The role of mechanosensitive ion channels in osteoarthritis pain

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February, 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science.

Abstract

Osteoarthritis (OA) is a disabling and prevalent condition affecting 27 million US citizens and 4.2 million Canadians. Pain is the primary symptom of OA and it is manifested as mechanical hypersensitivity to joint palpation/movement, although the underlying mechanisms are poorly understood. This project aims to examine changes that occur in mechanosensitive ion channels (MSCs) of joint innervating pain-sensing neurons (nociceptors) during OA. These channels transduce mechanical stimuli into electrical signals. Using a mouse model that produces OA-like joint pathophysiology and pain, we assess if alteration in the gating properties of MSCs in joint-innervating nociceptors is a contributor to OA pain. We hypothesize that MSCs are sensitized to mechanical stimulation in OA and blocking their activity will reduce pain behaviours in OA mice. Using acutely dissociated neurons from healthy and OA mice, we performed electrophysiology on knee-innervating nociceptors to examine the properties of MSCs. Our data shows that MSCs from OA nociceptors have a reduced mechanical activation threshold, which results in an increased mechanically-evoked current. Analysis of the frequency of patches containing MSCs demonstrates that nociceptors of OA mice do not have an increased density of active MSCs at the membrane. Furthermore, single channel iV curves demonstrate that it is likely that the same MSC is present in both naïve and disease states. Lastly, GsTMx4, a blocker of MSCs, blocks MSCs present in knee-innervating nociceptors. Finally, in behavioral experiments, intra-articular injection of GsMTx4 in the OA knee produces significant analgesia. In summary, our model for OA pain shows that in nociceptors, MSCs are sensitized to mechanical stimuli and contribute to OA pain. Furthermore, targeting these channels in OA knees may be a valuable therapeutic strategy for managing OA pain.

Résumé

L'arthrose est une maladie invalidante touchant 27 millions de personnes aux États-Unis et 4.2 millions de Canadiens. Elle est caractérisée principalement par une douleur, qui se manifeste par une hypersensibilité aux stimulations mécaniques suite à un mouvement ou à la palpation des jointures. Les mécanismes sous-jacents cette hypersensibilité demeurent cependant inexpliqués. Le présent projet vise à examiner les changements induits par l'arthrose dans l'activité des canaux mécanosensibles (MSCs) exprimés dans les neurones répondant aux stimuli douloureux (nocicepteurs) qui innervent les articulations. Ces canaux convertissent les stimuli mécaniques en signaux électriques. A l'aide d'un modèle murin d'arthrose, qui reproduit la physiopathologie et la douleur articulaire, nous examinons si des altérations dans l'activité des MSCs présents dans les nocicepteurs des articulations est un facteur important pour la douleur arthritique. Notre hypothèse est que l'arthrose sensibilise les MSCs aux stimulations mécaniques, et que de bloquer l'activité de ces canaux chez les souris arthritiques réduira non-seulement cette hypersensibilité au niveau cellulaire, mais également leurs comportements associés à la douleur. En utilisant des neurones dissociés provenant de souris en santé et de souris arthritiques, nous avons effectué des enregistrements électrophysiologiques sur les nocicepteurs des genoux. Nos données démontrent que ces nocicepteurs, dont l'activation est mécanique, ont un seuil d'excitabilité minimum réduit, et que cette hypersensibilité accroit également les courants engendrés par les stimuli mécaniques. L'analyse des courbes iV des canaux ioniques indiquent que les MSCs présents dans les cellules en santé soient les mêmes que ceux des cellules arthritiques. De plus, nous avons démontré que le peptide GsTMx4 (extrait de l'araignée *Grammostola spatulata* et qui a pour propriété de bloquer les MSCs) est efficace pour bloquer les MSCs des récepteurs nociceptifs des genoux. Finalement, une injection de GsMTx4 avant la flexion ou l'extension de l'articulation réduit de

façon significative les scores de douleurs des souris arthritiques. En résumé, notre modèle de douleur arthritique démontre donc que les MSCs exprimés par les nocicepteurs sont hypersensibilisés aux stimuli mécaniques et contribuent significativement à la douleur arthritique. Nos données suggèrent qu'un traitement ciblant spécifiquement les MSCs pourrait avoir des effets bénéfiques chez les patients souffrant d'arthrose en réduisant leurs douleurs.

Acknowledgements

Most importantly, I would like to thank Dr. Reza Sharif-Naeini for all his support and guidance throughout my Master's project and thesis writing. I was blessed to have worked with such a passionate and skilled teacher, who was always responsive and willing to tend to my questions and concerns. Dr. Sharif-Naeini has opened up many opportunities for me not only within research but in many other respects, including health care and academia.

During my time, I was able to interact with many current and past members of the lab. I would like to specially mention both Steven Li Fraine and Albena Davidova. Steven has been a warm and caring friend, often around to keep me company during long nights of electrophysiology. He always lent an open ear and I appreciated our conversations. Albena, with her vast array of underappreciated lab skills, has also been an important friend and the caretaker of my and everyone else's experiments. Without her help in the animal facility and at the bench, not many things would get done.

Lastly, I want to thank my family and friends who have supported me during my time as a graduate student. There has been difficulty and frustration but they helped me see through it all and persevere. I would like to thank in particular, Giulia Alberini, my long-time significant other for putting up with my rants and ramblings. You especially have helped me transform into a better, kinder version of myself. Thank you all for the encouragement and I hope I can return the favour in the future.

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Legend

12-HPETE	12-hydroperoxyeicosaenoic acid
ACLT	Anterior cruciate ligament transection
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
bFGF	basic fibroblast growth factor
BSP	Bone sialoprotein
CFA	Complete Freund's Adjuvant
cFOS	proto-oncogene, murine osteosarcoma viral oncogene homolog
CGRP	Calcitonin-gene related peptide
CIPA	Congenital insensitivity to pain with anhydrosis
CNS	Central nervous system
COMP	Cartilage oligomeric matrix protein
CS	Central sensitization
DEG/ENaC	Degenerin/epithelial sodium channel
DMM	Destabilization of the medial meniscus
DmPiezo	Drosophila melanogaster Piezo protein
DRG	Dorsal root ganglia
ERK	Extracellular-signal-regulated kinase
GAPDH	Glyceraldehyde-3- phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GsMTx4	*Grammostola spatulata* toxin peptide 4
HT	High threshold
IASP	International Association for the Study of Pain
ICOAP	the Intermittent and Constant OA Pain test
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IB4	Isolectin B4
KOOS	Knee injury and Osteoarthritis Outcome Score
LT	Low threshold
MIA	Monoiodoacetate
mL	Millilitres
mM	Millimolar
mm Hg	Millimetres of mercury
MMP	Matrix metalloprotease
Mrg	Mas-related G-protein
MrgprD	Mrg-coupled receptor D
MRI	Magnetic resonance imaging
MSCs	Mechanosensitive ion channels
mV	Millivolts
MΩ, GΩ	MegaOhm, GigaOhm
NADA	N-Arachidonoyl dopamine
$N_{av}1.7$	Voltage-gated sodium channel, type IX, α subunit encoded by *SCN9A* gene
Na_v 1.8	Voltage-gated sodium channel, type X, α subunit encoded by *SCN10A* gene

$N_{av}1.9$	Voltage-gated sodium channel, type XI, α subunit encoded by *SCN11A* gene
NGF	Nerve growth factor
NSAIDs	Non-steroidal anti-inflammatory drugs
N-terminal	Amino-terminal
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OMERACT	Outcome Measures in Rheumatology
P2XRs	Purinergic receptors
pA	Picoamperes
RA	Rheumatoid arthritis
SEM	Standard error of the mean
SNRIs	Serotonin–norepinephrine reuptake inhibitors
SON	Supraoptic nucleus
SP	Substance P
TNF-α	Tumor necrosis factor alpha
TRAAK	also known as KCNK4, TWIK-related arachidonic acid-stimulated K+ channel
Trek1, Trek2	also known as KCNK2, KCNK10, TWIK-related potassium channel 1 and 2
TRPM8	Transient receptor potential, subfamily M, member 8
TRPV1	Transient receptor potential, vanilloid subtype 1
TrkA	Tropomyosin receptor kinase A
VAS/NRS	Visual analog scale/numerical rating scale
VEGF	Vascular endothelial growth factor
VGCC	Voltage gated calcium channels
VPN	Ventral posterior nucleus of the thalamus
WOMAC	Western Ontario and McMaster Universities Arthritis Index

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Osteoarthritis

Osteoarthritis (OA) is a highly prevalent and debilitating disease. It affects roughly 27 million US adults and 4.2 million Canadians aged 15 or older (*1, 2*). With projected increases in the aging population, this affected population will only continue to grow in Canada and the US (*1*). By 2040, it is estimated that 10 million Canadians (1 in 4) will struggle with OA (*3*). With such a prevalence of OA in the mature workforce, there is a heavy disease burden on the socioeconomic balance of the country. The total economic burden of OA, including both direct and indirect costs to healthcare, is estimated to be almost $30 billion/year (*3*). A closer look at the impact of OA shows that 80% of patients suffer from some level of movement limitation and a quarter of OA patients are unable to complete their daily activities due to their illness (*4*). The typical pathophysiology of OA includes inflammation of the synovial membrane (synovitis) and meniscus, cartilage degradation, bone remodelling dysfunction (formation of bone spurs, i.e. osteophytes), and bone marrow & peri-articular lesions (*2*). Along with these physical symptoms and pain – both thermal and mechanical pain – are secondary effects such as obesity, heart disease, type II diabetes, depression, and poverty, all of which compound the societal costs of OA (*2*).

1.1.1 Pathophysiology of Osteoarthritis

The primary site of OA pathology resides within the synovial joints of the body (*5*). Synovial joints, being the most abundant type of joint in the body, are characterized by a fluid-filled cavity (joint cavity) that enables the joint to undergo a wide range of movements. The lining membrane of the synovial fluid (called the synovium) is composed of macrophages and fibroblasts that

forms a pliable capsule that encloses the synovial joint and, in conjunction with the articular cartilage that lines the condyles of the two contacting bones, allow for the smooth, buffered movement of the joint.

OA manifests as a general failure of the whole joint. While degradation of cartilage and death of cartilage-producing chondrocytes in the articular cartilage are hallmarks of the disease, OA is a complex host of maladaptive events. These include osteophyte formation, synovial inflammation, subchondral bone loss and meniscal inflammation and damage (**Figure 1-1a**) (*6*). Moreover, in both mild and severe cases of tibiofemoral OA, there is evidence of increased vascularization and innervation of subchondral bone and articular cartilage (*7*). This is of particular poignancy in terms of pain sensation; in the normal knee joint, innervation of the knee never breaches the articular cartilage. With this cohort of mechanical failures and physiological issues, it is likely that each factor compounds the severity of the others in the context of OA (*8*).

Recently, there have been important advances in our understanding of the progression of OA. Firstly, joint injuries can precipitate the aforementioned cascade of events. Both acute and repetitive stress to loaded joints have been linked to development of OA in those joints (*9*). These stresses can result in soft tissue damage within the joint to the articular cartilage, meniscus, ligaments and tendons. The injuries can accumulate and result in joint instability and abnormal loading patterns (*10*). Secondly, biomarkers derived from the degrading cartilage and/or inflamed synovium provided important insight on the progression of OA. Peptide portions of cartilage-composing type II collagen, ω6 and ω3 polyunsaturated fatty acids (eicosanoids), and serum cartilage oligomeric matrix protein all have implications in their predictive ability for the stage of OA as well as the severity of symptoms of the disease (*11-14*).

However, the most prominent symptom of OA is pain in the joint and surrounding tissues. Often the primary symptom reported by the patients, it can manifest in several forms, including both heat and cold hypersensitivity, and more importantly mechanical hypersensitivity to joint movement and touch. Mechanically hypersensitivity can be further sub-divided into two categories: mechanical allodynia and mechanical hyperalgesia. Allodynia refers to painful responses to previously innocuous stimuli, while hyperalgesia refers to the exaggerated response to painful stimuli. Allodynic responses can be either spontaneous – maintaining a low to moderate level of consistent pain – or evoked via normal activities, movements or innocuous stimuli such as touch. As pain symptoms worsen, there is the progressive loss of function of the joint concomitant with psychological distress and lowered quality of life (*2, 10*). Early forays into imaging the histopathological progression of OA indicated a lack of correlation between observed joint structural changes and reported pain (*15*). However, more advanced MRI imaging techniques have provided promising new pathways to identify and correlate pain with soft-tissue damage and subchondral bone reformation (*16-18*).

1.1.2 Causes of Osteoarthritis

Outside of injury and chronic stress to the joint, the causes of osteoarthritis are largely unknown. It is a disease that predominantly affects weight-bearing joints such as the knee (*5*). Due to the elusive nature of the cause of OA, much of the research focuses on the early identification of osteoarthritis, and pre-emptive assessment/treatment of the disease. New biomarkers, discovered through large proteomic searches, for early stage OA are currently under investigation (*19*). Alongside such efforts, new clinical classifications – driven from insights in the clinic – have been designed to assist in the determination of the efficacy of novel biomarkers (*20*).

Many risk factors have been associated with the development of the disease. OA is more prevalent among women and individuals 65 years old or older, (*5*). Sex-independent factors including obesity, joint misalignment, muscle weakness, joint trauma and surgery, and biochemical abnormalities such genetic predisposition and other metabolic disorders comprise the breadth of associated risk factors for OA development (*5, 6*). However, it is important to reiterate the presence of one or more of these risk factors do not guarantee OA development, nor does the absence of any of these risk factors exclude the potential of OA development. While there needs to be more research into the biology and etiology of OA, it has been proposed that the variation in risk in onset and progression of OA is due to individual joint mechanics. The specific genetic predisposition and particular joint defects are to blame for the progression of OA on an individual basis (*6*).

1.2 Pain

The International Association for the Study of Pain (IASP) describes pain as: "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (*21*). Outside of the immediate physical response, pain is a rather complex subjective phenomenon that includes psychological and social factors (*22*). Under normal conditions, the pain experience indicates immediate or expected error in behavior or bodily movement (i.e. bending a joint beyond its movement limit, chest pain due to ischemia, stepping on a needle). In this sense, pain is seen as a protective reflex, warning the organism about danger and impending harm (*23*). Thus, pain is as critical a survival mechanism as sight or thirst: maintaining bodily integrity (*24*). This depiction of pain's role becomes abundantly apparent in extreme conditions of pain insensitivity, such as congenital insensitivity to pain with

anhydrosis (CIPA), where there is total neglect of broken limbs, self-mutilation, auto-amputation, and corneal scarring, all of which leads to early death (*25*).

In essence, pain can be divided into three categories: acute, inflammatory, and pathological (*24*). Short-term or immediate pain (i.e. acute pain) serves a protective role, signalling to the individual to withdraw from the threat, rest to allow for tissue healing, or seek help (*2*). Inflammatory pain is a more persistent type of protective pain. Inflamed tissues provide heightened sensitivity following tissue damage (*24*). This is critical to allow for prolonged recovery following severe injuries, and prevent the individual from incurring further damage during the time. On the contrary, pathological pain serves no reasonable protective role. It is maladaptive; a result of nervous system malfunction or abnormality (*24*). However, it should be noted that there are many similarities between the physiology of inflammatory and pathological pain. The terminals of pain-sensing neurons, nociceptors, can become sensitized during both types of pain (*10*). This may be due to reduced threshold for pain signalling, along with the increase in the duration, amplitude and spatial distribution of pain signalling (*24, 26*). Furthermore, the axons may become hyperexcitable, generating spontaneous action potentials, and cell soma can change their protein trafficking and expression (*24*). Centrally, the spinal cord circuitry may also be modulated, having synapses strengthened or displaying morphological change in number or density of synapses (*24*). These sensitization effects culminate in allodynia and hyperalgesia. However, it is important to separate the role of inflammatory pain from pathological pain: while both can be forms of chronic pain, pathological pain can occur without inflammation or the presence of a prior injury, therefore its experience is no warning of threat but rather the result of substantial changes in the plasticity of the nociceptive system (*2, 23*).

Within the perspective of OA, both inflammatory pain and pathological pain can exist. The earlier phases of the disease correspond to the typical inflammatory pain which is marked by synovitis. However, in the absence of physiological correspondence, pain can still persist as a result of pathological changes in the pain signalling pathway. Interestingly, several genetic components identified in pain hypersensitivity and the development of chronic pain, may contribute to this pathological pain aspect in OA patients (*27-29*).

