Involvement of Lysophosphatidic Acid in Peripheral Neuropathy and Osteoarthritic Pain in Rats

by

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DEDICATION PAGE

I would like to dedicate this to my parents for their continuous support, which means more to be than they will ever know. Also, to all of the animals who valiantly gave their lives in the pursuit of science.

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ABSTRACT

A recent study discovered elevated levels of lysophosphatidic acid (LPA) in the synovial fluid of OA patients (Eli Lilly, unpublished). LPA is required for the initiation of neuropathic pain (Inoue, 2004), and therefore, elevated levels are indicative of a neuropathic pain state. The present study attempted to determine: if 1) LPA causes neuronal damage to joint afferents, and 2) if LPA is responsible for a neuropathic pain component in OA. The experimental OA model monosodium iodoacetate (MIA) was found to cause demyelination to the saphenous nerve at 14 days post-treatment. Selective LPA antagonism prevented this damage, implicating LPA in this novel pain state. The present study concluded that 1) neuropathic pain is a component of OA, and 2) LPA facilitates the initiation of this neuropathic pain. These new findings will allow better understanding of disease etiology and may lead to the emergence of an entirely new line of OA therapeutics.

LIST OF ABBREVIATIONS USED

AMPA	α-amino-3-hydroxy-5-methyl-4
ANOVA	Analysis of Variance
Ca_v	Voltage-gated calcium channel
CNS	Central nervous system
CPP	Condition placed paradigm
DMOAD	Disease-modifying osteoarthritis drug
dH20	Distilled water
DRG	Dorsal root ganglion
GPCR	G-protein coupled receptor
H_20	Water
HA	Hyaluronic acid
i.a.	Intraatricular
i.p.	Intraperitoneal
i.t.	Intrathecal
K_v	Voltage-gated potassium channel
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
MIA	Monosodium iodoacetate
Na_v	Voltage-gated sodium channel
NK1	Neurokinin 1
NS	Nociceptive specific
NSAID	Non-steroidal anti-inflammatory drug
NMDA	N-Methyl-D-aspartic acid
OA	Osteoarthritis
P2X	Purinergic
PAG	Periaqueductal grey
PAR	Protease-activated receptor
PB	Parabrachial area
PGE_2	Prostaglandin E2
PNS	Peripheral nervous system
RVM	Rostral ventromedial medulla
SD	Sprague-Dawley
SEM	Standard error of the mean
TEM	Transmission electron microscope
TRPV1	Transient receptor potential vanilloid 1
VEH	Vehicle
VIP	Vasoactive intestinal peptide
WDR	Wide dynamic range

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CHAPTER 1 INTRODUCTION

Osteoarthritis (OA) is the most prevalent musculoskeletal disorder in the world. Once considered a disease to primarily affect the elderly, disease onset is now understood to be initiated by factors independent of age. It has recently been reported that 30% of adults possess disease symptoms (Zhang, 2010). At an OA consensus conference (Toronto, ON, 2000), patients reported their primary concern was finding relief from the debilitating pain that negatively affects their quality of life. OA pain is the number one reason for physician visits (Hadler, 1992) and a large subpopulation of OA patients receives no analgesic effects from current pharmacotheraputic treatments (Neame et al., 2004; Towheed et al., 2006). One explanation for the lack of efficacy of analgesics, known to attenuate inflammatory states, is the presence of an additional pain state. Hochman et al. have determined 34% of OA patients experience neuropathic pain (Hochman et al., 2010). Additionally, a large percentage of OA patients who have received arthroplasty surgery experience post-surgical resting pain (Wylde et al., 2011). This further supports the presence of a neuropathic component to OA pain. The presence of this additional pain state may explain the lack of efficacy of analgesics, as opioid receptors have been demonstrated to be downregulated following peripheral nerve injury (Zhang et al., 1998).

Together, these findings create the pressing need for the development of Disease Modifying Osteoarthritic Drugs (DMOADs). To date, a lack of understanding of disease etiology has resulted in a deficiency of disease-specific therapeutics. With the incidence of OA increasing and the average age of disease onset lowering, there is a pressing need for a complete understanding of disease etiology and the development of DMOADs.

1.1 PAIN

1.1.1 Overview of Pain

Although pain is often thought of in a negative context, the sensation of pain is an adaptive trait that facilitates survival. Acute pain (nociceptive pain) warns individuals of dangers in their surroundings by causing an aversive sensation that dissipates when the harmful stimuli are removed; thus, preventing any further damage. Nociceptive pain is facilitated through activation of nociceptors (high-threshold free nerve endings located in the periphery) by noxious chemical, thermal, and mechanical stimuli.

Chronic pain, conversely, is maladaptive and impedes the quality of life. Chronic pain is defined as any pain that lasts longer than 3 months ("Classification of chronic pain. Descriptions of chronic pain syndromes and definitions of pain terms. Prepared by the International Association for the Study of Pain, Subcommittee on Taxonomy," 1986); much longer than required for tissue to fully regenerate following an injury.

Our current understanding of pain mechanisms is largely due to the Gate Theory proposed by Melzack and Wall (Melzack et al., 1965). Although this theory has been revised, it was the advent of the modern pain era, transitioning pain research away from the previous Descarte notions. Rene Descarte hypothesized that peripheral nerves directly connect sensations in the periphery to the brain, allowing for instantaneous registration of pain. “Just as by pulling at one end of a rope one makes to strike at the same instant a bell which hangs at the other end.” (Descartes, 1644).

Sherrington (1911) amended the Decarte theory and proposed there were specific subclasses of afferent neurons that he termed nociceptors. This revolutionary thinking led the way for Melzack and Wall’s Gate Theory. In brief, Gate Theory postulates that there are several sites of propagation within the nociceptive system. Modulation of stimuli occurs at each site to facilitate its onward propagation or impedes it from registering as a painful sensation. The main sites of modulation along the pain pathway include: the periphery, spinal cord dorsal horn, and the brain.

1.1.2 Afferent Neurons

There are two types of primary afferent sensory nerves; the first mediate innocuous sensations that are generated by low-threshold (innocuous) stimuli; the second mediate painful sensations that are generated by high-threshold (noxious) stimuli.

Of all primary afferent neurons, proprioceptors are encapsulated by the thickest myelin sheath, allowing for rapid conduction of action potentials. Within the proprioceptive class of afferents are the Aα fibres which innervate muscles, tendons, and joints, relaying interoceptive information, and Aβ fibres, which relay sensory information regarding spatial position and tension of the organs or extremities they innervate. Aβ fibres are unique from other primary afferents in that they are encapsulated by structures such as Pacinian corpuscles, Meissner's corpuscles, Merkel's disks, and Ruffini's corpuscles at somatic sensory terminals. These structures allow for the specialized detection and transduction of unique low threshold stimuli.

The second class of primary afferent neurons is termed nociceptors and is comprised of the thinly myelinated Aδ fibers and the unmyelinated C fibers. Nociceptors have four major functional components: firstly the peripheral terminal which registers noxious stimuli and transduces signals into depolarizing currents and ultimately action potentials (Hunt et al., 2001); secondly, the axon that conducts action potentials toward the central nervous system (CNS); thirdly, the cell body that controls the identity of the neurons through expression of various transcription factors; and lastly, the central terminal, which forms a synapse with second order neurons in the CNS. These action potentials, which travel to the spinal cord and onto higher centres of the brain, are capable of eliciting withdrawal reflexes, the conscious perception of pain, and emotional effects (Woolf et al., 2000).

Although both Aδ and C fibres are involved in the nociceptive system, signaling methods differ between fibres. In primary afferent signaling, form equals function, as the thinly myelinated Aδ fibres are capable of transducing noxious stimuli at a higher velocity, and therefore, are responsible for first pain (sharp and intense). Aδ fibres are capable of responding to mechanical and thermal stimuli. Conversely, unmyelinated C fibres, that comprise the majority of nociceptive fibres transmit signals at a much slower

velocity and are responsible for second pain (dull and aching) (Julius et al., 2001). C fibres are capable of responding to mechanical, thermal, and chemical stimuli.

1.1.3 Ascending Pain Pathway

Primary afferent neurons register stimuli in the periphery and transmit the nociceptive signal through the ascending pain pathway. The peripheral terminals (free nerve endings) of the primary afferent neurons typically reside in the skin, muscle, bone, joints, and viscera (Julius & Basbaum, 2001). Pain signaling travels through the axon to the nucleus (dorsal root ganglion) and onto the central terminal, which resides in the dorsal horn of the spinal cord. Rexed (1958) divided the gray matter of the cat spinal cord into parallel laminae for identification of afferent insertion; this scheme is widely used for most species today (Todd, 2002). Primary afferents differ in regard to laminae in which they terminate. Nociceptors, Aδ and C fibres, typically bifurcate superficially and synapse with the substantia gelatinosa or the Rexeds' laminae I and II. Lower threshold Aδ fibres travel and synapse at a deeper level of the dorsal horn, while proprioceptors (Aβ) typically innervate deeper laminae (III-VI) (Figure 1.1).

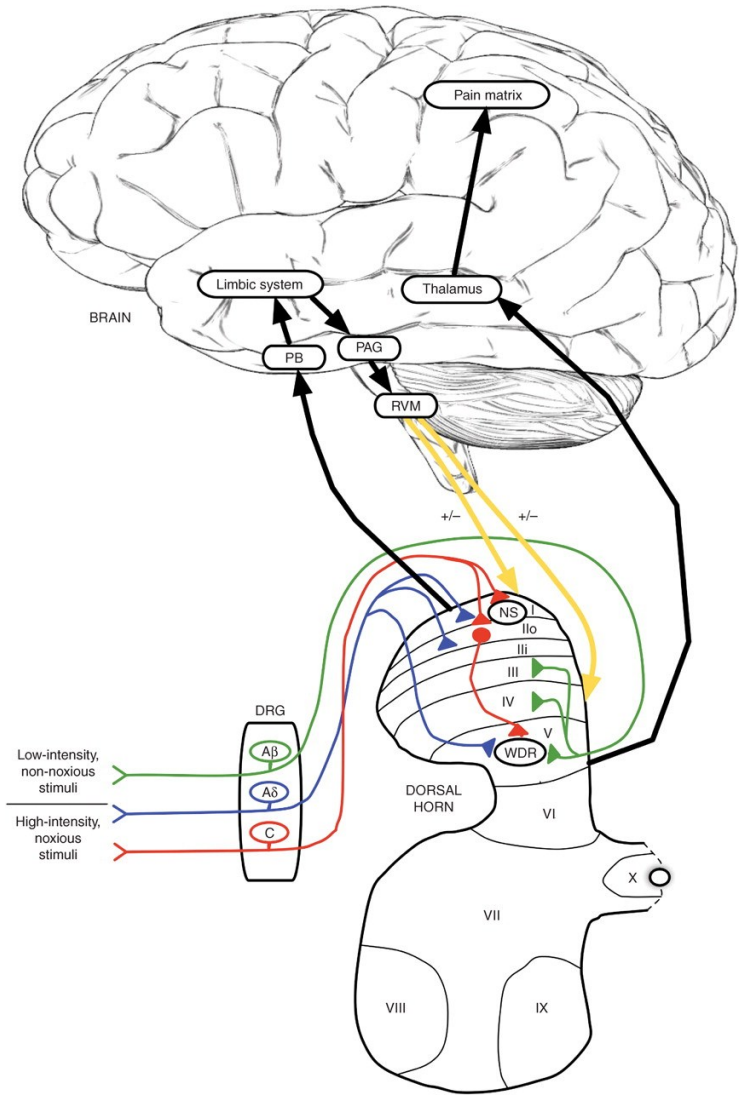


Figure 1.1 Overview of the ascending nociceptive system. Peripheral stimuli is transduced into action potentials by the free nerve ending of afferent fibres. These action potentials travel along the axon (through the dorsal root ganglion) toward the central terminal. The central terminals of the three classes of afferent fibres terminate in different laminae of the dorsal horn. The nociceptors tend to terminate in the superficial laminae (laminae I, II) while the proprioceptors terminate in the deeper laminae (laminea III- VI). Central terminals synapse with second order neurons that transmit signals to higher orders of the brain. Nociceptive stimuli travel through the spinothalamic tract to the thalamus and onto the cortex, where they register pain. Nociceptive specific (NS) cells, wide dynamic range (WDR) neurons, parabrachial area (PB), periaqueductal grey (PAG), and rostral ventromedial medulla (RVM) (Image adapted from D'Mello, 2008).

Sensory neurons are capable of synapsing with three cell types located within the spinal cord: 1) excitatory interneurons that synapse on second order projection neurons; 2) second order projection neurons that project to the brain through either the spinothalamic, spinoreticular, or spinomesencephalic tract; 3) inhibitory interneurons which modulate nociceptive input.

1.1.3.1 Wind-up

Wind up is a phenomenon arising from synaptic plasticity in which consistent noxious stimuli evoke an increased perception of pain. Within the dorsal horn of the spinal cord, there are three main types of neurons that synapse with first order neurons. The first class of neurons synapse with nociceptors and fire action potentials in response to painful stimuli registered in the periphery. The second respond to proprioceptive information sent from the periphery, involving innocuous sensations, and synapse with Aβ fibres. Wide dynamic range neurons, a third type of neuron in the dorsal horn, synapse with both nociceptors and proprioceptors. Wide dynamic range neurons differentiate incoming sensory input from the various classes of afferent neurons through incoming stimulus intensity. Proprioceptors generate moderate frequencies of action potentials, whereas nociceptors induce intense frequencies of action potentials. Wind up occurs when a constant barrage of noxious stimuli elicits an increased transmission of wide dynamic range neurons toward the higher centers of the brain (Dickenson et al., 1987). During wind-up, repeated stimulation of wide dynamic range neurons elicits an increase of evoked response and post-discharge with each stimulus.

1.1.3.2 Central Sensitization

Central sensitization shares identical molecular mechanisms with wind-up; however, where as wind-up is brought on through constant low stimuli activity of C fibres, plasticity leading to central sensitization is initiated through high intensity C fiber signaling. Peripheral nerve damage (neuropathy) and inflammatory pain states also elicit plasticity in the CNS. This plasticity may lead to a heightened response to painful stimuli

(hyperalgesia) or a painful response to an innocuous stimulus (allodynia) (Bridges et al., 2001).

1.1.3.3 Pain Signaling in the Dorsal Horn

The spinal cord is the initial site of convergence and modulation of nociceptive signaling (Melzack & Wall, 1965). Due to modulation, the response from output cells does not necessarily match the stimuli received from the periphery. Various factors within the dorsal horn may affect the response of output cells; interneurons, descending controls, and various neurotransmitters are capable of modulating signals.

The major excitatory amino acid in the CNS is glutamate. It is present throughout the entire nervous system and acts in the nociceptive signaling pathway. Glutamate is released from pre-synaptic spinal neurons as a result of noxious stimuli. It then binds to one (or several) receptors located in the post-synaptic terminal, including α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid (AMPA) receptors, *N*-methyl-D-aspartate (NMDA) receptors, and metabotropic glutamate receptors (mGluR) (Dingledine et al., 1999). It has also been discovered that glutamate has the ability to bind to kainate receptors located on the pre-synaptic terminal (Lucifora et al., 2006). The binding and subsequent activation of one (or several) subclasses of receptors causes membrane depolarization. This membrane depolarization is mediated through a large influx of Ca^{2+}; once inside the cell, the Ca^{2+} activates various effectors and initiates downstream changes.

Another primary excitatory signaling peptide in the nociceptive system is substance P, which is released pre-synaptically and binds to the neurokinin-1 (NK1) receptor on post-synaptic spinal neurons. Substance P is released from presynaptic afferent terminals in response to noxious stimuli and encodes moderate to intense stimuli (Yaksh et al., 1980). The expression of substance P also increases in response to peripheral inflammation (Donaldson et al., 1992).

1.1.4 Ion channels in the Nociceptive Pathway

1.1.4.1 Voltage-gated Sodium Channels

Voltage-gated ion channels are responsible for the generation and propagation of action potentials along the nociceptive pathway. Therefore, expression of the membrane

proteins determines the excitability of a neuron. Voltage-gated sodium channels (Na_v) are responsible for the rapid upstroke of the action potential, while voltage-gated potassium channels (K_v) mediate the repolarizing phase of the action potential. Voltage-gated calcium channels (Ca_v) do little to directly mediate the action potential, however indirectly influence membrane excitability through secondary processes within cells (Bridges et al., 2001).

Voltage-gated sodium channels possess an α-subunit, responsible for the influx of sodium into the cell, and at least one β-subunit, which modulates gating and channel kinetics (Read et al., 2008). Joint innervating afferent neurons express high amounts of Na_v. Hyperalgesia can result from aberrant activity, expression, and influence of Na_v channel auxiliary subunits.

Peripheral nerve damage has been shown to alter the expression of voltage-gated ion channels. Alteration of Na_v expression, following peripheral nerve damage, causes a state of hyperexcitability leading to the generation of aberrant action potentials. These abnormal action potentials travel toward the CNS, resulting in the perception of pain (Matzner et al., 1994).

1.1.4.2 Calcium Channels

Voltage-gated calcium channels (Ca_v) expressed on the presynaptic terminal regulate neurotransmitter release, neuronal plasticity, and gene expression (Cao, 2006). Activity of voltage-gated calcium channels on the presynaptic terminals within the dorsal horn has been demonstrated to play a vital role in the pain pathway. Deletion of the N-type calcium channel gene reduces inflammatory and neuropathic pain behavior (Saegusa et al., 2001). Expressed in the superficial laminar of the spinal cord and DRG neurons, calcium T channels have also been implicated in nociceptive signaling, modulating spinal excitability and sensitization caused by repetitive C fibre firing (Ikeda et al., 2003). Additionally, peripheral nerve injury elicits an upregulation the $Ca_v2.2$ and $\alpha_2\delta$ subunits in the DRG, implicating a significant role in neuropathic pain (McGivern, 2006).

1.1.4.3 Transient Receptor Potential Channels

Temperature sensitive transient receptor potential ion channels facilitate the transmission of information regarding temperature in the periphery to the CNS. The ion

channel responsible for transmitting noxious heat stimuli, activated by temperatures exceeding 43°C, is the transient receptor potential vanilloid 1 (TRPV1) receptor. TRPV1 is also activated by capsaicin (the spicy component of chili peppers), bradykinin, PGE_2, anandamide, and nerve growth factor. TRPV1 channel activation is essential for the generation of thermal nociception (Davis et al., 2000) and inflammatory nociception (Huang et al., 2006). Recently TRPV1 has also been determined to mediate mechanical sensitivity arising from repeated stimuli (Walder et al., 2012).

1.2 PAIN STATES

1.2.1 Inflammatory Pain

Although nociceptive pain is adaptive, in chronic settings pain is no longer protective, several examples of these chronic conditions include inflammatory and neuropathic pain. Inflammatory pain is typically associated with disease and is generated through an increased response to inflammatory mediators by peripheral terminals in nociceptors. These inflammatory mediators include bradykinin, prostaglandins, neuropeptides, and cytokines and act to augment-nociceptive responses through several mechanisms (Schaible et al., 2002). First, mediators bind to and directly activate receptors on the nociceptive terminal to generate action potentials and subsequent propagation along the nociceptive pathway in the absence of peripheral stimuli. Second, these inflammatory mediators lower the threshold of activation in the afferent nerves causing mild innocuous stimuli to elicit action potentials that ultimately result in the sensation of pain (peripheral sensitization). Many sensitizing agents work in parallel, causing great difficulty in ameliorating their responses through pharmacological intervention. However, preventing prostaglandin E2 synthesis, through inhibition of cyclooxygenase-2, is the basic mechanism of action for nonsteroidal anti-inflammatory drugs (NSAIDs).

Inflammatory agents exert sensitizing effects on nociceptors through intracellular signaling cascades initiated through their binding to membrane receptors. Multiple intracellular signaling cascades include protein kinase C, protein kinase A, phosphoinositide 3-kinase, and mitogen-activated protein kinases (Woolf et al., 2007)

and are capable of initiating plasticity. Peripheral sensitization is ultimately attributable to downstream effectors of these cascades, including the phosphorylation of transient receptor potential ion channels and voltage-gated sodium channels, which lowers the threshold and alters the kinetics of signaling.

