already providing a maximum effect, and therefore could not be enhanced further in combination. To address this possibility, a suboptimal dose of myrcene could have been tested in combination with low dose CBD. An observed analgesic or anti-inflammatory response in the presence of two suboptimal doses of compounds may have given a better insight into any synergistic potential, as described by Ben-Shabat et al. (1998). The absence of synergism may also be explained by the receptor mechanisms involved with the two compounds. CBD can act on TRPV1 receptors to desensitize nociceptive firing (Iannotti et al. 2014), whereas myrcene is not known to act on TRPV1 receptors. Therefore, a suboptimal dose of CBD may not have been enough to fully desensitize the receptor to induce analgesia through inhibition of algogenic and pro-inflammatory mediators (Ahluwalia et al. 2003; Iannotti et al. 2014). CBD has also been described as a negative allosteric modulator of the CB_1R (Laprairie et al. 2015). Despite previous preclinical studies and clinical observations suggesting that CBD can enhance the analgesic effects of THC, which acts via CB_1R and CB_2Rs like myrcene, it is also possible that a negative allosteric modulation of the CB_1R by CBD is reducing any potential therapeutic enhancements (Laprairie et al. 2015).

Studies have previously reported synergistic effects when combining a low dose of a phytocannabinoid with an active dose of an opioid. Specifically, morphine was combined with an inactive dose of THC to enhance nociception in the tail-flick test (Smith et al. 1998). This evidence does not use the same compounds as our research. Moreover, although the mechanisms of these compounds may be similar, involvement of TRPV1 receptor mechanisms of CBD as reported by Philpott et al. (2017) may account for the differences.

Our work also corroborates previous findings demonstrating that purified myrcene exhibited similar analgesic trends to that of the total essential oil, suggesting that the actions of myrcene are not enhanced by other terpenoids or other plant-derived molecules in the oil (Paula-Freire et al. 2013).

6.4   Summary

Our results show that acute administration of myrcene can reduce FCA-induced mechanical allodynia and can prevent leukocyte rolling. The anti-rolling actions of

myrcene appear to be mediated via cannabinoid receptors, but the analgesic actions could not be confirmed to be endocannabinoid system dependent. Repeated administration of myrcene demonstrated enhanced anti-inflammatory actions and sustained improvements in mechanical allodynia, but this had no effect on systemic cytokine levels or joint damage. The effects of myrcene showed similar efficacy to diclofenac. Finally, the therapeutic effects of myrcene were not enhanced by administering the terpene in combination with low dose CBD.

6.5   Study limitations

6.5.1   Model

The monoarthritic profile of our model may limit its translatability to the human condition which is known for its bilateral and systemic manifestation (Lee and Weinblatt 2001). Confinement of the inflammatory pathophysiology to a single joint allowed for inter-limb comparisons of relevant, validated pain and inflammatory measures (Deuis et al. 2017). Additionally, the inflammatory propagation of our model and the parameters we chose to investigate focus primarily on the innate immune response (oedema, leukocyte trafficking, etc.), even though we acknowledge that the adaptive immune response is also a contributor to RA (Firestein 2003). Similarly, FCA does not present with auto-antibodies derived from the adaptive immune response that are prevalent in the human conditions (Taurog et al. 1988). Effects on the innate immune response are still relevant in determining the anti-inflammatory potential of myrcene, however, the scope and translatability to the human condition may be limited.

Despite random allocation of treatments in our study, inoculation with FCA showed varying inflammatory and algogenic responses, despite random allocation of treatments. Variance in arthritic responses could be attributed to different antigen levels in the injection since the bacteria were suspended in paraffin oil. Therefore, comparing analgesia and anti-inflammatory potential using raw values was without merit as the groups were inherently different at baseline. To mitigate variations within the model, the data were presented as percent baseline on the given testing day.

