Development of a mouse model for the study of osteoarthritis pain

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DEVELOPMENT OF A MOUSE MODEL FOR THE STUDY OF OSTEOARTHRITIS PAIN

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Abstract

Murine models of osteoarthritis (OA) provide a potentially powerful tool to elucidate mechanisms responsible for OA pain. However, few studies have examined pain behaviours in relevant OA models in mice. Several models including spontaneous, surgical and chemically induced OA were explored before selecting partial medial meniscectomy of C57Bl/6 mice for in-depth study. The development of degenerative joint disease was confirmed using histology which illustrated progressive cartilage damage in a time dependent manner. Pain was assessed by monitoring weight bearing, mechanical hyperalgesia, cold allodynia, mechanical allodynia and vocalisation in response to knee compression for 12 weeks post-surgery. No significant weight bearing deficits were observed during the course of the study. Significant mechanical allodynia was present in the ipsilateral hind limb from 9 weeks following surgery. Hind limb mechanical hyperalgesia and cold allodynia, and increased vocalisation in response to knee compression developed in the ipsilateral limb in two phases. An early phase of hypersensitivities lasted for up to 3 weeks and was reversed by treatment with an NSAID, diclofenac. Pain then resolved for several weeks followed by a second phase of NSAID-insensitive pain after 6 weeks post-surgery. During this phase, all pain behaviours could be reversed by morphine. In contrast, other analgesic drugs (paracetamol, gabapentin and tramadol) had selective effects on only one or two modalities. Pain fluctuated during the second phase with transient periods of reduced pain. At these times underlying hypersensitivities could be unmasked by administration of naloxone indicating that reduced pain was due to endogenous opioids. The role of specific pain receptors in OA pain were investigated in this model of OA using genetically modified mice, novel analgesics and calcium imaging. Partial medial meniscectomy has proven to be a useful tool for the investigation of the pathophysiology of OA pain and also for the discovery of potential new therapeutics.

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List of Abbreviations

4HNE	4-hydroxynonenal
ACC	anterior cingulate cortex
ACLT	anterior cruciate ligament transection
ADAMTS	a disintegrin and metalloproteinase with thrombospadin-1 domains
AITC	allyl isothiocyanate
AMG-9810	[(E)-3-(4-t-Butylphenyl)-N-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylamide],
AMPA	α-amino-3hydroxyl-5-methyl-4-isoxazolepropionic acid
AMTB	N-(3-aminopropyl)-2{[3-methylphenyl)methyl]oxy}-N-(2-thienylmethyl)benzamide hydrochloride
AP-18	4-(4-Chlorophenyl)-3-methyl-3-buten-2-one oxime
ATP	adenosine triphosphate
BCTC	(N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide)
BDNF	brain derived neutrophic factor
BMP	bone morphogenetic protein
Ca^{2+}	calcium ion
CaMKII	calmodulin dependent protein kinase II
CCI	chronic constriction injury
CFA	complete freunds adjuvant
CGRP	calcitonin gene related peptide
CHO	chinese hamster ovary
CO_2	carbon dioxide

CNS	central nervous system
Contra	contralateral
COX	cyclooxygenase
CT	computed tomography
DMM	destabilisation of the medial meniscus
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FDA	food and drug administration
GA	general anaesthesia
GNTI	5'-Guanidinonaltrindole
HC-030031	2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide
HEK	human embryonic kidney
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
i.a.	intra-articular
IASP	international association for the study of pain
iNOS	inducible nitric oxide synthase
i.p.	intra-peritoneal
i.v.	intravenous
ipsi	ipsilateral
IGF	insulin growth factor
IL	interleukin

Ipsi	ipsilateral
K^+	potassium ion
KCL	potassium chloride
L	left
LFC	lateral femoral condyle
LTP	lateral tibial plateau
MAPK	mitogen activated protein kinase
Mg^{2+}	magnesium ion
MEM	minimum essential medium
MFC	medial femoral condyle
mGluR	metabotropic glutamate receptor
MIA	monosodium iodoacetate
MMP	metalloproteinase
MRI	magnetic resonance imaging
MTP	medial tibial plateau
Na^{2+}	sodium ion
NGF	nerve growth factor
NK	neurokinin
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSAID	non steroidal anti-inflammatory drug
OA	osteoarthritis
OARSI	osteoarthritis research society international
OVX	ovariectomised mice

PGE	prostaglandin
PKC	protein kinase II
PMM	partial medial meniscectomy
p.o.	per os
PP	paw pressure
PPI	proton pump inhibitor
PWL	paw withdrawal latency
PWT	paw withdrawal threshold
R	right
RNA	ribose nucleic acid
s.c.	subcutaneous
SEM	standard error of the mean
SOVX	sham ovariectomised mice
SP	substance P
TENS	transcutaneous electrical nerve stimulation
TNF	tumour necrosis factor
Trk	tyrosine receptor kinase
TRP	transient receptor potential
TRPA1	ankyrin TRP family
TRPM	melastatin TRP family
TRPV	vanilloid TRP family
VF	von Frey
WDR	wide dynamic range

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Publications Relating to Thesis

C.B. Knights, C. Gentry, S. Bevan. (2012) Partial medial meniscectomy produces osteoarthritis pain related behaviours in female C57BL/6 mice. PAIN. **153:** 281-292

Chapter 1

Introduction

1 Introduction

1.1 Osteoarthritis pain – an unmet need

Osteoarthritis (OA), a degenerative joint disease affecting di-arthroidal joints, is the most common articular disorder and is frequently cited as the leading cause of persistent musculoskeletal pain. It is increasingly common in the aging Western society, with a major health economic impact (Yelin and Callahan 1995). Current therapies to help alleviate joint pain are only partially effective and certain drugs produce unwanted negative side effects, thereby limiting their use in the long-term. Whilst there are considerable insights into the mechanisms underlying tissue remodelling, there is poor understanding of the link between disease pathology and pain. Studying the aetiopathogenesis of human OA is difficult due to the limited availability of diseased tissues, particularly in the early stages of OA and the poor sensitivity and specificity of diagnostic tools such as radiology. This has led to great interest in the use of animal models (Ameye and Young 2006). The majority of these animal models have focused on structural damage and changes in gait and mobility and have not addressed the issue of pain. The aims of the studies reported in this thesis were to establish and validate a murine model for the study of OA pain that can facilitate the study of the neuronal mechanisms underlying OA pain and the development of new therapeutic drugs.

OA can be viewed as the clinical and pathological outcome of a range of disorders that result in structural and functional failure of synovial joints (Nuki 1999). OA disease is a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage and subchondral bone (Eyre, Barton et al. 2004), see Figure 1-1. The disease processes affect the entire joint, including the articular cartilage, subchondral bone, ligaments, capsule, synovial membrane, and peri-articular muscles.

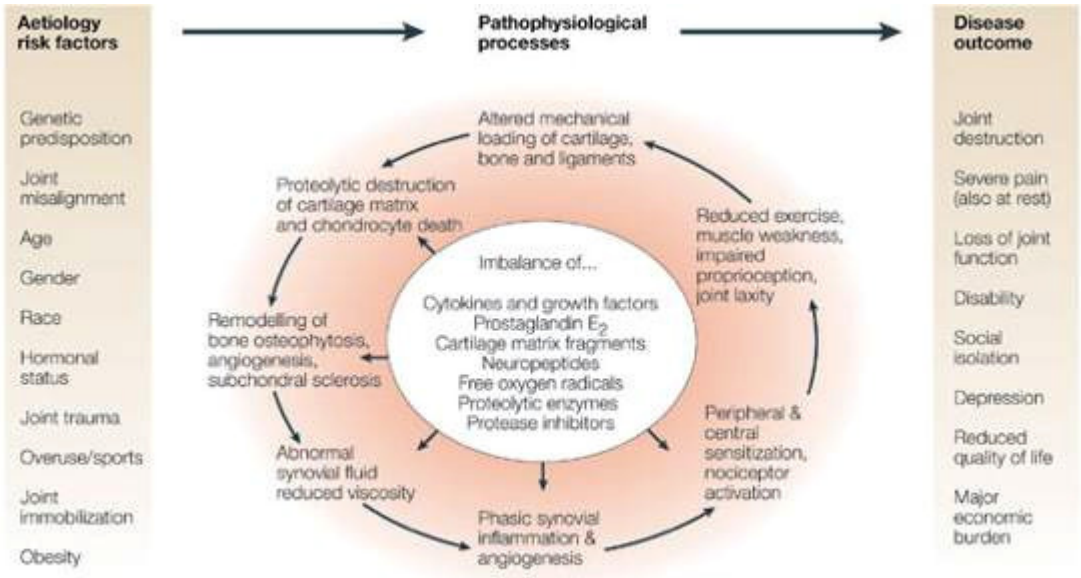


Figure 1-1 The development of OA

Relationship between aetiological factors (left), pathophysiological processes (central) and disease outcome (right). The pathophysiological processes influence and often amplify each other in a vicious cycle. Taken from Wieland, Michaelis et al. (2005).

In normal joints, the ends of the bones are covered in hyaline cartilage, a firm, smooth, visco-elastic tissue that provides a protective layer that distributes load across the whole joint surface, reducing the risk of biomechanical damage (Eyre 2002). Cartilage is non-vascularised so its nutrition is fully dependent on the diffusion of substances from the synovial fluid produced by the synovium (Muir 1995). Histologically, articular cartilage can be divided into four separate zones: a superficial zone, which lies next to the joint space, a transitional zone, a radial zone and the zone of calcified cartilage. The tidemark is the interface between non-calcified and calcified cartilage (Figure 1-2).

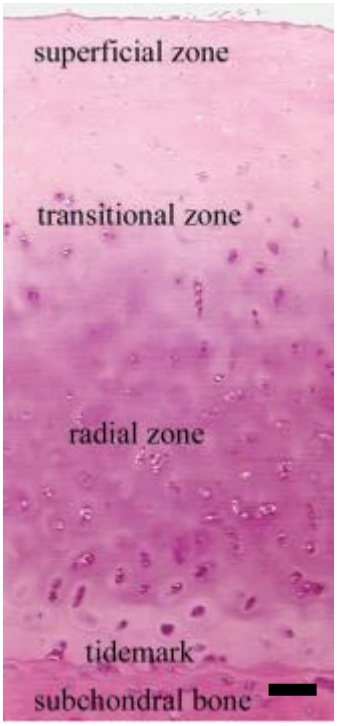


Figure 1-2 Normal cartilage histology.

Histological evaluation of a normal cartilage sample of articular cartilage stained with Haematoxylin and Eosin, Scale Bar: 70 µm. Taken from Tesche and Miosge (2005).

Cartilage consists of chondrocytes and extracellular matrix. The abundant extracellular matrix of articular cartilage is composed of collagen and proteoglycan. Normal articular cartilage contains types II, III, VI, IX, X, XI, XII and XIV collagens, the most abundant being collagen type II (Mayne and Brewton 1993; Eyre 2002). Proteoglycans such as aggrecan, consist of a central core protein with one or more glycosaminoglycans side chains. The resilience, integrity and function of articular cartilage depends on the composition of the abundant extracellular matrix which is synthesized by the chondrocytes (Hardingham, Bayliss et al. 1992).

The synthetic activity of chondrocytes and their release of inflammatory cytokines is influenced by biomechanical stresses on the joint and also inflammatory cytokines produced by other joint tissues such as the synovium (Ding, Heying et al. 2010; Torzilli, Bhargava et al. 2010). In the normal joint, equilibrium exists between anabolic and catabolic processes regulating the production and degradation of articular cartilage. Cyclical loading of the joint, for example, stimulates chondrocytes to increase production and release of Insulin growth factor 1 (IGF1) and bone morphogenetic proteins (BMPs) that stimulate cartilage generation and remodelling (Matsumoto, Gargosky et al. 1996; Sandell and Aigner 2001; Fan, Chubinskaya et

al. 2004). Normal loading of the joint also inhibits cartilage matrix degradation via decreased production of specific cytokines such as interleukin-1 (IL-1) (Torzilli, Bhargava et al. 2010). Injurious joint loading, however, results in depletion of proteoglycans, increased expression of inflammatory mediators, cartilage-degrading proteinases, and stress induced intracellular signals (Ding, Heying et al. 2010). In cases of OA, the equilibrium is shifted in favour of cartilage degradation. Stress-induced and inflammation-induced signalling, transcriptional and post-transcriptional events cause a phenotypic shift, aberrant expression of inflammation-related genes and chondrocyte apoptosis (Goldring, Otero et al. 2011). For example, the pro-inflammatory cytokine interleukin-1β (IL-1β) converts pro-matrix metalloproteinases (MMPs) into their active form. MMPs such as MMP-13, cleave collagen and A Disintegrin and Metalloproteinase (ADAM) with Thrombospondin-1 Domains (ADAMTS)-4 and 5 cleave aggrecan, compromising the integrity of the extracellular matrix. IL-1β and tumour-necrosis factor-α (TNFα) stimulate further inflammation by inducing overexpression of cyclooxygenase-2 (COX2) and prostaglandin-E2 (PGE2) in the joint (Fahmi, Pelletier et al. 2002; Martel-Pelletier, Pelletier et al. 2003). Increased synthesis of tissue-destructive proteinases, chondrocyte apoptosis, and inadequate synthesis of components of the extracellular matrix, leads to breakdown of the extracellular matrix of the articular cartilage producing fibrillation, fissures, ulceration, and full thickness cartilage loss particularly in areas of increased load (Figure 1-3). There is also sclerosis and eburnation of subchondral bone and osteophyte formation due to release of BMPs (Brandt and Slowman-Kovacs 1986). Synovitis is common in early and late stage disease due to the presence of cartilage degradation products, and involves infiltration of mononuclear cells into the synovial membrane and production of further proinflammatory mediators, including IL-1β, TNF-α, and chemokines (Smith, Triantafillou et al. 1997; Haywood, McWilliams et al. 2003; Sellam and Berenbaum 2010). OA can arise in any synovial joint in the body, but is most common in the hands, knees, hips, and spine with multiple joints usually affected (Dieppe and Lohmander 2005).

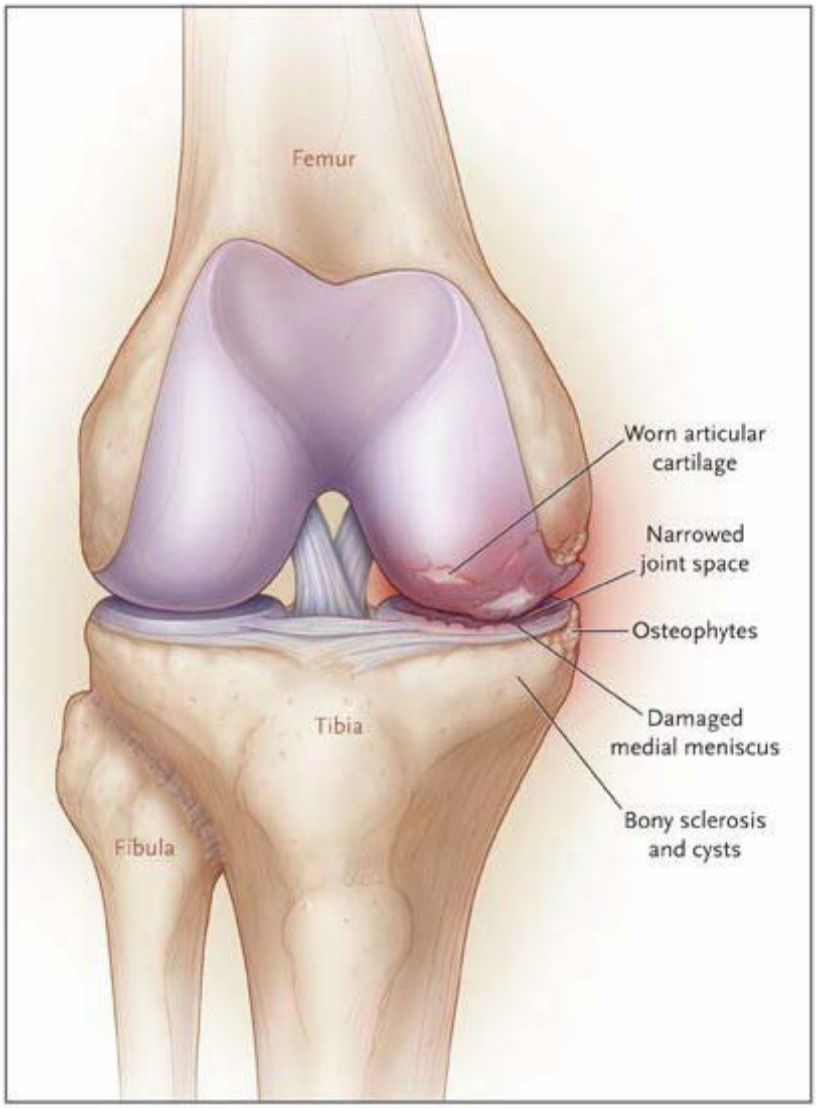


Figure 1-3 OA of the medial side of the knee. Taken from Felson (2006).

1.1.1 Aetiology

OA has a multi-factorial aetiology and can be considered the product of interplay between systemic and local factors (Zhang and Jordan 2008). Primary OA is typically thought to be a disease of "wear and tear" and to an extent is part of the ageing process. It is generally considered to be the result of articular cartilage damage from repetitive micro trauma that occurs throughout life. Secondary OA follows an identifiable underlying cause such as infection or traumatic injury to the joint. Genetic, hereditary, nutritional metabolic, pre-existing articular disease and lifestyle factors may contribute in some cases (Jacobson, Girish et al. 2008), see Figure 1-1.

1.1.2 Symptoms associated with OA

OA may cause pain, physical disability and psychological distress (Guccione 1994), although many people with structural changes consistent with OA are asymptomatic (Hannan, Felson et al. 2000). The reasons for this lack of correlation between disease severity and the level of reported pain and disability are unknown. When clinically evident, OA is characterized by joint pain, tenderness, restricted range of movement, crepitus and occasional effusion (Hunter, McDougall et al. 2008). OA joint pain is usually local, with insidious onset; aching, with episodic stabbing pain. The joint pain of OA is typically described as being exacerbated by activity and relieved by rest, however, up to 50% patients report rest pain (Creamer 2000). Neuropathic pain symptoms have also been reported in patients with OA including burning pain, pins and needles and numbness (Hawker, Stewart et al. 2008).

1.1.3 Diagnosis

The diagnosis of OA is made on the basis of the patient's history and physical examination. When disease is advanced, radiographic features include narrowing of the joint space, osteophytes and sclerosis of the subchondral bone. Since radiographs are notoriously insensitive to the earliest pathologic features of OA, the absence of positive radiographic findings are not interpreted as absence of the disease. Moreover, many individuals with radiographic features consistent with OA do not have pain or discomfort. The role of radiography is to confirm clinical suspicion and rule out other conditions (Guermazi, Eckstein et al. 2009). Magnetic resonance imaging (MRI) and computed tomography (CT) are more sensitive imaging modalities and are particularly helpful in discriminating between OA and other causes of joint pain such as osteochronditis dissecans (Hunter, McDougall et al. 2008).

1.1.4 Common treatments available for OA

There are currently no disease modifying agents or effective preventative strategies for OA. Therapies are therefore targeted towards controlling symptoms, maintaining function and reducing further joint damage (Kidd 2006). The management of OA is symptomatic up to the point of serious joint dysfunction, when surgical approaches are used. Osteoarthritis Research Society International (OARSI) produced twenty-five recommendations for the management of hip and knee OA based on critical appraisal of existing guidelines, systematic review of research evidence and the consensus opinions of an international, multidisciplinary group of experts (Zhang, Moskowitz et al. 2008). Recommendations for pharmacological and non-pharmacological modalities of OA pain management can be summarised as follows:

Patients should be educated on the importance of changes in lifestyle, exercise, pacing of activities, weight reduction, and other measures to unload the damaged joint(s). Regular phone contact can improve their clinical status. Patients with symptomatic knee OA may benefit from referral to a physical therapist for evaluation and instruction in appropriate exercises to reduce pain and improve functional capacity and be provided with assistive devices such as canes and walkers, as appropriate. Patients with hip and knee OA should undertake regular aerobic, muscle strengthening and range of motion exercises. Knee braces can reduce pain, improve stability and diminish the risk of falling in patients with varus/valgus instability and every patient should receive advice concerning appropriate footwear. Transcutaneous electrical nerve stimulation (TENS) and acupuncture can help with short-term pain control in some patients with hip or knee OA. For pharmacological modalities, the following recommendations were made: The drug of first choice is paracetamol, which if effective is the preferred long term oral analgesic. If paracetamol does not produce adequate pain relief, additional or alternative therapies are considered. Non-steroidal anti-inflammatory drugs (NSAIDs) are recommended at the lowest effective dose but their long-term use is avoided if possible due to the risk of gastrointestinal ulceration. In patients with increased risk of gastrointestinal side effects, either a COX-2 selective agent or a non-selective NSAID with co-prescription of a proton pump inhibitor (PPI) or misoprostol for gastric protection may be considered. Topical NSAIDs and capsaicin may be useful as adjunctive and alternatives to oral analgesics. Intra-articular (i.a.) injections with corticosteroids are considered particularly when patients have moderate to severe pain not responding satisfactorily to oral analgesic/anti-inflammatory agents. The use of weak opioids and narcotic analgesics are reserved for the treatment of refractory pain in patients with OA where other pharmacological agents have been ineffective, or are contraindicated. Stronger opioids should only be used for the management of severe pain in exceptional circumstances.

The guidelines provide core recommendations that can be followed and adapted for individual patients. The guidelines, highlight the lack of a universally effective management strategy and that a multimodal approach to OA needs to be utilised to be effective.

For patients with intractable symptoms, more invasive measures such as joint replacement may offer an effective long-term solution (McHugh and Luker 2009). Joint replacement, however, is an invasive procedure with a substantial recovery period and modest options for revision when needed (Matthews and Hunter 2011).

Also, persistent pain has been reported in joint replacement patients following surgery. For example, up to 8% of primary total hip replacement patients complain of persistent moderate to severe pain 2 years postoperatively which may be due to sensitization of the peripheral or central nervous system (Singh and Lewallen 2010).

Unsatisfactory efficacy of currently available treatment options has left an ongoing and increasingly unmet medical need. One treatment satisfaction survey reported inadequate pain relief by 73% of 2,000 primary care physicians and 63% of 30,000 patients (Crichton and Green 2002) highlighting a need for better management of OA pain. Further research is needed to produce more effective pain relief strategies for OA and to produce better pharmacological therapies that do not produce adverse effects when administered chronically.

1.1.5 Prognosis for OA

OA is generally regarded as a progressive, irreversible disease that ultimately leads to joint failure. However, the rate and degree of progression of affected joints is unpredictable and many cases are associated with minor symptoms or are asymptomatic (Hugues 2008).

1.2 Pain

1.2.1 Definition of pain

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey 2007). Acute pain has a protective role that reduces damage to the body since the pain perceived is directly related to the presence, intensity, or duration of stimuli. Chronic pain however does not serve the same defensive purpose since neither the intensity nor quality of chronic pain is related to the degree of tissue damage and may persist long after the resolution of the initial insult (Dray and Read 2007). In chronic pain conditions, such as OA, pain can arise spontaneously, can be elicited by a stimulus that does not normally provoke pain (allodynia), can be exaggerated and prolonged in response to a stimulus that normally provokes pain (hyperalgesia), and can spread beyond the site of injury (secondary hyperalgesia) due to peripheral and central sensitization (Latremoliere and Woolf 2009). The pain in symptomatic cases of OA can be considered to be a complex integration of sensory, affective and cognitive processes that involves a number of abnormal cellular mechanisms at both peripheral (joints) and central (spinal and supraspinal) levels of the nervous system (Dieppe and Lohmander 2005).

1.2.2 Sensory innervation of the joint

The primary function of joint sensory nerves is to detect and transmit mechanical information from the joint to the central nervous system. Sensory information from muscle and joint is involved in the sense of movement and position and the detection of noxious stimuli (Schaible 2004). The mammalian knee joint is supplied by 2 major articular nerves: the posterior articular nerve, a branch of the tibial nerve, and the medial articular nerve, which arises from the femoral nerve. Together these account for 80-90% of the neurons supplying the joint. The musculature, articular capsule, synovium, tendons, menisci, ligaments, periosteum and subchondral bone of the joint have a rich nerve supply, whereas normal articular hyaline cartilage is considered aneural (Theriault, Marshall et al. 1993). In studies using conscious patients, direct stimulation of fibrous structures with innocuous mechanical stimuli created pressure sensations. Pain was elicited when noxious mechanical, thermal, and chemical stimuli were applied to the fibrous structures such as ligaments and fibrous cartilage. No pain was elicited by stimulation of cartilage, and stimulation of normal synovial tissue only rarely evoked pain (Kellgren and Ball 1950). The lack of sensory neurons within the cartilage and poor correlation between the radiological signs of OA and the occurrence of joint pain has led to much discussion as to the site and nature of OA pain. OA symptoms of joint pain, swelling and stiffness are suggestive of some local inflammation, and episodic synovitis can coincide with occurrences of increased pain (Benito, Veale et al. 2005). However, OA is not considered a classical inflammatory arthropathy, due to the absence of neutrophils in the synovial fluid and the lack of systemic manifestations of inflammation (Pelletier, Martel-Pelletier et al. 2001; Pelletier, Raynauld et al. 2008). Whilst normal articular cartilage is considered aneural (Kellgren and Ball 1950; Dye, Vaupel et al. 1998), a recent study reported the observation of vascularisation of cartilage with accompanying sensory and sympathetic nerves within the vascular channels, in samples taken from patients undergoing total knee replacement and from elderly patients at post mortem that had evidence of OA (Suri, Gill et al. 2007). Perivascular and free nerve fibres were also observed within the subchondral bone marrow and within the marrow cavities of osteophytes. Vascularisation and the associated innervation of articular cartilage may contribute to knee pain in OA (Suri, Gill et al. 2007). Studies have also found some correlation between magnetic resonance imaging findings of synovial hypertrophy, synovial effusions and subchondral bone marrow oedema to incidences of increased OA pain. Bone marrow lesions including bone marrow necrosis, bone marrow fibrosis and bone marrow oedema, can often be visualized by MRI in subjects with OA (Felson,

Chaisson et al. 2001; Felson, McLaughlin et al. 2003; Hunter, Zhang et al. 2006). For example, bone marrow lesions were found in 272 (77.5%) out of 351 people with painful knees compared with 15 (30%) out of 50 with no knee pain (P<0.001) (Felson, Chaisson et al. 2001). In another study of bone marrow lesions in OA subjects categorized by painful and non-painful OA groups, larger lesions (>1cm^2) were significantly more frequent in the painful knee OA group (Sowers, Hayes et al. 2003). The participants with larger bone marrow lesions were also more likely to have full-thickness cartilage defects, adjacent sub cortical bone abnormalities and more pain. These findings suggest that the subchondral bone represents a potential source of nociceptive pain in patients with OA.

1.2.3 Acute pain

In the normal joint, large diameter myelinated nerve fibres (A beta) encode and transmit proprioceptive signals, which can be interpreted as being either dynamic or static (Schaible, Ebersberger et al. 2002). Nociceptive nerve fibres are typically small in diameter and are either unmyelinated (C) or thinly myelinated with an unmyelinated 'free' nerve ending (A delta). Most nociceptors are polymodal, responding to noxious mechanical stimuli (painful pressure, squeezing or cutting the tissue), to noxious thermal stimuli (heat or cold), and to chemical stimuli (Schaible, Ebersberger et al. 2002). In the rat and cat, it has been shown that 80% of all knee joint afferent nerve fibres are nociceptive (Hildebrand 1991, Langford 1983). Polymodal nociceptors in normal tissues have high thresholds and are not excited by gentle stimuli. They are activated by noxious mechanical, thermal and chemical stimuli in general via the activation of ion channels such as transient receptor potential (TRP) and purinergic channels that transduce these stimuli to elicit action potentials (Schaible and Richter 2004; Basbaum, Bautista et al. 2009; Gold and Gebhart 2010). Action potentials are conducted to the dorsal horn of the spinal cord where the neurons synapse onto second order pain projection neurons generally within the superficial laminae (I and II) (Craig 1995) and to the deep laminae V-VII (Schaible and Grubb 1993). Neurotransmitters such as glutamate and substance P are responsible for synaptic communication between nociceptors and pain projection neurons. Glutamate binds and activates α-amino-3hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA and kainate receptors as well as metabotropic glutamate receptors (Berthele, Boxall et al. 1999; Woolf 2004). Substance P is released from primary nociceptive afferents (Lawson, Crepps et al. 1997) and binds to and activates the neurokinin 1 receptor (NK1R). These spinal cord nociceptive neurotransmitters and their receptors are critical for activating second-order neurons, which communicate to supraspinal pain-processing centres

such as the brain stem, thalamus, somatosensory cortex, insular cortex and anterior cingulate cortex (ACC) (Zhuo, Wu et al. 2011) to elicit reflexive and protective responses to avoid potential or further tissue damage (Hunt and Mantyh 2001).

1.2.4 Chronic pain and peripheral sensitisation

Chronic pain results from inflammation and/or trauma to peripheral nerve(s), tissue(s), or the central nervous system (CNS) and may arise as a complication in numerous medical conditions such as diabetic neuropathy, chemotherapy-induced pain, postsurgical pain, and OA pain. Inflammation and local tissue damage exposes the high-threshold primary sensory neurons to inflammatory mediators including nerve growth factor (NGF), ATP, bradykinin, nitric oxide (NO) and prostanoids (Ji, Befort et al. 2002) together with several neuromediators such as substance P and calcitonin gene related peptide (McDougall 2006). These Inflammatory mediators act on ionotropic receptors such as $5HT_3$ and P2X3 receptors and also metabotropic membrane receptors that activate secondary messenger systems. The gating properties of ion channels are subsequently modified (Hucho and Levine 2007) resulting in a lowering of the neurons' excitation threshold. Depending on the mediator, the sensitization of nociceptors can be induced within minutes or hours (for example, by phosphorylation of ion channels in the membrane) (McCleskey and Gold 1999; Mizumura, Sugiura et al. 2009; St Pierre M 2009). Some signals are also transported along the axons of sensory nerve fibres to the cell body within the dorsal root ganglion where they affect transcription and/or translation, increasing expression of particular genes and the generation of proteins from messenger RNA. The proteins are transported down the terminal where they are able to contribute to the increased sensitivity of the terminal to peripheral stimuli via changes in the expression of key receptors, ion channels and enzymes (Ji 2004; Devor 2006). In addition, inflammation recruits "silent nociceptors" for activation (Schmidt, Schmelz et al. 1995). These C-fibres are inexcitable by noxious mechanical or thermal stimuli in normal tissue but inflammation sensitizes them to become responsive to stimuli enabling them to contribute to the generation of arthritis pain (Schaible, Schmelz et al. 2006). The lowering of the activation threshold causes nociceptors to become excited by gentle stimuli that do not normally activate them such as joint movements in the working range or gentle palpation of the joint or surrounding muscle (allodynia). Sensitized nociceptors also show an increased response to noxious stimuli (hyperalgesia). Increased responsiveness and reduced threshold of nociceptors to stimulation of their receptive fields is called peripheral sensitization (IASP 2011). This peripheral

sensitization occurs at the site of tissue injury and generally requires ongoing peripheral pathology for its maintenance (Hucho and Levine 2007).

1.2.5 Chronic pain and abnormalities in central pain processing

Abnormalities in central pain processing can also occur and include abnormalities in the descending facilitatory and inhibitory pain pathways, central sensitization and glial activation.

1.2.5.1 Descending facilitatory and inhibitory pain pathways

The descending pain pathways descend from the brainstem, thalamus and cortical structures, and modulate sensory input from primary afferent fibres and projection neurons in the dorsal horn of the spinal cord (Cervero, Schaible et al. 1991; Schaible, Neugebauer et al. 1991). Most spinal cord neurons with joint input are tonically inhibited by descending inhibitory systems that keep the spinal cord under continuous control. The best characterized descending analgesic pathways are the serotonergic–noradrenergic pathway and the opioidergic pathway. These pathways lead to the release of serotonin, norepinephrine and endogenous opioids (β-endorphins, enkephalins and dynorphins) which inhibit the release of excitatory neurotransmitters such as glutamate. These pathways are activated in response to noxious stimuli, leading to a widespread decrease in pain sensitivity after exposure to an acutely painful stimulus (Le Bars, Rivot et al. 1980). In chronic pain syndromes, descending inhibitory pathways are often impaired or absent (Kosek and Ordeberg 2000). For example, healthy control patients will show an increased pressure pain threshold (decreased pain sensitivity) following the application of a heterotopic noxious stimulus. In patients with chronic musculoskeletal pain such as OA, the pressure pain threshold does not increase indicating impairment of the diffuse noxious inhibitory control pathway (Kosek and Ordeberg 2000). Impairment of the descending inhibition lowers the excitation threshold of spinal cord neurons to joint nociceptive input, increases ongoing discharges and increases the receptive fields of neurons (Cervero, Schaible et al. 1991; Schaible, Neugebauer et al. 1991). Descending facilitatory pathways may also be involved in centrally mediated chronic pain pathways whereby enhanced activity down descending pain pathways leads to generalized increases in sensory sensitivity. For example, neuropathic pain states in rodents have been associated with an enhanced descending facilitatory control of mechanical responses of spinal neurones, mediated through the activation of spinal 5HT3 receptors. These excitatory influences are likely to contribute to the development and maintenance of central sensitisation in the spinal cord, and the behavioural manifestation of tactile allodynia (Suzuki and Dickenson 2004).

However, these pathways are not well established in human studies (Vanegas and Schaible 2004).

1.2.5.2 Central sensitisation

In addition to descending inhibitory and facilitatory pathways, central sensitisation also leads to enhanced CNS neuron excitability and increased transmission of pain signals. Central sensitization is defined by IASP as: "an increased responsiveness of nociceptive neurons in the central nervous system to their normal or sub threshold afferent input" and can result from increased neuronal activity from the periphery. Like peripheral sensitization, central sensitization occurs in 2 phases, an immediate but relatively transient phase that depends on changes to existing proteins and a slower onset but longer lasting phase that depends on new gene expression. Central sensitization involves sensitization of pain projection neurons in the superficial laminae of the spinal cord dorsal horn as well as wide dynamic range neurons (WDR) located in deeper lamina (Woolf 2004). As a consequence of peripheral sensitization, there is enhanced release of intraspinal signal molecules, including the excitatory amino acid synaptic transmitter glutamate. Glutamate acts on ionotropic, AMPA, kainate and NMDA receptors, as well as metabotropic receptors (G-protein-coupled receptors) (Woolf 2004). The AMPA receptors are responsible for the baseline response to noxious stimuli. When glutamate binds to AMPA receptors located on the postsynaptic membrane, they permit a mixed flow of Na+ and K+ across the cell membrane, causing a depolarization of the postsynaptic membrane. This depolarization is called the excitatory postsynaptic potential. NMDA receptors are normally inactive due to a voltage dependent blockade of the receptor/ion channel pore by Mg^{2+} (Luo, Seeburg et al. 2008; Baumbauer, Young et al. 2009). NMDA receptors therefore rely on sufficient membrane depolarization via the action of glutamate (for example via AMPA receptors) to decrease the electrical force pulling Mg^{2+} ions into the pore. Removal of the voltage dependent block rapidly boosts synaptic efficacy and allows entry of Ca^{2+} into the neuron. This in turn activates calcium-sensitive intracellular signal cascades that lead to phosphorylation of NMDA receptors and activation of extracellular signal-related kinases including calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Sweatt 1999). The protein kinases phosphorylate existing AMPA receptors to increase their activity and also mediate the insertion of additional AMPA receptors into the postsynaptic membrane (Malenka and Bear 2004). By increasing the efficiency and number of AMPA receptors at the synapse, future excitatory stimuli generate larger postsynaptic responses. Persistent depolarization by glutamate (and other intraspinal signal molecules) can also lead to

the activation of protein kinases such as Mitogen Activated Protein Kinase (MAPK) which can induce changes in gene expression and protein synthesis providing longer term central sensitization. These plasticity changes result in an increased synaptic efficacy due to increased pre-synaptic excitatory transmitter release, amplification of the post synaptic response or increased membrane excitability. This enables the recruitment of normally sub-threshold inputs to supra-threshold action potentials generating an increased or augmented action potential output (Latremoliere and Woolf 2009).

Central sensitization, in contrast to peripheral sensitization, leads to the assimilation of novel inputs to nociceptive pathways such as large low-threshold mechanoreceptor myelinated fibres to produce Aβ fibre–mediated pain (Woolf and Salter 2000). Neurons show increased responses to innocuous (allodynia) and noxious stimuli (hyperalgesia) and there is an expansion of their total receptor fields so that pain hypersensitivity is felt beyond the area of tissue damage and neurons exhibit enhanced responses to adjacent or even remote non-inflamed tissue (secondary hyperalgesia) (Schaible, Ebersberger et al. 2002).

1.2.5.3 Glial activation

Glial cells, including astrocytes and microglia have close interactions with neurons and thus modulate pain transmission. They are emerging as important players in the initiation and maintenance of chronic pain (Scholz and Woolf 2007; Suter, Wen et al. 2007; Milligan and Watkins 2009). Glial activation in rodent models of chronic pain, including neuropathic and inflammatory pain show an early and transient microglial response followed by more durable astrocytic changes (Romero-Sandoval, Chai et al. 2008; Scholz, Abele et al. 2008).

Microglia are activated and recruited by numerous signals including ATP and NO (Davalos, Grutzendler et al. 2005; Nimmerjahn, Kirchhoff et al. 2005; Duan, Sahley et al. 2009), cytokines, and chemokines, some of which are released by injured sensory neurons and others by microglial cells themselves, or by astrocytes and T-cells (DeLeo and Yezierski 2001; Watkins, Milligan et al. 2001; Watkins and Maier 2002; Abbadie, Lindia et al. 2003; Dominguez, Rivat et al. 2008; Milligan, Sloane et al. 2008; Abbadie, Bhangoo et al. 2009). Astrocytes become activated after peripheral nerve injury (Garrison, Dougherty et al. 1994; Ji, Gereau et al. 2009; Milligan and Watkins 2009) with a slower onset and more prolonged time course than microglia, and may play more of a role in the maintenance of neuropathic pain hypersensitivity than microglia.

Glial activation leads to activation of protein kinases via phosphorylation events (De Leo, Tawfik et al. 2006) initiating downstream cascades including activation of NF-κβ, a cytokine nuclear transcription factor, leading to the production of proinflammatory cytokines such as IL-1β and TNF-*α*, as well as certain chemokines (Hashizume, DeLeo et al. 2000; Ledeboer, Gamanos et al. 2005; De Leo, Tawfik et al. 2006; Watkins, Hutchinson et al. 2007; Ji, Gereau et al. 2009; Milligan and Watkins 2009). In the spinal cord, IL-1β and TNF-*α* can directly excite neurons by binding to their receptors on spinal neurons and indirectly via cytokine induced release of additional excitatory mediators such as prostaglandins and nitric oxide (NO). Thus, spinal glial activation establishes a feed forward loop that leads to further protein kinase signalling, NF-κβ activation, and increased NO, cytokine, and chemokine production which contribute to ongoing pathological pain (Meller and Gebhart 1993; Meller, Dykstra et al. 1994; Bhat, Zhang et al. 1998; Levy, Hoke et al. 1999; Levy and Zochodne 2004).

1.2.6 Peripheral and central processing abnormalities in OA patients

The relative contribution of peripheral sensitization and abnormalities in central processing to OA pain is unknown and may differ for individual patients. Studies involving the intra-articular administration of local anaesthetic into osteoarthritic hip and knee joints have shown greatly reduced pain in approximately 60% to 80% of patients supporting a peripheral drive to pain (Creamer, Hunt et al. 1996; Crawford, Gie et al. 1998). Many studies have shown evidence for peripheral sensitization with OA patients having lower mechanical and thermal pain thresholds compared to healthy controls at sites close to affected joints (Wessel 1995; Farrell, Gibson et al. 2000). However, studies have also shown that OA can produce pain at clinically distant sites or over a widespread area providing support for the role of central pain mechanisms in OA. O'Driscoll et al. (1974) reported lower pain pressure thresholds in the forehead region (O'Driscoll and Jayson 1974) and Kosek et al. (2000) reported contralateral joint pain (Kosek and Ordeberg 2000) in patients suffering from hip OA consistent with the presence of secondary hyperalgesia. In 2001, Bajaj et al. infused hypertonic saline into the anterior tibialis muscles of 14 OA patients and 14 healthy controls (Bajaj, Graven-Nielsen et al. 2001). OA patients reported a greater increase in pain intensity over a larger pain area compared to control subjects. Imamura et al. (2008) evaluated the presence of hyperalgesia in patients scheduled for a total knee replacement due to knee OA with refractory pain by measuring pressure pain threshold measurements at sites across the lower back and legs. Patients with knee OA had significantly lower pressure pain thresholds over all evaluated structures compared to healthy controls (Imamura et al. 2008)

indicating a level of central sensitization leading to secondary hyperalgesia. Central sensitization can also lead to temporal summation in OA patients. In a study examining the effects of repeated pressure stimulation on pain sensitivity, temporal summation at the knee and tibialis anterior muscle was significantly greater among patients with knee OA compared with controls (Arendt-Nielsen, Nie et al. 2010).

Impairment of spinal descending inhibitory systems has been reported in studies of OA patients (Kosek and Ordeberg, 2000; Arendt-Nielsen, Nie et al. 2010). Kosek and Ordeberg measured the effect of a heterotopic noxious conditioning stimulus (using the upper extremity sub maximal effort tourniquet test) on pressure pain thresholds over an area contralateral to the maximally painful area in 15 patients with painful hip OA. These authors reported an increased pressure pain threshold during the tourniquet test in healthy controls which was interpreted as tourniquet-induced diffuse noxious inhibitory control modulating input from deep nociceptive afferents triggered by the tourniquet application. In contrast, no modulation of pressure pain sensitivity was induced by the tourniquet in the OA patients suggesting a dysfunction in the descending inhibitory control systems. Arendt-Nielsen et al. (2010) also reported no significant increase in pressure pain threshold at the peripatellar region when ischemic compression of the left arm was used as a heterotopic noxious conditioning stimulation for evoking diffuse noxious inhibitory control in knee OA patients. However, the pressure pain threshold did increase significantly in healthy control patients. OA patients therefore exhibited a loss of descending analgesic activity compared to healthy controls.

These studies suggest that OA pain, historically considered a peripheral entity, is probably also modulated via widespread mechanisms controlled by the CNS. Abnormalities in central pain processing may account for the poor correlation between peripheral joint damage and joint pain (Bedson and Croft 2008) and may explain why so many patients with OA have residual pain that is not effectively treated with drugs targeting peripheral or inflammatory pathways.

1.3 Challenges and new directions

The predominant symptom of OA is pain, yet current management strategies do not meet the expectations of patients (Crichton and Green 2002). Currently available therapeutics have modest efficacy and have toxicity profiles that are ill-suited to long-term administration for this chronic disease. For example, gastrointestinal complications are associated with the use of NSAIDs and there are cardiovascular concerns with selective COX-2 inhibitors (Petit-Zeman 2004). Recent development

efforts to address these problems include re-formulations, alternative dosing regimens and drug combinations aimed at reducing side effect profiles (Matthews and Hunter 2011). Drugs used for other indications are also under clinical investigation for the relief of OA pain. An example of this is duloxetine, a selective serotonin and norepinephrine reuptake inhibitor which was originally marketed as an anti-depressant and has been used for neuropathic pain conditions such as diabetic peripheral neuropathy (Ormseth, Scholz et al. 2011). Duloxetine has been shown to provide significant pain relief and improved function in patients with knee OA compared to placebo over a 13-week trial period (Chappell, Desaiah et al. 2011) and has been recently approved by the United States Food and Drug Administration to treat chronic musculoskeletal pain, including discomfort from OA and chronic lower back pain.

