Mechanosensitive ion channels in osteoarthritis pain

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Abstract

Osteoarthritis (OA) is a debilitating disease affecting nearly 5 million of Canadians, with an economic impact of over $30 billion each year in direct and indirect costs. Pain is the prominent symptom of OA, yet its underlying mechanisms are not completely understood, leading to a lack of effective pain therapies. OA pain is manifested by a hypersensitivity to mechanical stimuli such as joint movement or palpation. Although the mechanical sensitization of pain-sensing nerve fibers, known as nociceptors, innervating the joint has been documented, the molecular and cellular mechanisms underlying this sensitization are currently unknown. The peripheral terminals of nociceptors express mechanosensitive ion channels (MSCs) that convert mechanical stimuli into depolarizing potentials. Our central hypothesis is that the sensitization of nociceptors to mechanical stimuli in OA can be explained by changes in the properties of these MSCs, whereby their activation threshold is reduced during the disease. Using the monoiodoacetate model of OA in mice, we demonstrate that mice develop chronic mechanical allodynia. Furthermore, cell–attached electrophysiological recordings of nociceptors isolated from OA mice indicate an increase in the current elicited by mechanical stimuli, as well as a reduced activation threshold of MSCs, when compared to naïve mice. An understanding of the molecular bases for these changes is presently impossible because the identity of the genes encoding MSCs is unknown. Interestingly, in a previous screen for MSC candidates, we identified five transmembrane proteins of unknown function (TMEMs). Our results indicate these candidates are expressed in sensory neurons and the mRNA of three of them is increased in OA. When expressed in COS-7 cells, two of these candidates, TMEM5B and TMEM1, caused an increase and decrease in cellular mechanosensitivity. This project will lead to a better understanding of the molecular mechanisms underlying arthritis pain.

Résumé

L'ostéoarthrite (OA) est une maladie débilitante affectant près de 5 millions de Canadiens et ayant un impact économique de plus de 30 milliards de dollars en coûts directs et indirects. Bien que le symptôme principal de l'OA est la douleur, les mécanismes qui en sont responsables ne sont pas compris, et par conséquent, les thérapies effectives contre la douleur sont manquantes. La douleur causée par l'OA est manifestée par une hypersensibilité aux stimuli mécaniques tels le mouvement des jointures ou la palpation. Bien que la sensibilisation mécanique des fibres nerveuses qui détectent la douleur (nocicepteurs) innervant la jointure ait été documentée, les mécanismes moléculaires et cellulaires de base de la sensibilisation ne sont pas compris. Les terminaisons périphériques des nocicepteurs expriment des canaux ioniques mécanosensibles (MSCs) responsables de la conversion des stimuli mécaniques en potentiels dépolarisants. Notre hypothèse centrale est que durant l'OA, la sensibilisation des nocicepteurs aux stimuli mécaniques peut être attribuée aux changements des propriétés de base des MSCs, où leur seuil d'activation est réduit lors de la maladie. Nous démontrons, en utilisant le modèle de monoiodoacetate de l'OA chez la souris, que cette dernière développe une allodynie mécanique chronique. De plus, les enregistrements électrophysiologiques en configuration cellule-attachée faites à partir des nociceptors isolés des souris OA indiquent une augmentation du courant élicité par les stimuli mécaniques, ainsi qu'une réduction dans le seuil d'activation des MSCs en comparaison aux souris naïves. Une compréhension de la base moléculaire de ces changements est présentement impossible car l'identité des gènes qui encodent les MSCs n'est pas connue. Dans des expériences antérieures, au cours desquelles nous avons fait un criblage différentiel afin d'identifier des candidats de MSC, nous avons identifié cinq protéines transmembranaires de fonctions inconnues (TMEMs). Nos résultats indiquent que ces candidats sont exprimés dans les

neurones sensoriels et que l'ARNm de trois de ces derniers augmente durant l'OA. Lorsqu'exprimés dans les cellules COS-7, deux des candidats (TMEM5B et TMEM1) ont causé une augmentation et une diminution de la mécanosensibilité cellulaire. Ce projet nous donnera une meilleure compréhension des mécanismes moléculaires de base de la douleur arthritique.

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Legend

$	Dollars
%	Percent
<	Less than
12-HPETE	12-hydroperoxyeicosaenoic acid
ANOVA	Analysis of variance
ASICs	Acid-sensing ion channels
ATP	Adenosine triphosphate
BMPs	Bone morphogenetic proteins
℃	Degrees Celsius
C. elegans	Caenorhabditis elegans
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freud's adjuvant
CGRP	Calcitonin-gene related peptide
CIPA	Congenital insensitivity to pain with anhydrosis
CMHs	C-fibers that are both mechanically and heat sensitive
CNS	Central nervous system
COS-7	African green monkey kidney cells
COX-2	Cyclooxygenase-2
DEG/ENaCs	Degenerin/epithelial sodium channels
DMEM	Dulbecco's modified eagle medium
DmPiezo	Drosophila melanogaster Piezo protein
dNTPs	Deoxynucleotide triphosphates
DRG	Dorsal root ganglia
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
h	Hours
HBSS	Hank's buffered saline solution
HEK293	Human embryonic kidney cell line
IASP	International Association for the Study of Pain
IB4	Isolectin B4
MDa	Megadaltons
MEC	Mechanosensory proteins
MIA	Monoiodoacetate
mL	Millilitres
mM	Millimolar
mmHg	Millimetres of mercury
MmPiezo	Mammalian mouse Piezo protein
mRNA	Messenger ribonucleic acid
ms	Milliseconds
MSCs	Mechanosensitive ion channels
mV	Millivolts
MΩ	MegaOhm
NADA	N-Arachidonoyl dopamine

NGF	Nerve growth factor
NO	Nitric oxide
NOMPC	No mechanoreceptor potential C
NSAIDs	Non-steroidal anti-inflammatory drugs
N-terminal	Amino-terminal
OA	Osteoarthritis
P2X	Purinergic receptors
pA	Picoamperes
PC2	Polycystin-2
RT-PCR	Reverse transcription polymerase chain rection
s	Seconds
SEM	Standard error of the mean
siRNA	Short-interfering ribonucleic acid
SP	Substance P
TMDs	Transmembrane domains
TMEM	Transmembrane proteins; mechanosensitive ion channel candidates
TNFα	Tumor necrosis factor alpha
TRP	Transient receptor potential
TRPA	Transient receptor potential, ankyrin subtype
TRPM	Transient receptor potential, melastatin subtype
TRPN	Transient receptor potential, no mechanoreceptor potential C subtype
TRPV	Transient receptor potential, vanilloid subtype
UV	Ultraviolet
α	Alpha
β	Beta
δ	Delta
μg	Micrograms
μL	Microliters

Chapter 1 - Introduction

1.1 Osteoarthritis

Arthritis is a disease affecting 4.6 million Canadians, of which approximately 4.4 million have osteoarthritis (OA)[1]. Due to Canada's aging population, these numbers only continue to grow[1]. The government of Canada suggests 100 000 new cases of OA are expected annually for the next 30 years, of which half will be among Canada's mature workforce[1]. By 2040, more than 10 million Canadians (1 in 4) will suffer from OA[2]. The socioeconomic impact of this disease is immense: considering both direct and indirect costs, the total economic burden of OA was estimated to be $27.5 billion in 2010[1]. OA is a leading cause of disability; 80% of patients experience some degree of movement limitations and 25% are unable to complete their daily activities[2]. Consequently, OA is often accompanied by secondary effects such as obesity, heart disease, type II diabetes, depression, and poverty – all of which add to the economic cost of arthritis.

1.1.1 Causes of knee osteoarthritis

OA is a disease of the joints that most often affects weight bearing joints such as the knee[3]. Despite the lack of understanding of the etiology of knee OA, several studies have suggested certain risk factors to be associated with the disease. OA is more prevalent in women and in older individuals (65 years and up), yet other factors independent of age and sex can influence the incidence of the disease[3]. These include both mechanical stresses, such as obesity, misalignment, muscle weakness, joint trauma and surgery, as well as biochemical abnormalities,

such as genetic predisposition and other metabolic disorders[3,4]. Despite their correlation with the disease, these risk factors do not definitively predict who will develop OA, and conversely the absence of these factors does not exclude the possibility of developing OA later in life. Therefore, the variation in risk of onset and progression of OA is due to joint mechanics; genetic predisposition and defects in joint anatomy are to blame[4].

1.1.2 Physiology of OA joint damage

The mammalian joint is a point at which two or more bones make contact. Synovial joints are the most abundant joint in the body; they are characterized by a fluid-containing synovial cavity (joint cavity) that allows a wide range of movement. Synovial joints are hinge joints that also contain bones (femur, tibia, patella), ligaments and cartilage, and are the primary site of OA incidence[3] (Figure 1-1).

OA is generally accepted as a failure of the whole joint, not just a disease of the cartilage[3]. The risk factors described above contribute to local mechanical stress on the joint, which over time induces several structural changes to the whole joint, including loss of articular cartilage, osteophyte development, synovial inflammation, subchondral bone loss, and meniscal damage[4] (Figure 1-2). It is likely that the three affected tissues – bone, cartilage and synovium – are each a manifestation of the OA joint failure, where the failure of one tissue exacerbates the disease in the others[5]. The link between injuries (both acute trauma and repetitive stress) to the joint and development of OA is well documented[7]. Typically these events result in soft tissue

damage within the joint. For example, damaged articular ligaments have been shown to cause joint instability and abnormal loading patterns of the joint[9].

Despite the structural changes described in OA, the predominant and often the first symptom of OA is pain of the joint and surrounding tissue. It is this episodic or chronic pain experienced with OA that causes loss of joint mobility and function, and results in the psychological distress and impaired quality of life described above[9]. Interestingly, there is a lack of correlation between observed joint structural changes and reported pain in knee OA[10]. Some patients experience the classic structural changes associated with OA, yet never experience pain, whereas others experience pain with very few joint structure symptoms.

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"[11]. Pain is a multidimensional phenomenon, encompassing the physical responses, emotional reactions, rational thoughts, social influences, and spiritual feelings associated with pain[12]. As a physical response, Charles Sherrington described pain as a protective reflex; it warns the organism about danger and impending harm[12]. In essence, pain is a survival mechanism just as much as thirst or sight; it is essential for maintaining bodily integrity[13]. This is best understood through analysis of the extreme: patients with congenital insensitivity to pain with anhydrosis (CIPA) exhibit self-mutilation, auto-amputation, and corneal scarring, among others, leading to early death[14].

Pain can be classified into three categories: acute, inflammatory, and pathological[13]. Acute pain is considered nociceptive; it is the protective mechanism in normal tissue, where high threshold physical and chemical noxious stimuli that can potentially cause tissue damage are detected[15]. This pain triggers essential avoidance reactions that are necessary for survival[16]. Inflammatory pain is also adaptive and protective[13], providing heightened sensitivity following tissue damage. Inflammatory pain is necessary to allow recovery and prevent further damage by discouraging movement and physical contact with the injured region. Pathological pain is maladaptive, and is a result of nervous system abnormality[13]. Both inflammatory and pathological pains are symptoms of injury and disease. These types of pain are different from acute pain in that they are triggered either spontaneously or by normally innocuous stimuli[13,16]. This phenomenon suggests there are substantial changes and plasticity in the nociceptive system. The terminals of nociceptors, pain transmitting neurons, become sensitized during inflammation; axons become more hyperexcitable (generating spontaneous action potentials), cell bodies undergo dramatic changes in protein expression and trafficking, and synapses in the spinal cord are modified (increasing their strength or undergoing structural reorganization)[13]. Such changes also occur in the spinal cord and brain, culminating in central sensitization – thresholds for generating pain decrease, and the duration, amplitude and spatial distribution increase[13,17]. Sensitization has been described as an uncoupling of nociceptive pain from its absolute need for noxious stimuli[13].

Although both play a role in OA pain, there exist differences between inflammatory and pathological pain: "the former represents hypersensitivity in reaction to a defined peripheral pathology, whereas the latter is the result of altered neural processing"[13]. Interestingly, genetic

components have been identified that play roles in the pain hypersensitivity and development of chronic pain[18,19,20].

1.2.1 Knee OA pain

The importance of pain in joints as a protective mechanism is demonstrated in neuropathic arthropathy patients, where loss of nociception results in joint degradation[13,21]. Knee joints are richly innervated by sensory and sympathetic nerves, which serve to detect mechanical stimuli and regulate joint blood flow, respectively[9]. Nociceptors innervate many parts of the joint, including the capsule, ligaments, menisci, periosteum and subchondral bone[22,23,24]. Current hypotheses suggest the shear strain on the free nerve terminals causes the opening of mechanically-gated ion channels[9].

The characteristic inflammation in OA is caused mainly by synovitis, which is the first symptom of OA, appearing in the very early stages of the disease (subclinical stages). It has been shown that increases in synovium mass and decreases in joint space are correlated with the severity of OA[25]. Synovitis causes thickening of the synovial membrane and infiltration of leukocytes along the synovium interior[8]. Many of the symptoms and structural changes in OA are directly attributed to synovitis[26]. During knee joint injury or inflammation, the fibrous capsule that contains synovial fluid becomes increasingly permeable to plasma proteins and inflammatory cells (monocytes), which leak into the intra-articular space via the synovial vasculature[27]. This is coupled to a fluid increase in the joint and cause oedema. The rise in fluid significantly increases the intra-articular pressure, which in turn causes burst firing of articular

afferents that is characteristic of OA; the frequency of these neuronal discharges is proportional to the pressure[9].

The synovial membrane contains synoviocytes with high metabolic activity. In healthy conditions, these cells provide nutrition to the chondrocytes and remove products and metabolites of matrix degradation[26]. However, in OA joints, products of cartilage breakdown in the synovial fluid are phagocytosed by synoviocytes, which amplify the inflammation and cause the production and release of catabolic and proinflammatory mediators by the synoviocytes[26] (Figure 1-3). This process leads to production of proteolytic enzymes responsible for cartilage breakdown, creating a positive-feedback loop that is further amplified by the presence of leukocytes[26]. The inflamed synovium also houses macrophages that contribute to the formation of osteophytes via their release of bone morphogenetic proteins (BMPs), which are multi-functional growth factors imperative for postnatal bone growth[26].