1.2.1 Neurophysiology of Pain and OA Pain

Pain is transduced via pain-sensing neurons in the periphery – nociceptors. Nociceptors come in many varieties, marked by the distinct expression of certain populations of channel proteins or canonical signalling proteins. The major division of nociceptors distinguishes between peptidergic and non-peptidergic subsets. At the embryonic stage of development, neural crest cells destined to be nociceptors express the NGF receptor TrkA (**Figure 1-2a**)(*30*). Following sensory neurogenesis, differentiation occurs among these cells, where a subpopulation silences their TrkA expression and expresses Ret, a receptor tyrosine kinase that allows for the expression of glial cell-derived growth factor (GDNF)(*30*). These GDNF-expressing cells compose the non-peptidergic population of nociceptors, whereas the population of nociceptors that continue expressing TrkA compose the peptidergic nociceptor population. Further along development, both populations express distinct sets of proteins that continue to differentiate the groups. Peptidergic nociceptors express calcitonin gene-related protein (CGRP) and TRPV1 (*30*). Non-peptidergic nociceptors specifically bind isolectin B4 (IB4), an extract from the plant *Griffonia simplicifolia*, and the Mrg-coupled receptor D (MrgprD) (*30*).

In terms of pain perception, nociceptors span a large breath of modalities. Thermal nociceptors are activated by noxious heat or cold at various temperatures. The classical channels in these thermal nociceptors responsible for the transduction of these stimuli are TRPV1 (for noxious heat) and TRPM8 (for noxious cold) (*31*). Chemosensitive nociceptors detect noxious chemicals, with the typical receptors being P2XRs, TRPA1, and TRPV1 (via capsaicin activation) (*31, 32*). Mechanosensitive nociceptors detect noxious mechanical stimuli with typical receptors being TREK1, TREK2, TRAAK, TRPA1, and other novel receptors still under investigation (*31*). Importantly, these modalities can overlap even within individual nociceptors, such that many different types of stimuli may activate a single nociceptor – such nociceptors are considered polymodal (*31*).

These nociceptors, with their soma in the DRG, have central terminals which make contact in the superficial dorsal horn of the spinal cord, projecting mainly in lamina I, II, and III. From there, pain projection neurons will travel along the anterolateral pathway and terminate at the ventral posterior nucleus (VPN) of the thalamus. Relevant VPN projections reach multiple cortical targets that process the various components of the pain signal (*10*). These cortical areas include the primary somatosensory cortex, secondary somatosensory cortex, parietal operculum, insula, anterior cingulate cortex, prefrontal cortex and amygdala (*33*). Of equal importance in the sensation of pain are the descending pain modulatory pathways. These are composed of the peri-aqueductal grey and rostral ventromedial medulla, which release noradrenaline and serotonin onto the aforementioned spinal circuitry and modulates the sensory experience of pain at the spinal level (*34*). These descending projections can be either excitatory or inhibitory, resulting in the amplification or attenuation of the pain response.

OA pain originates from affected synovial joints and primarily knee joints (*5*). These joints are densely innervated by nociceptors and their nerve terminals end in the capsule, ligaments, menisci, periosteum and subchondral bone – essentially, every major tissue aside from the articular cartilage (*35-37*). Contemporary hypotheses suggest that the shear forces on the free nerve terminals of these nociceptors causes the opening of mechanically-gated ion channels which signal pain (*10*). In the context of OA pain, synovial inflammation, cartilage degradation and central sensitization contribute to the errant pain signalling.

The characteristic synovitis of OA is the primary contributor to the inflammatory component of OA pain. The progression of OA in a patient is assessed via change increase in synovium mass and decrease in joint space(*9*). Synovitis results in the thickening of the synovial membrane and this allows for the entry of leukocytes into the synovium interior; many of the symptoms and structural changes in OA can be directly attributable to synovitis (*38*). Furthermore, during injury and inflammation, the capsule, containing synovial fluid, becomes increasingly permeable to inflammatory cells and plasma proteins, subsequently leaking into the intra-articular space through the synovial vasculature (*39*). As seen in rheumatoid arthritis (RA), this can result in an overall increase in fluid volume in the joint (oedema), and significantly increased intra-articular pressure, which cause burst firing of articular afferents (*10*). Of note, the frequencies of this firing are proportional to the pressure (*10*). Whether or not there is similar increase in intra-articular pressure is unclear, although primitive examples of immobilization induced OA in rabbits suggest that this may be the case (*40*).

Degradation of intra-articular cartilage contributes to the inflammatory response present in OA. Synoviocytes, responsible for feeding chondrocytes and the removal of metabolic products of matrix degradation are over-activated in the OA setting. Their activity,

paradoxically, results in the production and release of further catabolic and proinflammatory mediators (*38*). The result is a positive feedback loop, further compounded by the presence of leukocytes that amplify the signal generated from the inflammatory cytokines. This prolonged exposure to pro-inflammatory mediators will eventually lead to central sensitization.

Central sensitization (CS) is well documented in human OA patients (*41-44*). According to Woolf (2011), CS is "operationally defined as an amplification of neural signalling within the CNS that elicits pain hypersensitivity" (*45*). Specifically, the presence of dynamic tactile allodynia, secondary punctuate/pressure hyperalgesia, temporal summation and sensory after-effects all indicate potential CS in an OA patient. Mechanistically, CS can manifest as a loss of descending pain inhibitory mechanisms and increase of temporal and spatial summation at the spinal level (*46, 47*). The positive effects of centrally acting drugs (*48*), use of neuropathic pain descriptors (*43, 44*), and results from many functional brain neuroimaging studies (*42, 49*) support the presence of CS in a large subpopulation of patients suffering from OA.

The effect of these physiological changes on joint-innervating nociceptors is allodynia and hyperalgesia. The pro-inflammatory cytokines released by immunocytes, synoviocytes and vascular endothelium contribute to an "inflammatory soup" that increasingly sensitizes the neighbouring nerve terminals (*10*). Experiments done *in vitro* and *in vivo* confirm the notion that exposure to these pro-inflammatory cytokines results in elevated nociceptors sensitivity (*50-53*). One explanation for the increase in the sensitivity of nociceptors may the activation of silent nociceptors. In normal conditions, there exists a population of nociceptors that do not seem to respond to any mode of pain stimuli, termed silent nociceptors. Under pathological conditions such as OA, it is possible that they may become active and contribute to a larger pain response (*54*). Another possibility is that there is increased innervation and density of innervation to the

knee joint. Indeed, it has been shown that in mild and severe cases of human knee OA, there is increased innervation and irrigation of the knee joint and invasion of the nerve terminals into the remaining articular cartilage (*7*). This is also sensible in light of the fact that only peptidergic fibres innervate the knee joint (*55*). Peptidergic nociceptors express TrkA, an NGF receptor. Among the proinflammatory cytokines which are highly elevated in OA is NGF, and its primary action is as a chemo-attractant for neurons. The high levels of NGF would thus help to explain the increased density and area of innervation by peptidergic nociceptors in the knee joint. Despite these favourable theories, the full context of nociception in OA has yet to be elucidated.

1.2.2 Assessment of OA pain

OA pain manifests as two distinct types: "one that was intermittent but generally severe or intense, and another that was a persistent background pain or aching" (*2*). The intensity of this pain varies from patient to patient, and within a single patient there are daily variations in the report of their pain intensities as well (*2*). One in three patients report the pain as either burning, tingling, or a "pins and needles" quality (*43*). Such descriptors often lead to an assessment of a neuropathic pain component in OA, although specific nerve lesions have not been identified in the disease (*2*).

With such a diversity of pain descriptions in the disease, capturing all aspects of the pain experience via patient report is often unmet or tenuous. The standards for the evaluation of knee OA pain include the visual analog scale/numerical rating scale (VAS/NRS), the Western Ontario and McMaster Universities Arthritis Index (WOMAC) and the Knee injury and Osteoarthritis Outcome Score (KOOS) (*2, 56, 57*). In an attempt to better categorize the pain, both WOMAC and KOOS assess pain associated with specific activities. More recently, OARSI and

OMERACT have collaborated to create the Intermittent and Constant OA Pain (ICOAP) test. Improving and expanding on previous instruments, the test addresses "frequency of pain, intensity, effects on sleep and quality of life, degree of frustration and upset or worried feelings associated with the pain, … and whether the intermittent pain occurs without warning or with a trigger" (*58*). However, these efforts have yet to fully address the issues of OA pain in fatigue, sleep, and cognition (*2*).

1.2.3 Treatment of OA pain in the clinic

First-line treatment options for OA pain are non-steroidal anti-inflammatory drugs (NSAIDs) (**Figure 1-2a,b**). As recently reported, the effect size of many NSAIDs are relatively small and a large portion of OA patient receive no benefit at all as a result of its administration (*59*). In the same meta-analysis, it was reported that the numerous dissuading side-effects such as dyspepsia, nausea, vomiting, diarrhoea and rash result in many patients discontinuing NSAID usage, especially in light of its remarkably limited efficacy. As a consequence of this relatively weak first-line treatment, many new biological therapies are under investigation.

Serotonin–norepinephrine reuptake inhibitors (SNRIs) such as duloxetine have been shown to be efficacious in treating OA pain. A few studies have shown that duloxetine at 60 mg twice daily resulted in statistically significant pain relief when used as either a monotherapy or an adjunctive therapy to existing analgesics (titration to a higher dosage amount did not result in a significantly greater amount of analgesia) (*48, 60-62*). Side-effects of duloxetine treatment in OA pain mirror those present in patients administered duloxetine for major depressive disorder or general anxiety disorder; they included increase in blood pressure and heart rate, elevated liver function tests, nausea, dry mouth, fatigue, constipation, dizziness and increased sweating (*63*).

Duloxetine acts specifically to increase cerebral levels of serotonin, norepinephrine, and, to a lesser extent, dopamine. By selective blockade of the reuptake of these molecules in the CNS, it amplifies the molecules' roles in the CNS as modulators of descending pain pathways (**Figure 1-2c**) (*64*).

A bottom-up approach to treating OA pain which has shown promising success has been anti-NGF therapy. Clinical trials of antibodies to NGF (commercially known as tanezumab) showed mean analgesia between 45-62% compared to the 22% of placebo (*65-69*). Impressively, the only reported side-effect of tanezumab administration was paresthesia: a sensation of tingling, tickling, pricking, or burning of a person's skin with no apparent long-term physical effect (*65*). Targeting NGF is a particularly attractive option as it is highly elevated in OA joints and has been shown to critical to the induction and progression of many pain mechanisms present in OA (**Figure 1-2a**). NGF has been shown to increase peripheral hypersensitivity, potentiation of TRPV1, and upregulation of pro-nociceptive proteins such as mitogen protein kinase p38, substance P (SP), TRPV1, and Na_v 1.8 (*70-74*).

Finally, when all other treatments fail, traditional morphine and other opioid analgesics are used to treat OA pain (**Figure 1-2b,c**). Opioid analgesics are generally reserved for late-stage osteoarthritis sufferers who have incapacitating levels of pain. While effective at abolishing the pain, the associated side-effects are serious and often debilitating in themselves. These include constipation, nausea, dizziness, confusion and abuse potential (*75*). Alarmingly, chronic exposure to opioids can sometimes lead to opioid-induced hyperalgesia, where the individual experiences general nociceptive sensitization (*76*). This paradoxical hyperalgesia further complicates possible prescriptions for opioids in the treatment of OA pain.

1.2.4 Experimental Treatments for OA Pain

There are several avenues of investigation that are being explored in the treatment of OA pain. Among the more typical approaches involve the blocking of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in hopes of reaching a similar effect of anti-NGF therapy. There are mixed results from clinical and experimental trials with anti-TNF therapies. Reports of infliximab (an anti-TNF antibody) in a rabbit model of osteoarthritis demonstrates that injection of infliximab into affected joints slowed progression of the disease assessed via physiological changes in the concentrations of pro-inflammatory cytokines as well as reduced the morphological changes that accompany the disease (*77*). Early analysis of human patients injected with adalimumab (another type of anti-TNF antibody) demonstrated that anti-TNF may be useful in reducing symptoms of secondary OA in the hand, although anti-TNF did not show to significantly reduce the associated inflammation (*78*). However, it has been recently shown that adalimumab administration did not significantly alleviate pain in hand OA when compared to NSAIDs or placebo (*79*). Overall, more research is needed to dissect out the benefits of anti-TNF therapy in treating OA.

In an effort to generate more disease-modifying drugs to remedy the physiological symptoms of OA, injections of glucosamine and chondroitin, both naturally occurring constituents of articular cartilage, are being investigated for their potential benefits. A recent review of several studies has shown that injection of both components into affected joint increased joint function and reduced pain by 28% and 21%, respectively (*80, 81*). The exact mechanism of action of both glucosamine and chondroitin is unclear, although it is suggested that their simple presence may be enough for the depleted population of chondrocytes to help in the rebuilding of the damaged cartilage matrix (*81*).

Similarly, hyaluronic acid – a large molecular weight glycosaminoglycan that is present in the synovial fluid of both normal and OA joints – is being investigated for its potential restorative properties. The acid is meant to serve as a viscous supplement to the synovial fluid to ease movement of the joint and reduce the pain involved with joint use. Results indicate that while hyaluronic acid produces significant benefit compared to placebo, its effect size is comparable with NSAIDs and therefore do not supplant any of the traditional pathways for OA treatment, but may be considered as an adjunctive therapy (*82*).

1.3 Critical Proteins involved in OA Pain

1.3.1 Pain transmission and voltage gated cation channels

Much work has been done to show that within the population of nociceptors there exists an exclusive set of voltage-gated cation channels which propagate the pain signal. Through this determination, the therapeutic approach would be to silence specifically voltage gated cation channels in nociceptor to reduce pain. In models of chronic pain and inflammatory pain, it has been shown within a specific subpopulation of DRG neurons (those that innervate the affected joint, delineated via a retrograde tracer) that the expressions of Na_v 1.7, Na_v 1.8, and Na_v 1.9 are increased (*83*). Narrowing down, it was shown that Na_v 1.8 was responsible for the transduction of mechanical pain in a rodent model of OA (*84*). Blocking the activity of Na_v 1.8 significantly reduces the pain generated in models of neuropathic and inflammatory pain (*85*). Overall, the blocking of Na_v 1.8 may prove to be beneficial in the treatment of OA pain while not necessarily being specific to the disease.

Other voltage-gated cation channels under investigation include N-type, P-type and T-type voltage gated calcium channels (VGCCs). The role of high-threshold calcium channels in the transduction of inflammatory pain has been confirmed in models of knee inflammation (*86*). Of particular note, mice lacking the N-type VGCC show suppression of both neuropathic and inflammatory pain responses (*87*). Drugs that target these types of VGCCs, such as gabapentin and ziconotide, have shown significant benefit in reducing levels of chronic pain, although their efficacy in OA pain has yet to be determined (*88*).

1.3.2 Heat Pain: TRPV1

The importance of TRPV1 in sensing noxious heat and capsaicin has been well established in the literature. TRPV1 plays a pivotal role in nociception and thus is an established target for pain therapy (*89, 90*). TRPV1 has an expression profile that is almost exclusively limited to nociceptors and demonstrates a couple key characteristics: Its threshold for noxious heat is >43ºC (considered painful heat in humans) and it is ligand-gated – allowing passage of primarily calcium ions following the binding of capsaicin (*53*). Evidence for these characteristics come from classical experiments showing that TRPV1 knockout mice display attenuated response to noxious heat, which also indicates its contribution but non-necessity in noxious heat-sensing (*91*). Capsaicin was first used to identify TRPV1 as a channel and still remains as its most potent agonist (*92*).

Modulation of TRPV1 activity can occur through many pathways. Firstly, it is important to note that TRPV1 may also be activated in low pH conditions (less than 5.9), similar to the environment generated in inflamed tissues (*93*). Furthermore, inflammatory mediators such as arachidonic acid metabolites (e.g. 12-hydroperoxyeicosaenoic acid (12-HPETE)) and endocannabinoids (anandamide and N-Arachidonoyl dopamine (NADA)) can sensitize or de-

sensitize TRPV1 (*93*). More indirectly, the activity of bradykinin, prostaglandin E2, extracellular ATP, and NGF all can culminate in the sensitization of TRPV1 (*93*).

The contribution of TRPV1 to OA is beginning to be uncovered. In adjuvant-induced models of chronic arthritis, it was shown that TRPV1 is involved in the development of mechanical hyperalgesia (*94*). Using a chemical model of OA (intra-articular injection of MIA, which inhibits GAPDH and glycolysis resulting in chondrocyte cell death) in rats, it was shown that intra-articular blockade of TRPV1 via a pharmacological antagonist abolished OA-related sensitization and restored proper weight-bearing (animals with knee pain put less weight on the affected knee, causing an increase in the weight put on the healthy knee) (*95*). Previously, oral administration of TRPV1 antagonist resulted in on-target-induced hyperthermia in both humans and animal models, which halted clinical trials (*96*). However, a recent animal study showed that intra-articular injection of a different TRPV1 antagonist (JNJ-17203212) did not affect body temperature, suggesting a new potential therapeutic benefit for anti-TRPV1 therapy (*95*). Moreover, it has been shown that genetic variants of the gene encoding TRPV1 in humans increase the risk of patients developing knee osteoarthritis (*97*). All these results further enforces the notion of TRPV1 being a key player in the onset and progression of OA pain.