One promising area of progress in OA pain management is the development of targeted therapies for pain. Small drug molecules that modulate the activity of targets such as ion channels and receptors that act as primary transducers to excite or sensitize neurons (e.g. TRPV1, bradykinin) may offer new opportunities to interfere with the development and/or maintenance of OA pain. The next sections discuss the potential analgesic targets relevant to the studies in this thesis. These targets were selected based on known or potential relevance to OA or other chronic pain disorders indicated by previously published studies or in house (unpublished) data and the availability of specific inhibitors, antibodies or genetically modified mice.

1.3.1 Nerve Growth Factor

One of the most exciting pain relief targets to emerge are neurotrophins such as nerve growth factor (NGF). Neurotrophins and their receptors represent an important family of regulatory proteins essential for sensory nerve development, survival and the determination of neurochemical phenotype and the regulation of excitability (Sah, Ossipo et al. 2003; Zweifel, Kuruvilla et al. 2005). NGF is a small hormone produced by multiple structural, inflammatory and immune cell types following injury, inflammation or disease (Lei and Parada 2007). NGF activates multiple pain signalling pathways and is critical for the development of sensory neurons though not for neuron survival or repair following injury (Lei and Parada 2007). Binding of NGF to its high affinity tyrosine receptor kinase A (TrkA) and/or its low affinity p75 receptor, expressed on peripheral sensory nerves, can initiate intracellular signalling cascades and receptor--ligand trafficking activity to the nerve cell body to produce gene expression changes (Lei and Parada 2007). NGF binding

to TrkA is known to enhance TRPV1 signalling acutely (Zhang, Huang et al. 2005) and can induce expression of neuronal sensitizing factors calcitonin gene-related peptide (CGRP) and substance P (Zhang, Huang et al. 2005). NGF therefore regulates sensory neuron excitability and is an important mediator of injury-induced nociceptive and neuropathic pain (Ro, Chen et al. 1999; Theodosiou, Rush et al. 1999; Hefti, Rosenthal et al. 2006).

In humans, mutations in TrkA genes are linked to congenital insensitivity to pain (Indo, Tsuruta et al. 1996) and both NGF and *trkA*$^{-/-}$ mice have shown to have decreased responses to noxious stimuli (Crowley, Spencer et al. 1994; Smeyne, Klein et al. 1994). Transgenic mice over-expressing NGF demonstrate hyperalgesic pain behaviours (Davis, Lewin et al. 1993; Stucky, Koltzenburg et al. 1999) and administration of NGF in animals induces both rapid and long-lasting hyperalgesia (Lewin, Ritter et al. 1993; Andreev, Dimitrieva et al. 1995). NGF antibodies have been shown to reverse established allodynia in inflammatory/neuropathic pain models (Wild, Bian et al. 2007). In Complete Freund's Adjuvant (CFA)-induced hind-paw inflammation, the spinal nerve ligation model of neuropathic pain and streptozotocin-induced diabetic neuropathic pain models, a single intraperitoneal injection of a polyclonal anti-NGF antibody reversed established tactile allodynia from approximately day 3 to day 7 after treatment in rats (Wild, Bian et al. 2007). Similarly, in mice, a monoclonal anti-NGF antibody reversed established tactile allodynia in the CCI model of neuropathic pain (Wild, Bian et al. 2007).

Beneficial effects of reagents targeting TrkA have also been reported in animal models of OA pain and, importantly, in human clinical trials using OA patients. In a mouse model of OA, pre-treatment with soluble TrkA receptor, TrkAD5, (that will bind to and remove NGF, reducing available NGF concentration) prevented weight bearing deficits in the ipsilateral hind limb in the first 3 days following surgery to destabilize the medial meniscus (early phase) compared to control treated mice (McNamee, Burleigh et al. 2010). When administered at 12 weeks post-surgery (late phase), TrkAD5 administration significantly reversed chronic weight bearing deficits in the ipsilateral hind limb compared to the vehicle control mice (McNamee, Burleigh et al. 2010).

The anti-NGF monoclonal antibody, tanezumab, recently entered clinical trials for OA pain management (Lane, Schnitzer et al. 2010). In this double-blind randomized trial, 450 patients with painful knee OA were randomly assigned to receive intravenous treatment with tanezumab or a placebo. Over the 4-month study period, the NGF inhibitor demonstrated marked pain relief, producing improvements in all

efficacy measures. For example, those who received the active drug experienced a 45–62% reduction in pain intensity on walking, compared with only 22% in those who received placebo ($P<0.001$). Unfortunately, adverse effects were more common in patients who received any dose of the NGF inhibitor than those who received the placebo. 14% of patients on the active drug reported peripheral sensory symptoms, including paresthesia, compared with only 4% of the placebo group. In addition, a rapidly destructive arthritis was seen in 16 tanezumab-treated subjects, either in the knee, hip or shoulder, and all 16 required joint replacements. The U.S. Food and Drug Administration (FDA) suspended trials of tanezumab for OA in 2010 although an FDA advisory committee recently voted to allow the trials to continue (Lane, Schnitzer et al. 2010).

1.3.2 Tumour Necrosis Factor

Tumour Necrosis Factor-alpha (TNFα) is one of the major cytokines involved in the pathophysiology of OA and is up-regulated by activated synovial cells, mononuclear cells, schwann cells and chondrocytes during the cascade of events initiated by inflammatory stimuli.

TNFα can bind two receptors, TNF-R1 (expressed by most tissues) and TNF-R2 (expressed only by cells of the immune system). TNFα can directly sensitize neurons leading to a rapid increase in neuronal hyper-excitability (Czeschik, Hagenacker et al. 2008). TNFα can also induce activation of the MAPK pathways. For example, TNFα has been shown to cause persistent hyperalgesia in rats by long-lasting sensitization of joint nociceptors via the p38 MAPK system (Jin and Gereau 2006; Richter, Natura et al. 2010). TNF-α is also known to contribute to neuronal apoptosis via TNFR1 (Micheau and Tschopp 2003; Thorburn 2004) and the caspase signaling pathway (Micheau and Tschopp 2003). Binding of TNFα can also lead to activation of the transcription factor NF-κB which mediates the transcription of a vast array of proteins involved in cell survival and proliferation, the inflammatory response and anti-apoptotic factors. TNFα-induced production of Interleukins IL1, IL6 and IL8 (Pollock, McFarlane et al. 2002; Ohtori, Takahashi et al. 2004) stimulates endothelial cells to express adhesion molecules that attract leukocytes, increasing the production of metalloproteinases by synovial macrophages, fibroblasts, osteoclasts, and chondrocytes; and suppressing the synthesis of cartilage proteoglycans (Choy and Panayi 2001). Anti-TNF has become a common treatment for rheumatoid arthritis (Vinay and Kwon 2011), and has been shown to reverse weight bearing deficits in the first 3 days following surgery to destabilize the medial meniscus in a mouse model of OA (McNamee,

Burleigh et al. 2010). However, Anti-TNF therapy was not found to be efficacious at reversing weight bearing deficits when administered in the late OA pain phase of this model (McNamee, Burleigh et al. 2010). In an open label pilot study, no significant improvement in pain was found after 12 weeks of anti-TNF treatment (adalimumab) in 12 patients suffering from erosive OA (Magnano, Chakravarty et al. 2007). There is one case report, however, of successful treatment using anti-TNF treatment in a patient with severe knee OA pain that was refractory to NSAIDs (Grunke and Schulze-Koops 2006). The patient reported greatly improved symptoms following treatment with the human TNF antibody adalimumab, although therapy with a COX-2 inhibitor was initiated subsequently indicating that pain relief remained inadequate (Grunke and Schulze-Koops 2006). From the studies discussed above, TNFα does not appear to play a significant role in chronic OA pain. Antagonism of TNFα is therefore unlikely to be an effective treatment for OA pain.

1.3.3 Bradykinin

Kinins such as bradykinin are produced after tissue injury and are involved in the inflammatory response causing vasodilation, plasma extravasation, cell migration, pain and hyperalgesia (Dray 1997). Bradykinin acts on B1 and B2 G protein coupled receptors to assist in the initiation and maintenance of inflammation, the excitation and sensitization of sensory nerve fibres, and acts synergistically to potentiate the effects of proinflammatory cytokines (Sellam and Berenbaum 2010).

B1 G protein-coupled receptors are up-regulated in dorsal root ganglia (and other tissues) following injury (Levy and Zochodne 2000; Fox, Wotherspoon et al. 2003; Eisenbarth, Rukwied et al. 2004; Ferreira, Beirith et al. 2005), inflammation (Fox, Wotherspoon et al. 2003) or following exposure to neurotrophin glial-derived neurotrophic factor (GDNF) (Vellani, Zachrisson et al. 2004) and are activated by the active metabolite of bradykinin, des-Arg^{9}-bradykinin.

Bradykinin causes nociceptor activation and sensitization via B2 G protein-coupled receptors which are also expressed in a variety of tissues including synovial lining cells, fibroblasts, and endothelial cells of blood vessels in patients affected by OA (Bond, Lemon et al. 1997; Cassim, Naidoo et al. 1997; Dray 1997). Kinins initiate a cascade of secondary events, including prostanoid and nitric oxide production, phosphorylation of signalling proteins and sensitization of sensory transducers such as TRPV1 (Marceau, Hess et al. 1998). Targeting of bradykinin receptors should therefore enable a reduction in inflammation and sensitization, and provide effective analgesia in painful conditions. Several B1 antagonists have been studied in rodent

models of neuropathic and inflammatory pain. Peptide B1 antagonists, R715 and R954 showed an effective reversal of thermal hyperalgesia in a rat model of diabetic peripheral neuropathy induced by streptozocin (Gabra and Sirois 2003). Non-peptide B1 antagonist SSR240612 also reversed thermal hyperalgesia following UV radiation of the rat hind paw and in a rat CCI model of peripheral neuropathy (Gougat, Ferrari et al. 2004) and decreased nocifensive responses following intraplantar injection of formalin in mice (Gougat, Ferrari et al. 2004). The peptide B2 antagonist, Icabitant (des-Arg10 HOE-140) has also shown anti-hyperalgesic activity in a rat model of acute inflammation initiated by intraplantar injection of CFA (Gabra and Sirois 2003).

A role for bradykinin has also been implicated in OA pain using an anterior cruciate ligament transection model of OA in rats (Kaufman, Zaouter et al. 2011). Post-surgical weight bearing deficits were reported by Kaufman et al. to improve faster in R-954 (a peptide B1 antagonist) treated rats compared to saline injected rats. Intra-articular injection of R-954 was also associated with less subchondral bone remodelling, greater cartilage thickness and increased levels of cartilage proteoglycans and type II collagen compared to control rats. Kaufman et al. did not confirm the presence of pain by reversal of the weight bearing deficits with a known analgesic such as morphine, and therefore, the contribution of joint instability to the recorded weight bearing deficits is unknown. However, further studies are indicated to explore the therapeutic potential of B1 antagonism for reducing pain sensitivities and/or progression of disease in OA.

B2 antagonists have also shown efficacy in models of joint pain. Systemic administration of Bradyzide (a non-peptide B2 antagonist), significantly reversed mechanical hyperalgesia induced by intra-articular injection of CFA (Burgess, Perkins et al. 2000). In addition, intra-articular injection of either non-peptide B2 antagonist MEN16132 or peptide B2 antagonist Icabitant, led to the significant reversal of weight bearing deficits induced by monosodium iodoacetate (MIA) in a rat model of OA (Cialdai, Giuliani et al. 2009). Icabitant has also been used in a clinical trial using patients with symptomatic knee OA. Treatment by intra-articular injection of Icabitant was found to produce a significant reduction in pain intensity at rest and during activity (Song, Althoff et al. 2009).

Bradykinin receptors may prove to be valuable targets for the development of new analgesics. Difficulties have arisen, however, in species dependent differences in the pharmacological profile of the bradykinin receptors. For example, the B_2 receptor antagonist bradyzide, is reported to be a high affinity antagonist for

the rat B_2 receptor, but exhibits significantly lower affinity at the human B_2 receptor (Burgess, Perkins et al. 2000). This will provide difficulties in translating results of pre-clinical studies in rodents to human clinical trials. One approach may be to formulate human specific compounds based on the efficacy of bradykinin antagonists in animal models in rodent pre-clinical trials. However, safety studies using the human specific compounds would not be able to test for mechanism based adverse events in rats and dogs (the main toxicity/safety species used).

1.3.4 TRPV1

One of the ion channels that has been targeted for the development of novel analgesics is TRPV1, a polymodal nociceptor which is gated by capsaicin, noxious heat (>45°C), acidic pH (<5.3), and regulated either directly or indirectly by a variety of inflammatory agents, including protons, bradykinin, ATP, PGE_2, 12-lipoxygenase products, protease-activated receptor-2, anandamide, and NGF (Rosenbaum and Simon 2007). Activation of TRPV1 triggers an influx of calcium and sodium ions, depolarizing the neurons towards the potential for action potential initiation and transmission of neural impulses (Szallasi, Cortright et al. 2007). This sensory activation leads to a release of molecules associated with pain transmission such as glutamate, calcitonin gene-related peptide (CGRP) and substance P (Planells-Cases, Garcia-Sanz et al. 2005). Phosphorylation of TRPV1 by mediators including bradykinin, nerve growth factor and prostaglandins such as PGE_2 lowers the threshold for thermal activation of the channels and therefore results in sensitization of the nerves at normal physiological temperatures (Prescott and Julius 2003; Moriyama, Higashi et al. 2005).

The efficacy of TRPV1 antagonists in pain models has been investigated. For example, the TRPV1 antagonist, BCTC, produced anti-hyperalgesic effects in models of inflammatory and neuropathic pain (Pomonis, Harrison et al. 2003). In rats with CFA induced inflammation, BCTC significantly reduced the accompanying thermal and mechanical hyperalgesia when administered orally. BCTC also reduced mechanical hyperalgesia and tactile allodynia 2 weeks after constriction of the sciatic nerve which has been used as a model of neuropathic pain in rats. Another TRPV1 antagonist, AMG9810, has been shown to reverse thermal and mechanical hyperalgesia in a model of inflammatory pain induced by intraplantar injection of CFA (Gavva, Tamir et al. 2005).

A TRPV1 antagonist has also shown efficacy in a rat model of OA pain. Administration of TRPV1 receptor antagonist, A-889425, alleviated grip force impairment 3 weeks after MIA injection and significantly reduced the responses of

WDR and nociceptive specific neurons to 300 g von Frey hair stimulation of the knee joint in OA rats, but not control animals (Chu, Chandran et al. 2011). In addition, A-889425 also reduced the elevated spontaneous firing of WDR neurons in OA rats but did not alter spontaneous firing in sham rats. These findings indicate an important role of TRPV1 in OA pain including the production of spontaneous pain behaviours (Chu, Chandran et al. 2011).

However, clinical trials involving TRPV1 inhibitors have identified significant adverse effects on thermoregulation with increases in core body temperature and a reduced sensitivity to noxious heat (Gavva, Treanor et al. 2008; Krarup, Ny et al. 2011; Rowbotham, Nothaft et al. 2011) leading to discontinuation of the trials and concerns for the future of TRPV1 inhibitors as a potential treatment for chronic OA pain.

1.3.5 TRPA1

Other TRP channels (TRPV3, TRPV4, TRPA1) have also been suggested to be involved in transduction of noxious stimuli. TRPA1 (Transient receptor potential ankyrin subfamily, member 1) is highly expressed in sensory neurons of dorsal root ganglion (DRG), nodose ganglion and trigeminal ganglion neurons (Story, Peier et al. 2003; Nagata, Duggan et al. 2005) and is reported to be co-expressed with TRPV1 (Story, Peier et al. 2003; Bautista, Movahed et al. 2005; Kobayashi, Fukuoka et al. 2005; Nagata, Duggan et al. 2005). The functional expression of TRPA1 in human synovial cells has also been demonstrated (Kochukov, McNearney et al. 2006). TRPA1 is activated by noxious cold (≤17ºC), mustard oil (AITC), allicin, cinnamaldehyde, formalin and icilin but can also be sensitized by inflammatory mediators, including bradykinin (Bandell, Story et al. 2004), known to be significantly elevated in osteoarthritic synovial fluid (Melmon, Webster et al. 1967; Bond, Lemon et al. 1997; Nishimura, Segami et al. 2002). Tissue damage and inflammation can also initiate the release of several other endogenous compounds that are known to activate TRPA1 and induce pain behaviours in mice including 4-hydroxynonenal (4-HNE), reactive prostaglandins and hydrogen peroxide (Trevisani, Siemens et al. 2007; Andersson, Gentry et al. 2008; Cruz-Orengo, Dhaka et al. 2008). For example, intraplantar injection of 4-HNE causes lifting and licking of the affected paw, whilst these pain behaviours are greatly reduced in *trpa1*$^{-/-}$ mice (Trevisani, Siemens et al. 2007). In rodents, a selective TRPA1 antagonist, HC-030031, reduced nocifensive behaviour following AITC or formalin injection into the paw of rats. HC-030031 also increased paw withdrawal thresholds (PWTs) in the Randall Selitto test in a model of chronic inflammatory pain induced by intraplantar injection

of CFA and in a spinal nerve ligation model of neuropathic pain (spinal nerve ligation) (McNamara, Mandel-Brehm et al. 2007; Eid, Crown et al. 2008). Intrathecal TRPA1-targeted antisense oligonucleotides have also been shown to dramatically reduce cold hypersensitivity that was induced by spinal nerve ligation as a model of neuropathic pain in rats (Katsura, Obata et al. 2006). These preclinical data highlight TRPA1 antagonists as a promising new approach for the treatment of acute and chronic pain.

1.3.6 TRPM8

The transient receptor potential channel, melastatin type 8 (TRPM8) is a non-selective cation channel expressed in 5-10% of sensory primary afferent neurons of the DRG and at slightly higher levels in the Trigeminal Ganglia (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002). TRPM8 has a temperature threshold of ~25°C when expressed heterologously in mammalian cells such as Chinese Hamster Ovary (CHO) and Human Embryonic Kidney 293 (HEK293) cells and can also activated by exogenous ligands such as menthol and icilin (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002). TRPM8 agonism with menthol has been shown to increase PWTs (decrease pain sensitivities) to von Frey hairs and noxious thermal stimuli in the chronic constriction injury model of neuropathic pain (Proudfoot, Garry et al. 2006). However, TRPM8 antagonism may also provide relief from some forms of pain. For example, it has been shown that TRPM8 antagonist AMTB was effective in reversing established pain in a model of overactive bladder syndrome (Lashinger, Steiginga et al. 2008) and TRPM8 antagonists reduced cold allodynia in the CCI model of neuropathic pain (Parks, Parsons et al. 2011; Knowlton, Daniels et al. 2011) and CFA-induced inflammatory model of pain (Knowlton, Daniels et al. 2011). *Trpm8*$^{-/-}$ mice failed to develop cold allodynia measured using the acetone response test in either the CCI model or after injection of CFA into the hind paw (Colburn, Lubin et al. 2007; Parks, Parsons et al. 2011). Furthermore, unpublished pilot studies in the Bevan laboratory using TRPM8 antagonist, AMTB, and siRNA to reduce TRPM8 expression led to lower mechanical as well as cold sensitivity suggesting that TRPM8 antagonism could potentially affect joint pain.

The observation that both TRPM8 agonists and TRPM8 antagonists may be useful for the treatment of pain indicates a potentially useful approach to the treatment of disorders such as OA in which there continues to be a high unmet medical need.

1.4 Animal models of OA

Studying the aetiopathogenesis of human OA is challenging. Humans have extensive genetic heterogeneity and varied nutritional, biochemical and pharmacological history. Furthermore, identification of early disease is difficult due to the poor sensitivity of diagnostic tools and the relative inaccessibility of diseased and control tissues for sampling (Ameye and Young 2006). This has led to great interest in the use of model systems. Model systems are systems that are used to simplify complex phenomena and to understand otherwise elusive processes (Richardson 1984). Ex vivo models such as biochemical, cell or organ culture models can provide insight into the mechanisms for functional events within joint tissues. They are most useful for understanding short term biological events isolated from the physiological influences of adjacent structures and general metabolism and are inherently more highly controlled than animal models (Pritzker 1994). Ex vivo models cannot simulate the structural changes which occur in joint tissues over months to years, however. An animal model for human disease can be defined as "a homogenous set of animals which have an inherited, naturally acquired, or experimentally induced biological process, amenable to scientific investigation, that in one or more respects resembles the disease in humans" (Wessler 1976). Animal models of OA can be used to study how the complex structural changes in tissues evolve over time spontaneously or following experimental injury, and to determine how different factors may initiate, promote, or otherwise regulate these changes (Pritzker 1994). The selection of an animal model may be influenced by many factors (Table 1-1) and thorough characterisation of the model is required to validate it.

Table 1-1 Factors influencing the selection of animal models of OA. Adapted from Altman and Dean (1990)

Factors Influencing The Selection Of Animal Models of OA
Similarity to human disease
Quantity of tissue required for study
Cost of animal purchase and husbandry
Size of the animal and ease of handling
Initiating event (e.g. trauma, chemical, hormone, etc.)
Reproducibility of OA (e.g. location)
Consistency of changes
Animal age and genetic background

Animal models also can be used to develop disease modifying analgesic drugs through the study of pain behaviours. Chronic pain models demonstrate various hypersensitivities that resemble the array of symptoms seen clinically. These hypersensitivities may be evoked (by application of a particular stimulus) and measured directly at the site of tissue injury or as referred pain measures at distal sites. Thermal sensitivities (heat or cold) are usually measured from the latency to withdraw the limb from the stimulus. Mechanical hypersensitivity in an affected hind limb can be estimated by measuring the static distribution of weight borne by the two hind limbs or by dynamic analysis of gait in ambulatory animals. Mechanical hypersensitivities can also be measured by paw pressure thresholds (mechanical hyperalgesia), von Frey hair thresholds (tactile mechanical allodynia) and latencies for removal from a brush stimulus (dynamic mechanical allodynia). Other types of mechanical stimuli include compression of a joint with measurement of subsequent vocalization or withdrawal of the limb (Mogil 2009). Pain behaviours indicating the presence of hypersensitivities can also be displayed spontaneously (as changes in natural behaviour such as grooming and burrowing). It is essential that pain behaviours representing particular hypersensitivities can be recorded consistently to enable the determination of the effect of interventions in pre-clinical trials (Mogil 2009).

1.4.1 Mouse models of OA

Given the high cost of maintaining larger species and their greater drug requirements, smaller animal models are preferred for preliminary studies. The mouse is the primary species for the generation of genetically modified animals, including animals with gene deletions and gene over-expression. These features make the mouse a good choice for the development of an OA model. However, there are relatively few studies of OA in mice compared to other species.

1.4.2 Spontaneous OA

Models of OA can be classified as either spontaneous or induced. Spontaneous OA has been studied in mice, guinea pigs, Syrian hamsters and dogs (Walton 1977; Bendele and Hulman 1988; Liu, Burton-Wurster et al. 2003). Spontaneous OA occurs in the knee joints of several strains of mice including C57Bl/6, BALB/c, DBA/1 and STR/ort with an increased incidence and severity as they age (Walton 1977; Walton 1979; Nordling, Karlsson-Parra et al. 1992). Reports of spontaneously occurring OA in mice have been limited to descriptions of joint pathology, using histology, radiology, and Computed Tomography (CT) to document cartilage erosion, sclerosis of the subchondral bone and osteophyte formation. Spontaneous OA in mice has a high variability in the age of onset and severity of joint pathology compared to guinea pigs who develop predictable knee OA at a relatively young age (Bendele and Hulman 1988; Bendele, White et al. 1989). The disease variability of spontaneous OA in mice has generally precluded their use for pharmaceutical testing or pathogenesis studies. To the author's knowledge, there have been no reports of OA pain behaviours originating from spontaneously occurring OA pain in mice.

The development of transgenic technology has made possible the generation of targeted gene-mutated mouse lines suitable for use in experimental OA research. For example, mutations in several human genes coding cartilage specific collagens have been shown to be responsible for evoking OA. Mice harbouring these genes (e.g. mutations in cartilage collagen types II and IX) develop early-onset OA and are potentially promising models of OA (Helminen, Saamanen et al. 2002). These models will help increase our understanding of cartilage development and growth and perhaps be useful in the search for mechanisms of cartilage repair. They may also be useful for the development and testing of disease modifying drugs that delay or reverse progression of the morphological changes that occur in degenerative joint disease. However, mice showing severe and early chrondrodysplasia and OA may not be ideal representations of human idiopathic OA that has a slow, insidious onset

and further testing in other animal models will be likely required to validate experimental results.

1.4.3 Induced OA

1.4.3.1 Chemical induction of OA

Intra-articular injections of chemicals that interfere with cartilage metabolism have also been successful in causing the degeneration of cartilage and the development of OA. Methods for chemical destruction of cartilage have included intra-articular injection of papain, collagenase and MIA amongst others (van der Kraan, Vitters et al. 1989). One of the best characterized models is the injection of the metabolic inhibitor MIA into the joint which was first described in 1987 (Kalbhen 1987). MIA inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase in chondrocytes, resulting in disruption of glycolysis and eventually cell death (Guzman, Evans et al. 2003). The progressive loss of chondrocytes leads to histological and morphological changes of the articular cartilage, closely resembling those seen in OA patients (Janusz, Hookfin et al. 2001; Janusz, Bendele et al. 2002). Intra-articular injection of MIA has been reported in studies of C57Bl/6, C57Bl/10 and BALB/c mice (van Osch, van der Kraan et al. 1994; Harvey and Dickenson 2009; Hadipour-Jahromy and Mozaffari-Kermani 2010; Sniekers, Weinans et al. 2010). Most of these studies have focussed on joint pathology rather than pain. Following intra-articular injection of MIA into murine knee joints, knee swelling and inflammatory cell infiltration have been reported immediately following surgery and up to day 3 post-surgery (van Osch, van der Kraan et al. 1994; van der Kraan, Vitters et al. 1989). Findings such as disorganization of chondrocytes, erosion and fibrillation of the cartilage surface, subchondral bone exposure and loss of proteoglycan in cartilage are reported to affect the ipsilateral femorotibial joint from day 14 post MIA injection (Hadipour-Jahromy and Mozaffari-Kermani 2010). These histopathological lesions are time dependent with greater joint degeneration noted at later time points (Hadipour-Jahromy and Mozaffari-Kermani 2010). Histological findings are generally mild with respect to cartilage erosion of the femorotibial joint although small osteophytes have been reported (van der Kraan, Vitters et al. 1989). Following MIA injection, femoropatellar joint cartilage erosion also occurs (van der Kraan, Vitters et al. 1989; van Osch, van der Kraan et al. 1994) with a marked decrease in proteoglycan synthesis (van Osch, van der Kraan et al. 1994) and depletion of safranin O staining from day 7 post-surgery (van der Kraan, Vitters et al. 1989; van Osch, van der Kraan et al. 1994) representing loss of proteoglycan and erosion of patellar cartilage. In contrast to the femorotibial joint, marked

osteophytosis of the femoropatellar joint is noted from day 7 post-MIA injection (van der Kraan, Vitters et al. 1989; van Osch, van der Kraan et al. 1994).

Pain in a murine MIA-induced model of OA was described by Harvey et al. (2009). A persistent significant mechanical allodynia developed in the ipsilateral hind limb following MIA injection with significantly decreased PWTs in response to stimulation with von Frey hairs, at all time points (up to day 28) compared to the contralateral hind limb or control mice. Thermal hypersensitivity did not develop following MIA injection at any time point studied and a significantly decreased latency to fall using the rotarod was noted only at day 14 post MIA injection compared to control mice (Harvey and Dickenson, 2009).

In summary, intra-articular injection of MIA in mice appears to produce degenerative joint disease lesions characteristic of OA in a time-dependent manner. In terms of OA pain, persistent mechanical allodynia of the ipsilateral hind paw is the only pain sensitivity to be reported. A more thorough evaluation of pain behaviours, with reversal using known analgesics is required to characterise the murine MIA model further.

In rats, the MIA model of OA has been widely used. There are many reports of the histopathological findings in knee joints following injection of MIA and many studies also include reports of spontaneous or evoked pain behaviours. Fewer studies, however, confirm the presence of joint pain rather than joint instability by reversal of the behaviours using known analgesics. A summary of the behavioural studies in rats that include studies of the effects of common analgesics used to treat OA pain follows:

In 2003, Bove et al., provided the first report of the development of persistent weight bearing deficits following intra-articular injection of MIA in the ipsilateral hind limb in a 2 week study of male Wistar rats (Bove, Calcaterra et al. 2003). Persistent weight bearing deficits have also been reported in studies ranging from 2 weeks up to 10 weeks in duration (Pomonis, Boulet et al. 2005). These deficits can be significantly reduced by the administration of opioid drugs such as morphine and tramadol (Pomonis, Boulet et al. 2005; Combe, Bramwell et al. 2004) and paracetamol (Bove, Calcaterra et al. 2003) confirming the presence of hind limb pain. The response of weight bearing deficits to anti-inflammatory drugs varies between studies. Bove et al. (2003) reported that deficits were significantly reduced by the administration of the NSAID naproxen, and COX-2 inhibitor, rofecoxib. However, Pomonis et al. (2005) reported that the administration of the NSAID, indomethacin or COX-2

inhibitor, celecoxib, did not significantly reverse weight bearing deficits unless administered as part of a chronic dosing regime. The variable response to anti-inflammatory drugs may indicate that ongoing inflammation does not drive knee pain in rat models of MIA induced OA.

The development of persistent mechanical allodynia to von Frey filament stimulation of the ipsilateral hind limb of rats injected with MIA has been reported in several studies (Fernihough, Gentry et al. 2004; Beyreuther, Callizot et al. 2007). Mechanical allodynia was significantly decreased following administration of morphine (Beyreuther, Callizot et al. 2007; Fernihough, Gentry et al. 2004; Combe, Bramwell et al. 2004) and tramadol (Combe, Bramwell et al. 2004). However, diclofenac (an NSAID) and paracetamol treatment have proved to be ineffective at reversing established mechanical allodynia in MIA treated rats (Beyreuther, Callizot et al. 2007; Fernihough, Gentry et al. 2004).

Persistent mechanical hyperalgesia evoked by paw pressure stimulation has been recorded from day 3 following MIA injection in rats (Beyreuther, Callizot et al. 2007; Fernihough, Gentry et al. 2004). Mechanical hyperalgesia was significantly reduced by morphine at all time points tested (Beyreuther, Callizot et al. 2007; Fernihough, Gentry et al. 2004), however, paracetamol was only effective at day 3 post-surgery (Fernihough, Gentry et al. 2004). Variable responses have been reported in response to diclofenac with reversal of mechanical hyperalgesia only observed at early time points (day 3 post-surgery) being reported by Fernihough et al. (2004) whilst it was effective at all time points tested (up to day 14) by Beyreuther et al (2007).

Further pain measures were recorded by Vonsy, Ghandehari et al. (2009) who examined cooling hypersensitivity (acetone drop test) and latency to fall (rotarod) in male Sprague-Dawley rats in addition to tactile mechanical allodynia. Whilst a morphine responsive mechanical allodynia developed over the 2 week study period, there was no significant difference in the latency to fall in MIA injected rats compared to sham rats. The acetone drop test produced a trend towards cooling hypersensitivity in MIA injected rats over the 2 week period although this was not statistically significant.

Spontaneous activity levels and dynamic gait analysis have also been used as indicators for OA pain in rats (Nagase, Kumakura et al. 2011). Nagase et al. showed that morphine and tramadol could significantly reverse decreased levels of spontaneous activity in MIA injected rats compared to control animals. However, no

significant effect was noted following administration of naproxen or paracetamol compared with the control group. Administration of celecoxib also failed to reverse decreased spontaneous activity in MIA treated rats (Nagase, Kumakura et al. 2011) and alterations in hind limb gait using CatWalk dynamic gait analysis (Ferland, Laverty et al. 2011) unless administered within the first 3 days following MIA injection.

The MIA model has recently been used to test several novel analgesics aimed at increasing our understanding of the pain pathways involved in degenerative joint disease (Rahman, Bauer et al. 2009; Sagar, Burston et al. 2011). Selective TRPV1 antagonist A-889425 reversed hind limb grip force impairment in a rat model of OA pain (Chu, Chandran et al. 2011) and bradykinin B2 antagonists, MEN16132 and Icatibant, reversed weight bearing deficits (Cialdai, Giuliani et al. 2009) indicating roles for TRPV1 and bradykinin in OA pain.

Overall, rodent studies show that OA pain induced by intra-articular injection of MIA produces rapid onset of robust pain behaviours that may persist for up to 10 weeks. Whilst further testing is required in mice, pain behaviours in rats are consistently reversed by opioid analgesics such as morphine and tramadol but have varying responses to anti-inflammatory drugs such as naproxen and diclofenac, selective COX-2 inhibitors such as rofecoxib and celecoxib and other drugs such as paracetamol. The varying responses to anti-inflammatory drugs may be due to differing levels of inflammation present in the joint at the time of drug administration. For example, infiltration of joint tissues with inflammatory cells seen on histopathology and greater efficacy of anti-inflammatory drugs were consistently observed within the initial post-injection time period but not at later time points (Nagase, Kumakura et al. 2011; Fernihough, Gentry et al. 2004; van Osch, van der Kraan et al. 1994; van der Kraan, Vitters et al. 1989; Bove, Calcaterra et al. 2003). This suggests that early pain behaviours were related to joint inflammation evoked by MIA actions in the joint rather than a direct measure of OA pain and that ongoing pain due the presence of OA has a less significant inflammatory component.

1.4.3.2 Surgical induction of OA

Surgical models offer many advantages to spontaneous models of OA including faster onset of disease, decreased variability, and decreased dependence on genetic background (Glasson, Blanchet et al. 2007). OA can be induced in animal knee joints by reducing ligamentous support (transection of the cranial cruciate ligament or collateral ligaments) with or without removing all or part of each meniscus. The meniscus can also be partially removed by procedures such as

partial medial meniscectomy (Fernihough, Gentry et al. 2004; Welch, Cowan et al. 2009) or destabilized by cutting the meniscotibial ligament (Inglis, McNamee et al. 2008) without disruption to the cruciate or collateral ligaments. Surgically induced models of OA are widely accepted in a number of species including rabbits (Fahlgren, Messner et al. 2003), sheep (Ghosh, Sutherland et al. 1990) goats (Laurent, O'Byrne et al. 2006) and rats (Janusz, Bendele et al. 2002). These types of surgeries aim to mimic injuries occurring in humans such as cruciate ligament rupture and meniscal tears that may subsequently result in the development of OA. Injuries to the menisci are a common orthopaedic problem and can result from trauma or from degeneration (Baker, Peckham et al. 1985). Traumatic meniscal tears typically occur from twisting type injuries to the knee and can vary in size, location and stability, although the majority of tears in humans affect the medial meniscus (Campbell, Sanders et al. 2001). Surgical excision of a tear (partial or total meniscectomy) is the most common treatment particularly for tears involving the central, less vascular portion of the meniscus (Gallacher, Gilbert et al. 2010). Development of OA is a common complication following meniscectomy and the degree of degenerative joint disease is positively correlated with the amount of meniscus removed (Andersson-Molina, Karlsson et al. 2002).

There have been relatively few surgically induced mouse models of OA reported in the literature. The first was published in 1996, where Visco et al described partial medial meniscectomy (PMM) and medial collateral ligament transection as a method of inducing degenerative joint disease (Visco DM 1996) which was confirmed by histology. Histopathological findings were also explored by Kamekura et al. (2005) who produced 4 different mouse models exhibiting various speeds of OA progression utilising different combinations of ligament transection and meniscectomy (Kamekura, Hoshi et al. 2005). They included anterior cruciate ligament resection with or without medial meniscectomy, medial meniscectomy with medial collateral ligament resection or complete disruption of the stifle including anterior and posterior cruciate ligament transection, bilateral collateral ligament transection, medial and lateral meniscectomy and patellar ligament resection. Radiological and histological analyses of the knee joints at time points up to 12 weeks post surgery, showed time dependent joint destruction and osteophyte formation with speed of onset and severity correlating with the degree of induced joint instability.

Destabilization of the medial meniscus (DMM) by transection of the meniscotibial ligament was first described in detail by Glasson, Blanchet et al. (2007) who

compared the histological progression of degenerative joint disease following DMM in mice with those who underwent transection of their anterior cruciate ligament (ACLT). Similar to the previous study by Kamekura et al. (2005), the ACLT model produced severe and rapid degenerative joint lesions including chondrogenesis of the joint capsule and, in some cases, severe subchondral erosion of the posterior tibial plateau which has not been reported in cases of spontaneous OA in mice. The DMM model, however, produced milder degrees of cartilage erosion that increased with time following surgery. The ACLT model was therefore considered to be inferior to the DMM model since the subsequent cartilage lesions were too severe to be representative of spontaneous human OA and the greater surgical proficiency required increased the risk of iatrogenic damage. The DMM model has since been the main model for studies examining features of OA pain, novel therapeutics and the effects of joint destabilization in genetically modified mice.

Pain behaviours accompanying histological lesions consistent with knee OA in a murine surgical model of OA were first reported by Inglis et al. (2008) who investigated the onset of both cartilage degeneration and pain using the DMM model. Significant levels of degenerative joint disease were observed from 2 weeks post-surgery compared to naïve control mice and progressed over the 12 week study period. Reduced weight bearing of the ipsilateral hind limb was recorded at day 3 in sham and DMM mice which was attributed to post-surgical inflammation, and at weeks 12 to 16 post-surgery in the DMM mice, which was attributed to the development of OA pain. Measures of spontaneous activity (such as speed of movement, distance travelled and climbing) were decreased in DMM mice compared to sham-operated animals at 8 weeks post-surgery (Inglis, McNamee et al. 2008) and both weight bearing deficits and decreased spontaneous activity measures were reversible using analgesics, celecoxib and morphine, confirming the presence of joint pain. DMM surgery did not, however, alter the overnight home cage locomotor activity of mice recorded up to 8 weeks post-DMM surgery in another study (Malfait, Ritchie et al. 2010). Malfait et al. did report both ipsilateral and contralateral mechanical allodynia from day 28 post-DMM surgery compared to sham-operated mice which was reversible using analgesics, morphine and paracetamol (Malfait, Ritchie et al. 2010). However, changes in gait (tested up to day 42), mechanical sensitivity of the tail and thermal hyperalgesia of the hind paws (tested up until day 56 post-surgery) were not observed (Malfait, Ritchie et al. 2010).

These two studies illustrate the development of degenerative joint disease and accompanying changes in weight bearing, spontaneous activity and development of

mechanical allodynia in the hind paws following DMM surgery. Behavioural changes were reversible with known analgesics confirming the presence of pain rather than joint instability alone. However, it is difficult to interpret the degree of pain caused by the destabilized meniscus, compared to that caused by degenerative joint disease within the knee. The DMM model is also disadvantaged by the 8 week (decreased spontaneous activity) and 12 week (weight bearing deficits) delay for certain pain behaviours to become established and therefore requires a prolonged period of post-surgery measurements in order to fully characterise the development of OA pain and response to different analgesics (Inglis, McNamee et al. 2008). Experiments with the opioid receptor antagonists naloxone and naloxone methiodide, suggest that endogenous opioids play a role in the temporal disparity between the appearance of joint degeneration and the presence of pain behaviours (Inglis, McNamee et al. 2008) and further investigation into the endogenous opioid system and OA pain is warranted.

A wide variety of OA animal models have been described suggesting that none of the existing models are ideal or that many models are required to fulfil differences in human OA due to different aetiologies and characteristics of the disease. Pain is the predominant feature in clinical OA; however, the majority of animal models have focused on structural damage and changes in gait and mobility and have not addressed the issue of pain. The development of models of OA in mice has lagged many years behind those in other species perhaps due to challenges that arise with working with smaller animals that require careful handling and intricate surgery. Current models such as the MIA model and DMM model produce pain behaviours and histopathological findings in mice consistent with OA but their clinical relevance is compromised by the potential non-specific effects of MIA or the continued presence of a destabilized meniscus within the joint cavity.

1.5 Overview

This introduction has identified a need for a clinically relevant mouse model for human OA pain that can be used to further our understanding of OA disease pathophysiology, and for the development of new analgesic therapies. The following results chapters describe the investigation of three potential mouse models of OA pain (MIA, partial medial meniscectomy and spontaneous OA in the STR/ort mouse). One of these models is then selected for further characterisation and validation using a range of behavioural and pharmacological tests. The analgesic/anti-hyperalgesic effects of tool compounds that inhibit specific molecular targets were tested using the chosen model of OA pain and the effect of deleting selected genes on the development of OA pain were determined using genetically modified mice. Finally, retrograde neuronal back labelling techniques were used to identify joint afferents from mice with OA pain enabling some functional properties of the neurons to be examined using calcium imaging studies.

1.6 Aims to be addressed during these PhD studies

The PhD studies were designed to establish a mouse model of OA pain for the study of peripheral neuronal mechanisms and potential sites for therapeutic intervention.

To achieve this, a number of study areas were explored:

- Examination of chemical, surgical and spontaneous murine models of OA prior to the selection and validation of a clinically relevant murine model of OA pain for further study
- Identification of the importance of selected ion channels for the transduction/transmission of joint pain in vivo using genetically modified mice and a murine model of OA pain
- Identification of the importance of selected mediators and ion channels for the transduction/transmission of joint pain *in vivo* using tool compounds
- Identification of the importance of selected mediators and ion channels for the transduction/transmission of joint pain using calcium imaging of joint afferent neurons from a murine model of OA pain

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Chemicals, solutions and media

All chemicals were analytical grade, purchased from Sigma-Aldrich (Poole, UK), Invitrogen (Paisley, UK) Fisher (Loughborough, UK) or VWR (Lutterworth, UK) unless stated otherwise. Cell culture media were purchased from Invitrogen (Paisley, UK). Solutions were made using de-ionized water and were autoclaved or filter sterilised where required.

2.2 Reagents/Compounds

Buprenorphine hydrochloride, diclofenac sodium, morphine sulphate, tramadol, lamotrigine, paracetamol, naloxone hydrochloride and naloxone methodide were obtained from Sigma-Aldrich Ltd (Poole, UK). Celecoxib and gabapentin were obtained from Molekula, Shaftesbury, UK. Naltrindole, Nalxonazine and 5'-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5,α-epoxy-3,14dihydroxy-6,7-2'3'-indolomorphinan dihydrochloride (GNTI) were obtained from Tocris Bioscience (Ellisville, USA). Trk A inhibitors CE-245677 and AZ-23 were kindly donated by Pfizer Inc. UK (Sandwich, UK). Duloxetine was purchased from Toronto Research Chemicals (North York, Canada). HC-030031 was obtained from Chembridge Corporation (San Diego, USA). N-(3-aminopropyl)-2{[3-methylphenyl)methyl]oxy}-N-(2-thienylmethyl)benzamide hydrochloride (AMTB) was synthesized by Simon Lewis (University of Bath, UK). A TNF chimeric neutralizing protein (murine TNF receptor - type II Fc fusion protein) plus a class matched negative control reagent (anti–Elmeria tenella, muIgG2a) were kindly donated by Janet Nicholson (Pfizer Inc. UK, Sandwich, UK). Bradykinin receptor 2 antagonist, Bradyzide, was obtained from Novartis, Switzerland. Capsaicin was purchased from Sigma (St. Louis, USA). Icilin was obtained from Biomol (Exeter, UK) and Allyl isothiocyanate (AITC) was obtained from Sigma-Aldrich (Poole, UK).

2.3 Animals

Animals were housed in a temperature controlled environment on a 12h light/dark cycle with access to food and water ad libitum. All animal procedures were performed in accordance with the Home Office Animals (Scientific) Act 1986 (UK) regulations (project licence PPL 70/6637) and approved by the Kings College London Ethical Review Committee.

The following animals were purchased from Harlan UK Ltd., (Bicester, UK):

- 8-10 week old female Wistar rats weighing approximately 140g ± 10g.