Inflammation of the knee joint induces peripheral and central nervous system changes so that the pain threshold is lowered, causing allodynia (a painful response to a normally innocuous stimulus) and hyperalgesia (a heightened pain intensity to a normally painful stimulus). Inflammatory mediators are thought to be partially responsible for this, as they are released into the joint by surrounding tissue including nerves, immunocytes, synoviocytes and vascular endothelium[9]. These inflammatory factors sensitize local sensory neurons, causing hyperexcitability in the OA joint. Indeed, sensory neuron firing in OA can be replicated when inflammatory agents are applied to normal joints[9]. One cause for this is the activation of silent nociceptors – sensory fibers that are dormant in normal conditions but become active in pathological conditions such as OA[28]. It was also shown that chemical induction of acute

synovitis reduced the activation threshold of nociceptors in knee joint afferents, causing mechanical hypersensitivity[29]. The authors also reported an increase in firing frequency during normal and extreme ranges of joint movement after synovitis. Noxious stimuli applied to the joint increase the nociceptors' firing rate, resulting in the allodynia and hyperalgesia seen in OA[30]. The lowering of activation threshold, increased firing rate, and spontaneous firing of joint sensory neurons was observed in adjuvant-induced chronic arthritis as well as monoiodoacetate-induced OA in rats[31,32]. Despite the advances in our understanding of the role on nociceptors in OA, current treatment of OA pain remains incomplete.

1.2.2 Treatment of OA pain

Treatment of OA pain has proven difficult, if non-existent[33]. Joint structure-modifying drugs, such as glucosamine sulfate and chondroitin sulfate, have shown no proven effect[3,34]. Few drugs are currently used in the treatment of OA pain, however, their chronic use is limited by their efficacy and harmful side effects[3,9]. For example, many non-steroidal anti-inflammatory drugs (NSAIDs) target cyclooxygenase-2 (COX-2), yet their alleviation of OA pain is limited and they have gastrointestinal side effects due to the adverse COX-1 activation. Newer, highly selective COX-2 inhibitors reduce the side effects seen with normal NSAIDs, yet their efficacy in treating pain remains the same[33]. Therefore, it is important to develop disease-modifying therapies that have an efficient analgesic profile. Contemporary pain management has made little progress developing novel therapeutic targets and treatments. At the heart of the problem is our poor understanding of the molecular mechanisms of joint pain; in order to have better targets for pain management, we need a better understanding of the mechanisms involved in pain.

1.2.3 Neurophysiology of pain

Most organs are innervated by nociceptors and can thus detect noxious stimuli[16,35]. The pain is detected by nociceptor sensory afferents whose cell somas are located in the dorsal root ganglia (DRG). The peripheral terminals of these neurons innervate their target organs while their central terminals enter the spinal cord to synapse onto second order neurons[36]. There exist two major types of nociceptors[36]. The first type is the medium diameter, lightly myelinated Aδ-fibers, and the second type is the small diameter, unmyelinated C-fibers. Aδ-fibers generate the acute pain response; they encode a well-localized "first" or fast pain response[36]. These fibers are further divided into two types. Type I are high-threshold mechanical nociceptors (HTMs) that respond to both mechanical and chemical stimulation, but have a very high heat threshold (>50°C)[36]. They will sensitize to mechanical and thermal stimuli following tissue injury[36]. Type II Aδ-fibers have a much lower heat threshold, but a very high mechanical threshold[36]. It is proposed that Type II Aδ-fibers mediate the "first" response to noxious heat, and type I are responsible for the "first" response of intense mechanical stimuli[36].

Contrary to Aδ-fibers, C-fibers encode the "second" or slow pain that is poorly localized[36]. C-fibers also have subclasses, and like myelinated afferents, they are polymodal in their sensitivity (both heat and mechanically sensitive; CMHs)[16,36,37]. Silent nociceptors are C-fibers that are heat sensitive but mechanically insensitive. These C-fibers develop mechanosensitivity only following injury and are more responsive to chemical stimuli (capsaicin or histamine) compared to CMHs[28]. This suggests they may become mechanically activated during inflammation[36]. C-fibers are not exclusively nociceptors: some C-fibers are responsive to

cooling or gentle touch but not chemical or heat stimulation (therefore mediate pleasant touch)[36]. C-fibers responsible for nociception express molecular markers that identify their function. Peptidergic C-nociceptors release neuropeptides such as substance P (SP), neurokinin A and calcitonin-gene related peptide (CGRP). They also express the neurotrophin receptor TrkA, which responds to nerve growth factor (NGF)[38]. Non-peptidergic C-nociceptors express the neurotrophin receptor c-Ret, which is the receptor for glial-derived neurotrophic factor (GDNF)[39]. A large percentage of c-Ret positive cells also bind IB4 isolectin, express G protein-coupled receptors of the Mrg family and purinergic receptors such as P2X3[36,40]. Nociceptors can also be classified by their expression of ion channels (or associated proteins) that are known to confer sensitivity to specific stimuli. For example, neurons sensitive to acidity express ASICs, neurons sensitive to chemical irritants express TRPA1, and heat- and cold-sensing neurons express TRPV1 and TRPM8, respectively[36].

Sensory neuron axon terminals project to different laminae of the spinal cord, depending on the stimulus they encode. Innocuous sensory afferents project to deeper layers of the spinal cord, whereas nociceptors are thought to project to superficial lamina, most notably lamina I and II. It is in this region that they synapse with second order neurons, where the signal propagates up to the brain. The dynamic interactions of excitatory and inhibitory interneurons of the dorsal horn lead to the modification of the afferent signal. Due to their ease of access, modulation of sensory neurons input and output is best studied in the skin. There have been two long-standing theories about the function of sensory neurons. The specificity theory suggests that individual sensory neurons encode specific modalities in a direct-line from the skin to the brain, referred to as labelled lines[37]. These primary sensory fibers respond to specific stimuli such as heat, cold and pain; the identification of specific sensory receptors and channels for each type of stimuli

has provided further evidence for the specificity theory[41]. Pattern theories, such as the gate control theory, propose that pain sensations are products of collective primary sensory inputs that are modulated by descending inputs from the rostral ventral medulla[37,41]. It is now understood that there is synergism between the two theories: labelled lines do exist, but they crosstalk to generate and shape somatosensory perception[42].

During inflammatory conditions such as OA, it is generally accepted that C-fibers become sensitized to mechanical stimuli. The underlying mechanisms for this sensitization can be via changes in the function of voltage gated ion channels or of the mechanotransduction apparatus in the nerve terminals of joint nociceptors[43,44,45]. The central components of this apparatus are mechanosensitive ion channels (MSCs) that convert mechanical forces in to electrical signals. Indeed, nociceptors express MSCs that constitute the first site of mechanically-induced excitation. The role of these channels in pain transmission may therefore be as fundamental to OA pain as the role of photoreceptors is to vision.

1.3 Mechanotransduction

Mechanotransduction is a fundamental process that is central to many physiological functions such as our senses of touch (including pain) and hearing, as well as our ability to regulate osmolarity (myogenic tone)[46,47]. Mechanotransduction is a property of sensory afferents, which are alternatively known as mechanoreceptors. Mechanoreceptors are tuned to detect specific stimuli, a property that is established at the nerve terminal. The sensory nerve endings express sensor molecules – ion channels, or proteins linked to ion channels. When a

mechanoreceptor is stimulated with its corresponding stimulus, MSCs are opened, causing a depolarizing potential due to influx of sodium and calcium ions[16]. This depolarization travels down the axon to reach the action potential trigger zone. If the depolarization is above the threshold of activation, an action potential is generated and conducted to the spinal cord dorsal horn[16].

1.3.1 Mechanosensitive ion channels

MSCs are the ion channels that underlie the mechanoreceptor stimulus detection. These proteins are embedded in the membrane and are thus subject to the anisotropic nature of the bilayer; the polar head groups and the long-chain fatty acids of the bilayer provide a force profile on embedded proteins[48,49]. As a result, any tension in the membrane disrupts this force profile, which may make it more energetically favorable for the protein to assume a new conformation[50]. This dynamic relationship between the lipid bilayer and membrane-soluble proteins forms the basis for the "bilayer model" of channel opening[51]. This model suggests that mechanical stimulation changes membrane tension, thus opening ion channels that signal the presence of mechanical stimuli. Another model proposed for the gating of MSCs is the tethered model. This model involves the physical interaction of the ion channel with structural proteins in the cytoskeleton or extracellular matrix[51]. It is thought that the structural scaffolding is what is sensitive to the mechanical stimulation, where movement of these structures opens the ion channels. These proteins pull (via the tether) with enough force to overcome the force profile of the lipid bilayer.

1.4 Ion channels involved in pain

1.4.1 Transient receptor potential ion channels

Transient receptor potential (TRP) channels are MSC candidates and are part of a large superfamily of proteins that possess homologs in organisms such as the worm, fly, mouse and human, suggesting a highly conserved protein[52]. These TRP channels are mostly non-selective cation channels that are weakly voltage-sensitive and are involved in a wide variety of cellular signalling processes, including mechanotransduction.

Drosophila possess sensory bristles that cover their body and mediate touch sensitivity. A TRPN1 channel known as NOMPC (no mechanoreceptor potential C) was identified as a candidate MSC that underlies Drosophila touch sensitivity through a genetic screen for touch insensitive animals[53]. Additionally, the presence of 29 ankyrin repeats at the amino-terminus of TRPN1/NOMPC may serve as tension transmission structures to the pore-forming region[46]. It was shown that three out of four mutant nompC alleles all but eliminated the transient current response to bristle deflection[52]. However, the presence of stimulus-mediated currents in a fourth mutant affecting the extracellular loop between the third and fourth transmembrane domains suggests that TRPN1/NOMPC is involved in the amplification process of the true MSC[52,54]. The evidence for TRPN1 in the amplification of mechanical inputs was also reported in the auditory systems of Drosophila, some fish and amphibians[53,54,55].

The mammalian homologue of TRPN1 that possesses the N-terminal ankyrin repeat is TRPA1[46]. It has been characterized as a possible MSC, as the C. elegans ortholog of mouse

TRPA1 is expressed in some mechanosensory neurons and contributes to neural responses of these cells to touch. TRPA1 was at first considered the sensor for noxious cold (<18°C), but there is no consensus that it really is a noxious cold sensor[16,36]. It is activated by bradykinin and pungent ingredients of mustard oil and garlic[56]. TRPA1 has also been shown to be involved in the nociceptive process, being present in small Aδ and C-fiber afferents; TRPA1 knockout mice show an increase in mechanical pain threshold[57]. Despite data showing TRPA1 is mechanosensitive, it has yet to be concluded whether TRPA1 is an MSC or an accessory to the mechanotransduction process, whereby it amplifies or modulates the signal from the MSC as seen in TRPN1.

The vanilloid channel subtypes (TRPV), due to their similarity with known C. elegans MSCs, are important candidates for MSC activity[58]. The TRPV1 channel plays an important role in nociception and is a constant target for pain drug therapy[46,59]. TRPV1 is a ligand-gated ion channel that when stimulated, becomes permeant to cations (particularly calcium). TRPV1 is almost exclusively expressed in nociceptors and has several key characteristics: It is a heat sensor activated at temperatures above 43°C, a temperature considered painful for humans[16]. This is supported by evidence that TRPV1 knockout mice show attenuated responses to noxious heat[36], suggesting the importance, but not necessity, of TRPV1 in noxious heat sensing. TRPV1 is also activated by capsaicin and ethanol (applied to a wound) that elicit burning pain[60]. TRPV1 is activated by low pH conditions (<5.9), such as those seen in inflamed tissue, as well as by inflammatory mediators such as arachidonic acid metabolites (produced by lipoxygenases such as 12-hydroperoxyeicosaenoic acid (12-HPETE), and by endocannabinoids such as anandamide and N-Arachidonoyl dopamine (NADA)[60]. Additionally, TRPV1 is indirectly sensitized by bradykinin, prostaglandin E2, extracellular ATP, glutamate, proteases, and NGF[56,60]. At the

cellular level, sensitization may be a result of increased TRPV1 expression, protein phosphorylation, and the release of TRPV1-inhibition (by phosphatidyl-inositol-4,5-biphosphate)[16]. Such sensitization results in the lowering of the temperature threshold, causing activation of TRPV1 by normal body temperatures (thermal hypersensitivity)[61]. TRPV1 knockout mice show reduced inflammation-induced thermal hyperalgesia in carrageenan- or complete Freund's adjuvant (CFA)-induced inflammation[56,60]. One of the issues of TRPV1 antagonists as therapeutic agents is that inflammatory pain in humans usually translates to mechanical (and not thermal) hyperalgesia (meaning the sensitization of nociceptive system to mechanical stimulation)[59]. More recently, it was demonstrated that TRPV1 was involved in the development of mechanical hyperalgesia in adjuvant-induced chronic arthritis[56]. Therefore the effectiveness of TRPV1 antagonists for treatment of mechanical hyperalgesia (seen in OA) remains questionable.

Other TRP channels are present in sensory neurons, and some colocalize with TRPV1, although their role and significance in pain is much less understood. TRPV2 is a candidate MSC since heterologously-expressed TRPV2 increased mechanosensitivity in Chinese hamster ovary cells[62]. TRPV2 is also activated by high temperatures (~>52°C), and may act as the heat sensor in high temperature-threshold Aδ nociceptors[16]. TRPV2 is colocalized with TRPV1 in just a small percentage of DRG neurons, whereas it predominantly expressed in medium diameter sensory neurons[54,56]. TRPV2 is also upregulated in DRG neurons following intra-plantar injection of CFA[56]. However, TRPV2 knockout mice showed normal behavioral responses to noxious heat and mechanical stimuli, and electrophysiological recordings showed C- and Aδ-fibers responded normally to mechanical stimuli[63]. Another TRPV channel, TRPV4, has been implicated in hearing, osmosensitivity, and nociception. TRPV4 is activated by innocuous

temperatures (>27°C), and has been implicated in mechanical hyperalgesia following exposure to inflammatory mediators[16,36]. TRPV4 is activated by phorbolester, low pH, citrate, endocannabinoids, arachidonic acid metabolites, and NO[61]. As an MSC candidate, it was found that TRPV4 lacked inherent MSC function, since knockout only has modest effects on mice mechanosensation, despite the phenotype having a reduced sensitivity to noxious mechanical stimuli[46]. Additionally, the long latency of activation (suggesting the implication of second messengers), and required upstream metabolite for proper function, imply TRPV4 may have a modulatory function in the mechanotransduction process[46,64].

Sensory neurons express stretch-sensitive ion channels that have been proposed to be involved in mammalian mechanotransduction. The canonical TRP channel TRPC1 was first thought to be activated by membrane stretch in Xenopus oocytes and was found to be expressed in somatosensory neurons[65]. Heterologous expression of TRPC1 significantly increased mechanosensitivity, while knockdown of TRPC1 abolished endogenous mechanosensitivity in Xenopus oocytes[65]. However upon further inspection, it was concluded that TRPC1 is in fact not mechanosensitive and is not directly gated by membrane stretch[66]. Another stretch-sensitive channel candidate, the two-pore potassium channel TREK-1, has been shown to be involved in touch[54]. TREK-1 is expressed in a subset of C-fibre nociceptors and there is evidence to suggest these channels regulate firing responses of mechanoreceptors[46]. TREK-1 knockout mice demonstrate an increased sensitivity to low-threshold mechanical stimuli, but they maintained normal sensitivity to noxious pressure[67]. However, it remains to be determined whether these channels are direct transducers of mechanical stimuli or regulators of neuronal excitability[46].