1.2.1.1 Phenotypic Switches

The cell bodies in the DRG of afferent nerves maintain contact with the peripheral terminals through retrograde transmission. Peripheral inflammation produces retrograde signals that cause an increased transcription of neuropeptides, brain derived neurotrophic factor, and sodium channels. Furthermore, retrograde signaling mediates the upregulation of transient receptor potential channels, which augment peripheral sensitization (Huang et al., 2006).

1.2.2 Neuropathic Pain

Neuropathic pain arises due to damage to the nervous system. It is autonomous of peripheral tissue damage or inflammation and lasts long after the damage to the nervous system is repaired. Peripheral neuropathic pain, resulting from damage to the peripheral nervous system, may be mediated through mechanical trauma, metabolic disease, neurotoxic chemicals, infection, or tumor invasion (Costigan et al., 2009).

Neuropathic pain is the registration of nociceptive stimuli by the CNS in the absence of noxious, or any, stimuli in the periphery. The perception of nociceptive stimuli is generated largely through atypical expression of ion channels in afferent neurons resulting in the unprovoked generation of action potentials. Similar to how atypical retrograde transmission in inflammatory conditions may lead to altered phenotype expression in cells within the DRG, loss of communication with the periphery through axotomy of the peripheral nerve has also been demonstrated to elicit altered phenotypes.

In biological conditions, afferent impulses are generated solely at the sensory endings, in skin and other innervated tissues, in response to appropriate sensory stimulation. Following peripheral nerve damage, however, ectopic activity can occur at multiple sites along the nociceptive pathway. This ectopic activity is considered to mediate the resting pain experienced in neuropathic states. Aberrant activity can initiate

in the site of axonal injury or in the cell bodies of the dorsal root ganglion (Amir et al., 2005). This ectopic activity is largely attributable to atypical expression of voltage-gated sodium channels. Ephatic transmission (cross-excitement) can also occur between the cell bodies in the DRG and neighbouring nerve fibres, causing quiescent neurons to initiate ectopic signaling (Wu et al., 2002).

1.2.2.1 Changes in Ion Channel Expression

Action potential transmission from peripheral receptors to the higher centres of the brain occurs through a wave of depolarization and subsequent release of neurotransmitters. The release of neurotransmitters at afferent nerve synapses is mainly influenced through Na_v, Ca_v, and K_v expression. Following peripheral nerve axotomy, there is a downregulation of the K_v channel TRESK (Tulleuda et al., 2011), and upregulation of $Na_v1.3$ (Kim et al., 2001) and calcium channel 2.2 and $\alpha2\text{-}\delta_1$ subunits through retrograde signaling (McGivern, 2006). These phenotypic changes create a hyperpolarized state and excess release of the neurotransmitters responsible for initiating hyperalgesia.

1.2.2.2 Peripheral and Central Sensitization

A hallmark symptom of neuropathies is allodynia (painful response to a nonpainful stimuli) and can be brought on through peripheral and central sensitization (Bridges et al., 2001). Macrophages and T cells become upregulated in damaged nerves sensitizing ion channels or altering gene regulation. This post-translational modulation alters the activation threshold and kinetics leading to heightened peripheral sensitization (Woolf & Salter, 2000).

Plasticity occurring within the CNS is also common in neuropathies. The molecular mechanisms of action include the release of glutamate and substance P from the pre-synaptic nociceptive afferent nerve terminal in the spinal dorsal horn, which then bind to NMDA-receptors and NK1 receptors. The binding of these neuropeptides to the post-synaptic terminal causes the influx of Ca^{2+}, leading to the propagation of an action potential toward the higher centres of the brain. Reduction of the inhibitory transmission system at the segmental level (e.g. gamma-aminobutyric acid, glycine) (Baba et al., 2003) as well as the descending inhibitory system, mediated by the rostral ventromedial

medulla, contribute to central sensitization (De Felice et al., 2011). Disinhibition leaves dorsal horn neurons susceptible to activation of all stimuli from A-fibres, including innocuous stimuli.

Table 1. Mechanisms Of Neuropathic Pain

Evoked changes	Consequence
Altered Phenotype	Nerve axotomy leads to loss of retrograde signaling with the periphery, resulting in transcriptional changes.
Aberrant Signaling	Upregulation of Na_v expression, down regulation of K channels, and altered expression of Ca_v mediates unprovoked generation of action potentials.
Ephatic Signaling	Silent afferent neurons, neighbouring a damaged nerve, can be recruited into aberrant activity, contributing to the stimuli received by the CNS.
Peripheral and Central Sensitization	DRG neurons experience alterations to membrane potential, bringing them closer to firing threshold. Afferent barrage of stimulus following nerve damage leads to wind-up, a key feature of central sensitization.

Compiled from Bridges et al. 2001

1.3 OSTEOARTHRITIS

Osteoarthritis (OA) is the most prevalent musculoskeletal disorder in the world affecting over 30% of the adult population (Haugen et al., 2011). This disease is primarily characterized by chronic pain and there are currently no disease modifying osteoarthritis drugs (DMOADs) that can attenuate disease progression. Current pharmacotherapy strategies act to manage pain associated with the disease; however, long-term consumption of analgesics is associated with a wide range of severe side effects. This creates the pressing need for development of DMOADs that will prevent disease progression and alleviate the suffering of millions.

1.3.1 Joint structure

The knee joint is a hinge joint that is required to accommodate a variety of stresses that occur on a daily basis. The three osseous structures of the knee joint are the femur (distal femur condyle), tibia (proximal tibial plateaus), and patella. The femur is characterized by two strong bone protrusions (femoral condyles), which are covered with cartilage (Figure 1.2). The femoral condyles rest into grooves atop the proximal tibial plateaus. Two articular disks, termed the medial meniscus and lateral meniscus, reside in the center of the knee joint fused with the synovial membrane laterally. Although the joint surfaces between the femur and tibia are complementary, they are not congruent, and the menisci act as a connector between the two bones that prevents rubbing between them and offers additional protection by acting as shock absorbers.

The osseous structures within the knee joint are secured by multiple ligament structures, which act in concert to prevent the bones from rubbing together. Lateral ligaments (lateral collateral ligament and medial collateral ligament) offer support to the outside of the knee joint from varus and valgus movements, and cruciate ligaments (anterior cruciate ligament and posterior cruciate ligament) are located within the knee joint, providing anteroposterior stability to the joint and allowing for hinge-like movements.

Articular cartilage is a smooth, lubricated tissue that aids in protecting the bone from the biomechanical forces elicited during the loading phase during gait

(Wang et al., 2011). Approximately 75% of the wet weight in articular cartilage is H_2O and 70% of its dry weight is collagen. The remaining amount of dry weight is termed the noncollagenous matrix, and is stabilized by link-proteins and comprised mostly of proteoglycan aggrecan and hyaluronan (Plaas et al., 1983). The aggrecan is composed of 10% core protein and 90% chondroitin sulfate (Wang et al., 2011).

Chondrocytes of the articular cartilage are organized into three layers: superficial, middle, and deep. Collagens form a fibrous (mesh-like) network that is densely populated by aggregan and chondrocytic cells which mediate articular cartilage homeostasis. Chondrocytic cells secrete and degrade matrix components in response to external influences, including: growth factors, cytokines, and biomechanical insults (Wang et al., 2011).

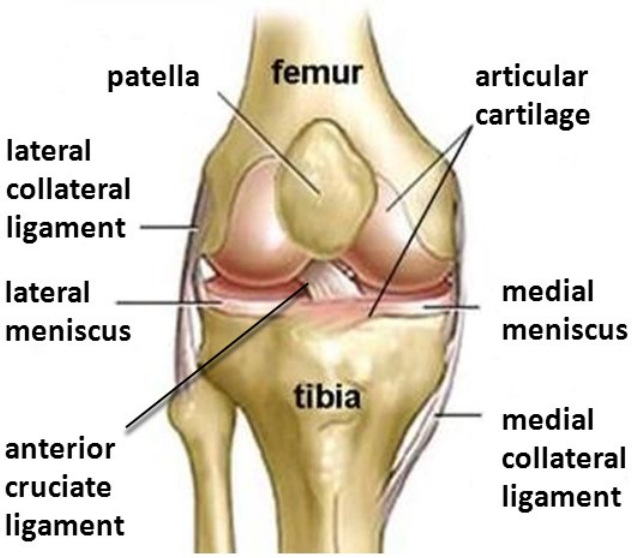


Figure 1.2 Diagram of the knee joint. The knee joint is comprised of three osseous structures; the patella, the distal femoral condyle, and the proximal tibial plateaus. Two articular disks reside between the femoral condyles and tibial plateaus termed the medial and lateral menisci. The menisci offer shock absorption and mediate contact between the femur and tibia. The bone structures within the joint are supported through external (lateral collateral ligament and medial collateral ligament) and internal (anterior cruciate ligament and posterior cruciate ligaments) ligaments. The ligaments offer stability within the joint and prevent the osseous structures from rubbing together or breaking apart. (Image adapted from Yin, 2010).

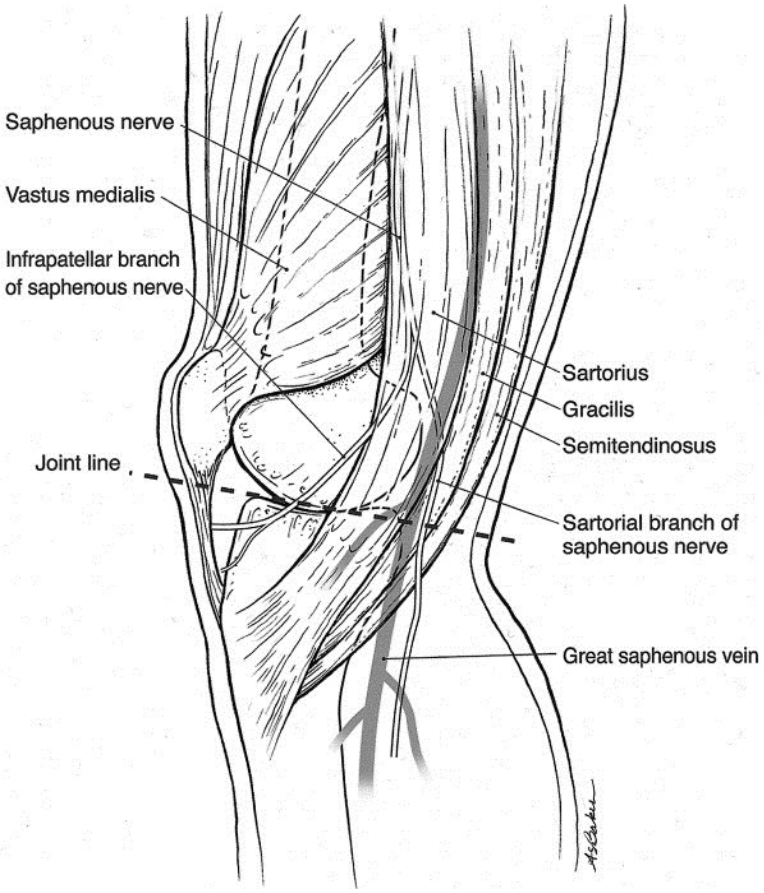


Figure 1.3 Innervation of the knee joint. The saphenous nerve is a sensory nerve that travels from the cutaneous portion of the foot through the entire length of the leg. The sartorial branch of the saphenous nerve innervates the knee joint (Image adapted from Dunaway, 2005).

1.3.2 Disease Etiology

Our understanding of the disease progression of OA has evolved significantly over the past several decades. Once thought to be restricted to the articular cartilage of diarthrodial joints, OA is now understood to be a 'whole joint disease'. OA affects the synovium, periarticular muscle, meniscus, as well as nerves and ligaments of the diseased joint (Brandt, 2008). However, the exact pathogenesis remains unelucidated; many factors including joint trauma, genetics, age, sex, altered biomechanics and hormonal factors have been linked to the onset of OA.

OA can affect all joints but primarily affects the hands, knees, and hips. Two primary features characterize the disease: 1) the progressive damage of articular cartilage, 2) bone remodeling or formation (osteophytes) (Grynpas et al., 1991). Synovial inflammation and damage to ligaments, tendons, and menisci are also commonly associated with disease progression.

The aetiology of OA has traditionally been defined as the failed attempt to repair damage caused to the joint through excessive strain on joint tissues. The body's innate repair mechanisms are unable to repair this damage with the underlying stressor still engaged. Natural repair mechanisms circumvent this problem through the remodeling of the subchondral bone that acts to alleviate the strain placed on the joint, however, this bone remodeling results in joint pain (Brandt et al., 2008).

Mechanical loading has been strongly linked to the initiation of OA; the high onset of OA following an anterior-cruciate ligament (ACL) transection is markedly attenuated when the joint is stabilized immediately after surgery (Palmoski et al., 1982). However, strong arguments have been made for other factors in disease progression including: chemokines, cytokines and subsequent inflammation.

The cartilage-centric disease pathology theory includes mechanical stress or inflammatory mediators affecting quiescent chondrocytes, changing their dynamics, causing release of matrix metalloproteinases and prostaglandins, leading to rapid degradation and erosion of bone. This theory of OA etiology aligns with the classic symptom of cartilage erosion, as mechanical stress has been demonstrated to induce the release of various factors, including: extracellular matrix degrading enzymes (i.e. matrix

metalloproteinases and aggrecanases) (Cawston et al., 2006; Song et al., 2007), proinflammatory cytokines (e.g. interleukin-1, tumor necrosis factor) (Kapoor et al., 2011), and chemokines (e.g. growth regulated oncogene α, IL-8, monocyte chemotactic protein 1), which have all been demonstrated to potentiate the inflammatory response.

1.3.3 OA Pain

The urgency to develop treatments that specifically target OA pain is great, as OA patients rank pain the number one symptom impairing their quality of life (OA Consensus Conference, Toronto, 2000). However, to develop DMOADs, it is necessary to gain a better understanding of the etiology and cellular mechanisms involved in the pain generated by the disease.

OA has traditionally been viewed as a degenerative disorder; however, clinically, there is discordance between radiographical diagnosis of OA and joint pain (Hannan et al., 2000). Patients presenting with severe disease progression, including robust bone marrow lesions and osteophyte formation, often report not experiencing joint pain. Conversely, patients demonstrating minor radiographical alterations report severe pain. This enigma adds complexity to OA pain and further research of underlying pain mechanisms is required.

1.3.3.1 Joint Innervation

Myelinated Aβ, thinly myelinated Aδ, unmyelinated C, and postganglionic sympathetic nerve fibres innervate diarthrodial joints. Aβ fibres innervate the capsule, fat pad, ligaments, menisci and periosteum; Aδ and C nerve fibres innervate the capsule, ligaments, menisci, and periosteum(Freeman et al., 1967; McDougall et al., 1997). Of all bone structures in the joint, the periosteum possesses the highest sensory and sympathetic innervation, which is speculated to even increase during disease states.

Aβ fibres, innervating the periosteum, become activated primarily through innocuous movements within the working range of the joint. However, the majority of Aδ (50%) and C (70%) fibres become activated through nociceptive stimuli (Schaible et al., 1993). Afferent fibres demonstrate plasticity during inflammation, with Aβ fibres exhibiting heightened responsiveness, and high-threshold nociceptive afferents becoming

sensitized and subsequently activated, in response to normal working movements of the joint (Schaible et al., 1985).

1.3.4 Inflammation

The discovery of a low-grade inflammatory component to OA has occurred over the past decade, and it is now widely believed that OA pain is mediated through both peripheral and central sensitization. Most clinical cases of OA present with a substantial amount of inflammation. This inflammation is driven by a wide-variety of inflammatory factors, including tumor necrosis factor-α, interleukin 6, interleukin 1β, calcitonin gene related peptide, substance P, vasoactive intestinal peptide, nerve growth factor, prostaglandin E_2, glutamate, serotonin, that are elevated in synovial fluid of OA patients (Read & Dray, 2008).

1.3.4.1 Synovitis

The synovial membrane is a thin tissue barrier around the joint that acts to regulate the influx of nutrients and the efflux of articular matrix turnover (Brandt et al., 2008). Articular cartilage is avascular and aneural, therefore, all extracellular molecules must diffuse through the synovial membrane to enter the joint. In inflammatory joint conditions, such as rheumatoid arthritis, the inflammatory mediators that facilitate disease cross the synovial membrane to elicit effects within the joint. The synovial membrane is vulnerable to inflammation, and hyperplasia of the lining cell layer of the synovial membrane is indicative of an inflammatory environment within the joint.

Although early studies, using primitive diagnostic techniques, failed to determine a correlation between global joint synovitis and knee OA, recent evidence has found a linkage between the two. Utilizing contrast-enhanced MRI technology, synovitis has been correlated to knee OA disease progression and pain with synovitis conferring a 9.2-fold increased odds ratio of pain (Baker et al., 2010). Unlike rheumatoid arthritis, which demonstrates robust synovitis, knee OA synovitis is localized to the cruciate ligament and the suprapatellar region of the diseased joint (Baker et al., 2010). Joint swelling has been a traditional clinical symptom of OA, and in light of these recent findings, it is now believed to be caused, in part, by synovitis (Berenbaum, 2013).

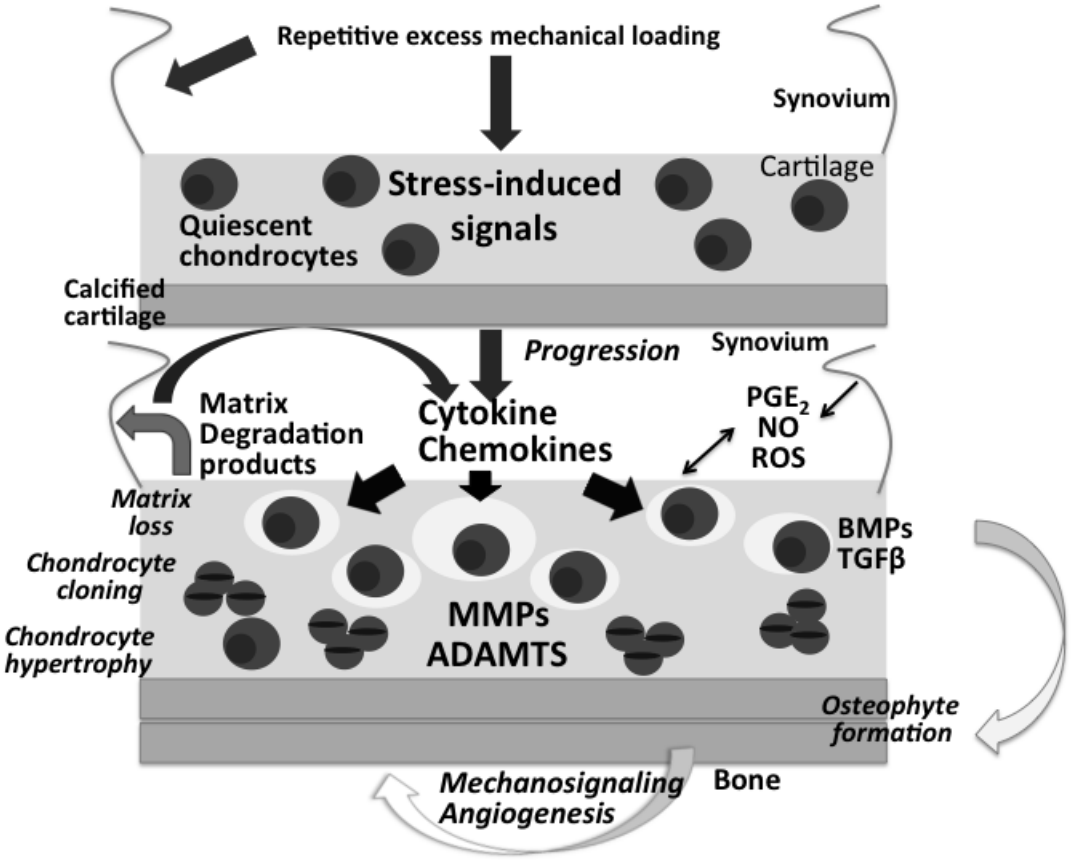


Figure 1.3 Overview of disease etiology in OA. Abnormal joint loading and excessive compression of articular cartilage alters the phenotype of quiescent chondrocytes, causing the release of pro-inflammatory mediators into the joint. The release of inflammatory mediators facilitates joint degradation, remodeling, and ultimately pain. (Image adapted from Goldring, 2007).