- 4-10 week old female C57Bl/6 mice weighing approximately 18g ± 2g
- 8 week old male CBA mice weighing approximately 22 ± 2g

2.3.1 *Trpa1*$^{-/-}$ mice

Trpa1$^{-/-}$ mice were bred from Heterozygotic, *trpa+/-* mice that were kindly provided by Drs. Kelvin Kwan (Harvard Medical School, Boston, MA) and David Corey (Harvard Medical School, Boston, MA) and backcrossed for 10 generations on to a C57Bl/6 background. Male and female homozygote *trpa1*$^{-/-}$ and *trpa1*$^{+/+}$ wild-type littermates were indistinguishable and normal in overall appearance and viability.

2.3.2 STR/ort mice

16-19 week old male STR/ort Mice were provided by Dr Andrew Pitsilides at the Royal Veterinary College, London, UK. The STR/ort mice were originally derived from the Mason/Chambers group. The parent STR/1N strain was isolated by Dr George E Jay, Jr in 1951 as a spontaneous mutant of STR/1N arising in the F29 generation. STR/ort mice were derived from the parent STR/1N strain following a period of non-inbreeding and inbreeding.

2.4 Surgery

All surgery was carried out under isoflurane/O2 inhalation general anaesthesia unless otherwise stated. Peri-operative analgesia was not used unless specified. Injections were made using 26Gx3/8 needles and a Hamilton syringe.

2.4.1 Induction of acute inflammation

2.4.1.1 Induction of acute joint inflammation

Anaesthetized mice were positioned in dorsal recumbency and the left leg flexed at a 90º angle. Inflammation was induced by injection of 10µl of 0.1% w/v CFA (Fisher Scientific UK Ltd, Loughborough, UK) in mineral oil through the patellar ligament. Sham operated animals received an injection of 10µl of mineral oil instead of CFA.

2.4.1.2 Induction of acute paw inflammation

Conscious, mice were restrained and held in dorsal recumbency. 10µl of 0.1% w/v CFA in mineral oil was injected into the plantar surface of the paw.

2.4.2 Induction of OA

2.4.2.1 Chemical induction of OA

Under general anaesthesia, mice were positioned in dorsal recumbency. The left leg was flexed at a 90º angle. Joint damage was induced by injection of 10µl of

(various concentrations) MIA (Fisher Scientific UK Ltd, Loughborough, UK) in physiological saline through the patellar ligament. To control for the effects of intra-articular injection, another group of mice received an injection of 10µl of 0.9% sterile saline instead of MIA following the same protocol.

2.4.2.2 Surgical induction of OA by partial medial meniscectomy

Mice were positioned in dorsal recumbency. The left leg was shaved and the skin cleaned with antiseptic. With the leg held in an extended position a medial para-patellar skin incision (approximately 3mm) and arthrotomy was performed using a no. 11 scalpel blade (Figure 2-1). In contrast to previously described partial meniscectomy surgery (Welch, Cowan et al. 2009), the collateral ligaments were left intact and the patellar was not displaced. With the aid of a light microscope and angled 23 gauge needle, the medial meniscus was freed from its attachments to the margin of the tibial plateau and approximately half of the medial meniscus was removed (~1mm of tissue). The skin incision was closed with a wound closure clip. Sham operated animals were treated in the same way with a skin incision and arthrotomy being performed; however, the meniscus was left intact. The wound closure clip was removed with the animals remaining conscious once the skin incision had healed 7-10, days following surgery.

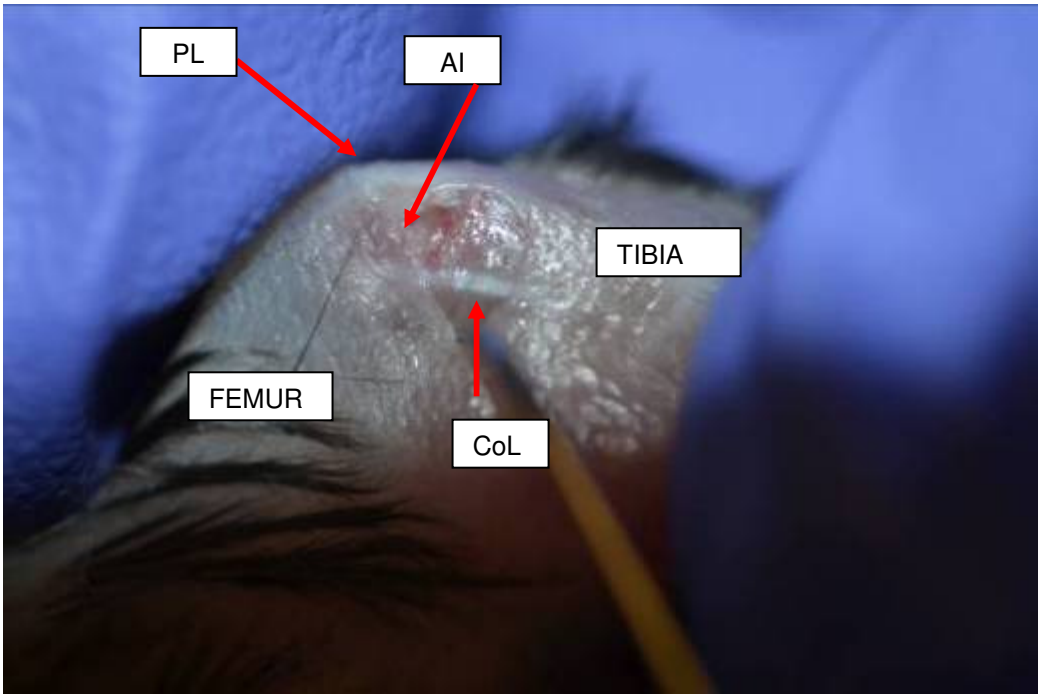


Figure 2-1 Surgical approach for partial medial meniscectomy of the left knee.
Left femorotibial joint of a C57Bl/6 mouse where (PL) represents the patellar ligament, (CoL) represents the medial collateral ligament, (AI) represents the location of the arthrotomy incision and medial meniscus.

2.4.3 Removal of Gonads

2.4.3.1 Ovariectomy

Anaesthetized mice were placed in lateral recumbency and 0.3mg/kg buprenorphine administered s.c. The dorsal and lateral mid-lumbar region was shaved and prepared aseptically. A dorsal midline skin incision was made caudal to the posterior border of the ribs. Blunt dissection was used to tunnel subcutaneously lateral to the skin incision and the muscles of the posterior abdominal wall were separated to allow entry to the abdominal cavity. The ovary was located in a fat pad just beneath the muscles (Figure 2-2). Using forceps, the peri-ovarian fat was gently grasped to lift and exteriorize the ovary. Mosquito forceps were used to crush the fallopian tube and cranial-most part of the uterine horn distal to the ovary, being careful not to crush or contact the ovary. A single ligature of 5-0 Silk (Ethicon Inc, UK) was tied around the ovarian pedicle. The ovary was removed by cutting between the clamped area and the ligature. The uterine horn was returned into the abdomen, and the process was repeated on the other side. The skin incision was closed using wound clips. Sham operated animals were treated in the same way with a bilateral laparotomy being performed; the ovaries were identified and exteriorized before being replaced in the abdomen intact. The wound closure clip was removed with the animals remaining conscious once the skin incision had healed 7-10 days following surgery.

Figure 2-2 Identification of the left ovary by left dorsal-ventral laparotomy

2.5 Behaviour

2.5.1 General

In the week prior to surgery, the animals were handled daily to adapt to the laboratory, apparatus and behavioural tests. All experiments were performed at the same time of the day to avoid diurnal variation in behavioural readings.

Measurements for pain behaviour in mice were taken before and at regular intervals following the induction of OA. During initial time course studies, readings were also taken from sham operated animals for comparison. All measurements were performed with the operator blinded to the treatment. The general behaviour and appearance of operated and sham operated animals were indistinguishable from each other. Behavioural tests always followed the same sequence for each set of experiments: von Frey 1, paw pressure 2, cold plate 3, knee compressions 4. When weight bearing measurements were taken, these were performed as the first test in the sequence. In experiments where thermal hyperalgesia was included, this was performed as the final test and only a single leg was measured since this test induces rapid learning of avoidance behaviour and artificially decreased paw withdrawal latencies (PWLs) for the second leg. The sequence of measurements was chosen to minimise any risk of sensitization to subsequent tests.

2.5.2 Weight bearing

The difference in weight borne by the ipsilateral compared to the contralateral hind limb was measured using a Dual Channel Weight Averager (Linton Instrumentation, Diss, Norfolk, UK), see Figure 2-3 . Mice were individually placed in to a Perspex chamber designed to so that the weight of the animal was borne on the hind limbs with each limb resting on a separate transducer pad. The transducer pad records over three seconds the distribution of the animals' body weight on each paw. Animals were allowed to acclimate to the equipment on at least 2 occasions prior to taking the measurements. At least 2 separate measurements were made for each animal at each time point, and the result expressed as the mean percentage of the weight placed on the operated limb versus the contralateral un-operated limb.

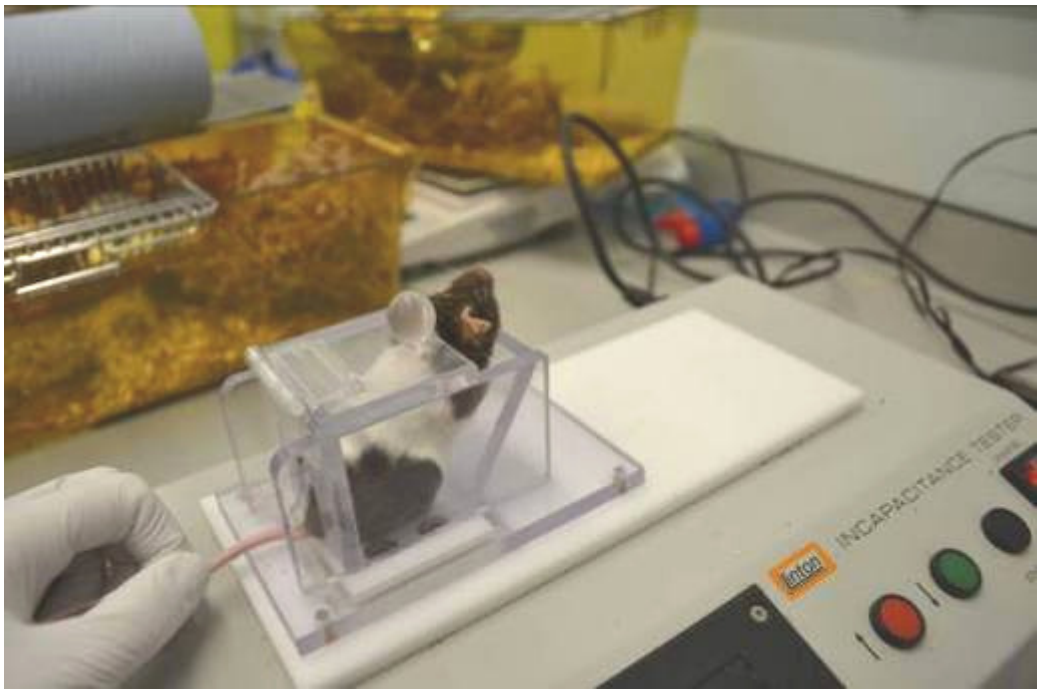


Figure 2-3 Demonstration of hind limb weight bearing measurements on a STR/ort mouse using a Dual Channel Weight Averager

2.5.3 Weight bearing following either hind limb passive range of motion or flexed knee compression

In some experiments weight bearing was measured after knee manipulations. The general procedure was as described in the previous section with the following modifications prior to taking the measurements. In one set of experiments, each mouse was gently restrained and the ipsilateral knee was flexed and extended through its full range of motion a total of ten times before immediately placing the mouse in the Dual Channel Weight Averager. 1 measurement was made for each animal.

In a second set of experiments, the thigh was held in a fixed position and the operator's thumb and forefinger were used to compress the ipsilateral knee in a flexed position with moderate force (Figure 2-4). This was repeated 5 times at 2 second intervals and the animal was then immediately placed in the Dual Channel Weight Averager. One measurement of weight borne by the ipsilateral and contralateral hind limbs was made once both hind limbs rested on the transducer pads.

Figure 2-4 Compression of the knee whilst held in a flexed position

2.5.4 Mechanical sensitivity (von Frey)

Mechanical sensitivity was assessed by measuring withdrawal thresholds to calibrated von Frey filaments. Animals were placed into a Perspex chamber with a metal grid floor giving access to the underside of their paws and allowed to acclimatise prior to the start of the experiment (Figure 2-5). Mechanical allodynia was tested by touching the plantar surface of the animals' hind paw with von Frey filaments in ascending order of force for up to 6 seconds. A range of filaments from 0.04g to 1.4g were used. A positive response was noted if the paw was sharply withdrawn or there was flinching upon removal of the hair. Once a positive withdrawal response was established, the paw was re-tested starting with the next descending von Frey hair until no response occurred (Chaplan, Bach et al. 1994). The lowest amount of force required to elicit a response was recorded as the paw withdrawal threshold (PWT) in grams.

Figure 2-5 Mechanical allodynia measurement using von Frey hairs

2.5.5 Mechanical hyperalgesia (paw pressure)

Mechanical sensitivity was also assessed by measuring PWT to an increasing pressure stimulus placed on the dorsal surface of the hind paw (Randall and Selitto 1957) using an analgesymeter (7200, Ugo-Basile, Milan, Italy) employing a dome shaped probe and a cut off of 150g (Figure 2-6). Withdrawal thresholds were measured on ipsilateral and contralateral paws, prior to and then at regular intervals following the induction of OA. The data are expressed as the PWT in grams.

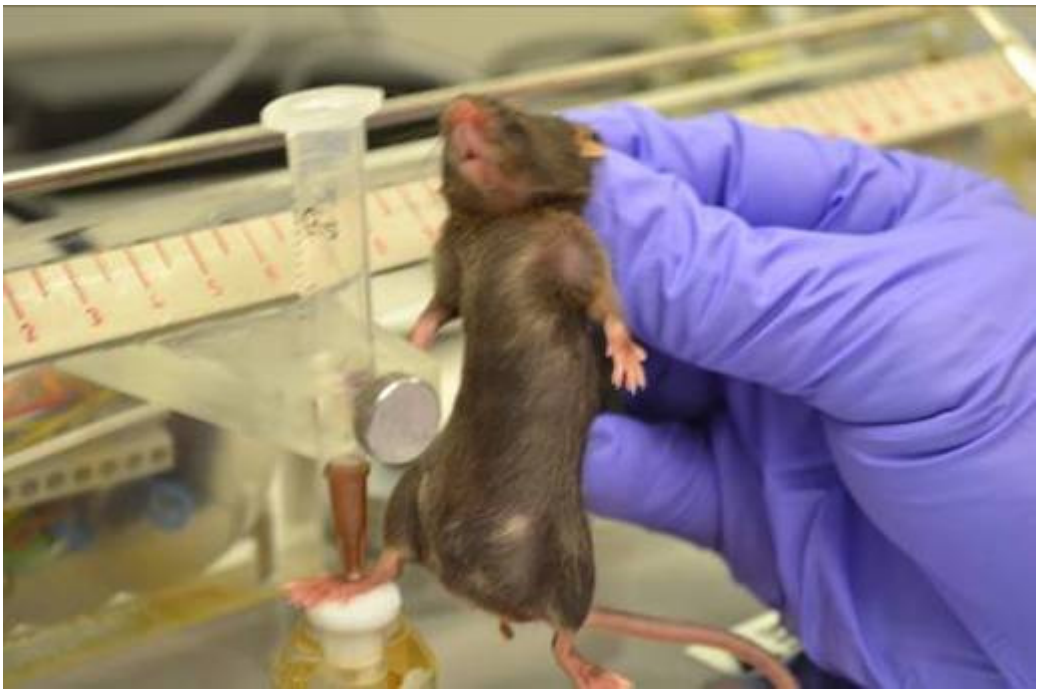


Figure 2-6 Demonstration of mechanical hyperalgesia measurement using an analgesymeter

2.5.6 Cold sensitivity

Cold sensitivity was assessed using the method described by (Gentry, Stoakley et al. 2010), using a commercially available cold-plate (Ugo Basile, Milan), see Figure 2-7 . The cold-plate was set to a pre-calibrated surface temperature and allowed to stabilize for 5 minutes prior to testing. Paw withdrawal latencies (PWLs) were determined with the cold-plate set at 10℃. The animals were lightly restrained and each hind paw in turn placed onto the surface of the cold-plate. The end point was taken as the withdrawal of the paw and recorded as the withdrawal latency for the ipsilateral (operated leg) or the contralateral (un-operated leg) paw. A maximum cut-off of 30 seconds was used for each paw. The data are expressed as the PWL in seconds.

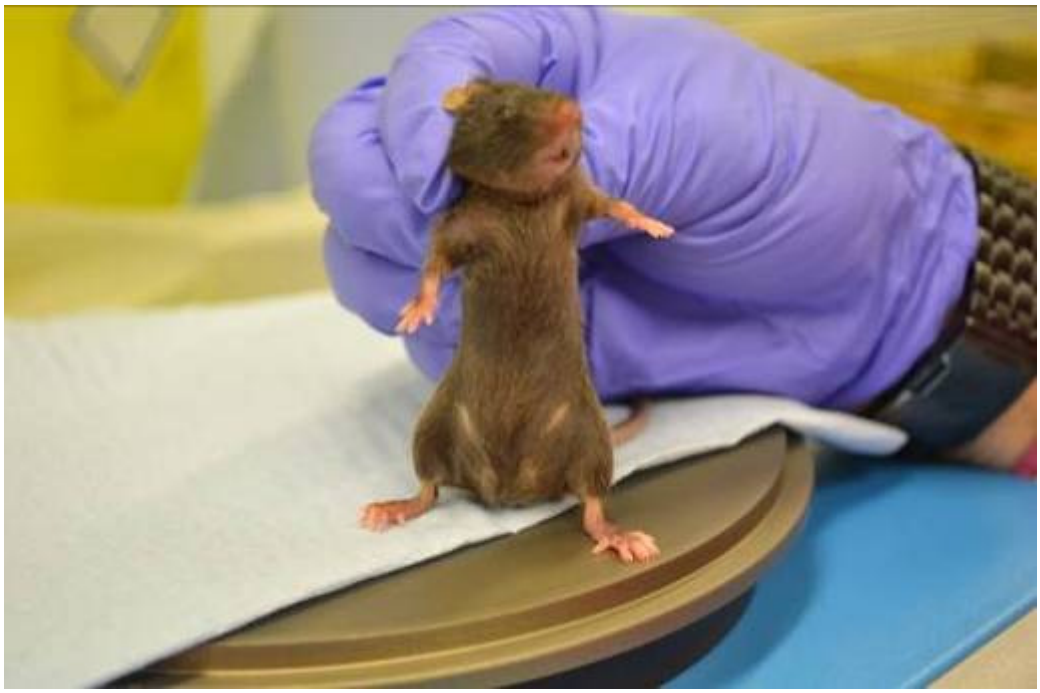


Figure 2-7 Demonstration of cold sensitivity measurement using a cold-plate

Cold-plate temperature of 10ºC was chosen to assess cold allodynia based on previous in-house experiments performed by Clive Gentry whereby cold sensitivity was assessed in 2 groups of 6 mice using a cold plate (Figure 2-8). Measurements were performed on the same day with an interval of at least 5 minutes between each reading. Readings were taken on alternate paws in the order (temperature/paw):10L, 15R, 5L, 2R, 20L, 25R, 35L, 45R, 50L, 55R. At ambient temperatures (23℃- 37.5℃), mice did not withdraw in response to plate temperature. At 10℃, naive mice consistently produced a rapid withdrawal of the hind limb after approximately 12 seconds.

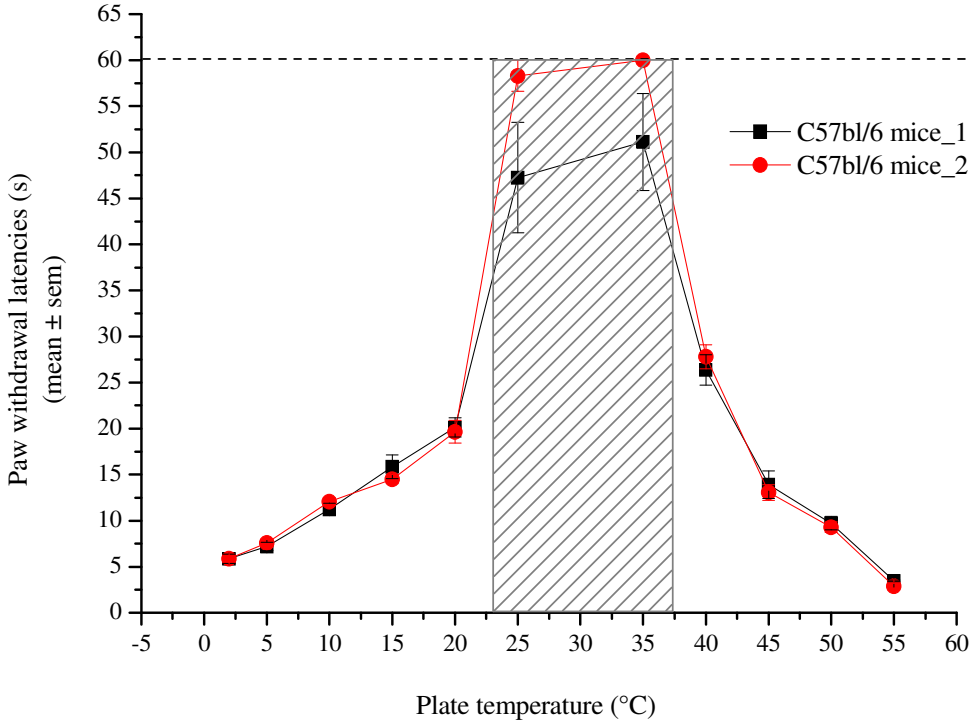


Figure 2-8 Temperature response curve

Paw withdrawal latencies of 2 groups of C57Bl/6 mice to stimulation at various temperatures. Each point represents the mean ± SEM of 6 mice. Data provided by C. Gentry 2011.

2.5.7 Vocalisations evoked by passive flexion and extension of the knee joint

The mice were gently restrained with one hand. With the thigh in a fixed position the operator's thumb and forefinger were used to flex and extend the knee joint through its full range of motion passively 10 times. The number of audible vocalisations for each limb was recorded.

2.5.8 Vocalisations evoked by flexed knee compression

The procedure was carried out as described in the previous section except that the mouse's knee was compressed in a flexed with moderate force that produced no sign of pain/avoidance behaviour in naïve mice (Figure 2-4). This was repeated 10 times at 2 second intervals starting with the contra-lateral leg and the number of audible vocalisations for each limb recorded.

2.5.9 Heat sensitivity

Heat sensitivity was assessed by measuring PWL using a commercially available calibrated hot-plate (Ugo Basile, Milan). PWL were determined with the hot-plate

set at 50℃ to provide a noxious heat stimulus. The animals were lightly restrained and the left hind paw placed onto the surface of the hot-plate. The end point was taken as the withdrawal of the paw and recorded as the withdrawal latency. A maximum cut-off of 30 seconds was used for each paw. The data are expressed as the PWL in seconds.

2.6 Articular Joint Histology

2.6.1 Tissue preparation – rapid decalcification

Animals were killed by cervical dislocation, the knee joints dissected out and the surrounding muscle trimmed. Tissues were fixed overnight with 10x volume of 4% w/v paraformaldehyde (VWR International, Lutterworth, UK) in physiological saline and then decalcified for a maximum of 16 hours in water containing 7% w/v $AlCl_3.6H_2O$, 5% Formic acid and 8.5% Hydrochloric acid. The decalcified knee joints were washed overnight in 0.1M phosphate buffer solution, pH 7.4, and then cryopreserved in 20% w/v sucrose in 0.1M phosphate buffer solution for a further 24 hours. The tissue was frontally embedded in Tissue-Tek (VWR International, Lutterworth, UK) and then frozen at -80ºC. Frontal embedding was chosen to allow concurrent evaluation of the medial and lateral femorotibal joints. Anterior-posterior coronal sections (frontal sections) of each knee were cut using a Bright cryostat (Huntingdon Cambridgeshire, UK). 12µm sections were harvested at 80µm intervals encompassing the whole joint surface. Histology sections were stored at -80ºC before use when necessary.

2.6.2 Tissue preparation – slow decalcification

Animals were killed by cervical dislocation, the knee joints dissected out and the surrounding muscle trimmed. Tissues were fixed overnight with 10x volume of 4% w/v paraformaldehyde (VWR International, Lutterworth, UK) in physiological saline. The knee joints were then rinsed in distilled water and then decalcified for 10 days in 14% tetra sodium EDTA (VWR International, Lutterworth, UK) w/v in distilled water (pH 7.6 adjusted with glacial acetic acid). The decalcified knee joints were washed overnight in 0.1M phosphate buffer solution, pH 7.4, and then cryopreserved in 20% w/v sucrose in 0.1M phosphate buffer solution for a further 24 hours. The tissue was frontally embedded in Tissue-Tek (VWR International, Lutterworth, UK) and then frozen at -80ºC. Frontal embedding was chosen to allow concurrent evaluation of the medial and lateral femorotibal joints. Anterior-posterior coronal sections (frontal sections) of each knee were cut using a Bright cryostat (Huntingdon Cambridgeshire, UK). 12µm sections were harvested at 80µm intervals

encompassing the whole joint surface. Histology sections were stored at -80ºC before use when necessary.

2.6.3 Toluidine blue staining

Joint sections were dipped in 0.1% w/v toluidine blue (Sigma-Aldrich, Gillingham, UK) in distilled water for 30 seconds, and then washed thoroughly in distilled water. They were sequentially dehydrated through 70, 90 and 100% ethanol. Finally, sections were cleared in xylene (VWR International, Lutterworth, UK) for 2 minutes, and mounted in DPX mountant (VWR International, Lutterworth, UK) before being photographed using a Nikon Eclipse E800 microscope with a JVC colour digital camera attached.

2.6.4 Safranin O/Fast green staining

Joint sections were immersed in 0.1% w/v safranin O (Sigma-Aldrich, Gillingham, UK) in distilled water for 5 minutes before being rinsed thoroughly in distilled water. The sections were dipped in 0.02% fast green (Sigma-aldrich, Gillingham, UK) w/v in ethanol for 2 minutes. Sections were then dipped briefly in 0.1% acetic acid in before being rinsed in distilled water. The sections were allowed to dry before being sequentially dehydrated through 70, 90 and 100% ethanol, cleared in xylene for 2 minutes, and mounted in DPX mountant before being photographed.

2.6.5 OA scoring

Joint sections were randomised and scored using the method described by (Glasson, Chambers et al. 2010). Cartilage damage in each quadrant (medial femoral condyle, medial tibial plateau, lateral femoral condyle, lateral tibial plateau) was scored on a scale of 0-6 with 0 representing normal joint cartilage (**Figure 2-9**). A score of 0.5 = loss of proteoglycan with an intact surface, 1 = superficial fibrillation without loss of cartilage, 2 = vertical clefts and loss of surface lamina (any % or joint surface area) 3 = vertical clefts/erosion to the calcified layer lesion for 1-25% of the quadrant width, 4 = lesion reaches the calcified cartilage for 25-50% of the quadrant width, 5 = lesion reaches the calcified cartilage for 50-75% of the quadrant width, and 6 = lesion reaches the calcified cartilage for >75% of the quadrant width.

To produce the combined average joint score, the quadrant scores across the sections of each mouse were averaged. Then this score was averaged for all mice in the group. The total of the 4 quadrants produced the combined average score for each observer. The observer's scores were averaged to calculate the final score for the group. The maximum possible score was 24.

For the maximal joint score, the highest score for each quadrant of each mouse was averaged and then summed to provide a maximal joint score for each observer. The mean of the observer's scores was calculated to produce the combined maximal joint score for the group. The maximum possible score was 24.

The rapid decalcification process did not enable the assessment of changes to the subchondral bone which is not a component of this histological scoring system.

Initially 3 observers were used for the partial medial meniscectomy histopathology study to investigate whether consistent joint scores could be produced. For histopathology studies investigating the MIA model and spontaneous OA using STR/ort mice, a single observer was used. The scorers were provided with a description of the OA grading system along with representative images of OA in a mouse knee (Figure 2-9). Images for each mouse were randomised and blinded by an independent person using a random number generator. The intra-class correlation coefficient was calculated to be 0.90 indicating an excellent level of agreement between the observers. For all other studies, a single observer was used to score randomised and blinded mouse sections. In an effort to control the inherent subjectivity of the assessment technique, all sections were graded by the same observer on two separate occasions, two weeks apart. The interclass correlation coefficient was 0.94 or greater in all cases indicating an excellent level of reproducibility. The mean value of the two scorings was used for the final score.

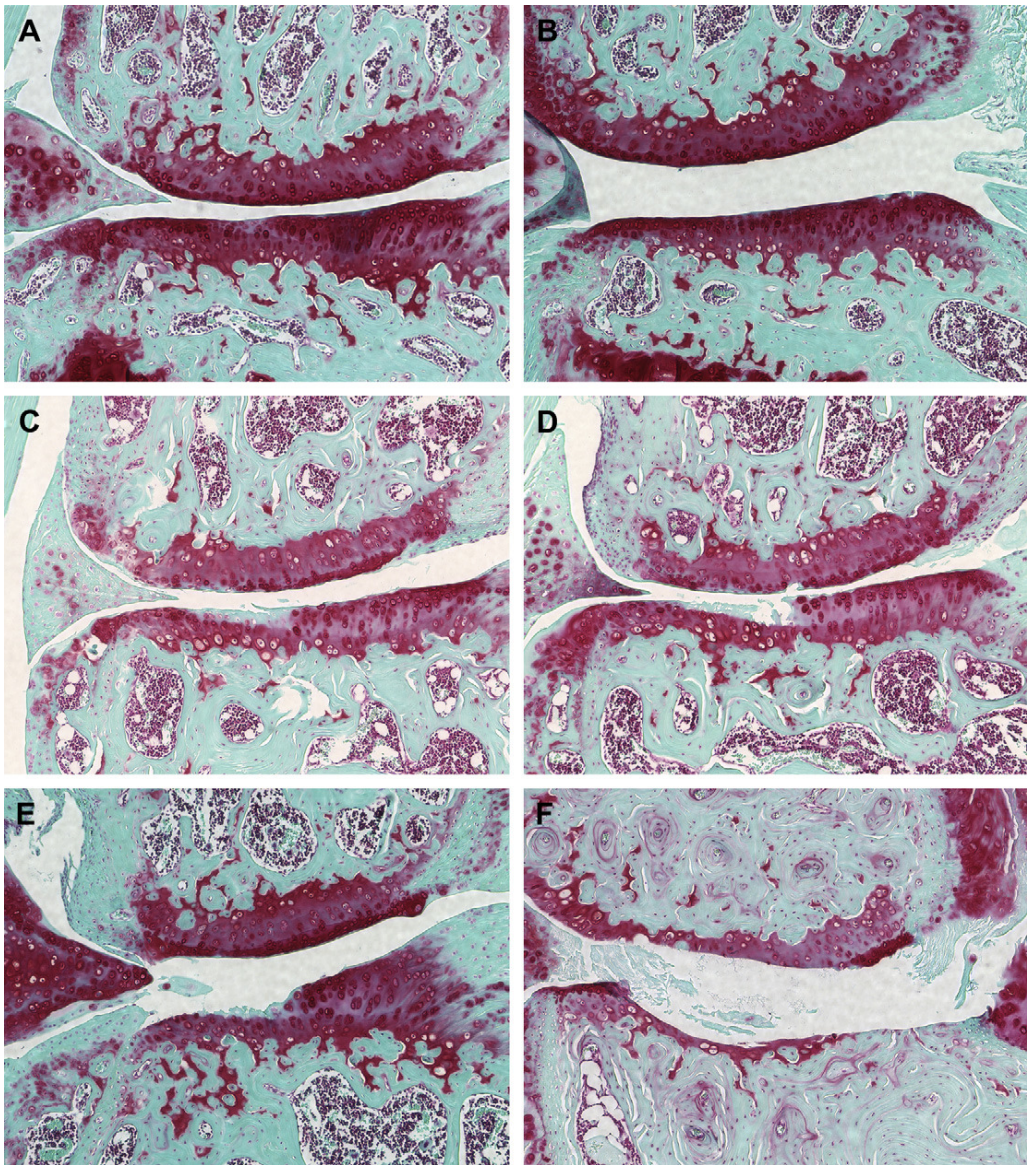


Figure 2-9 Histopathology examples for joint scoring

Safranin-O photomicrographs showing the medial femoral condyle (MFC) (above) and medial tibial plateau (MTP) (below) and the medial meniscus (left), displaying a variety of OA severity and semi-quantitative scores. First score represents MFC, second score is MTP; (A) 0, 0.5; (B) 0, 1; (C) 0.5, 2; (D) 3, 3; (E) 0, 4; (F) 5, 6. (Glasson, Chambers et al. 2010)

2.6.5.1 Osteophyte scoring

The presence of an osteophyte within a particular quadrant was assigned a score of 1. To produce the combined average joint score, the quadrant scores (0 or 1) across the sections of each mouse were averaged. Then this score was averaged across the mice. The total of the 4 quadrants produced the combined average score for each observer. The observer's scores were averaged to calculate the final score for the group. The maximum possible score was 4.

2.7 Pharmacological studies

Following the induction of OA, the response of pain behaviours to the administration of various analgesic drugs or experimental compounds was recorded.

2.7.1 Control group

Behavioural data gathered from the medial meniscectomy model of OA was sufficiently reliable to justify the reduction in number of animals used for in vivo experiments by excluding a sham surgery control group. Animals receiving the drug vehicle only served as a control group.

2.7.2 Analgesic drug dose and formulation

Drug doses, routes of administration and intervals between drug treatment and subsequent behaviour readings, were chosen based on published pharmacodynamic data and/or previous in-house experiments in mice or rats to optimize the treatment effect whilst minimizing the potential for adverse effects e.g. sedation, excitation and gastrointestinal ulceration (Table 2-1 and Table 2-2). In studies where groups of mice were given more than one analgesic drug, a minimum of 1 week “wash-out” period was employed to reduce or eliminate any residual effects on pain behaviours from previously administered drugs.

Table 2-1 Analgesic drugs

Analgesic drugs used to test the presence of pain behaviours in mice following the induction of degenerative joint disease. The total drug volume in all cases was 0.2ml.

Drug	Dose	Route	Treatment – Testing Interval	Vehicle
Diclofenac	10mg/kg	i.p.	1hr	0.9% sterile saline
Morphine	4mg/kg Or 6mg/kg	s.c.	1hr	0.9% sterile saline
Celecoxib	30mg/kg	p.o.	1hr	0.5% methyl cellulose
Gabapentin	60mg/kg	p.o.	1.5hr	0.5% methyl cellulose
Paracetamol	300mg/kg	s.c.	1hr	0.9% sterile saline
Lamotrigine	30mg/kg	p.o.	1.5hr	0.5% methyl cellulose
Tramadol	50mg/kg	s.c.	1hr	0.9% sterile saline
Duloxetine	30mg/kg	i.p.	0.5hr	0.9% sterile saline

Table 2-2 Novel investigational drugs

Novel analgesic drugs used to assess the role of specific receptors in OA pain behaviours in mice

Drug	Dose	Route	Treatment – Testing Interval	Total Volume	Vehicle
AMTB	10mg/kg	i.p.	1hr	0.2ml	1% DMSO/0.9% saline
AMG-9810	100mg/kg	i.p.	1hr	0.5ml	80% DMSO/saline
HC-030031	300mg/kg	p.o.	1hr	0.2ml	0.5% methyl cellulose
CE-245677	30mg/kg	p.o	2hr	0.2ml	0.5% methyl cellulose
AZ-23	30mg/kg	p.o	2hr	0.2ml	0.5% methyl cellulose/0.1% Tween80
AP-18	50mg/kg	i.a.	1hr	5µl	1%DMSO/0.5% Tween80/saline
Bradyzide	2mg/kg	p.o.	1hr	0.2ml	0.5% methyl cellulose
Anti-TNF	200µg per mouse every 48hr	i.p.	0, 24hr, 72hr, 168hr	0.2ml	0.9% saline

2.7.2.1 Opioid antagonists

Opioid antagonists were used to assess the influence of endogenous opioids on OA pain behaviours. They were dissolved in 0.9% sterile saline, with a total volume of 0.2ml. The treatment-testing interval was 1 hour for each drug. Naloxone and Naloxone methodide were administered using a dose of 2.5mg/kg i.p, Naltrindole was administered using a dose of 5mg/kg s.c., Naloxonazine with a dose of 20mg/kg s.c. and GNTI 0.3mg/kg s.c.

2.7.3 Randomisation and blinding

Behavioural measurements were performed with the observer blinded to the groups of mice e.g. sham meniscectomy versus meniscectomy. This was achieved by an independent person substituting cage labels with cards marked A or B. The groups

of mice otherwise appeared identical (with the exception of the STR/ort and CBA mice).

Where the behavioural measurements were being recorded in response to administration of a particular drug, pre and post-drug behavioural tests always followed the same sequence: von Frey filaments 1, paw pressure 2, cold plate 3, knee compressions 4. Drug treatments were randomised within groups of mice so that within each cage, individual mice were assigned to receive either the drug or vehicle. Each mouse tail was numbered 1-10 and treatments (a = drug or b = vehicle) assigned to produce 2 groups with similar pre-drug behavioural readings. The person administering the treatments to the mice and the observer performing the behavioural readings were blinded to the code. The mice were administered the drug or vehicle and tested in the same order (1-10) to help reduce any variation in time interval between dosing and testing of each drug between the mice.

2.8 Retrograde Neuronal Tracers

Fluorescent retrograde neuronal tracers were injected into knee joints of anaesthetized mice so that sensory neurons innervating the joint could be identified within the dorsal root ganglia (DRG) using appropriate UV filters. Injections were carried out using a 26Gx3/8 needle and Hamilton syringe. Table 2-3 and Table 2-4 show the fluorescent neuronal tracers used in studies for rats and mice.

Table 2-3 Fluorescent retrograde neuronal tracers used in rats

Neuronal tracer	Interval	Volume	Formulation	Peak Excitation wavelength (pH 7.4) (nm)	Peak Emission wavelength (pH 7.4) (nm)
Fast blue (Sigma-Aldrich, Poole, UK)	5 days	20µl	1% w/v in 0.2% v/v DMSO/distilled water	360	410
DiI (Invitrogen, Paisley, UK)	5 days	20µl	2% w/v DMSO	549	565
Fluorogold (Sigma-Aldrich, Poole, UK)	5 days	20µl	2% w/v in sterile physiological saline	370	565

Table 2-4 Fluorescent retrograde neuronal tracers used in mice

Neuronal tracer	Interval	Volume	Formulation	Peak Excitation wavelength (pH 7.4) (nm)	Peak Emission wavelength (pH 7.4) (nm)
Fluoro-emerald (Invitrogen, Paisley, UK)	3 to 7 days	10µl	0.1-10% w/v in sterile physiological saline	490	518
Fluorogold (Sigma-Aldrich, Poole, UK)	3 days	10µl	0.1%-2% w/v in sterile physiological saline	370	565

2.8.1 Intra-articular injection

Animals were positioned in dorsal recumbency and the left leg was flexed at a 90º angle. 10µl (20µl in rats) of the neuronal tracer was injected through the patellar ligament under general aneaesthesia.

2.8.2 Intra-articular injection validation

In order to detect extra-articular leakage of dyes injected into the knee joint, histology was performed following completion of the study. The methods used were as described in section 2.6.1 except that the tissue was laterally embedded in Tissue-Tek. Lateral embedding was chosen to allow concurrent evaluation of dye within the joint and also within the soft tissue outside of the joint capsule. Sections were viewed using fluorescent microscopy with appropriate UV filters for the particular neuronal tracer dye used.

2.8.3 Extra-articular Injection of Fluorogold

In order to examine the possible labelling pattern of neurons innervating the area around the joint, experiments were repeated with 10µl (total volume) of the neuronal tracer injected into the skin, subcutaneous tissues and muscle surrounding the left knee joint.

2.8.4 Identification of specific dorsal root ganglia by identification of the lumbar vertebrae and tracing the path of the sciatic nerve

Specific DRGs were identified by tracing the path of the sciatic nerve which projects from spinal nerves at lumbar vertebrae L3, L4, L5 (Rigaud, Gemes et al. 2008) and by counting the lumbar vertebrae from the last rib. Mice were killed by cervical dislocation. The skin overlying the dorsal spine was removed and spinal column dissected so that the thoracic and lumbar spinal cord could be visualised clearly. The most cranial vertebra that lacked an articulation with a rib at its cranial margin was denoted as the first lumbar (L1) vertebra. In addition, a 5mm caudo-lateral incision was made in the skin over the left thigh. The sciatic nerve was identified and its path traced cranially beneath the wing of the ileum until it divided into the spinal nerves that feed into the spinal cord via DRGs at L3, L4 and L5. Individual DRG were identified from these landmarks (see Figure 2-10).

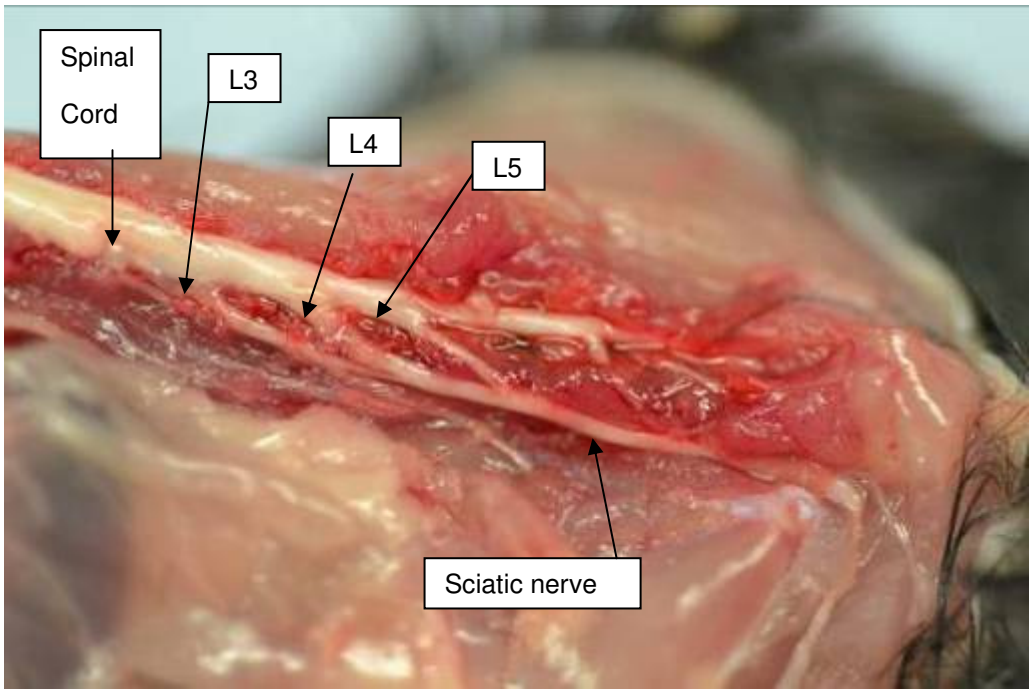


Figure 2-10 Identification of specific DRGs

The path of the sciatic nerve is traced to the spinal nerve branches at L3, L4 and L5 in order to identify specific DRG. Arrows point to individual lumbar DRG.

2.8.5 Collection of DRGs prior to histology and microscopy

DRG were collected aseptically and placed in individual eppendorf tubes containing 4% neutral buffered formalin and left overnight. For mice that had received an intra-articular injection of a fluorescent retrograde neuronal tracer dye, ipsilateral and contralateral thoraco lumbar DRG were collected separately. After fixation, the DRG were transferred to 20% sucrose for cryopreservation. After 24hrs, the DRG were individually embedded in Tissue-Tek and frozen at -80ºC. The DRGs were cut on a Bright cryostat in 10µm sections and thaw mounted onto Superfrost plus slides.