1.3.3 Chemical Pain: P2X Receptors

Ligand-gated purinergic receptors are non-selective cation channels, activated via binding of ATP. In the context of pain, it has been shown that extracellular ATP can act as a pain mediator in certain tissues (*53*). Specifically in models of rheumatoid and osteoarthritis, it is suggested that P2X4 expression in synovial fibroblasts can lead to the induction and expression of brain-derived neurotrophic factor (BDNF), a neuromodulator involved in nociceptive hypersensitivity (*98*). Moreover, ERK-positive neurons – the phosphorylation of which leads to

the sensitization of nociceptive neurons both in the DRG and the spinal cord - co-express P2X3 and, in an arthritis model (complete Freund's adjuvant), this percentage of co-expression significantly increased (*99*). In the same study, blockade of P2X3 via TNP-ATP – a selective antagonist of the $P2X_1$, $P2X_2$, and $P2X_{2/3}$ subunits – suppressed pain behaviours as well as the induction of phosphorylated ERK positive DRG neurons.

1.3.4 Mechanical Pain: Piezo2 and other novel proteins

Despite ongoing investigation, the identity of the MSC(s) that underlies nociception in various pathologies and models has yet to be determined. The discovery of Piezo1 and Piezo2 as distinct components of mechanically gated cation channels sparked interest in their possible roles in pain and chronic pain illnesses (*100*). One breakthrough was the identification of Dmpiezo (the single Piezo member in *Drosophila melanogaster* (fruit fly)) as being required for mechanical nociception (*101*). Knockdown of Dmpiezo in degenerin/epithelial sodium channel (DEG/ENaC) expressing mechanically responsive nociceptors was sufficient to impair responses to noxious mechanical stimuli.

As for Piezo2 and chronic pain, only indirect connections have been made. Via bradykinin activation, it has been shown that Piezo2 current is increased and its inactivation is slowed in a class of rapidly-adapting mechanically activated neurons of the DRG (*102*). Therefore, the presence of one type of inflammatory signal is sufficient in increasing mechanically-evoked current via Piezo2 in a subpopulation of Piezo2 expressing neurons. The relevance of this increase in more physiologically relevant disease models and whether this

population of neurons participates in the pain signalling of those disease models has yet to be determined.

Early attempts of categorizing mechanically-gated responses of DRG neurons have helped to outline potential channels that may play important roles in nociception and chronic pain disease. Two classes of mechanically-evoked responses were discovered in cultured DRG cells: one responding at high thresholds (HT) and another at low thresholds (LT) of mechanical stimulation (*103*). Importantly, this study also showed that the HT responses generally corresponded with cells of smaller soma diameter – a general indicator of nociceptive neurons. A follow-up study demonstrated that these high-threshold MSCs are linear non-specific cation channels that have an approximate conductance of 14pS (*104*). Overall, these studies have helped to outline the potential profile of the MSC(s) that may underlie regular nociception as well as pathological nociception.

1.4 Inflammation and Osteoarthritis

1.4.1 Inflammatory profile of OA

The great confusion of OA progression is the causal relationships of its defining inflammatory characteristics. Is the degradation of the cartilaginous articular matrix a result of excess signalling via the hypertrophic synovium and invading immune cells? Or is the synovial membrane inflammation the result of cartilage degradation and subchondral bone lesions, which releases abnormal levels of inflammatory metabolites? Perhaps there are mechanisms yet to be discovered that precipitate the aforementioned events? These are open questions that have yet to be sufficiently addressed.

As aforementioned, pre-diagnostic (symptomatic OA that has yet to be diagnosed) OA is marked by inflammation of the synovial membrane – synovitis. There is hyperplasia of the cell layer that comprises the inner lining of the synovial membrane which is accompanied by the focal infiltration of lymphocytes and monocytes (*105*). Inflammatory cells such as macrophages directly and indirectly promote the vascularization of the inflamed synovial membrane. Macrophages themselves can secrete pro-angiogenic factors, but also stimulate endothelial cells and fibroblasts, to produce vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and other molecules that further enhance angiogenesis (*106*). The permeability of the blood vessel and up-regulation of adhesion molecules concomitant with angiogenesis perpetuates the inflammatory response (*107*). Thus synovitis and angiogenesis may persist through all stages of osteoarthritis.

Cartilage degradation is a classical symptom of OA, although the mechanisms underlying its initiation are unknown. There is breakdown of collagen and proteoglycans, which hyper-activate resident macrophages and synovial macrophages, resulting in the release of pro-inflammatory cytokines (*105*). It is theorized that, at the onset of OA, there is an increase in both anabolic and catabolic activity in chondrocytes. The initial compensatory mechanisms, being increased synthesis of matrix molecules and proliferation of deeper layer chondrocytes, are overwhelmed by the precipitating loss of cartilage and changes in the chemical environment surrounding the cartilage (*105*). This culminates in the apoptosis of chondrocytes. Becoming more pronounced over time, there is fibrillation of the superficial cartilage layers, fissuring and diminished cartilage thickness. Eventually all cartilage is lost and the subchondral bone plate is left completely exposed (*105*).

Changes to the subchondral bone seem to closely mirror the deleterious progression of the articular cartilage. Both cartilage oligomeric matrix protein (COMP) and bone sialoprotein (BSP) increase in parallel in people with early osteoarthritis (*105*). The main events occurring within subchondral bone are bone marrow lesions, progressive increase in subchondral bone plate thickness, and the formation of new bone at the joint margins – named osteophytes (*108*). Moreover, late stage OA can be characterized by significant aseptic bone necrosis and bone cysts as a result of the inflammatory synovial fluid having direct access to the bone marrow (*105*).

The meniscus is not spared from OA progression. Being composed of cartilaginous materials very much similar to the articular cartilage, menisci undergo tearing, fissuring, fragmentation, maceration or complete destruction (*105*). Over time, there appears to be a gradual reduction in the Type I collagen content and increase in the proteoglycan content. Peri-meniscal synovitis and calcification to the outer peripheral portions of the meniscus results in the reduced tensile strength of the meniscus (*105*). Consequently, the meniscus is less able to withstand loading of weight during normal movement and results in the aforementioned physical events.

1.4.2 Positive feedback: Pro-inflammatory cytokines

Invading immune cells, resident macrophages and synovial macrophages all contribute to the growing concentrations of pro-inflammatory cytokines present in OA. Factors such as TNF-α, IL-1β, IL-6 are highly upregulated in OA joints and thought to mediate the progression and pain associated with the disease (**Figure 1-1b**) (*38*). In patients with knee OA, radiographic scoring of the OA knee and pain-related scores have been associated with the levels of pro-inflammatory cytokines present in the synovial fluid (*109*).

The defining characteristic of all major pro-inflammatory cytokines associated with OA is that they all feedback to stimulate more production of pro-inflammatory cytokines. TNF-α is elevated in the synovial membrane and fluid, the subchondral bone, and the articular cartilage (*51*). It acts to suppress the synthesis of proteoglycans, link proteins and type II collagen in chondrocytes, while stimulating the release of matrix metalloproteases (MMPs, which breakdown the cartilage matrix) and Il-6 and Il-8 (*51*). Il-1β and Il-6 both perform similar activities in suppression of cartilage synthesis pathways, while promoting expression of MMPs and themselves.

1.4.3 Mechanical allodynia and hyperalgesia

Mechanical hypersensitivity has been well documented in reports of human OA. Mechanically evoked pain is a hallmark of OA, especially for those patients that suffer from triggering pain (*2*). The pain progresses from limited mobility of the joints to the eventual total immobility of the affected joints. This mirrors the progressive change in gait of OA sufferers as they near disability. Moreover, secondary allodynia is also present in many cases of OA, where distal joints become hypersensitive as well, despite not showing any physiological symptoms of OA (*2*).

Through animal models, many of the characteristics of mechanical hypersensitivity have been recapitulated. In total, animal models have been able to reproduce the particular mechanical allodynia and hyperalgesia, altered gait, diminished grip force, spontaneous pain, and secondary allodynia present in OA reports (*110*). Through the study of these animal models, new insights can be made on the underlying mechanisms of these pain events. Of particular note are the

mechanosensitive ion channels that transduce the mechanical pain signal, and the potential modulation of those channels via the actin cytoskeleton.

1.4.3.1 Mechanosensitive ion channels

MSCs are present in nociceptors that innervate potential OA joints. While the identity of the MSCs functioning in the OA context is unclear, it is the aim of this project to demonstrate that a significant portion of the mechanically evoked pain found in a model of OA is due to the direct sensitization of MSCs in OA joint innervating nociceptors. By blocking these MSCs with GsMTx4 – a specific blocker of MSCs – we aim to demonstrate that not only is the MSC activity reduced in OA nociceptors but also the mechanically evoked pain is attenuated. With the validation of the sensitization of MSCs in an OA context, it may be of therapeutic benefit to target MSCs for the treatment of OA pain.

1.4.3.2 Actin cytoskeleton and modulation of MSC sensitivity

Other groups have demonstrated the importance of the actin cytoskeleton in modulating the sensitivity of MSCs in other sensory contexts (*111-114*). Within the supraoptic nucleus (SON), osmoreceptors that express mechanosensitive ion channels were amenable to modulation via rearrangement of the actin cytoskeleton (*115*). Rigidification of the actin cytoskeleton using jasplakinolide, which promotes actin polymerization significantly increased the mechanosensitivity index of SON neurons and amplified the depolarizing and excitatory effects of hypertonic stimuli (*115*). Conversely, application of cytochalasin-D, a compound that depolymerizes actin filaments had the opposite effects in these neurons (*115*). Regarding mechanosensation in the DRG, it has been shown that treatment with cytochalasin D reduces all types of mechanically evoked current (*103*). This suggests that actin cytoskeletal elements in

general modulate the sensitivity of MSCs in DRG neurons. In total, the prevalence of actin cytoskeletal components modulating the sensitivity of MSCs warrants the investigation of its role in the possible hypersensitivity of MSCs in an OA setting.

1.5 Modelling Osteoarthritis

1.5.1 Validity of animal models of OA

While it is critical to have continual clinical reports on OA as it is presented in reality, animals models of OA provide a discerning power in the discovery of OA mechanisms. As described in Malfait et al., 2013,

> "Clinical studies provide important data on *association* between clinical symptoms (i.e. pain) and particular tissue pathologies, genetic differences (e.g. SNPs), psychological determinants, etc… Ascribing a *causal relationship* between a specific molecular, cellular or pathological event and OA pain, requires … modification of that factor with a measureable change in the onset, severity or progression of the pain. In the absence of such interventions … OA pain needs to be investigated in preclinical models where such factors … can be targeted" (*110*)

The array of models emulating OA pain faces a more rigorous standard that those simply used to emulate the structural correlates of OA progression (*116*). While each on its own has not yet been shown to encompass the full breadth of OA pain symptoms, together they form an amalgam that is amenable to a vast array of quantitative and qualitative assessment strategies. These include electrophysiology, von Frey hair algesiometry, evoked pain tests, vocalization recording, pressure application measurement, gait analysis, spontaneous pain behavioral tests, activity-based assessments and facial expression analysis (*110*).

1.5.2 Pharmacological Models of OA

Pharmacological methods of inducing OA-like symptoms and pain are preferred for their rather rapid induction phase (on average a few days to a couple weeks) and consistent pain sensitization (*110*). However, it is by the same coin that criticism is levelled on the method; the induction is too rapid to be considered physiological relevant to the disease as it is presented in humans.

Fortunately, compounds such as sodium monoiodoacetate (MIA), bacterial collagenase, and papain reliably reproduce both physiological and behavioral markers of OA progression. MIA is injected into the intra-articular space such that only the lining chondrocytes within the articular cartilage receive the active ingredient. MIA works by inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and, thus, glycolysis. This results in the death of the chondrocytes in the articular cartilage, precipitating the progressive cartilage matrix degradation and associated physiological symptoms of OA. MIA is the most widely used compound for the emulation of OA pain. It has been shown to display the progressive mechanical hypersensitivity in affected joints with changes in weight-bearing, gait, diminished grip force, altered sleep cycle, and spontaneous pain – all of which reflect symptoms reported by human OA patients (*117-120*). Moreover, histological sections of injected joints display cartilage degradation, meniscal damage, synovitis, and osteophyte formation (*119, 121*).

Less work has been done in outlining the effects of collagenase and papain induced osteoarthritis. What is known for certain is that both produce a comparable level of mechanical hypersensitivity (*122*). Collagenase injected knees do appear to display mild symptoms of OA progression (with the exception of bone lesions appearing 3 weeks after injection), although these symptoms varied based on the sex and strain of mice used (*123, 124*). With time, both compounds have seen less and less use with the growing compendium on MIA.

1.5.3 Surgical Models of OA

In terms of physiological relevance, surgical models arguably provide greater correspondence between clinical OA and the experimental animals. Surgical models of OA are the earliest models of experimental OA and continue to be widely used today (*125*). Importantly, the induction phase of most surgical OA models reflects much more the slow chronic degradation that is present in human reports of OA. Destabilization of the Medial Meniscus (DMM) and Anterior cruciate ligament transection (ACLT) both require a minimum of 8 weeks to fully plateau in pain and physiology phenotype, with some animal models requiring up to 3 months for the full severity of the model to be induced (*122*).

DMM models have been shown to generate mechanically allodynia rather early during the induction phase, which persists for 16 weeks; interestingly, thermal allodynia does not coincide with mechanical allodynia, requiring up to 8 weeks for presentation (*126*). There are also accompanying changes in gait and weight bearing and secondary allodynia (*110*). The histopathology of DMM shows that the physiological symptoms of DMM are relatively mild in comparison to chemical models of OA. Loss of superficial articular cartilage and fibrillation of remaining cartilage was observed, while there was no ectopic chondrogenesis or ectopic bone formation (i.e. osteophytes) (*127*).

Comparatively, ACLT appears to provide histopathological evidence of severe OA. Erosion of the growth plate, chondrogenesis and osteophyte formation were present along with the enhanced degradation of the articular cartilage which appeared to be more widespread and severe in the ACLT model (*127*). Mechanically allodynia and gait changes accompanied ACLT in experimental rat and dog models (*110*).

While surgical models seem to provide induction of OA along a more realistic timescale, these models fail to capture mechanisms responsible for the pathogenesis of degenerative OA. Since the OA is induced via traumatic intervention, it can be argued that this model still does not capture the circumstances of human OA as there are an enormous portion of idiopathic, or non-injury related cases of OA (*122*). Only by changing the genetics or the living conditions of the experimental models can we assess the mechanisms of OA pathogenesis.

1.5.4 Genetic Models of OA

Genetic models of OA can be explored in two ways. Mice which have knockouts of genes potentially critical in OA progression may undergo certain models of OA induction and show reduction of OA symptoms. Conversely, overexpression of pro-degradative proteins and inflammatory markers or knockout of protective genes may be assessed for their OA-like symptomology.

Loss of specific MMPs (MMP-13) has shown to attenuate articular cartilage erosion induced by surgical section of the meniscus (*128*). Generally, the role of MMPs is the cleavage of cartilage matrix molecules. MMP13 preferentially cleaves type II collagen, the most abundant cartilage matrix molecule in the articular cartilage and cartilage endplate of the intervertebral disc (*129*). Additional targets of MMP-13 cleavage include aggrecan, types IV and IX collagen, gelatin, osteonectin and perlecan – all cartilage components (*130*). Clinical reports of patients with OA associated articular cartilage destruction had high MMP-13 expression (*131*). As a corollary, overexpression of MMP-13 in transgenic mice produced spontaneous articular cartilage destruction via excessive type II collage cleavage and loss of aggrecan (*132*). Indeed, the presence of MMP-13 is critical in the cartilage destruction associated with OA progression.

Another particularly interesting family of proteins involved in OA progression is the disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family of proteins. Generally, ADAMTS proteins are peptidases, with each family member cleaving specific peptides present in the ECM. Within OA, ADAMTS-4 and ADAMTS-5 seem to play important roles as aggrecanases, breaking down aggrecan present in the meniscus and articular cartilage (*133*). Both proteins cleave the aggrecan core protein at the Glu373-ALA374 bond (*134*). In normal joints there is a finely controlled balance of tissue inhibitor of matrix metalloproteases (TIMP-3) and aggrecanases, but in the OA state there is a shift towards overexpression of both ADAMTS-4 and ADAMTS-5 (*133*). Single knockout of the *Adamts5* gene or double knockout of the *Adamts4* and *Adamts5* genes protect animals from cartilage degradation in both surgery-induced and pharmacologically-induced models of knee OA (*135-137*). Importantly, it has also been shown that ADAMTS-5 knockout mice do not develop mechanical allodynia following the induction of OA via DMM (*126*).