1.3.5 Experimental Models

Similar to other experimental models of disease, the purpose of OA models is to recapitulate clinical symptoms in animals. There are several methods in which this is successfully accomplished (Table 2). It is important to note, models used to study the pathophysiology and regulation of structural joint damage are distinct from the models employed to investigate OA pain. Several of the widely used pre-clinical models are highlighted in Table 2.

Surgical - a traditional method of eliciting OA is through surgical induction. Various surgical techniques may be employed (Table 2), ranging from eliciting menisci damage, to the more popular model of anterior cruciate ligament transection model (Pond et al., 1973). These surgical models result in a high rate of disease onset.

Spontaneous - similar to humans, specific animals display a high onset of naturally occurring OA that arises without the requirement of chemicals or surgical intervention (Table 2). A species of animal utilized for their high prevalence (~90%) of naturally occurring OA is the Dunkin-Hartley guinea pig. Disease onset occurs at 6 months of age with extensive joint damage by 18 months. Although the mechanism of disease pathophysiology remains elusive, it is hypothesized to occur due to the large body weight and abnormal joint loading (Bendele, 2001).

Chemical - The recent advent of chemical introduction into the joint has allowed for consistent recapitulation of the clinical symptoms experienced in OA. Intraarticular injection (i.a.) disease models allow for easy induction and variance in symptoms. The introduction of monosodium iodoacetate (MIA) into the knee joint causes extensive destruction of chondrocytes and rapid degradation of articular cartilage (Bove et al., 2003). MIA is the most widely used experimental model of OA pain, with an estimated 50% of OA pain research employing it (Little et al., 2012).

Table 2- Preclinical animal models of OA

Animal Model	Species	Outcome measures
Surgically Induced		
Anterior cruciate ligament transection	Rat, Dog, Rabbit	Gait analysis
Medial collateral ligament transection	Rat	HL weight distribution (incapacitance)
Chemically-induced		
Monosodium iodoacetate	Rat, mouse	HL weight distribution (incapacitance), mechanical allodynia, mechanosensitivity, grip strength meter, thermal hyperalgesia, mechanical hyperalgesia, gait analysis
Collagenase	Mouse	Visual gate analysis, joint tenderness
Papain	Rat, mouse	HL weight distribution (incapacitance), mechanical allodynia, mechanosensitivity, thermal hyperalgesia, mechanical hyperalgesia,
Spontaneous		
Age	Guinea pig	Mechanosensitivity
Obesity	Mouse	Motor coordination (rotarod), grip strength meter, gait analysis
Genetic		
Col9a1 (-/-)	Mouse	Motor coordination (rotarod), gait analysis, mechanical allodynia, thermal hyperalgesia, grip strength

Modified from: Little et al., 2012.

1.3.6 Current OA Treatments

Acetaminophen is often used in early stages of OA, as it has demonstrated efficacy attenuating disease symptoms and is relatively safe. Previous studies determined acetaminophen possessed a similar efficacy as NSAIDs in the reduction of effusion and synovial tissue inflammation of knee OA (Brandt et al., 2006). Although the exact mechanisms of action have not been fully elucidated, analgesic effects are believed to occur through inhibition of COX enzymes. Most recently it has been hypothesized that acetaminophen exerts analgesic effects through binding with transient receptor potential cation channel, subfamily A, member 1 in the spinal cord, attenuating nociceptive signaling in the superficial lamina (Andersson et al., 2011).

Non-selective NSAIDs are often used as a second-line treatment for moderate OA pain. Non-selective NSAIDs inhibit COX expression, and subsequent conversion of arachidonic acid to prostaglandins (Figure 1.4) (Cheng et al., 2012). Although NSAIDs are successful at achieving analgesia through COX-2 inhibition, COX-1 is responsible for maintaining homeostasis in a variety of cellular processes, including platelet aggregation and gastrointestinal epithelium generation. As non-selective NSAIDs inhibit both COX enzymes significant side effects accompany their long-term use.

Synthetic opioid agonists (i.e.- codeine) have been shown to be effective as an adjuvant therapy to NSAIDs or an efficacious alternative for individuals unable to tolerate NSAIDs. Opioids bind to three primary receptors (μ, κ, and δ) located in the periphery, the spinal column, supraspinally, and in the gastrointestinal tract. Mechanistically, activation of peripheral opioid receptors causes sensory neuron hyperpolarization, attenuating the hyperexcitability caused through inflammation and injury (Hurley et al., 2000). However, opioid use is accompanied by a wide range of side effects including: constipation, nausea, and respiratory depression ("Recommendations for the medical management of osteoarthritis of the hip and knee: 2000 update. American College of Rheumatology Subcommittee on Osteoarthritis Guidelines," 2000).

Topical NSAIDs (e.g. Voltaren®, Pennsaid®) act peripherally through similar cellular mechanisms as oral NSAIDs and possess a similar efficacy (Heyneman et al., 2000).

Producing an equal analgesic response with a much lower risk of GI and renal toxicity makes topical NSAIDs an attractive therapy in the treatment for OA.

Viscosupplementation of hyaluronic acid (HA), a polysaccharide and major component of synovial fluid and cartilage, is a novel treatment for OA (Marshall, 2000). HA is synthesized by chondrocytes and synoviocytes and released into the synovial space, aggregating on ligament surfaces and cartilage. HA facilitates synovial fluid homeostasis and aids in lubricating the joint and shock absorption (Marshall, 2000). Healthy individuals are estimated to possess 2.5-4.0 mg/mL of HA within the knee joint, while this amount is downregulated to 1-2 mg/mL during OA disease progression (Marshall, 2000). A novel therapeutic treatment in OA is the reintroduction of HA into the joint through intra-articular injection (Marshall, 2000). Reintroduction of the polysaccharide increases the elasticity and viscosity of the synovial fluid, lowering the biomechanical impact on the joint.

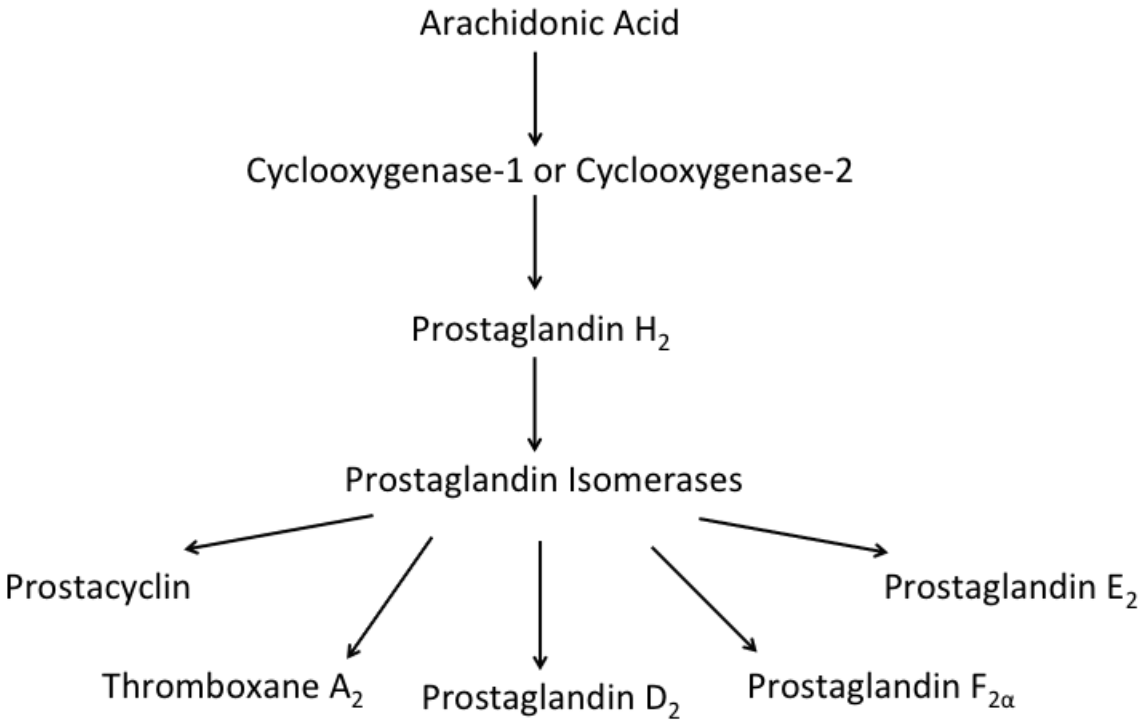


Figure 1.4 Arachidonic acid signaling cascade. Cyclooxygenase 1 (COX-1) is constitutively expressed in a wide variety of tissues, comparatively cyclooxygenase 2 (COX-2) proteins are upregulated through proinflammatory cytokines at the site of inflammation. COX-1 and COX-2 act to catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) that can then be further metabolized into various eicosanoids.

1.4 LYSOPHOSPHATIDIC ACID

Lysophosphatidic acid (LPA), the simplest phospholipid, is composed of a glycerol backbone, a phosphate, and a fatty acid. LPA has been found to be involved in a wide range of both physiological and pathological conditions (Okudaira et al., 2010). Naturally present in fluids throughout the body, LPA binds to a class of cell surface G-protein coupled receptors (GPCRs) through which it elicits a wide range of effects including: cell migration, cell proliferation, vasodilation, inflammation, and neuropathic pain (Ueda, 2008).

1.4.1 LPA Cell Signaling

To date, five GPCRs specific for LPA have been discovered (Table 3). The endothelial differentiation gene (EDG) subgroup is comprised of three receptors: LPA_1 (EDG2), LPA_2 (EDG4), LPA_3 (EDG7). The second subclass is composed of two receptors: LPA_4 (GPR23, p2y9), LPA_5 (GPR92) (Aoki et al., 2008). The orphan GPCR GPR87 has been proposed as a possible sixth receptor in this subclass, however further characterization is required (Choi et al., 2010).

All five LPA receptors are type I, rhodopsin-like G-protein coupled receptors that activate different downstream signaling pathways (Choi et al., 2010)(Figure 1.5). Activation of $G_{\alpha q}/_{11}$ leads to the stimulation of phospholipase C β isoform (PLC β), which either catalyzes the production of inositol (1,4,5) triphosphate, triggering the release of intra-cellular Ca^{2+} stores, or diacylglycerol which activates protein kinase C (PKC) (Yuan et al., 2003).

Phosphorylation of $G_{\alpha 12/13}$ activates Ras homolog gene family (Rho) members, leading to activation of Rho-dependent kinase (Rock), which then stimulates c-jun N-terminal kinase (JNK) causing the formation of stress fibres. The activation of Rho also causes an upregulation of serum response factor (SRF) (Riobo et al., 2005).

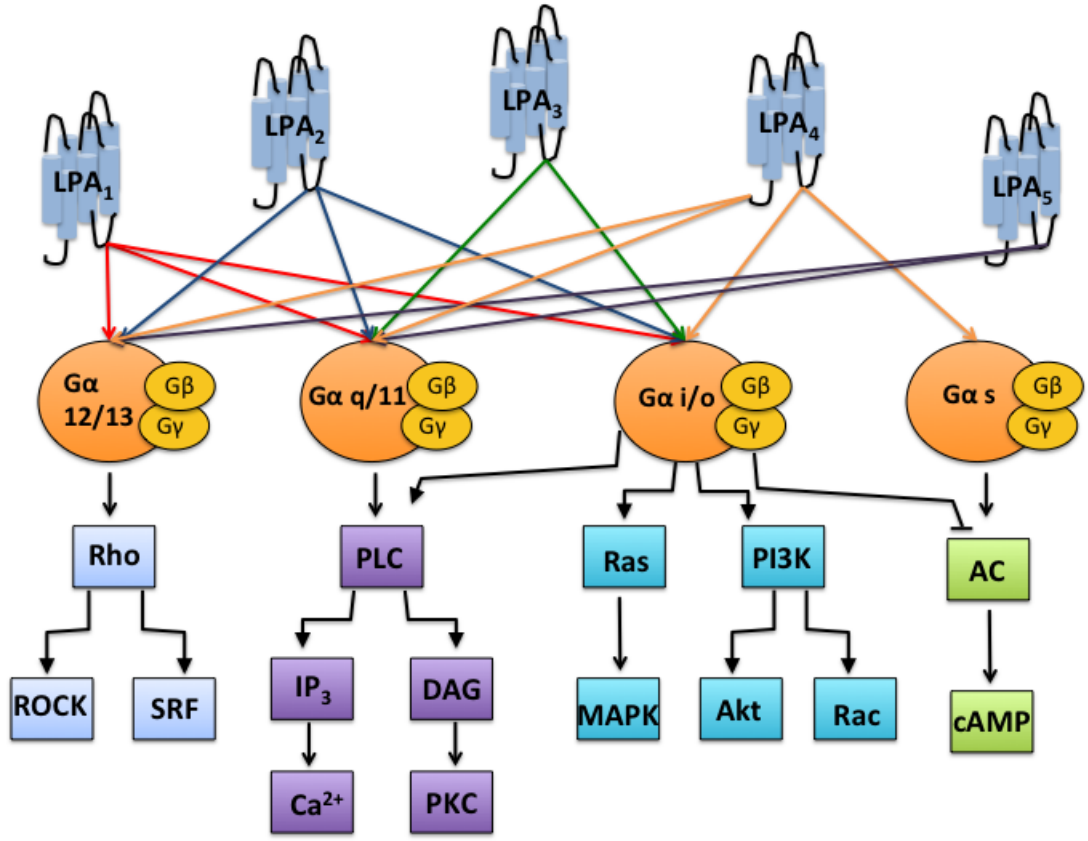


Figure 1.5 Intracellular signaling cascades initiated via LPA receptor activation. Binding of extracellular LPA activates membrane bound LPA GPCR receptors. Activation of these receptors phosphorylate one or more G-proteins which are capable of initiating a host of intracellular signaling cascades which can result in: increased release of intracellular Ca^{2+} stores, activation of RHO-Rock signaling pathway, up regulation of cAMP, amongst others. (Image adapted from Choi, 2010).

LPA receptors are widely distributed throughout a host of systems. LPA_1 is highly expressed in the nervous system; LPA_2 demonstrates robust expression in immune organs (thymus and spleen), while LPA_3 is widely expressed within reproductive organs including the uterus and the testis (Okudaira et al., 2010). LPA_4 and LPA_5 are widely expressed at low amounts. Relevant for neuropathic pain, LPA receptors are expressed on myelin forming cells, astrocytes, and neuronal cells within the CNS and peripheral nervous system (PNS) (Table 4) (Noguchi et al., 2009).

1.4.2 Production of LPA

LPA is produced both intracellularly and extracellularly and determined to be present in abundance in serum with minute amounts in plasma (Okudaira et al., 2010). Due to this discovery, researchers have speculated that LPA is produced during serum formation. LPA can be produced in two ways: Phospholipids or diacylglycerol produce phosphatidic acid (PA), which is converted into LPA; or phospholipid, is converted into lysophospholipids, which is then converted into LPA.

The first method of LPA production occurs intracellularly. The initial step in this pathway, production of phosphatidic acid, is mediated by diacylglyceral kinase (DGK) and phospholipase D (PLD). The second step in this pathway, conversion of phosphatidic acid to LPA, is mediated by phosphatidic acid-selective phospholipase A_1 (PA-$PLA_{1\alpha}$) (Sonoda et al., 2002). The second method occurs in extracellular body fluids (e.g. serum, plasma, etc.) and is initiated by the intracellular production of phosphatidylcholine (PC) that is converted into lysophosphatidyl choline (LPC). The lysophosphatidyl choline is cleaved from the membrane into the extracellular space and hydrolyzed by the ectoenzyme autotaxin (ATX), which possesses phospholipase D activity, resulting in LPA and choline (Okudaira et al., 2010).

Table 3- LPA receptor subtypes

	Synonym	Coupling G protein	Cellular response	Physiological function	Pathological function
EDG subgroup					
LPA1	EDG2	Gi/o, Gq, G12/13	Proliferation, chemotaxis, fibre stretching, anti-apoptosis	Brain development	Neuropathic pain, lung fibrosis, renal fibrosis, metastasis of cancer cells
LPA2	EDG4	Gi/o, Gq, G12/13	Proliferation, chemotaxis, fibre stretching, anti-apoptosis	?	Protection against radiation
LPA3	EDG7	Gi/o, Gq	Proliferation, chemotaxis	Embryo implantation	?
P2Y subgroup					
LPA4	GPR23, P2Y9	Gs, Gi/o, Gq, G12/13	Stress fibre formation, neurite extraction, cell adhesion	?	?
LPA5	GPR92	Gi/o, Gq	Stress fibre formation, neurite extraction	?	?
P2Y5	P2RY5	Gs, Gi/o	-	Hair growth	?

Table adapted from Aoki et al., 2008

1.4.3 Neuropathic pain

Over the past decade researchers have examined neuropathic experimental models and concluded that demyelination occurs in all models (Ueda et al., 2013). Due to its elicitation of demyelination, LPA has been characterized as the distinct chemical signature in the initiation of neuropathic pain (Ueda et al., 2013).

1.4.3.1 LPA Mediated Demyelination

Intense noxious stimuli responsible for the initiation of neuropathic pain have been discovered to cause the release of excitatory neurotransmitters (glutamate and substance P) from the pre-synaptic terminal of the primary afferent nociceptor neurons. The release of these neurotransmitters, and the subsequent activation of the ionotropic NMDA subtype receptor and metatropic NK1 receptor on the postsynaptic terminal, has been discovered to produce the release of LPA (Inoue et al., 2008).

In addition to the pleiotropic effects that LPA elicits, the presence of LPA in the CNS initiates demyelination, via activation of the LPA_1 receptor, and ultimately neuropathic pain (Inoue et al., 2004). A single intrathecal (i.t.) injection of LPA has been shown to elicit tactile allodynia and thermal hyperalgesia, and subsequent dorsal horn demyelination (Inoue et al., 2004). Furthermore, these hallmark traits of neuropathic pain are absent in LPA_1-/- mice that receive identical treatments.

As peripheral nerve demyelination occurs in both mononeuropathic and polyneuropathy states, LPA's involvement in peripheral nerve demyelination, through disruption of Schwann cells (Inoue et al., 2004) and down-regulation of myelin proteins (Fujita et al., 2007), further implicates LPA as the biological signature for neuropathic pain. Additionally, it has been discovered that LPA_1 signaling up-regulates calcium channel $\alpha 2\text{-}\delta_1$ subunits in DRG neurons one week after partial sciatic nerve injury (Inoue et al., 2004).

One hallmark feature of neuropathic pain is central sensitization. $EphrinB_1$, a glycosyl-phosphatidylinositol-lined transmembrane protein-ligand located on the presynaptic nerve terminal, has been characterized to modulate the Ca^{2+}-gating of NMDA-type glutamate receptors (Takasu et al., 2002). This regulation is hypothesized to be responsible for the removal of Mg^{2+} from NMDA allowing glutamate binding and subsequent Ca^{2+}-gating without the requirement of post-synaptic membrane depolarization. $EphrinB_1$ has been found to be elevated through activation of LPA_1

receptor signaling (Uchida et al., 2009). As NMDA-type glutamate receptor activity is a key characteristic of synaptic plasticity (Wu et al., 2009), this linkage further defines LPA's role in neuropathic pain.