1.4.2 Acid-sensing ion channels

Acid-sensing ion channels (ASICs) are members of the degenerin/epithelial sodium channels (DEG/ENaCs) and were discovered based on their homology with previously identified mechanosensory (MEC) proteins in C.elegans[58]. As their name suggests, they are highly selective for sodium. ASICs are expressed in many DRG neurons, including nociceptors, and were candidates for a mechanotransduction channel[36,54]. Due to their sensitivity to acidic extracellular environments, they may play also role in inflammatory pain where tissue acidosis often occurs[16]. They may be most important in skeletal muscle and heart, where impaired circulation causes immediate pain[36]. Three members of the ASIC family are expressed in mechanoreceptors and nociceptors[46]. ASIC1 is present in most somatosensory neurons, and despite knockout phenotypes having increased visceral mechanotransduction, no cutaneous mechanotransduction effects were noticed[68]. ASIC2 and ASIC3 are coexpressed in medium and large diameter DRG neurons, as well as at the peripheral terminals of cutaneous mechanoreceptors[46]. However, knockout of ASIC2 and ASIC3 show limited effects on mechanotransduction[68]. Taken together, ASICs may play a general role in neuronal excitability rather than specific roles in mechanotransduction, or may help modulate the mechanotransduction process through association with more specialized mechanotransduction molecules.

1.4.3 P2X receptors

Extracellular ATP has been shown to act as a pain mediator in some tissues[16]. Ligand-gated purinergic receptors (P2X2 and P2X3) are non-selective cation channels that are activated by ATP. The release of ATP from damaged cells and keratinocytes of inflamed skin may underlie inflammatory hyperalgesia[61]. The calcium influx depolarizes cells, causing secondary calcium influx via voltage-gated calcium channels.

1.4.4 Voltage-gated cation channels

Voltage gated sodium channels are fundamental to action potential generation and conductance. Local anaesthetics work by blocking voltage-gated sodium channels, thus having an analgesic effect[61]. However, this effect occurs in all nerve fibers (including touch receptors, motor neurons, and thermoreceptors) and as such local anaesthetics are not a sufficient long-term treatment for pain[16]. The discovery of differential expression of subset sodium channels in nociceptive neurons allows for the possibility of targeting voltage-gated sodium channels as pain treatments.

1.4.5 Piezo proteins

The most recent and promising finding in the field of mechanotransduction has been the work done by the Patapoutian group on Piezo proteins. The Piezo proteins were discovered while characterizing the genes involved in the rapidly adapting currents in mechanosensitive neuroblastoma cells[69]. By knocking down candidate MSC Piezo1, they observed a significant decrease in mechanically activated current. Overexpression of mouse Piezo1 in various cell types

also conferred mechanically activated currents. A Piezo homolog conferred mechanosensitivity when heterologously expressed in various cell types and was labelled Piezo2. The Piezo subtypes are differentially expressed: Piezo1 is barely detectable in DRG neurons whereas Piezo2 is quite abundant. In situ hybridization shows approximately 20% of adult mouse DRGs contain Piezo2 mRNA. As such, Piezo2 has potential roles in touch and pain[69].

The group then looked at the role of the Drosophila melanogaster Piezo protein (DmPiezo) in nociception. Their studies revealed that DmPiezo is at least partly responsible for mechanical nociception in the fly, having demonstrated mechanosensitive properties while overexpressed in human embryonic kidney cells (HEK293T) and eliminating mechanical nociceptive response in fly knockouts[70]. DmPiezo's diverse expression in sensory neurons and non-neuronal tissues alike is consistent with that of Piezo1 and Piezo2 in mice. DmPiezo knockout phenotypes in Drosophila larvae showed only mechanical nociception insensitivity (no thermal nociception or gentle touch deficits), and DmPiezo knockdown showed a significant reduction in noxious mechanosensitivity. This work showed Piezo is an essential factor in mechanotransduction in the entire organism and further implicates the mammalian Piezo2 in pain transduction, since Piezo2-expressing DRGs were concluded to be both mechanosensitive and nociceptive.

Further analysis by the same group demonstrated that the piezo proteins are extremely large, with mouse Piezo1 (MmPiezo1) being reported as having 120-160 transmembrane domains and a molecular weight of approximately 1.2 MDa in the active mammalian homotetramer[71]. Overexpression of MmPiezo1 in human cell line shows a mechanically activated channel with unique biophysical properties. Interestingly this protein appears to utilize

a novel channel structure, as MmPiezo1 retains its mechanosensitivity in reconstituted lipid bilayers despite lacking any classical ion channel domains. MmPiezo1 remains sensitive to the channel blocker ruthenium red in this synthetic membrane, an important feature in characterizing MSCs. These results conclude Piezos are in fact MSCs, but their exact role in mechanosensation remains unsolved.

1.5 Models of OA in mice

In order to study OA pain, animal models are needed to decipher the underlying mechanisms and to validate therapeutic targets[27]. OA can arise either spontaneously in certain strains of mice and guinea pig or can be induced chemically or surgically[71,72].

1.5.1 Monoiodoacetate

Monosodium iodoacetate (MIA) is a chemical inducer of OA. Intra-articular injection of MIA produces pathological symptoms similar to OA, including cartilage degradation/loss, inflammation, subchondral bone alterations, and osteophyte formation (Figure 1-4) [72,73,74]. MIA works as an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, which induces apoptosis in chondrocytes in vivo and in vitro[74]. This model is a well-characterized and established preclinical model of OA[74]. Intra-articular injection of MIA also induces behavioral responses consistent with the manifestation of pain, seen as changes in hind-limb weight bearing in rats and mice[72].

1.5.2 Dissection of the medial meniscus

Surgical induction of OA is most often done by transection of the medial collateral ligament and an incision is made in the meniscus. 3-6 weeks post-surgery, chondrocyte loss and osteophyte formation occurs, where the rapid degeneration of the cartilage produces pathology similar to those of human OA[75].

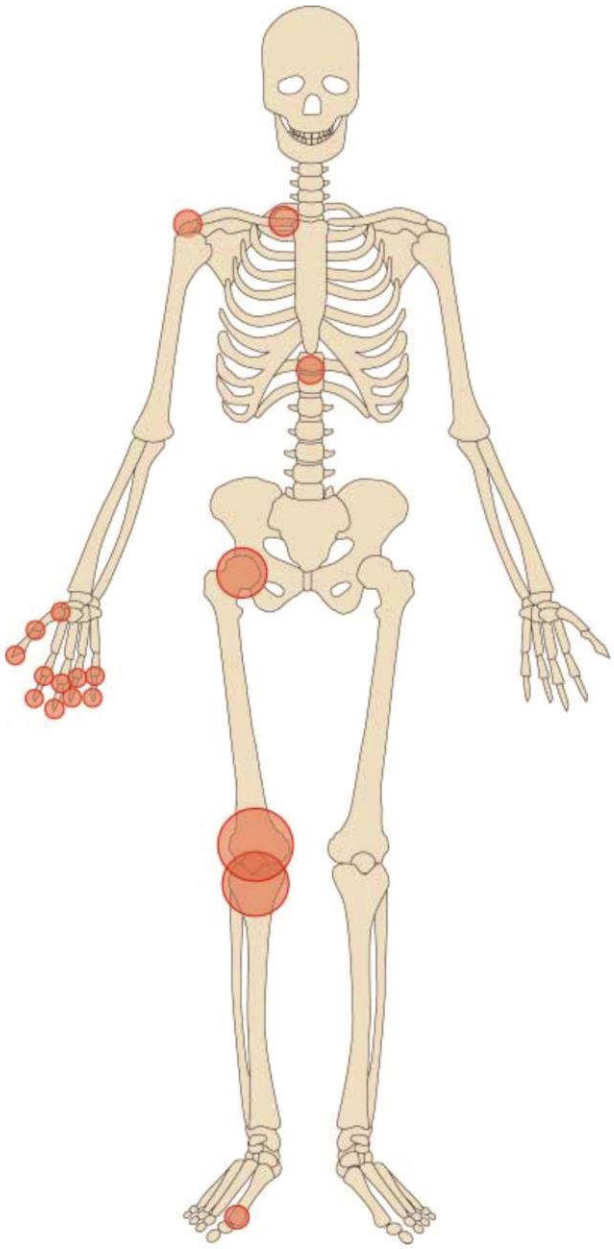

Figure 1-1: The knee is the joint most commonly affected by OA. The hip, shoulder, spine and toes are less frequently affected. OA has a slow, insidious onset and mostly affects only one or a few joints (in contrast to rheumatoid arthritis, which is a systemic multi-joint disease). Adapted from Wieland et al. 2006.

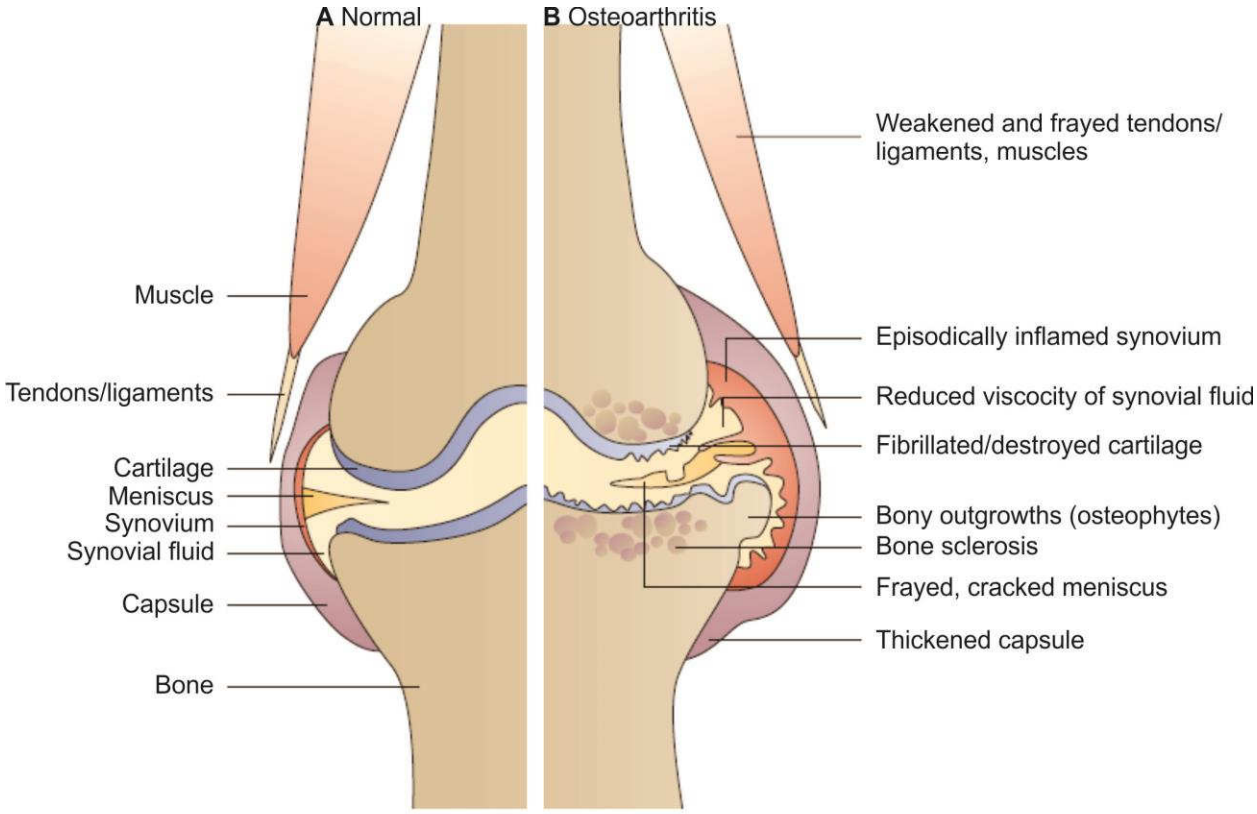

Figure 1-2: Articular structures of the knee joint that are affected in OA. (**A**) Healthy joint is shown: normal cartilage without any fissures, or signs of synovial inflammation. (**B**) OA joint showing early focal degenerate lesions and 'fibrillated' cartilage, as well as remodelling of bone. This can lead to bony outgrowth and subchondral sclerosis. Adapted from Wieland et al. 2006.

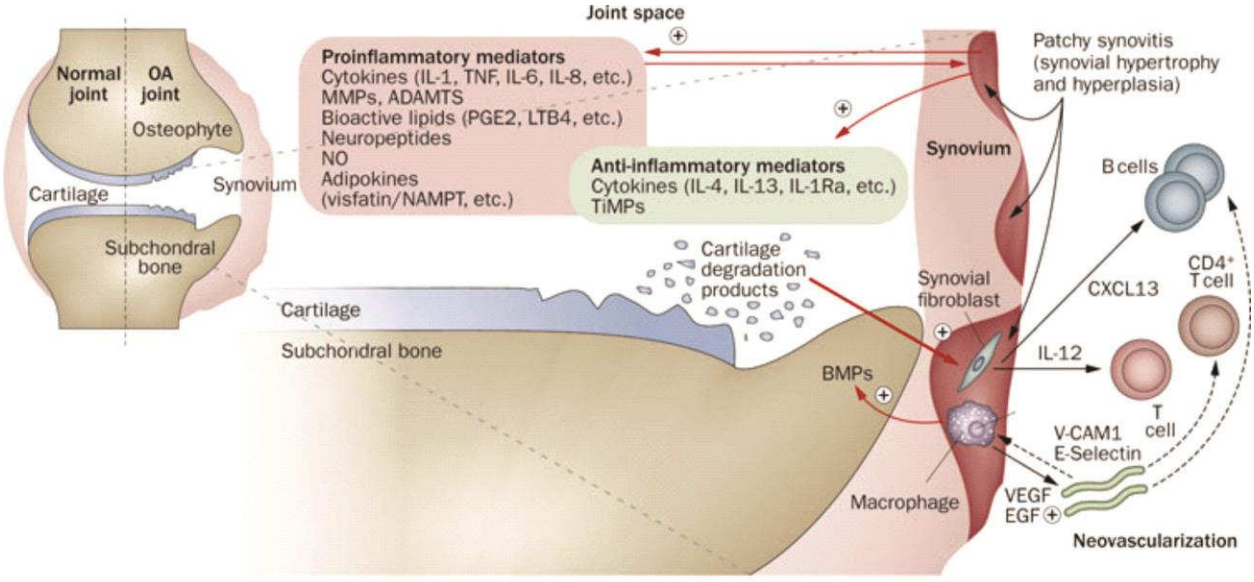

Figure 1-3: Involvement of the synovium in OA pathophysiology. Products of cartilage breakdown that are released into the synovial fluid are phagocytosed by synovial cells, amplifying synovial inflammation. In turn, activated synovial cells in the inflamed synovium produce catabolic and proinflammatory mediators that lead to excess production of the proteolytic enzymes responsible for cartilage breakdown, creating a positive feedback loop. The inflammatory response is amplified by activated synovial T cells, B cells and infiltrating macrophages. To counteract this inflammatory response, the synovium and cartilage may produce anti-inflammatory cytokines. In addition to these effects on cartilage inflammation and breakdown, the inflamed synovium contributes to the formation of osteophytes via BMPs. Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BMP, bone morphogenetic protein; CCL2, CC-chemokine ligand 2; CXCL13, CXC-chemokine ligand 13; EGF, endothelial growth factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; LIF, leukemia inhibitory factor; LTB4, leukotriene B4; MMP, matrix metalloproteinase; NAMPT, nicotinamide phosphoribosyl transferase; NO, nitric oxide; NGF, nerve growth factor; OA, osteoarthritis; PGE2, prostaglandin E2; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; vCAM-1, vascular cell adhesion molecule 1; vEGF, vascular endothelial growth factor. Adapted from Sellam & Berenbaum, 2010.