1.5.5 Other Models of OA and inflammatory pain

Naturally progressing models of OA and diet-induced OA represent the animal models that most closely match human degenerative OA in terms of pathophysiology. Several breeds of dogs can develop spontaneous OA at various ages depending on their genetic predisposition (*122*). Interestingly, *Cynomolgus* macaques seem to develop early OA lesions by age 10 and severe lesions prior to age 15 (*138, 139*). Importantly, it has been shown that these aged-related models of OA display similar histopathology with those from DMM models of OA pain, giving ground to both approaches as representative models of OA progression (*140*). Recently, it has been suggested that horses, with their high prevalence of OA development, may also be viewed as another viable animal model for the study of human OA. The equine carpal osteochondral

fragment model demonstrates the typical pain and histopathology of OA progression, which is reproducible (*141*). Moreover, obesity-associated OA has been studied in diet-induced mouse models of OA. In these mice, mechanical hypersensitivity accompanied with changes in locomotion and anxiety-like behaviors were observed (*142*). The load-bearing joints of these mice also underwent typical OA morphological changes and there was impaired musculoskeletal force generation and motor function.

Other chronic pain models such as kaolin, complete Freund's Adjuvant (CFA), and carrageenan have been used to characterize many aspects of acute and inflammatory pain, although their connection with OA is tenuous at best. Kaolin, used in the induction of monoarthritis, is hydrated aluminum silicate. It is used to inflict mechanical damage to the cartilage, emulating the destruction of articular cartilage found in OA (*143*). Lambda carrageenan type IV is a mixture of sulfated polysaccharides extracted from the red seaweed *Gigartina*. Intra-articular injection of either kaolin or carrageenan produced acute onset of inflammation within hours, followed by behavioural changes and neuroplasticity (*143*). Since the pain occurs so rapidly and the pathology of the knee differs from the typical path of OA, both compounds are most likely better suited for the study of inflammatory pain mechanisms in the periphery and CNS.

Lastly, CFA is composed of heat-killed *Mycobacterium tuberculosis* in paraffin oil. Since it is a suspension of previously potent bacteria, the inflammatory response is mainly an immune-mediated one. Arthritis produced via injection of CFA follows much more closely the progression of RA rather than OA (*144*). CFA induced arthritis is accompanied by lesions of the eyes, ears, nose, skin, and genitals, in addition to anorexia and weight loss (*144*) .

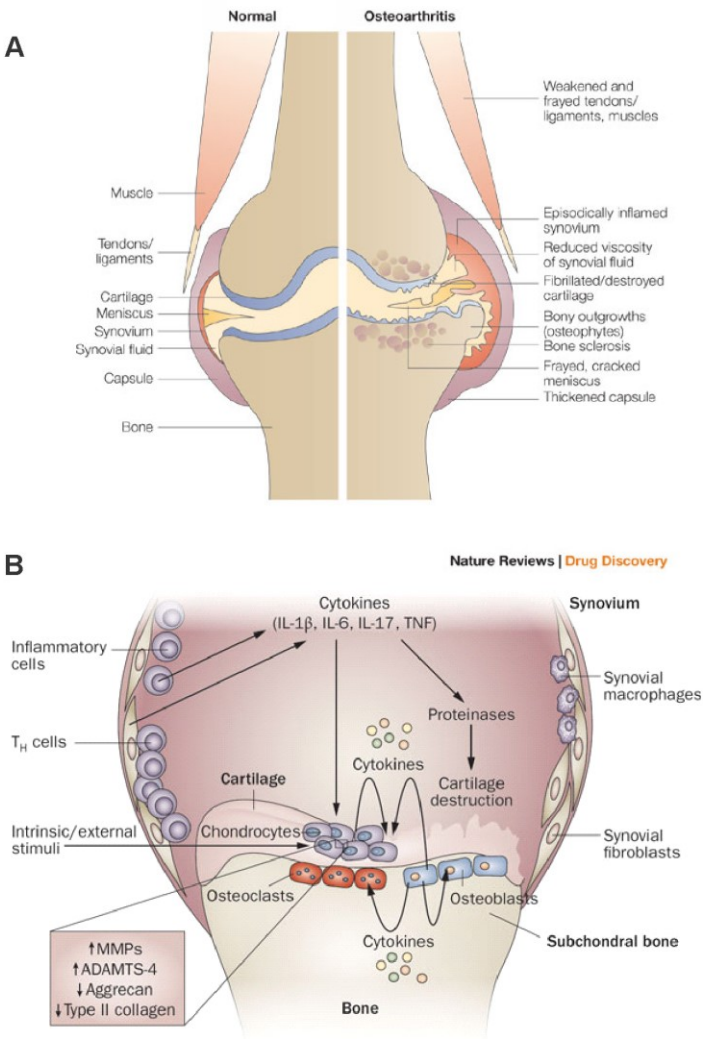

Fig 1-1. Pathophysiology of knee OA
A. Major structural and tissue changes in OA progression. This includes fibrillation and destruction of the articular cartilage, fraying and cracking of the meniscus, osteophyte formation and inflammation of the synovium.
B. Elevated levels of proinflammatory cytokines contribute to the progression of OA. TNF-α, Il-1ß, and Il-6 all contribute to the pathogenesis of OA through downregulation of anabolic events and upregulation of catabolic and inflammatory events. These events culminate in the structural and tissue damage highlighted in **(A)**. Abbreviations: IL - interleukin, TNF - tumor necrosis factor, MMP - matrix metalloproteinase, ADAMTS - a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs.

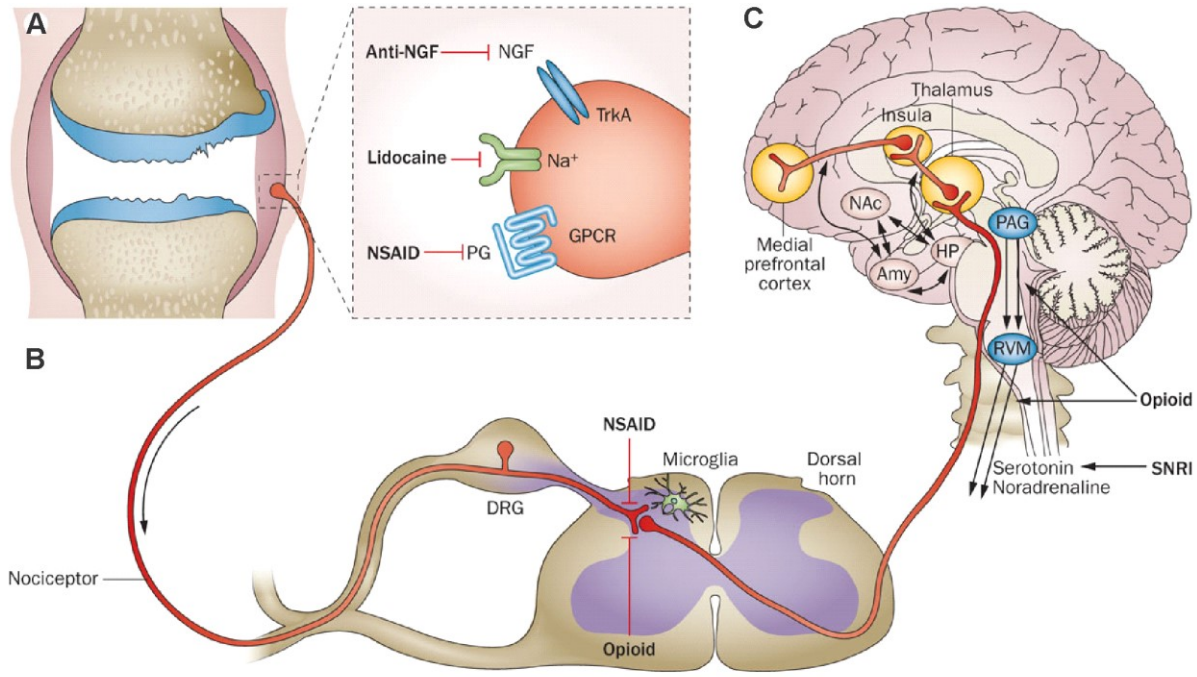

Fig 1-2. The pain pathway and various sites of action for different analgesics. **A.** Painful stimuli are detected in the periphery through open nerve endings. Several analgesics have been shown to target pain via receptors expressed at these nociceptor terminals. **B.** The projections from DRG nociceptors into the superfical dorsal horn terminate on either pain projection neurons (which are commissural) or inter-neurons (not shown). In OA progression, central sensitization can occur via strengthening of these synapses, loss of inhibitory mechanisms (not shown), and/or activation of microglia. NSAIDs and opioids can exert their action at the level of the dorsal horn. **C.** Pain projection neurons travel within the anterior column along what is known as the spinothalamic tract, ultimately terminating in the ventral posterior nucleus of the thalamus. From there, projections can go to several key cortical areas including primary somatosensory cortex, secondary somatosensory cortex, parietal operculum, insula, anterior cingulate cortex, prefrontal cortex and amygdala. Lastly, descending pain modulatory pathways release noradrenaline and serotonin to modulate the response of pain neurons at the spinal level. Abbreviations: Amy, amygdala; DRG, dorsal root ganglion; GPCR, G-protein-coupled receptor; HP, hippocampus; NAc, nucleus accumbens; NGF, nerve growth factor; PAG, peri-aqueductal grey; PG, prostaglandin; RVM, rostral ventromedial medulla; SNRI, serotonin–noradrenaline reuptake inhibitor.

CHAPTER 2: METHODS

2.1 Generation of transgenic animals

Transgenic mice were produced from C57/BL6 mice (Jackson Laboratories). A transgenic mouse containing the promoter of Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) followed by the gene encoding Cre recombinase (B6.129-*Trpv1*$^{tm1(cre)Bbm}$/J, Stock number: 017769) was crossed to another mice expressing nuclear or cytosolic Green Fluorescent Protein (nuGFP or cytoGFP, Rosa 26 locus) with a STOP cassette just upstream flanked by 2 loxP sites (B6.129-*Gt(ROSA)26Sor*^{tm1Joe}/J, Stock number: 008606). The resulting progeny were Trpv1::cre x nuGFP and Trpv1::cre x cytoGFP with Cre expression in all TRPV1 expressing cells and therefore fluorescing these cells in green. As previously shown, the expression of TRPV1 is restricted to primarily peptidergic nociceptors (*145*).

2.2 Monoiodoacetate induction of Osteoarthritis

Under brief, light isoflurane anaesthesia (3% isoflurane diffused in 100% oxygen for anaesthesia induction, 2% for maintenance), a 5µl intra-articular injection using a 10 µl Hamilton syringe was performed, with the needle passing behind the patellar ligament into the joint space of the left knee. Animals received varying concentrations of monoiodoacetate (MIA) dissolved in sterile physiological saline: 0 µg/µl (saline only), 5 µg/µl, 10 µg/µl, 20 µg/µl, and 50 µg/µl. In total, each MIA group receives either 0 µg, 25 µg, 50 µg, 100 µg, or 250 µg of MIA. Concentrations were based around previously established values in the literature of the MIA model that demonstrates both physiological and behavioral change (*110, 146*). Animals were randomly assigned to each injection group and both sexes were included in the control and experimental groups.

2.3 Co-labelling of knee-innervating nociceptors

Previous studies have demonstrated the presence of TRPV1 in nociceptors (*145, 147*). To identify the population of nociceptors that innervate the knee, we employed a 2-step approach to highlight this subpopulation. Firstly, it is important to note that only IB4- neurons innervate the knee joint in a rat model, which indicates that only TrkA+ neurons (which include peptidergic nociceptors) innervate the knee joint (*55*). One confounding factor is that TRPV1 expression is in nearly half of all neurons at the embryonic stage and only restricts down to the peptidergic C-fibers by adulthood. Due to this expression pattern of our Trpv1::cre x nuGFP and Trpv1::cre x cytoGFP mice, this would result in half of all DRG neurons being fluoresced in green; GFP expression is sensitive but not specific for knee innervating nociceptors (DJ Cavanaugh, 2011). To narrow down the population, we use a retrograde tracer, Fast DiI (Life Technologies) to help label neurons that innervate the knee. 7 – 10 days prior to recording, mice were injected in the knee joint both ipsilateral and contralateral to MIA injection with 5µl of DiI using a 10µl Hamilton syringe, according to the same protocol as the MIA injection. This retrograde tracer will invade all knee innervating neurons and label the soma of these cells in the dorsal root ganglion (DRG). This, along with the aforementioned fact that only peptidergic nociceptors innervate the knee joint, allows us to assert that co-labelled DRG neurons (GFP and DiI) will demarcate all peptidergic C-fibers that innervate the knee.

2.4 Behavioral Testing

Movement-induced behavior responses were evaluated by the Knee-Bend test (*121*). Both saline and MIA-injected (at 25 µg, 50 µg, 100 µg, and 250 µg MIA to the left knee) were tested both in the ipsilateral knee receiving the injection, as well as the contralateral knee as a

measure of the normal level of responsiveness to the test. Testing was done on days 0 (prior to MIA injection), 1, 2, 5, 7, 14, 21 and 28 post injection. The experimenter was blind to the condition of each mouse undergoing the test. The Knee-bend test consists of 5 alternating flexion and extension repetitions to the knee of the animal. Each flexion and extension is done up to the physiological limits of the knee flexion/extension. The response of the mouse to each flexion and extension was scored as follows: 0 – no response to any kind of movement of the joint; 0.5 – struggle/resistance to maximal flexion/extension; 1 – struggle/resistance to moderate flexion/extension (any range of knee motion below maximal flexion or extension) or vocalizations to maximal flexion/extension; 2 –vocalizations to moderate flexion/extensions. A maximal extension corresponds to the full 180º extension of the knee joint, while a maximal flexion corresponds to the physical limit of flexion of the knee (approximately a 30º angle bend). The sum of these responses has a maximum value of 20 and this represents the animals Knee-bend score, an indication of their movement-induced pain. The contralateral knee was always tested first, in order to avoid sensitization of the responses of the mouse from manipulating the injected knee. Results for ipsilateral knees are presented, contralateral knees did not have significantly different values at any time point for all groups (data not shown).

2.5 Histological Sections

MIA injected (0 µg, 100 µg, 250 µg in 5 µl saline) mice were perfused and knees were harvested and cleaned of excess muscle and skin tissue before allowing them to soak in decalcifying solution (10% EDTA, pH 7.2) for 3 weeks. Knees were then paraffin-embedded and mid-Coronal sections (8 µm in thickness) of the ipsilateral and contralateral knee (relative to MIA injection) were stained and observed for histopathology of MIA-induced joint degradation. Mid-coronal sections were selected based on the availability of observable articular cartilage

along the tibial and femoral condyles as well as presence of meniscus and synovial membrane. A standard Hematoxylin and eosin stain was performed to identify major morphological components of the knee as well as highlighting nuclei of cells. Toluidine blue stains were performed on the sections following standard protocols, after deparaffinising the slides. Toluidine blue stains acidic tissue components, therefore highlighting nuclei acids as well as polysaccharides, which are richly abundant in normal articular cartilage and menisci.

2.6 Acute dorsal root ganglia dissociation

Approximately 1 month post MIA injection and 7-10 days post DiI injection; the dorsal root ganglia (DRGs) from transgenic mice were dissected and prepared for electrophysiology. From our own preliminary studies and corroborated by finding from other groups, the lumbar 3rd and 4th DRG (L3 and L4) are removed from mice, as these contain the majority of knee innervating nociceptors (*148*). The ventral and dorsal roots are trimmed along with the exiting peripheral nerve, leaving only the DRG itself. A few small cuts are made to the DRG sac to allow entry of digestive enzymes.

Ipsilateral and contralateral DRGs (relative to MIA injection) are separated into two 1ml Eppendorf tubes. The DRGs are initially stored in Hank's Balanced Salt Solution (HBSS, Wisent), but following trimming of roots and peripheral nerve, they are transferred to a solution of 0.25% trypsin in EDTA and incubated on a rotator for 20 minutes at 37ºC. DRGs are then transferred into a solution of collagenase (2 mg/ml) and dispase II (5 mg/ml) in HBSS and incubated on a rotator for 30-40 minutes at 37 ºC, until DRGs are well-digested and there is only a small portion of the sac still intact in solution. The solution is spun down on a centrifuge for 2 minutes at 800 rpm.

The enzymatic solution is removed and 200 µl of DRG culture media is added. The DRG media follows previous standards and consists of DMEM/Hams-F12 medium (Life Technologies, Gaithersburg, MD, USA) containing 10% heat-inactivated horse serum (Biochrome), 20 mM glutamine, 0.8% glucose, 100 U penicillin and 100 mg ml^{-1} streptomycin (Life Technologies) (*149*). DRG neurons are mechanically separated by trituration through fire-polished Pasteur pipettes. The suspension is spot-plated on poly-L-lysine (100 µg/ml) and laminin (20 µg/ml) coated 35 mm glass-bottom Fluorodishes (World Precision Instruments, Sarasota, FL, USA) or Ibi-treated 35 mm µ-dishes (Ibidi, Ingersoll, ON, CA) and incubated at 37ºC. 1 hour following plating, an additional 700 µl of DRG culture media is added to the dishes to provide sufficient nutrients to dishes while they incubate prior to recording.