2.8.5.1 Identification of fluorogold positive neurons within DRGs

In order to objectively identify fluorogold containing neurons and non-fluorogold neurons, all of the images were viewed and photographs taken using the same exposure and light intensity. In order to objectively distinguish between a positive and negatively staining cell body, the maximum intensity of auto fluorescence produced by negative control DRG sections from mice that had not been injected with fluorogold was measured from photographs using the same settings. Outlines of individual cells were identified using Image Pro software (Bethesda, USA) and the mean pixel intensity for 100 cells was recorded. Since the amount of fluorogold

fluorescence varied between individual neurons, all neurons fluorescing with intensity greater than this threshold were considered positive for fluorogold.

2.8.5.2 Toluidine blue staining of lumbar DRGs

10µm DRG sections thaw mounted on Superfrost plus slides were allowed to dry before being dipped in 0.1% w/v toluidine blue (Sigma-aldrich, Gillingham, UK) in distilled water for 30 seconds, and then washed thoroughly in distilled water. The sections were then mounted using Vectashield (Vector laboratories Inc, Peterborough, UK) and viewed under UV microscopy.

2.8.6 Collection and preparation of DRG neurons for calcium imaging analysis

Mice were killed by cervical dislocation and ipsilateral spinal ganglia L2-L4 were identified (see 2.8.4) and removed aseptically from the lumbar region of the spinal cord. Ganglia were incubated in 2.5% collagenase in MEM containing 1% penicillin and streptomycin for 3 hours (Invitrogen, Paisley, UK) at 37℃ in a humidified incubator gassed with 5% CO_2 in air. This was followed by 20 minute incubation with 0.25% trypsin in MEM. Trypsin was removed by a 10ml MEM wash containing 10% FBS and centrifugation at 1000 revolutions min^{-1} for 5 minutes to overwhelm the enzyme with substrate. The pellet containing DRG neurons was re-suspended in MEM containing 1% penicillin and streptomycin, 10% FBS and 0.05% DNase. Neurons were then dissociated mechanically by trituration with a flame polished Pasteur pipette to obtain a single cell suspension. The cell suspension was centrifuged through a 2ml cushion of sterile 15% w/v bovine serum albumin (free of essential fatty acids) in MEM at 1000 revolutions min^{-1} for 10 minutes. The cells were re-suspended in an appropriate volume of MEM containing 10%FBS, 50ngml^{-1} NGF and 10µM cytosine arabinoside added to reduce the growth of non-neuronal cells. The neurons were then plated at high density (~80%) onto sterile 13mm glass coverslips previously coated with 10µgml^{-1} poly-D-lysine and maintained at 37℃ in a humidified incubator gassed with 5% CO_2. Cells were studied 15 to 24 hours after dissociation.

2.9 Calcium imaging

2.9.1 Microfluorometric measurement of intracellular calcium concentration

Changes in intracellular calcium concentration were monitored using a microscope based system and a calcium indicator dye (either Fura-2 or Fluo-4). For all calcium imaging experiments the dissociated DRG neurons were loaded with either Fura-2 or Fluo-4 for 1 hour prior to experimentation using the acetoxymethyl (AM) esters.

The AM esters allows the indicator to passively diffuse across cell membranes, once inside the cell, these ester bonds are cleaved by intracellular esterases to yield a cell-impermeant fluorescent indicator. Fura-2 or Fluo-4 were loaded in extracellular solution supplemented with 0.01% pluronic acid and 1mM probenecid. Pluronic acid was used to improve the solubility of the fluorescent dyes and probenecid included to reduce the efflux of the acidic forms of the dyes by organic-anion transporters located in cell membranes.

Fura-2 (Invitrogen, Paisley, UK) is a UV excitable, ratiometric calcium indicator dye. Upon binding to calcium, the excitation spectrum for fura-2 changes with an increased emission when the dye is excited at 340nm and a reduced emission evoked by 380nm excitation. Fluo-4AM (Invitrogen, Paisley, UK) was also used to measure changes in calcium concentration. Fluo-4 is an excitable, non-ratiometric calcium indicator dye. Upon binding to calcium, fluo-4 exhibits a large fluorescence intensity increase when excited at 490nm.

Neuronal cells from dissociated DRG cultures were plated onto 13mm glass cover slips which formed the base of the perfusion chamber (volume~1ml). The chamber was mounted on to the stage of an inverted microscope (Nikon Diaphot) and cells were viewed using a 10x Fluor objective with a numerical aperture of 0.5. The neuronal cells were perfused with solutions supplied from one of 8 reservoirs, which allowed different compounds to be applied sequentially to the neurons.

Fura-2 and Fluo-4 signals were measured using a RatioMaster Fluorescence Microscopy System (PTI). Cells were excited by light generated by a xenon-arc lamp which was passed alternately through one of two monochromonators (DeltaRam high speed monochromator, PTI) to transmit light of the pre-selected wavelengths (340nm and 380nm, ± 2nm for Fura-2 and 480nm ± 2nm for Fluo-4). The emitted light was filtered by a long pass optical filter (>510nm) and captured by a cooled CCD camera (PTI CoolOne). Exposure length was equal for each excitation wavelength and determined by the user to ensure adequate signal without saturation of the camera (typically 100-400msec).

PTI imagemaster software 5.0 (Media Cybernetics) served as the user interface during the experiments to monitor the fluorescence intensity data and was also used to mark individual neurons for analysis. The intensity time-base data for regions of interest was exported into Microsoft Excel (Microsoft) for further analysis and then into Origin (Origin Lab, version 7.5) for graphical representation of the results.

2.9.2 Perfusion of DRGs during calcium imaging studies

The isolated cells were perfused with solutions supplied from one of 8 reservoirs, which allowed different compounds to be applied sequentially to the neurons. Extracellular solution was applied to the DRG before the application of any agonists to establish baseline intracellular calcium concentrations. The composition of the extracellular solution was as follows (in mM), NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 10 titrated to pH7.4 with NaOH. Extracellular solution was also applied between applications of each solution to wash out the agonists and to allow the calcium concentrations to return to baseline levels where possible. In this way the influence of the previous agonist application on the response of the neurons to the next agonist was minimized. Concentrations of the agonists described in Table 2-5 were chosen to provide robust responses whilst ensuring specificity for the ion channel being studied.

Solutions containing TRP channel agonists and a high concentration of KCl to depolarize all viable neurons were made up in extracellular solution as described above. Each agonist was applied for 1 minute followed by a 3 minute wash in extracellular solution. The order of capsaicin and AITC application was varied to facilitate identification of neurons that responded to only one of these agonists. This was often necessary as the intracellular calcium concentration remained at very high levels in some cells after washout of either compound. The orders of application were (A). Icilin, AITC, capsaicin, KCl, and (B) Icilin, capsaicin, AITC, KCl.

Table 2-5 Solutions used to stimulate DRG neurons in calcium imaging experiments

Compound	Concentration	Company	Preparation
Icilin	1 μM	Biomol (Exeter, UK)	Stock solution was prepared in DMSO*.
Allyl isothiocyanate (AITC)	100 μM	Sigma-Aldrich (Gillingham, UK)	AITC prepared directly into extracellular solution.
Capsaicin	1 μM	Sigma (St. Louis, MO)	Stock solution was prepared in DMSO*.
Potassium chloride	50 mM	Sigma (St. Louis, MO)	KCl prepared directly into extracellular solution.

*Stock solutions of 10mM Icilin, and 10mM Capsaicin, were made up in DMSO, Calbiochem (Darmstadt, Germany), aliquoted and stored at -20°C. Dilutions from these aliquots into physiological extracellular solution were made daily for use in experiments (maximum final DMSO concentrations of 0.01%). A master stock of 500μM AITC, was made daily in extracellular solution and further diluted in extracellular solution to obtain the final concentration.

The intracellular calcium concentration response was measured by the increase in 340/380nm signal (Fura) or increase in fluorescence intensity with 480nm excitation (Fluo-4). The average signal 5 seconds prior to the application of each agonist was subtracted from the maximum signal evoked by the agonist. A positive response was designated if the difference between the signals was greater than the normal fluctuation in baseline calcium ratio. The normal fluctuation in calcium ratio was determined by estimating the maximum and minimum signal during application of extracellular solution at the beginning of each experiment.

2.9.3 Calcium imaging studies on identified joint afferents

In these experiments, neurons innervating the knee joint were first identified by excitation at the wavelength appropriate for the specific retrograde neuronal tracer used (370nm for fluorogold, 490nm for fluoro-emerald). A photograph was taken to record the location of the labelled cells so that the joint afferents could be marked and the calcium responses of these neurons distinguished from those of other

neuronal and non-neuronal cells. The wavelength was then changed to the appropriate excitation wavelength for the calcium indicator dye being used and the responses to different stimuli recorded.

2.10 Statistics

Where appropriate data was presented as mean ± standard error of the mean (SEM), which was calculated using Excel (Microsoft Excel 2003, Washington, USA). Statistical analysis was performed in order to investigate the probability that the results of an experiment occurred by chance. $P<0.05$ was set as the level of statistical significance.

Normality was assessed using the Shapiro-Wilk test or the D'Agostino-Pearson Omnibus test as appropriate using STASTICA (version 7.1, StatSoft Inc, OK, USA) or GraphPad Prism (version 5.04 for Windows, GraphPad Software, California USA). The D'Agostino-Pearson test was chosen preferentially in cases where results included data with identical values such as those generated from von Frey experiments.

To assess intra observer agreement for histopathology analysis where multiple observers were used, the intra-observer correlation coefficient was calculated using SPSS (version 20.0.0., IBM, NY, USA). To assess reproducibility of histopathology results, a test re-test procedure was performed whereby joint sections were scored twice, 2 weeks apart and the inter-class correlation coefficient calculated. The intra-class correlation coefficient was calculated to be 0.90 indicating an excellent level of agreement between the observers. The interclass correlation coefficient was 0.94 or greater in all cases indicating an excellent level of reproducibility.

In order to assess whether the means of two independent groups were statistically different from each other either the unpaired t-test was used (parametric data) or the Mann Whitney test was used (non-parametric data). Statistical significance was calculated using STASTICA (version 7.1, StatSoft Inc, OK, USA).

To assess whether means of 2 or more groups were significant compared to each other, a one-way ANOVA followed by Tukey's post hoc test was used. To assess whether means of 2 or more groups were significant compared to a control group (e.g. joint score of OA mice at different times post-surgery compared to naïve mice) a one-way ANOVA followed by Dunnett's post hoc test was used. The post hoc analysis determined which means were significantly different from each other. These analyses was performed using GraphPad Prism (version 5.04 for Windows,

GraphPad Software, California USA) or STASTICA (version 7.1, StatSoft Inc, OK, USA).

For experiments where groups received identical treatments over a period of time repeated measures ANOVA followed by Tukey's post hoc test was used. Statistical significance was calculated using STASTICA (version 7.1, StatSoft Inc, OK, USA).

The chi squared test was used to determine significant differences between categorical data, such as the percentage of neurons responding in a given condition. The chi squared test was performed using GraphPad Prism (version 5.04 for Windows, GraphPad Software, California USA).

Chapter 3

Developing a Mouse Model of Osteoarthritis

3 Developing a mouse model of OA

3.1 Introduction

The mouse is the primary species for the generation of genetically modified animals, including animals with gene deletions and gene over-expression. These features make the mouse the preferred species to characterize the in vivo significance of a particular gene. Mice have additional advantages as animal models due to their lower maintenance costs and drug requirements.

OA is a degenerative disease that occurs with ageing but it can also be induced chemically or surgically in animal models that offer many advantages including faster onset of disease, decreased variability, and decreased dependence on genetic background (Glasson, Blanchet et al. 2007). Methods for chemical destruction of cartilage resulting in the development of OA in mice have included intra-articular injection of papain, collagenase and MIA (van der Kraan, Vitters et al. 1989; Harvey and Dickenson 2009) amongst others.

OA can also be induced surgically by destabilizing the knee joint. Several murine surgical models of OA have been studied in the past including destabilization of the medial meniscus (DMM) (Glasson, Blanchet et al. 2007; Inglis, McNamee et al. 2008), transection of the anterior cruciate ligament (Glasson, Blanchet et al. 2007) and removal of the medial meniscus with or without transection of the collateral ligaments (Kamekura, Hoshi et al. 2005, Welch et al. 2009).

Spontaneous OA has been studied in strains of mice including C57Bl/6, BALB/c (Stoop, van der Kraan et al. 1999) and STR/ort mice (Walton 1977; Mason, Chambers et al. 2001). The STR/ort mouse spontaneously develops degenerative changes of the knee joints. Lesions include loss of hyaline cartilage, osteophyte formation, calcification and ossification of cruciate ligaments and chondroid metaplasia (Walton 1977). Around 85% of male mice develop such lesions by 35 weeks of age (Chambers, Bayliss et al. 1997). However, accompanying pain behaviours have not been studied previously in STR/ort mice.

In the studies described in this Chapter, three potential models of OA were explored in mice using techniques to assess joint histopathology and the development of accompanying joint pain behaviours. The establishment of a reliable and clinically relevant mouse model of OA will provide a useful tool to help achieve a greater understanding for the mechanisms responsible for OA pain.

3.2 Aims

- Establish a suitable mouse model of OA
- Use a histological scoring system to assess joint pathology to confirm the presence of cartilage degeneration in murine models of OA.
- Use behavioural analysis for the assessment of OA pain behaviours

3.3 Results

3.3.1 Knee joint histology in naïve animals

Histological staining protocols using Safranin O/fast green and toluidine blue were developed to enable assessment of cartilage degeneration in models of OA. 8-10 week old female Wistar rats were killed by cervical dislocation and the knee joints prepared for histology. Rat knee joints were used initially since the larger structures are less fragile during tissue preparation. Safranin O/fast green and toluidine blue protocols were compared for consistency in staining and preservation of the architecture. Both stains produced joint sections with good structure integrity, allowing assessment of the cartilage and underlying subchondral bone. Safranin O/fast green provided excellent staining of the chondrocytes and proteglycans within the cartilage and enabled visualisation of the junction between calcified and non-calcified cartilage (Figure 3-1). Toluidine blue also produced good quality staining showing a uniform thickness of articular cartilage with intact cartilage proteoglycans staining an intense dark purple/blue (Figure 3-2). These staining techniques were then used to examine tissues from mice. Figure 3-3 shows sections of normal mouse knee joints stained with either Safranin O/Fast green or toluidine blue. In mice, both staining protocols produced sections that enabled the assessment of joint cartilage. However, the more complex protocol (greater number of steps) required by Safranin O/fast green resulted in poorer preservation of joint structures. Therefore, toluidine blue staining was chosen for histological analysis of the mouse model of OA.

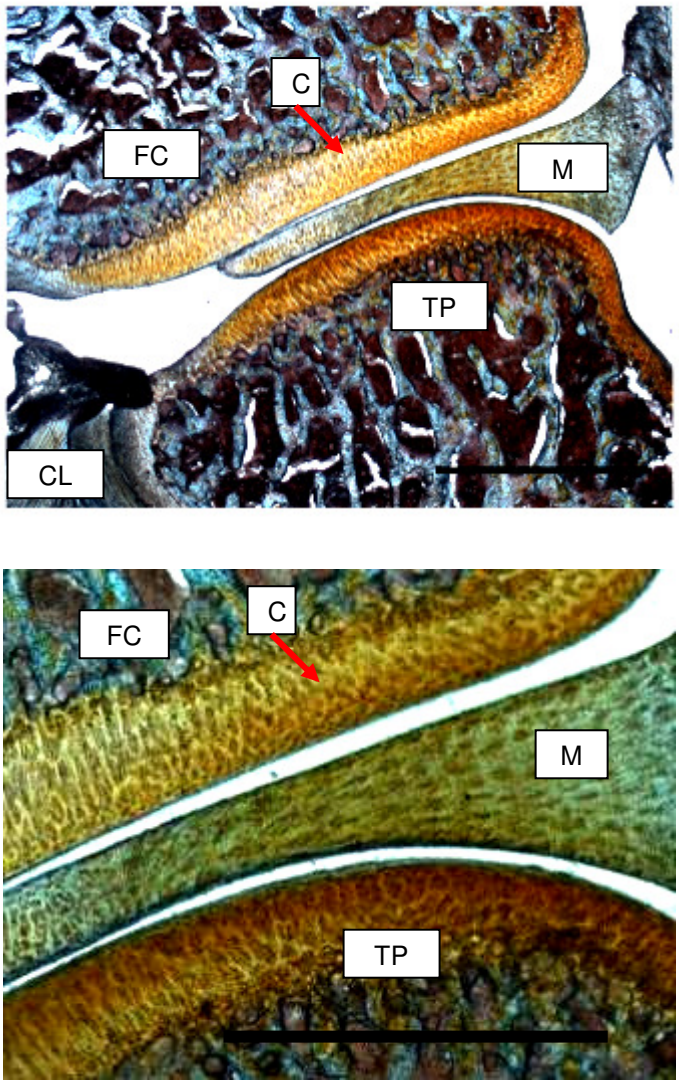


Figure 3-1 Photomicrographs showing representative 12µm thick frontal sections through the femorotibial joint of a rat stained with safranin O/fast green.

The section shows cartilage (C); meniscus (M); cruciate ligament (CL); femoral condyle (FC) and tibial plateau (TP). Scale bar: 1mm

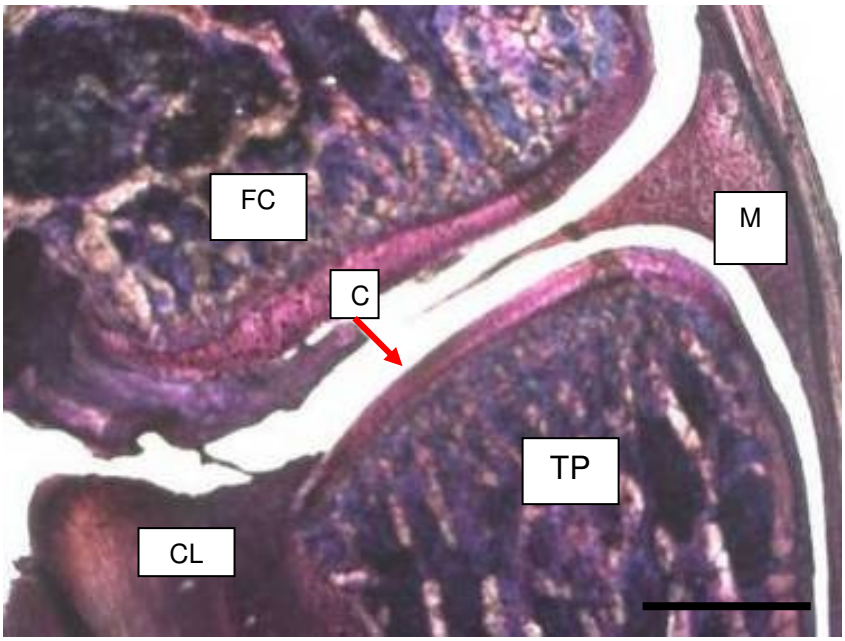


Figure 3-2 Photomicrographs showing a representative 12µm thick frontal section through the femorotibial joint of a rat stained with toluidine blue.

The section shows cartilage (C); meniscus (M); cruciate ligament (CL); femoral condyle (FC) and tibial plateau (TP). Scale bar: 1mm

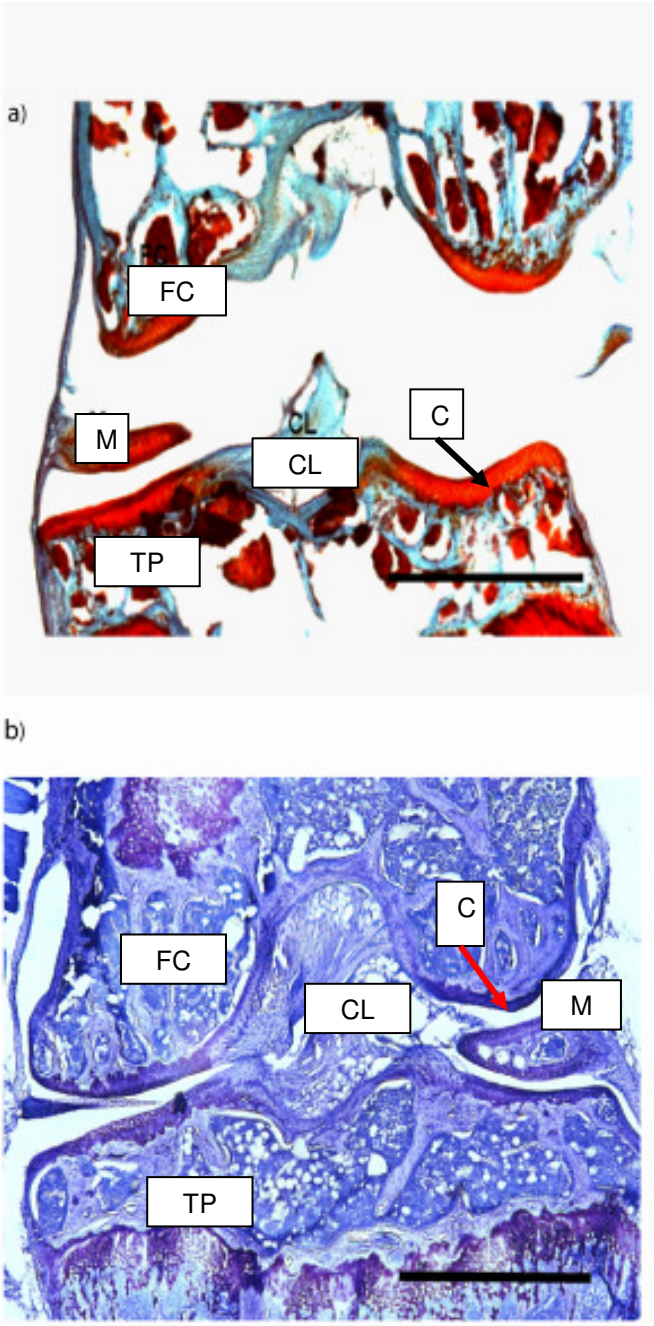


Figure 3-3 Photomicrographs showing representative 12µm thick sections through the femorotibial space of a C57Bl/6 mouse

Sections of the knee joint of a mouse stained with safranin O/fast green a) and toluidine blue b) with cartilage (C); meniscus (M); cruciate ligament (CL); femoral condyle (FC) and tibial plateau (TP). Scale bar: 1mm

3.3.2 Chemical Induction of OA by intra-articular injection of monosodium iodoacetate (MIA)

Intra-articular injection of MIA can cause the degeneration of cartilage and the development of OA by inhibition of the activity of glyceraldehyde-3-phosphate dehydrogenase in chondrocytes, resulting in disruption of glycolysis and eventually cell death (Dunham, Hoedt-Schmidt et al. 1993; Guzman, Evans et al. 2003).

3.3.2.1 Preliminary experiment for induction of OA by intra-articular injection of MIA

A range of doses for MIA were initially tested for the induction of OA pain behaviours. 10 week old female C57Bl/6 mice received a 10µl intra-articular injection of 20% (2mg), 10% (1mg) or 5% (0.5mg) w/v MIA in 0.9% sterile saline or the vehicle alone into the left femorotibial joint space under general anaesthesia (n=6 mice per group).

Mice who received intra-articular injection of 20% w/v MIA died within a few minutes of injection and this dose was not repeated. Mice receiving the other concentrations of MIA showed signs of stress on recovery (piloerect fur, hunched posture) from the general anaesthetic but no further deaths were recorded in this study.

Pain behaviours were recorded prior to (day 0), and at day 7 and day 14 after MIA injection. At day 0, there was no significant difference in PWT or PWL measured using the paw pressure test and cold plate test, respectively, between the groups (Figure 3-4). At day 7, PWTs decreased significantly in the ipsilateral hind paw of mice injected with 5% MIA ($P<0.001$) and 10% w/v MIA ($P<0.001$) compared to control animals. This mechanical hyperalgesia persisted at day 14 in 5% w/v MIA injected mice ($P<0.001$) and 10% w/v MIA injected mice ($P<0.001$). The mice also developed a cold sensitivity with significantly decreased PWLs in the ipsilateral hind limb at day 7 ($P=0.046$, $P=0.045$) and day 14 ($P=0.036$, $P=0.0015$) in 5% and 10% w/v MIA injected mice respectively, compared to saline injected controls. There was no significant change in pain behaviours from baseline measurements in the saline injected animals or in the contralateral limb of MIA injected mice. Injection of 10% w/v MIA was chosen as the dose for further study as a potential model for the induction of OA pain behaviours since it produced the most robust hypersensitivities in this study.

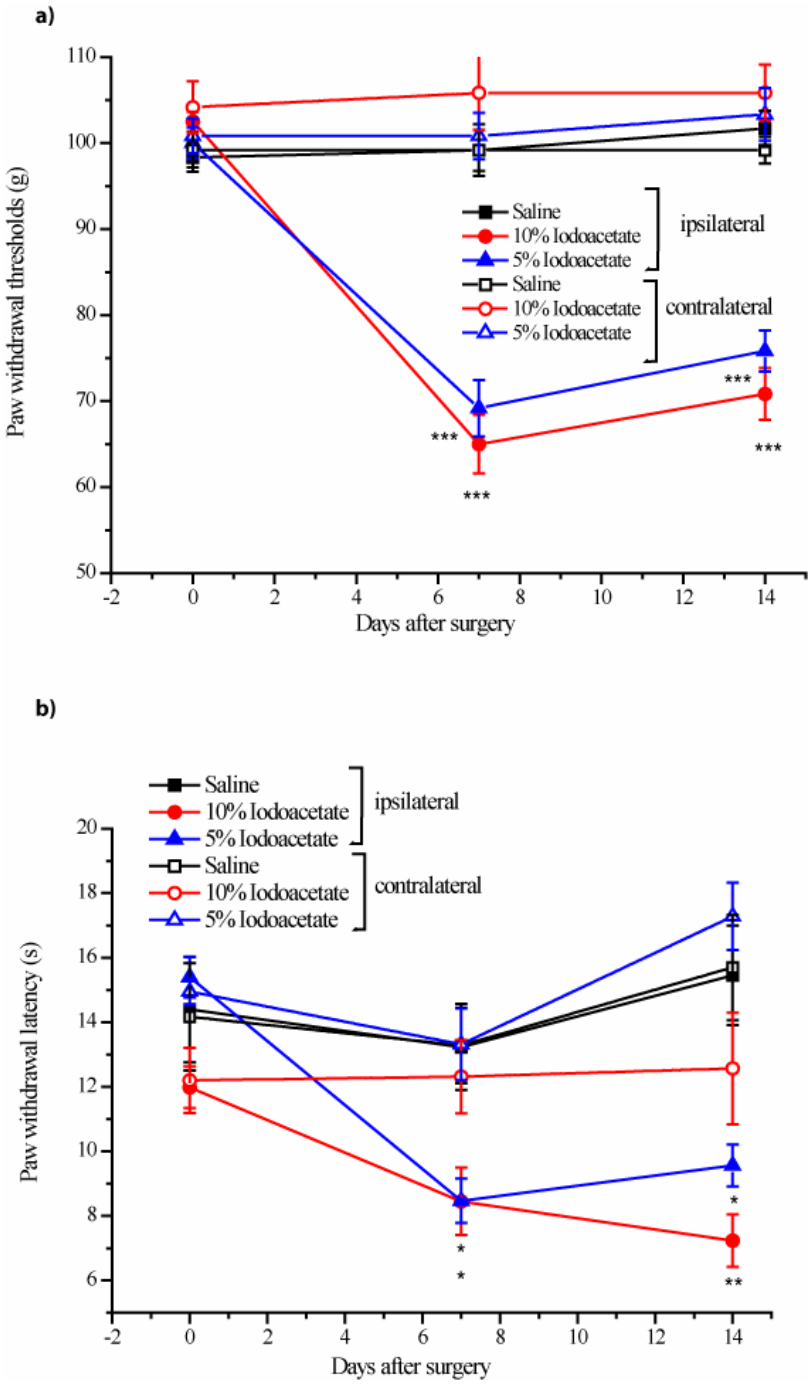


Figure 3-4 The effect of intra-articular injection of MIA in female C57Bl/6 mice on pain behaviours.

Test for the development of mechanical hyperalgesia a) cold allodynia b) are shown. Each point represents the mean value ± SEM of six mice. Statistics: MIA injected mice versus saline injected control mice by repeated measures ANOVA followed by Tukey's Test, * P<0.05, ** P<0.01, *** P<0.001

3.3.2.2 Induction of OA by intra-articular injection of 10% w/v MIA in 0.9% saline

8-10 week old female C57Bl/6 mice received an intra-articular injection of 10% w/v MIA in 0.9% sterile saline or the vehicle alone (n=22 per group). On recovery from general anaesthesia, the mice showed signs of stress following MIA injection but appeared to make a full recovery within 3 hours, however, 41% of the MIA injected mice died overnight (within 24 hours of MIA injection). The remaining mice had a visible ipsilateral hind limb lameness which resolved within 5 days but otherwise appeared healthy. The study was completed using a reduced number of mice for histological analyses with only one time point studied (16 weeks post MIA injection; n=3 per group). 10 mice per group were used for behavioural analyses.

Pre-treatment weights for saline injected mice and MIA injected mice were 17.73g ± 0.18g and 17.64 ± 0.22g, respectively. At 16 weeks post treatment, there was no significant difference in body weight gain between the two groups (saline injected mice, 23.29g ± 0.23g; MIA injected mice 23.05g ± 0.36g).

3.3.2.2.1 Joint Pathology

In order to confirm joint cartilage damage produced by intra-articular injection of 10% MIA, joint histology was performed. Table 3-1 shows the average joint score of 3 mice, 16 weeks following intra-articular injection of 10% w/v MIA and representative images are contained in Table 3-5 . Cartilage damage was scored according to the method described in section 2.6.5. The ipsilateral knee joints showed superficial fibrillation of the cartilage over all areas of the joint surface. In focal areas, moderate cartilage lesions were present with erosions extending to the calcified cartilage. The combined average joint score was 5.37 ± 0.37 with a maximal ipsilateral joint score of 10.67 ± 0.67 of a possible joint score of 24 which was significant compared to the contralateral limb which showed average and maximal joint scores of 0.67 ± 0.13 (P=0.0068) and 2.33 ± 0.33 (P=0.0079) respectively. The most severe cartilage lesions were located on the medial tibial plateau of the ipsilateral knee joint. A sample of control animals that were injected with 0.9% sterile saline were also assessed histologically. At 16 weeks, the control mice did not show significant signs of cartilage erosion with a combined average joint score of 0.33 ± 0.00 and a maximal ipsilateral joint score of 1.50 ± 0.17 compared to the contralateral limb which showed average and maximal joint scores of 0.23 ± 0.03 and 1.17 ± 0.17 respectively. These results suggest that cartilage damage was related to the MIA rather than the intra-articular injection itself.

Table 3-1 Quantitative joint pathology for female C57Bl/6 mice 16 weeks post 0.9% saline injection (sham) or intra-articular injection of 10% MIA.

Average joint scores a) maximal joint scores b) where L= operated left leg R= un-operated right leg. Values represent the mean value ± SEM of three mice.

a)	Average LTP (/6)	Average LFC (/6)	Average MTP (/6)	Average MFC (/6)	Combined Average Joint Score (/24)
Sham Week 16 L	0.00 ± 0.00	0.17 ± 0.03	0.03 ± 0.03	0.13 ± 0.00	0.33 ± 0.00
Sham Week 16 R	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.03	0.23 ± 0.03
MIA Week 16 L	0.77 ± 0.17	1.40 ± 0.07	1.67 ± 0.00	1.53 ± 0.13	5.37 ± 0.37
MIA Week 16 R	0.10 ± 0.05	0.17 ± 0.05	0.03 ± 0.05	0.37 ± 0.14	0.67 ± 0.13
b)	**Maximal LTP (/6)**	**Maximal LFC (/6)**	**Maximal MTP (/6)**	**Maximal MFC (/6)**	**Combined Maximal Joint Score (/24)**
Sham Week 16 L	0.00 ± 0.00	0.33 ± 0.00	0.17 ± 0.17	1.00 ± 0.33	1.5 ± 0.17
Sham Week 16 R	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.00	1.17 ± 0.17
MIA Week 16 L	2.17 ± 0.17	2.17 ± 0.17	3.50 ± 0.17	2.83 ± 0.17	10.67 ± 0.67
MIA Week 16 R	0.50 ± 0.17	0.50 ± 0.17	0.17 ± 0.17	1.17 ± 0.17	2.33 ± 0.33

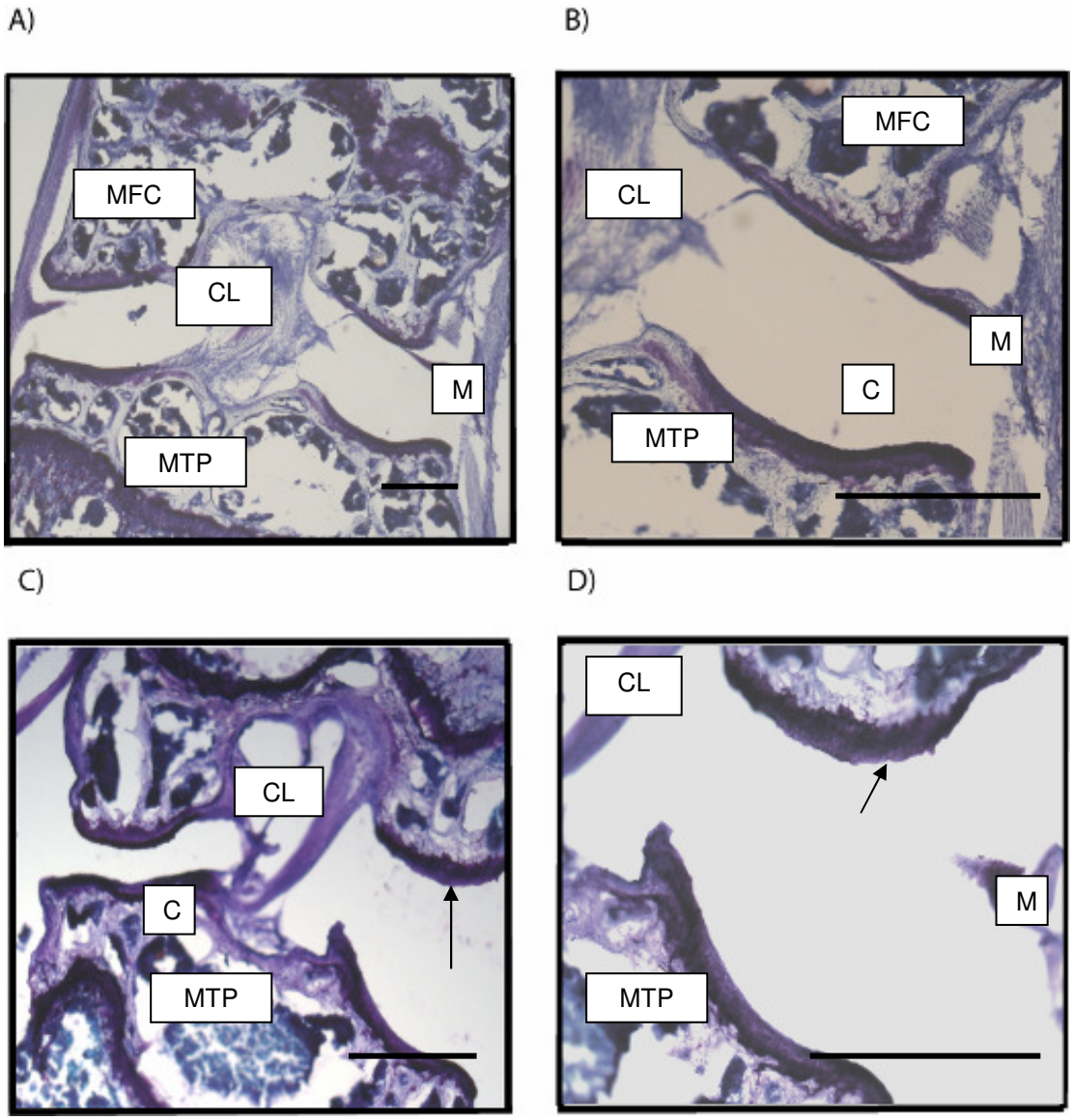


Figure 3-5 Representative photomicrographs of 12μm thick frontal sections of the medial femorotibial joint of female C57Bl/6 mice following intra-articular injection of 10% MIA.

Sections stained with toluidine blue with medial meniscus (M) to the right; cruciate ligament (CL) to the left; medial femoral condyle (MFC) at the top and tibial plateau (MTP) at the bottom. Ipsilateral knee joint at low power 16 weeks post 0.9% saline injection A) Ipsilateral knee joint at higher power 16 weeks post 0.9% saline injection B) Ipsilateral knee joint at low power 16 weeks post 10% MIA injection C) Ipsilateral knee joint at higher power 16 weeks post 10% MIA injection D). (↑) represents ulceration of cartilage into the superficial or middle zones. Scale bar: 1mm

3.3.2.2.2 Behaviour

Measurements were taken to compare the pain behaviours in groups of 10 female 8-10 week old C57Bl/6 mice injected with either 10% w/v MIA in 0.9% sterile saline or 0.9% sterile saline (control group) into the left knee joint. Measurements were performed at regular intervals following MIA injection. Results are shown in Figure 3-6 and Figure 3-7.

The effect of MIA on the distribution of weight borne on the ipsilateral and contralateral hind limbs was examined as a potential measure of pain (Figure 3-6a). Assessment of weight distribution showed a significant decrease in the weight borne by the ipsilateral hind limb at day 3 (P=0.005), day 14 (P=0.010), day 21 (P=0.013), day 28 (P=0.014), day 91 (P=0.010), and day 98 (P=0.029) following intra-articular injection of MIA compared to sham animals. A high variability was present in this particular measurement in both groups of animals which may reflect the inquisitive nature of mice.

The lowest force required to elicit a response using von Frey filaments was assessed before and after intra-articular injections (Figure 3-6b). Pre-treatment PWTs were similar in both sham (0.60 ± 0.11g) and MIA injected animals (0.64 ± 0.07g). Control animals maintained a mean PWT of at least 0.54g ± 0.03g throughout the study period. MIA injected animals developed significant levels of mechanical allodynia in the ipsilateral hind limb from day 14 when a PWT of 0.43g ± 0.05g was recorded, compared to sham animals (P= 0.030). The lowest force required to elicit a response was recorded at day 105 with a value of 0.36g ± 0.06g. No significant changes from baseline were observed in the contralateral limb of MIA injected animals at any time point studied.

Mechanical hyperalgesia following injection of MIA was assessed using an analgesymeter to apply an increasing pressure stimulus on the dorsal surface of the hind paw (Figure 3-7a). Pre-treatment PWTs to a noxious stimulus were similar in both control (96.5g ± 1.67g) and MIA injected animals (100g ± 1.67g). Control animals maintained a mean PWT of at least 96.5g ± 1.67g throughout the 105 day study. In contrast, MIA injected mice showed significant levels of hyperalgesia from day 3 with a mean PWT of 77g ± 3.59g (P=0.00031). PWTs returned to control levels at day 77 and 84 (102.2g ± 1.69 and 102.2g ± 2.22g respectively) before significantly decreasing again at day 91 (P=0.00036). Significant levels of hyperalgesia persisted for the remainder of the study. A significant contralateral mechanical hyperalgesia developed in the MIA injected mice compared to control

animals at day 42 (P=0.00018) but this contralateral effect was not noted at the other time points measured in this study.

Behavioural cold sensitivity following intra-articular injection of MIA was assessed using a 10ºC cold plate (Figure 3-7b). PWLs to this cold stimulus were similar in both groups of mice before treatment (control, 11.75s ± 0.76s and MIA injected animals, 12.30s ± 0.65s). The control animals maintained a mean PWL of at least 9.97s throughout the 105 day study. MIA injected mice showed significant levels of allodynia from day 3 with a PWL of 7.86s ± 0.55s. Significant levels of allodynia persisted until day 70 and day 77 where PWLs returned to control levels. Significant levels of allodynia were present again at day 90 (P=0.00037) and persisted for the remainder of the study. A significant contralateral cold allodynia was noted in the MIA injected mice compared to sham animals from day 21 (P=0.00085) to around day 40. Contralateral PWLs returned to control levels at day 70 and day 77 and reached significant allodynia levels again at day 91 (P=0.0039) and day 98 (P=0.042).

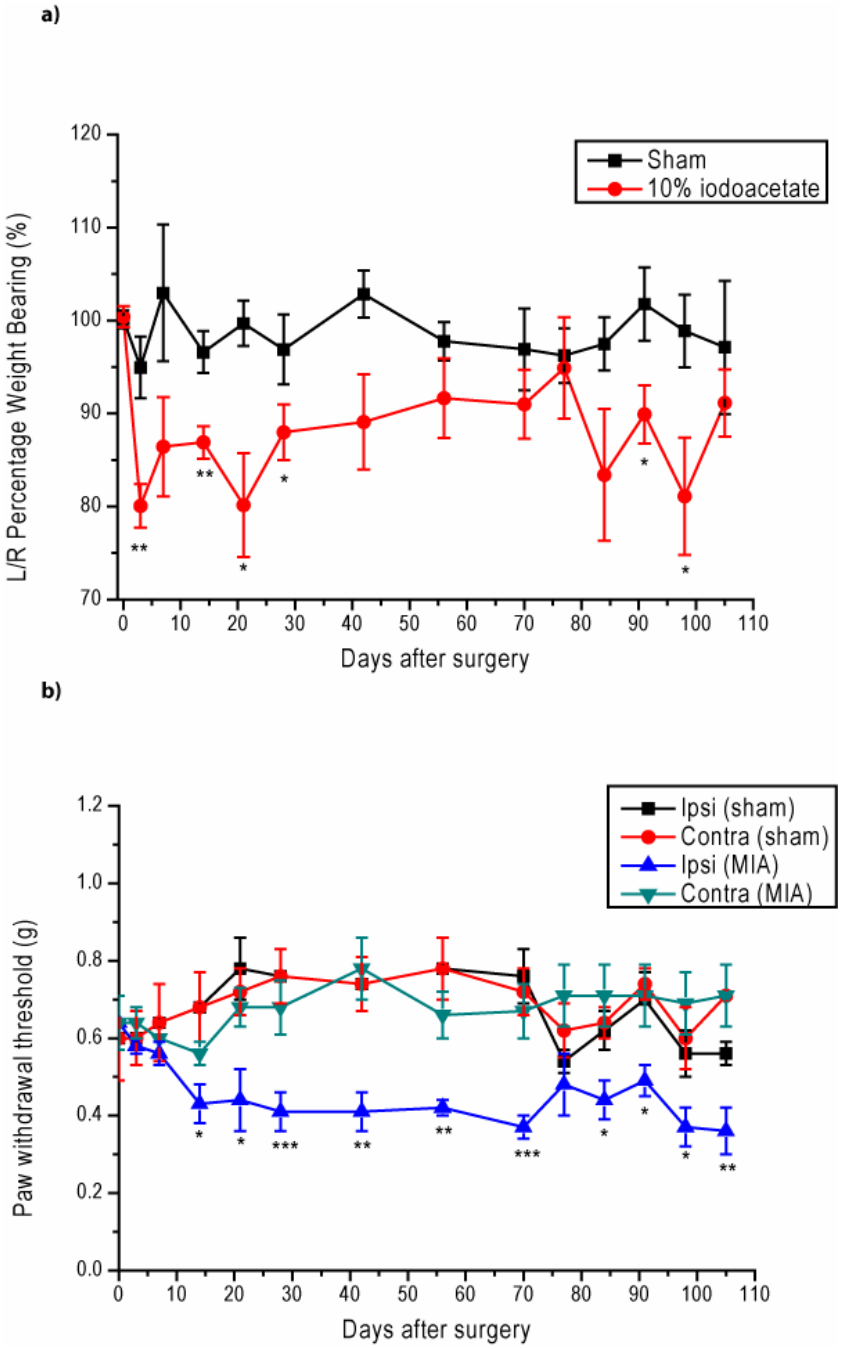


Figure 3-6 The effect of OA induction on pain behaviours following intra-articular injection of 10% w/v MIA in female C57Bl/6 mice.