6.5.2   Pain Techniques

An inherent limitation of rodent pain behavioural analysis is that outcome measures make inferences based on nociceptive parameters and not pain directly (Mogil 2009). With this caveat, a limitation of dynamic weight bearing analysis is the requirement for the animal to be ambulatory or distinctly placing weight on the hind paws (Deuis et al. 2017). Animals injected with FCA tend to have reduced locomotor activity (Matson et al. 2006), and anecdotally prefer to lean or lay down in the chamber. If the animal is unwilling to directly weight bear, more weight may be placed on areas such as the tail or lower abdomen. This limits our ability to detect a specific weight bearing deficit between ipsilateral and contralateral hind paws. The overhead camera placement may also be a limitation during both weight bearing analysis and locomotion investigations (Piel et al. 2014). Other set ups such as CATWALK use an underneath position that may give a better perspective when validating hind paws (Piel et al. 2014; Deuis et al. 2017). Additionally, FCA animals may also rear solely on the contralateral paw as observed in our study, which may go undetected with an overhead camera. Photobeam Activity Systems have also been implemented in activity set ups to detect movement when the beams are interrupted, but this approach still poses its own limitations such as incomplete actions that manage to break the beam's surface (Piel et al. 2014; Deuis et al. 2017).

Measurement of mechanical allodynia with von Frey filaments also requires the test subject to weight bear on the treated paw (Deuis et al. 2017). FCA animals tend to favour their ipsilateral paw and may not weight bear or use the plantar surface to weight bear (Quadros et al. 2015). This makes applying the filament difficult. Additionally, 15g is used as the force cut off for rats as higher forces can elevate the paw irrespective of withdrawal response (Chaplan et al. 1994). This cut off limits the full analgesic potential of a test reagent.

6.5.3   Inflammation techniques

Techniques used to measure and quantify inflammation may introduce study limitations within our study. Surgical cannulations, and exposure of the knee joint took place in order to perform intravital microscopy and blood flow analysis. This involved

removal of the skin surrounding the area and blunt dissection of the fascia proximal to the joint. These procedures may inherently introduce inflammation. This could potentially provide an explanation as to why rolling leukocytes exist even in naïve, healthy animals involved in our study. However, the surgery was completed in all animal cohorts and the data was represented as percent baseline to account for differences.

The exposure of the microvasculature of the joint over time may also be a source of inflammation. During the experiment, attempts were made to maintain physiological conditions and to prevent the joint from drying out as Atherton and Born recommend (1972). However, our set up is not equipped with constant perfusion of the exposed joint. This may have been an explanation for the increase in adherent leukocytes in naïve or vehicle cohorts.

Furthermore, our analysis investigated leukocyte trafficking through nuclear labelling with rhodamine 6G. The non-selective nature of the dye prevents us from making any conclusions as to the types of leukocytes involved in the transmigration process in the FCA model (Brackmann 2000).

6.5.4   Drugs

The solubility of myrcene proved to be a limitation in our study. Based on the limited existing literature (Paula-Freire et al. 2013), we originally dissolved myrcene in soy bean oil. Every other compound used in our study was soluble in a DMSO, cremophor, and saline solution. Despite this potential limitation, the treatments were always compared to their respective vehicle.

DMSO can also act as an analgesic/anti-inflammatory on its own (Elisia et al. 2016), albeit at higher concentrations than used in our study. This may have influenced comparisons between this vehicle and other treatments within this study.

6.5.5   Analysis

Due to significant between group variation in baseline pain and inflammation scores, the data were presented as percent baseline. The statistics were also run on the percent baseline data due to intergroup variability that would have skewed the statistical testing of differences.

Other limitations involving analysis of the data were evident in withdrawal threshold data using von Frey hair algesiometry. Due to the nature of the procedure, there is a maximum cut-off measurement that inherently precludes the data from achieving normality. Despite a lack of Gaussian distribution, two-way parametric ANOVAs were carried out in the absence of a non-parametric equivalent to assess the effect of treatment over time.