Table 4- LPA Receptor Expression and Physiological Responses in the Nervous System

Cell Type	Receptor Expression	Responses to LPA
Neural progenitor cell	LPA1, LPA2	Proliferation Differentiation Inhibition of differentiation Neurite retraction/ cell rounding Early conductance change
Neuron	LPA2	Neurite retraction/ cell rounding Growth cone collapse/ repulsive turning Inhibition of migration Survival Apoptosis
Oligodendrocyte	LPA1	Process formation (maturation) Process retraction/ cell rounding
Astrocyte	LPA1-5	Proliferation Impaired glutamate uptake ROS production Cytoskeletal changes Neuronal differentiation factor release
Microglia	LPA1, LPA3	Metabolic activity increase (proliferation) Chemokinesis enhancement
Schwann cell	LPA1, LPA2	Survival Differentiation Cytoskeletal changes Demyelination

Table adapted from Noguchi et al., 2009

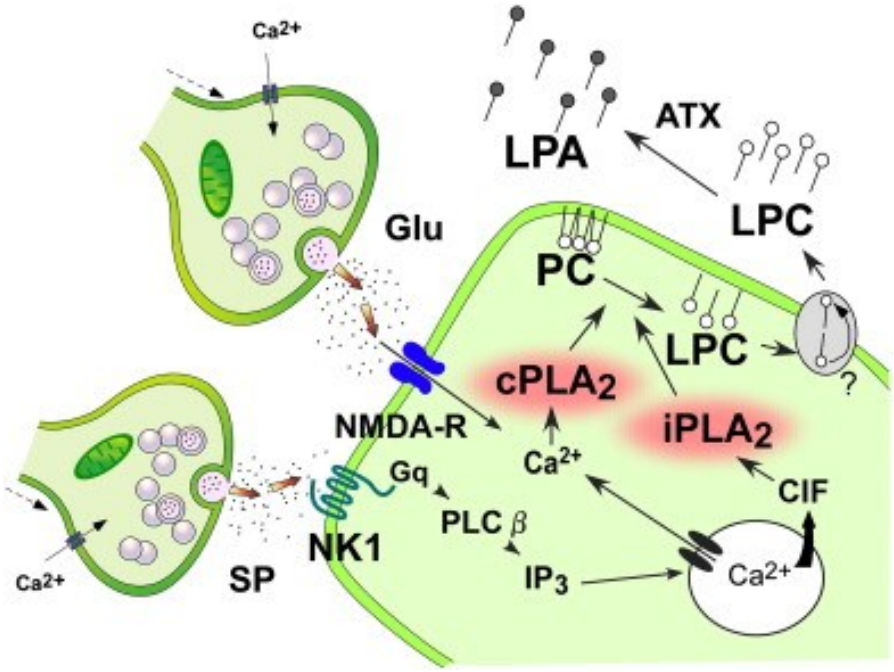


Figure 1.6 De novo synthesis of LPA initiated through primary afferent activation. Release of substance P (SP) and glutamate (Glu) from pre-synaptic afferent nerve terminal and subsequent binding to membrane bound NK1 and NMDA-R receptors of the post-synaptic membrane causes the intracellular signaling cascade: beginning with stimulation of phospholipase C β isoform (PLCβ) which catalyzes the production of inositol (1,4,5) triphosphate, leading to the upregulation of calcium (Ca^{2+}). Upregulation of intracellular Ca^{2+} causes the production of calcium-dependent/ cytosolic phospholipase A2 ($cPLA_2$) and calcium-independent/ intracellular phospholipase A2 ($iPLA_2$) that simultaneously catalyze the conversion of membrane phosphatidyl choline (PC) to lysophosphatidyl choline (LPC). LPC is released into the extracellular space where it is hydrolyzed by autotaxin (ATX) and converted into lysophosphatidic acid (LPA). (Image adapted from Ueda, 2013).

1.4.4 LPA_{1-3} receptor antagonist (Ki16425)

Previous studies have characterized Ki16425 to selectively inhibit acute LPA-induced actions including Ca^{2+} release, inositol phosphate response, and cAMP responses (Ohta et al., 2003). Ki61425 was additionally determined to ameliorate chronic LPA-induced actions (e.g. neuropathic pain, cell migration) (Ohta et al., 2003). Furthermore, it has been established that Ki16425 preferentially attenuated LPA_1 and LPA_3 actions without impeding the cellular actions of other LPA receptors. The antagonist was also found to be specific for the binding of LPA, as the cellular effects of sphingosine-1-phosphate, lysophosphatidylcholine, ATP, bradykinin, and epidermal growth factor were not attenuated through its administration.

1.5 GENERAL HYPOTHESIS AND RATIONALE

1.5.1 Rationale

Current pharmacotherapeutic treatments have failed to ameliorate joint pain accompanying OA in a large subpopulation of patients. As these analgesics (NSAIDs) are efficacious in inflammatory pain states, the lack of efficacy in OA raises the concern of another pain state being present. Several studies have proposed a neuropathic component to OA pain, and the first clinical linkage was presented with the finding of heightened levels of LPA in the synovial fluid of OA. LPA, a key mediator in the initiation of neuropathic pain, could be an exogenous mediator of a neuropathic component in OA pain and a potential target for therapeutic development.

1.5.2 Hypothesis

There is a neuropathic component to OA pain. Furthermore, this neuropathic pain is initiated and mediated through the activation of LPA_{1-3} receptors.

1.5.3 Project Objectives

The purpose of the present study was to investigate the role of LPA in the etiopathogenesis of OA in SD rats. We attempted to determine if LPA causes neuronal damage to myelinated joint afferents, and whether LPA is responsible for a neuropathic

pain component in OA. Introduction of LPA or MIA into the knee joint was used as a model of OA and incapacitance weight bearing and von Frey hair algesiometry were considered as correlates of pain in all experiments.

1) In the first part of this study, we sought to characterize a novel model of nociception through i.a. injection of LPA.

2) In the second part of this study, systemic delivery (i.p. injection) of a selective receptor antagonist was used to determine: whether blockade of LPA_{1-3} receptors (with the selective antagonist Ki16425) attenuates peripheral nerve demyelination and reverses acute nociception caused by i.a. administration of LPA.

3) The third portion of this study sought to determine if a single i.a. injection of LPA could elicit chronic nociception.

4) In the last portion of this study, systemic delivery of the selective LPA antagonist Ki16425 was used to assess whether blockade of LPA_{1-3} receptors attenuates peripheral nerve demyelination, and whether it reverses nociception cause by i.a. administration of MIA.

CHAPTER 2 MATERIALS AND METHODS

2.1 ANIMALS

All experiments conducted were approved by the University Committee on Laboratory Animals at Dalhousie University (Halifax, Nova Scotia, Canada) and performed in compliance with the ethical guidelines of the Canadian Council on Animals Care. Male Sprague-Dawley rats (Charles River Laboratories, Quebec, Canada), weighing 150-200 g at the time of experimental induction, were used for behavioral and histological assessments. Upon arrival, animals were housed in pairs and placed on a 12-hour light/dark cycle in a temperature-controlled room (22 ± 1°C) and allowed access to food and water *ad libitum*. Animals were allowed one week for habituation upon their arrival to the Carleton Animal Care Facility before experimental testing began.

2.2 EXPERIMENTAL MODEL INDUCTION

2.2.1 Intra-articular Knee Injection

Intra-articular (i.a.) knee injections were used to assess the physiological changes attributed to administration of either LPA or MIA into the knee joint. All injections were performed using aseptic technique while animals were deeply anesthetized by isoflurane inhalation (2-5% isoflurane; 100% O_2 1L/min). The right knees of the animals were shaved with clippers and 70% ethanol and iodine was then applied to the skin. Upon induction of anesthesia (i.e. loss of pedal withdrawal reflex), a 25-guage needle was introduced into the knee joint through the patellar ligament (Figure 2.1). Once the needle was correctly positioned between the tibial plateau and femoral condyles, 50 µl of test substance was injection into the joint, followed by multiple flexion/extensions of the knee joint to ensure proper fluid distribution within the aqueous environment. After the procedure, animals were placed into a recovery suite and observed before being returned to the Carleton Animal Care Facility. Proper technique of i.a. injection was verified by injecting aniline blue dye (dissolved in H_2O). Animals that received control dye injections were not used in any experiments.

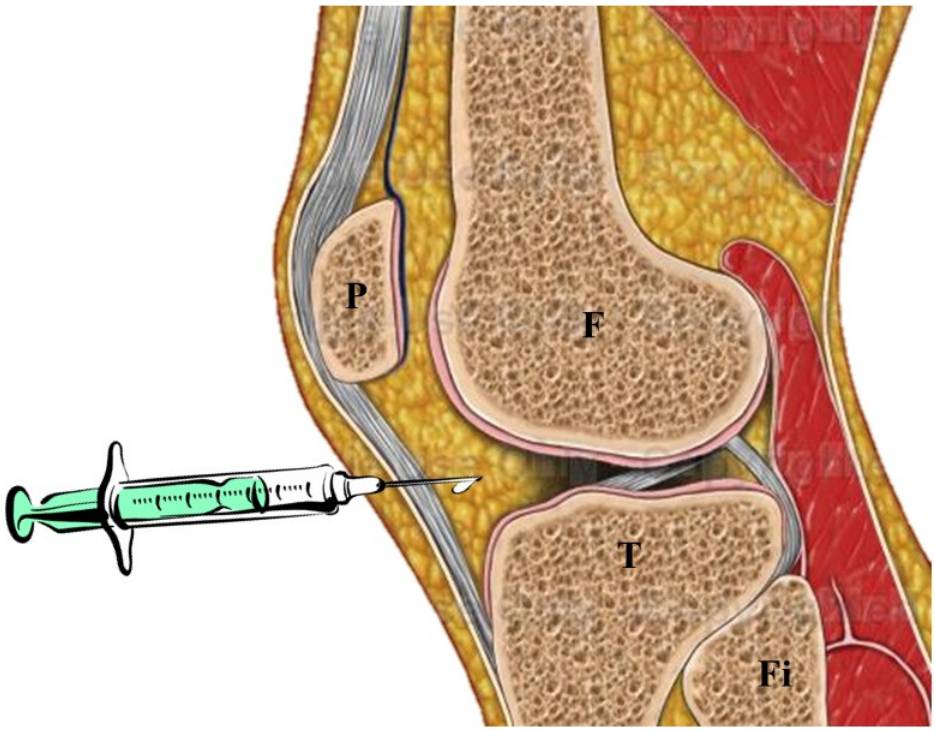


Figure 2.1 Illustration of an intra-articular injection into the knee joint. A 25-gauge needle is introduced into the joint through the patella tendon. Once positioned between the femur and tibia, 50 µl of test substance is injected into the synovial cavity of the joint. The needle is removed and the area is swabbed with 70% ethanol. The injected knee is moved into flexion and extension several times to allow for proper fluid dispersion. Patella (P) femur (F) and tibia (T) and fibula (Fi). Image adapted from www.Findlaw.com.

2.3 BEHAVIORAL ANALYSIS

Behavioral analysis was used to determine the initiation of pain states caused by the administration of test substances into the knee joint. All behavioral assessments were conducted in an area void of noise or other external factors that could cause disruption to the assessment. The testing area remained under constant illumination and was kept at constant temperature (22 ± 1°C) for the duration of testing. Animals underwent training and habitualization procedures for the incapacitance weight-bearing apparatus and von Frey algesiometry. As standing solely on the hindlimbs, in a foreign apparatus, is not an innate ability for rats, training was required to garner accurate weight-bearing measurements. Additionally, upon introduction to novel environments, rodents initiate innate exploratory behaviours. Habitulization to the algesiometry Plexiglass chambers was required for the cessation of this exploration behaviour allowing for accurate paw withdrawal recordings.

2.3.1 Weight-bearing assessment

An incapacitance apparatus (Linton, Norfolk, United Kingdom) was used to measure hindlimb weight-bearing and to assess joint pain. Prior to recordings, rats were carefully positioned into the Plexiglass tube so each hindpaw rested on a separate force plate transducer and care was taken to ensure that the rats' weight was not dissipated onto the Plexiglass tube (Figure 2.2). The incapacitance tester is a dual-channel weighted averager that calculated the amount of weight (measured in grams) each hindlimb exerted over a 3-second time interval. The percentage of weight borne on the ipsilateral (right) leg was calculated using the following equation:

% weight on ipsilateral leg =

[weight on right leg / (weight on right leg + weight on left leg)] x 100

Three readings were taken for each animal per time point, and the average of these three measurements was used in analysis.

2.3.2 Pain withdrawal reflex

An automated von Frey hair algesiometer (Ugo-Basile, Milan, Italy) was used to assess secondary allodynia. Animals were placed into a Plexiglass chamber (20 x 20 x 14 cm) with a metal mesh bottom and allowed to move about freely. Upon cessation of exploratory behavior, an automated blunt microfilament was positioned under the plantar surface of the animal's hindpaw which rose toward the paw at a constant speed (Figure 2.3). The initial force exerted by the microfilament was below the threshold of detection and increased steadily until a withdrawal reflex was caused. The apparatus automatically recorded the force required to elicit a withdrawal response in the animal. A maximum force of 50 g and a ramp speed of 4.5 g/s were used for all algesiometry tests.

The percentage of force required to elicit a paw withdrawal response after treatments, compared to naive baseline measurements, was calculated using the following formula:

% change from baseline

= [(force required after treatment – force required prior to treatment)/ force prior to treatment] x 100

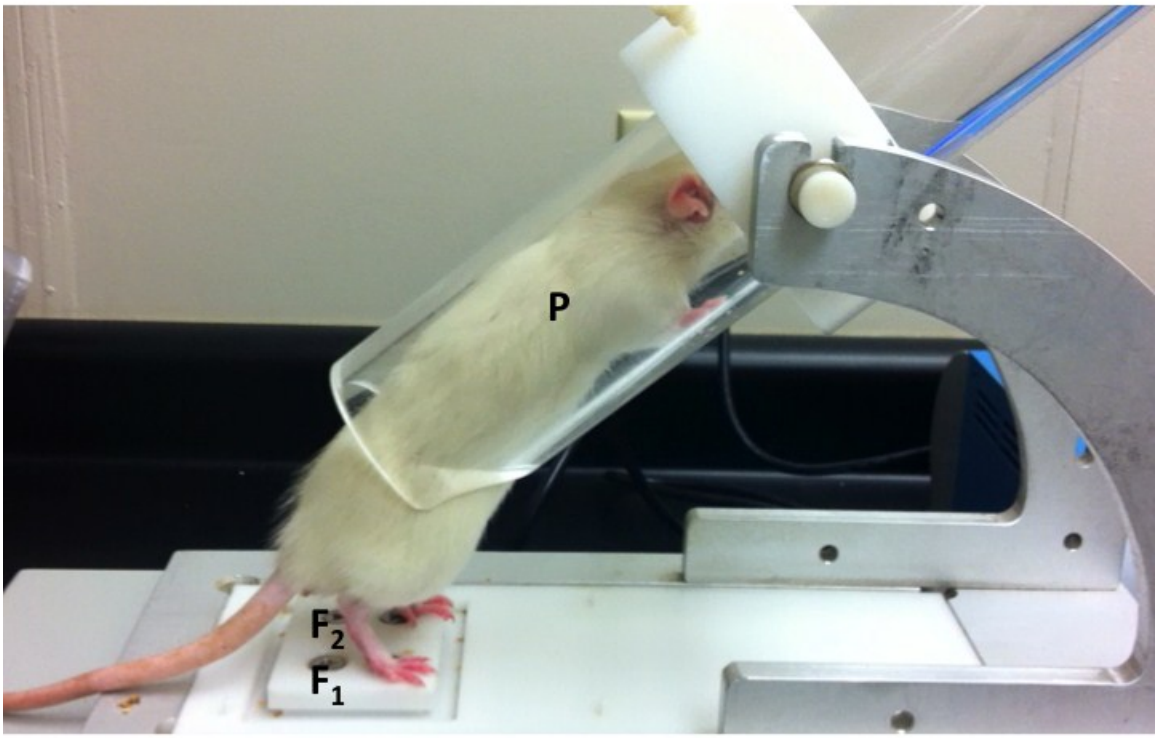


Figure 2.2 Incapacitance tester evaluates musculoskeletal discomfort by assessing hindlimb weight-bearing of animals. Each paw was placed on a separate force plate transducer that measured the amount of weight (g) placed on it over a 3 second period. Measurements obtained can be indicative of musculoskeletal discomfort in the animal. Ipsilateral force plate transducer (F_1), contralateral force plate transducer (F_2), Plexiglass holding tube (P).

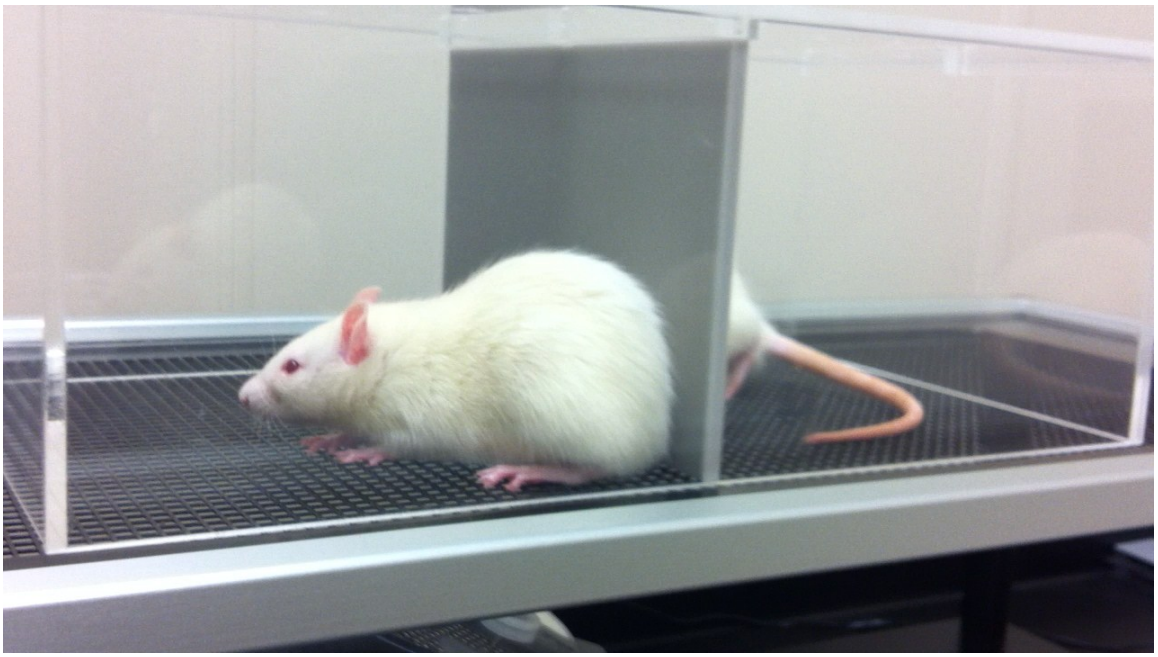


Figure 2.3 Von Frey hair algesiometry measures secondary allodynia in animals. Algesiometer raises a blunt microfilament toward the hindpaw of the animal. The force exerted by the microfilament is initially below the threshold of detection but gradually increases with constant speed until a withdrawal reflex is elicited. The force required by the algesiometer to elicit this withdrawal reflex is automatically recorded. Von Frey algesiometry is a measurement of secondary allodynia.

2.4 DRUGS

Lysophosphatidic acid, monosodium iodoacetate, and 2-hydroxypropyl-β-cyclodextrin, were purchased from Sigma-Aldrich Ltd. (Oakville, Ontario, Canada). Ki16425 was graciously donated by Eli Lilly (Indianapolis, IN, USA). All drugs were prepared fresh on the day of administration. Lysophosphatidic acid was dissolved in 0.9% saline and ethanol (5%) to final concentrations of 0.1, 1, and 3 mg/ml. 3 mg of monosodium iodoacetate was dissolved in 0.9% saline (50 µL). Ki16425 and 2-hydroxypropyl-β-cyclodextrin were dissolved in dH_2O.