2.7 Electrophysiology

All recordings (cell-attached, whole-cell, outside-out) were made on co-labelled knee-innervating nociceptors (GFP and DiI labelled). Electrophysiology was performed on neurons 2-6 hours after plating. Both whole-cell and Cell-attached recordings were made using 10 cm long glass pipettes (AM Systems, 8250, 1.5 mm OD, 0.86 ID) pulled by a Model P-97 flaming/brown micropipette puller (Sutter Instrument Co., Novato, CA, USA) into recording electrodes using a custom protocol. Whole-cell recordings were made with electrodes fire-polished to a resistance of 3–5 MΩ. Cell-attached recordings were made with electrodes fire-polished to a resistance of 1.5–2.4 MΩ.

Extracellular neuronal bath solution, pH 7.4 and 303 mOsm, consisted of (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, and Glucose 10. For whole-cell recordings, the recording electrode contained a solution of (in mM): K-Gluconate 123, KCl 10, $MgCl_2$ 1,

HEPES 10, EGTA 1, $CaCl_2$ 0.1, K_2ATP 1, Na_4ATp 0.2, Glucose 4 at pH 7.20 and 302 mOsm. For cell-attached recordings, the recording electrode was filled with the same solution as the extracellular neuronal bath.

Whole-cell and outside-out recordings were made using fire-polished glass electrodes (AM Systems, Glass Borosilicate, 1.5 mm OD, 0.86 ID) with a resistance of 3–5 MΩ. Electrodes were pulled using a custom protocol l. The extracellular solution consisted of NaCl 140 mM, $MgCl_2$ 1, $CaCl_2$ 2, KCl 4, glucose 4 and Hepes 10 (pH 7.4), and electrodes were filled with solution containing KCl 110, Na^+ 10, $MgCl_2$ 1, EGTA 1 and HEPES 10 mM (pH 7.3).

Observations were made on Olympus IX91 inverted microscope with a MultiClamp 700B (Axon CNS, Molecular Devices, Sunnyvale, CA, USA) as amplifier and Digidata 1440A (Molecular Devices) as digitizer. Membrane current and voltage were amplified and acquired via the MultiClamp and Digidata and were sampled at 10 kHz; signals were recorded with Clampex 10 and MultiClamp 700B software and analysed with ClampFit 10. Pipette and membrane capacitance were compensated for with the auto function of the Clampex 10. A micromanipulator and electrode manifold (MP-225, Sutter Instruments) was used to bring loaded electrodes near the membrane of the cell. The electrode manifold was also connected to a high-speed pressure clamp and Pressure-Vacuum pump (HSPC, ALA Scientific) for pressure-pulse recording protocols, such that pressure stimuli were fed directly through the recording electrode. Mechanical stimulations were programmed by Clampex 10, and performed by the HSPC, with less that 10ms rise time from no applied pressure to intended magnitude of pressure. To take images of cell soma, a CCD camera (QImaging QI Click) was connected to the microscope and taken at 10x objective magnification. Images were saved and analyzed with Olympus MetaMorph Advanced. The threshold amplitude for taking an opening event was set at 50%. The

minimum duration of open events was set at 100 μs, in concordance with previous set standards (*103*). For the majority of cell-attached recordings, membrane voltage was held at -80 mV to generate more discernable mechanically evoked channel openings. Each patch was subject to 10 sweeps of incrementally increasing negative pressure from 0 mm Hg to -100 mm Hg, at 10 mm Hg increments. Each sweep is 3 seconds, with the onset of the pressure command at 0.6 seconds and the offset at 1.6 s. Sweeps in which the cell membrane is ruptured or disturbed – as seen through the recording – during the exposure to negative pressure are not analysed. For the establishment of an IV curve of recorded MSCs, cells were held at vary voltages and then subject to the threshold pressure stimulus of that particular cell to elicit single channel openings. Membrane voltages were set from -120 mV to -40 mV, at intervals of 20 mV (command voltages were from -65 mV to +15 mV, based on the average resting membrane potential assessed in Figure 2). Higher holding voltages resulted in non-specific channel activity as well as destabilization of the patch seal and were excluded from the analysis. Pipette solution remained as external bath solution, for both regular cell-attached recordings as well as those intended for IV curve analysis.

Whole-cell recordings were analyzed to generate the resting membrane potential of the cell. Procedurally, a GΩ seal is created with the electrode and, manually, a quick pulse of negative pressure is applied to the patch to rupture the membrane, making the contents of the cell continuous with the pipette solution. Fast and slow capacitance values were compensated for with the auto functions present in Clampex 10. 15 second recordings in current clamp mode, with an injection of 0pA of current was analyzed and averaged to assess the resting membrane potential of the cell.

For Outside-Out recordings, a GΩ seal was first formed on a knee innervating nociceptor. Manually, a quick pulse of negative pressure is given to rupture the patched membrane and the electrode is then carefully lifted from the cell adhered onto the Fluorodish. In current clamp mode with no applied current, gap-free recordings were done to ensure that the portions of membrane attached to the lifted electrode had reconfigured to form an outside-out patch of membrane (positive recordings displayed smooth traces that did not vary greatly in voltage nor have noise levels above ~1 pA). In voltage clamp mode, the membrane was held at -80 mV to match the parameters of the previous cell-attached recordings. Patches were subjected incrementally increasing levels of positive pressure until they reach a threshold opening (increments of 5 mm Hg at a time). It is important to note that this corresponding positive pressure threshold does not reflect the activation threshold of the cell that this patch of membrane belongs to, as the underlying intracellular structures and proteins have been disrupted as a result of achieving the outside-patch. Once threshold is reached, the patch is subject to that threshold pressure for 10 sweeps, with timings the same as that of the cell-attached recordings. A perfusion system was set-up in the dish so that a constant slow stream of external bath or 5 μM GsMTx4 dissolved in external bath is being directed towards the recording electrode, with the end of the perfusion line ~2-3 mm away from the electrode. An initial 10-sweep pressure pulse protocol is performed in the presence of external bath only. Following ~30 s of GsMTx4 perfusion (to allow for adequate GsMTx4 insertion into the membrane), another 10-sweep protocol is performed. Finally, 2 minutes after wash with external bath solution, a final 10-sweep protocol is performed on the same outside-out patch.

Analysis of mechanically evoked current in cell-attached and outside-out recordings was performed on ClampFit. Traces were baselined before setting cursors at the onset and offset of

pressure. Statistics were run on the region bounded by these cursors, where we extracted the average current as a result of pressure. Mean open time for outside-out recordings was performed on every opening validated by our selection protocol. Cursors were placed at the base of the onset and offset of channel opening at the difference in time (with resolution up to 100 μs) determined that particular instance of channel open time.

2.8 Conditioned place preference testing

Animals are injected with 100 μg MIA to induce OA of the left knee 1 month prior to behavioural recording. A custom made plexi-glass open-top, open-bottom 3-chamber housing unit (55.5x20.5x20cm), in concordance with previous mouse CPP models (*146*), was created to contain the mice during the recording of movement. The two middle walls of the chamber are reversible with a little hemi-spherical entrance on one end to allow for the passage of mice between chambers. The end-chambers (20.5x20.5x20cm) had contrasting walls that differentiate each chamber. One end-chamber has 2 completely white walls and 2 completely black walls, in alternating order. The other end-chamber had 4 walls displaying thick stripes of black and white. Before each recording session, new absorbent bench pad is placed underneath the end-chamber to reduce confounding scents left behind from mice during previous sessions. The middle chamber (14.5x20.5x20cm) has no absorbent bench pad underneath and a bright mini-flashlight illuminates the chamber, reducing latent time spent in the middle, white chamber.

Animals are habituated for 2 days before assessing baseline preference values. With the reversible walls allowing for passage of the mice, a recording of 15 minutes made on a JVC camcorder is taken from a top-down view of the chamber. Mice are scored for their time spent in each room, with the total of all 3 rooms equalling 15 minutes. Following

baseline, mice are removed from the experiment if their show a >10% preference difference for one end-chamber than the other. Notably, no mice were removed for these experiments.

The CPP protocol is a 3-day procedure. Day 1 involves baseline recordings as aforementioned. During day 2, mice are anaesthetized under light isoflurane and 10 µl of treatment solution (either 50 µM GsMTx4 in saline or saline alone as a control) is injected into the ipsilateral knee). The mice are then placed into one of the two end-chambers (one mouse at a time) with the reversible walls such that no passage between chambers can be made and the choice of end-chamber is counterbalanced for the mouse's initial biases. They awake and are free to explore the chamber for 30 minutes before being returned to their cages. 4 hours following the first injection, the procedure is repeated but with saline alone. The mice are placed in the opposite end-chamber for 30 minutes before being returned to their cages. Day 3 is a repeat of Day 1's procedure, with the mice allowed to freely roam all 3 chambers during a 15 minute recording.

2.9 Nervous tissue immunohistochemistry

DRGs and spinal cords are collected from perfused mice and placed in 4% PFA for 2 hours as post-fixation. Following that, the tissues are placed in 30% sucrose as cryoprotection and left for 3 – 14 days. Both DRG and spinal sections are made on a cryostat (Leica CM3050 S, Leica Biosystems, Concord, ON, CA) using optimal cutting temperature compound (TissueTek O.C.T. medium, VWR, Ville Mont-Royal, QC, CA) to hold the specimen in place. DRG sections are made at the thickness of 14 µm and spinal cord sections are made at the thickness of 50 µm. Spinal cords were pre-cut to contain the majority of the lumbar enlargement (projection site of L3-L5 DRG, which contain knee-innervating nociceptors).

For actin-stains, DRG sections were taken from Trpv1::cre x tdTomato mice. Therefore half of all DRG neurons should be fluoresced by the tdTomato protein. 1 week prior to dissection, both ipsilateral and contralateral knees were injected with Fluorogold (Fluorochrome, LLC) which is illuminated via wide-band UV light. Lastly, a standard actin stain protocol with Acti-Stain 488 Phalloidin (Cytoskeleton, Inc.) to fluoresce polymerized actin filaments. Images were taken at 60x oil-immersion objection on a confocal microscope (LSM 510 Meta Confocor2 Confocal Microscope) and analyzed with MetaMorph Advanced (Olympus).

For quantification of cFOS signal in the superficial dorsal horn, 100µg MIA injected mice (1 month progression) underwent knee flexion-extension under light isoflurane anaesthesia for 10 minutes. 90 minutes later, they are perfused and their spinal cords are sectioned as aforementioned. Spinal cords sections were placed in PBS before being mounted on SuperFrost Plus microscope slides (Fisher Scientific). A nickel-intensified diaminobenzidine (DAB) reaction against a biotin-conjugated anti-cFOS antibody was performed following established protocols (*150*). Images were taken through bright-field and dark-field objectives at 20x on a wide-field and fluorescence microscope (Zeiss Imager.Z1) via AxioVision and analysis was performed using ImageJ.

2.10 Dorsal root ganglion cell culture

Following the same protocol for 2.6 (Acute dorsal root ganglia dissociation), we plate triturated DRG cells onto ibi-treated 35 mm µ-dishes (Ibidi, Ingersoll, ON, CA) and fed with a custom mixture designed to nourish DRG cells (*149*). DRG media consisted of DMEM/Hams-F12 medium (Life Technologies, Gaithersburg, MD, USA) containing 10% heat-inactivated horse serum (Biochrom), 20 mm glutamine, 0.8% glucose, 100 U penicillin and 100 mg ml−1

streptomycin (Life Technologies). Cultures are placed in a 37ºC incubator kept at 5% CO_2 content and humidified with a water basin.

Experimental cultures had 50 ng/ml TNF-α mixed in with their culture media, corresponding to similar values used in previous reports on effects of TNF-α on the DRG (*50*). These dishes were cultured for 1 week, with the media changed every 2-3 days. It is important to note that these values are higher than those discovered in human OA patients and experimental models of OA. Levels of solubilized TNF-α in the synovial fluid taken from OA patients undergoing total knee arthroplasty hovered around 2.5 pg/ml (*151, 152*). Homogenized knees from rat models of OA demonstrated a peak value of 2.6 pg/mg of TNF-α protein (*109*). Despite the comparatively low concentrations of TNF-α in the synovial fluid of OA patients, recent reports indicate that TNF-α concentrations present in in the articular cartilage are much higher, on the order of several ng/g of cartilage tissue (*153*). Moreover, the concentration of TNF-α when normalized to single chondrocyte production in native OA tissue resulted in 4ng/mg of DNA (*153*). Therefore, though our experimental concentration of TNF-α may be high, it is within 1 order of magnitude of physiological TNF-α in OA. Due to brief nature of our culture, in comparison to the chronic nature of OA, this increase in concentration may be justified.

2.11 Statistical analysis

Results are represented as ± SEM. All results are first tested for equal variance and normality (F-test for equal variance. The D'Agostino and Pearson omnibus tests for normality was used when sample size for all groups exceeded 7, otherwise the Kolmogorov-Smirnov normality test was used). Following the passing or failing of these variance and normality tests, appropriate parametric or non-parametric tests were applied. For two populations, either paired or unpaired Student t-tests were used for parametric populations, and Wilcoxon rank sum test for

non-parametric data. For populations of three or more, One-way ANOVA was used for parametric data and Kruskal-Wallis was used for non-parametric data. For comparisons of groups along two axes, Two-way ANOVA was performed. Bonferroni post-hoc tests were used for comparisons among several means. Differences were considered significant for $p < 0.05$ (*), with $p < 0.01$ (**) and $p < 0.001$ (***) also being noted.

CHAPTER 3: RESULTS

The primary aim of this project was to assess one aspect of the pain associated the OA progression: mechanical allodynia. Though there are indeed reports of mechanical hyperalgesia, heat and cold hypersensitivity, and spontaneous pain (which will be addressed somewhat in the later portions of the results) in human OA patients or experimental OA models (*154-156*), the mechanisms underlying mechanical allodynia in OA are poorly understood.

Using a knee OA model (MIA injection), which is a well-documented and established model, we assess whether the mechanical allodynia present in this model is due to changes in MSCs that innervate the knee joint.

3.1 Animals

All animals were tested and euthanized between the ages of 2 months to 4 months old. Both male and female mice were used and well-distributed in both behavioural trials and electrophysiological recordings to ensure results indicate a robust response that is not specific to one sex. For initial knee flexion-extension trials, 41 animals were used to set the baseline results (**Figure 2-1**). For knee flexion-extension following injection of either 5% lidocaine, 50 μM GsMTx4 or their vehicles, 34 animals were split among the different groups. For all electrophysiological techniques, knee-innervating nociceptors were recorded from 69 animals which received either 0 μg, 100 μg or 250 μg MIA.

3.2 Validation of MIA model in mice

Assessment of primary mechanical allodynia in OA pain was done using the Knee Flexion and Extension protocol (see methods) previously established for the MIA model of OA

pain (*119, 121*). Each mouse was tested 3 days prior to MIA injection to assess a baseline response (Day 0, **Figure 2-1**). Following injections, behavioural scores are recorded on subsequent days and are plotted in **Figure 2-1**. By Day 5 onwards all MIA injection groups, except the 25 µg group display a significant increase in pain score (100 µg MIA and 250 µg MIA groups show significant increase in pain score by day 2, significance not shown). Our observations indicate that in this model, mechanical allodynia sets in rapidly and persists until the last day of testing (day 28, **Figure 2-1**), confirming other reports using this model (*157-161*).

To assess whether the mechanical allodynia correlates with joint degradation, mid-coronal sections of the ipsilateral and contralateral knees were stained with a general nuclear stain and also for proteoglycans and glycosaminoglycans, which are structural components of the articular cartilage (**Figure 2-2**). In MIA injected ipsilateral knees, hematoxylin and eosin (HE) stains display enlargement of the meniscus as well as some loss of the nuclear stain within the articular cartilage indicating loss of chondrocytes (**Figure 2-2**, arrows in HE stains). The contralateral knee displays a visible continuous band of articular cartilage, which is also present in the ipsilateral knee of saline-injected mice (**Figure 2-2**, "0 µg IPSI"). Importantly, MIA-injected knees (both 100 µg and 250 µg) show considerable loss of articular cartilage, as is shown in the lack of the deep purple stain surrounding the subchondral bone along the joint (**Figure 2-2**, brackets in Toluidine blue stains).

3.3 Resting Membrane Potential does not change during OA

It has been reported in models of inflammation that there is a decrease in the expression of background K^+ channels (*162*). The net effect of such a decrease would be a depolarization of the resting membrane potential, thus making it easier for receptor generated depolarisations to

reach the action potential threshold. To determine whether knee-innervating nociceptors have a similar depolarisation in resting membrane potential following induction of OA, we performed whole-cell patch clamp current clamp recordings. Our results show that there was no significant difference in resting membrane potential between control and OA nociceptors (**Figure 2-3**). Importantly, there were also no differences in ipsilateral and contralateral knee-innervating nociceptors, relative to the site of MIA injection. These findings suggest that the behavioural pathology we saw was not due to a general change in the resting membrane potential. We therefore examined the characteristics of the MSCs that are expressed in these nociceptors.