6.6   Future directions

6.6.1   Is myrcene a peripherally acting therapeutic?

Based on myrcene's potential to act as a sedative (Do Vale et al. 2002), our study administered the drug subcutaneously over the affected area to mimic local administration. We also recognize that subcutaneous administrations are considered systemic routes. Although we postulate that myrcene may be acting peripherally based on the lack of central adverse effects, this needs to be confirmed. Other studies have previously administered the test drug contralaterally to see if any analgesic or anti-inflammatory effects occur systemically (Philpott et al. 2017). A lack of analgesic or anti-inflammatory action to the ipsilateral limb when given contralaterally would suggest that the drug is in fact restricted to a peripheral site of action (Philpott et al. 2017). These future studies would add to previous literature suggesting that local administration of myrcene could act peripherally while avoiding any sedative or euphoric concerns.

6.6.2   What is the mechanism of myrcene analgesia in joints?

Based on our findings, we could not conclude that the endocannabinoid system was involved in mediating the analgesic effects of myrcene. Previous literature posits that the endogenous opioid system may be responsible for the analgesic actions of myrcene in acute pain models (Rao et al. 1990; Paula-Freire et al. 2013). Our next step would be to investigate whether myrcene is exerting analgesic properties through agonism of peripheral opioid or adrenoreceptors in the FCA model. To do this, we would administer either the opioid receptor antagonist naloxone or the adrenoreceptor antagonist yohimbine together with myrcene to examine any alteration in effects (Rao et al. 1990; Paula-Freire et al. 2013). It would also be noteworthy to use a peripherally restricted

opioid antagonist such as naloxone methiodide to confirm peripheral opioid analgesia as described by Katsuyama et al. (2013). These future studies would determine whether these mechanisms are involved in chronic disease models.

6.6.3   How is the extravasation pathway being controlled by myrcene?

Although our findings show evidence for CB_2R control of leukocyte trafficking in the presence of myrcene, the exact mechanism has not yet been elucidated. To investigate this matter, molecular levels of selectins (P-, E-selectin) or adhesion molecules (ICAM-1, VCAM-1) could be examined (Muller 2011). Similar studies have performed such analysis, investigating a CB_2R agonist (Lehmann et al. 2012). They showed that CB_2R agonism reduced leukocyte adherence as well as levels of ICAM and VCAM in a model of experimental sepsis (Lehmann et al. 2012). Lower levels of these molecules would infer that myrcene is altering the proliferation of molecules responsible for leukocyte-endothelial interactions during inflammation including leukocyte rolling, adhesion and ultimately extravasation.

6.6.4   Can our existing combination therapy be improved?

The combination investigated in our study used an active dose of myrcene with a low dose of CBD because previous studies have shown that an inactive dose of a cannabinoid could enhance the nociception provided by an analgesic (Smith et al. 1998; Richardson 2000). We also hypothesized that combination of myrcene with CBD may enhance anti-inflammatory actions due to CBD's negative allosteric modulation of the CB_1R. (Laprairie et al. 2015). Future studies could look to improve this combination by exploring an inactive dose of myrcene as well as a therapeutic dose of CBD based on our model and route of administration. Combining an optimal dose of CBD with an inactive dose of myrcene may provide receptor desensitization (Philpott et al. 2017), coupled with additional analgesia provided by myrcene. Additionally, Ben-Shabat et al. (1998) propose exploring synergism using two inactive doses. Moreover, limited literature exists on *in vitro* terpene-terpene synergism and therefore warrants further investigation in the search for a desirable therapeutic based on an entourage effect.

6.7   Conclusions

Our findings show that both acute treatment with myrcene can modulate evoked pain behaviour and prevent leukocyte rolling. Chronic administration of myrcene can also modulate evoked pain and local knee joint inflammation in the FCA model of rheumatoid arthritis. Our results substantiate therapeutic targeting of the endocannabinoid system using isolated cannabis constituents. The lack of adverse centrally-mediated side effects may support the therapeutic use of myrcene over euphoric cannabinoid and opioid treatments as well as unsafe NSAIDs. As such, myrcene may be a suitable alternative option for managing pain and inflammation alongside pre-existing disease modifying agents. However, more work needs to be carried out to further clarify the analgesic mechanisms, its synergistic potential with other cannabis constituents, and to fully harness therapeutic dosing levels.

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