The effect on weight bearing a) and mechanical allodynia b). Each point represents the mean value ± SEM of ten mice. Statistics: MIA injected mice versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test. * P<0.05, ** P<0.01, *** P< 0.001

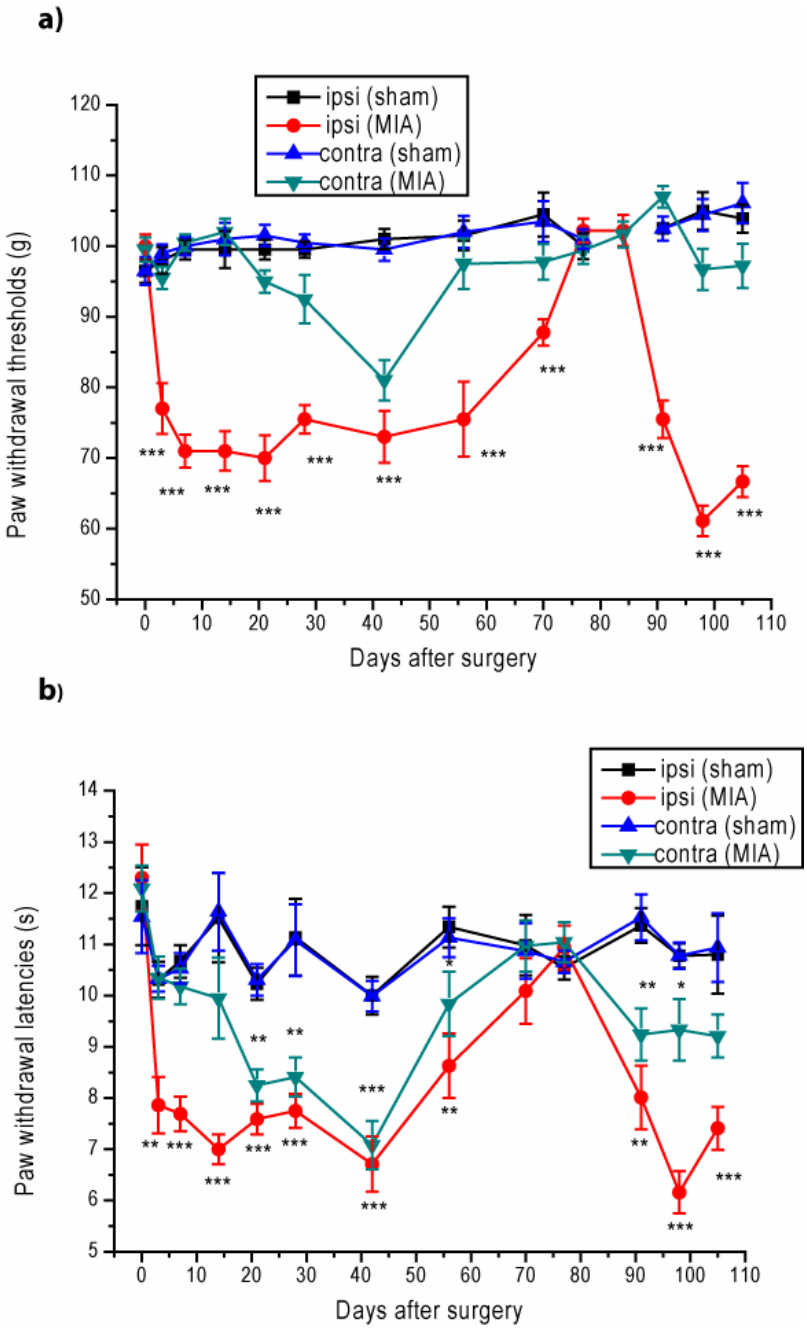


Figure 3-7 The effect of OA induction on pain behaviours following intra-articular injection of 10% w/v MIA in female C57Bl/6 mice.

Test for the development of mechanical hyperalgesia a) and cold allodynia b). Each point represents the mean value ± SEM of ten mice. Statistics: MIA injected mice versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test. *P<0.05, **P<0.01, ***P< 0.001

3.3.2.2.3 Comments on this model

Injection of 10% w/v MIA into the femorotibial joint space of female C57Bl/6 mice produces cartilage lesions and accompanying alterations in weight bearing and evoked pain behaviours that are consistent with the induction of mild-moderate OA. For two of the pain measures (mechanical hyperalgesia and cold allodynia) there were later time points where the hypersensitivities were temporarily reduced and PWTs and PWLs returned to the levels seen in control mice. This loss of sensitivity requires further investigation. The toxic nature of MIA in mice, however, limits its potential for use in experimental studies of mice with a fine balance between inducing cartilage damage and persistent pain sensitivities and causing death of the animals. The high mortality rate associated with this dosage made it inappropriate for further investigation.

3.3.2.3 Induction of OA by intra-articular injection of 1% w/v MIA in 0.9% saline

In this series of experiments, 8-10 week old female C57Bl/6 mice received an intra-articular injection of 1% w/v MIA in 0.9% sterile saline. Mice exhibited signs of stress following MIA injection with a hunched posture and piloerect fur but were ambulating and eating normally within 3 hours. The mice had a visible ipsilateral hind limb lameness which resolved within 5 days. No signs of spontaneous nociceptive behaviour, impaired locomotion or distress were observed following the initial 5 days of study. At the beginning of the time course study, the mean weights for saline injected mice and MIA injected mice were 17.59g ± 0.18g and 17.66 ± 0.27g. At 16 weeks post treatment, there was no significant difference in body weight gain between the two groups (saline injected mice, 23.39g ± 0.23g; MIA injected mice 22.80g ± 0.31g).

3.3.2.3.1 Joint Pathology

In order to assess the degree of joint damage produced by intra-articular injection of 1% MIA, joint histology was performed at 0, 28, 56 and 84 days following MIA injection. At each time point, 3 mice were killed by cervical dislocation and the ipsilateral and contralateral knees dissected, decalcified and stained for analysis.

Representative images of knee joint histology are contained in Figure 3-8 and quantitative joint scoring in Table 3-2 and Table 3-3. At week 4, mild OA lesions were present on the ipsilateral knee joints with superficial fibrillation of the cartilage. At week 4, joint scores for the ipsilateral knee joint were not significantly different compared to naïve or contralateral knee joint scores. By weeks 8 and 12 mild-moderate cartilage lesions were noted with cartilage erosions extending into the mid

and deep zone. The most severe lesions were recorded in the medial femoral condyle and medial tibial plateau. The combined average joint score at week 12 was 3.40 ± 0.33 with a maximal joint score of 7.83 ± 1.17. The contralateral knee joints showed no significant cartilage lesions by week 12 compared to naïve animals with a combined average joint score of 0.03 ± 0.03 of a total possible joint score of 24 and maximal joint score of 1.17 ± 0.17. Cartilage lesions in the ipsilateral hind limb were significant compared to the contralateral hind limbs at weeks 8 and 12 (P=0.008 and P=0.027 respectively, see Figure 3-9).

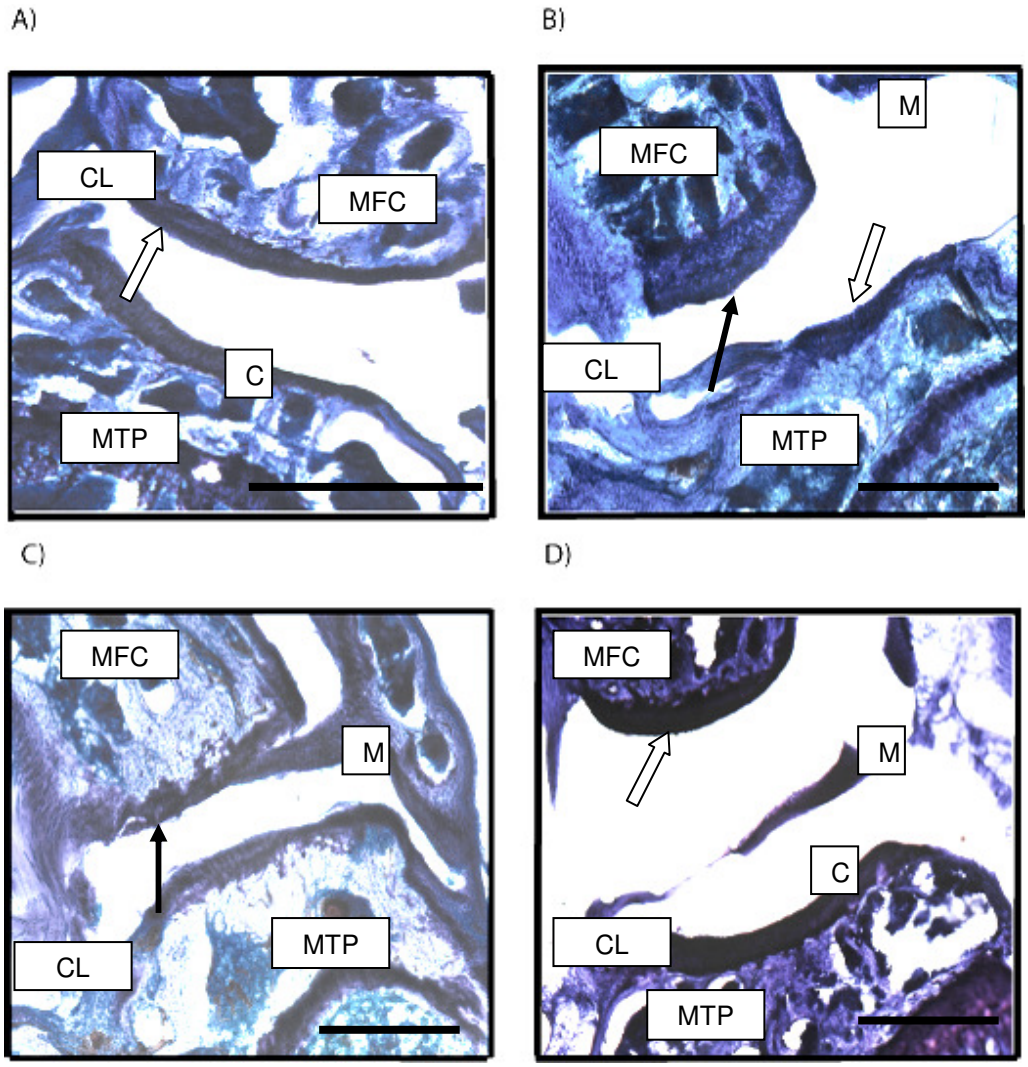


Figure 3-8 Representative photomicrographs of 12µm thick frontal sections of the medial femorotibial joint of female C57Bl/6 mice following intra-articular injection of 1% MIA. Sections stained with toluidine blue with medial meniscus (M) to the right; cruciate ligament (CL) to the left; medial femoral condyle (MFC) at the top and tibial plateau (MTP) at the bottom. Ipsilateral knee week 4 post MIA injection A) Ipsilateral knee week 8 post MIA injection B) Ipsilateral knee week 12 post surgery C) Contralateral knee week 12 post surgery D) (⇐) represents mild fibrillation of the surface of the cartilage (↑) represents ulceration of cartilage into the middle zones. Scale bar: 1mm

Table 3-2 Summary table displaying average joint scores for female C57Bl/6 mice 12 weeks post intra-articular injection of 1% w/v MIA.

Where L= operated left leg R= un-operated right leg. Values represent the mean value ± SEM of three mice.

	Average LTP (/6)	**Average LFC (/6)**	**Average MTP (/6)**	**Average MFC (/6)**	**Combined Average Joint Score (/24)**
Naïve L	0.03 (± 0.03)	0.00 (± 0.00)	0.03 (± 0.03)	0.17 (± 0.11)	0.23 (± 0.03)
Week 4 L	0.20 (± 0.20)	0.30 (± 0.10)	0.00 (± 0.0)	0.30 (± 0.10)	0.80 (± 0.20)
Week 8 L	0.60 (± 0.20)	0.83 (± 0.23)	1.80 (± 0.04)	1.59 (± 0.01)	4.82 (± 0.38)
Week 12 L	0.27 (± 0.13)	0.40 (± 0.13)	1.07 (± 0.00)	1.67 (± 0.07)	3.40 (± 0.33)
Naïve R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
Week 4 R	0.00 (± 0.00)	0.00 (± 0.00)	0.07 (± 0.07)	0.00 (± 0.00)	0.07 (± 0.07)
Week 8 R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
Week 12 R	0.00 (± 0.00)	0.03 (± 0.03)	0.00 (± 0.00)	0.00 (± 0.00)	0.03 (± 0.03)

Table 3-3 Summary table displaying maximal joint scores for female C57Bl/6 mice 12 weeks post intra-articular injection of 1% w/v MIA.

Where L= operated left leg R= un-operated right leg. Values represent the mean value ± SEM of three mice.

	Maximal LTP (/6)	**Maximal LFC (/6)**	**Maximal MTP (/6)**	**Maximal MFC (/6)**	**Combined Maximal Joint Score (/24)**
Naïve L	0.17 (± 0.17)	0.00 (± 0.00)	0.33 (± 0.00)	0.67 (± 0.00)	1.17 (± 0.17)
Week 4 L	0.17 (± 0.17)	0.33 (± 0.33)	0.00 (± 0.00)	0.50 (± 0.17)	1.00 (± 1.00)
Week 8 L	1.17 (± 0.17)	1.33 (± 0.00)	2.67 (± 0.33)	2.17 (± 0.17)	7.33 (± 0.67)
Week 12 L	1.00 (± 0.67)	1.00 (± 0.00)	2.83 (± 0.17)	3.00 (± 0.33)	7.83 (± 1.17)
Naïve R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
Week 4 R	0.00 (± 0.00)	0.00 (± 0.00)	0.33 (± 0.33)	0.00 (± 0.00)	0.33 (± 0.33)
Week 8 R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
Week 12 R	0.00 (± 0.00)	0.17 (± 0.17)	0.17 (± 0.17)	0.33 (± 0.33)	0.67 (± 0.33)

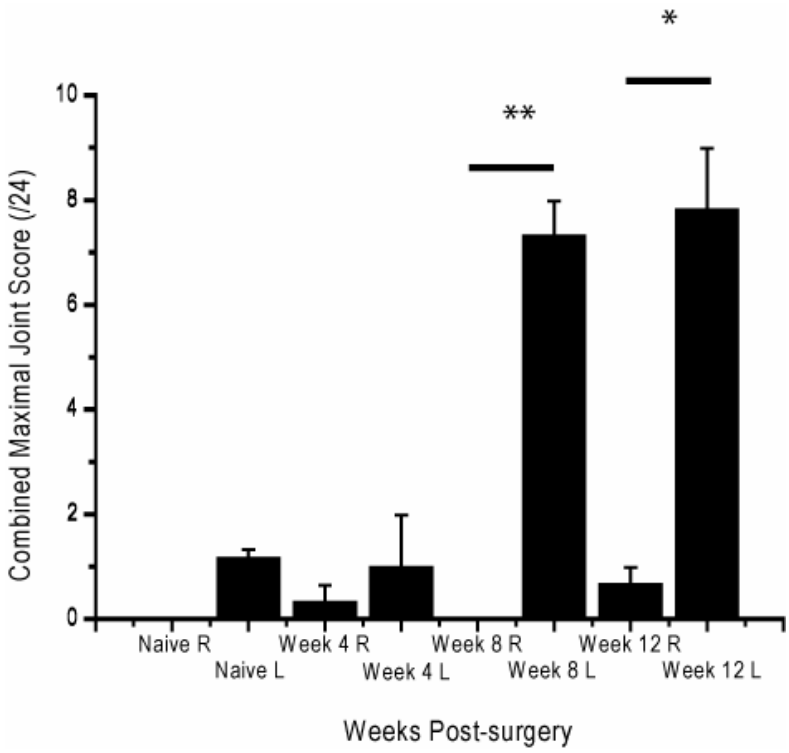


Figure 3-9 Quantitative scoring of osteoarthritic joint pathology following intra-articular injection of 1% MIA in female C57Bl/6 mice.

Each point represents the mean value ± SEM of three mice where L= operated left leg R= un-operated right leg. * P<0.05, ** P<0.01 by unpaired t-test.

3.3.2.3.2 Behaviour

Pain behaviours were assessed in a group of 10 female C57Bl/6 injected with 0.9% sterile saline (control group) and 10 female C57Bl/6 mice injected with 1% w/v MIA in 0.9% sterile saline into the left knee joint. Measurements were performed at regular intervals following MIA injection.

Assessment of weight distribution showed no significant decrease in the weight borne by the ipsilateral hind limb following intra-articular injection of MIA compared to saline injected control animals (Figure 3-10a).

Pre-MIA injection PWTs tested using von Frey filaments were identical in both saline (0.80g ± 0.07g) and MIA injected animals (0.80 ± 0.09g) (Figure 3-10b). Control mice maintained an ipsilateral PWT of at least 0.56g ± 0.06g throughout the study period. MIA injected animals developed significant levels of mechanical allodynia in the ipsilateral hind limb at day 5 (P=0.00023) with a mean PWT of 0.42g ± 0.02g

compared to the ipsilateral hind limb of control animals who had a PWT of 0.82g ± 0.08g. PWT returned to the levels seen in control animals day 14 post-MIA injection and then decreased again at later time points. Significant levels of mechanical allodynia were present at day 28 (P=0.023), day 63 (P=0.0060) day 98 (P=0.013) and day 105 (P=0.00048) compared to the ipsilateral hind limb of sham animals. The lowest force required to elicit a response was recorded at day 105 post MIA injection with a PWT of 0.30g ± 0.04g. No significant changes from baseline were observed in the contralateral limb of MIA injected animals at any time point studied when compared to the ipsilateral or contralateral limb of saline injected mice.

Intra-articular injection of 1% MIA led to the development of mechanical hyperaglesia. Pre-injection PWTs were similar in both control (96.0g ± 1.45g) and MIA injected animals (98.0g ± 1.86g) (Figure 3-11a). Saline injected animals maintained a PWT of at least 96.0g ± 1.86g throughout the 105 day study. MIA injected mice showed significant levels of hyperalgesia from day 5 with a PWT of 76.5g ± 4.15g (P=0.00022). This mechanical hypersensitivity was not maintained constantly, however, and PWT increased to sham levels at day 84 (102.0g ± 3.74g) before returning to significant levels at day 105 (P=0.00016). No significant contralateral mechanical hyperalgesia developed in the MIA injected mice compared to saline injected animals during this study.

Behavioural cold hypersensitivity (cold allodynia) was assessed using a 10ºC cold plate. Pre-treatment PWLs to this cold stimulus were similar in both groups of mice (control, 12.15s ± 0.93s; MIA 13.08s ± 0.78s) (Figure 3-11b). Saline injected animals maintained a mean PWL of at least 10.84s throughout the 105 day study. MIA injected mice showed significant levels of allodynia from day 14 when a PWL of 8.78s ± 0.51s was recorded. As reported above for mechanical hyperalgesia, cold hypersensitivity was not constant. Significant levels of allodynia persisted until day 84 when PWL returned to control levels but significant levels of allodynia were present again at day 105 (P= 0.00018). A significant contralateral hind limb cold allodynia was noted in the MIA injected mice compared to saline injected animals at day 42 (P=0.0079) and day 105 (P=0.018).

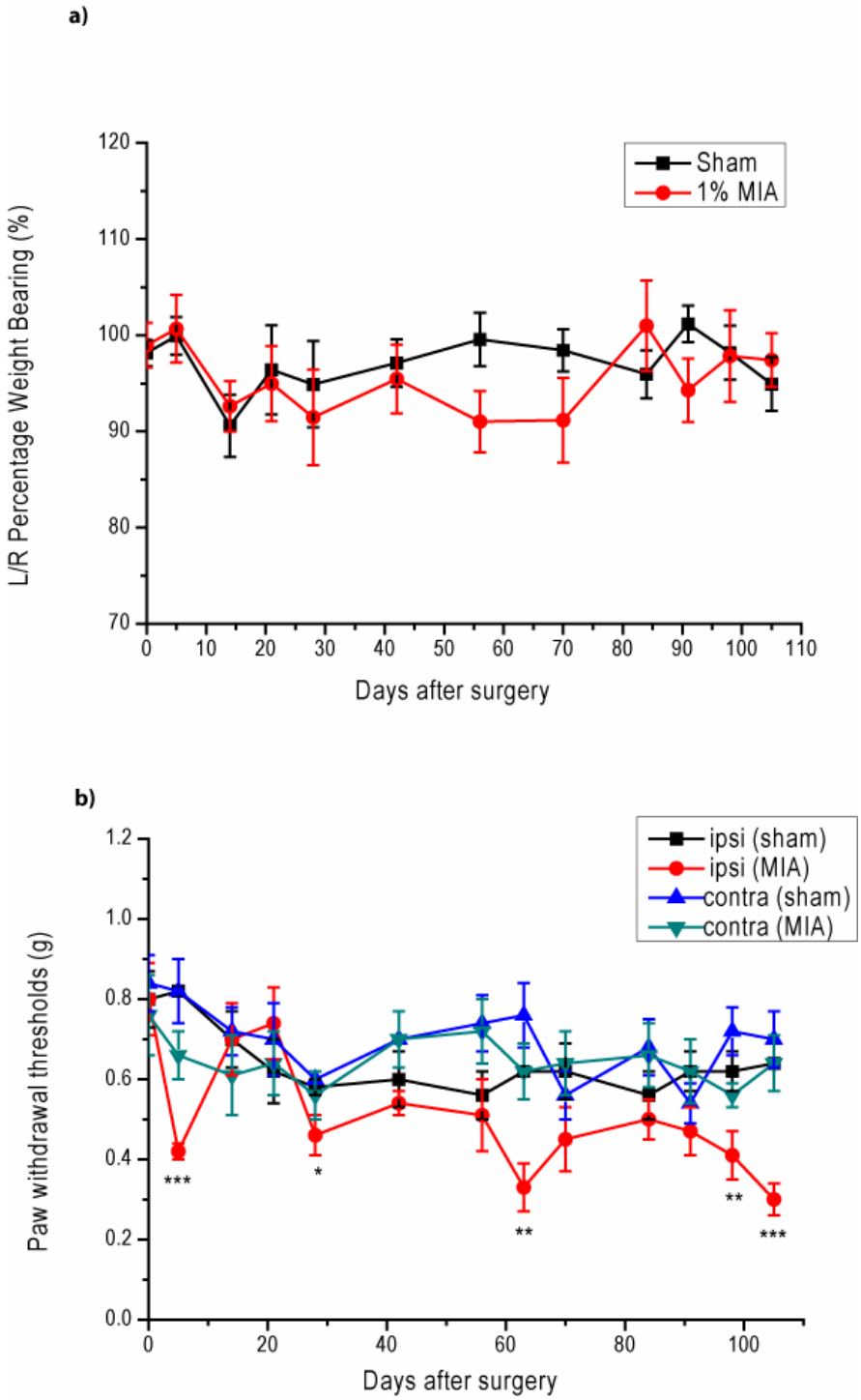


Figure 3-10 The effect of OA induction on pain behaviours following intra-articular injection of 1% w/v MIA in female C57Bl/6 mice.

The effect on weight bearing a) and mechanical allodynia b). Each point represents the mean value ± SEM of ten mice. Statistics: MIA injected mice versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test. * P<0.05, ** P<0.01, *** P<0.001

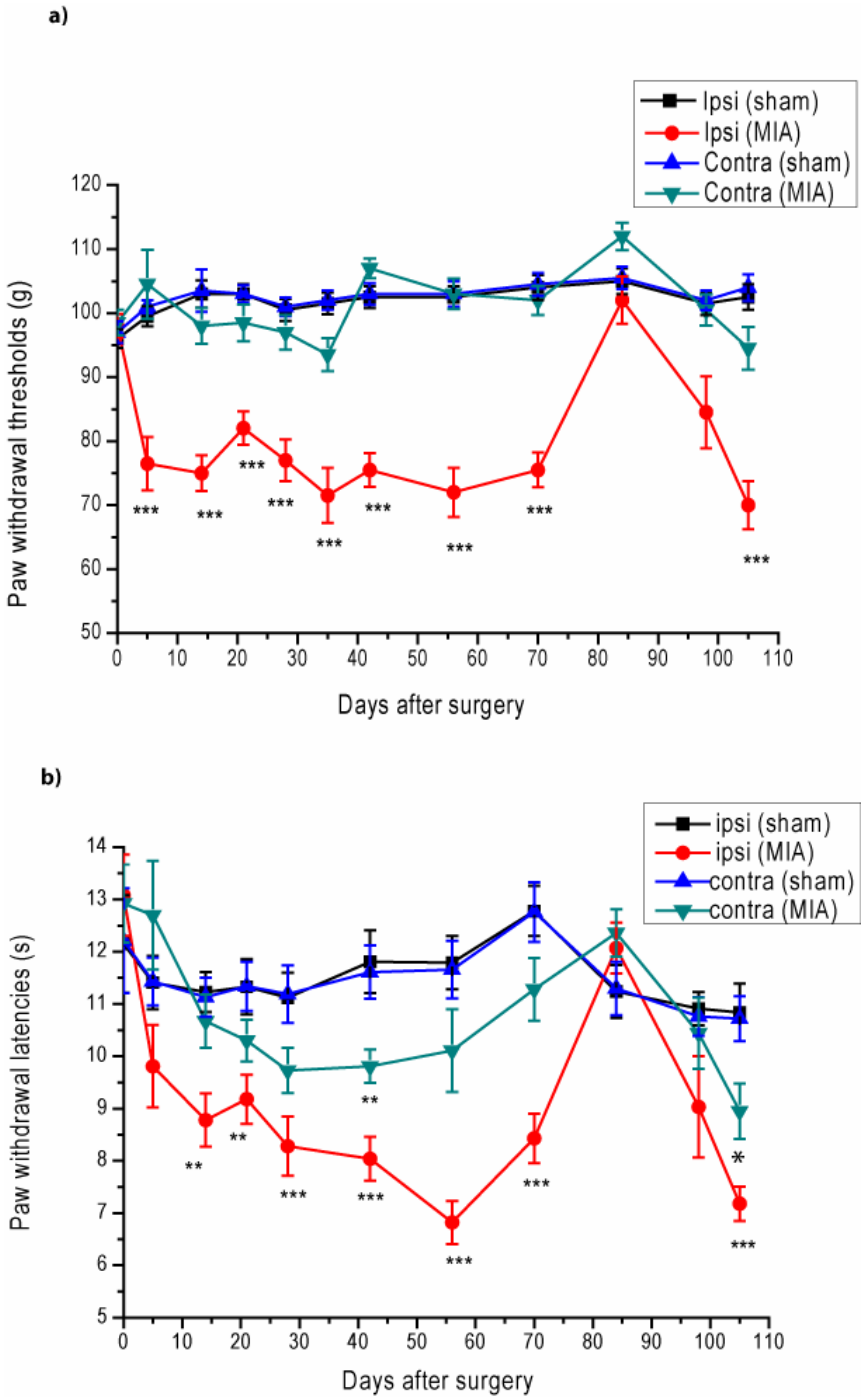


Figure 3-11 The effect of OA induction on pain behaviours following intra-articular injection of 1% w/v MIA in female C57Bl/6 mice.

The effect on mechanical hyperalgesia c) and cold allodynia d). Each point represents the mean value ± SEM of ten mice. Statistics: MIA injected mice versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test, ** P<0.01, *** P< 0.001.

3.3.2.3.3 Influence of endogenous opioids

As mentioned above, a marked reduction in mechanical hyperalgesia and cold allodynia was noted at day 84 following intra-articular injection of 1% MIA. Endogenous opioids have been reported to mask OA induced pain behaviours in a murine surgical DMM model (Inglis, McNamee et al. 2008), therefore naloxone (2.5mg/kg i.p.) was administered at this time point to investigate whether the absence of pain was due to the influence of endogenous opioids (Figure 3-12). Naloxone (2.5mg/kg i.p.) or the vehicle (0.9% saline) was administered to randomly assigned mice (n=5 per group) and behavioural measurements repeated after 1 hour. The administration of naloxone resulted in a significant return of mechanical hyperalgesia (P=0.049) and cold allodynia (P=0.0090) pain behaviours compared to pre-drug withdrawal thresholds. Naloxone had no effect on weight bearing and von Frey measurements at this time point in MIA treated mice and did not affect any of the pain behaviours in vehicle treated animals.

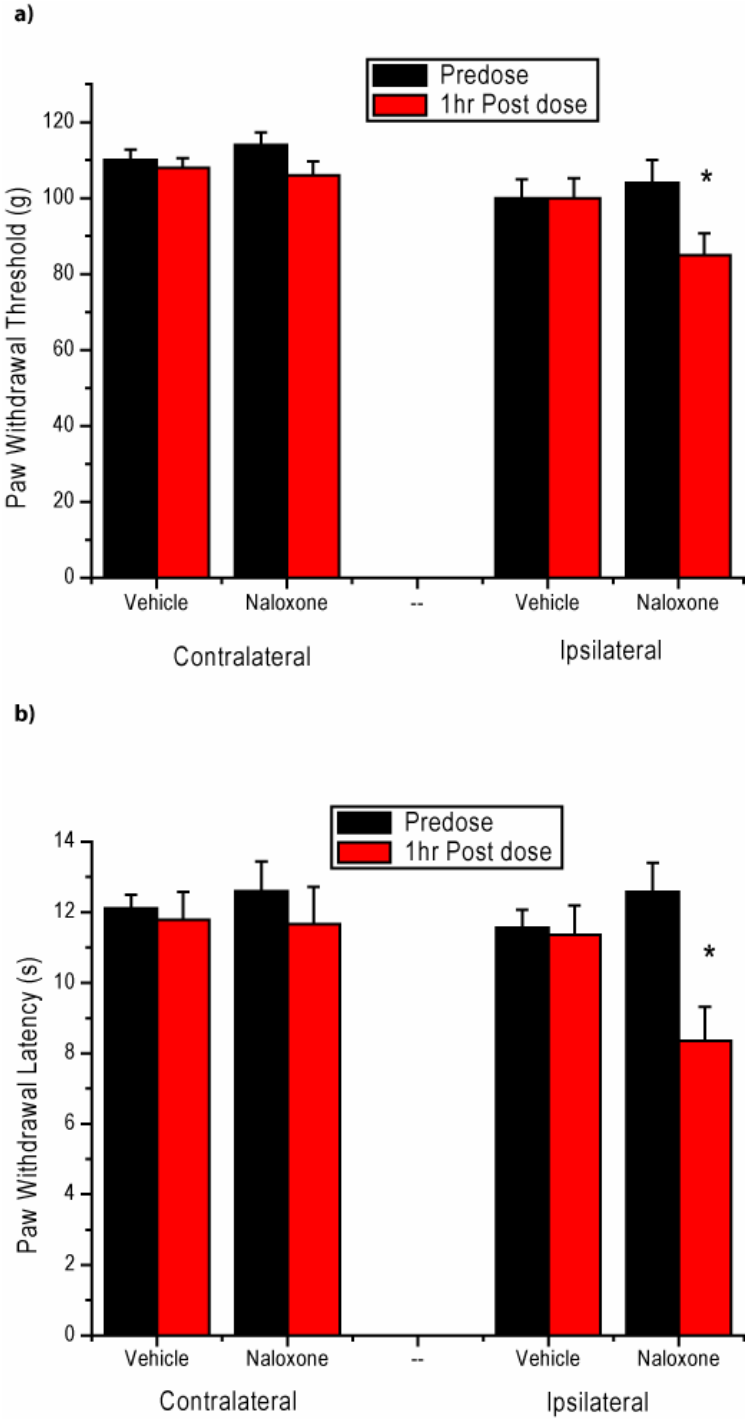


Figure 3-12 The effect of 2.5mg/kg i.p. naloxone on pain behaviours at day 84 following intra-articular injection of 1% MIA in female C57Bl/6 mice.

The effect of naloxone on mechanical hyperalgesia a) cold allodynia b). Each point represents the mean value ± SEM of five mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *P<0.05

3.3.2.3.4 Reduction of pain behaviour by an analgesic drug, morphine

A new cohort of mice underwent intra-articular injection of 1% MIA in order to confirm that the alterations in pain behaviours were due to the presence of joint pain.

The development of pain related behaviours followed the same time course as the previous study with animals exhibiting similar levels of mechanical hyperalgesia and cold allodynia 2 weeks following intra-articular injection of 1% MIA. Morphine (6mg/kg s.c.) or the vehicle (0.9% saline) was administered to randomly assigned mice and behavioural measurements repeated after 1 hour. Following drug administration, the mice did not exhibit any signs of visible sedation or hyperactivity. Weight bearing deficits were not present and therefore not tested in this study. Ipsilateral mechanical allodynia ($P=0.0048$), mechanical hyperalgesia ($P<0.0001$) and cold allodynia ($P=0.0003$) were reversed significantly by morphine compared to pre-treatment levels (Figure 3-13 and Figure 3-14). Ipsilateral PWTs and latencies were increased beyond the pre-drug levels representing an anti-nociceptive effect of the morphine. Contralateral PWTs to cold and paw pressure stimuli were also increased in the contralateral hind limb following morphine administration indicating a systemic analgesic effect.

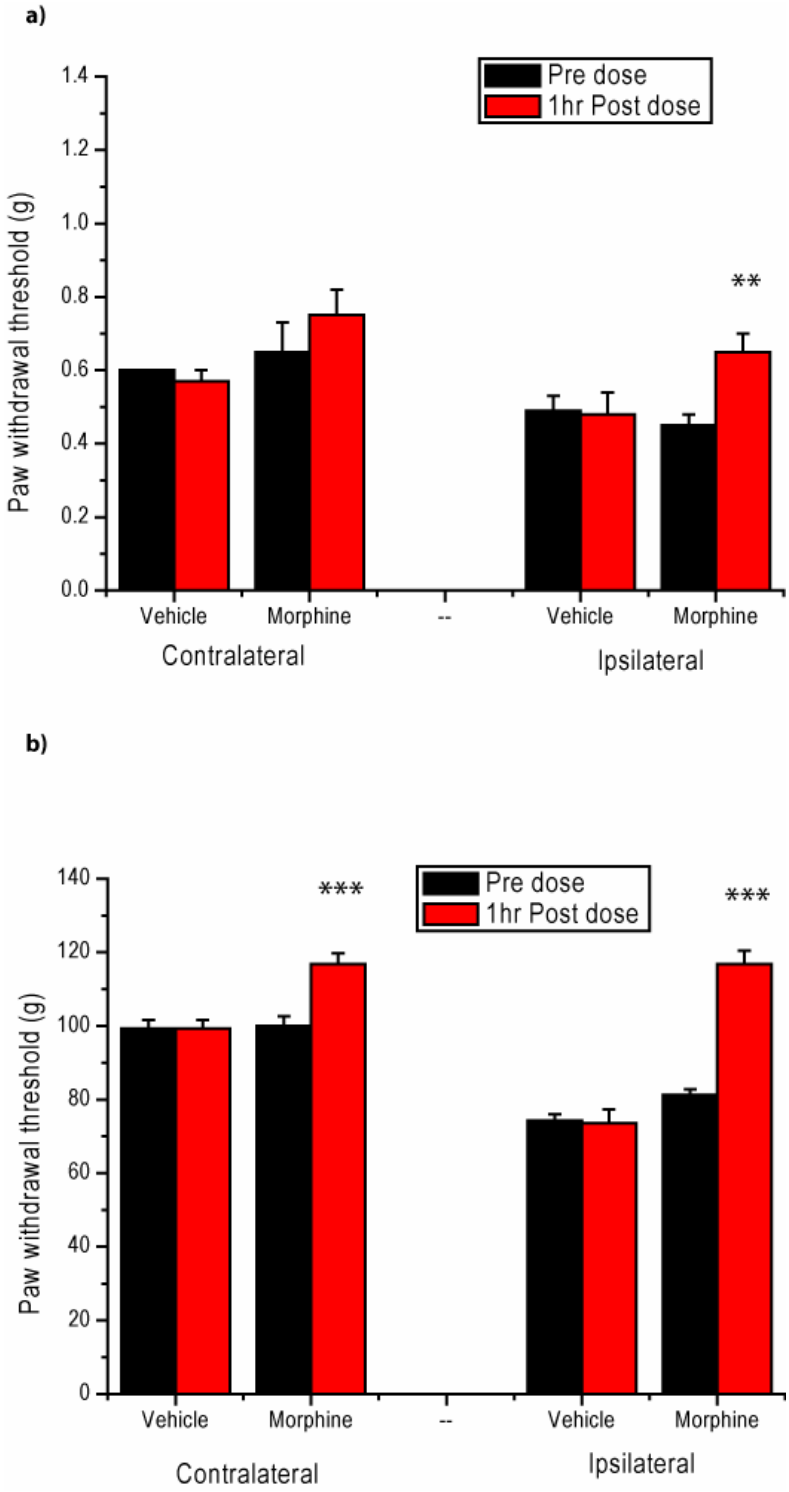


Figure 3-13 The effect of 6mg/kg s.c. morphine on pain behaviours at day 14 following intra-articular injection of 1% MIA in female C57Bl/6 mice.

The effect of morphine on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01, *** P<0.001

a)

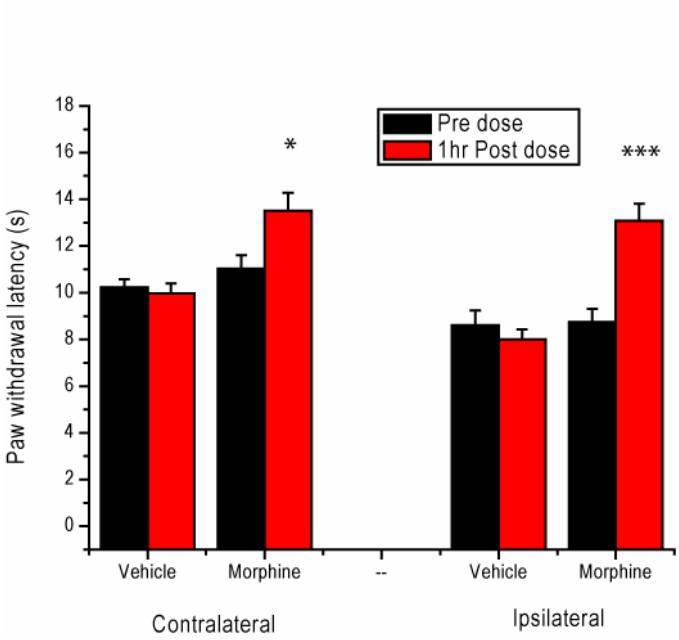


Figure 3-14 The effect of 6mg/kg s.c. morphine on pain behaviours at day 14 following intra-articular injection of 1% MIA in female C57Bl/6 mice.

The effect of morphine on cold allodynia a). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *P<0.05, *** P<0.001

3.3.2.3.5 Comments

Injection of 1% w/v MIA into the femorotibial joint space of C57Bl/6 mice produces cartilage lesions and accompanying alterations in evoked pain behaviours that are consistent with the induction of mild-moderate OA. The onset of pain behaviours was rapid enabling the testing of therapeutic compounds from day 14 onwards. The general health of the animals over the time course of the study was good and the mice showed no signs of spontaneous pain.

3.3.3 Development of a direct measure of knee pain/evoked pain

The majority of behavioural measures of joint pain used in in-vivo studies test referred pain rather than providing a direct measure of joint pain. Two new techniques to measure evoked knee pain as a reflection of OA pain were therefore examined.

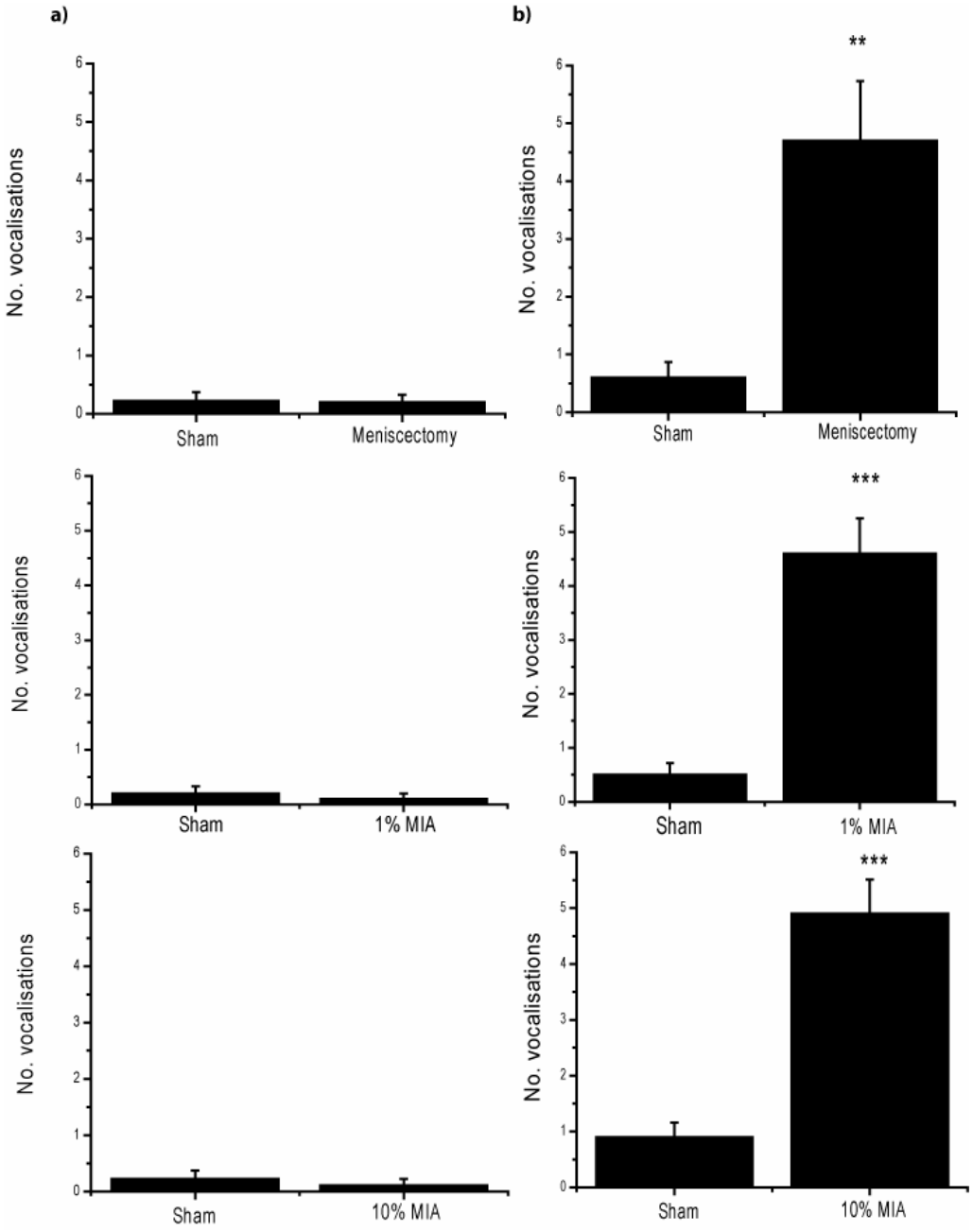


Figure 3-15 The effect of knee manipulation on vocalisations

Vocalisations during a) 10 passive flexion and extensions of the ipsilateral knee joint and b) 10 compressions of the ipsilateral knee when held in a flexed position. Measurements recorded at 16 weeks intra-articular injection of 1% MIA in female C57Bl/6 mice. Each point represents the mean value ± SEM of ten mice. Statistics: MIA versus control animals by unpaired t-test, ** P< 0.01. *** P< 0.001

3.3.3.1 Vocalisations in response to passive flexion/extension

Female C57Bl/6 mice who had received intra-articular injection with either 1% or 10% w/v MIA 16 weeks previously or who underwent partial medial meniscectomy 16 weeks (see section 3.3.4) previously (n=10 per group) were tested to investigate vocalisation as a pain response to knee joint manipulation as described in 2.5.7. Mice in the MIA and meniscectomy groups vocalised less than once per 10 passive knee joint manipulations (Figure 3-15a). There was no significant difference between control animals and those in any of the treatment groups tested. During these experiments, all mice appeared comfortable during manipulation of the ipsilateral hind limb with no flinching or other indicators of pain or distress.