2.5 DRUGS DOSING AND ADMINISTRATION

2.5.1 LPA Dose-response

After animals were habitualized to the apparatuses, naive baseline behavioral measurements were attained prior to treatment on day 0. After naive measurements were recorded, animals were administered an i.a. injection of LPA (5 µg, 50 µg, or 150 µg) or an equal volume (50 µl) of vehicle control into the right knee joint. A second round of behavioral measurements was conducted 24 h after i.a. injection (Figure 2.4).

2.5.2 LPA co-treated with LPA antagonist

After training and habitualization, naive baseline behavioral measurements were attained on day 0. Upon completion of baseline measurements, half of the animals were treated with a prophylactic systemic dose of $LPA_{1\text{-}3}$ antagonist Ki16425 (30 mg/kg i.p.) and the other half treated with a vehicle control. One hour after treatment all animals were administered an i.a. injection of LPA (50 µg) into the right knee joint. A second round of behavioral measurements were conducted in all animals 24 h after the i.a. injection of LPA (Figure 2.5).

2.5.3 Chronic LPA

Similar to experimental models outlined above, naive baseline behavioral measurements were recorded on day 0. Once behavioral measurements were attained, animals were administered an i.a. injection of LPA (50 µg or 150 µg) into

the right knee joint. Subsequent behavioral measurements were conducted on days 3, 7, 14, and 21 (Figure 2.6).

2.5.4 MIA co-treated with LPA antagonist

Upon recording of naive baseline measurements on day 0, half of the animals were treated with a prophylactic systemic dose (i.p.) of the LPA_{1-3} antagonist Ki16425 (30 mg/kg). The remaining half of the animals were administered a vehicle-paired control. One hour post-treatment, all animals received an i.a. injection of MIA (3 mg) into the right knee joint. A secondary round of behavioral analyses were conducted on day 14 (Figure 2.7).

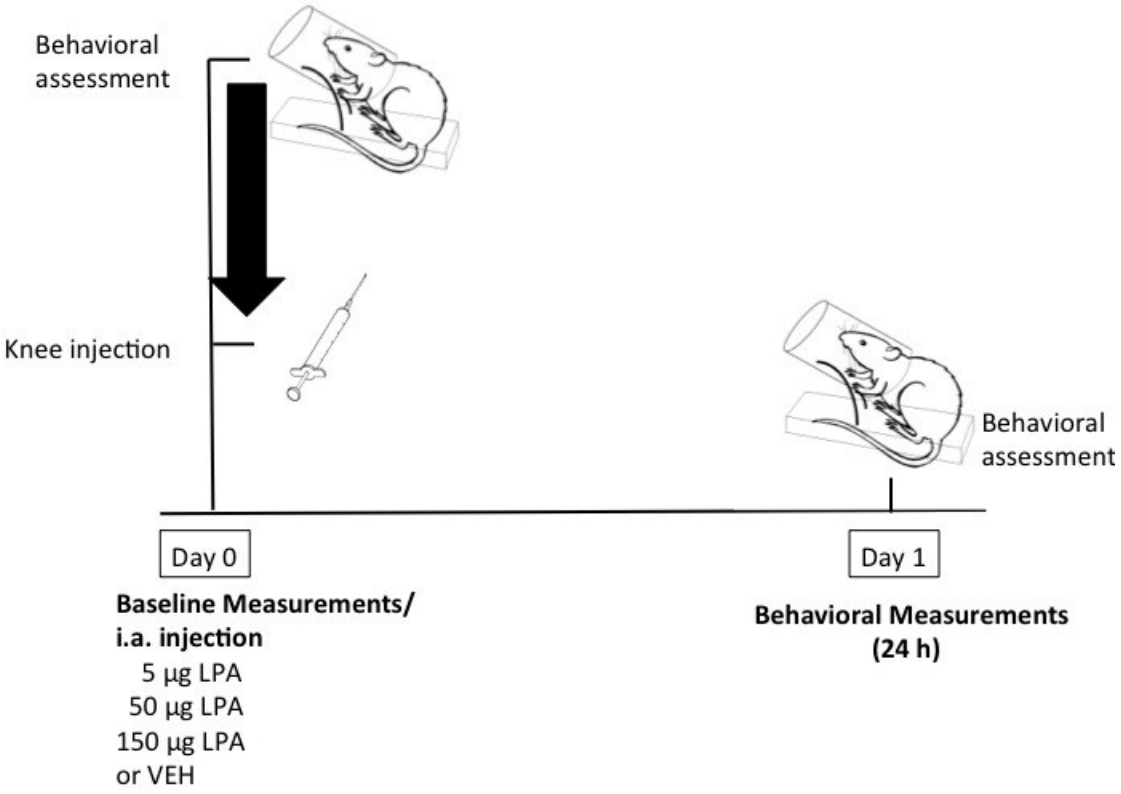


Figure 2.4 Behavioral assessment and injection schedule for LPA (i.a.) dose response experiments. Prior to treatments, baseline behavioral assessment was completed in naive animals. Rats then received an i.a. injection (50 µl) of vehicle, 5 µg, 50 µg, or 150 µg of LPA. Behavioral scoring was assessed again at 24 h post-injection.

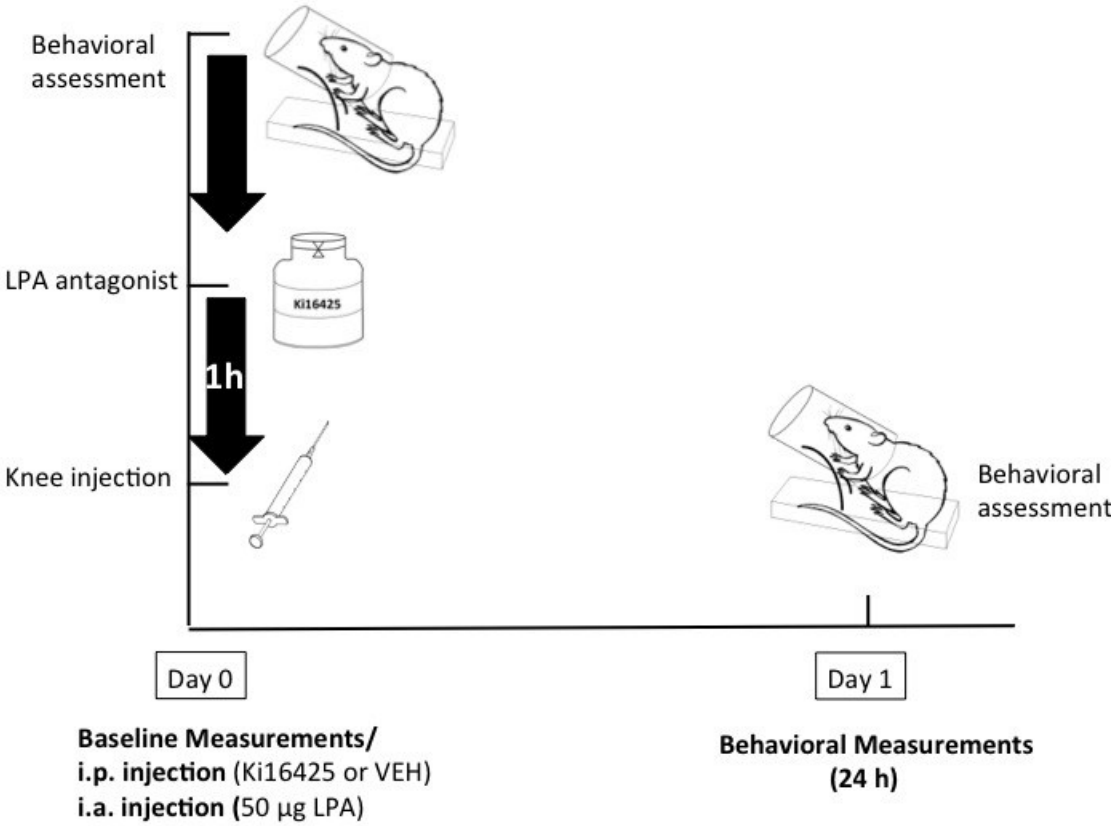


Figure 2.5 Behavioral assessment and injection schedule for LPA (i.a.) + Ki16425 (i.p.) experiments. Prior to treatments, baseline behavioral assessment was completed in naive animals. Rats then received a prophylactic dose of Ki16425 (or vehicle) 1 h prior to the i.a. injection (50 µl) of LPA (50 µg). Behavioral scoring was assessed again 24 h post-injection.

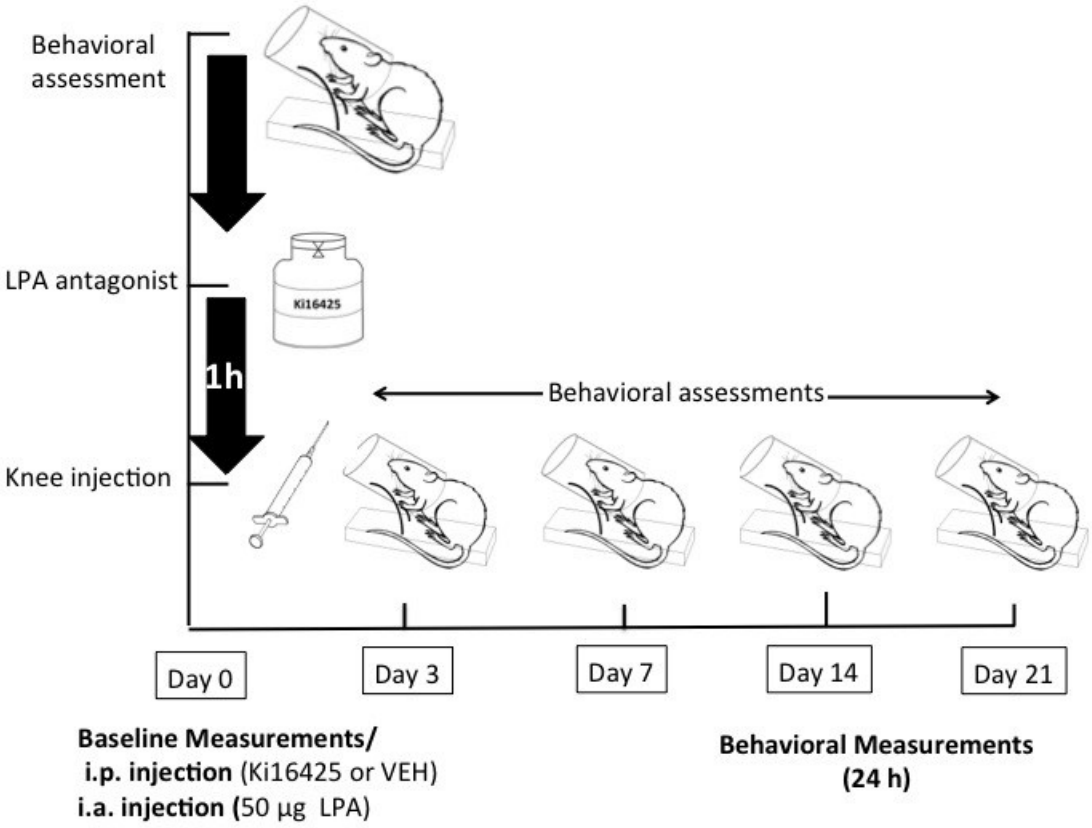


Figure 2.6 Behavioral assessment and injection schedule for chronic LPA (i.a.) experiments. Prior to treatments, baseline behavioral assessments were completed in naive animals. Rats then received an i.a. injection (50 µl) of LPA (50 µg). Behavioral scoring was assessed again on Day 3, Day 7, Day 14, and Day 21.

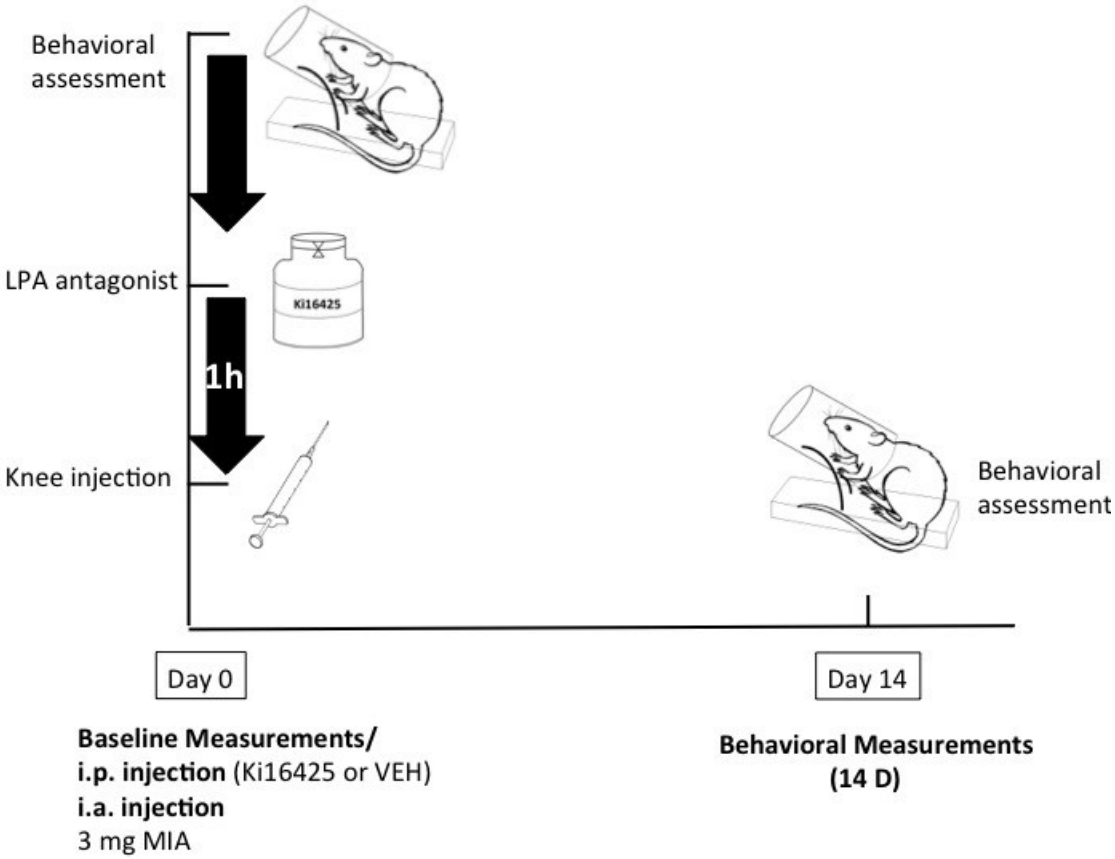


Figure 2.7 Behavioral assessment and injection schedule for MIA (i.a.) + Ki16425 (i.p.) experiments. Prior to treatments, baseline behavioral assessments were completed in naive animals. Rats then received a prophylactic dose of Ki16425 (or vehicle) 1 h prior to an i.a. injection (50 µl) of MIA (3 mg). Behavioral scoring was assessed again on day 14.

2.6 TISSUE PROCESSING

2.6.1 Saphenous nerve extraction

All animals were humanely euthanized through sodium pentobarbital overdose. An incision along the interior portion of the quadriceps (spanning from above the knee joint to the peritoneal cavity) was made. Superficial adipose tissues were bluntly dissected and cleared in order to isolate the saphenous nerve. Once isolated, the portion of the saphenous nerve running superior to the joint capsule and inferior to the peritoneal cavity was excised. The nerve was partitioned into three equal portions, termed distal, medial, and proximal.

2.6.2 Paraffin embedding

Nerve tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2-4 h. Samples were then washed and stored in glycine (0.2 g per 100 mL PBS) prior to embedding. Samples were dehydrated by a series of ascending ethanol passages and then embedded in paraffin. Following embedding, samples were transversely sectioned (5 μm) onto Superfrost slides using a RM2135 microtome (Leica Microsystems, USA) and allowed to dry overnight in a humidified chamber (40°C).

2.6.3 Toluidine blue staining

Tissue sections were mounted onto slides and deparaffinated and rehydrated in a series of graded ethanol solutions (xylene (x 4), 100% (x 3), 95% (3), 70%). Slides were immersed in toluidine blue working solution and rinsed with distilled water (x 3). Samples were then dehydrated rapidly through the ascending ethanol passages 95%, 100% (x 2), xylene (x 2) and coverslipped.

2.6.4 Hematoxylin & Eosin staining

Slides containing the tissue were deparaffinated and rehydrated with a decreasing ethanol passage (previously described in section 2.6.3). Samples were immersed in 0.1% hematoxylin and then underwent a series of rinses of: tap water, Scott's H_2O, tap water, 0.2% nitric acid, tap water, Scott's H_2O, and finally tap water. A counter stain of Eosin Y

was applied and the samples were then dehydrated with ascending ethanol passages (previously described in section 2.6.3) and coverslipped.

2.6.5 EM post-fixation

The right saphenous nerves of each group (n=8) were fixed in 2.5% glutaraldehyde diluted with 0.1 M sodium cacodylate buffer at 4°C for 2 h, administered three rinses of 0.1 M sodium cacodylate buffer, and fixed in 1% buffered osmium tetroxide (2 h, 4°C). Samples were rinsed with distilled water and placed in 0.25% uranyl acetate overnight (4°C). Following overnight fixation, samples were then dehydrated with a graduated series of acetone (50%, 70% (x2), 95% (x2), 100% (x2), dried 100%) and infiltrated with Epon Araldite Resin. Prior to sectioning, samples were embedded in 100% Epon Araldite Resin and placed in an oven to cure (48 h, 60°C). Epoxy embedded tissue samples were cut into thin sections (110 nm) using a LKB Huxley Ultramicrotome (Bromma, Sweden) with a diamond knife and placed on 300 mesh copper grids. Prior to viewing, sections were contrasted with 2% aqueous uranyl acetate and lead citrate. Following processing, ultrathin sections were viewed using a JEOL JEM 1230 Transmission Electron Microscope (Tokyo, Japan) (x 2500 magnification) and images captured by a Hamamatsu ORCA-HR digital camera.

2.7 DATA ANALYSIS

2.7.1 Quantification of demyelination

To determine the thickness of myelination of the afferent neurons, digital photographs (2500x magnification) were analyzed. The myelin sheath thickness was measured using an area-based G-ratio (Figure 2.8), calculated by the ImageJ G-ratio Calculator (National Institutes of Health, USA).

2.7.2 ImageJ G-ratio calculator

The two steps in G-ratio analysis are “Image preparation” and “Analysis”; these steps involve the application of several filters and processing procedures.
Image preparation: Initially the image being analyzed was converted to 8-bit (black & white). A median filter was then applied which allowed for better neuron edge detection

by the elimination of background noise. Finally, an additional user-defined filter was applied; throughout this present study the Shanbag filter was utilized for clarity of neuron edges.

Analysis: A neuron was selected for analysis by clicking on the centre of it, the program then measured the inner area and inner perimeter (a1 and p1) (Figure 2.8 & 2.9). The program then identified the myelin border and measured the total area and outer perimeter (a2 and p2).

2.7.2.1 Methods used for G-ratio calculation

The G-ratio calculator automatically computes two values for each neuron analyzed. For perfectly spherical neurons, the perimeter based G-ratio and the area based G-ratio would be identical. However, due to demyelinating processes, a large majority of neurons undergo morphological alterations. The area based G-ratio is a better representation of myelination as it is shape-independent, and was utilized in all analyses within the present study.

The perimeter based G-ratio is calculated: $\text{pG-ratio} = \frac{p1}{p2}$

The area based G-ratio is calculated: $\text{aG-ratio} = \sqrt{\frac{a1}{a2}}$

Saphenous nerves of three animals in each treatment group were analyzed; a cross-section at the proximal, medial, and distal levels of each nerve examined. Three images were captured at each cross-section, and within each digital image the G-ratio of 3 neurons were analyzed. To ensure an unbiased analysis, measurements were conducted without the knowledge of treatment groups.