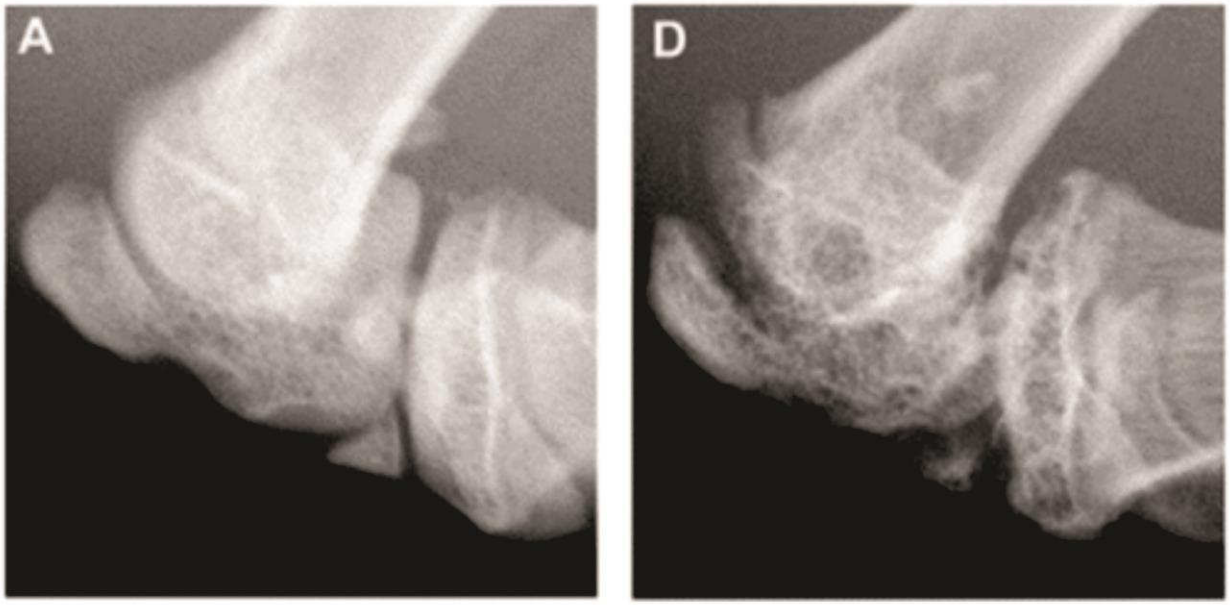

Figure 1-4: Intra-articular injection of MIA results in joint degeneration. High resolution radiographs of knee joints 29 days after injection of saline (**A**) or 3 mg MIA (**D**) demonstrates patellar displacement and rough edges of the tibia and femur, indicative of bone lysis and swelling in rats that received 3 mg MIA. Adapted from Pomonis et al. 2005.

Chapter 2 - Methods

2.1 MIA-induction of OA

Mouse aged 6-8 weeks were anaesthetized using isoflurane. An incision was made in the shaved skin of the left knee. 5 μL of 5% MIA (Sigma, I2512) was injected into the intra-articular space of the flexed knee joint. The skin around the incision was stapled and the mice were placed back in their cage for at least 21 days to allow for the chronic phase of OA pain to set in.

2.2 DRG dissociation

Mice (aged 8-10 weeks) were anaesthetized using isoflurane. Cervical dislocation was performed, followed by removal of the DRGs. Lumbar DRGs L2-L4 were dissected and placed in cold Hank's Buffered Saline Solution (HBSS; Wisent, 311-516-CL). If the mouse was treated with MIA, the ipsilateral and contralateral DRGs were placed in separate eppendorf tubes, and the remaining procedure was carried out for both conditions. DRGs were trimmed under a dissection microscope to remove as much of the remaining axons as possible. A small incision was made in the bulb of the DRG to allow access for the digestion enzymes. DRGs were first placed in 0.25 % trypsin (Wisent, 325-043-EL) for 10 minutes at 37°C, followed by incubation in a 2 mg/mL collagenase IA (MP chemicals, 195109) solution for 45 minutes at 37°C, after which they were placed in Dulbecco's Modified Eagle Medium (DMEM; Wisent, 319-005-CL) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. 100 μL of medium was added for each dish to be used. DRGs were triturated with 3-4 fire polished glass Pasteur

pipettes. Cells were plated on glass-bottom dishes coated with 0.01% poly-L-lysine and 1 μg/mL laminin. 100 μL of cells were placed on the dishes and incubated in at 37°C with 5% CO_2 for at least 45 minutes. Cells were used within 6 hours of plating. If cells were being cultured, an additional 1mL of media was added to each dish with 25 μg/mL NGF.

2.3 Cell culture

A cell line of fibroblast-like African green monkey kidney cells (COS-7) were used. TMEM constructs were transfected using Lipofectamine (Invitrogen, 18324-012). 5 μg DNA was added to COS-7 cells, at a confluency of approximately 50%. Cells were incubated with the transfection media for 5-6 hours at 37°C with 5% CO_2, after which the regular DMEM media (10% FBS, 1% penicillin/streptomycin) was added. Cells were split the next day into glass bottom dishes for electrophysiology recordings.

2.4 Electrophysiology

Pipette solution contained 150 mM NaCl, 5 mM KCl, 1 mM CaCl2, and 10 mM HEPES (pH 7.4 with NaOH). Cell-attached recordings in DRG neurons were performed using the same bath solution as in the recording electrode. For recordings performed with COS-7 cells, the bath medium contained 155 mM KCl, 5 mM EGTA, 3 mM $MgCl_2$, and 10 mM HEPES 10 (pH 7.2 with KOH). Electrodes were pulled and fire-polished to resistances of 1.5-2.0 MΩ.

2.5 Activation of MSCs

In the cell attached configuration, neurons were voltage-clamped at -80 mV. Brief pulses of negative pressure were applied through the recording electrode. The pulse duration was 400 ms, with each sweep increasing by 10 mmHg negative pressure. Each run contained 11 sweeps, with the final pulse being -100 mmHg.

2.6 Single-cell PCR

RT-PCR was conducted on total RNA extracted from single acutely-dissociated DRG neurons. Gene-specific forward and reverse oligonucleotide primers were designed from unique sequences in different exons of the both TMEM1 and TMEM5 genes. β-actin was amplified in the same samples to monitor template amount in each reaction. 20 μL PCR reactions were carried out containing 2 μL of cDNA synthesized from single cell RNA, 0.1 μL Phusion DNA polymerase (Thermo scientific, F-530S), 4 μL 5x Phusion buffer, 0.4 μL dNTPs, and 0.6 μL primers. PCR products were run on a 10% agarose gel with ethidium bromide. Gels were analyzed by UV light.

2.7 Statistical Analysis

Results are represented as mean ± SEM. Statistical significance was tested using t-tests for comparison of paired means and by ANOVA and Tukey post-hoc test for comparisons among several means. Differences were considered significant for $p < 0.05$ (*).

Chapter 3 – Results

The epidemic of OA desperately requires research into the mechanisms of OA pain. With mechanical allodynia being a major symptom of OA, we directed our research towards its underlying molecular mechanisms. Our central hypothesis is that mechanical allodynia is due to the sensitization of MSCs in nociceptors, such that they become activated by innocuous stimuli.

Studies of the mechanisms underlying mechanical sensitization of articular nociceptors in OA require animal models that replicate OA joint pathology and associated pain symptoms. The MIA model is a particularly useful OA model for the study of pain and analgesic drug effects because it is reproducible and mimics the pathological and pain features of human OA[27]. As the model progresses into the third week post-MIA injection, the subchondral bone becomes exposed, generating joint impairment and associated pain symptoms[72]. The ladder includes ongoing pain as well as mechanical allodynia of the ipsilateral knee. Mechanical allodynia is also present in the ipsilateral hindpaw, indicating distal secondary allodynia, a common observation in human OA[76,77,78,79,80,81,82]. More importantly, it is possible to isolate articular nociceptors from MIA-injected mice and study the molecular mechanisms responsible for their increased mechanosensitivity in acute or cultured preparations.

3.1 MIA-induced OA symptoms in mice

Our experiments indicate that intra-articular injection of MIA produces a secondary mechanical allodynia that persists for as long as four weeks after injection (Figure 3-1) as verified by von Frey behavioral experiments performed by Hossein Taheri and Behrang Sharif.

Mice injected with MIA gained weight over time in a similar manner as naïve animals. Injection of MIA did not cause a deterioration of the animals' health as evidenced by the similar motor function and grooming patterns between groups. The intra-articular injection of MIA in the left knee of mice (aged 6-8 weeks) caused a significant reduction in mechanical paw withdrawal threshold after 1 day (54 ± 5 % of pre-MIA thresholds in ipsilateral DRG neurons compared to 104 ± 3 % in contralateral, N= 20 for each group; $p<0.01$). This effect was maintained into the chronic phase of the OA, where the lowest mechanical paw withdrawal threshold occurred at day 21 (31 ± 5 % in ipsilateral DRG neurons compared to 108 ± 3 % in contralateral; $p<0.001$).

3.2 Changes in the activity of MSCs in sensory neurons isolated from naïve or OA mice

Sensitization of nociceptors to mechanical stimuli has been proposed as a mechanism underlying mechanical allodynia. Sensitized nociceptors have a reduced threshold for activation by mechanical stimuli. We hypothesized that if this sensitization is due to MSCs, we would expect these channels to have a reduced activation threshold. Indeed, MSC activation occurs at lower pressures in neurons isolated from OA mice (Figure 3-2 A). Quantification of the minimum pressure required to elicit the first MSC opening revealed that MSCs in OA activate at significantly lower pressures (Figure 3-2 B; -48 ± 4.7 mmHg, N=39, in ipsilateral DRG neurons compared to -57 ± 4.9 mmHg, N=30, in contralateral). An advantage of the cell attached approach is that is acts as a survey of the cell membrane in terms of channel expression level. Given the scarcity of MSCs in native neurons, only half of the patches are considered active, indicating they have at least MSC (Figure 3-2 C; 52 ± 6.7 %, N=67). Therefore, any increase in the percentage of active patches would suggest an increase in the number of MSCs at the

membrane. Our results demonstrate that the number of active patches is significantly higher in sensory neurons isolated from OA versus naïve mice (Figure 3-2 C; 65.5 ± 6.1 %, N=43, versus 50.8 ± 6.6 %, N=59; p = 0.04). Together, these findings suggest that in OA, an increase in the number of MSCs and a decrease in their activation threshold may explain the mechanical hypersensitivity of DRG neurons.

Despite our results indicating that the sensitivity of MSCs is enhanced in nociceptors after OA, further progress is hampered by our lack of knowledge on the molecular identity of the MSCs. In a recent study on a mechanosensitive smooth muscle cell line, it was identified that the membrane protein polycystin-2 (PC2) is an inhibitory modulator of MSCs (Figure 3-3)[47]. Speculating that PC2 might interact with endogenous MSCs found in these cells, the authors performed a proteomic screen of membrane proteins interacting with PC2, and identified five candidates with multiple transmembrane domains (TMDs) of unknown function (termed TMEMs). The candidate proteins have multiple TMDs and interact with a modulator of MSCs (PC2), and could therefore be interesting candidates for the MSCs expressed in sensory neurons. To determine whether these candidates are expressed in sensory neurons, we isolated mRNA from mouse DRGs to examine the expression of the TMEMs. Our results demonstrate that the mRNA of all 5 TMEMs is present in DRG neurons (Figure 3-4). Therefore these proteins remained interesting MSC candidates.

If these TMEMs are involved in mechanosensitivity, we would expect their expression to change in animal models of chronic pain associated with mechanical hypersensitivity. We isolated mRNA from ipsilateral and contralateral DRGs from OA mice 3-weeks post MIA injection as well as in naïve mice. Our results indicate that the expression of TMEM1,

TMEM5A, and TMEM5B is significantly increased in OA (Figure 3-5; relative units normalized to GAPDH; naïve vs. OA: TMEM1: 0.128 ± 0.006 vs. 0.172 ± 0.013;p = 0.004; TMEM5A: 0.01 ± 0.0005 vs. 0.014 ± 0.002; p = 0.02; TMEM5B: 0.0105 ± 0.001 vs. 0.0137 ± 0.001; p = 0.009). Therefore these proteins remain MSC candidates as they clearly are present in diseased phenotype.

3.3 Can TMEMs form MSCs in heterologous systems?

A common limitation in testing of MSC candidates is that all known cell lines express endogenous MSCs. Therefore, when one has to test MSCs, the baseline mechanosensitivity of the expression system must be taken into consideration. Our results indicate that the epithelial cell line COS-7, commonly used for the study of MSCs, expresses endogenous mechanosensitive channels (Figure 3-6)[47]. To determine whether the identified TMEMs can form mechanosensitive channels or modulators of MSCs, we transfected them in COS-7 cells. Our results indicate that of the 5 TMEMs, only 2 of them had an effect on the activity of MSCs in COS-7 cells (Figure 3-7). TMEM5B caused a significant increase in cellular mechanosensitivity, while TMEM1 caused a significant decrease (Figure 3-8 A). Expression of these TMEMs neither affected the percent of active patches (Figure 3-8 B; mock: 93 ± 2.7 %; TMEM5B: 96 ± 2.0 %, p=0.345; TMEM1: 91 ± 3.4 %, p=0.892) nor the threshold of first channel opening (Figure 3-8 C; mock: -21 ± 1.6 mmHg; TMEM5B: -23 ± 1.3 mmHg, p=0.527; TMEM1: -25 ± 2.3 mmHg, p=0.143). Therefore, TMEM5B remains a candidate MSC since it increased activity in COS-7 cells, while TMEM1 is likely a modulator of mechanosensitivity since it decreased activity.