3.3.3.2 Compression of the knee in a fixed position

In other experiments, vocalisation in response to knee joint compression was investigated as described in 2.5.8. There were significantly more vocalisations in animals who had received partial medial meniscectomy ($P=0.0012$), intra-articular injection of 1% w/v MIA ($P<0.0001$) and intra-articular injection of 10% w/v MIA ($P<0.0001$) when compared to their respective control groups (Figure 3-15b). Vocalisations in partial medial meniscectomy, 10% MIA and 1% MIA were similar with mean values of 4.7 ± 1.03, 4.89 ± 0.61, 5.60 ± 0.65 vocalisations, respectively. This method appears to provide a good indicator of joint pain in C57Bl/6 mice.

3.3.4 Induction of OA by partial medial meniscectomy

In humans the medial meniscus is most vulnerable to injury due to its firm attachment to the tibia and traumatic injury commonly leads to early onset OA, (Campbell, Sanders et al. 2001). Partial medial meniscectomy was therefore chosen as a potential surgical model of OA pain that may reflect human OA pathophysiology.

3.3.4.1 General

Female 8-10 week old C57Bl/6 mice underwent partial medial meniscectomy according to the methods described in 2.4.2.2. All mice recovered quickly from surgery with ipsilateral hind limb lameness resolving within 24 hours. Animals showed a good state of health and no signs of spontaneous nociceptive behaviour, impaired locomotion or distress were observed at any time point during the study. At the beginning of the time course study, the mean weights for sham operated mice and meniscectomized mice were 16.95g ± 0.15g and 17.19 ± 0.26g, respectively. At 16 weeks post treatment, there was no significant difference in body weight gain between the two groups (sham operated mice, 22.46g ± 0.20g; meniscectomized mice 22.77g ± 0.29g).

3.3.4.2 Joint Pathology

Figure 3-16 shows representative images of the histological findings. 4 weeks post-meniscectomy, mild OA lesions were observed across all joint surfaces with superficial fibrillation of the cartilage and some vertical cleft formation. By week 8, more severe lesions were apparent, particularly in the medial compartment with the presence of full thickness cartilage erosions. At 12 weeks post-meniscectomy, extensive full thickness cartilage erosions were present affecting large areas of the medial tibial plateau and medial femoral condyle. The combined average joint score at week 12 was 10.77 ± 0.95 (Table 3-4) with a maximal joint score of 13.22 ± 1.82 (Table 3-5). The contralateral knee joints showed no significant cartilage lesions at week 12 with a combined average joint score of 0.37 ± 0.19 of a total possible joint score of 24.

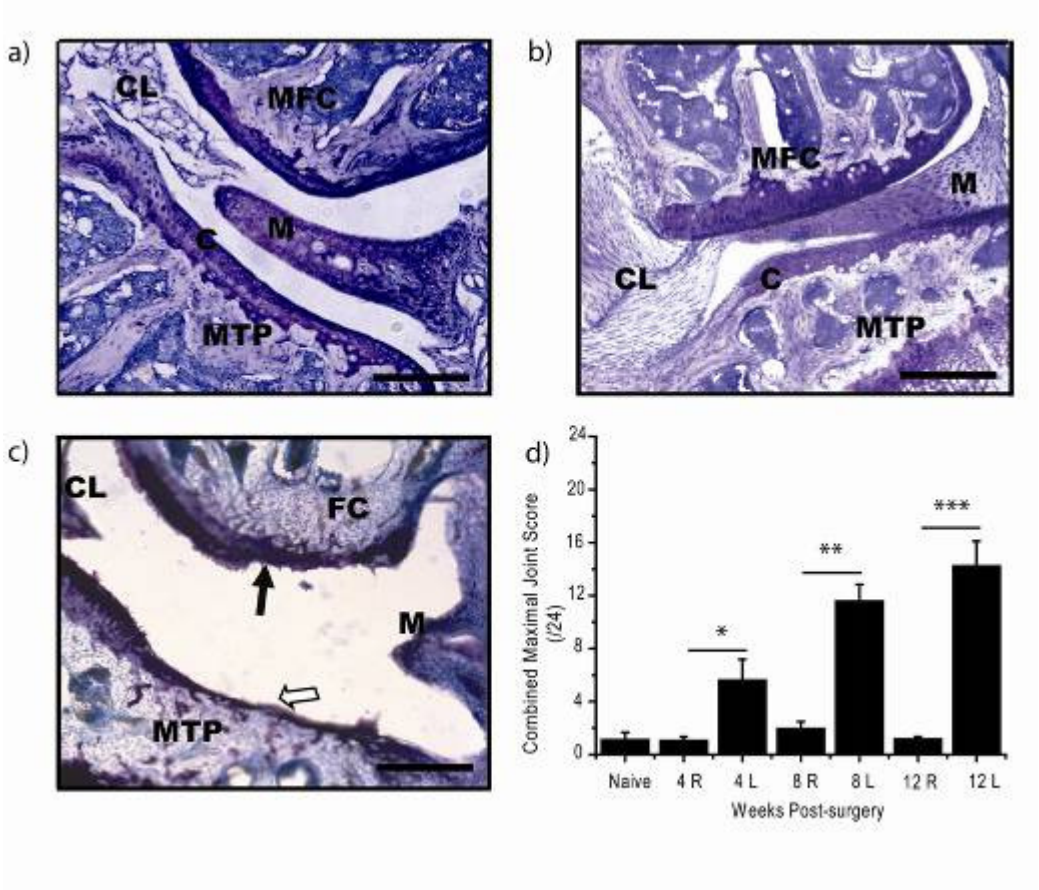


Figure 3-16 Osteoarthritic pathology following partial medial meniscectomy in female C57Bl/6 mice.

Representative 12µm thick sections (a-c) through the medial knee joint stained with toluidine blue with medial meniscus (M) to the right; cruciate ligament (CL) to the left; medial femoral condyle (MFC) at the top and tibial plateau (MTP) at the bottom. a) full thickness cartilage (C) of a surgically naïve mouse b) full thickness cartilage (C) of the contralateral knee joint 12 weeks post-meniscectomy c) severe ulceration (⇐) and focal full depth loss of cartilage (↑) of the ipsilateral knee joint 12 weeks post-meniscectomy. Scale bar: 1 mm. d) Quantitative scoring of osteoarthritic joint pathology. Each point represents the mean value ± SEM of three mice where L= operated left leg R= un-operated right leg. Statistics: L versus R by unpaired t-test, * P<0.05, ** P<0.01, *** P<0.001.

Table 3-4 Summary table displaying average joint scores for female C57Bl/6 mice following partial medial meniscectomy.

Average joint scores where L= operated left leg R= un-operated right leg. Values represent the mean value ± SEM of three mice.

	Average LTP (/6)	**Average LFC (/6)**	**Average MTP (/6)**	**Average MFC (/6)**	**Combined Average Joint Score (/24)**
Naïve	0.23 (± 0.18)	0.06 (± 0.06)	0.16 (± 0.07)	0.01 (± 0.01)	0.46 (± 0.31)
Week 4 L	0.53 (± 0.20)	0.96 (± 0.26)	0.66 (± 0.28)	0.83 (± 0.33)	2.99 (± 0.98)
Week 8 L	0.29 (± 0.67)	1.87 (± 0.74)	2.48 (± 0.12)	1.92 (± 0.36)	6.55 (± 1.26)
Week 12 L	1.00 (± 0.57)	0.43 (± 0.15)	4.56 (± 0.17)	4.78 (± 0.17)	10.77 (± 0.95)
Week 4 R	0.14 (± 0.04)	0.07 (± 0.04)	0.07 (± 0.03)	0.14 (± 0.11)	0.41 (± 0.12)
Week 8 R	0.33 (± 0.03)	0.06 (± 0.04)	0.19 (± 0.06)	0.26 (± 0.13)	0.53 (± 0.19)
Week 12 R	0.03 (± 0.02)	0.16 (± 0.08)	0.07 (± 0.04)	0.11 (± 0.06)	0.37 (± 0.19)

Table 3-5 Summary table displaying maximal joint scores for female C57Bl/6 mice following partial medial meniscectomy.

Average joint scores where L= operated left leg R= un-operated right leg. Values represent the mean value ± SEM of three mice.

	Maximal LTP (/6)	Maximal LFC (/6)	Maximal MTP (/6)	Maximal MFC (/6)	Combined Maximal Joint Score (/24)
Naïve	0.33 (± 0.17)	0.17 (± 0.17)	0.44 (± 0.15)	0.06 (± 0.06)	1.00 (± 0.51)
Week 4 L	1.00 (± 0.38)	1.89 (± 0.48)	1.22 (± 0.48)	1.50 (± 0.44)	5.61 (± 1.58)
Week 8 L	0.72 (± 0.39)	3.00 (± 1.26)	3.83 (± 0.25)	4.11 (± 0.73)	11.67 (± 2.46)
Week 12 L	2.11 (± 0.87)	1.56 (± 0.48)	4.22 (± 0.44)	5.33 (± 0.19)	13.22 (± 1.82)
Week 4 R	0.39 (± 0.11)	0.11 (± 0.06)	0.17 (± 0.17)	0.39 (± 0.39)	1.06 (± 0.40)
Week 8 R	0.17 (± 0.17)	0.17 (± 0.10)	0.56 (± 0.22)	0.61 (± 0.36)	1.50 (± 0.59)
Week 12 R	0.17 (± 0.10)	0.50 (± 0.25)	0.11 (± 0.11)	0.33 (± 0.19)	1.11 (± 0.56)

3.3.4.3 Behaviour

Pain behaviours were assessed at regular intervals to compare a group of 10 female C57Bl/6 mice who underwent sham meniscectomy surgery with 10 female C57Bl/6 mice who underwent partial medial meniscectomy surgery.

Figure 3-17a, shows the effect of partial medial meniscectomy on weight bearing. Although there was a trend for a weight bearing difference in the meniscectomy group there was, in general, no significant reduction in weight borne by the affected limb was not observed when compared to sham animals during the 84 day study. The maximum change from sham operated animals was seen at day 35 when the mean percentage weight on the ipsilateral limb dropped to 86.0% (P=0.023). No statistically significant difference in weight bearing was measured at any other time point.

Figure 3-17b, shows the effects of meniscectomy on PWT evoked by von Frey filament stimulation. Before surgery PWTs were similar in sham (0.64 ± 0.04g) and meniscectomised animals (0.56 ± 0.03g). Sham operated animals developed an initial ipsilateral mechanical allodynia that was evident at day 7 after surgery with a mean PWT of 0.38 ± 0.02g. PWT then returned to baseline levels at day 21 and no further allodynia was observed in these mice during the study. Meniscectomised mice developed a similar level of ipsilateral allodynia to sham operated animals 7 days after surgery with mean PWT reduced to 0.45 ± 0.06g. PWT remained at this level until day 56 when a second phase of hypersensitivity was noted with PWTs further reduced to 0.24 ± 0.07g ($P<0.00016$ compared to sham mice). Greater degrees of hypersensitivity were noted at 70 and 84 days with the lowest PWT (0.14 ± 0.03g) recorded at day 70. No significant changes in PWT from pre-surgery levels were observed in the contralateral limb at any time point studied.

Pre-surgery PWTs measured using the paw pressure test were similar in both sham operated (107.5 ±1.71g) and meniscectomised animals (107.0 ± 1.53g). In the sham operated group, PWT was maintained throughout the 84 day study with a lowest measured value of 95.5 ± 1.89g. In contrast, meniscectomised mice showed two phases of mechanical hyperalgesia (Figure 3-18a). During the first phase, PWT declined to a minimum at day 7 (80.5 ± 3.61g) and then returned at days 21 and 28 to the levels seen in sham operated mice. Following this, a second phase of hypersensitivity developed. In this second phase, PWT was reduced to 60-75g for the remainder of the study. A significant mechanical hyperalgesia also developed in the contralateral limb of meniscectomised mice compared to that of sham operated animals from day 56 onwards ($P<0.001$).

Behavioural cold hypersensitivity (cold allodynia) following meniscectomy was assessed using a 10ºC cold plate (Figure 3-18b). Before surgery the PWLs to the 10℃ cold stimulus were similar in both groups (sham operated, 13.59 ± 0.66s; meniscectomised animals, 12.65 ± 0.92s). PWLs were maintained at greater than 9.83s in the sham operated group throughout the 84 day study. In contrast, the meniscectomised mice showed a biphasic cold hypersensitivity profile. PWLs declined to a minimum at day 14 (9.14 ± 0.68s) followed by a return to sham operated levels at day 21 and 28. PWLs then declined for a second time and remained at low levels for the remainder of the study. Meniscectomised mice developed a significant contralateral cold allodynia compared to sham animals from day 56 onwards ($P<0.01$).

Direct knee pain following meniscectomy was assessed by counting the number of vocalisations in response to 10 compressions of each knee when held in a flexed position (Figure 3-19a). A low number of vocalisations in response to 10 compressions of each knee was noted prior to surgery (sham operated, 0.5 ± 0.27 and meniscectomised mice, 0.1 ± 0.1). 7 days after surgery, compression of the ipsilateral knee evoked 2.2 ± 0.48 vocalisations in sham operated mice but this returned to baseline levels by day 14 and stayed at this level for the remainder of the study. No significant increase in the number of vocalizations was observed for the contralateral hind limb.

Meniscectomised mice showed two phases of hypersensitivity to knee compression. During the first phase vocalisations increased to a maximum at day 7 (4.1 ± 0.81) followed by a return to baseline at day 21 and 28. By day 35 the number of vocalisations had increased to 3.1 ± 0.6 and remained at significantly higher levels compared to sham operated mice for the remainder of the study.

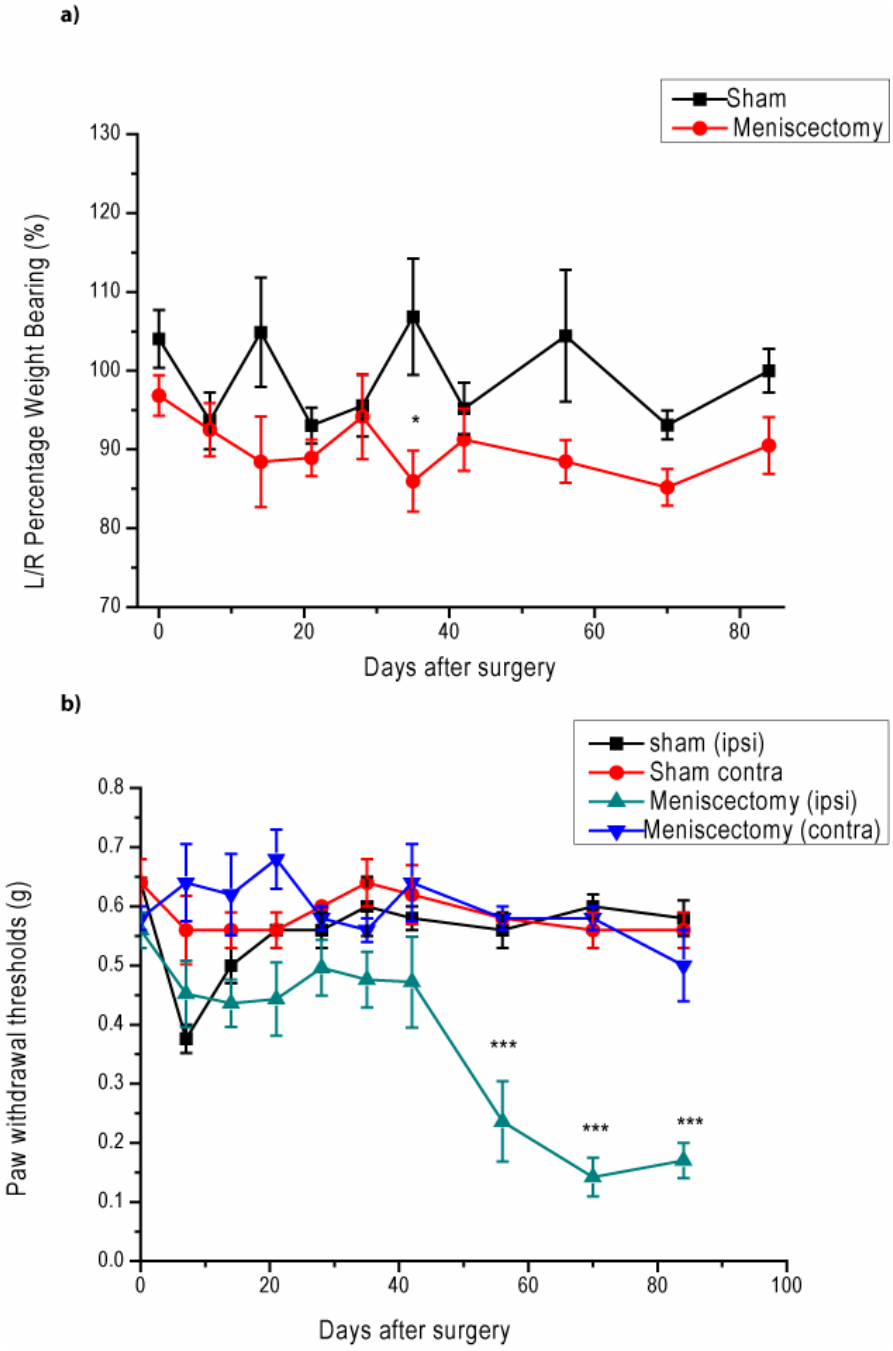


Figure 3-17 The effect of OA induction on pain behaviours following partial medial meniscectomy in female C57Bl/6 mice.

The effect on weight bearing a) and mechanical allodynia b). Each point represents the mean value ± SEM of ten mice. Statistics: Meniscectomy versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test, * P<0.05. *** P<0.001.

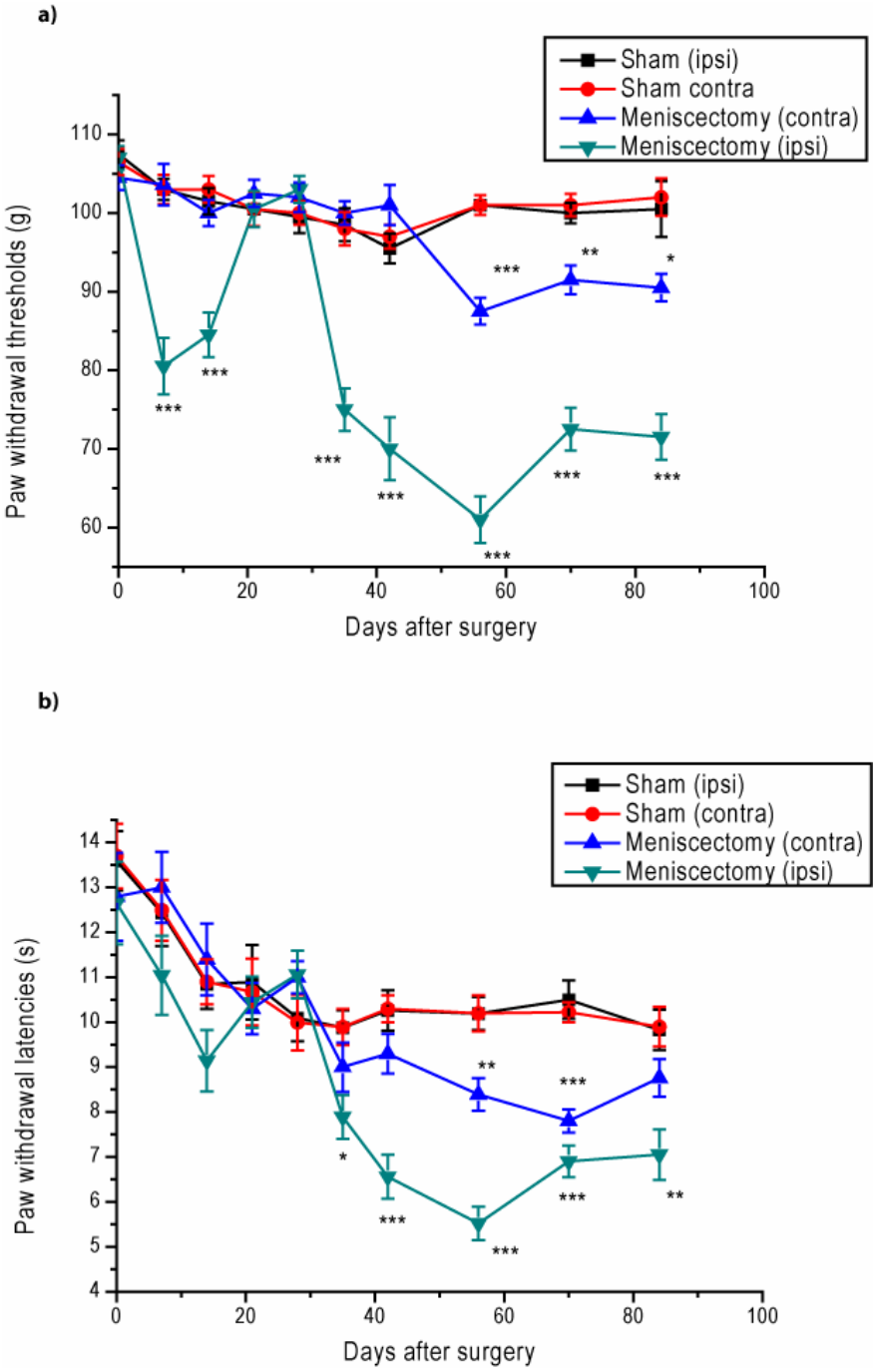


Figure 3-18 The effect of OA induction on pain behaviours following partial medial meniscectomy in C57Bl/6 mice.

The effect on mechanical hyperalgesia a) and cold allodynia b). Each point represents the mean value ± SEM of ten mice. Statistics: Meniscectomy versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test, * P<0.05. ** P<0.01. *** P<0.001

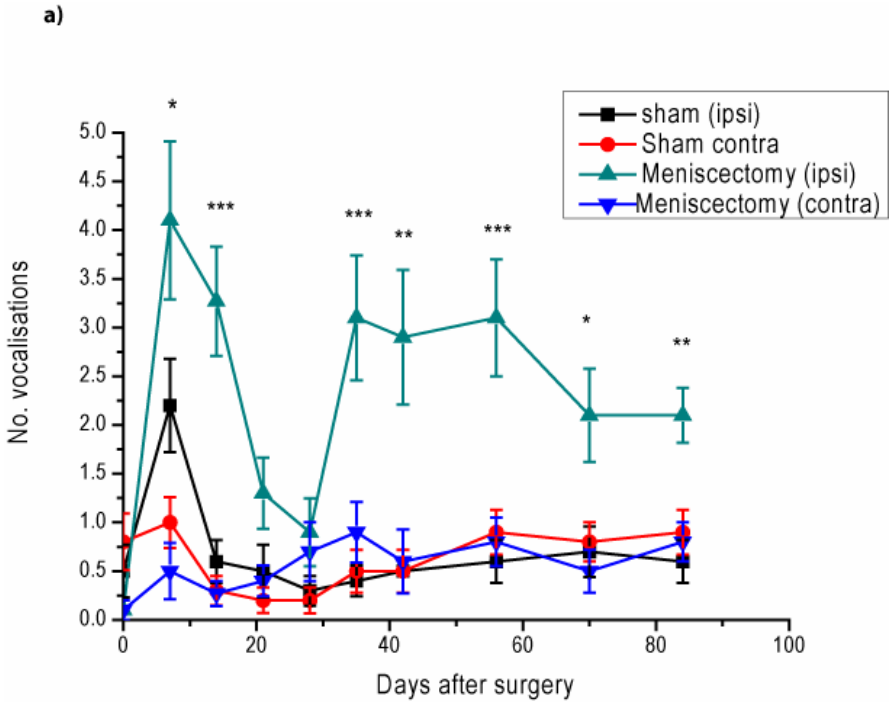


Figure 3-19 The effect of OA induction on pain behaviours following partial medial meniscectomy in female C57Bl/6 mice.

The effect on vocalisations during knee compressions a). Each point represents the mean value ± SEM of ten mice. Statistics: Meniscectomy versus sham-operated controls by repeated measures ANOVA followed by Tukey's Test, * P<0.05. ** P<0.01. *** P<0.001

3.3.4.3.1 Reduction of pain behaviour by an analgesic drug, morphine

A new cohort of mice underwent partial medial meniscectomy in order to confirm that the alterations in pain behaviours were due to the presence of joint pain.

The development of pain related behaviours followed the same time course as the previous study with animals exhibiting similar levels of mechanical hyperalgesia and cold allodynia 2 weeks following partial medial meniscectomy. Morphine (6mg/kg s.c.) or the vehicle (0.9% saline) was administered to randomly assigned mice and behavioural measurements repeated after 1 hour. Following drug administration, the mice did not exhibit any signs of visible sedation or hyperactivity. Weight bearing deficits were not present and therefore not tested in this study. Ipsilateral mechanical allodynia (P=0.0032), mechanical hyperalgesia (P=0.00016) and cold allodynia (P=0.0080) were reversed significantly by morphine compared to pre-treatment levels (Figure 3-20 and Figure 3-21) indicating an anti-nociceptive effect

of the morphine and the reversal of joint pain. However, post-treatment ipsilateral and contralateral withdrawal thresholds and latencies were increased beyond baseline thresholds following morphine administration for mechanical allodynia (P=0.029), and mechanical hyperalgesia (P=0.00024) indicating a systemic analgesic effect. Subsequently, the dose of morphine was reduced to 4mg/kg for all later pharmacological studies.

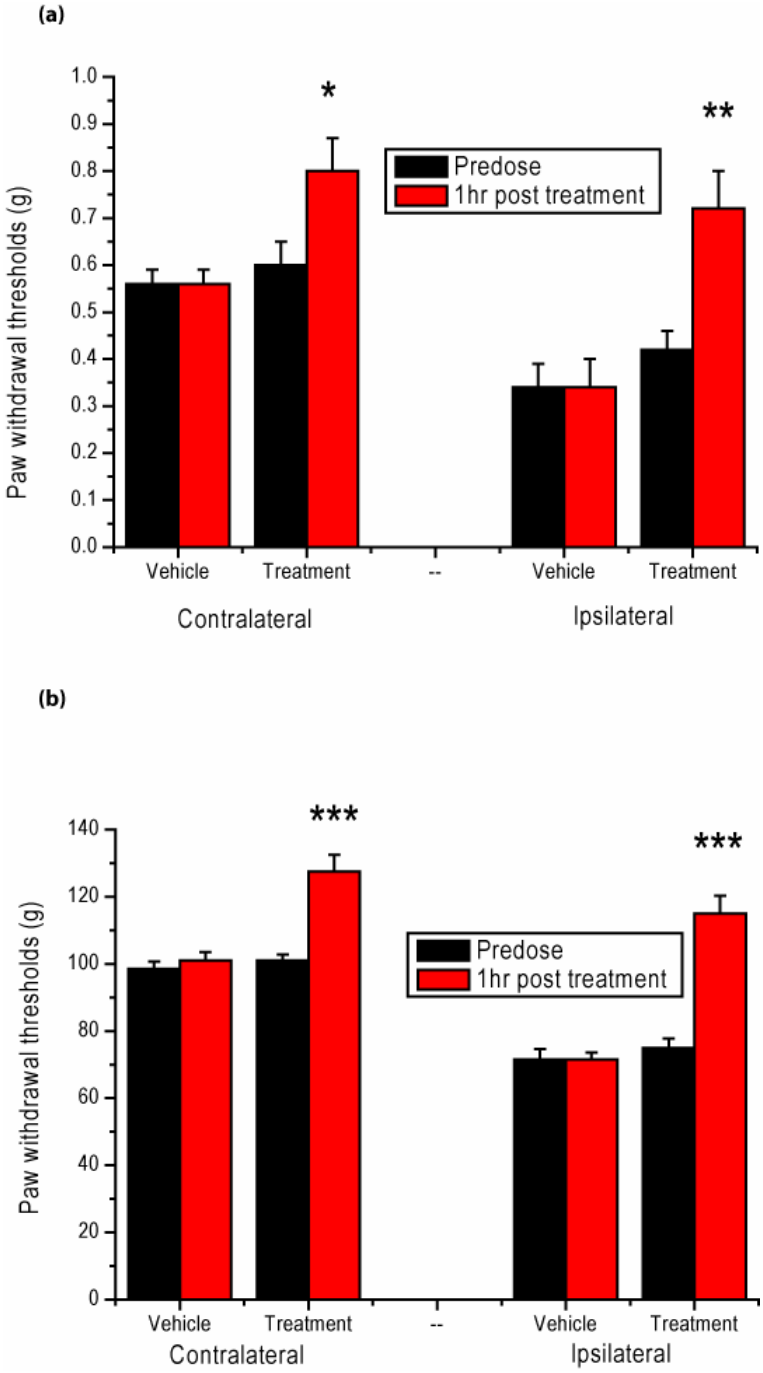


Figure 3-20 The effect of 6mg/kg s.c. morphine on pain behaviours in female C57Bl/6 mice at day 14 post partial medial meniscectomy.

The effect of morphine on mechanical allodynia a) and mechanical hyperalgesia b). Each point represents the mean value ± SEM of 10 mice. Statistics: Pre dose versus post dose by unpaired t-test, * P<0.05, ** P<0.01, *** P<0.001

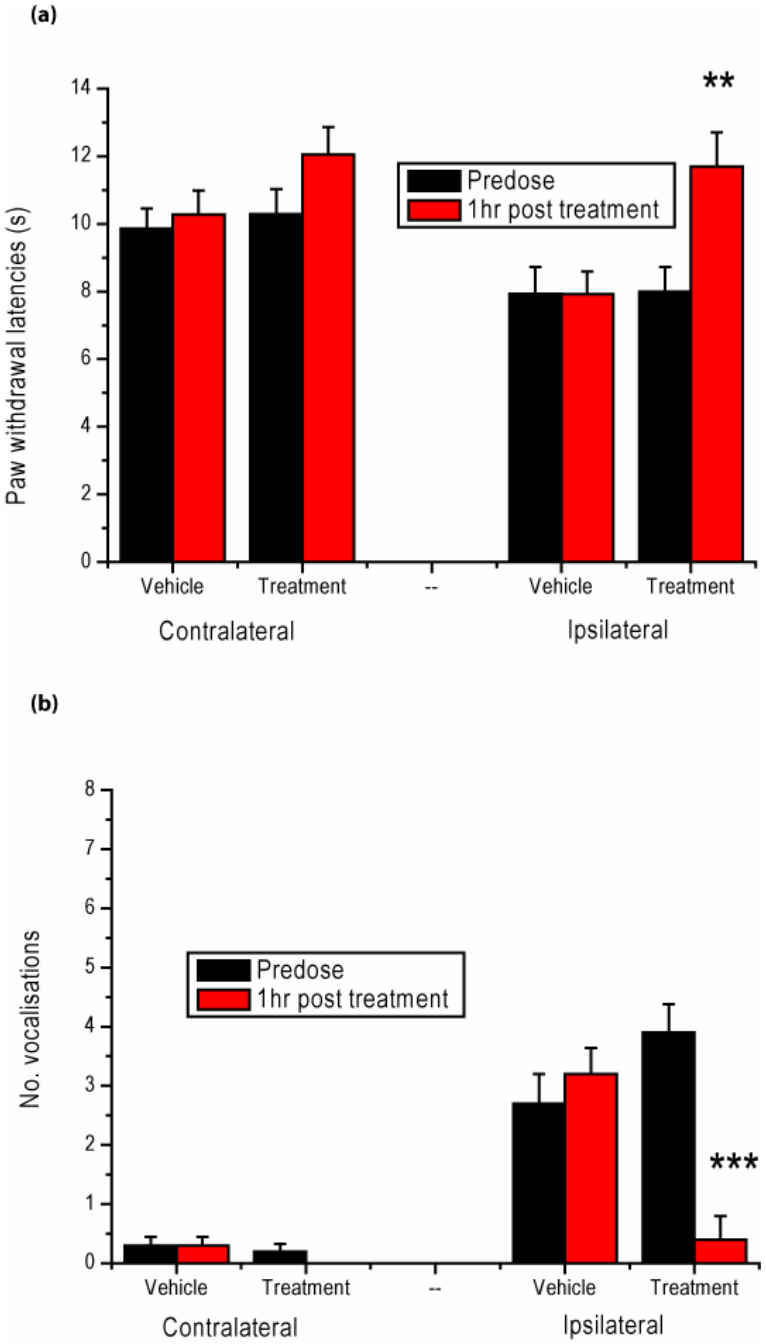


Figure 3-21 The effect of 6mg/kg s.c. morphine on pain behaviours in female C57Bl/6 mice at day 14 post partial medial meniscectomy.

The effect of morphine on cold allodynia a) and vocalisations in response to knee compression b). Each point represents the mean value ± SEM of 10 mice. Statistics: Pre dose versus post dose by unpaired t-test, ** P<0.01, *** P<0.001

3.3.4.4 Comments

Partial medial meniscectomy of female C57Bl/6 mice produces moderate-severe cartilage lesions and accompanying alterations in evoked pain behaviours that are consistent with the induction of OA and reversible with morphine administration. The onset of pain behaviours was slower than the MIA model, requiring 5-6 weeks to establish significant pain sensitivities.

3.3.5 Validation of weight bearing testing

No weight bearing differences were observed in either the 1% w/v MIA or meniscectomy OA models. To ensure the technique was adequate, 6 female C57Bl/6 mice (8 weeks of age) received an intra-articular injection of 10µl of 1% w/v CFA in mineral oil into the left femorotibial joint to create an acutely painful inflammatory response in the ipsilateral knee joint which has been shown to cause weight bearing deficits in mice and rats (Barton, McQueen et al. 2006; Staton, Wilson et al. 2007). A further 6 mice received an intra-articular injection of 10µl of mineral oil to act as control animals. Weight bearing and von Frey measurements were performed prior to injection and at day 4 and day 8 following injection. Figure 3-22 shows that animals that received an injection of CFA developed a significant weight bearing deficit at both day 4 ($P<0.0001$) and day 8 ($P<0.0001$) compared to pre-dose measurements. Significantly less weight was borne on the ipsilateral hind limb. No weight bearing deficits were recorded in control animals. A significant decrease in PWTs in response to von Frey filaments was also noted at day 4 ($P=0.0039$) and day 8 ($P=0.011$) compared to pre-dose readings. No significant difference was recorded in control animals. This experiment demonstrated that the experimental technique is able to detect weight bearing deficits in mice with inflammatory joint pain.

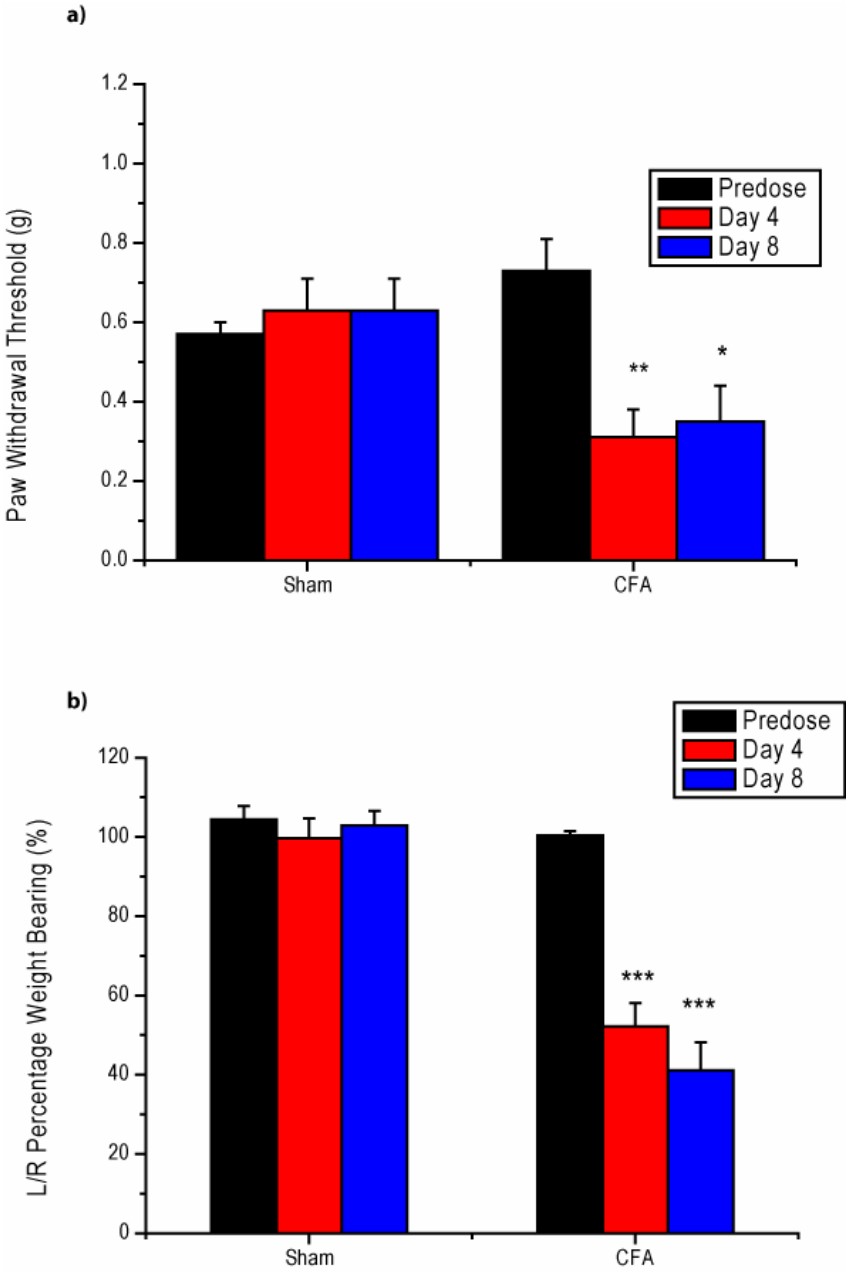


Figure 3-22 The effect of intra-articular injection of 1% w/v CFA in mineral oil on pain behaviours.

The effect on mechanical allodynia a) and weight bearing measurements b) of the hind limbs. Each point represents the mean value ± SEM of six mice. Statistics: CFA treated animals versus sham animals by ANOVA followed by Tukey's post hoc test, * $P<0.05$. ** $P<0.01$. *** $P<0.001$

3.3.6 Weight bearing following joint manipulations

At 16 weeks post surgery, 10 meniscectomised mice and 10 sham operated mice were tested to determine whether flexion and extension of the ipsilateral hind limb could exacerbate weight bearing differences between the ipsilateral and contralateral limb according to 2.5.3. The mice did not vocalise or object to moving the ipsilateral knee joint through its full range of motion. Figure 3-23a shows that no significant difference in weight bearing recordings was found following 10 passive flexion/extensions of the knee joint. The mice were also evaluated to see whether prior compression of the ipsilateral knee joint whilst held in a flexed position could exacerbate any weight bearing difference. The mice showed obvious signs of discomfort during the procedure and vocalised when the knees were compressed. When placed in the weight bearing chamber the mice were visibly stressed with a hunched posture, shaking and piloerect fur. However, despite showing visible signs of discomfort in the ipsilateral hind limb, no significant weight bearing difference was found (Figure 3-23b).

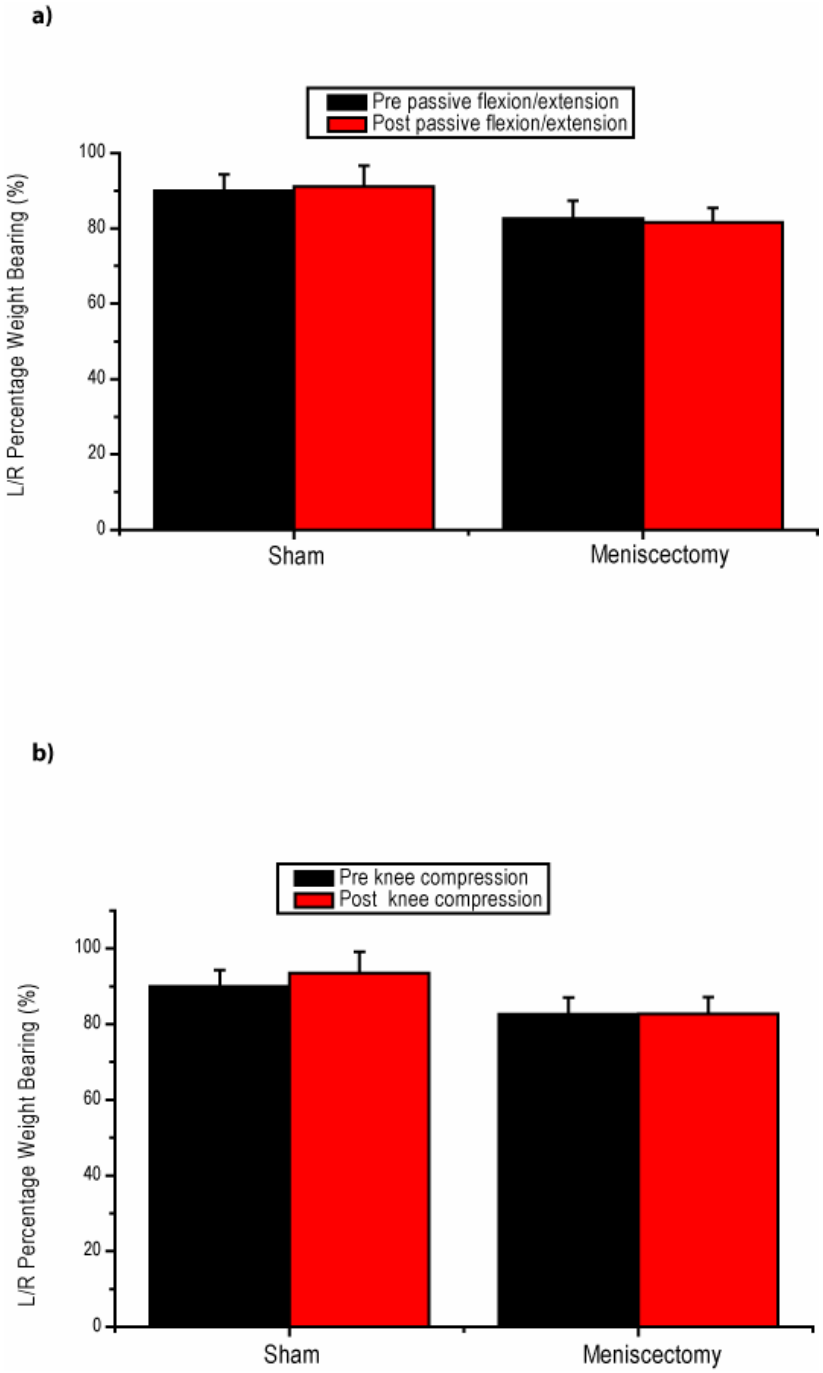


Figure 3-23 The effect of knee manipulation on weight bearing measurements.

The effect on weight bearing of the hind limbs following a) 10 passive flexion and extensions of the ipsilateral knee joint and b) 10 compressions of the ipsilateral knee when held in a fixed flexed position. Measurements recorded 16 weeks post-partial medial meniscectomy. Each point represents the mean value ± SEM of ten mice.