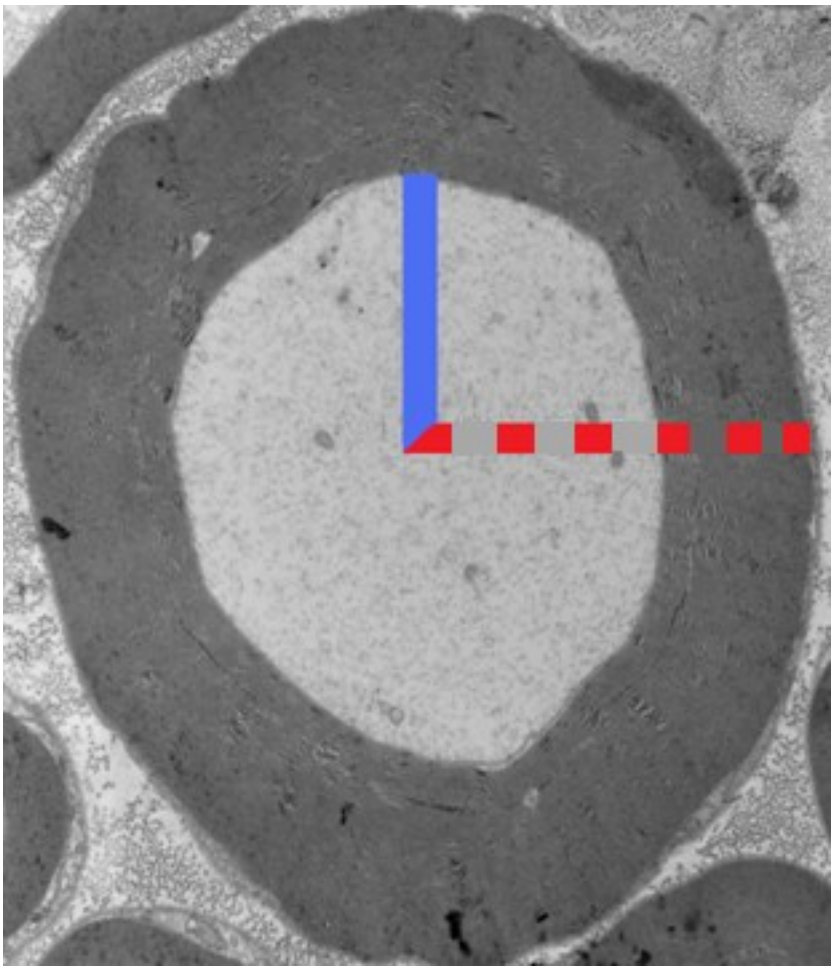


Figure 2.8 TEM nerve analysis quantifying afferent myelination using the G-ratio. The area-based G-ratio is determined by dividing the internal axonal area (a1) (blue) by the total axonal area (a2) (red) to give a representation of the myelin present. This ratio can be compared between treatment groups to quantify demyelinating processes and amelioration there of.

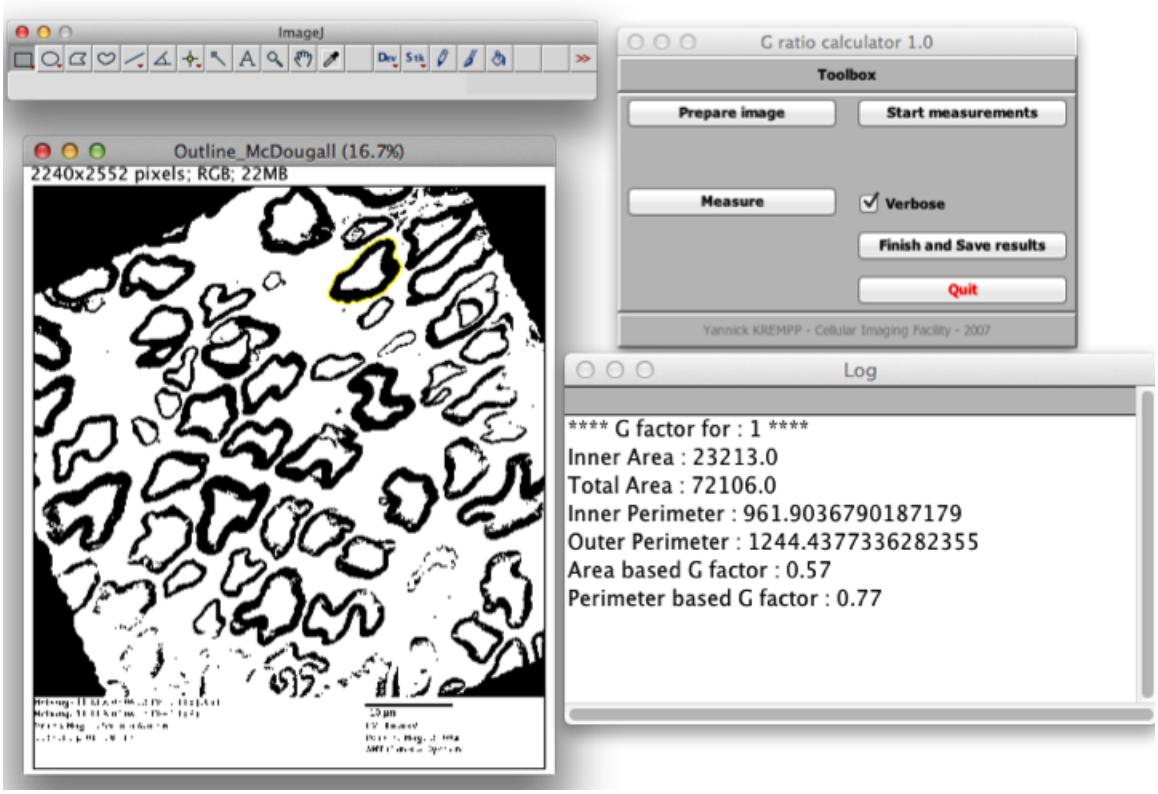


Figure 2.9 Analysis of TEM sections through ImageJ G-ratio Calculator. TEM digital images were opened with ImageJ G-ratio Calculator, following the application of several filters, the G-ratio calculator calculated both the perimeter based G-ratio and the area based G-ratio. The area based G-ratio was used for statistical analysis, as demyelinated neurons undergo structural alterations and this measurement is shape-independent.

2.8 STATISTICAL ANALYSIS

Data analysis was performed using Graphpad Prism software (version 5.0) and presented as mean ± standard error of the mean (SEM). In studies only including two groups, a Student's t-test was performed. In multiple treatments with normal distribution and equal variance, a one-way analysis of variance was performed. The Bonferroni post-hoc test was utilized in all groups. A value of $P < 0.05$ was determined to be statistically significant.

2.9 ROBUSTNESS OF MEASUREMENTS TECHNIQUE

Extensive efforts were made to minimize variability of behavioral assessments among experimental cohorts. Behavioral analyses were conducted by the same researcher, within the same room (void of noise), and at the same time of day, to ensure reproducibility between experimental groups.

Over the course of the study, the average naive baseline measurement of ipsilateral hindlimb weight-bearing in all experimental groups (n = 79) was determined to be 51.03% with a standard deviation of 2.51. The low standard deviation indicates reproducibility among experimental groups. The average amount of force required to elicit a paw withdrawal response in naïve animals (n = 79) in all groups was found to be 35.04g, with a standard deviation of 8.64.

Although every effort was taken to ensure animals became habitualized to the algesiometer chamber, the operation of the algesiometer is a much more dynamic process compared to the incapacitance tester. The algesiometer apparatus often needs to be repositioned beneath the animal and the raising of the blunt microfilament elicits soft noise. In rare instances, these factors may influence the readings in timid animals and account for the large standard deviation.

The incapacitance weight-bearing apparatus is specifically designed to house a moderate-sized animal. As the animals grew large and had difficulty fitting into the holding tube comfortably, measurements became challenging. This caused a limitation to the length of our chronic studies (21 days).

CHAPTER 3 RESULTS

3.1 NOCICEPTIVE EFFECTS CAUSED BY AN INTRA-ARTICULAR INJECTION OF LPA

To determine if musculoskeletal discomfort arose from introduction of LPA into the knee joint, 5, 50, or 150 μg of LPA or vehicle controls were introduced into the joint of animals in several cohorts. Incapacitance measurements were conducted 24 h after the intra-articular (i.a.) injection of LPA and were compared to naive baseline measurements (Figure 3.1). I.a. administration of both 50 μg ($p < 0.001$; one-way ANOVA and Bonferonni post-hoc test) and 150 μg ($p < 0.05$; one-way ANOVA and Bonferonni post-hoc test) LPA caused a marked reduction (-4.59%) in the amount of weight distributed onto the injected (ipsilateral) hindlimb 24 h post injection compared to naive controls.

Conversely, no significant change in weight-bearing was noted in 5 μg or vehicle groups. This experiment demonstrates that an i.a. injection of LPA into the knee joint does induce a shift in weight-bearing 24 h post-injection, with the most significant changes resulting from administration of 50 μg.

In order to determine if peripheral administration of LPA causes secondary allodynia, tactile allodynia was measured in the hindpaw. In identical treatment groups, 5, 50, or 150 μg of LPA along with vehicle controls were administered into the knee joint and von Frey algesiometry was assessed 24 h post injection. Although no group was found to be statistically different than vehicle control, both 50 and 150 μg LPA treatment groups were trending on a reduction in the force required to elicit a withdrawal response when percent (%) change from baseline was calculated. On average, animals in the 50 μg treatment group demonstrated a 20% decrease in the force required to elicit a withdrawal reflex at 24 h compared to paired baseline measurements (Figure 3.2). Similarly, the 150 μg treatment group demonstrated a 10% decrease in force, on average, to elicit a withdrawal reflex 24 h after administration of LPA into the knee joint ($p > 0.05$; one-way ANOVA and Bonferonni post-hoc test) (Figure 3.2).

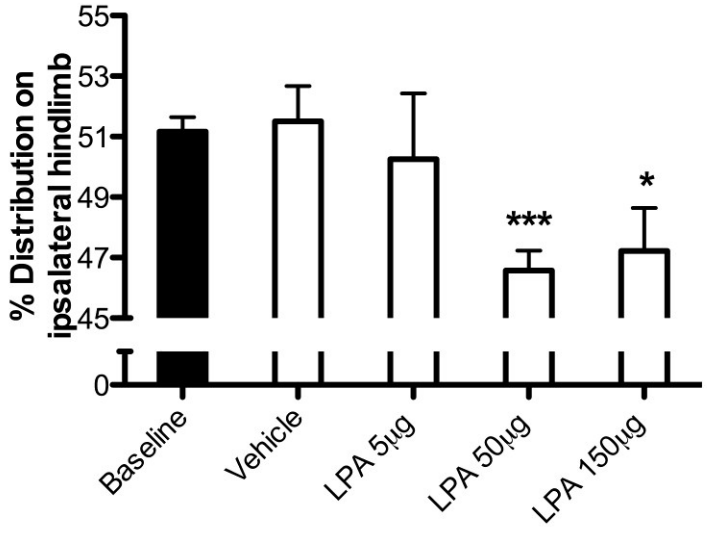


Figure 3.1 LPA causes disruption to hindpaw weight-bearing in normal rats. Histogram shows shift in hind limb weight-bearing at 24 h after intra-articular injection of LPA. Values represent the mean ± SEM (n=8 / group). ***$p<0.001$ compared to naïve controls, *$p<0.05$ compared to naive controls; one-way ANOVA and Bonferonni post-hoc test.

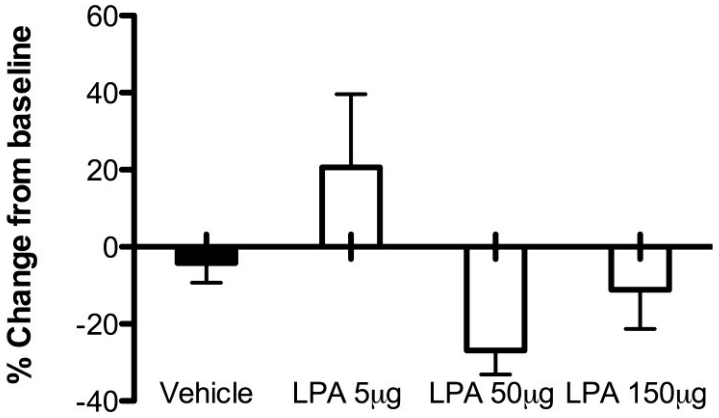


Figure 3.2 Force required to elicit withdrawal response after LPA injection. Histogram shows percent (%) change from baseline for force required to elicit a paw withdrawal 24 h after intra-articular injection of LPA (5 - 150 μg). Values represent the mean ± SEM (n=8 /group).

3.2 PREVENTION OF LPA-INDUCED NOCICEPTION THROUGH SYSTEMIC KI16425

In an attempt to characterize the mechanisms by which LPA elicits acute peripheral sensitization, animals were co-treated with the LPA_{1-3} antagonist Ki16425 (30 mg/kg, i.p.) and LPA (i.a.). Incapacitance weight-bearing and von Frey algesiometry behavioral assessment was conducted to assess the pain states mediated by LPA and the potential attenuation through Ki16425.

In these experiments, injection of LPA in vehicle-treated animals failed to elicit changes to ipsilateral hindlimb weight-bearing at 24 h (Figure 3.3). Although the Ki16425-treated animals maintained baseline levels of weight bearing on the ipsilateral hindlimb, due to the failure of the vehicle-treated control group to demonstrate peripheral sensitization at 24 h (Figure 3.3), the anti-nociceptive effects of the LPA antagonist could not be fully elucidated ($p > 0.05$; one-way ANOVA and Bonferonni post-hoc test).

Similarly, tactile allodynia was not present in animals administered LPA + vehicle at 24 h, as determined through algesiometry assessment (Figure 3.4). Visually, it can be observed that the percent (%) change from baseline in the force required to elicit a withdrawal reflex in vehicle-treated control animals (-15.3 %) was approximately twice as much as the Ki16425-treated animals (-9.5 %) ($p = 0.6275$; unpaired two-tailed students T-test). Although no statistical difference was found between treatment groups, the behavioral analyses are trending on significance and an increase in the number of animals tested may produce statistical significance.

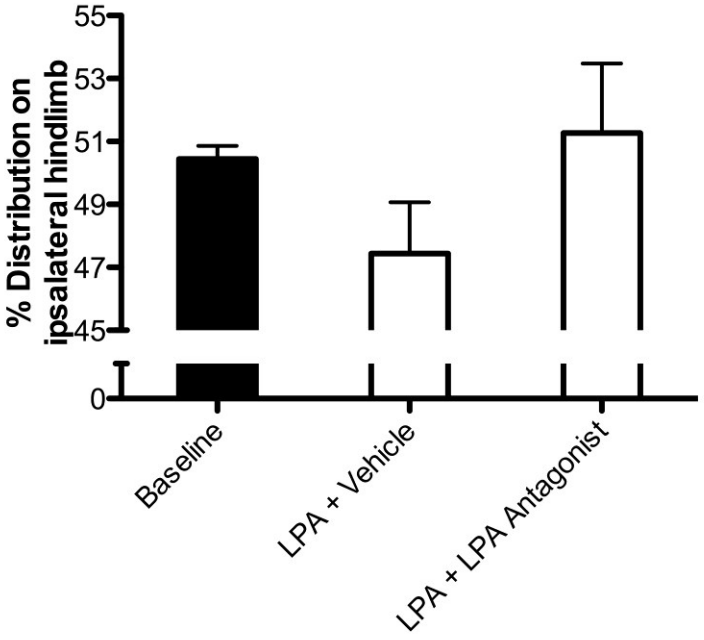


Figure 3.3 LPA antagonist does not prevent change in weight-bearing. Histogram shows a comparison of the effect of prophylactic treatment of the LPA antagonist Ki16425 (i.p.) 1 h before intra-articular injection of LPA (50 μg) on hind limb weight-bearing at 24 h post-LPA injection. Values represent the mean ± SEM (n= 8/ group). One-way ANOVA and Bonferonni post-hoc test.

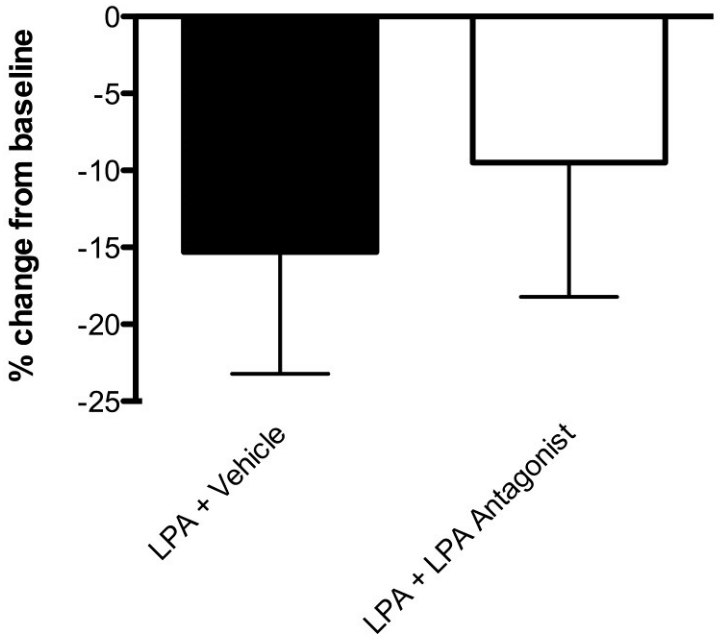
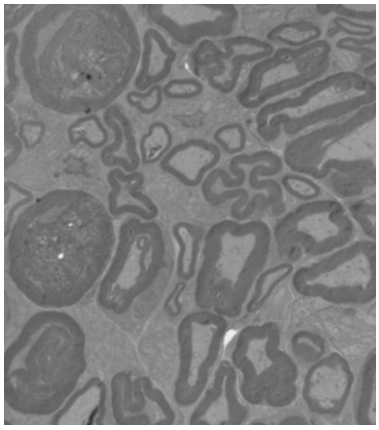


Figure 3.4 LPA antagonism does not prevent secondary allodynia. Histogram is a comparison of difference in percent change from baseline (force) required to elicit withdrawal 24 h after intra-articular injection of LPA (50 μg) in animals treated with either LPA antagonist Ki16425 (i.p. injection 1 h prior to LPA injection) or vehicle (i.p. injection 1 h prior to LPA injection). Values represent the mean ± SEM (n=8 /group). p = 0.6275; unpaired two-tailed students T-test.

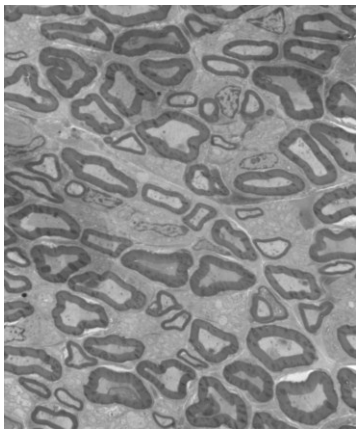
3.3 INTERACTIONS BETWEEN SYSTEMIC Ki16425 AND LPA-INDUCED AFFERENT DEMYELINATION

The above results implicated LPA in peripheral sensitization and we now sought to determine if this pain was neuropathic in nature. Initially, we set out to determine if the pain behaviour mediated by LPA was associated with demyelination of joint afferent axons. Previous research found centrally administered (i.t.) LPA to elicit pain through demyelination (Inoue, 2004); to determine if peripherally administered LPA elicits pain in a similar manner, Transmission Electron Microscope (TEM) analysis was utilized. It was determined that administration of LPA into the knee joint did not cause demyelination of local afferent axons in the saphenous nerve 24 h after LPA injection (Figure 3.5 and 3.6). The G-ratio for animals co-treated with the $LPA_{1\text{-}3}$ antagonist Ki16425 (i.p.) and LPA (i.a.) showed no statistical difference compared to animals administered LPA (i.a.) and a vehicle control (i.p.) ($p = 0.4253$; unpaired two-tailed students T-test) (Figure 3.6). Similar to the behavioral analysis, a reduction in demyelination (through G-ratio analysis) is trending and increasing the amount of animals analyzed may generate statistical significance.