3.4 TMEM1 and TMEM5 mRNA is expressed in different subpopulations of DRG neurons.

RNA extraction from DRG tissue indicated that all five TMEMs were expressed. However, the positive signal for the TMEMs could originate from Schwann cells surrounding the neurons. To verify that TMEMs are expressed in neurons, and determine which subset of DRG neurons expresses which of the two TMEMs shown to modulate mechanosensitivity; we performed single-cell experiments. Typically nociceptors are <25μm in diameter, whereas non-pain sensing neurons >25μm in diameter[15]. Neurons for this experiment were therefore selected based on this size criterion. Our results indicate that TMEM1 is expressed in small-diameter neurons, whereas TMEM5B is expressed in large-diameter neurons (Figure 3-9). Therefore, the expression of both TMEMs in DRG neurons suggests that these proteins may account for the mechanical hypersensitivity seen in OA.

Further experiments are required to determine the contribution of TMEM to the intrinsic mechanosensitivity of sensory neurons. In these experiments, sensory neurons are cultured in the presence of short interfering RNA (siRNA) targeting specific TMEMs or control siRNAs. To ensure an efficient knockdown, neurons have to be incubated for up to 72h with the siRNA. One potential limitation of this approach is that phenotypic changes may occur in neurons once in culture, such as losing the disease-related difference in MSC activity. To determine whether the changes in MSC activity in OA neurons are maintained after 3 days of culture, we recorded the activity of these channels in neurons isolated from naïve or OA mice. Interestingly, our preliminary results indicate that disease related changes in MSC activity persist even after 3 days in culture (Figure 3-10; 1.29 ± 0.55 pA at -70 mmHg in OA DRG neurons compared to 0.41 ±

0.19 pA at -70 mmHg in naïve; p=0.037). The low number of recordings may explain why only a single point was significantly different in this preliminary analysis, more recordings are to confirm this observation. Nonetheless, this indicates that it is possible to incubate sensory neurons with siRNA against TMEMs to determine whether these candidates contribute to the intrinsic mechanosensitivity of these neurons.

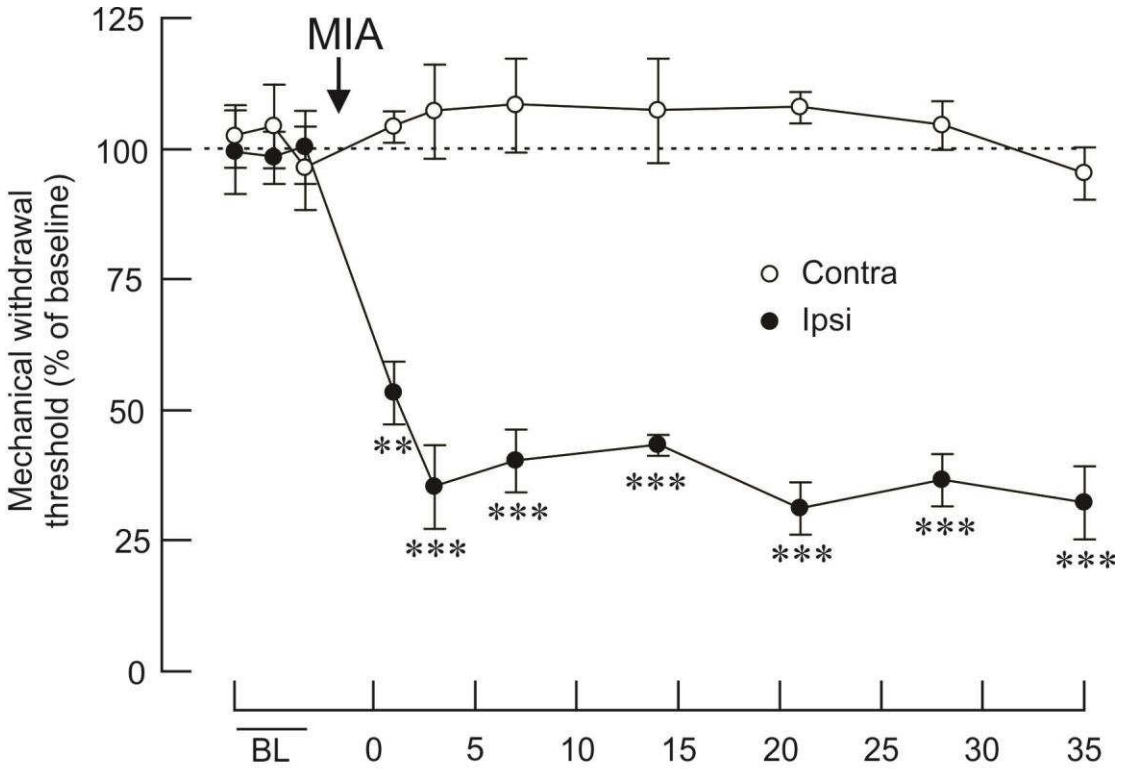

Figure 3-1. Unilateral injection of mono-iodoacetate (MIA) in the knee joint induces secondary mechanical allodynia in the hindpaw. Twenty C57BL/6 mice underwent unilateral (left, ipsilateral) knee injection of MIA. Mechanical withdrawal threshold are significantly reduced on the ipsilateral hindpaw compared to the naive. **=p<0.01; ***=p<0.001.

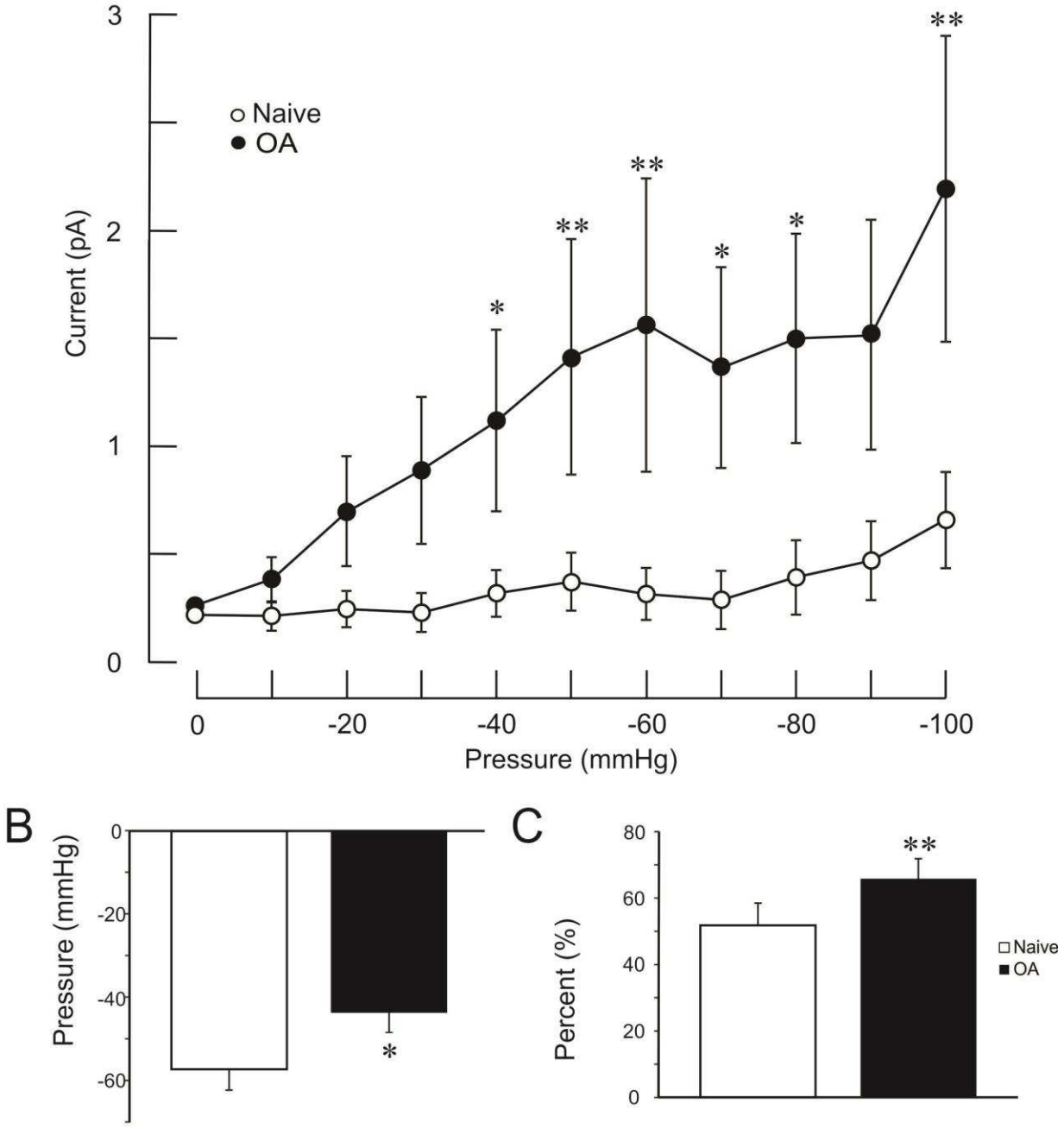

Figure 3-2: Ipsilateral sensory neurons show an increase in stimulus-mediated mean current response. Mechanically-activated currents in nociceptors isolated from OA (dark circles and bars) or naive (white circles and bars) mice. Recordings were performed in the cell-attached configuration. Patches were held at -80mV. (**A**) Stimulus response curves of mechanically-activated currents in response to a 0.4s pressure pulse in neurons of naive (n=68) and OA (n=69) mice. (**B**) Minimum pressure required to trigger the opening of the first mechanosensitive channel in neurons of naive (-57.3 ± 4.9 mm Hg, n=30) and OA (-43.8 ± 4.4 mm Hg, n=39) mice. (**C**) Percentage of patches in which at least one mechanosensitive channel was present in neurons of naive (50.8 ± 6.6%, n=59) and OA (65.5 ± 6.1%, n=43) mice. Statistical significance at *=p<0.05.

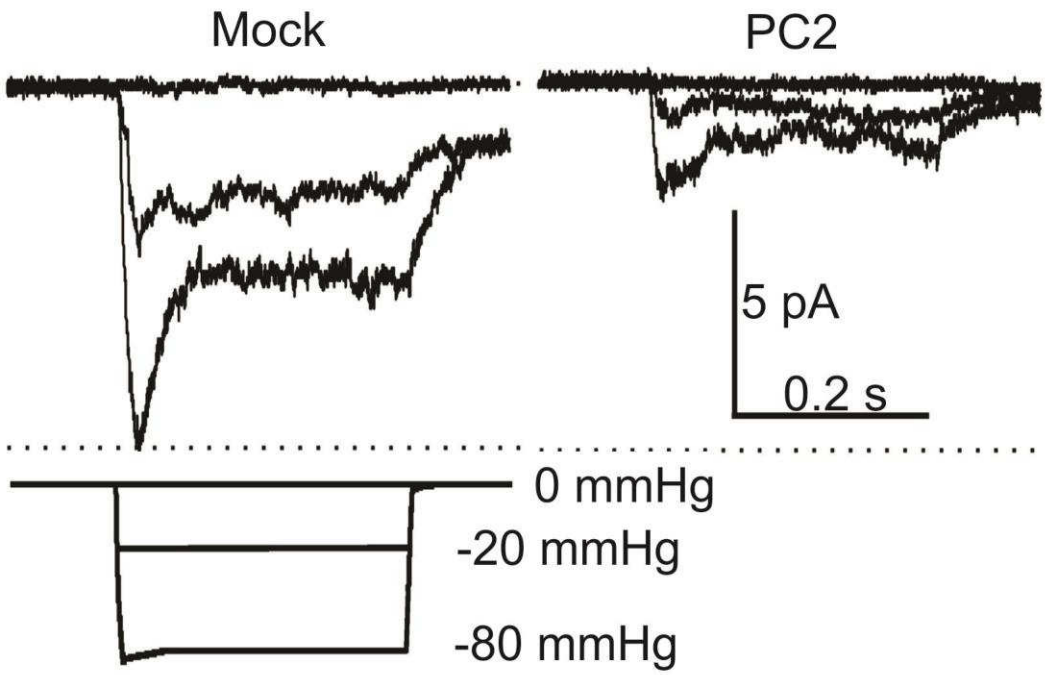

Figure 3-3: PC2 significantly reduces the endogenous MSCs. Mean mechanically activated currents in COS-7 cells transfected with Mock (upper left panel) or PC2 (upper right panel), at a holding potential of -80 mV in the cell attached configuration. Downward deflections (upper panels) represent the averaged current response of MSCs to negative pressure pulses (bottom panel) applied through the recording electrode. In the presence of PC2, there is a significant reduction in mechanically activated currents. Adapted from Sharif-Naeini et al. 2009 (48).