3.3.7 Development of spontaneous OA in STR/ort mice

3.3.7.1 General

STR/ort mice have been used as a model of spontaneously developing OA (Mason, Chambers et al. 2001) therefore they were studied to determine whether they develop accompanying OA pain behaviours. Female STR/ort mice have been reported to develop a much milder form of OA whereas 85% of 35 week old males show extensive OA-like lesions (Walton 1977). For this reason, male mice were used in this study. CBA mice are a similarly sized, docile strain of mouse that are often used as control mice for studies of STR/ort mice. CBA mice have also been reported to be resistant to the development of spontaneous OA (Gaffen, Gleave et al. 1995).

3.3.7.2 Behaviour

A time course study of pain behaviours was performed using a group of 13 male STR/ort (aged 16-19 weeks) and 10 age-matched male CBA mice over a 19 week period. Behaviour readings were performed every 2 weeks after week 1 of the study to test for the development of mechanical or cold sensitivities.

During the study, STR/ort mice remained heavier than CBA mice. Starting weights for the STR/ort and CBA mice were 34.4g ± 0.9g and 26.8g ± 0.5g, respectively. At the end of the study 19 weeks later, average weights for STR/ort and CBA mice were 41.7g ± 1.8g and 34.6g ± 0.7g, respectively.

At the beginning of the study, PWTs measured using von Frey filaments were similar in both CBA (0.88g ± 0.06g left hind paw, 0.92 ± 0.05g right hind paw) and STR/ort mice (1.00g ± 0.05g left hind paw, 0.97g ± 0.03g right hind paw) (Figure 3-24a). CBA mice maintained a mean average PWT of at least 0.88g ± 0.06g throughout the 19 week study period. STR/ort mice maintained a PWT of at least 0.69g ± 0.05g over the same time period.

Initial PWTs measured using the paw pressure test were also similar in both CBA and STR/ort mice (Figure 3-24b). Over the 19 week study period, neither group of mice developed mechanical hypersensitivity and mean PWTs remained greater than 145g ± 2.98g for CBA mice and 144g ± 2.48g for STR/ort mice.

Similar PWLs for cold sensitivity were noted in both CBA (23.61s ±1.64s left, 22.59s ± 1.76s right) and STR/ort mice (24.70s ± 0.72s left, 24.88s ± 0.72s right) at the beginning of the study (Figure 3-25a). CBA mice appeared to develop a level of cold hypersensitivity with PWLs dropping to their lowest reading 15 weeks into the

study with a PWL of 15.57s ± 1.06s left, 15.56s ± 1.21s right). In contrast, PWLs for the hind limbs of STR/ort mice remained at least 21.94s ± 1.40s throughout the study period.

The number of vocalisations in response to 10 compressions of each knee when held in a flexed position was recorded over the 19 week study period (Figure 3-25b). The CBA mice did not show any increase in vocalisations over the study period. The greatest number of vocalisations was recorded at week 6, where one mouse vocalised once and another mouse vocalized twice during the ten left knee compressions. No audible vocalisations were recorded from the STR/ort mice during the knee compression test over the 19 week study period.

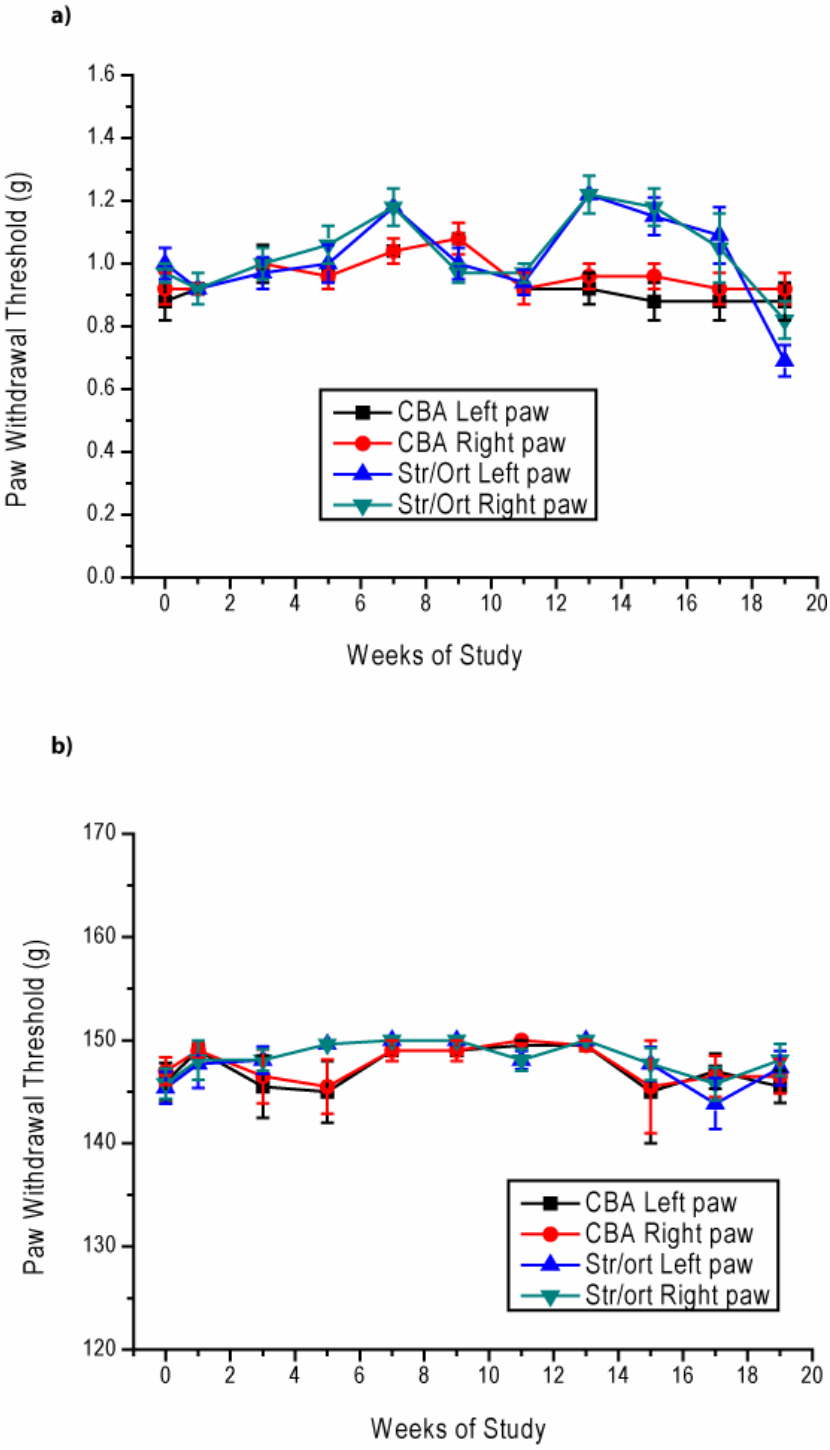


Figure 3-24 The development of pain behaviours in male CBA and male STR/ort mice. Test for the development of mechanical allodynia a) and mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten CBA mice and thirteen STR/ort mice.

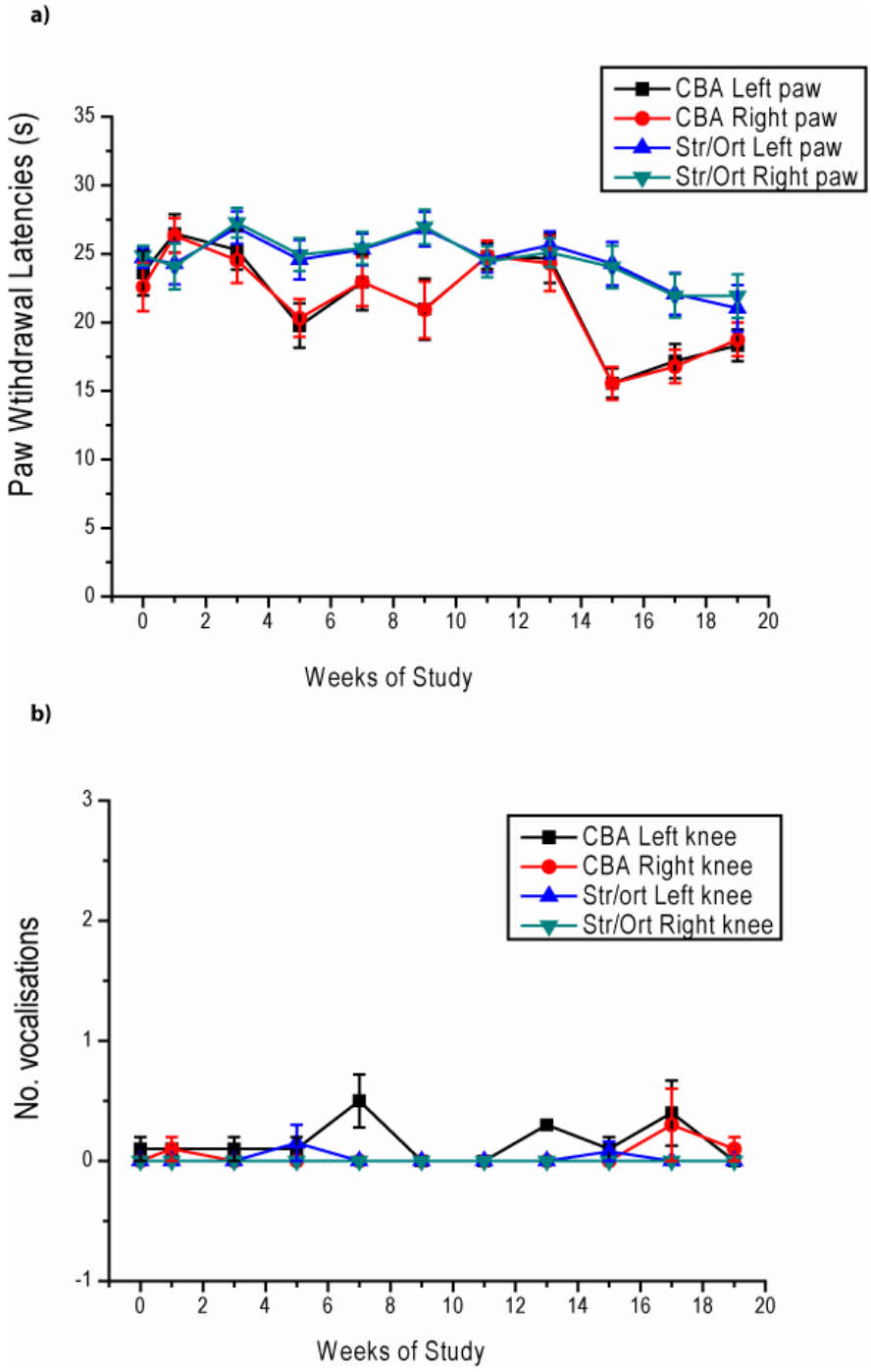


Figure 3-25 The development of pain behaviours in male CBA mice and male STR/ort mice.

Test for the development of cold allodynia a) and vocalisations during ten knee compressions b). Each point represents the mean value ± SEM of ten CBA mice and thirteen STR/ort mice.

3.3.7.3 The effect of naloxone on pain behaviours in CBA and STR/ort mice

During the behaviour time course for both STR/ort mice and CBA mice, it became apparent that pain behaviours were not changing dramatically over the initial 3 months of study. Therefore at week 13 (mice aged 29-32 weeks) 2.5mg/kg naloxone or sterile saline was administered intraperitoneally to test whether the absence of expected pain behaviours was due to the masking effect of endogenous opioids. Behavioural readings were taken before and 1 hour after administration. The results are shown in Figure 3-26, Figure 3-27, Figure 3-28 and Figure 3-29. There was no effect of naloxone in any of the behavioural tests in either STR/ort or CBA mice indicating that the absence of pain behaviours at this time point was not due to the effect of endogenous opioids.

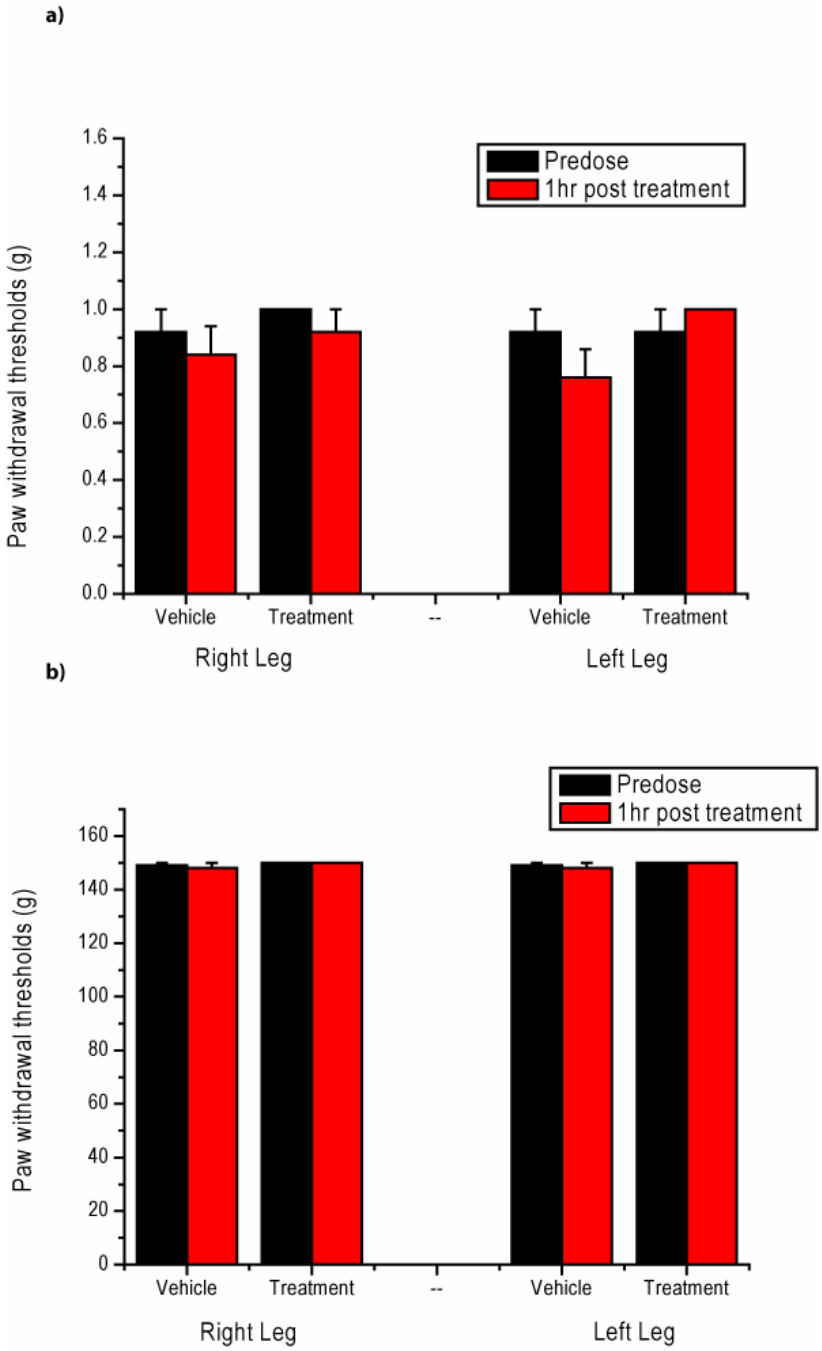


Figure 3-26 The effect of 2.5mg/kg i.p. naloxone on pain behaviours in male CBA mice at 13 weeks into the behavioural study (29-32 weeks of age).

The effect on mechanical allodynia a) and mechanical hyperalgesia b). Each point represents the mean value ± SEM of 5 mice.

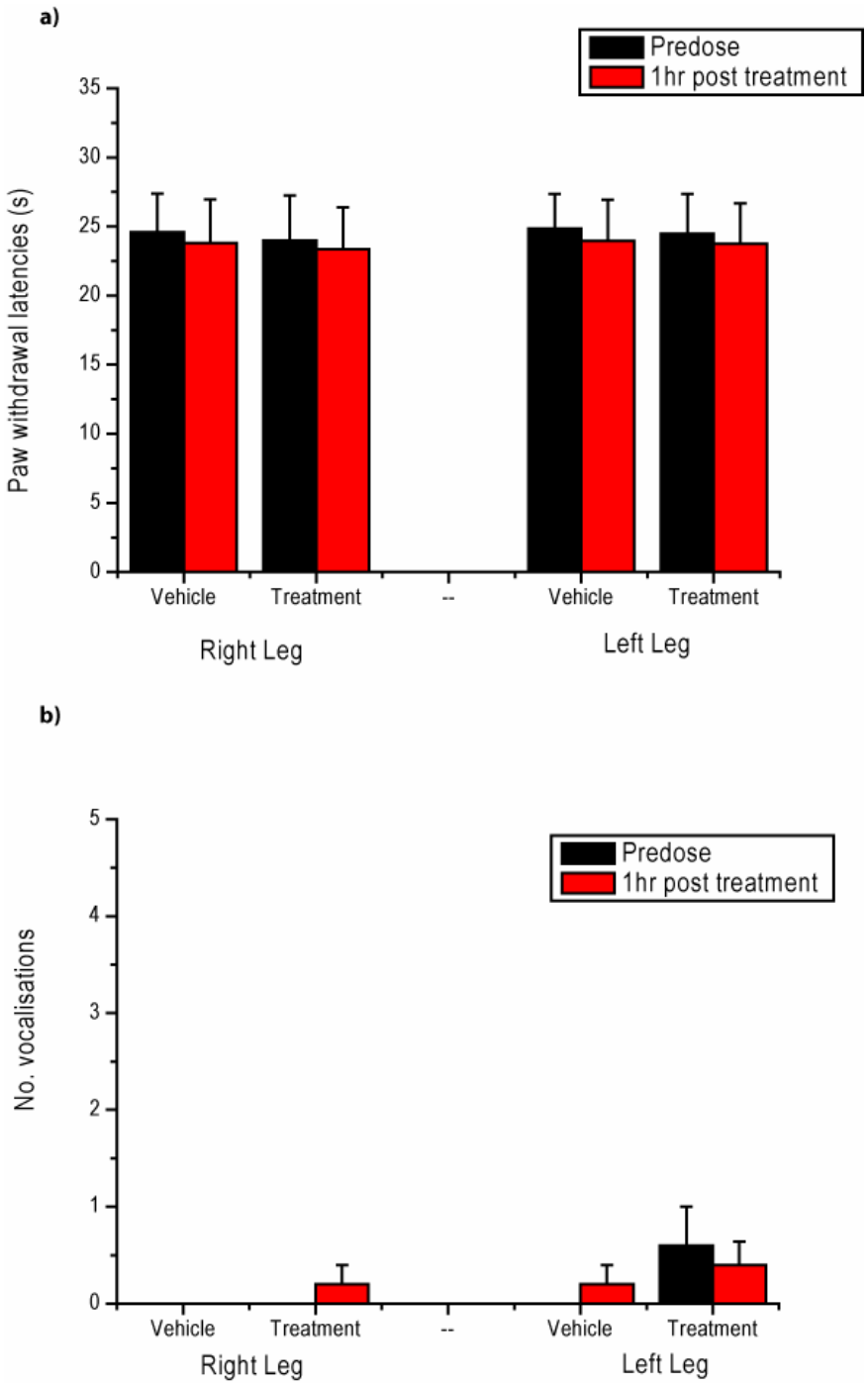


Figure 3-27 The effect of 2.5mg/kg i.p. naloxone on pain behaviours in male CBA mice at 13 weeks into the behavioural study (29-32 weeks of age).

The effect on cold allodynia a) and knee vocalisations b). Each point represents the mean value ± SEM of 5 mice.

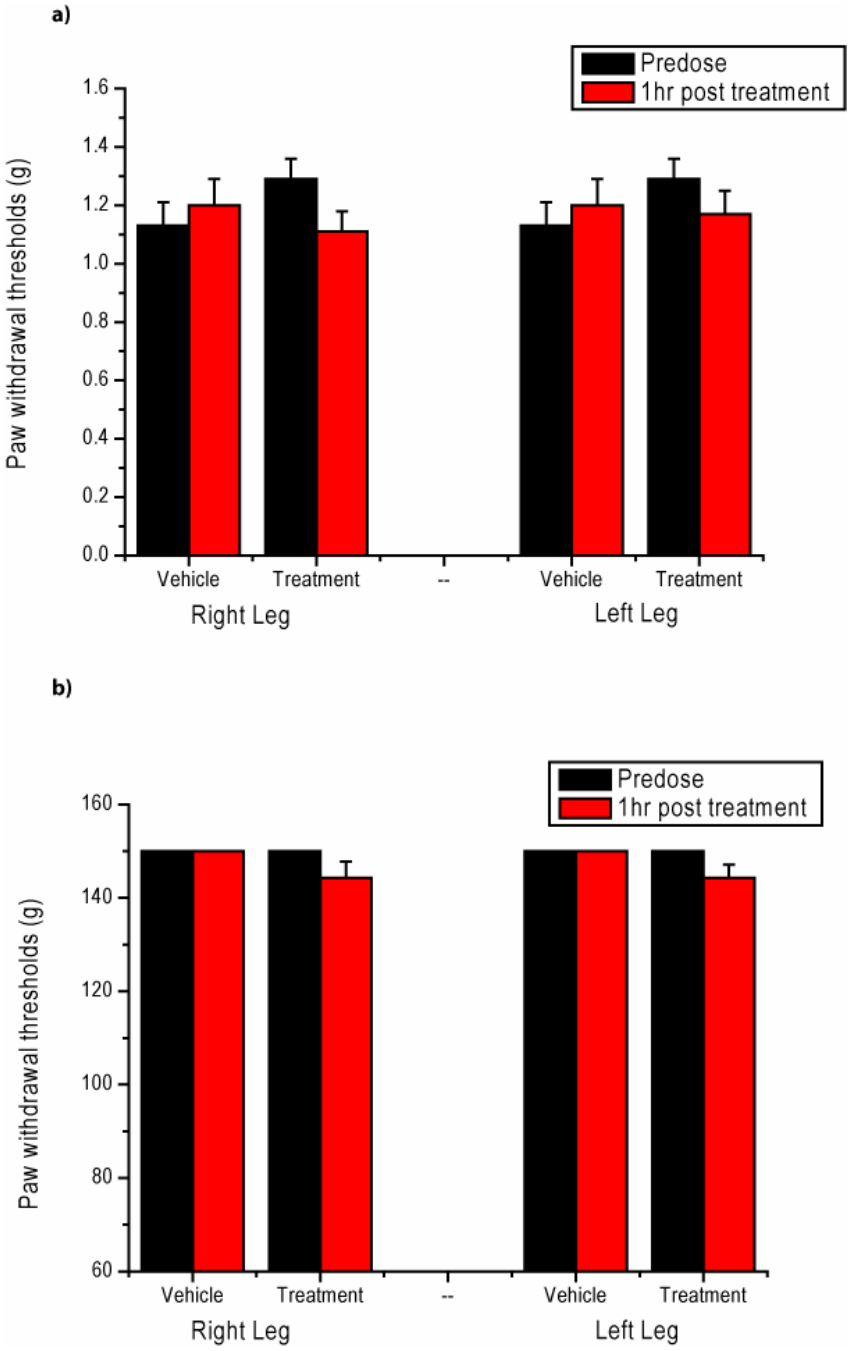


Figure 3-28 The effect of 2.5mg/kg i.p. naloxone on pain behaviours in STR/ort mice at 13 weeks into the behavioural study (29-32 weeks of age).

The effect on mechanical allodynia a) and mechanical hyperalgesia b). Each point represents the mean value ± SEM of 7 naloxone treated mice and 6 vehicle treated mice.

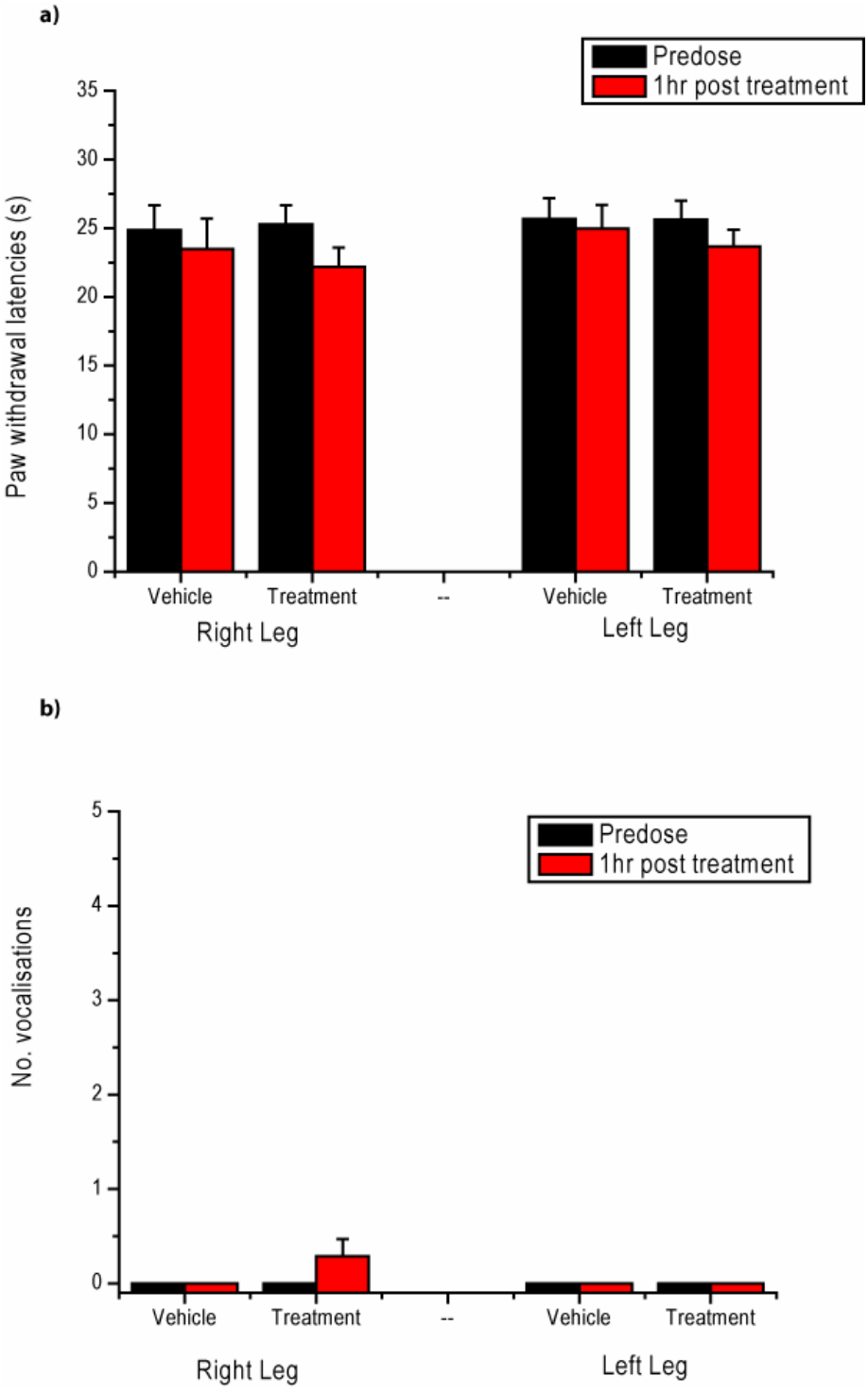


Figure 3-29 The effect of 2.5mg/kg i.p. naloxone on pain behaviours in STR/ort mice at 13 weeks into the behavioural study (29-32 weeks of age).

The effect of naloxone on mechanical allodynia a) and mechanical hyperalgesia b). Each point represents the mean value ± SEM of 7 naloxone treated mice and 6 vehicle treated mice.

3.3.7.4 Joint Pathology

Knee joints were collected from a sample of STR/ort and CBA mice (age 37-40 weeks) for confirmation of the development of OA lesions. Table 3-6 shows quantitative joint pathology scores and Figure 3-30 shows representative sections from both STR/ort mice and CBA mice at 37-40 weeks of age. CBA mice developed mild-moderate cartilage erosion. STR/ort mice developed severe and extensive cartilage erosion with large areas of full thickness damage. Both groups displayed the most severe lesions on the medial aspects of the joint surface with maximal joint scores of 2.5 ± 0.17 for the medial tibial plateau and 3.00 ± 0.33 for the medial femoral condyle of CBA mice and 5.50 ± 0.17 for the medial tibial plateau and 5.50 ± 0.17 for the medial femoral condyle of STR/ort mice. By studying the joint scores from the individual mice (Figure 3-31), it is clear that there was a large amount of variation in the degree of cartilage erosion between the mice. However, the STR/ort mice had significantly greater individual joint scores compared to CBA mice ($P<0.0001$) by unpaired t-test.

Table 3-6 Summary table displaying quantitative joint pathology of CBA and STR/ort mice at 37-40 weeks of age

Average joint scores a) maximal joint scores b) for male CBA and STR/ort mice where L= left knee R= right knee. Values represent the mean value ± SEM of three mice.

a)	Average LTP (/6)	Average LFC (/6)	Average MTP (/6)	Average MFC (/6)	Combined Average Joint Score (/24)
CBA L	0.61 (± 0.11)	0.61 (± 0.11)	0.89 (± 0.17)	0.86 (± 0.14)	2.97 (± 0.03)
CBA R	0.47 (± 0.03)	0.53 (± 0.08)	1.50 (± 0.06)	1.64 (± 0.08)	4.14 (± 0.19)
STR/ort L	2.41 (± 0.08)	2.62 (± 0.13)	3.96 (± 0.29)	3.59 (± 0.33)	12.56 (± 0.57)
STR/ort R	2.58 (± 0.08)	2.13 (± 0.16)	2.31 (± 0.19)	1.97 (± 0.08)	8.98 (± 0.19)
b)	**Maximal LTP (/6)**	**Maximal LFC (/6)**	**Maximal MTP (/6)**	**Maximal MFC (/6)**	**Combined Maximal Joint Score (/24)**
CBA L	1.67 (± 0.00)	2.33 (± 0.00)	1.67 (± 0.00)	2.17 (± 0.17)	7.83 (± 0.17)
CBA R	1.00 (± 0.00)	1.33 (± 0.00)	2.50 (± 0.17)	3.00 (± 0.33)	7.83 (± 0.17)
STR/ort L	4.17 (± 0.17)	4.67 (± 0.00)	5.50 (± 0.17)	5.50 (± 0.17)	19.83 (± 0.17)
STR/ort R	3.67 (± 0.33)	3.00 (± 0.00)	3.30 (± 0.08)	3.83 (± 0.17)	13.80 (± 0.58)

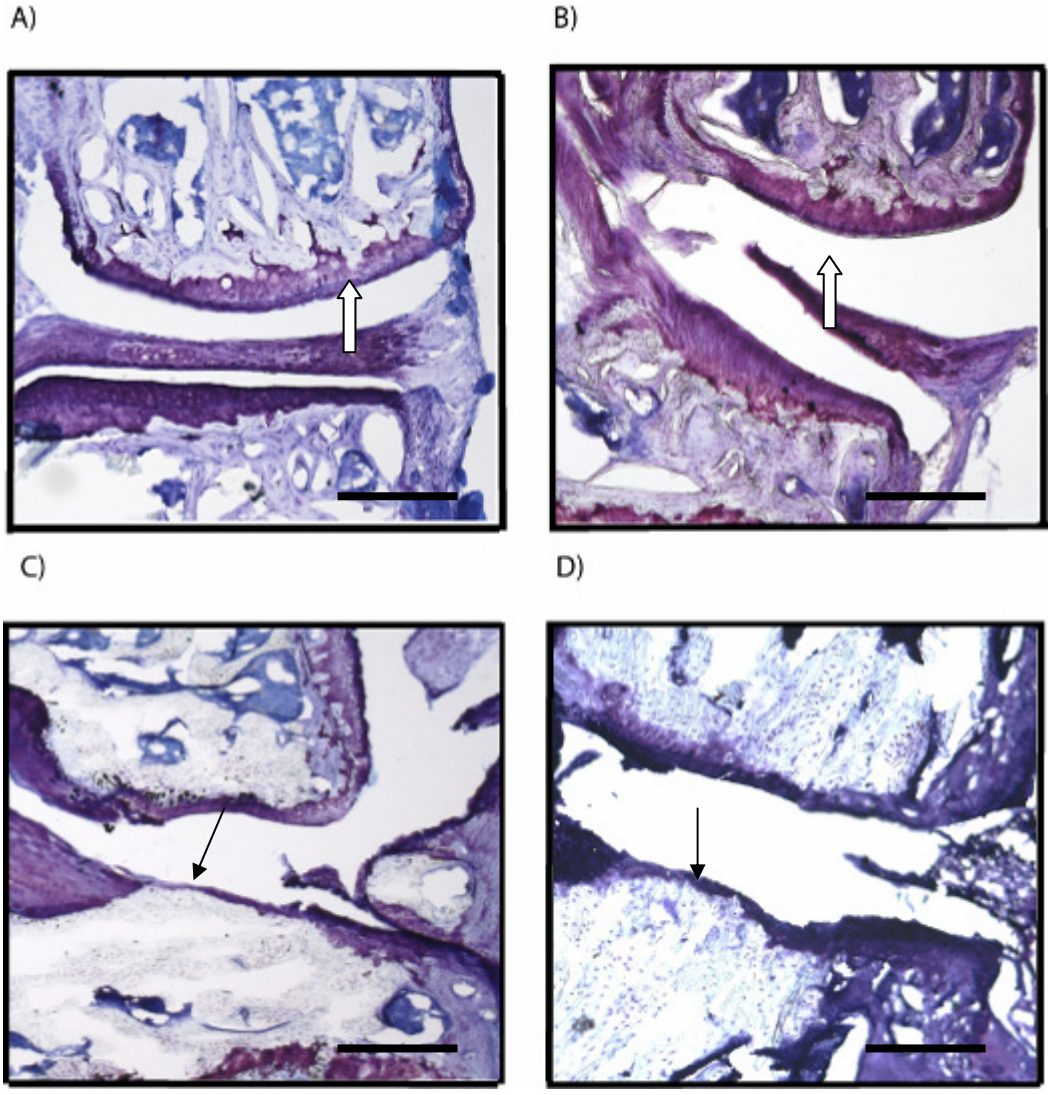


Figure 3-30 Representative 12µm thick section through the medial knee joint stained with toluidine blue showing spontaneous osteoarthritic pathology In the knee joints of 37-40 week old mice.

CBA L A), CBA R B), STR/ort L C) STR/ort R D) where L = left knee and R = R knee, (⇐) indicates mild fibrillation of the cartilage of the tibial plateau; (↑) points to full depth loss of cartilage of the femoral condyle. Scale bar: 1 mm.

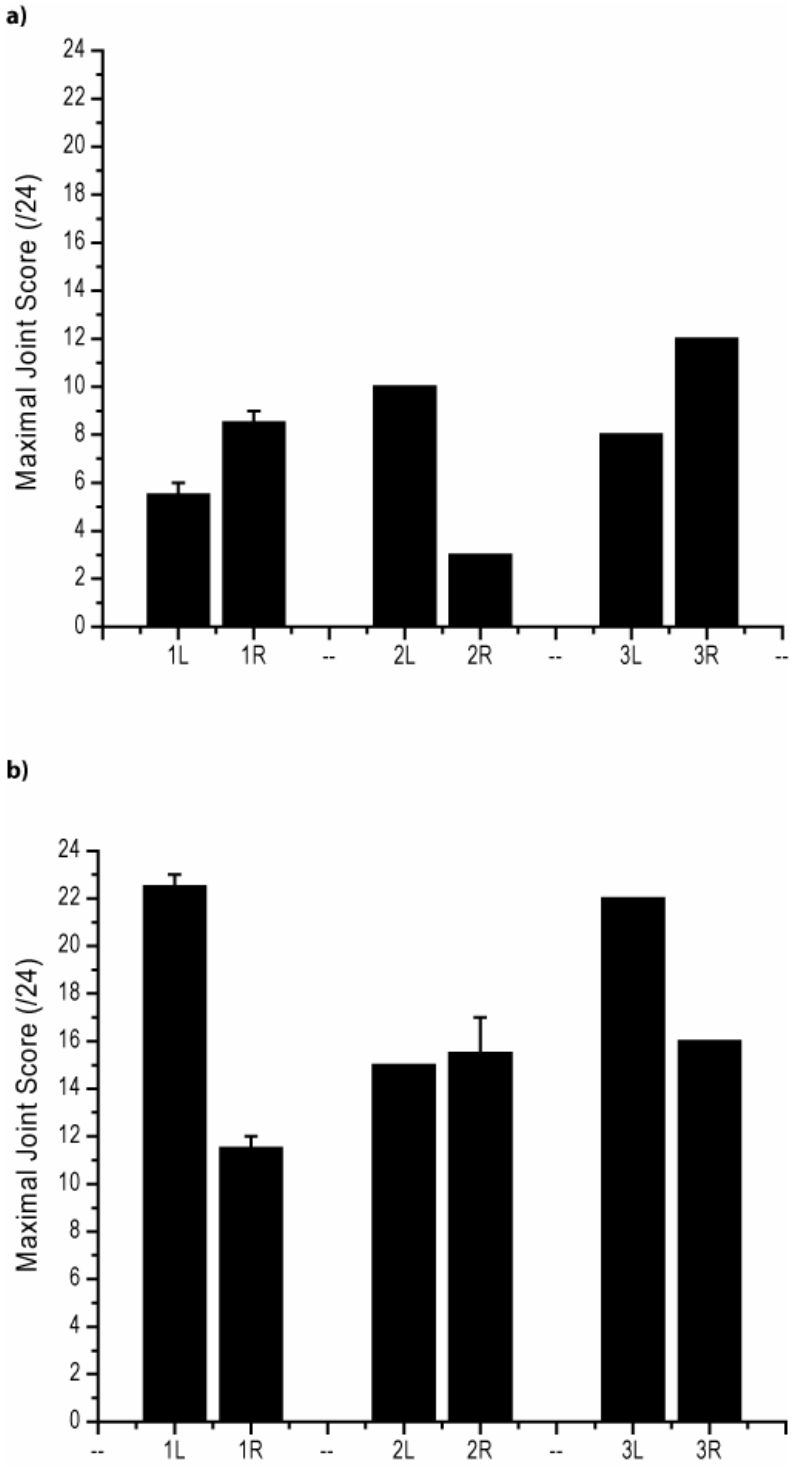


Figure 3-31 Individual Maximal Joint scores of three CBA mice a) three STR/ort mice b) at 37-40 weeks of age. Scores represent the maximal joint score of each mouse ± SEM

3.3.7.5 Validation of behavioural tests in STR/ort mice using CFA

The effect of CFA induced inflammation within the knee was examined to determine if the absence of pain behaviours in STR/ort with moderate-severe OA was due to pain insensitivity. To ensure that the behavioural tests could reveal pain when a significant painful stimulus was present, a small experiment was performed. Three 40-43 week old male STR/ort mice received an intra-articular 10µl injection of 1% w/v CFA in mineral oil under general anaesthesia. The contralateral leg was used as a control for comparison. Weight bearing, von Frey, paw pressure, cold allodynia and knee compression measurements were performed prior to injection and one and three days following injection.

Figure 3-32a) shows that animals who received an injection of CFA had decreased weight bearing on the ipsilateral hind limb at 24 hours post CFA injection with L/R percentage weight of 61.9% ± 10.1% compared to 92.4% ± 2.21 % prior to CFA injection (P=0.042, unpaired t-test). At 72hours post CFA injection, a more equal weight distribution was recorded on the hind limbs with L/R percentage of 84.9% ± 6.20. PWTs in response to von Frey filaments were significantly lower in the ipsilateral hind paw at 24hours (P=0.0495) but not at 72hours (post CFA injection (p=0.081) (Figure 3-32b). PWTs in the paw pressure test and PWL in the cold plate test were also significantly decreased in the ipsilateral hind paw compared to the contralateral hind paw at 72 hours post injection (PWT, P=0.00096; PWL, P=0.026, Figure 3-33a and Figure 3-33b), and were lower at 24 hours although the decreases were not statistically significant. Furthermore the number of vocalisations in response to 10 knee compressions were not significantly increased following CFA injection (Figure 3-34a) despite the animals flinching dramatically during the test. This measure is therefore probably not a good indicator of pain in male STR/ort mice.

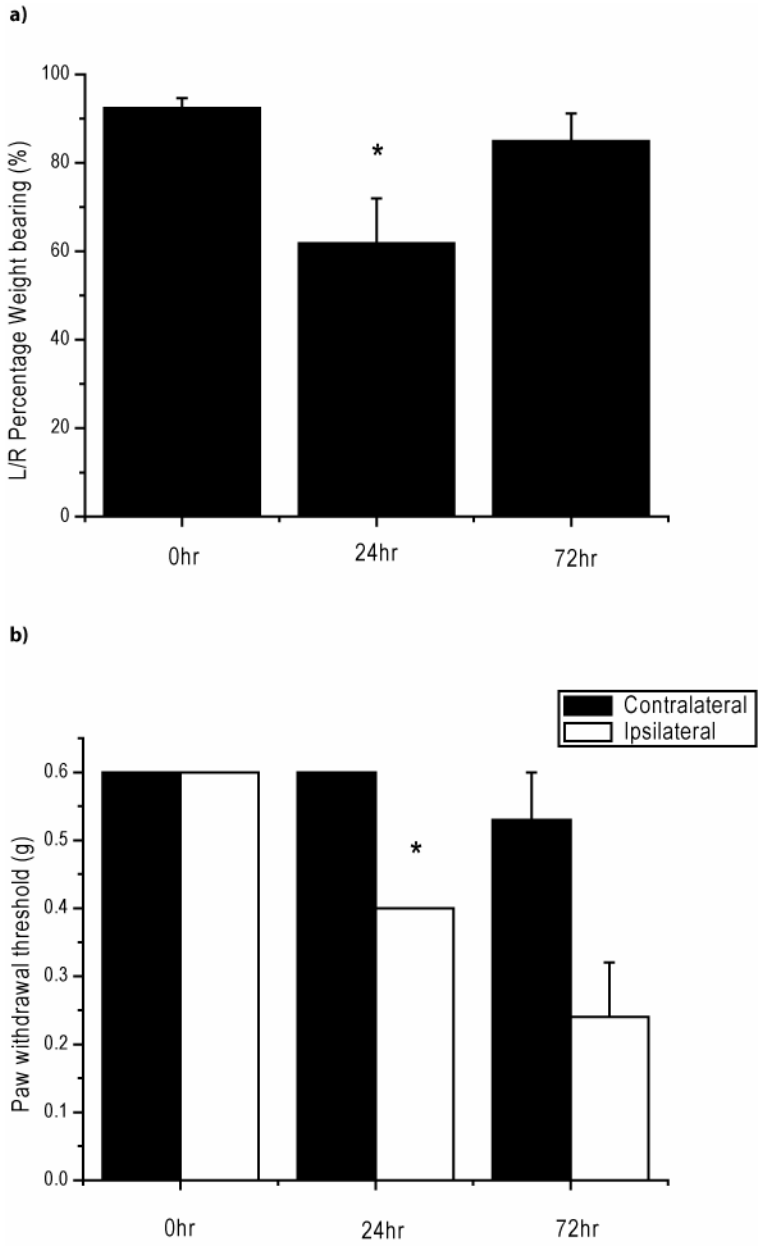


Figure 3-32 The effect of intra-articular injection of 1% w/v CFA in mineral oil into the knee joint on pain behaviours.

The effect on weight bearing a) mechanical allodynia b) of the hind limbs in 40-43 week old male STR/ort mice (n=3). Statistics: Pre dose versus post dose by unpaired t-test (weight bearing). Ipsilateral hind limb versus contralateral hind limb by Mann Whitney U test (mechanical allodynia). * $P<0.05$.