3.4 Activation Threshold is reduced in OA nociceptors

In a preliminary study, examining retrograde labelling of DRG neurons, we discovered that neurons from the L3 and L4 DRGs contained the highest proportion of knee innervating nociceptors. Thus neurons from the L3 and L4 DRGs were dissociated and we performed cell-attached recordings with a negative pressure-pulse protocol which exposed the patched portion of the membrane to increasing levels of negative pressure. The negative pressure would stretch the membrane. Though the sensory terminals were not present in our preparation, previous reports demonstrate that the mechanical stimulus response by cultured DRG neurons were identical whether induced at the periphery or at the cell soma (*149*). Representative traces show that OA nociceptors displayed MSCs which have a lower threshold to activation when compared to normal nociceptors (**Figure 2-4a**). MSCs from nociceptors of MIA-injected knees (both 100 μg and 250 μg MIA) displayed average activation thresholds (-35.22 mm Hg ± 2.55 and -32.89 mm Hg ±1.99) which are significantly lower than that of control (saline-injected, -65.38 mm Hg ± 2.6) and their respective contralateral knees (-62.54 mm Hg ± 2.31 and -64.48 mm Hg ± 2.39) (**Figure 2-4b**). Importantly, MSCs from contralateral knees of mice which received MIA did not

show significant change in their activation threshold when compared to control, nor were there any difference between the left and right knees of control mice. Given the reduced activation threshold specifically in OA nociceptors, we sought to analyze the altered mechanosensitive current profile within these neurons.

3.5 Mechanically evoked current is increased in OA nociceptors

Mechanically evoked currents from pressure pulse protocols were averaged to generate an average current profile for each injection group (**Figure 2-4d**). Both 100 μg and 250 μg MIA injected groups demonstrated mechanically evoked current significantly different from control as early as -10 mm Hg ($p<0.01$, **Figure 2-4e**). This is still consistent with the activation threshold values of MSCs from OA nociceptors (35.22 mm Hg ± 2.55 and 32.89 mm Hg ±1.99), as this is an average value including traces that demonstrate channel opening at weaker pressures. These increases in mechanically evoked current may be explained by an increase in the density of active MSCs present at the surface of knee-innervating nociceptors. We can assess this, in proxy, by looking that the frequency of active patches (recordings in which we see at least one mechanosensitive response at some non-zero pressure pulse) (**Figure 2-4c**). We see no change in frequency of active patches in both the naïve and OA conditions, although both are valued at a moderately high percentage of 60-70%. Interestingly, there is no significant difference in mechanically evoked current within MIA injected groups at any pressure point. Moreover, MSCs of OA nociceptors did not show significant difference in current amplitude for negative pressures greater than -60 mm Hg (data not shown), suggesting that mechanically evoked events that are suprathreshold of the naïve threshold for knee nociceptors do not undergo a similar sensitization.

3.6 *Single channel IV curves suggest the same MSC is present in OA and normal nociceptors*

To assess whether the change in activation threshold was due to the introduction of a novel channel or the modulation of existing MSCs, we performed our cell-attached pressure pulse protocols are various holding voltages (from -40 mV to -120 mV in 20 mV increments). Representative traces from the three experimental groups demonstrate similar single channel amplitudes at various holding voltages (**Figure 2-5a**, quantified in **2-5b**). Linear regression of the different groups generated the conductance values of the MSCs, which suggest that it is a non-selective cation channel (**Figure 2-5c**). During analysis, we did not note a significant presence of channel amplitudes that differed from the plotted current amplitude points. This suggested to us that there is likely to be only one type of MSC present and active in our recordings. Moreover, the values do not differ between MIA and control groups indicating that the same channel is present both in normal and OA conditions in knee innervating nociceptors. Therefore, this suggests that the sensitization present in our MIA model is not due to the introduction of a novel MSC. We now ask whether this MSC can be a target for pharmacological blockade.

3.7 MSCs in OA nociceptors are GsMTx4-sensitive

Previous studies have shown that MSCs in the hypothalamus and epithelial cells are sensitive to GsMTx4, a peptide extracted from *Grammostola rosea* (Chilean tarantula) toxin (*163, 164*). To assess its effectiveness on MSCs in DRG nociceptors, we performed outside-out patches on knee innervating nociceptors of OA knee joints. Notably, the activation threshold of MSCs in the outside-out configuration is reduced (data not shown) which suggests the involvement of intracellular molecules such as the actin cytoskeleton in modulating the sensitivity of the channel (*111*). Perfusion of GsTMx4 (5 μM) in the external bath significantly reduces the amount of mechanically evoked current, and this pharmacological blockade is

reversed immediately after washing away of the peptide (**Figure 2-6a,b**, $p < 0.001$). Interestingly, the mean open time of MSCs was also significantly reduced upon GsMTx4 perfused and this reduction was also recovered following wash (**Figure 2-6c**, $p < 0.001$). Overall, this confirms that the MSCs present at the membrane of knee innervating nociceptors are sensitive to GsMTx4 and that the method of GsMTx4 blockade is through the reduction of the probability of opening of MSCs as well as reduction in the mean open time of the channel.

3.8 Intra-articular injection of GsMTx4 attenuates evoked mechanical pain

We now understand that our OA model produces a behavioural and electrophysiological phenotype. Sensitization of MSCs is specific to the ipsilateral knee and this increased current can be selectively blocked by application of GsMTx4 in the bath. However, we have yet to determine whether blockade at the nerve terminals on OA knees can affect the pain behaviors demonstrated by our mice. To assess pain attenuation, we performed knee flexion-extension tests following intra-articular injection of GsMTx4. To first confirm that intra-articular injection will allow entry of our drug into knee innervating nociceptors, we injected our mice with 5% lidocaine as a control (**Figure 2-7, inset**). We see that lidocaine injection does produce a significant reduction in pain score as assessed from our knee flexion-extension test ($p < 0.05$). To investigate GsMTx4's effects, we first injected our animals with a 5 μM concentration in saline (**Figure 2-7**, grey bar), as it has been shown within our Outside-out recording method that 5 μM is sufficient for direct blockade of MSCs in knee nociceptors. This did not produce any analgesic effect in our model ($p > 0.05$). However, it is likely that much of the peptide was diffused and taken up into the surrounding non-nervous tissue, reducing its final effective concentration at the nerve terminal. We then increased our concentration of GsMTx4 to 50 μM and this produced a significant analgesic effect as assayed by knee flexion-extension. Importantly, we wanted to

ensure that our increased concentration did not lead to any non-specific systemic analgesic. Therefore, we injected in the contralateral knee the same concentration and dose (10 µl) of GsMTx4. This did not result in any reduction in pain score when the ipsilateral knee underwent flexion-extension. Thus, the effect we saw with ipsilateral injection of 50 µM GsMTx4 is a specific and local effect on the OA knee joint.

3.9 Preliminary: FOS stain reveals altered pain signaling in MIA model

To assess whether the MIA model causes functional change in the pain signalling via knee nociceptor input to the superficial dorsal horn, we performed immunohistochemistry against cFOS in the lumbar enlargement of the spinal cord of mice that recently underwent 10 minutes of flexion-extension of the OA knee. Early results suggest that MIA injection increases cFOS labelling specifically to the ipsilateral superficial dorsal horn compared to control. Lamina I-III of 100 µg and 250 µg MIA mice both display higher levels of FOS-positive cells (17.667 ± 2.466 and 15,650 ± 1.326 respectively) compared to control (3.586 + 0.396). Whether or not there is a significant difference between the 100 µg MIA group and the 250 µg MIA group has yet to be determined. Furthermore, it is still undetermined whether injection of GsMTx4 prior to knee flexion-extension will attenuate the increased cFOS labelling.

3.10 Preliminary: TRPV1 DRG neurons show mechanical sensitization in TNF

To determine whether TNF-α alone was sufficient to reduce the activation threshold of MSCs on naïve nociceptors, we dissociated and cultured DRG neurons in 50 ng/ml TNF-α. Interestingly, small diameter (<30µm) TRPV1 positive neurons that are cultured in 50 ng/ml TNF-α exhibit MSCs which have a reduced activation threshold compared to the same population of neurons cultured in DRG media alone (**Figure 3-8ab**). These activation thresholds

(-65.0 mm Hg ± 8.5 for control DRG neurons, -31.3 mm Hg ± 3.0 for TNF-α treated neurons) mirror those observed in the naïve and OA condition knee-innervating nociceptors and suggest that TNF-α alone is sufficient to induce OA-like mechanical hypersensitivity in DRG nociceptors. Moreover, though the sample size is relatively small, there appears to be a trend towards an increase in density of active MSCs in TNF-α treated TRPV1 expressing neurons, although the frequency of active patches appears to be lower in both DRG cultures (**Figure 3-8b**). Interestingly, the mechanically evoked current is significantly greater in TNF-treated DRG neurons, especially at subthreshold pressures (relative to naïve DRG neurons) (**Figure 3-8c**). This current profile is very similar to that of knee-innervating nociceptors in our OA model. However, whether or not this sensitization can be attenuated by cytochalasin D or whether naïve TRPV1 DRG neurons can be sensitized similarly with jasplakinolide has yet to be explored.

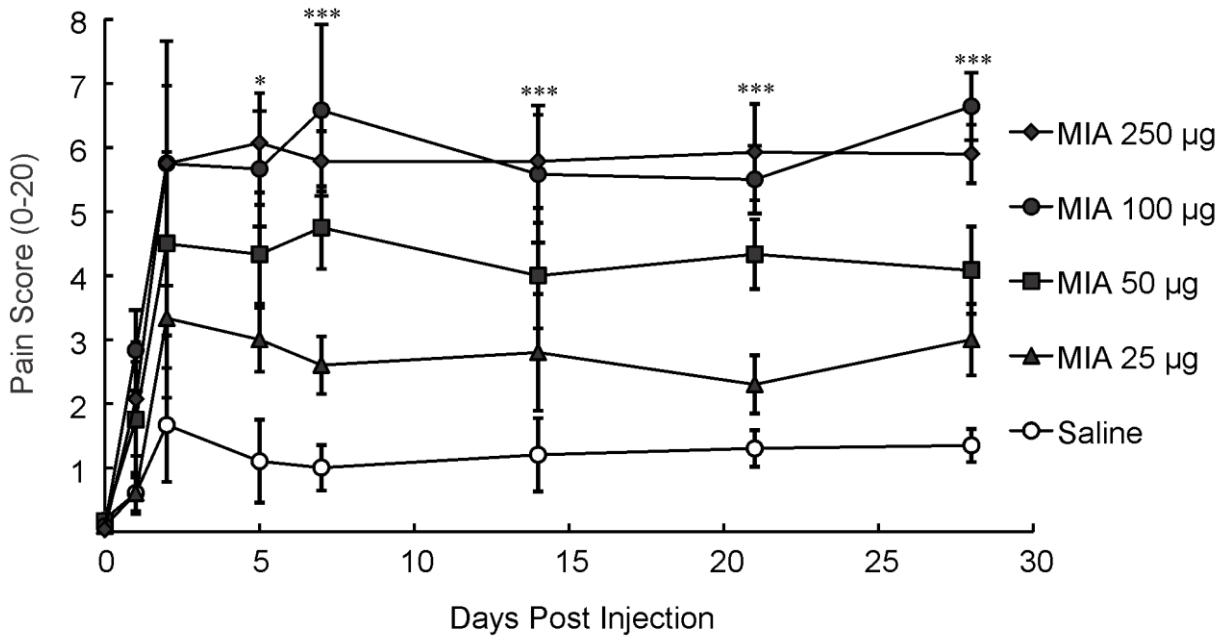

Fig 3-1. Knee Flexion Extension scores from saline, 25 µg, 50 µg, 100 µg, and 250 µg MIA injected mice. Only Ipsilateral knee scores are shown, contralateral knees from all groups did not differ in pain score at any stage (data not shown). There was a significant difference in in pain score for all MIA scores compared to control by day 5. Only the 25 µg MIA group returned to non-significant pain scores on days 14, and 21. N = 5-7 for all groups

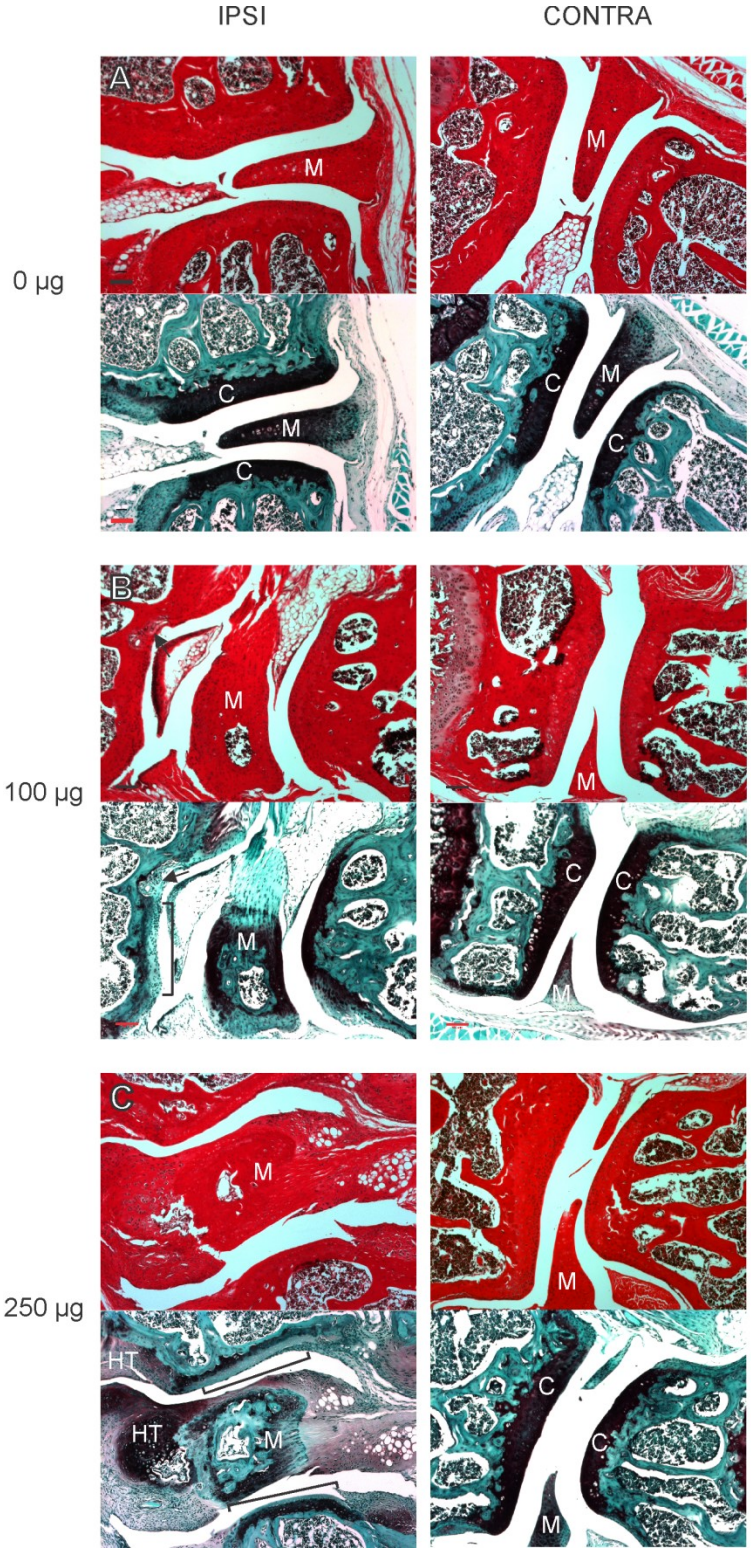

Fig 3-2. Representative histological sections from saline, 100 µg and 250 µg MIA injected mice (1 month post injection). Scale bar = 100 µm

A-C. upper panels show Hematoxylin and eosin stain as a general tissue stain, outlining cell nuclei. Lower panels show Toluidine blue stains for the articular cartilage and other glycosaminoglycan containing structures.

A. Control knees demonstrate a normal structure of cartilage (C) and meniscus (M).

B. OA progression results in loss of articular cartilage in the injected knee only. Furthermore, there is thickening of the meniscus and a hypertrophic zone developing in the subchondral bone (arrow).

C. The symptoms become more pronounced in the 250 µg MIA group as the cartilage destruction is more wide-spread, along both the femoral and tibial condyles.Moreover, there is even more exaggerated thickening of the meniscus along with hypertrophy of the chondrocytes along the meniscus and articular cartilage (HT).