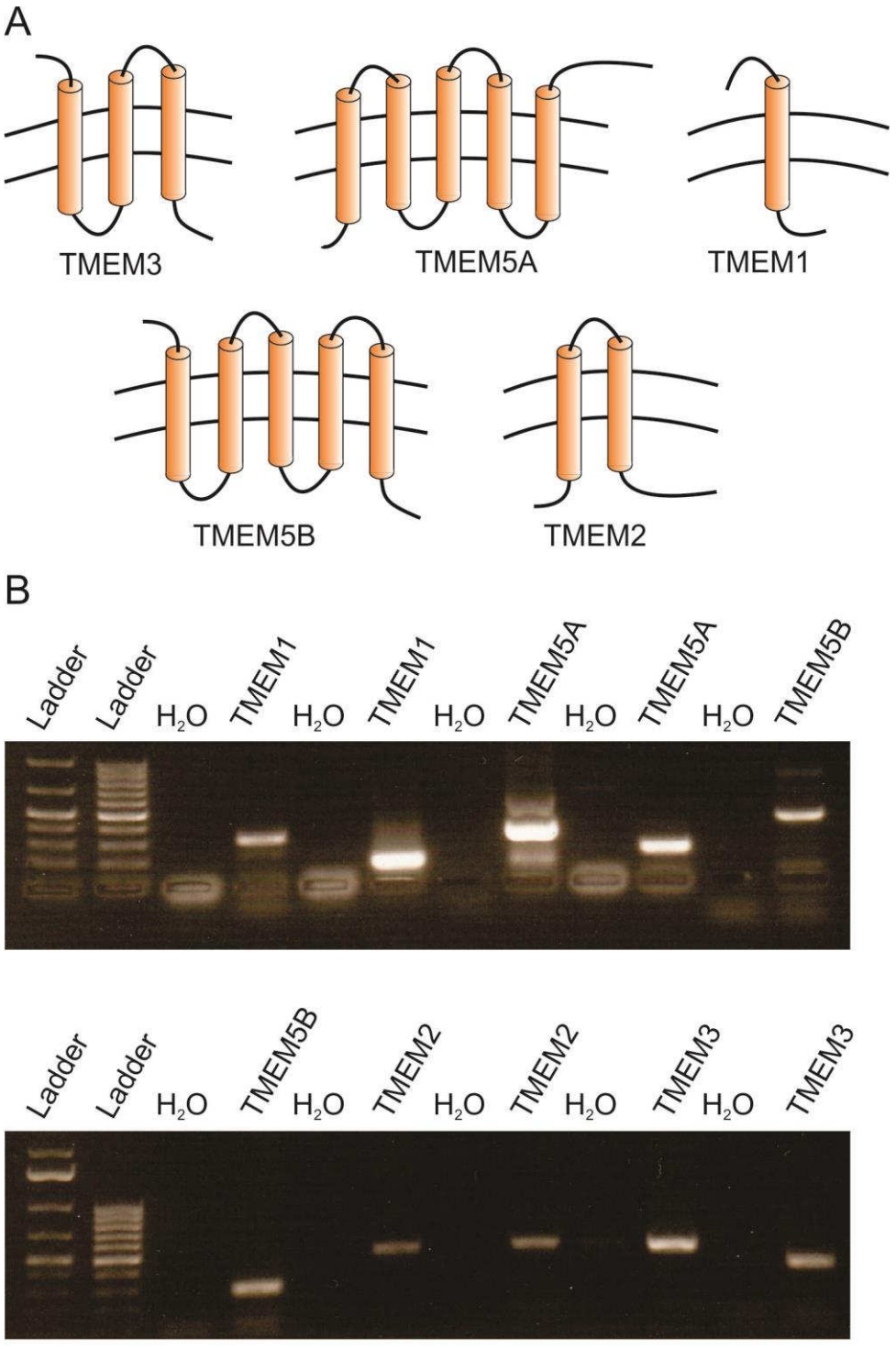

Figure 3-4: The identified TMEMs are expressed in sensory neurons. (**A**) Predicted membrane topology of the five TMEM proteins. (**B**) The messenger RNAs of all five TMEMs are expressed in dorsal root ganglia.

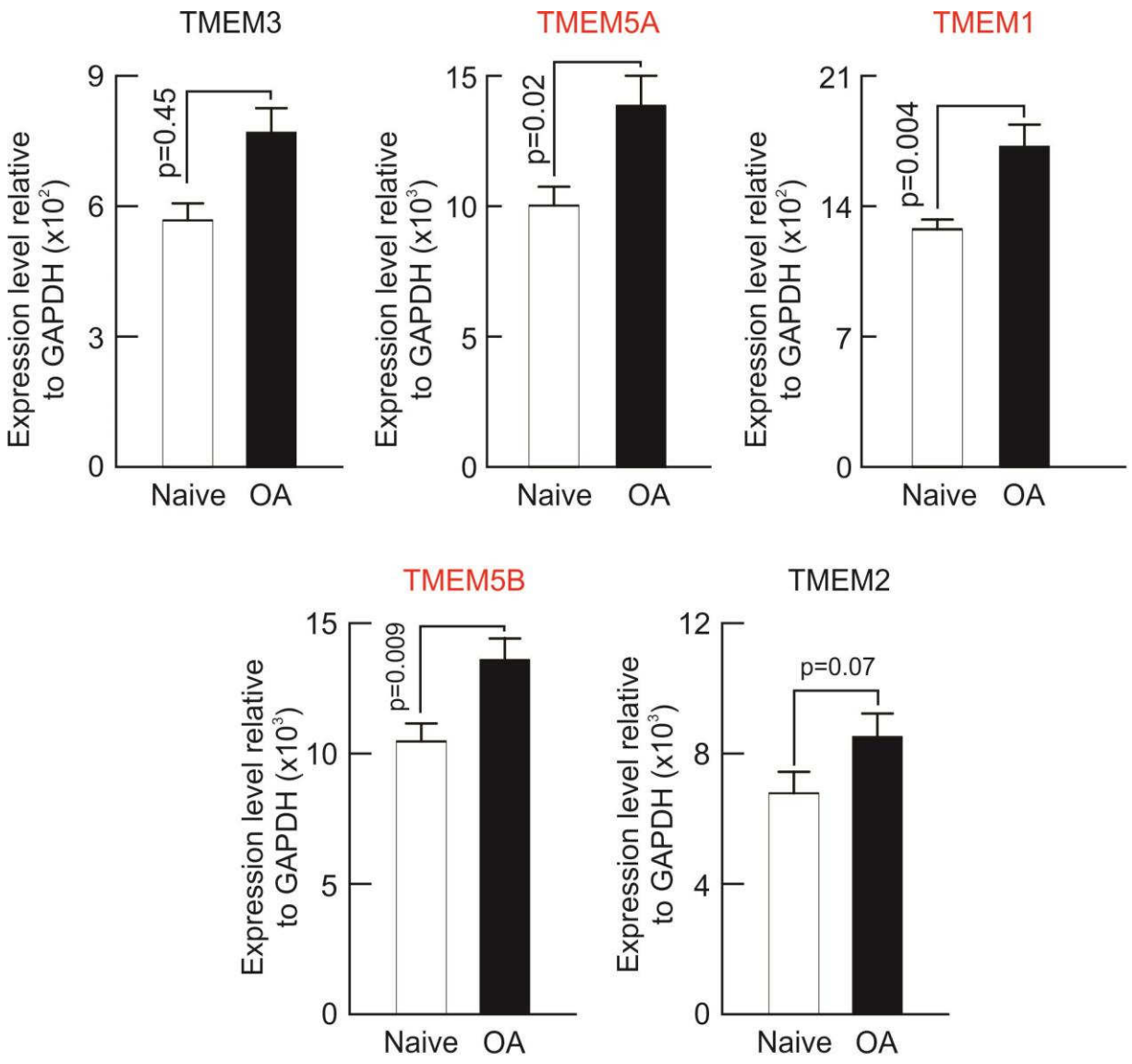

Figure 3-5: Changes in mRNA levels of the candidate TMEMs relative to GAPDH between naive (n=9) and OA (21-days post-MIA, n=10) mice. TMEMs indicated in red are those with a significant increase in mRNA expression compared to controls.

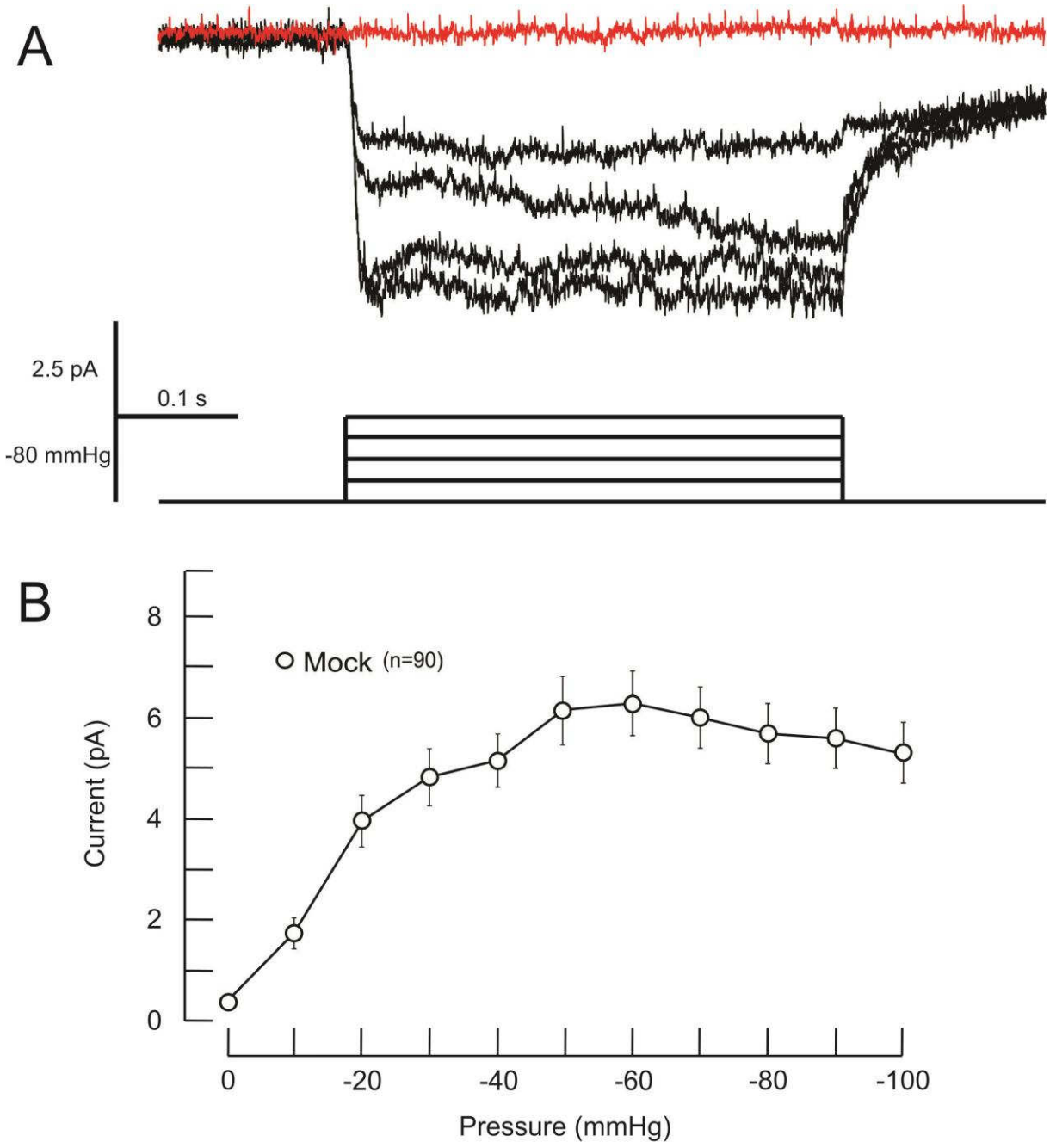

Figure 3-6: COS-7 cells express endogenous MSCs. Characterization of endogenous MSC currents in COS-7 cells. (**A**) Mean mechanically activated currents in COS-7 cells transfected with the empty expression vector (Mock) at the holding potential of -80mV in the cell attached configuration. (**B**) Mean (±s.e.m) current response as a function of pressure in Mock-transfected COS-7 cells.

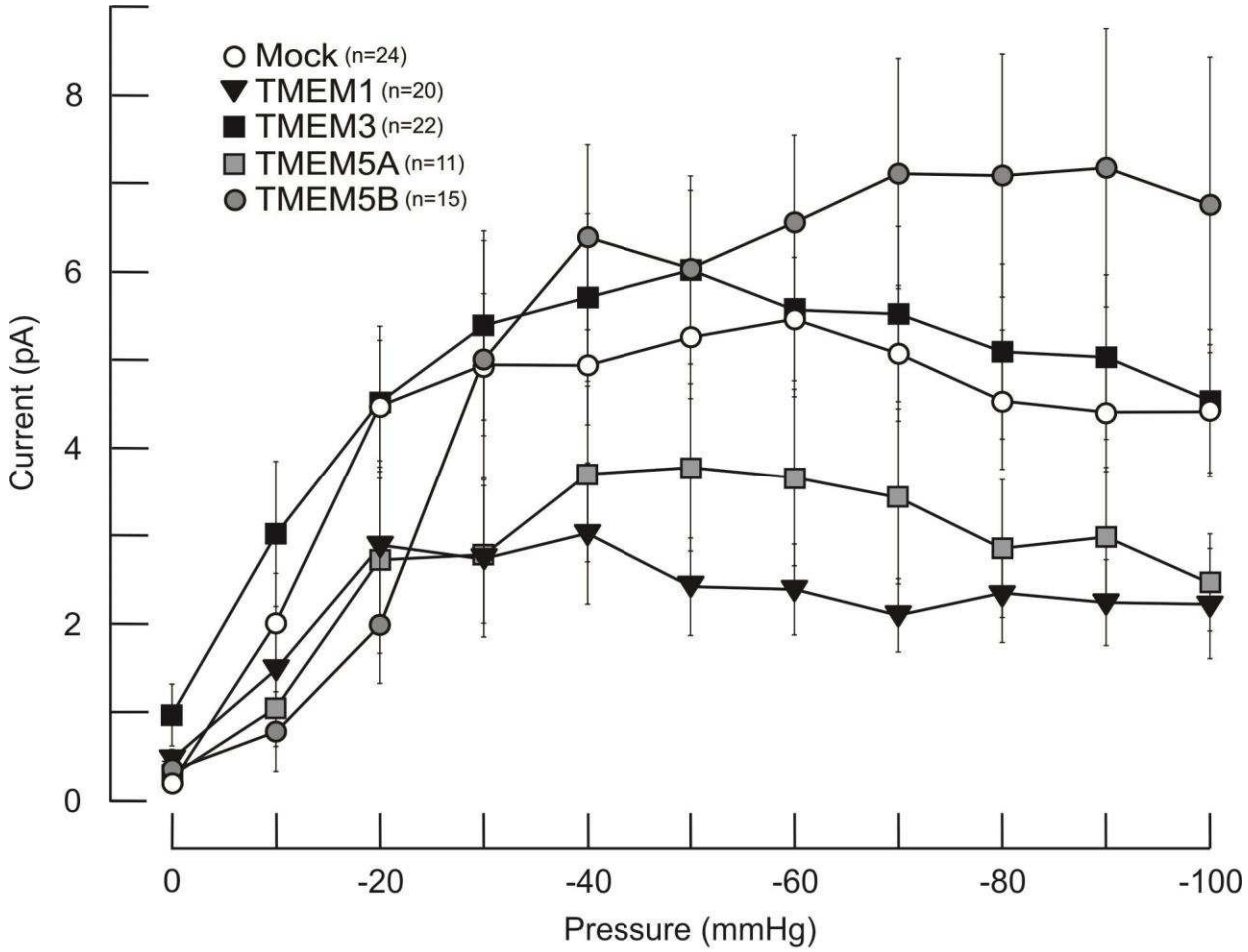

Figure 3-7: Cell-attached recordings show two of the four TMEMs affect cellular mechanosensitivity. COS-7 cells transfected with each TMEM are screened for their effects on cellular mechanosensitivity.