A) LPA + Vehicle



B) LPA + LPA Antagonist



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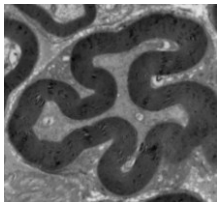

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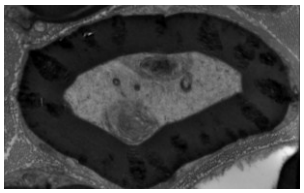


Figure 3.5 Transmission Electron Microscope cross sections of saphenous nerves (24 h). Transmission Electron Microscope (TEM) cross sections of saphenous nerves (110 nm) of animals administered with LPA (50 µg) and co-treated with A) Vehicle, or B) LPA antagonist Ki16425 (30 mg/kg; i.p.) at 2500x magnification and C) Vehicle, or, D) LPA antagonist Ki16425 (30 mg/kg; i.p.) at 15000x magnification.

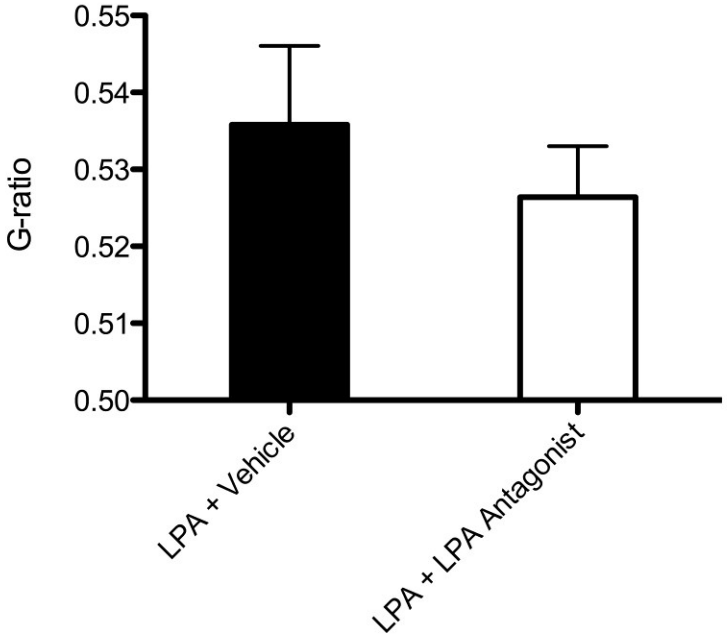


Figure 3.6 LPA antagonist does not impact peripheral nerve demyelination.
Histogram shows a comparison of myelination of saphenous nerves, as determined by G-ratio analysis, between LPA antagonist Ki16425-treated animals and vehicle-treated animals at 24 h post-LPA (50 μg) injection. Values represent the mean ± SEM (n= 91 for vehicle, n= 144 for LPA antagonist). $p = 0.4253$; unpaired two-tailed students T-test.

3.4 CHRONIC NOCICEPTIVE EFFECTS OF LPA

Administration of LPA into the knee joint appears to elicit acute nociception (Figure 3.1). However, as OA presents as a chronic condition clinically, to accurately represent this in a pre-clinical model, characterization of LPA's chronic effects in the knee joint was undertaken.

It was found that a single i.a. injection of LPA failed to maintain peripheral sensitization, at both 50 μg and 150 μg doses, as ipsilateral hindlimb weight-bearing returned to baseline levels 21 days post injection (Figure 3.7).

At the 50 μg dose the largest shift in weight-bearing onto the contralateral hindlimb, observed on day 3, was completely abolished by day 21, as measurements returned to baseline measurements. A150 μg i.a. injection of LPA into the knee joint failed to produce any significant changes in weight bearing throughout the course of the study (Figure 3.7).

Tactile allodynia was determined to reach a heightened level of sensitization at day 14 after injection of LPA (50 μg), although returning to baseline level by day 21 (Figure 3.8). A higher dose of LPA (150 μg) failed to initiate allodynia throughout the course of the study (Figure 3.8).

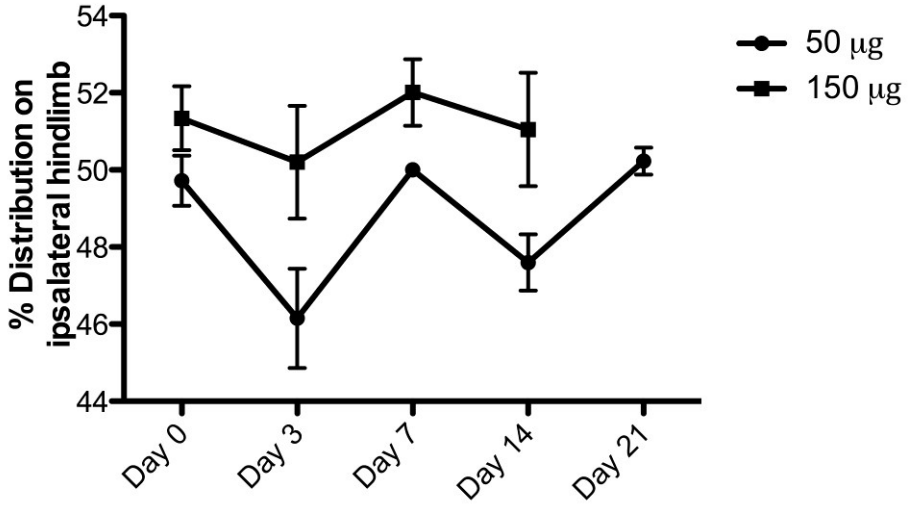


Figure 3.7 LPA does not induce chronic change in weight-bearing. Time course of the effect of intra-articular injection of LPA at 50 μg and 150 μg on distribution of hind limb weight-bearing. Values represent the mean ± SEM (n=8 for 50 μg and 150 μg). Repeated measures ANOVA.

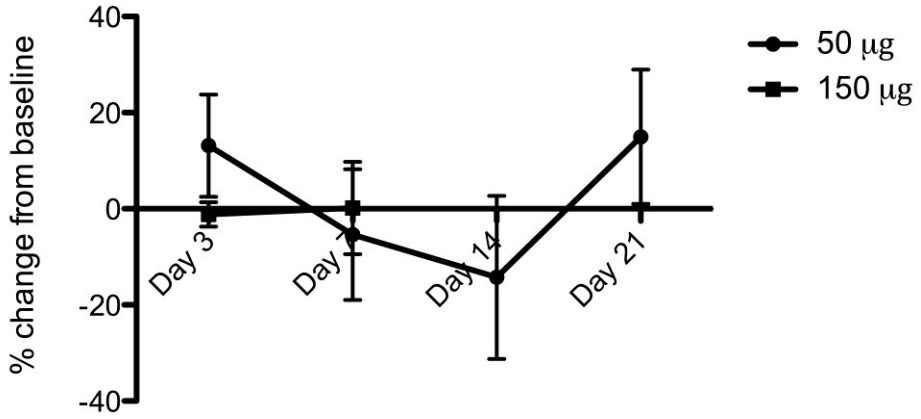


Figure 3.8 LPA does not induce chronic secondary allodynia. Time course of the effect of intra-articular injection of LPA at 50 μg and 150 μg on force required to elicit a paw withdrawal. Values represent the mean ± SEM (n=8 for 50 μg and 150 μg). Repeated measure ANOVA.

3.5 Ki16425 FAILED TO PREVENT MIA-INDUCED NOCICEPTION

Systemic administration of Ki16425 (30 mg/kg, i.p.), one hour prior to OA induction, failed to ameliorate MIA-induced hindlimb incapacitance (Figure 3.9). Marked decreases in the amount of force placed on the ipsilateral hindlimb of both Ki16425 (40.7 %) ($p < 0.001$; one-way ANOVA and Bonferonni post-hoc test) and vehicle-treated groups (42.09 %) ($p < 0.001$; one-way ANOVA and Bonferonni post-hoc test) (Figure 3.9) were noted 14 days after OA induction compared to naive baseline controls (51.92 %).

Similarly, systemic administration of Ki16425 (30 mg/kg) failed to prevent the secondary allodynia caused by MIA (Figure 3.10). The vehicle control group demonstrated a 24 % change from baseline in the amount of force require elicit a withdrawal response, compared with the Ki16425-treated group that demonstrated a 15 % change from baseline ($p = 0.6006$; unpaired two-tailed students T-test) (Figure 3.10).

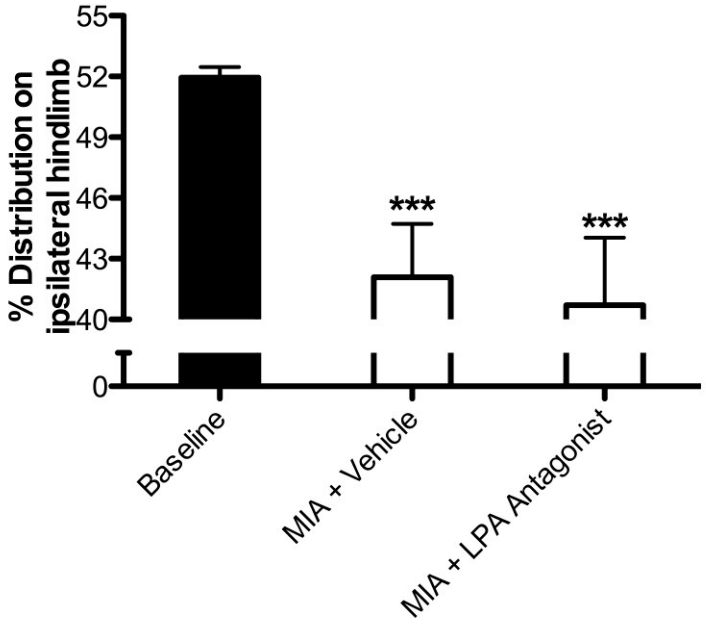


Figure 3.9 LPA antagonism does not prevent MIA-induced change in weight-bearing. Histogram shows a comparison of distribution in hind limb weight-bearing at 14 days post MIA injection (3 mg) in animals treated with the LPA antagonist Ki16425 or vehicle (i.p. injection at 1 h pre-MIA injection). Values represent the mean ± SEM (n=8 for all groups). ***$p<0.001$ compared to naïve baseline; one-way ANOVA and Bonferonni post-hoc test.

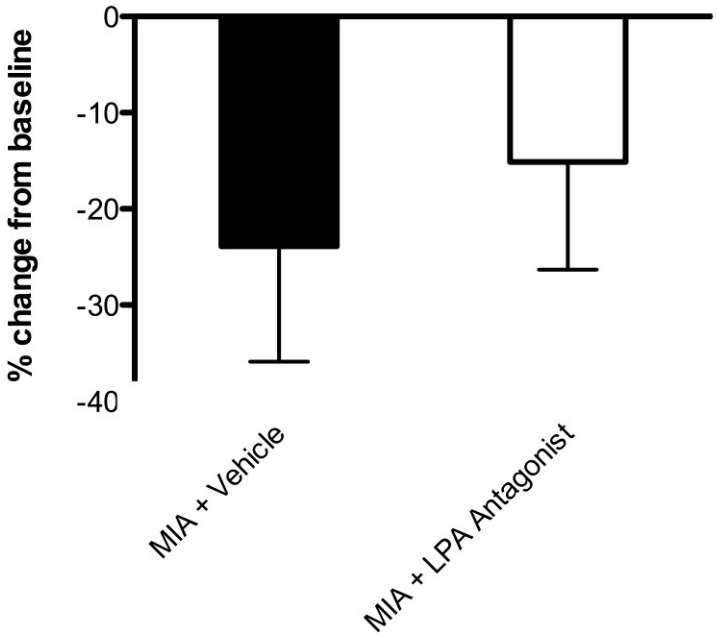
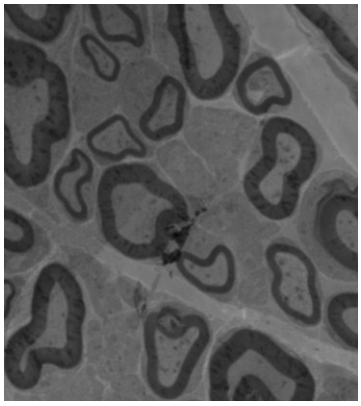


Figure 3.10 LPA antagonism does not prevent MIA-induced secondary allodynia. Histogram shows a comparison of percent (%) change from baseline in force required to elicit withdrawal at 14 days post MIA injection in animals treated with LPA antagonist Ki16425 or vehicle (i.p. injection 1 h pre-MIA injection). Values represent the mean ± SEM (n=8 /group). p = 0.6006; unpaired two-tailed students T-test.

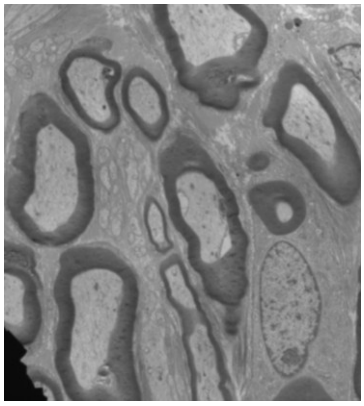
3.6 EFFECTS OF SYSTEMIC Ki16425 ON MIA-INDUCED PERIPHERAL NERVE DEMYELINATON

As determined through Transmission Electron Microscopy (TEM), i.a. injection of MIA caused observable demyelination of axons in the saphenous nerve (Figure 3.11). These morphological changes were quantified between Ki16425 treated animals (0.57) and vehicle controls (0.61) by G-ratio analysis and demyelination was confirmed (Figure 3.12). Systemic administration of LPA_{1-3} antagonist Ki16425 (30 mg/kg; i.p.) attenuated the demyelinating affect of MIA, indicating it is mediated through LPA_{1-3} receptors ($p < 0.0039$; unpaired two-tailed students T-test) (Figure 3.12).

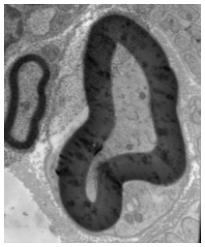
A) MIA + Vehicle



B) MIA + LPA Antagonist



C)



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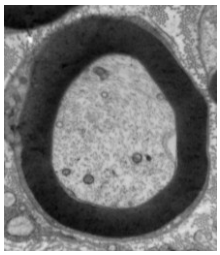

Figure 3.11 Transmission Electron Microscope of saphenous nerves of animals co-treated with Ki16425 and MIA (14 d). Transmission Electron Microscope (TEM) cross sections of saphenous nerves (110 nm) of animals administered with MIA (3 mg) and co-treated with A) Vehicle, or B) LPA antagonist Ki16425 (30 mg/kg; i.p.) at 2500x magnification and C) Vehicle, or, D) LPA antagonist Ki16425 (30 mg/kg; i.p.) at 15000x magnification.

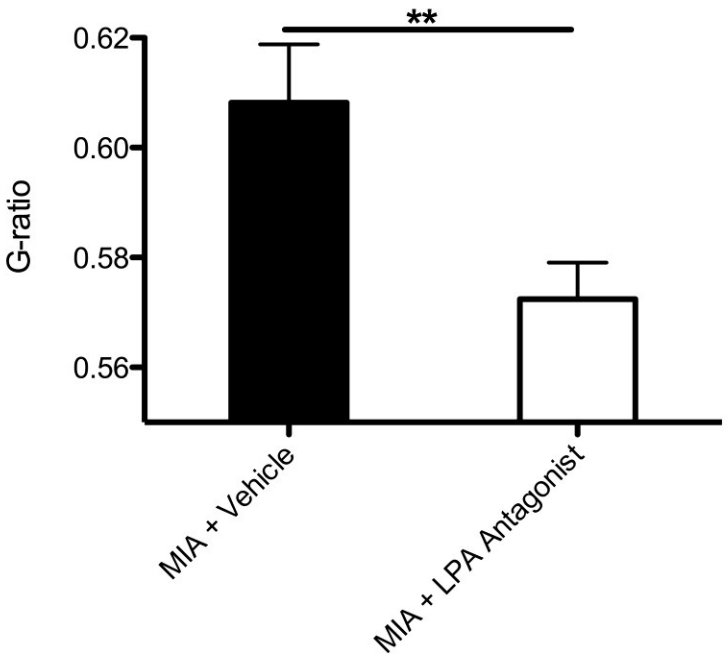

Figure 3.12 LPA antagonist Ki16425 inhibits peripheral nerve demyelination caused by MIA. Histogram shows a comparison of myelination of saphenous nerves, as determined by G-ratio analysis, in animals treated with either the LPA antagonist Ki16425 or vehicle treated animals 14-day post MIA injection. Values represent the mean ± SEM (n=55 for vehicle, n= 123 for antagonist). ** p = 0.0039; unpaired two-tailed students T-test.

CHAPTER 4 DISCUSSION

Lack of efficacy of NSAIDs and analgesics in OA patients raises concerns of an additional pain state present in the disease. Recently, our colleagues at Eli Lilly (Eli Lilly, Indianapolis, IN) discovered elevated levels of LPA in the synovial fluid of OA patients. LPA has been concluded to be a chemical signature for the initiation of neuropathic pain (Ueda, 2011). This finding is therefore a major clinical linkage between OA and neuropathic pain. In the present study, the novel experimental model of introducing LPA into the animal knee joint is characterized, and the effects of Ki16425, LPA_{1-3} antagonist are examined. Additionally, the potential therapeutic effects of Ki16425 in the OA disease model, MIA, are explored.

4.1 VALIDATION OF EXPERIMENTAL PROTOCOL

Behavioural effects of MIA in the present study were comparable to behavioural changes previously reported using a similar concentration of MIA (Bove et al., 2003). Previous research, employing retrograde tracers, has determined that joint capsule disruption during the administration of MIA does not cause MIA to diffuse to neighbouring tissues (Ferreira-Gomes et al., 2010). Therefore, MIA leaking to neighbouring tissues was unlikely to cause the demyelination induced by MIA in this study.

Incapacitance weight-bearing is a widely used, non-invasive method of assessing musculoskeletal discomfort in the femorotibial joints of animals. The apparatus has been extensively characterized to accurately model peripheral sensitization in MIA and various other pre-clinical models of pain (Bove et al., 2003). Additionally, von Frey algesiometry is a widely used method of modeling secondary allodynia and has been extensively characterized in models of arthritis (McDougall et al., 2006).

4.2 DRUG DOSING AND ADMINISTRATION

Previous studies guided our determination of doses of LPA (Inoue et al., 2004), MIA (Bove et al., 2003), and Ki16425 (Ma, Matsumoto, et al., 2009). We modeled our dosing of i.a. MIA injections from Bove et al. (2003) who found significant OA effects with 3mg i.a. injection. Finally, Ma et al. (2009) determined systemic administration of Ki16425 at 30mg/kg attenuated demyelinating LPA effects when administered 30 or 60 minutes prior to LPA injection, but not at 90 minutes (Ma, Matsumoto, et al., 2009).

4.3 PERIPHERAL ADMINISTRATION OF LPA

In the present study, the introduction of LPA into the knee joint was discovered to cause alterations to hindlimb weight-bearing at 24 h (Figure 3.1), indicative of peripheral sensitization. Although LPA was not found to cause secondary allodynia, there was a reduction in the force required to elicit a hindpaw withdrawal in the group administered 50 μg (-26.9%), compared to naive baseline measurements. Comparatively, only a minimal reduction in the amount of force required to elicit a hindpaw controls (-4.9%) was noted in vehicle controls.

A prophylactic dose of $LPA_{1\text{-}3}$ receptor antagonist Ki16425 (30mg/kg) was found to not inhibit alterations to hindlimb weight-bearing, caused by introduction of LPA into the knee joint, when compared to vehicle controls. Interestingly however, the LPA-induced alteration to weight-bearing demonstrated in the first study was not recapitulated in the second cohort of animals. Therefore, the absence of statistical significance between groups may not be due to the lack of efficacy of Ki16425, but rather, the absence of LPA-induced nociception in vehicle controls.