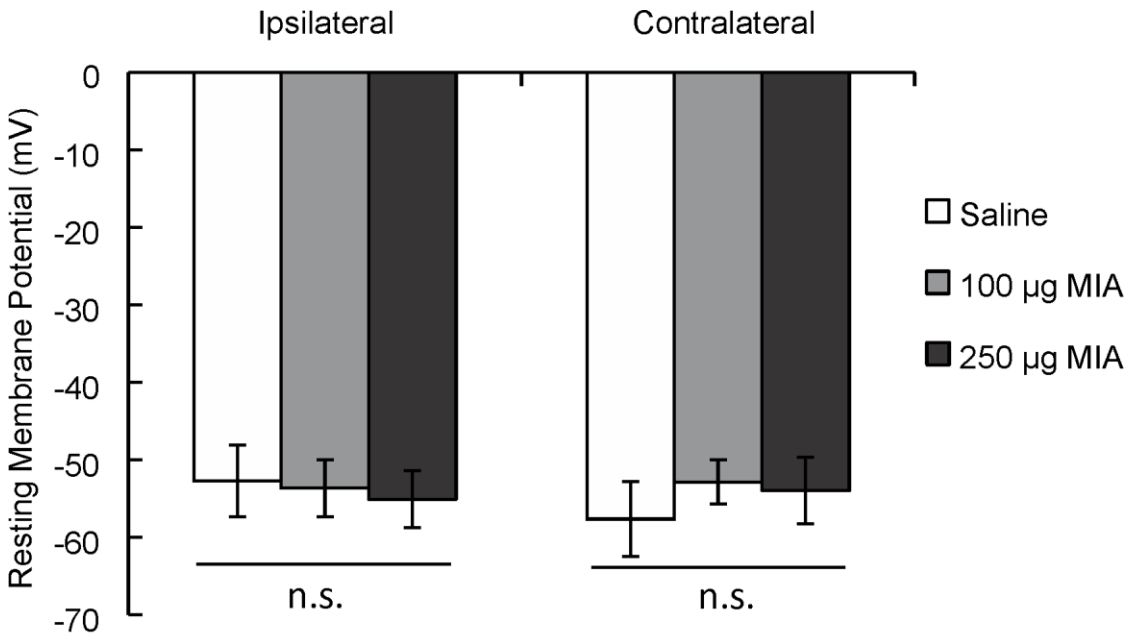

Fig 3-3. Whole-cell current clamp recording of knee innervating nociceptors. Command current was set to 0. There is no signficant difference among all groups of mice both in the ipsilateral knee as well as the contralateral knee. n.s. = not significant. n = 9-12 for all groups.

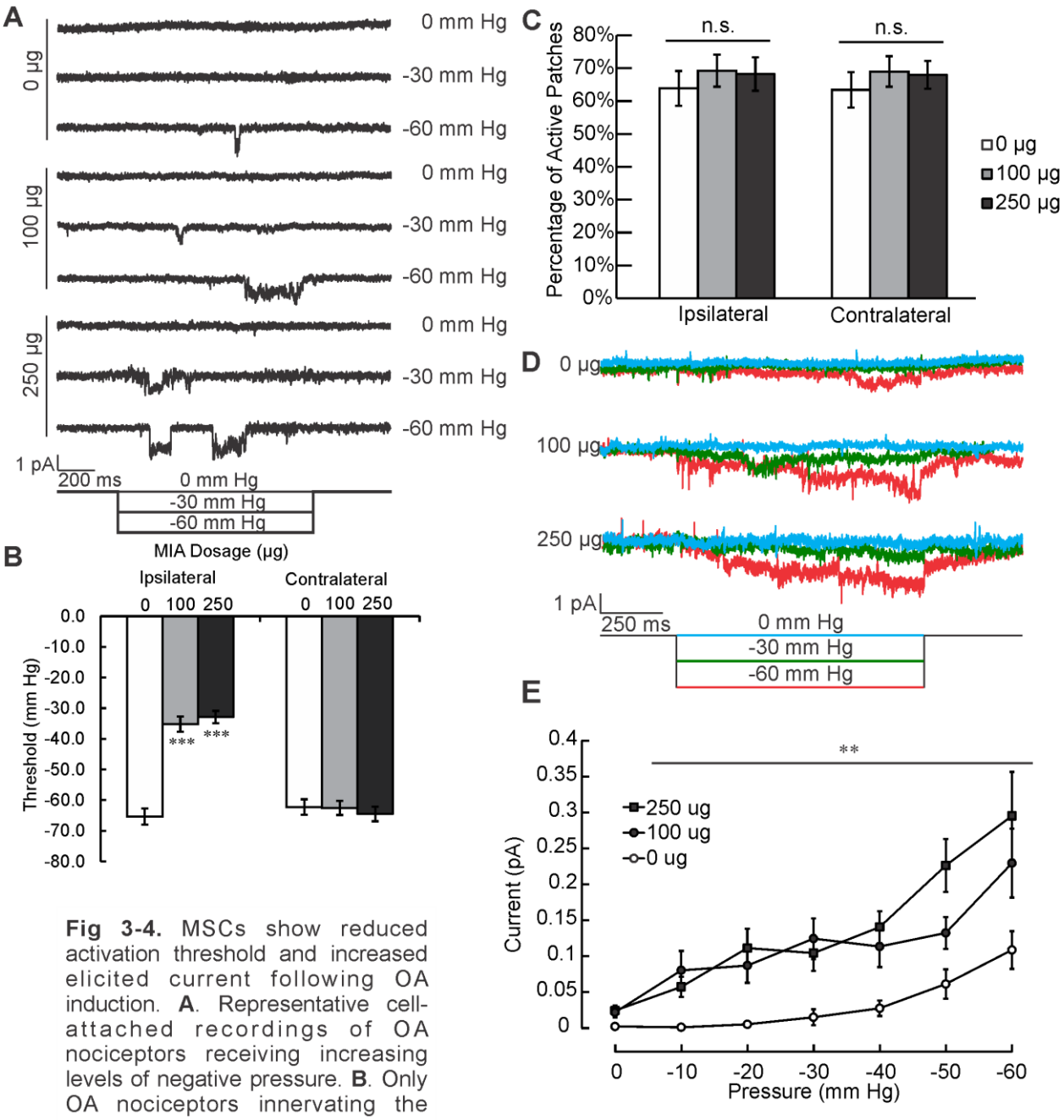

Fig 3-4. MSCs show reduced activation threshold and increased elicited current following OA induction. **A**. Representative cell-attached recordings of OA nociceptors receiving increasing levels of negative pressure. **B**. Only OA nociceptors innervating the injected knee (ipsilateral) display significantly reduced activation threshold (Kruskal-Wallis with Dunn's Multiple Comparison test). **C**. Frequency of active patches does not change across group suggesting sensitization is not due to increase in density of active MSCs present at membrane of OA nociceptors. **D**. Averaged traces of pressure-pulse recordings, color-coded according to level of negative pressure applied. **E**. Mechanically evoked current is significantly higher than control in MIA groups as early as -10 mm Hg (Two-way ANOVA with Bonferroni post-hoc comparisons). N = 82-120 for all groups

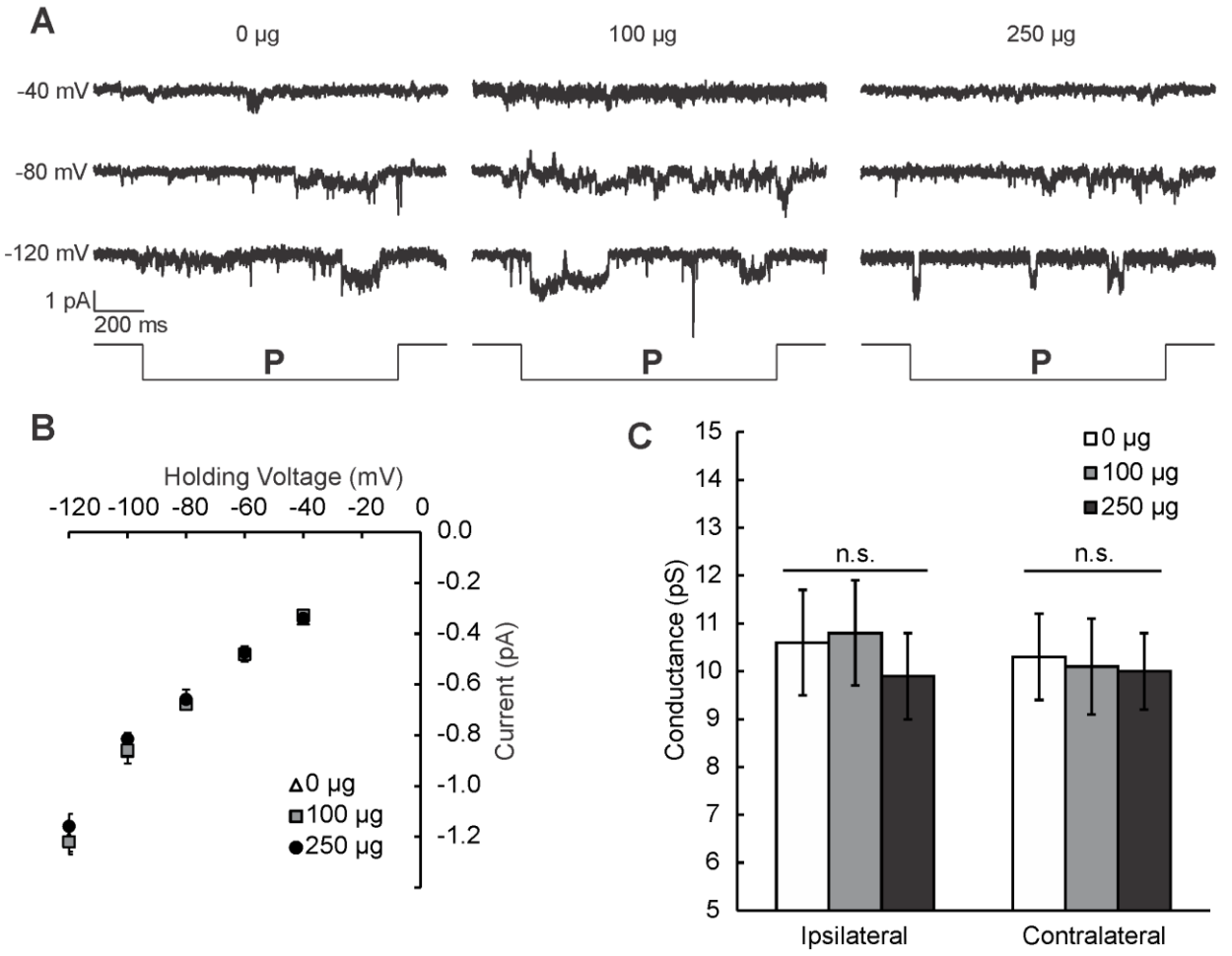

Fig 3-5. Single channel analysis suggests that the same MSC is present on knee innervating nociceptors both in the naïve state and OA state. **A**. Representative traces of pressure pulse recording held at various voltages. The presence of one kind of MSC at a specific amplitude is shown. **B**. Current amplitudes of MSCs from both control and MIA injected knee innervating nociceptors overlap. Comparison of linear regressions (i.e. conductances) gives **C**. which shows there is no significant difference in the conductance of MSCs from either MIA or saline injected mice (ANCOVA). N = 8-14 for all groups

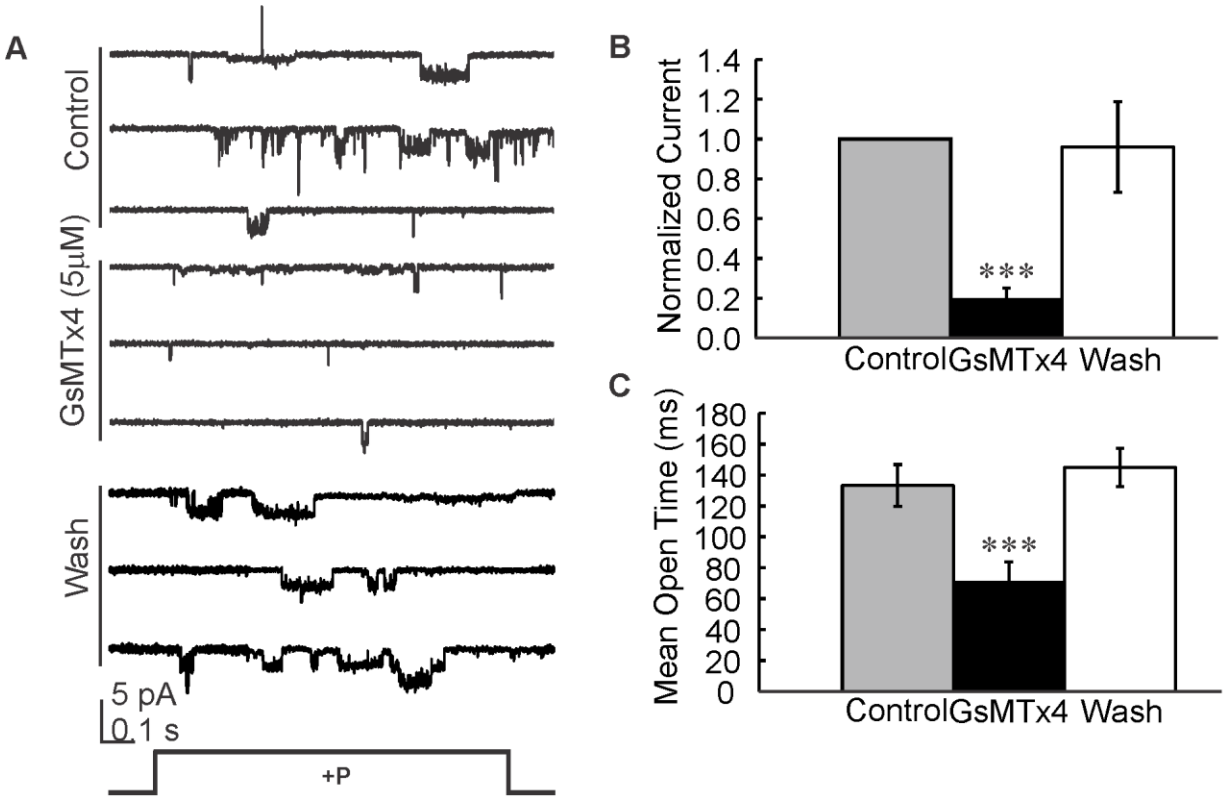

Fig 3-6. GsMTx4 is able to block MSC activity in knee innervating nociceptors while also reducing mean open time of the channel. **A**. Representative traces of outside recording protocol. An outside out patch is formed and the minimal positive pressure (+P) is applied to open MSCs. 5 μM GsMTx4 is perfused and the protocol is ran again. GsMTx4 is then washed and the same patch undergoes one final protocol run. **B**. Currents are normalized to the control trace. GsMTx4 significantly reduces the mechanically evoked current and this reduction is recovered following wash (One-way ANOVA, with Dunn's multiple comparisons, n = 17, 12, 7 for Control, GsMTx4 and Wash respectively. **C**. Mean open time is also significantly reduced via GsMTx4 perfusion. Likewise, wash recovers this reduction (Kruskal-Wallis with Dunn's multiple comparisons, n = 161, 85, 100 for Control, GsMTx4 and Wash respectively.