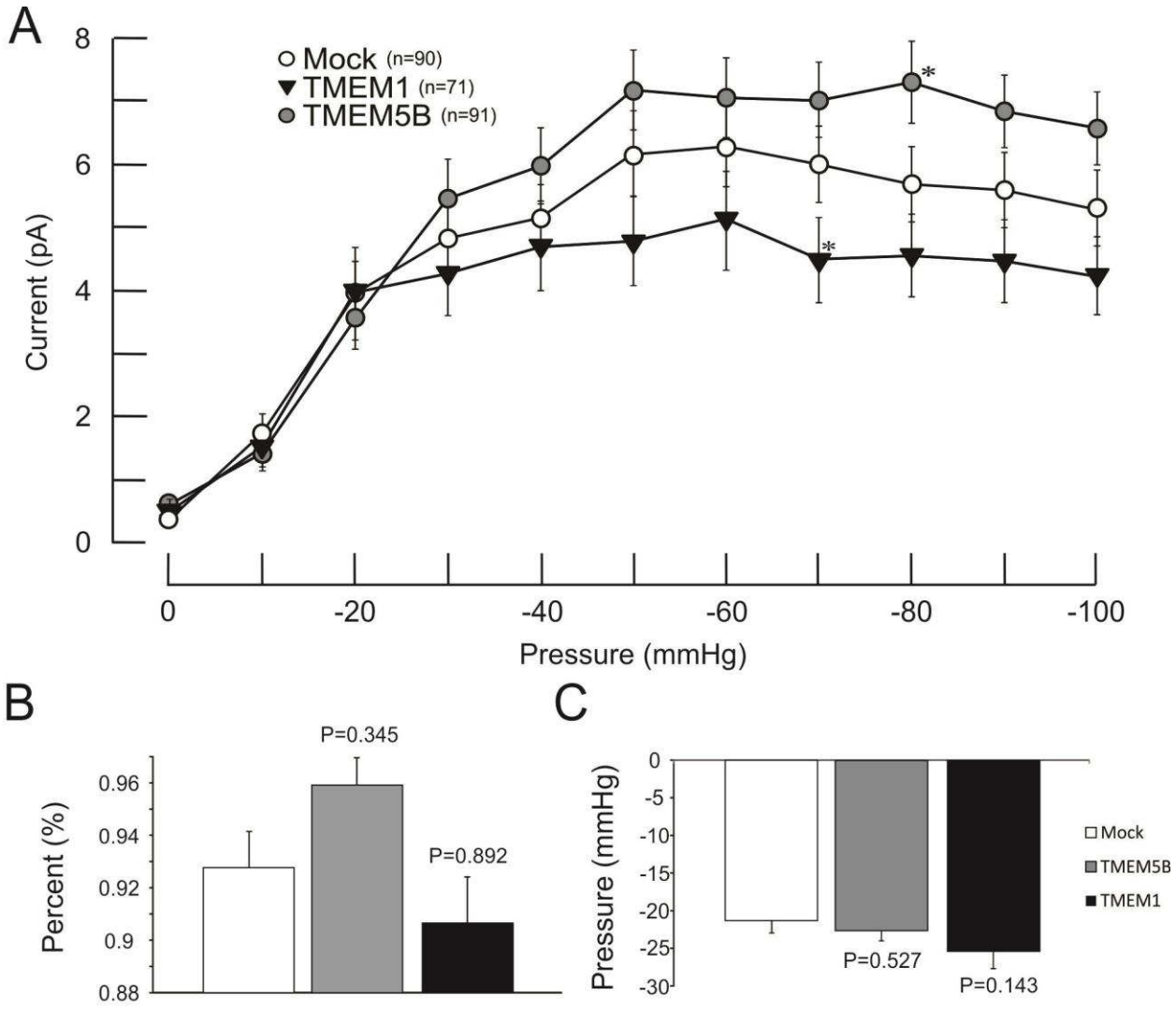

Figure 3-8: Effect of candidate TMEM proteins on COS-7 cellular mechanosensitivity. (**A**) Mean (± s.e.m) current response in Mock-, TMEM5B-, or TMEM1-transfected cells. *=p<0.05, when compared to Mock. Two Way ANOVA with Tukey post-hoc test. TMEM1 reduces COS-7 MA currents, whereas TMEM5B increases mechanically-activated currents. (**B**) Percent of active patches. P-values compared to Mock. (**C**) Average pressure causing the first channel opening. P-values compared to Mock.

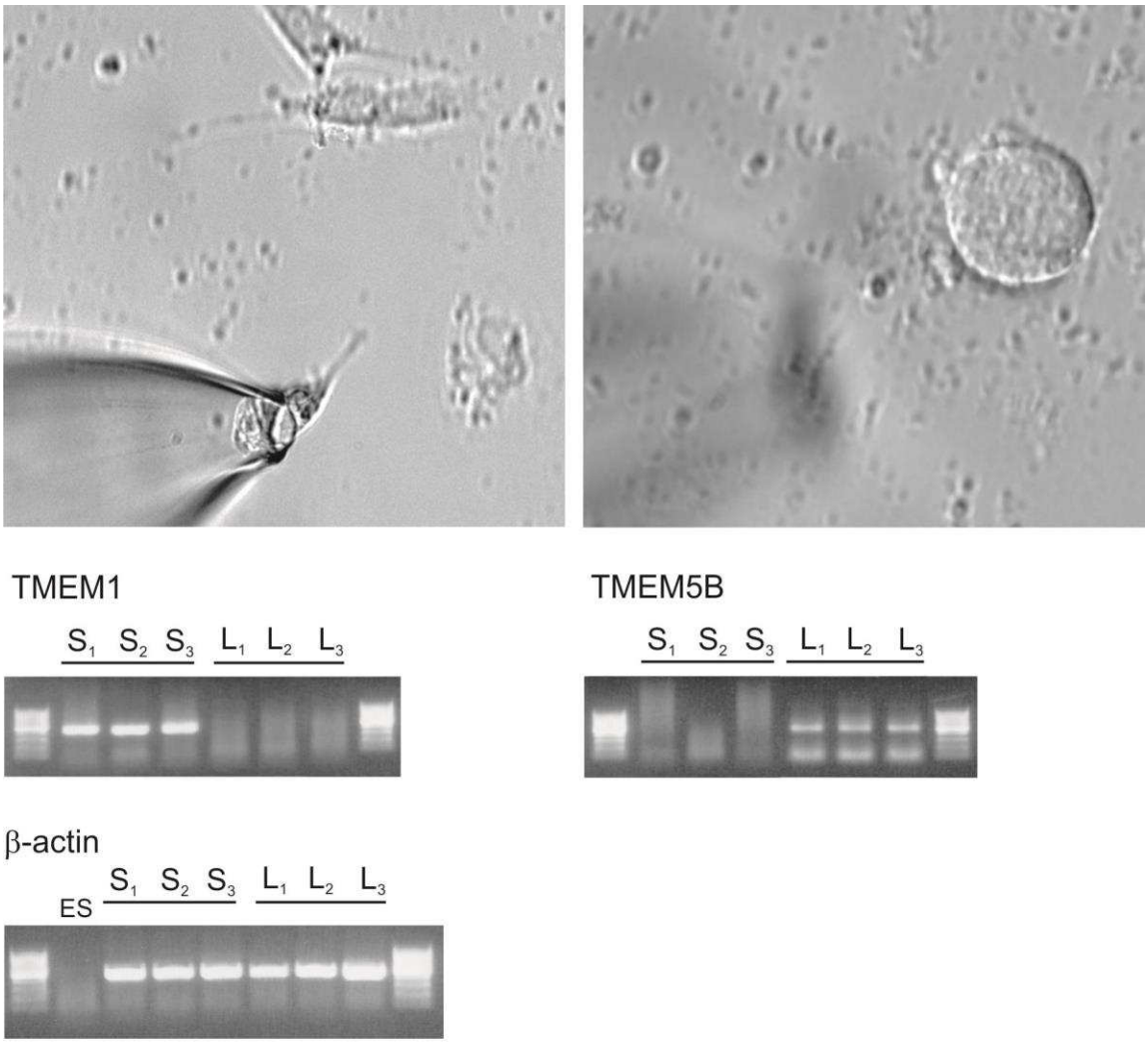

Figure 3-9: TMEM1 and TMEM5B are expressed in small and large diameter neurons, respectively. Photographs display a small diameter sensory neuron (left) entering a suction pipette, or a large diameter neuron (right) about to be collected by a suction pipette. Messenger RNA was extracted, converted to cDNA, and amplified by PCR using primers specific to TMEMs or β-actin. External solution (ES) collected near a cell was run as a negative control. cDNA from each cell was run in triplicates (S1 to S3, and L1 to L3; for small and large diameter neurons, respectively). β-actin was used as the positive control.

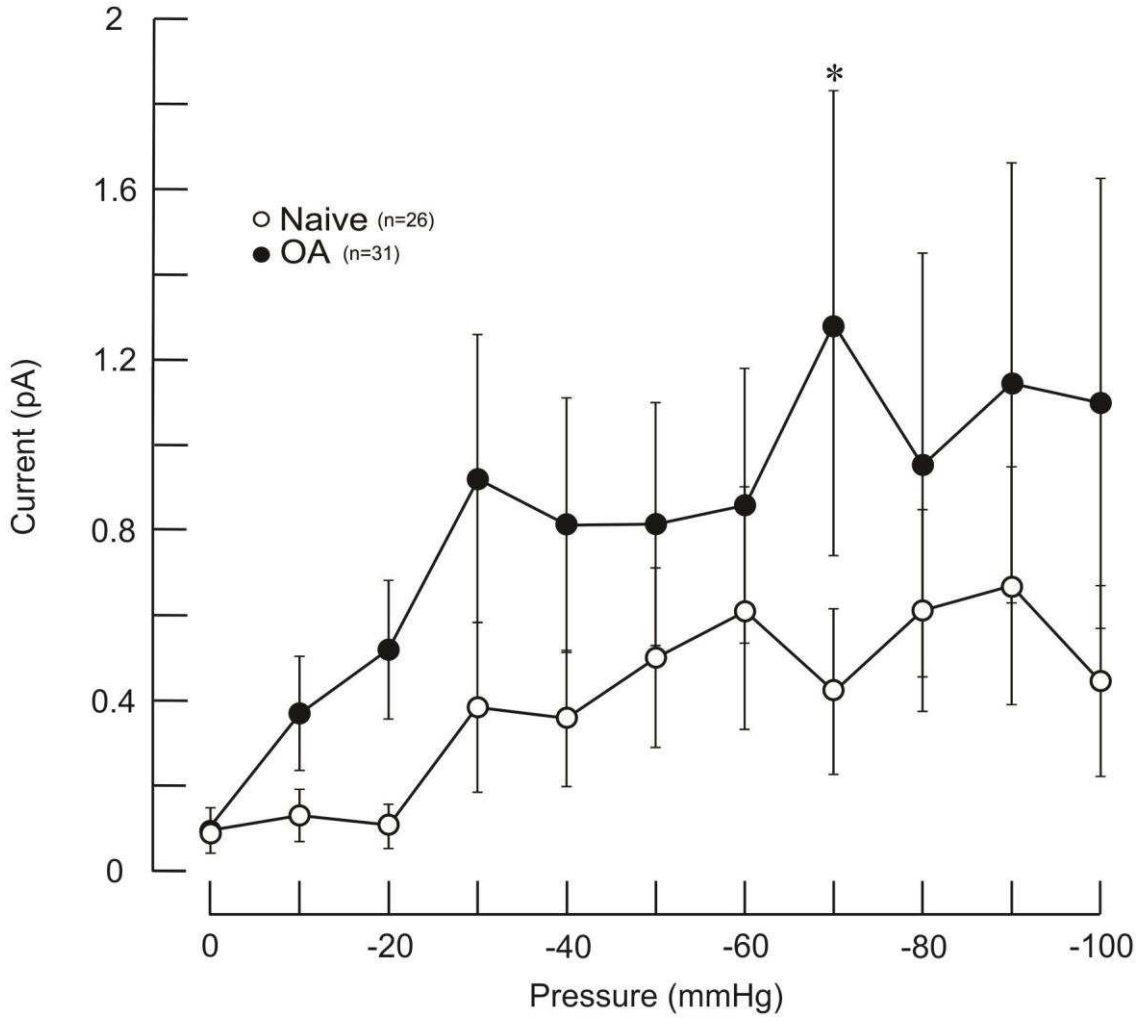

Figure 3-10: Disease-related changes in MSC activity are maintained after 3 days in culture. Mean (± s.e.m) MSC current in primary sensory neurons from OA (black circles) or naive (white circles) mice cultured for 72 hours. * =p<0.05

Chapter 4 – Discussion

4.1 Increased mechanosensitivity of OA neurons

The increase in mechanosensitivity of ipsilateral DRG neurons isolated from OA mice is a novel finding. This work may help identify the underlying molecular principles of the hypersensitivity of sensory neurons in OA. Our work on the mechanosensitivity of DRG neurons in OA shows that there are changes at the cellular level that result in the mechanosensitive phenotypes observed. We know that MIA-induced OA displays the inflammation and structural changes typically associated with OA. However, it is difficult to pinpoint what symptom of OA causes such differences in phenotype. It is known that inflammation sensitizes neurons, making them hypersensitive to mechanical stimuli, which may be the cause of the increased mechanosensitivity seen in our work[47]. It is also possible that structural changes in the joint, such as cartilage-degradation that exposes sensory neurons in the bone, which lead to the sensory neuron hypersensitivity[9].

Based on our findings, we propose several possibilities by which neurons in OA may become hypersensitive (Figure 4-1). Because of the increased current response to the mechanical stimulus, it stands to reason that there may be more channels at the membrane or there is a fundamental change of the existing channels on the membrane. For example, OA may trigger the upregulation of existing MSCs so that more of them were present at the membrane, thus a larger current would be detected and more patches would be active (Figure 4-1A). However, we can discard this possibility since more of the same channel cannot account for the lowered activation threshold. Conversely, OA can induce the upregulation of a modulatory enzyme, such as a

kinase, that either directly or indirectly phosphorylates the existing MSCs to increase their conductance, producing the characteristic MSC activity increase as shown (Figure 4-1Bi).

A third possibility that would account for the hypersensitivity of OA neurons is that there is an increase in MSC regulatory proteins. We know that the activation time of MSCs is fast, meaning it seems unlikely there is a signalling cascade that triggers MSC opening. However, it is understood that the cytoskeleton plays a role in MSC gating, possibly as a tension sensor[50]. Therefore, the increased presence of a regulatory/linker protein that tethers the ion channel to a cytoskeletal element may trigger the increased current. In this scenario, the cytoskeleton acts as the tension sensor, whose movement opens the channel gate (Figure 4-1Bii). Additionally, regulatory proteins embedded in the membrane (eg. β-subunit in VGSCs) can regulate membrane localization for ion channels[50] (Figure 4-1Bi). As such, it is plausible that regulatory proteins may interact with the MSCs and increase their conductance.