Ki16425 (30mg/kg) also failed to attenuate secondary allodynia when compared to vehicle controls (Figure 3.4). However, animals treated with Ki16425 demonstrated a much lower change in the force required to elicit paw withdrawal of -9.5% (from baseline) compared to vehicle controls (-15.9%). Although these values are not statistically significant, they are trending on significance. This difference amongst groups may be highlighted with the addition of animals into the cohort.

As LPA has previously been characterized to initiate neuropathic pain through demyelination (Inoue, 2004), myelinated joint afferents were examined following the introduction of LPA into the knee joint. Transmission electron microscopic (TEM)

analysis determined there to be no difference in the G-ratio of saphenous nerves of animals treated with LPA + Ki16425 (0.52) compared to LPA + vehicle controls (0.53) (Figure 3.6). However, the absence of effect may not be due to the inability of Ki16425 to offer protection from demyelination, but rather, the time course in which it was examined. LPA most likely requires longer than 24 h to cause demyelination, and in the present study, no significant difference was found when Ki16425-treated animals were compared to vehicle controls perhaps because the demyelinating effects of LPA had not been fully developed in the control animals.

Although the preliminary studies of LPA did not produce statistically significant findings, most trended on significance and will most likely find confirmation upon the addition of more animals into the experimental groups and the examination of demyelinating effects at a later time point.

4.4 LPA-INDUCED CHRONIC NOCICEPTION

The present study determined that a single i.a. injection of LPA (50μg or 150μg) failed to augment chronic nociception (Figure 3.7). Neither peripheral nor central sensitization was elicited in animals throughout the duration of the experiment. One explanation for the lack of effect is an inadequate amount of time for a single i.a. injection to elicit effects within the joint, as the knee joint is highly regulated by the lymphatic system. This strict regulation would cause exogenous LPA to be flushed from the synovial environment before it elicited activation of the LPA receptors. This regulation is also experienced in human synovial joints, and is the reason why i.a. therapies require constant re-administration to elicit full therapeutic effects (Allen et al., 2010).

Furthermore, LPA in the periphery may not utilize the deleterious feed-forward production mechanisms that occur in the CNS. Endogenous LPA production is known to occur through extracellular binding of LPA to the LPA_3 receptor (Ma, Uchida, et al., 2009). LPA_3 receptors are located primarily on microglia (Table 4), which become upregulated in the spinal cord following peripheral nerve damage (Eriksson et al., 1993). Upon activation, LPA_3 initiates an intracellular signaling cascade, which culminates in the conversion of PC to LPC, which is then released into the extracellular matrix and

hydrolyzed into LPA. This feed-forward production loop justifies how nociception, elicited through i.t. injections of exogenous LPA into the spinal column, is not recapitulated through an i.a. injection into the knee joint. Although LPA is known to exert effects in the periphery, a single i.a. injection most likely does not utilize the feed-forward production processes, as in the CNS, failing to recruit an adequate amount of LPA to initiate a persistent pain state. Future studies should examine repeated injections of LPA to ensure an adequate amount of the phospholipid is present in the joint to elicit actions.

4.5 NEUROPATHIC COMPONENT TO MIA EXPERIMENTAL MODEL

Previous studies have implicated a neuropathic component to the experimental OA MIA model. As LPA has been linked to the initiation of neuropathic pain (Inoue et al., 2004), in the present study we attempted to characterize it's role in disease progression.

Prophylactic treatment of Ki16425 failed to ameliorate MIA-induced alterations to weight-bearing (Figure 3.9). As MIA has been characterized to express robust bi-phasic inflammatory components, it stands to reason that the peripheral sensitization experienced in this model is mediated through factors other than LPA. However, animals treated with Ki16425 demonstrated a lower change from baseline values (-15.1%) compared to vehicle treated controls (-23.9%) (Figure 3.10). Although not statistically significant, these results are trending and with the addition of more animals into the cohorts, a significant difference will most likely be found.

Interestingly, upon TEM analysis of myelinated joint afferents, it was determined that introduction of MIA into the knee joint causes demyelination of peripheral nerves and this demyelination was attenuated by Ki16425. Through G-ratio analysis, it was determined that the saphenous nerves of animals administered a prophylactic dose of Ki16425 (30 mg/kg) possessed more myelin (0.57) than vehicle treated controls (0.61) two weeks after MIA induction (Figure 3.12).

Although it has been speculated widely in previous studies, this is the first time, to our knowledge, that the demyelinating effects of MIA have been quantified.

Furthermore, the present study characterized LPA_{1-3} receptors to mediate MIA-induced demyelination and concomitant neuropathic pain.

4.6 CURRENT UNDERSTANDING OF LPA

4.6.1 Effects of LPA in the periphery

Although LPA has previously been demonstrated to initiate neuropathic pain following i.t. administration into the spinal column (Inoue et al., 2004), this is the first study which examined its effects in the periphery. The finding that administration of LPA into the knee joint elicits nociception is novel in itself. Although, LPA was not demonstrated to generate central sensitization, the complete characteristics of LPA may not have been fully characterized within this study. Woolf et al. has determined central sensitization to occur after prolonged peripheral stimulation (Woolf & Salter, 2000). As behavioral testing was conducted 24 h after LPA injections, the present study may not have allowed enough time for peripheral nociception to generate concomitant central sensitization. Subsequent studies examining tactile allodynia at later time points will better characterize the central sensitization that accompanies LPA-induced nociception in the periphery.

4.6.2 Neuropathic pain in OA

Recent pre-clinical studies using both chemical (Ivanavicius et al., 2007; Okun et al., 2012; Orita et al., 2011; Thakur et al., 2012) and surgical OA induction (Im et al., 2010) as well as clinical studies (Hochman et al., 2010; Ohtori et al., 2012) have implicated a neuropathic pain component to OA pain. Ivanavicius et al. (2007) demonstrated injection of MIA into the femorotibial joint generated an upregulation of ATF-3 in DRG cells. As upregulation of ATF-3 is indicative of damage to joint afferents (Tsujino et al., 2000), this finding implies a neuropathic pain component exists in the MIA model. Similarly, clinical studies utilizing focus groups discovered OA patients use descriptors such as “pins & needles, tingling sensation” to describe the pain experienced. Interestingly, these descriptors were also commonly used in neuropathic diseases. The investigators determined that 34% of the OA patients included in the experiment experienced neuropathic pain (Hochman et al., 2010).

Although implicated, prior to the present study, the exact mechanisms through which MIA elicits damage to the peripheral nervous system remained unelucidated. As neuropathic pain is attributed to damage of primary afferent axons leading to the generation of ectopic action potentials and concomitant phenotypic changes (Bridges et al., 2001), the quantification of joint afferent demyelination induced by MIA has significant implications. For the first time, the preliminary mechanisms responsible for neuropathic pain have been confirmed to occur within an OA experimental model. As there has been a high correlation between experimental models and the clinical setting (Arendt-Nielsen et al., 2010), this finding ultimately provides evidence of a neuropathic component in human OA.

Despite the attenuation of peripheral nerve demyelination by Ki16425, the LPA antagonist did not attenuate the behavioural alterations, induced by MIA. This confirms earlier theories that various mediators are involved in MIA-induced nociception. Vasoactive intestinal peptide (VIP) is known to mediate MIA nociception (McDougall et al., 2006). Additionally, serine proteinases (e.g. thrombin, trypsin, and mast cell tryptase), which are released from mast cells during inflammation, have been implicated in the OA disease process. In addition to hydrolyzing proteins, proteinases are responsible for the activation of proteinase-activated receptors (PARs), which modulate joint sensitivity (McDougall et al., 2009) and have been linked to OA disease progression (Xiang et al., 2006).

The MIA experimental model is understood to model disease process through the inhibition of glycolysis, which leads to chondrocyte destruction (Bove et al., 2003). Following chondrocyte destruction, fibrin proteinaceous edema fluid expands the synovial membrane allowing it to become susceptible to infiltration by exogenous components including lymphocytes, macrophages, and plasma cells (Bove et al., 2003). The inflammatory component eventually subsides; however, not before robust chondrocyte destruction and collapse of necrotic cartilage within the joint has occurred. Following inflammation, increased osteoclast activity, the collapse of subchondral bone, and its replacement by fibrous tissue is observed. The robust expression of inflammatory mediators within the disease model justifies why a single antagonist did not ameliorate nociception.

One potential receptor for the mediation of MIA nociception is TRPV1. TRPV1 mediates several forms of nociception and its expression is elevated on the primary afferent neurons from the knee of rats with MIA-induced OA (Fernihough et al., 2005). Recently, LPA was discovered to cause TRPV1 receptor activation, independent of LPA receptors (Nieto-Posadas et al., 2012). TRPV1 is expressed on the peripheral terminal of nociceptors and has widely been understood to mediate nociceptive processing (Davis et al., 2000). Demonstration of LPA-mediated TRPV1 channel activation, analogous of metatrophic receptor activation, links LPA with acute nociception in addition to its characterized role in chronic neuropathic pain.

As LPA receptors are specific for LPA, the attenuation of demyelination caused by the MIA experimental model by an LPA antagonist, within the present study, confirms that LPA mediates, at least part of, the disease progression within this model.

In addition to pre-clinical models, there is evidence that TRPV1 mediates OA pain in the clinical setting. It has been elucidated that TRPV1 receptors are expressed on the chondrocytes in the articular cartilage and synovial fibroblasts of OA patients (Engler et al., 2007; Gavenis et al., 2009) and desensitization of TRPV1 receptors reduces chronic pain associated with OA (Remadevi et al., 2008).

4.6.3 Potential mechanisms of OA disease progression

4.6.3.1 LPA plays a role in bone formation

A key feature in OA disease progression that ultimately leads to pain is subchondral bone remodelling. LPA receptors are expressed on human skeletal cells and their activation leads to abnormal bone formation, as seen in OA (Zhang et al., 1999). This process is believed to occur through the activation of $LPA_{1\text{-}2}$ receptors, which are coupled to $G_{\alpha 12/13}$ and subsequently activate the Rho kinase (Rock) pathway, leading to cytoskeletal reorganization. Another mechanism by which LPA mediates upregulation of bone growth is through inhibition of osteocyte destruction (Grey et al., 2002).

In biological conditions, a large percentage of osteoblasts undergo apoptosis (65%), with only a minority of cells undergoing transformation into osteocytes (cells which compromise human bone). The disregulation of osteocyte formation may create bone spurs, commonly developed during OA disease progression.

4.6.3.2 LPA causes articular cartilage destruction

Chondrocytes, the only cells present in the articular cartilage, undergo strict regulation of apoptosis and proliferation in order to maintain healthy tissue. Disruption of this homeostasis leads to structural and chemical changes in the chondrocytes and cartilage, which ultimately result in a diseased state (Grynpas et al., 1991). LPA receptors are expressed on the surface of articular chondrocytes (Koolpe et al., 1998). Activation of LPA receptors in human articular chondrocytes leads to upregulation of calcium stores and subsequent structural alterations (Koolpe et al., 1998). LPA receptor signalling mediates both proliferation of chondrocytes and their increased destruction (Facchini et al., 2005).

4.7 LIMITATIONS

Behavioral measurements were a limitation within the present study. Despite reproducibility between groups (SD = 2.51; n = 79) in weight-bearing measurements, there was greater variance in the hindpaw withdrawal measurements (SD = 8.64; n = 79); and the possibility that several timid animals acted as outliers within these measurements exists. Although habitualization was conducted in every group, in rare instances, timid animals may have demonstrated fear-evoked responses, which would have negatively affected the behavioral data. Although, if this phenomenon had occurred, the large number of animals within each cohort should have compensated for this confounder.

The present study examined the saphenous nerve to determine the demyelinating effects of compounds administered into the knee joint. However, the saphenous nerve is a mixed sensory and motor nerve that innervates many structures including joints. Although it is possible that the demyelination observed within the present study was generated from non-joint afferents, they are most likely not responsible for these damaging effects, as the exogenous compounds were administered into the knee joint.

Quantitative analysis of nerve fibre morphology is a key investigative tool in peripheral nerve damage. Several geometrical parameters may be assessed to determine damage to peripheral nerves, however, the G-ratio has previously been found to be the only parameter that statistically correlates to nerve functionality (Kanaya et al., 1996). Five sources of bias exist when analyzing peripheral nerve morphology (Geuna et al.,

2001): 1) strain, gender, and age of the animals examined, 2) the point along the nerve axis that is examined, 3) the location of the sampling fields within the nerve cross-section, 4) the inclusion-exclusion rules for sampling fibres within the field, and 5) the method for measuring parameters.

The first two sources of bias are related to study design and have been managed in the present study by the examination of male Sprague-Dawley (SD) rats of the same age and examining several points along the saphenous nerve axis. The third source of bias was addressed by the sectioning of the nerve cross-section onto TEM metal grids the tissue was mounted on following fixation. Nerve fibres in several grids, spanning the entire width of the cross-section were examined in all samples. By using an examiner blinded to the treatment groups, to evaluate all of the nerve fibres within the study, consistency in the sampling of fibres between groups was ensured. Lastly, using a computer program to calculate G-ratios of nerve fibres ensured the measuring parameters were consistent amongst groups.

4.8 FUTURE DIRECTIONS

4.8.1 MIA concentration

The use of chemical-induced OA is heavily debated, as disease severity may be easily manipulated through alteration of the concentration of monosodium iodoacetate injected into the joint (Schuelert et al., 2009). Arguments for various concentrations have been made for the correct modeling of clinical symptoms. The concentration of MIA utilized in the present study (3 mg) is widely used in pre-clinical studies of OA pain. However, other concentrations have been noted to elicit a pain state that resembles neuropathic pain in animals. Okun et al. (2012) has previously demonstrated injection of 4.8 mg of MIA elicits a continuous pain state that is driven by primary afferent fibres. As the hypothesized effects of LPA is the induction of a neuropathic pain state in OA patients, a disease model which generates pain mediated through primary afferents would be an ideal model to characterize its effects.

Arguments have been made to the accuracy of high concentrations of MIA in modeling the clinical presentation of OA, as disease progression occurs at much faster

rates than in clinical settings. However, to thoroughly characterization the effects of LPA_{1-3} activation in OA, examination of the effects elicited through higher concentrations of MIA are worth investigating.

4.8.2 LPA_{1-3} antagonist Ki16425

Although dosing of Ki16425 in the present study is modeled from previous research, which found systemic administration of Ki16425 (30 mg/kg) to attenuate neuropathic pain elicited through LPA induction, the method of LPA delivery differs from those previously employed. As this is the first time the effects of peripherally administered LPA were examined, and the first time its actions on MIA progression were analyzed, alternative dosing of Ki16425 may be required to elicit complete antagonism of LPA receptors. Subsequent studies using additional doses of systemically administered Ki16425 are required to accurately characterize the potential therapeutic effects of the compound in OA pain.

LPA has been demonstrated to activate the peripheral TRPV1 receptor, a known mediator in the pain pathway; therefore, additional studies combining Ki16425 with a TRPV1 antagonist would be worth examining. Attenuation of LPA-induced acute nociception through a TRPV1 antagonist may further link peripheral LPA to the sensitization experienced in early OA progression.

4.8.3 Animal species

4.8.3.1 Strain differences

A more likely explanation for the lack of chronic pain experience through peripheral administration of LPA is the innate resistance in the strain of animal used in the present study. Although SD rats are a common strain for preclinical models, demonstrating a high induction rate of neuropathic pain (De Felice et al., 2011), their success has been strictly with surgical models. Previous research has found SD rats fail to demonstrate neuropathic pain through chemical induction (Banik et al., 2001; Zhu et al., 1998). As the LPA model is an attempt to generate neuropathic pain through chemical induction, specific genetic variances in SD rats may prevent the manifestation of pain. Therefore, further experimentation with additional strains that have

demonstrated vulnerability to chemical induction (i.e. Lewis rats) should be carried out to determine if peripheral administration of LPA does in fact initiate chronic pain.

4.8.3.2 LPA receptors

The five cognate G-protein cell surface receptors with affinity for LPA in humans are also expressed in mice; the expression of the receptors throughout organ systems in mice mirrors that in humans. Rats, however, have only been characterized to express LPA_{1-3}. Although LPA_{1-3} are the focus of the present study, LPA_5 has been implicated in the conversion of acute nociceptive pain to chronic nociceptive pain in mice (Lin et al., 2012). Expressed in the DRG and spinal cord dorsal horn neurons of mice, although exerting different mechanisms than LPA_1, activation of LPA_5 leads to neuropathic pain (Lin et al., 2012). The present study did not explore the roles of LPA_5 in the induction of neuropathic pain; however, the absence of this cognate receptor may explain the failure of pain induction in vehicle treated control groups (Figure 3.3). Therefore, future studies should utilize the mouse model to examine the role of LPA_5 in the neuropathic component of OA.

4.8.4 Behavioral assessment

Neuropathic pain is characterized by allodynia, hyperalgesia, and hyposensitivity in the clinical setting. Extensive testing has been utilized to ensure the accuracy of pre-clinical animal models of neuropathic pain and OA. Overall, the efficacy of therapeutics in animals, receiving a preclinical model of neuropathic pain or OA, has closely mirrored the efficacy demonstrated in the clinical setting. However, although potential drug candidates have achieved success at alleviating hyperalgesia and allodynia, patients still experienced neuropathic pain. It is therefore determined that the true cause of neuropathic pain is the 'pain at rest'.

Unfortunately, due to testing constraints, traditional behavioral analyses in animals have relied solely on the attenuation of stimulus-evoked pain. The advent of the condition placed paradigm (CPP) has allowed for the measurement of the resting pain experienced by animals (King et al., 2009). CPP utilizes the innate reward system associated with pain relief in animals to determine if a potential therapeutic alleviates

pain at rest. As resting pain is believed to be the source of clinical neuropathic pain, future studies should utilize CPP to characterize the effects of Ki16425 at alleviating resting pain.

Furthermore, electrophysiological studies examining the firing of neurons in the spinal cord, responsible for eliciting nociception, may be examined and directly quantified. Electrophysiological readings may then be correlated to CPP analysis to better represent the resting pain experienced by animals with neuropathic pain. Together, these will give a better representation of therapeutic effects of potential drugs at attenuating resting pain, which ultimately drives neuropathic pain in humans.

4.9 SUMMARY AND CONCLUSIONS

The present work examined the effects of exogenously administered LPA into the femorotibial joint and the potential therapeutic effects of the LPA antagonist Ki16425 in an experimental OA model.

Summary:

- I.a. injection of LPA (50 μg) causes disruption of hindlimb weight-bearing 24 h after injection, however does not cause secondary allodynia or demyelination of joint afferents.
- Ki16425 does not prevent the alterations to hindlimb weight-bearing caused by LPA.
- I.a. injection of MIA (3 mg) causes disruption of hindlimb weight-bearing, secondary allodynia, and demyelination of joint afferents.
- Ki16425 does not prevent alterations to hindlimb weight-bearing, nor secondary allodynia, but does prevent demyelination of joint afferents.

Conclusions:

- Therefore, demyelination is not required for changes in hindlimb weight-bearing or secondary allodynia induced by MIA or LPA.
- Alterations to hindlimb weight-bearing and secondary allodynia are LPA_{1-3} –independent when induced through MIA.

- Secondary allodynia is LPA independent.

Although differences exist between rodent species and humans, efficacy of therapeutics in experimental models of neuropathic pain have demonstrated high correlation to the efficacy demonstrated in patients experiencing neuropathic pain and also OA pain. Similarly, hyperalgesia and secondary allodynia (referred pain) induced in experimental animal models of OA, have been demonstrated in OA patients (Arendt-Nielson, 2010). Therefore, the evidence of LPA potentiating the aetiology of OA in animal models holds tremendous significance for its role in clinical OA. The findings in the present study, in conjunction with elevated levels of LPA in the synovial fluid of OA patients, presents strong evidence for the role of this phospholipid in the initiation of a neuropathic component in OA pain, through demyelination of joint afferents, and other potential mechanisms in disease progression.

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