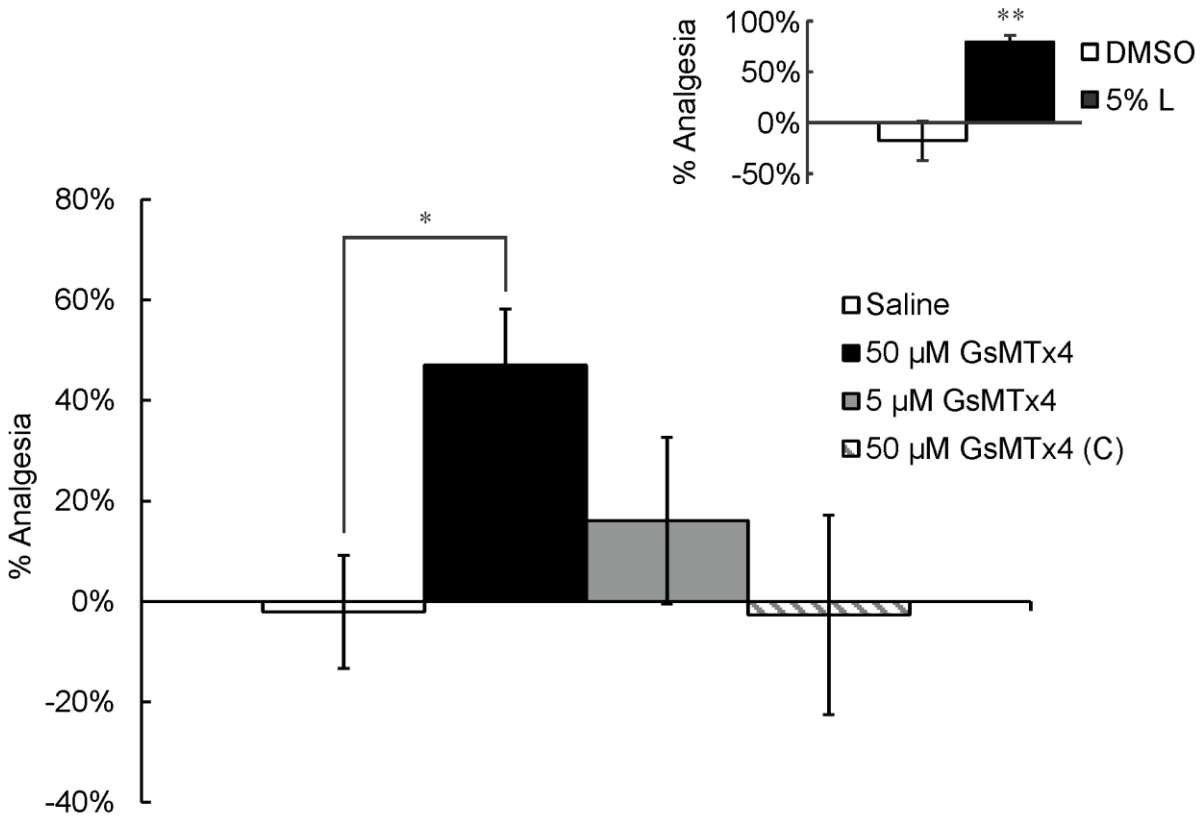

Fig 3-7. Intra-articular injection of GsMTx4 can attenuate pain scores from knee flexion-extension in 100 µg MIA injected mice. **(in-set)** Injection of 5% Lidocaine in DMSO provides a significantly larger percentage of analgesia compared to vehicle. Moreover, it confirms that intra-articular injections can reach the nerve terminals of knee innervating nociceptors.
50 µM GsmTx4 into the ipsilateral knee results in a significantly larger percentage of analgesia compared to vehicle (saline). 5 µM GsMTx4 is unable to provide significantly more analgesia compared to vehicle. Furthermore, 50 µM GsMTx4 injected into the contralateral knee (C) does not provide significant analgesia when knee flexion-extension is scored on the ipsilateral knee. (Kruskal-Wallis with Dunn's multiple comparisons). N = 6-10 for GsMTx4 injections, N = 9 for all groups in lidocaine trial.

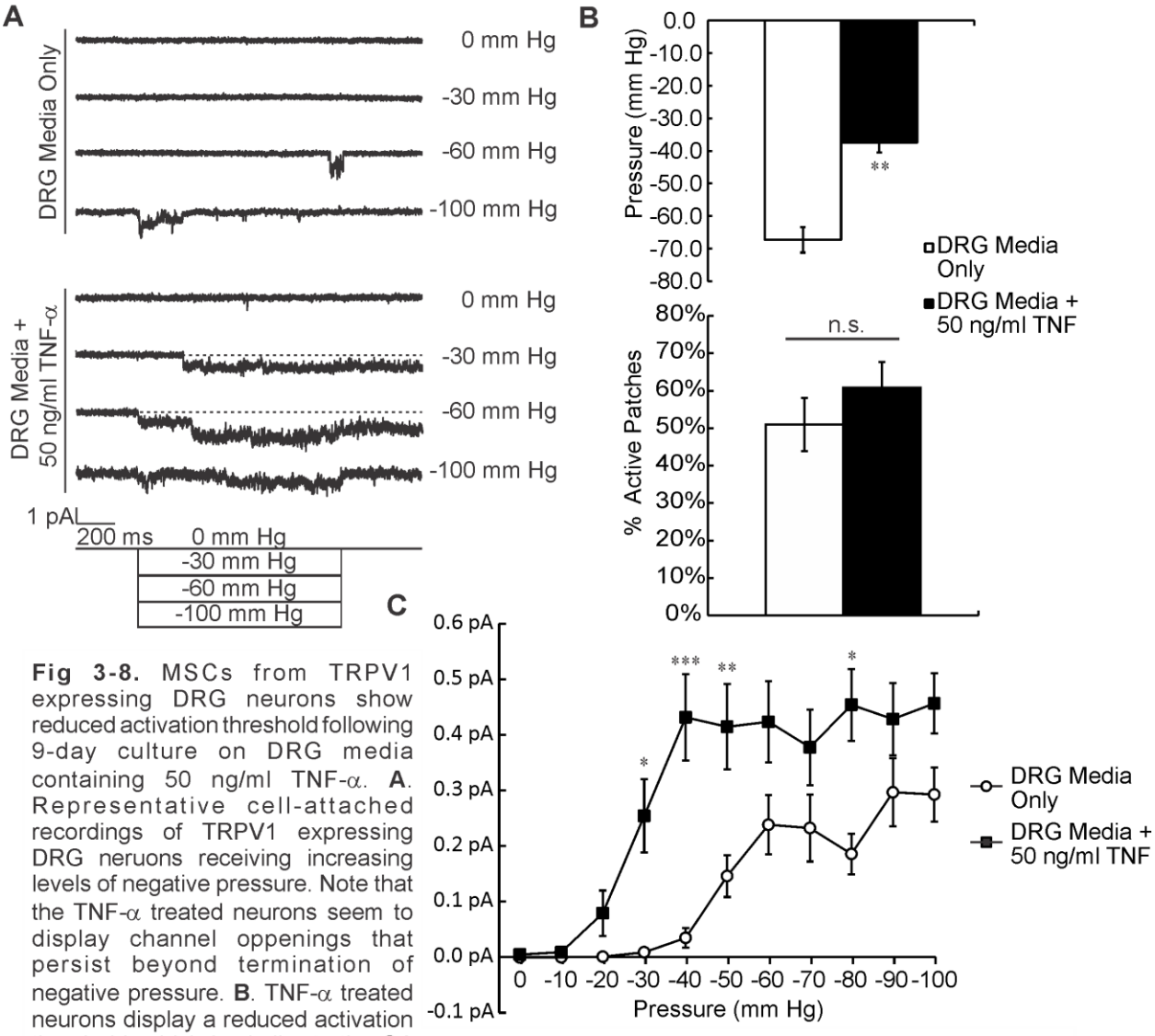

Fig 3-8. MSCs from TRPV1 expressing DRG neurons show reduced activation threshold following 9-day culture on DRG media containing 50 ng/ml TNF-α. **A**. Representative cell-attached recordings of TRPV1 expressing DRG neruons receiving increasing levels of negative pressure. Note that the TNF-α treated neurons seem to display channel oppenings that persist beyond termination of negative pressure. **B**. TNF-α treated neurons display a reduced activation threshold, which is similar to the OA model (One-way ANOVA with Bonferroni post-hoc test). Moreover, Frequency of active patches does not change following treatment of TNF-α (Fisher's exact test). **C**. Mechanically evoked current is signficantly higher inTNF-a treated TRPV1 expressing DRG neurons, especially in sub-threshold (relative to naïve TRPV1 expressing DRG neurons) pressures (Two-way ANOVA with Bonferroni post-hoc comparisons). N = 51 for all groups

CHAPTER 4: DISCUSSION

Osteoarthritis (OA) pain is composed of various components both at the periphery and centrally along the spinal cord. The very first site of mechanically evoked pain transduction is at the nerve terminals within affected joints. Using a pharmacological model of OA and recording using behavioral and electrophysiological approaches, we demonstrate that there is sensitization of MSCs in the neurons innervating the affected joint. Moreover, blocking these MSCs with a specific blocker, GsMTx4, actively attenuates the pain in our model. These results highlight a possible new target in the periphery for effective pain treatment in OA.

4.1 Profile of MSCs responding in OA nociceptors

MSCs are a wide class of proteins that have many roles throughout the CNS and peripheral nerves including osmoregulation, fine touch, proprioception and nociception. While the identity(s) of MSCs in nociceptors is currently unknown, previous studies have demonstrated that GsMTx4, a specific blocker of MSCs which include Piezo1 and Piezo2, reduces mechanical pain and actively protects articular chondrocytes from mechanically induced cell death (*165, 166*). Whether this action is through Piezo1 and Piezo2 remains unclear as Piezo2 is only expressed in 24% of TRPV1 positive DRG neurons where as Piezo1 is expressed only in very low quantities at the DRG(*100*). Moreover, previous reports indicate that GsMTx4 at 1 μM did not attenuate currents from slowly adapting DRG neurons, which are associated with nociceptive signalling (*167*). In this present study, we found that MSCs of TRPV1 expressing nociceptors which innervate the pathological knee are in fact sensitive to GsTMx4 at 5 μM and this blockade at the knee joint resulted in a significant reduction in pain response to mechanical stimuli. Given the other protective qualities reported on GsMtx4, it would be interesting to determine whether

exposure to GsMTx4 at the onset of OA may be beneficial in reducing the pathological progression of the disease at the joint as well and reducing overall pain response. In total, MSCs in OA nociceptors are susceptible to GsMTx4 blockade and this has significant attenuation in mechanically evoked pain.

In this study, we also performed single-channel analysis of the MSCs recorded in cell-attached configuration. Interestingly, our values for conductance (approximately 10 to 11 pS) of MSCs in both the naïve and OA conditions matched closely those reported by other groups (*103, 104*) regarding the DRG neurons which are of smaller diameter (10 to 20 μm) and high threshold (cell-attached recording with a -60mmHg minimum threshold), typically associated with C fibers and nociceptors. Moreover, our naïve condition and contralateral knee nociceptors reflect the same activation threshold as reported in these small-diameter high-threshold DRG neurons. Taken together, our results and previous results from others corroborate the fact that the MSCs responsible for mechanical pain signalling in OA are likely to be these normally high-threshold MSCs.

4.2 Other nociceptive targets for attenuation of mechanical allodynia

While mechanoreception plays an important role at the onset of mechanical pain signalling, there are other key factors to consider in the transmission of the pain signal. It has been reported that Na_v 1.8 is crucial to the propagation of the pain signal in chronic pain models, including the MIA model (*84, 85*). Blocking Na_v 1.8 activity significantly reduces firing rate specific to noxious mechanical stimuli and reduces MIA-induced mechanical hypersensitivity (*84*). However, less than half of all C-fibers express Na_v 1.8 and an even smaller percentage of A-δ fibers express the channel (*83, 168*). Furthermore, blocking these voltage-gated sodium

channels generally occurs through the intravenous route and would affect the nociceptive response in non-pathological areas. Our results show that intra-articular injection of 50 μM GsMTx4 directly into the synovial space of the knee is sufficient to attenuate the pain response. There is also no leakage of effect since GsMTx4 injection into the contralateral (relative to MIA injection) knee did not result in the same level of pain attenuation, suggesting that the analgesia induced by GsMTx4 is specific to site of injury and not a generalized effect in the mouse.

Indeed, there also exists a central sensitization component to OA progression. Previous clinical reports have shown that there exists a population of OA patients that experience centrally mediated pain sensitization (*42, 47, 155, 169, 170*). Interestingly, it has been shown in some knee OA patients that their degree of central sensitization, as assessed by electric stimulation of the knee, in part determined the degree of persistent pain following total knee replacement (*155, 171*). While it is important to continue assessing the contribution of central sensitization to the reported pain in OA, the safety and potential efficacy of peripheral pain analgesia provides an exciting new avenue for treatment of OA pain. Many opioid and SSRI analgesics used to treat moderate to severe OA pain have targets in the CNS and carry harmful side-effects that affect the physical and mental state of the patient (*62*). The risks for these harmful side-effects increase for the elderly, which comprise the majority of patients that suffer from OA pain. On the contrary, intra-articular injections of corticosteroids and hyaluronic acids show significant pain reduction with minimal lasting side-effects (*172, 173*). It should also be noted that many of the approved methods of pain management in OA focus on the care and exercise of the affected joints, including aerobics, knee braces, and weight loss for obese patients (*174*). Our study has shown that peripheral block of mechanically sensitive nociceptors is sufficient for attenuation of pain responses in a model of OA pain. Taken together, the evidence highlights the importance of

peripheral tissue health in the management of OA pain and indicates a strong continuous contribution of signalling from the periphery that drives pain throughout the progression of the disease.

4.3 Mechanisms underlying MSC sensitization

What our study has demonstrated is a clear sensitization of MSCs in OA nociceptors to weaker mechanical stimuli. However, the mechanisms underlying said sensitization are currently unknown. It has been shown previously that MSCs in purified lipid bilayers and xenopus oocytes that lack cytoskeletal components can still open in response to membrane tension (*175, 176*). However, there are also many reports of the role of the actin or microtubule cytoskeleton which modulates the sensitivity of MSCs in various sensory and developmental contexts including photoreception, osmoreception, and synaptogenesis (*111-114*). Early cell-attached recordings in oocytes showed MSC inactivation following repeated stimulation via pressure clamp (*177*). It was suggested that the repeated stimulation destroyed the integrity of the underlying cytoskeleton rendering MSCs less sensitive to mechanical pressure. This was later confirmed in astrocytes (*178*). With specific attention to mechanical nociception, application of cytochalasin-D, a compound which destabilizes f-actin into g-actin, mimics the effect of GsMTx4 in suppressing the sensitivity of MSCs in high-threshold small-diameter DRG neurons (*103*). Therefore, the modulation of the underlying actin cytoskeleton seems to be involved in the setting of the sensitivity of active MSCs on the membrane of nociceptors. Assessing whether or not there is change in the cytoskeleton in the OA disease state may help to elucidate the intracellular mechanisms underlying the mechanical hypersensitivity.

4.4 Future Experiments

Reports from RA have demonstrated that there is increased intra-articular pressure following onset of the disease (*10, 179*). Normal joints usually display an intra-articular pressure that is sub-atmosphere, ranging from -2 to -10 mm Hg (*180*). However in RA, this value increases dramatically to approximately 20 mm Hg following joint inflammation and oedema (*179*). Whether or not this same effect occurs in human cases of OA is unclear. Very primitive examples of experimental OA in rabbits demonstrated that there is increased pressure in the bone and joint of the osteoarthritis knee, following immobilization of that leg (*40*). If this were true in the human OA condition, then it implies that the increased pressure may activate MSCs in joint innervating nociceptors in the absence of external stimuli. This would then provide a meaningful physiological basis for the spontaneous pain reported in human OA. Moreover, it may be then possible to administer GsMTx4 for the relief of ongoing spontaneous pain, as it would block the underlying MSC activity. Our preliminary results from conditioned place preference testing show that there may be a preference for the GsTMx4 associated chamber. However, a control group that receives only saline for both chamber associations is needed to verify the change is significant.

While it is clear that the MIA model elicits increased pain behaviours, the demonstration of the precise functional signalling changes has yet to be made. Our cFOS stains of the superficial dorsal horn of the lumbar enlargement bolsters the implication that MIA increased pain signalling along the superficial dorsal horn. However we have yet to assess whether intra-articular GsTMx4 is sufficient at reducing this increased cFOS labelling.

Lastly, this project has only begun to touch upon the effects of TNF and the actin cytoskeleton in the modulation of MSC sensitivity in the context of OA pain. The MSCs present in TRPV1 expressing DRG neurons demonstrate a reduced activation threshold, and the changes in mechanically evoked current closely mimic those observed in our OA model. Importantly, this change has yet to be determined as a result of changes in the underlying actin cytoskeleton. Immunohistochemistry of DRGs from MIA mice which are triple labelled for TRPV1, knee-innervating neurons and actin may give important insight on the rearrangement of actin cytoskeleton in the MIA model of OA. This may confirm the presence of such rearrangement in the MIA model and if so, modulation of said cytoskeleton via pharmacological means will lend further evidence towards this claim. By treating OA nociceptors or TNF-α treated DRG neurons with cytochalasin-D, we can assess whether the mechanical sensitivity of the neurons are attenuated. Conversely, treating naïve nociceptors with jasplakinolide may induce OA-like sensitization of MSCs and once again demonstrate the potent ability of the actin cytoskeleton to modulate MSC sensitivity.

CHAPTER 5: CONCLUSION

In conclusion, we have established a new role for MSCs in nociceptors in the context of mechanical pain and osteoarthritis. We have characterized the change that occurs in knee-innervating nociceptors of arthritic joints and how the hypersensitivity in our OA model is linked to the hypersensitivity of MSCs in these neurons. The findings here underscore the potential of targeting MSCs and peripheral nerve terminals as a viable and safe alternative pain analgesia in OA. Ideally, the aim of targeting MSCs in the context of OA pain is to restore normal physiological sensitivity to the affected joint(s). However, simple antagonism of MSC function in nociceptors innervating affected joints may not truly restore physiologically normal function. We have shown that GsMTx4 is capable of reducing the mechanical hypersensitivity associated with OA, but there is also evidence that GsMTx4, injected intraperitoneally reduces baseline sensitivity in rats (*166*). While desensitization of sensitized MSCs may be favoured in OA, the over-compensation via antagonism of MSCs in nociceptors may also result in dangerous injuries such as hyper-extension. More work needs to be done in either fine-tuning treatment plans involving MSCs or generation of anti-allodynic treatments that target MSCs. Lastly, future studies into the factors inducing the onset of MSC hypersensitivity and the underlying mechanisms involved in its sensitization can lead to the development of more targeted and comprehensive OA pain treatment from the periphery.

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