One of the caveats of this work is that we are stimulating the cell membrane at the soma instead of at the nerve terminals where they detect the mechanical stimuli in vivo. Sensory nerve endings are impossible to record from in situ, since they are too small and embedded throughout the tissue. Fortunately, DRGs house the more accessible somata which express the relevant molecules[16]. Therefore, electrophysiological recordings are usually done on dissociated sensory neuron cell bodies. This data is combined with behavior studies as well as in situ experiments in order to provide a well-rounded picture. Despite this, there remains the possibility that subtle differences in expression of MSCs exist between soma and the nerve terminals[83]. However, it has been shown that mechanical stimulation of a neurite produced the same somatically-recorded current as that produced by mechanical stimulation of the soma[84]. Thus we cautiously assume

that channel gating in the neurite, and therefore in the nerve terminals, is the same as channel gating at the soma.

In order to assess the roles of our TMEMs in OA DRG neurons, we need to be able to reduce their expression in both control and OA DRG neurons using siRNA knockdown. siRNA knockdown of a protein requires at least 48 hours. However, one limitation to this approach is the potential change in phenotype when placing the cells in culture. Therefore, we cultured DRG neurons from both OA and naïve mice and tested their mechanosensitivity 72 hours after dissociation. We found that OA DRG neurons maintained their enhanced mechanosensitivity in culture, thus siRNA knockdown remains feasible.

Inflammatory mediators in the joint contribute to catabolic and nociceptive pathways[5]. When present in the joint, the inflammatory mediator tumor necrosis factor alpha (TNFα) causes the production of cartilage-degrading proteases and the sensitization of primary afferents to mechanical stimuli[85]. It has been shown that direct application of TNFα in the joint periphery induces pain in rats, an effect that can be reduced by the application of anti-inflammatory medications[86]. Therefore, in order to assess the effects of inflammatory mediators on the mechanosensitive response, we did try to apply TNFα in the culture media. We found no change in mechanosensitive response between control and TNFα on neuronal mechanosensitivity, although it was a small sample size. This does not mean that inflammatory factors do not play a role in mediating the mechanosensitive response in OA; since TNFα is not the only factor released during inflammation, we may be able to mimic the mechanosensitive phenotype found in OA with the application of others inflammatory factors. For example, it has been shown that bradykinin sensitizes native sensory neurons to mechanically-activated currents[87].

4.2 Mechanosensitive TMEMs

The identification of several TMEMs was the first step in identifying a novel MSC. Because the identity of MSCs has remained elusive, identifying new MSC candidates is something the field desperately needs, especially in the field of pain research. The initial screening of the TMEM candidate MSCs and their effects on mechanosensitivity showed that TMEM5B and TMEM1 increased and decreased mechanosensitivity in COS-7 cells, respectively. The characteristics of channel opening were analyzed through the percent of active patches and the pressure of first channel opening (Figure 3-8). If the channel was activated at different pressures in TMEM-transfected cells, then we could conclude that the TMEMs affected channel gating (Figure 4-2). If the number of active patches was altered in TMEM-transfected cells, then we could say that the TMEMs affected the number of MSCs at the membrane, or perhaps they affected channel gating. We were unlikely to see a difference in number of active patches since they were very high in both mock- and TMEM-transfected cells.

Based on what we know from the hydrophobicity plots and other ion channels, TMEM1 is most likely a modulator of MSCs. TMEM1 has only one membrane-spanning domain, and no ion channel whose structure we currently know has a single transmembrane domain. Therefore, since our data clearly shows that TMEM1 has an effect on mechanosensitivity, we can speculate the roles for this protein in the mechanotransduction process. For example, TMEM1 may act as a modulator of activity by preventing the MSCs from binding their regulatory proteins. On the other hand, TMEM5B is predicted to have 5 transmembrane domains. Since this TMEM shows an increase in mechanosensitivity, this protein remains a candidate as a novel MSC. It should be noted that just because both TMEM1 and TMEM5B have hydrophobic domains that we presume

will preferentially solubilize in the membrane, we cannot know whether they actually end up at the cell membrane. In order to clarify this, we must visualize their localization using microscopy. For example, we could create a hemagluttinin (HA) tagged-TMEM fusion protein and apply fluorescent HA antibodies to track the TMEMs cellular location.

Our data demonstrates that COS-7 cells express TMEM1 and TMEM5 (Data not shown). Ideally, we would have used a cell line that had little to no mechanosensitivity. This cell line would benefit our experiment since any mechanosensitivity detected would be a direct result of the transfected TMEMs. Therefore we additionally tested the endogenous mechanosensitivity of other cell lines (HEK293, F11), with none proving to have significantly less activity than COS-7 cells. However, if TMEM5B continues to remain a candidate MSC, then it will eventually be expressed in artificial bilayers (eg. liposomes) to detect its endogenous mechanosensitivity.

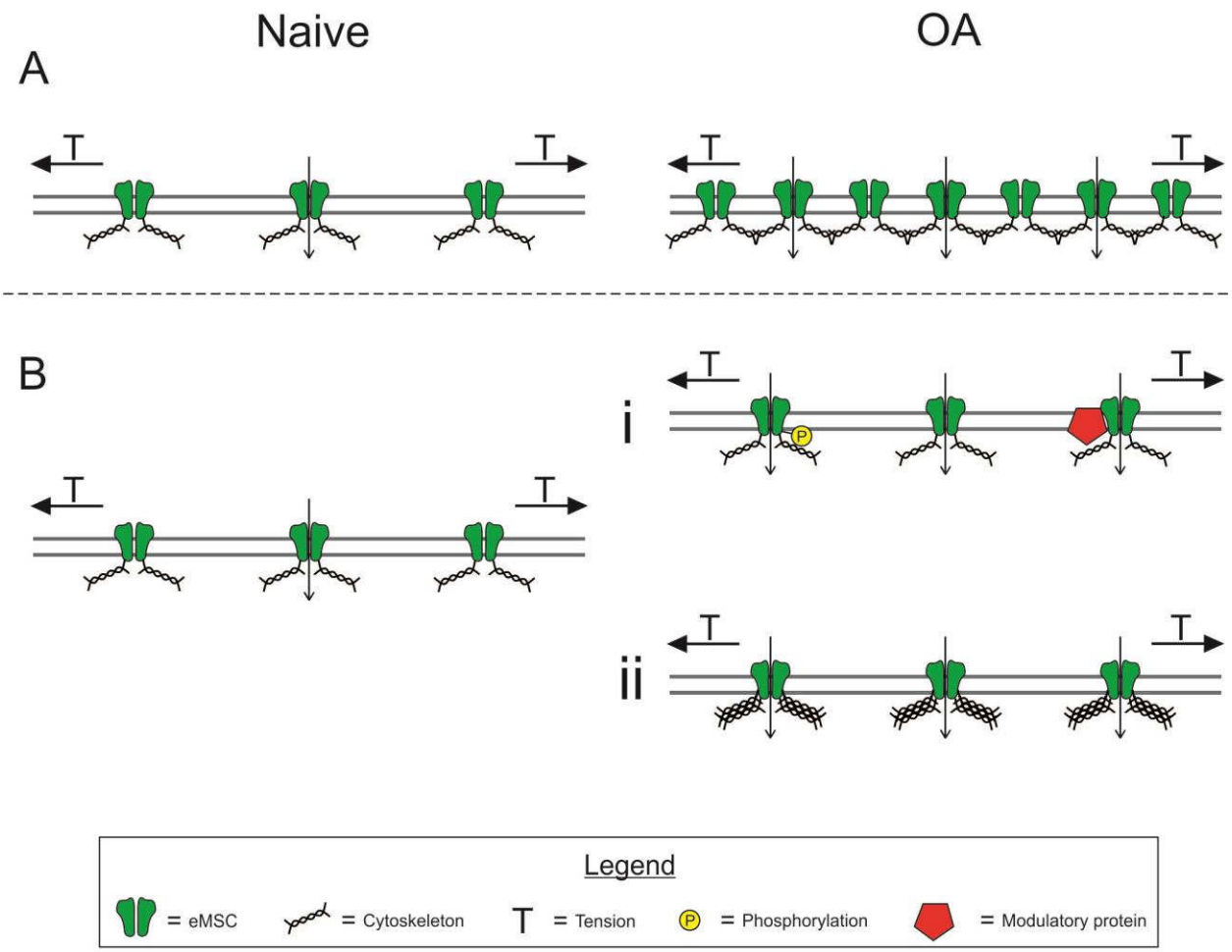

Figure 4-1: Hypothetical mechanisms explaining the increase in mechanosensitivity of DRG neurons in OA mice. (**A**) OA DRG neurons express more channels (right) than naive DRG neurons (left). This may be due to an upregulation of endogenous MSCs (eMSCs) in the pathological condition. (**B**) OA and naive DRG neurons maintain the same number of eMSCs, but there is some modulation of these eMSCs that alters the channel opening. For example, (**i**) the eMSCs are modulated (i.e. phosphorylation or modulatory protein) in OA so that more channels are opened during mechanical stimulation, or (**ii**) there is a tighter coupling between the MSCs and the tension-sensing cytoskeleton.

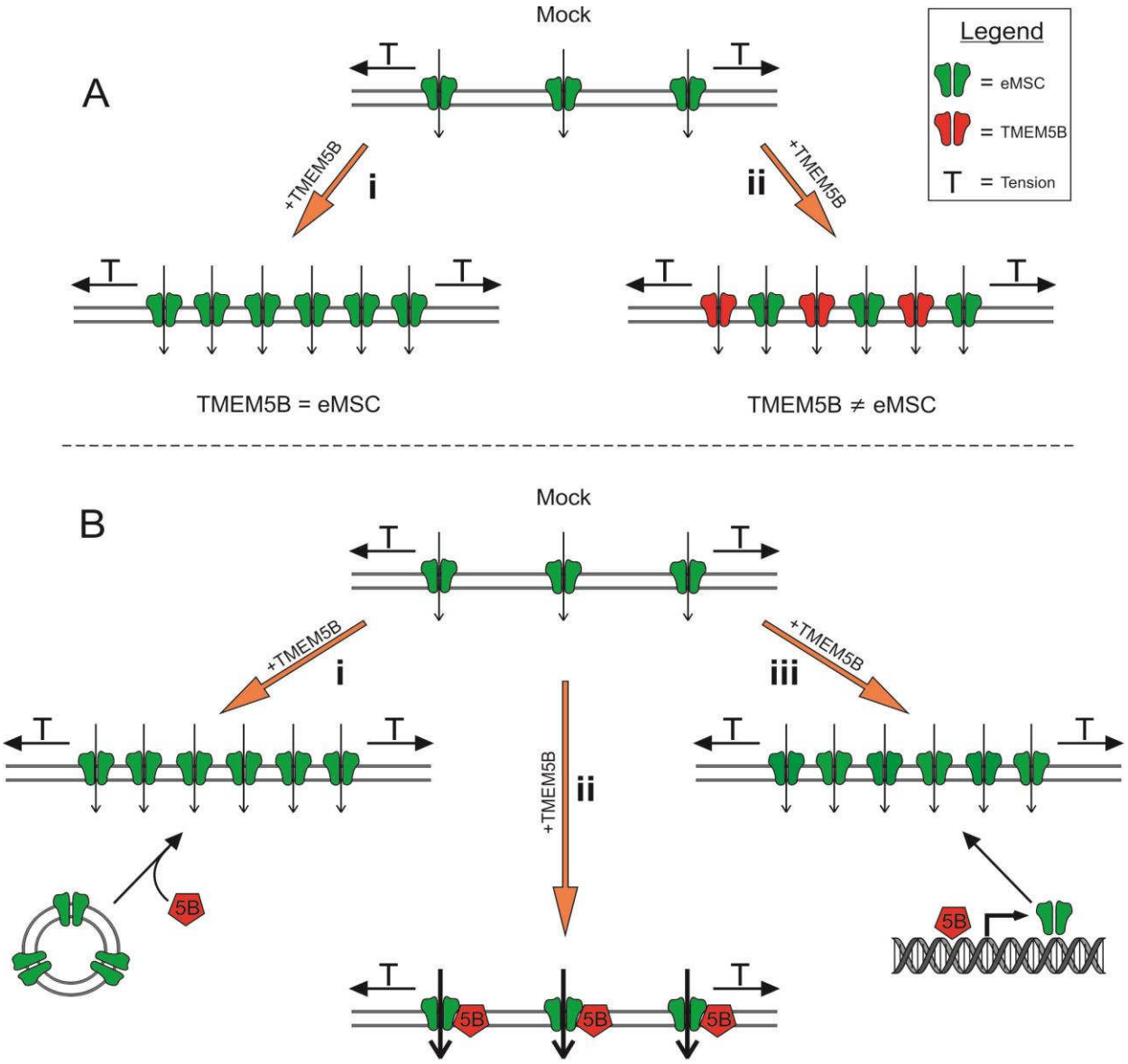

Figure 4-2: Possible scenarios explaining how TMEM5B increases cellular mechanosensitivity in COS-7 cells. (**A**) TMEM5B is an ion channel that is either (**i**) the eMSC, or (**ii**) a novel MSC in addition to the eMSC. In either scenario, there are more MSCs in TMEM5B-transfected cells compared to mock, accounting for the lowered activation threshold. (**B**) TMEM5B is not an ion channel, but rather an accessory protein that increases the number of eMSCs at the membrane. For example, TMEM5B may: (**i**) assist in the membrane localization of eMSC-containing vesicles, (**ii**) increase the conductance of the eMSCs by embedding in the membrane next to the eMSCs, or (**iii**) increase the expression of the eMSC.

Chapter 5 – Conclusion

Under normal circumstances, nociception and the perception of pain are present only at temperatures and pressures extreme enough to cause tissue injury, by toxic molecules, and by inflammatory mediators[15]. However during chronic pain conditions, less extreme stimuli provoke the firing of action potentials in nociceptors. Here we show that during OA, nociceptors are hypersensitive to mechanical stimuli. We propose that the hypersensitivity of DRG neurons is, at least in part, responsible for the mechanical allodynia in OA patients. However, further work on this project is limited by the lack of the molecular identity of MSCs involved in OA pain. In order to address this problem, we initiated a search for possible candidate MSCs. We showed that indeed our TMEMs were expressed in DRG neurons, and the mRNA of three of these TMEMs was increased in OA. We found that of these three TMEMs, TMEM5B and TMEM1 caused an increase and decrease in cellular mechanosensitivity in COS-7 cells, respectively. These two TMEMs will be investigated further as possible modulators of MSCs, or in the case of TMEM5B, a novel MSC. In summary, these findings will help identify the neurological basis for chronic pain in OA and may help elucidate the molecular basis for the pain in OA.

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Chapter 7 – Appendix

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Thanks
Craig