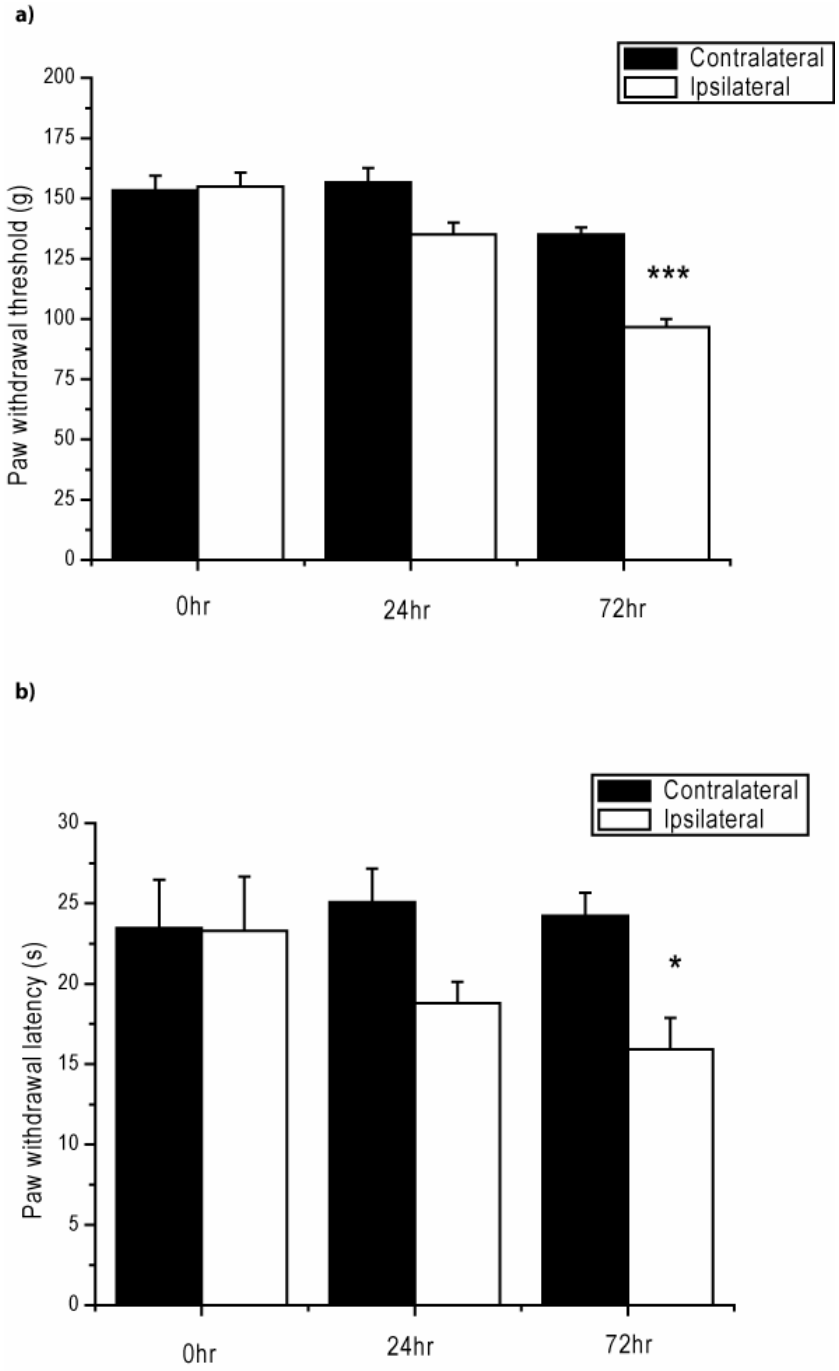


Figure 3-33 The effect of intra-articular injection of 1% w/v CFA in mineral oil into the knee joint on pain behaviours.

The effect on mechanical hyperalgesia a) cold allodynia b) of the hind limbs in 40-43 week old male STR/ort mice (n=3). Statistics: Ipsilateral hind limb versus contralateral hind limb by unpaired t-test. * $P<0.05$. *** $P<0.001$

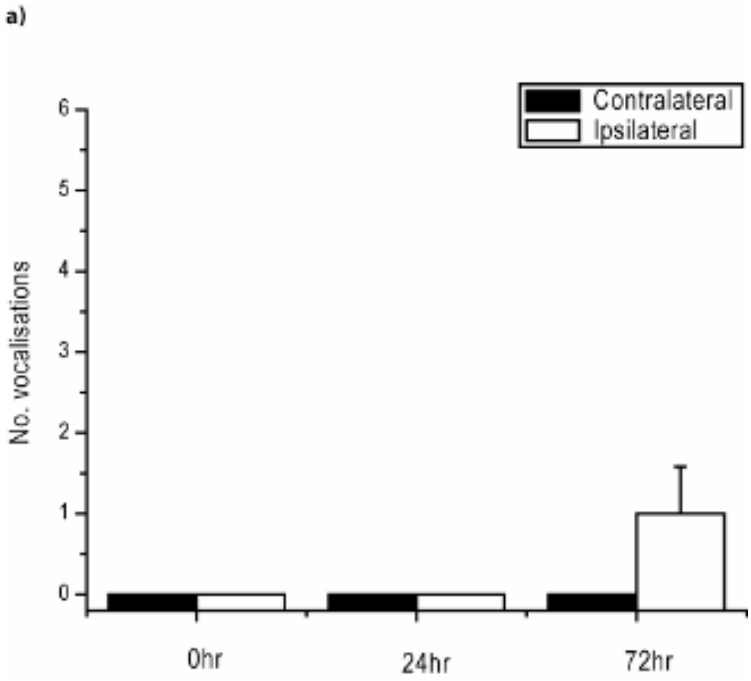


Figure 3-34 The effect of intra-articular injection of 1% w/v CFA in mineral oil into the knee joint on pain behaviours.

The effect on knee vocalisations a) in 40-43 week old male STR/ort mice (n=3).

3.3.7.6 Comments

All of the STR/ort and CBA mice appeared to maintain a good level of health. Individual STR/ort mice were noted to develop transient episodes of paw swelling which was not associated with an observable lameness, flinching or measurable pain in response to manipulation or squeezing of the hind paw or ankle (see 3.4.4, for further discussion).

3.4 Discussion

3.4.1 Joint pathology

Many changes occur in the articular cartilage as a result of the OA process in humans. Histological features in early OA include focal or generalized cartilage matrix swelling (oedema), proliferation of chondrocytes in the superficial zone, chondrocyte hypertrophy and chondrocyte death (Pritzker, Gay et al. 2006). Superficial fibrillation of cartilage characterized by microscopic cracks into the superficial zone develops and as the OA becomes more severe, increasingly deeper cartilage becomes involved. Ultimately, the cartilage becomes eroded completely and the subjacent bone becomes the articular surface (Collins 1953; Milgram 1983; Benito, Veale et al. 2005; Pritzker, Gay et al. 2006). Osteophyte formation at joint margins, proliferation of synovial cells and sclerosis of subchondral bone below the degenerated cartilage are also common features. All of these characteristics have been identified in animal models (Walton 1977; Brandt and Fife 1986; Brandt and Slowman-Kovacs 1986; Bendele, White et al. 1989; Kamekura, Hoshi et al. 2005).

In the current studies, a time dependent increase in cartilage damage was observed following intra-articular injection of MIA into the knee joint of C57Bl/6 mice, similar to previous reports (van der Kraan, Vitters et al. 1989; Hadipour-Jahromy and Mozaffari-Kermani 2010). Mild cartilage fibrillation was noted at week 4, but significant cartilage damage of the ipsilateral knee joint was not observed until week 8 post-treatment and was more severe at week 12. Whilst MIA has been shown to have a rapid and direct effect on chondrocyte metabolism resulting in the inhibition of proteoglycans synthesis (Dunham, Chambers et al. 1988; van Osch, van der Kraan et al. 1994), only mild cartilage lesions such as loss of proteglycans and fibrillation of the cartilage surface of the tibial plateaus have been reported previously in mice within the first 3 weeks post-treatment (van der Kraan, Vitters et al. 1989; Hadipour-Jahromy and Mozaffari-Kermani 2010). Cartilage lesions of greater severity have been recorded at later time points however. For example, ulceration of the cartilage from day 28 (Hadipour-Jahromy and Mozaffari-Kermani 2010), fissures of the cartilage from day 35 (van Osch, van der Kraan et al. 1994) and erosion of the non-calcified cartilage from day 42 onwards (van der Kraan, Vitters et al. 1989). These features were observed at week 8 post MIA treatment in the studies reported here, and are consistent with degenerative cartilage lesions observed in human OA (Janusz, Hookfin et al. 2001; Guingamp, Gegout-Pottie et al. 1997). More rapid and extensive cartilage damage has been reported following examination of the femoral-patellar joint compared to the femoral-tibial joint with

osteophytosis at day 7 and extensive loss of cartilage from the femoral-patellar joint by day 21 (van der Kraan, Vitters et al. 1989; van Osch, van der Kraan et al. 1994). It would be useful to study the femoral-patellar joint in our model of MIA induced OA in conjunction with behavioural hypersensitivity readings since histopatholgical lesions of the femoral-patellar joint may correlate better with the early onset of joint pain.

Following partial medial meniscectomy in C57Bl/6 mice, mild degenerative changes were noted 4 weeks after surgery and marked progressive structural modifications seen at 8 and 12 weeks. These findings were similar to the surgical model described by Welch et al. (2009), where partial medial meniscectomy in conjunction with medial collateral ligament transection in male C57Bl/6 mice also produced a time dependent increase in joint histopathology. Focal, superficial to deep fibrillation of the cartilage was described in the early stages (2-6 weeks post-surgery). In the later phases (6 weeks onwards), more diffuse lesions were described including vertical fissures through the transition zone and occasional delamination of the superficial layer. OA lesions were significant compared to control animals by week 6 (Welch, Cowan et al. 2009), in contrast to week 4 in the studies described in this thesis. In other surgical models of OA in mice, such as the DMM model, a slowly progressive cartilage degeneration was also reported with significant increases in joint pathology reported at week 2 (Inglis, McNamee et al. 2008) or week 4 (Glasson, Blanchet et al. 2007). In more severe models of OA induction where greater joint instability is induced, a much more rapid and severe progression of OA is reported compared to the partial medial meniscectomy model. For example, following anterior cruciate ligament transection, severe and rapid cartilage erosion including involvement of the subchondral bone was reported in 129S6/SvEv mice (Glasson, Blanchet et al. 2007) and C57Bl/6 mice (Kamekura, Hoshi et al. 2005). An aggressive time course for the development of OA lesions may be inappropriate for a model of OA which is generally slow in onset in humans. Partial medial meniscectomy therefore produces an appropriate model for the study of OA pain and associated joint pathology since cartilage degradation mirrors the more slowly-progressive human OA.

In male STR/ort mice, the presence of moderate-severe spontaneous osteoarthritic cartilage lesions were confirmed at 37-40 weeks of age. These findings were consistent with previous studies that reported the onset of mild OA lesions from 16 weeks of age with approximately 85% of male STR/ort mice showing histological evidence cartilage degeneration by 35 weeks of age (Walton 1977).

The rapid decalcification process used to generate the histological samples in the studies reported in this chapter meant that sclerosis of the subchondral bone and presence of osteophytes could not be assessed accurately. However, the development of progressive cartilage erosions were observed in both the MIA and partial medial meniscectomy models of OA and severe spontaneous cartilage lesions were seen in STR/ort mice that were consistent with other published studies of animal models and human cases of OA.

In this study, a simple method of scoring gross cartilage lesions was employed. More subtle changes at the cellular level were not examined. By limiting the investigation of joint pathology to easily definable lesions, a greater confidence in scoring results could be obtained. The intra-class correlation coefficient was calculated to be 0.90 showing an excellent level of agreement where multiple observers were used for histopathology studies. Reproducibility of results was also excellent with the interclass correlation coefficients calculated to be 0.94 or greater for each study when scoring was repeated twice with a 2 week interval. Histological analysis in this study was performed primarily to confirm the presence of cartilage lesions (spontaneous or surgically induced) characteristic of knee joints with OA. More in depth studies with histology performed by trained pathologists could be used to investigate more subtle changes in the cartilage, synovium and subchondral bone which may highlight similarities and differences between the models and for comparison to human cases of OA. However, this is particularly important for studies in which disease modifying drugs will be employed rather than OA pain which is the main focus of this thesis.

3.4.2 Pain measures

This study used a comprehensive range of behavioural tests to document OA pain behaviours. A summary table of the findings are presented in Table 3-7. These studies incorporated measures of direct knee pain (vocalisations in response to knee compression), weight bearing and measures of referred hyperalgesia (paw pressure test) and allodynia (von Frey and cold sensitivity). In the studies of female C57Bl/6 mice, the behavioural tests produced robust and sensitive measures allowing the recording of hypersensitivities in both the ipsilateral and contralateral hind limbs. Pharmacological investigation to further validate and characterize the pain behaviours was performed as described in chapter 4.

3.4.2.1 Weight bearing

Currently, there are no published reports on weight bearing measurements of the hind limbs following intra-articular injection of MIA in mice. In the studies described

here, significant weight bearing deficits were not observed following treatment with 1% MIA but were present following injection of 10% MIA. This finding may be attributable to the greater cartilage degradation in this model and therefore greater joint instability and pain, or as a result of a direct effect of MIA on joint sensory neurons. Partial medial meniscectomy also does not appear to produce significant weight bearing differences between the operated and un-operated limbs of C57Bl/6 mice studied up to 12 weeks post-surgery. This finding is consistent with partial medial meniscectomy in the rat which also does not produce a significant weight bearing deficit in the ipsilateral limb (Fernihough, Gentry et al. 2004). Destabilisation of the medial meniscus however, has been reported to cause a significant shift in body weight towards the unaffected hind limb of mice from 12 weeks onwards (Inglis, McNamee et al. 2008; McNamee, Burleigh et al. 2010). This difference may be attributable to greater pain caused by ongoing instability of the medial meniscus in the DMM model. Weight bearing deficits were not studied in the STR/ort mice since they develop spontaneous OA bilaterally.

3.4.2.2 Von frey filament testing

Von Frey filaments have been used clinically to assess mechanical allodynia in patients with chronic arthropathies such as OA and have shown a significant decrease in cutaneous pain thresholds (Hendiani, Westlund et al. 2003). Decreased PWTs in response to von Frey hairs were observed in the studies described here following intra-articular injection of MIA and partial medial meniscectomy of C57Bl/6 mice. In an iodoacetate induced murine model of OA, Harvey et al. (2009) also reported the development of mechanical allodynia in the ipsilateral hind paw of male and female MIA-injected C57Bl/6 mice at all observed time points for 28 days following intra-articular injection MIA (Harvey and Dickenson 2009). Similar to the MIA model described here, the pattern of hypersensitivity reported by Harvey et al. also suggested a slight biphasic profile with an initial phase of reduced withdrawal thresholds that declined to a minimum at day 4 followed by a slight increase at day 11. The first phase of hypersensitivity may be due to the onset of inflammation and the second phase a result of the induction of degenerative joint disease. Useful further studies would include testing the response of early and late pain behaviours to anti-inflammatory drugs such as diclofenac to examine the contribution of inflammation to joint pain during these two time periods.

Mechanical allodynia has also been reported in a recent article describing OA pain induced by DMM in C57Bl/6 mice (Malfait, Ritchie et al. 2010). Malfait et al describe significant levels of ipsilateral mechanical allodynia in male C57Bl/6 mice compared

to baseline thresholds from 2 weeks post surgery that persisted for the remainder of the 8 week study. In the study of female C57Bl/6 mice described here, persistent allodynia was not observed until 8 weeks post partial medial meniscectomy surgery. This disparity might be a reflection of the type of meniscal surgery or sex of the mice giving rise to a different pattern of hypersensitivity. Malfait et al also reported the development of both ipsilateral and contralateral mechanical allodynia in CD-1 mice following DMM. Contralateral hypersensitivity was not observed in the same study of male C57Bl/6 mice or in our study of female C57Bl/6 mice, which might also be a reflection of strain differences in pain behaviour development (Mogil, Wilson et al. 1999).

3.4.2.3 Paw pressure and cold sensitivity

Significant referred pain sensitivities of mechanical hyperalgesia and cold allodynia were found to develop in female C57Bl/6 mice following intra-articular injection of MIA or partial medial meniscectomy compared to sham animals. Pain in OA is often elicited by activities such as walking or stair climbing but is also associated with spontaneous pain (pain at rest), hyperalgesia, and referred pain (pain at distant sites) (Konttinen, Kemppinen et al. 1994; Schaible, Ebersberger et al. 2002). Referred pain is attributed to central hyperexcitability following increased input at the periphery (Gwilym, Keltner et al. 2009) and has been reported in many studies of OA pain in humans (O'Driscoll and Jayson 1974; Kosek and Ordeberg 2000; Bajaj, Graven-Nielsen et al. 2001; Imamura, Imamura et al. 2008). The development of mechanical hyperalgesia in the ipsilateral limb using the paw pressure test has been reported previously in rats following MIA injection but was not found to be significant following partial medial meniscectomy (Fernihough, Gentry et al. 2004). The studies reported here, were the first to identify the development of mechanical hyperalgesia in a murine model of OA.

Cold allodynia, an increased sensitivity to normally non-painful cool temperatures, is a characteristic feature of clinical neuropathic pain states (Verdugo and Ochoa 1992; Allchorne, Broom et al. 2005). Cold allodynia has been assessed previously using a MIA model of OA in rats whereby a cooling allodynia was measured using the acetone drop test (Vonsy, Ghandehari et al. 2009). Vonsy et al. showed a persistent cold allodynia in the ipsilateral hind paw from day 7; however, no contralateral effect was noted. In the current study, contralateral effects were observed in cold sensitivity and paw pressure measurements in the MIA and partial meniscectomy models but no other contralateral hypersensitivities were found. Contralateral effects have been previously reported in human cases of OA as well

as animal models. Kosek et a. (2000) reported that patients with unilateral painful hip OA show lower pain thresholds for pressure stimulation both at the affected hip and at the unaffected contralateral hip (Kosek and Ordeberg 2000). The presence of contralateral pain sensitivities suggests a component of central sensitisation underlying the development of OA pain behaviours.

3.4.2.4 Knee compressions

This is the first study to report a direct measure of knee pain (vocalisation in response to knee compression) in a surgically induced mouse model of OA. The technique is similar to clinical examination in human OA patients whereby knee palpation, flexion and extension are used to identify movement-evoked pain. The technique relies on mice producing audible vocalisations consistently in response to evoked pain and may be limited by the variability in the force applied and the lack of detection of ultrasonic vocalisations. The mice need to be handled regularly in the week prior to the initiation of testing to ensure that the mice are habituated to the gentle restraint used and are accustomed to manipulation of the hind limbs. The technique appears to produce a reliable indicator of knee pain in female C57Bl/6 pain with the number of vocalisations following a similar time course to paw pressure and cold sensitivity measurements. The lack of vocalisations in response to knee compressions in sham operated animals (tested at 16 weeks post MIA injection and from 14 days post partial meniscectomy) suggests that the vocalisations are specific for OA knee pain, however further validation is required.

Table 3-7 Summary table of behavioural sensitivities recorded from the hind limbs in 3 murine models of osteoarthritis pain

Behavioural test	**1% i.a. MIA in female C57Bl/6 mice** **(Ipsilateral hind limb)**	**Partial medial meniscectomy in female C57Bl/6 mice** **(ipsilateral hind limb)**	**Spontaneous OA in Male STR/ort mice** **(both hind limbs)**
Weight bearing	No significant findings (during 12 week study)	No significant findings (during 12 week study)	Not recorded
Von Frey	Biphasic pattern of sensitivity Significant mechanical allodynia at day 5, 28, 63, 98, 105	Persistent allodynia from day 56 onwards	No pattern of sensitivity (during 19 week study)
Paw pressure	Mechanical hyperalgesia from day 5 onwards	Biphasic pattern of sensitivity. Significant mechanical hyperalgesia at day 7,14, 28, 42, 56, day 70, day 84	No pattern of sensitivity (during 19 week study)
Cold sensitivity	Cold allodynia from day 5 onwards	Biphasic pattern of ipsilateral hindlimb sensitivity. Significant cold allodynia at day 7,14, 28, 42, 56, day 70, day 84	No pattern of sensitivity (during 19 week study)
Vocalizations in response to knee compressions	Significantly increased vocalizations at day 112 (only time point recorded)	Biphasic pattern of ipsilateral hindlimb sensitivity. Significant mechanical hyperalgesia at day 7,14, 28, 42, 56, day 70, day 84	No pattern of sensitivity (during 19 week study)

3.4.2.5 Reversal of pain behaviours following morphine administration

Mechanical allodynia, mechanical hyperalgesia, cold allodynia and vocalisations in response to knee compression were significantly decreased following the administration of 6mg/kg s.c. morphine representing an anti-nociceptive effect. Following morphine administration, mechanical hypersensitivity and cold allodynia readings were higher than baseline threshold measurements for both the ipsilateral and contralateral hind limbs indicating a systemic analgesic effect. The dose of morphine was therefore decreased to 4mg/kg in further studies (Chapter 4). The response to morphine indicates that the pain behaviours recorded are due to knee pain rather than instability, however, much greater pharmacological analysis is necessary to validate the models and characterise the OA pain. These pharmacological studies were performed for the partial medial meniscectomy model as described in Chapter 4. Further studies would also be useful to characterize the profile of pain behaviours in the MIA model.

3.4.3 Endogenous Opioids

Endogenous opioids have been reported to modulate pain behaviours in a mouse model of OA. Inglis et al. (2008) reported that administration of either naloxone or naloxone methodide led to significant weight bearing deficits 8 weeks post-surgery rather than 12 weeks post-surgery in mice with OA induced by DMM (Inglis, McNamee et al. 2008). In the current study, the influence of endogenous opioids appeared to vary at different times, with waxing and waning degrees of certain OA pain behaviours (mechanical hyperalgesia and cold sensitivity) in the MIA model. Further investigations are described in Chapter 4 to assess the influence of endogenous opioids on OA pain. These included testing the effect of administration of naloxone and naloxone methiodide in the partial medial meniscectomy model and testing specific opioid receptor antagonists to determine which receptors are most significant for the action of the endogenous opioids on OA pain.

3.4.3.1 Absence of pain in STR/ort mice

This study was designed to document pain behaviours that might be attributable to OA of the knee joint in STR/ort mice. Over the 19 week study, there was no recordable increase in pain sensitivities in either of the hind limbs despite the development of significant joint pathology. For repeated paw pressure and cold sensitivity measurements a cut-off of 150g and 30 seconds is employed to prevent repeated trauma to the soft tissues of the hind paw from the testing apparatus sensitizing the animals. Paw pressure measurements often reached the cut-off PWT for both CBA and STR/ort mice. This might mean that subtle changes in

mechanical hyperalgesia may have occurred but were not detectable in this study. Whilst this is a significant limitation in this study, the development of mechanical hyperalgesia in response to intra-articular injection of CFA shows that PWTs will decrease below cut-off levels when a significantly painful stimulus is present. Cold sensitivity measurements in both STR/ort mice and CBA mice were usually below the cut-off PWL threshold, however, no pattern of hypersensitivity developed during the study. The recording of vocalisations in response to knee compressions did not produce a reliable indicator of pain in STR/ort mice and alternatives should be considered in this strain of mouse.

STR/ort mice are an inbred strain of mice that are particularly susceptible to developing a severe form of spontaneous OA. It is possible that they also have an inbred resistance to OA pain. The apparent resistance to pain of STR/ort mice was also noted by Walton et al. (1978) whilst reporting on spontaneous ankle deformities that sometimes develop in STR/ort mice. Walton et al. observed that the mice continued to bear weight on deformed ankles, even in the presence of soft tissue swelling and showed no consistent response to skin pricking on the sole of the foot or between the digits. The absence of joint pain in some humans with radiographic evidence of knee joint pathology is also widely documented (Sahlstrom, Johnell et al. 1997; Dieppe and Lohmander 2005) and it is currently not known why there is a mismatch between joint pathology and pain. The STR/ort mouse may prove to be a useful tool to provide an insight into the absence of pain in cases of OA or for the development of disease modifying drugs. However, the extended time course and associated costs, concurrent metabolic disorders and structural deformities and lack of a "true" control group of mice makes this model of limited value.

3.4.4 Relevance and validity of the models

MIA is known to cause chondrocyte death by inhibition of glyceraldehyde-3-phosphate dehydrogenase which is necessary for glycolysis (Guzman, Evans et al. 2003) leading to progressive loss of chondrocytes and histological changes of the articular cartilage similar to those found in human OA (Guingamp, Gegout-Pottie et al. 1997). Progressive cartilage degradation and erosion consistent with OA were observed in the studies reported here from 4 weeks post-treatment, but significantly increased joint scores compared to naïve or contralateral knee joints were not recorded until week 8 post-treatment. Significant pain behaviours including mechanical allodynia, mechanical hyperalgesia and cold allodynia were recorded from day 14 post-treatment, however, which may indicate that early OA lesions were missed using the joint scoring techniques employed in this study or that the MIA was

directly inducing pain behaviours during the early post-treatment period. Whilst chondrocytes are particularly vulnerable to MIA, other cell types are almost certainly affected by this metabolic poison, and the extent to which actions on non-chondrocytic cells, including sensory neurons, influences the development of pain behaviour is yet to be determined. Significantly increased immunoreactivity of activating transcription factor (ATF)-3, a marker of nerve injury, was shown following MIA treatment in the L5 DRG on days 8 and 14 in a rat model of OA (Ivanavicius, Ball et al. 2007). However, it is currently unknown whether this effect is due to OA bone pathology leading to neuronal damage or as a direct effect of the MIA. Immunoreactivity of ATF-3 has not been reported in other models of OA. Further directions might include comparing ATF-3 in the MIA, partial medial meniscectomy and spontaneous OA models to identify whether increases in ATF-3 immunoreactivity within the DRG are associated with the presence of pain behaviours recorded in the ipsilateral or contralateral hind limbs.

The MIA model is also limited by its systemic toxicity. The use of MIA has not been studied extensively in mice compared to rats and this may reflect the toxic nature of the compound with a fine balance between inducing cartilage damage and persistent pain sensitivities and causing death of the animals.

The partial medial meniscectomy model aims to replicate the induction of early onset OA that occurs following a meniscal tear. Injuries to the menisci are a common orthopaedic problem and can result from trauma or from degeneration (Baker, Peckham et al. 1985). Traumatic meniscal tears typically occur from twisting type injuries to the knee and can vary in size, location and stability although the majority of tears in humans affect the medial meniscus (Campbell, Sanders et al. 2001). Surgical excision of a tear (partial or total meniscectomy) is the most common treatment technique particularly for tears involving the central, less vascular portion of the meniscus (Gallacher, Gilbert et al. 2010). Development of OA is a common complication following meniscectomy and the degree of degenerative joint disease is positively correlated with the amount of meniscus removed (Andersson-Molina, Karlsson et al. 2002). This study has shown that partial medial meniscectomy produces degenerative joint disease lesions consistent with OA.

Mild degenerative changes were noted 4 weeks after surgery and marked progressive structural modifications seen at 8 and 12 weeks. The pain hypersensitivities occurred in two phases. Initial hypersensitivity readings were apparent at 1-2 weeks and spanned a period with minor cartilage damage.

Hypersensitivities during this period are likely to have an inflammatory component due to tissue damage and inflammation related to the partial meniscectomy surgery. In contrast, the timing of the later pain behaviours (>5 weeks) may be attributable to the development of degenerative joint disease. The relatively slow development of structural changes following joint surgery and the associated increase in hypersensitivity mirrors the development of human OA and gives surgical models face validity for the study of OA pain.

Spontaneous OA replicates the slower onset degenerative process of naturally occurring spontaneous OA in humans and therefore may be considered a more clinically relevant model of OA. However, STR/ort mice are known to develop a number of other disorders that may occur concurrently or may even contribute to the development of spontaneous OA. These include obesity, hyperlipidemia and hyperinsulinemia which are known risk factors for the development of OA in humans (Altman 1981; Uchida, Urabe et al. 2009). STR/ort mice also develop structural deformities including patellofemoral joint dislocation and internal rotation of the tibia (Walton 1979; Schunke, Tillmann et al. 1988). Patellofemoral joint dislocation is reported to precede or at least coincide with early stages of OA and surgical stabilization of the patellae in the normal position lowers the incidence of OA in young STR/ort mice (Walton 1979). However, it remains unclear how patellar dislocation is induced and why such incidence differs between colonies of the STR/ort mice (Walton 1979; Schunke, Tillmann et al. 1988). Some male STR/ort mice also develop a spontaneous ankle deformity with calcification of the Achilles tendon (Walton 1978). However, there appears to be no correlation between ankle deformity and presence of OA in the knee joint (Walton 1978). The variation in concurrent disorders and structural deformities in colonies of STR/ort mice make it very difficult to produce unified groups of mice for controlled studies. Similar to the studies reported here, Mason et al. (2001) also found a large variation in histological joint scores between individual mice and between the left and right hind limbs (Mason, Chambers et al. 2001). Another limitation of the STR/ort mouse model of OA is that there is no “true” control group available. CBA mice have been used most frequently because knee OA has rarely developed in these mice (Walton and Elves 1979; Dunham, Chambers et al. 1990). In the studies reported here, CBA mice did develop a moderate level of cartilage degradation, although there was no consistent pattern of hypersensitivity when compared to the chemically or surgically induced models investigated.

3.5 Conclusion

This collection of studies has shown that we were able to produce three murine models of OA that allowed the assessment of OA joint pathology and OA pain behaviours. Whilst both partial medial meniscectomy and injection of MIA produce degenerative joint disease and measurable behavioural disturbances, partial medial meniscectomy has been chosen for further study since it produces the most clinically relevant model replicating the clinical scenario in humans whereby meniscal injury (e.g. due to a sports injury) and subsequent treatment by partial meniscectomy can lead to the development of early onset OA. Further work contained within this thesis will focus on the confirmation and characterization of the pain behaviours associated with the OA induced in this model.

Chapter 4

Characterisation and Validation of a Mouse Model of Osteoarthritis Pain

4 Characterisation and Validation of a Mouse Model of OA Pain

4.1 Introduction

The aetiology of OA is diverse and multi-factorial but pain is usually the main symptom and the first complaint of OA patients. According to OA pain treatment recommendations published by Osteoarthritis Research Society International (OARSI) (Zhang, Moskowitz et al. 2008), paracetamol should be the analgesic of first choice for mild/moderate pain, and if successful, should be used as the preferred long-term analgesic, because of its safety and effectiveness. NSAIDs are considered in patients unresponsive to paracetamol, and stronger analgesics, such as weak opioids (e.g. tramadol) and narcotic analgesics used in cases of refractory pain where other drugs have been ineffective or are contraindicated. Partial medial meniscectomy has been identified as an animal model with potential for producing a clinically relevant model of human OA pain. It was therefore important to validate and characterise the presence of OA pain by testing the response of pain behaviours to different classes of commonly used analgesic drugs.

4.1.1 Neuropathic pain drugs

Neuropathic pain drugs have been recently investigated for treatment of chronic pain syndromes such as OA. Neuropathic pain medications, such as anticonvulsants, tricyclic antidepressants, and serotonin-norepinephrine reuptake inhibitors may be effective, particularly in patients with neuropathic-like symptoms and/or those not responding to conventional analgesic therapy. They have been used for the treatment of neuropathic pain states such as diabetic peripheral neuropathy, trigeminal neuralgia and post-herpetic neuralgia (Segal and Rordorf 1996; Canavero and Bonicalzi 1997; Lunardi, Leandri et al. 1997; Eisenberg, Lurie et al. 2001) and therefore were investigated in the partial medial meniscectomy model of OA pain.

4.1.2 Endogenous opioids

Waxing and waning levels of pain behaviours were recorded in studies of OA induced by MIA and partial medial meniscectomy in C57Bl/6 mice (Chapter 3) that may be related to endogenous opioid tone. Endogenous opioids comprise enkephalins, endorphins, dynorphins and endomorphins (Horvath and Kekesi 2006; Bodnar 2010). These peptides and their receptors exist throughout the central and peripheral nervous systems and in other tissues and are involved in a diverse array of homeostatic functions and movement control as well as the processing of noxious

sensory input (Bodnar 2010). Endogenous opioids have been previously shown to modulate pain behaviours in a murine model of OA pain (Inglis, McNamee et al. 2008) and an absence of pain behaviours in the MIA model of OA could be reversed by administration of naloxone in the current studies (see 3.3.2.3.3). Therefore, the influence of the endogenous opioid system was investigated in OA pain induced by partial medial meniscectomy.

4.1.3 Sex and OA

Primary OA occurs in a larger number of females than males, and both prevalence and incidence increase in postmenopausal women (Srikanth, Fryer et al. 2005). It has also been reported that as with other pain syndromes (Fillingim, King et al. 2009), women experience greater OA-related pain and disability than do men (Keefe, Lefebvre et al. 2000; Theis, Helmick et al. 2007; Axford, Heron et al. 2008; Perrot, Poiraudeau et al. 2009). Therefore, a preliminary study was performed to gain an insight into the role that sex hormones play in OA induced by partial medial meniscectomy.

4.2 Aims

- To characterize joint pain induced by the medial meniscectomy model of OA by measuring the effect of different therapeutic agents on pain behaviours.
- To assess the role of the endogenous opioid system in pain behaviours induced by partial medial meniscectomy.
- To identify differences in the development of pain behaviours and articular pathology in male, female and ovariectomised female C57Bl/6 mice following partial medial meniscectomy

4.3 Results

4.3.1 General

Pharmacological studies were performed in order to confirm the presence of pain behaviours (rather than joint instability) and to characterise the response to different types of analgesic drugs. Following partial medial meniscectomy of female C57Bl/6 mice, pain behaviours were recorded weekly. In mice with significant pain behaviours, measurements were taken prior to and after administration of a drug. For each drug tested, a group of meniscectomised mice were randomly assigned to receive the drug vehicle to serve as a control. Diclofenac was tested in the first phase of hypersensitivity (day 7) and also in the second phase of hypersensitivity (from day 42 onwards) along with the other analgesic drugs. A minimum wash out period of 7 days was used between drug treatments. To monitor the effect of early analgesia on the development of OA pain behaviours, a separate group of meniscectomised mice did not receive any treatment for the first 6 weeks of the study.

Since the magnitude of the weight bearing change was not as robust a signal as other measures (mechanical hyperalgesia, allodynia, cold sensitivity and vocalisations in response to knee compressions) recorded in the previous partial medial meniscectomy behaviour studies, analysis was restricted to the latter behaviours.

4.3.2 Development of pain behaviours

Mice developed initial pain behaviours for mechanical hyperalgesia, cold allodynia and vocalisations in response to knee compression by day 7 and day 14 following partial medial meniscectomy, similarly to the results reported in Chapter 3. The time courses for the development of pain behaviours in 3 groups of the mice are shown in Figure 4-1 and Figure 4-2. Mice within groups 1 and 2 had been treated with diclofenac on day 7 post-surgery. In contrast, group 3 mice had not received any analgesic drugs during the first 6 weeks of the study. Time courses for the development of pain behaviours did not vary dramatically between the groups of mice indicating that early intervention with diclofenac did not have a significant influence on the development of later pain behaviours. Pain behaviours were diminished at day 21 and day 28 following surgery and were pronounced again by week 6. Persistent mechanical allodynia developed in the ipsilateral hind limb in all 3 groups of mice from day 56 onwards. A marked reduction in mechanical hyperalgesia, cold allodynia and knee vocalizations, but not mechanical allodynia

(Figure 4-1 and Figure 4-2), was also noted at several time points in the second phase of hypersensitivity. These discrete fluctuations in pain behaviour during the second phase of hypersensitivity were marked at days 63 and 82. These reductions in sensitivity persisted for 1-2 weeks before hypersensitivities returned. Fluctuations in hypersensitivity appeared to synchronise within individual cages of mice and may have hormonal or environmental trigger factors.

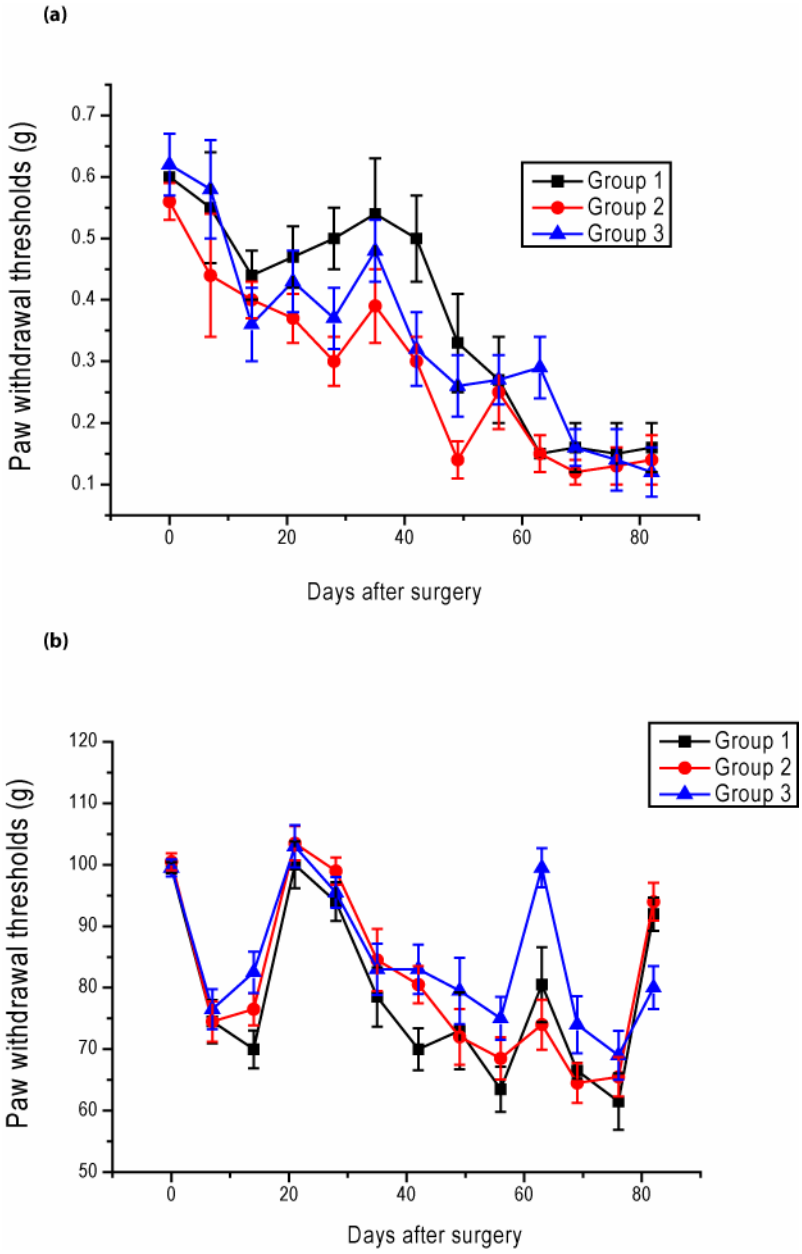


Figure 4-1 The effect of partial medial meniscectomy on 3 groups of female C57Bl/6 mice.

Test for the development of mechanical allodynia a) and mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Mice from within groups 1 and 2 were randomly assigned to receive analgesic drugs (or vehicle) from day 7 post-surgery. Group 3 were included in pharmacological studies from day 42 post-surgery to assess whether the time course for pain development would be significantly altered from early analgesic administration.

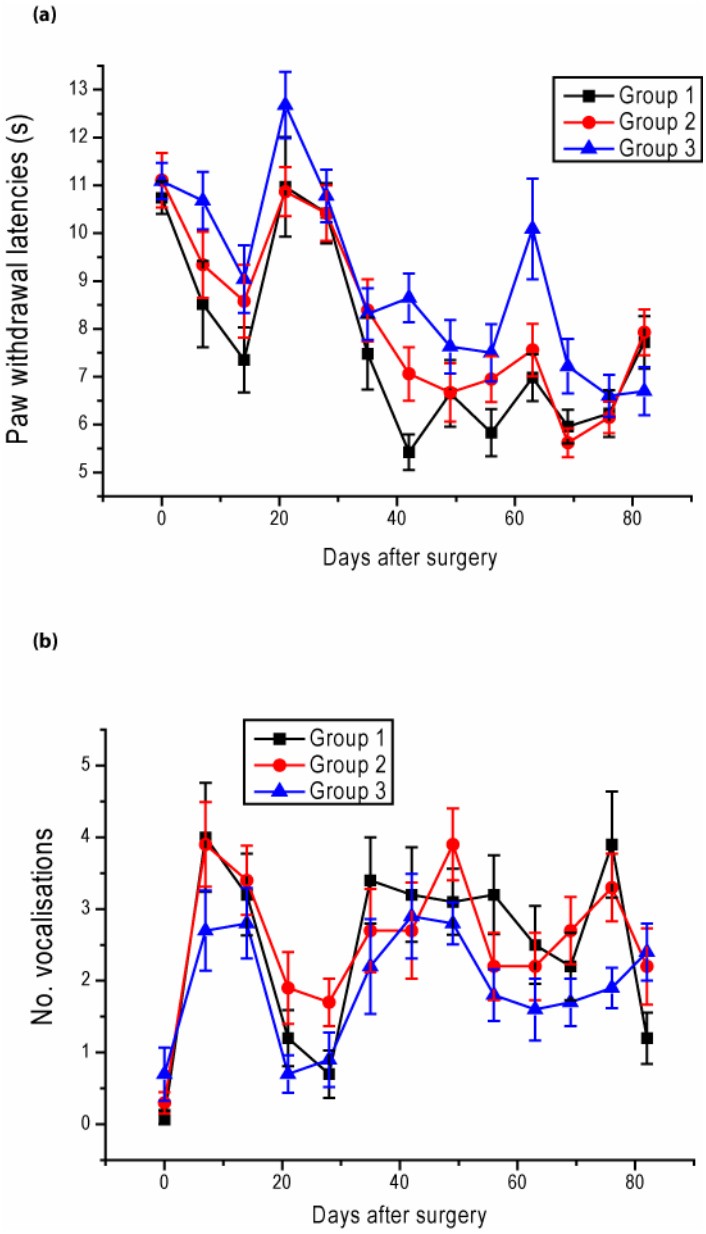


Figure 4-2 The effect of partial medial meniscectomy on 3 groups of female C57Bl/6 mice.

Test for the development of cold allodynia a) and vocalisations in response to knee compression b). Each point represents the mean value ± SEM of ten mice. Mice within groups 1 and 2 were randomly assigned to receive analgesic drugs from day 7 post-surgery. Group 3 were included in pharmacological studies from day 42 post-surgery to assess whether the time course for pain development would be significantly altered from early analgesic administration.

4.3.3 Role of inflammation in pain behaviours following medial meniscectomy

Diclofenac, a non-steroidal anti-inflammatory drug, commonly used in the management of OA pain, was used to assess the inflammatory component of the pain behaviours in the two major phases of this model. The ability of diclofenac to reduce pain behaviours in the early phase (day 7) and later phase (tested at day 42 after partial medial meniscectomy) was very different (Figure 4-3 to Figure 4-6). On day 7 after surgery, diclofenac (10mg/kg i.p.) had a marked effect decreasing mechanical hyperalgesia measured by the paw pressure method (Figure 4-3b, P=0.00058) and the number of vocalisations in response to knee compression (Figure 4-4b, P=0.0040). The reduction in cold hypersensitivity after diclofenac administration (Figure 4-4a) did not reach statistical significance and diclofenac had no effect on von Frey filament PWT (Figure 4-3a).

In contrast to the effect on day 7, treatments with diclofenac had no significant effect on any of the measured parameters when tested 6 weeks after surgery (Figure 4-5 and Figure 4-6). Similarly, no significant effect was seen following the administration of the COX-2 selective agent, celecoxib at day 72 following surgery (Figure 4-7 and Figure 4-8). These results suggest that early pain behaviours were related to post-surgical inflammation whereas later pain behaviours were due to degenerative joint disease which has a less significant inflammatory component. For this reason, the second phase of pain behaviours was the main focus for more extensive pharmacological studies.

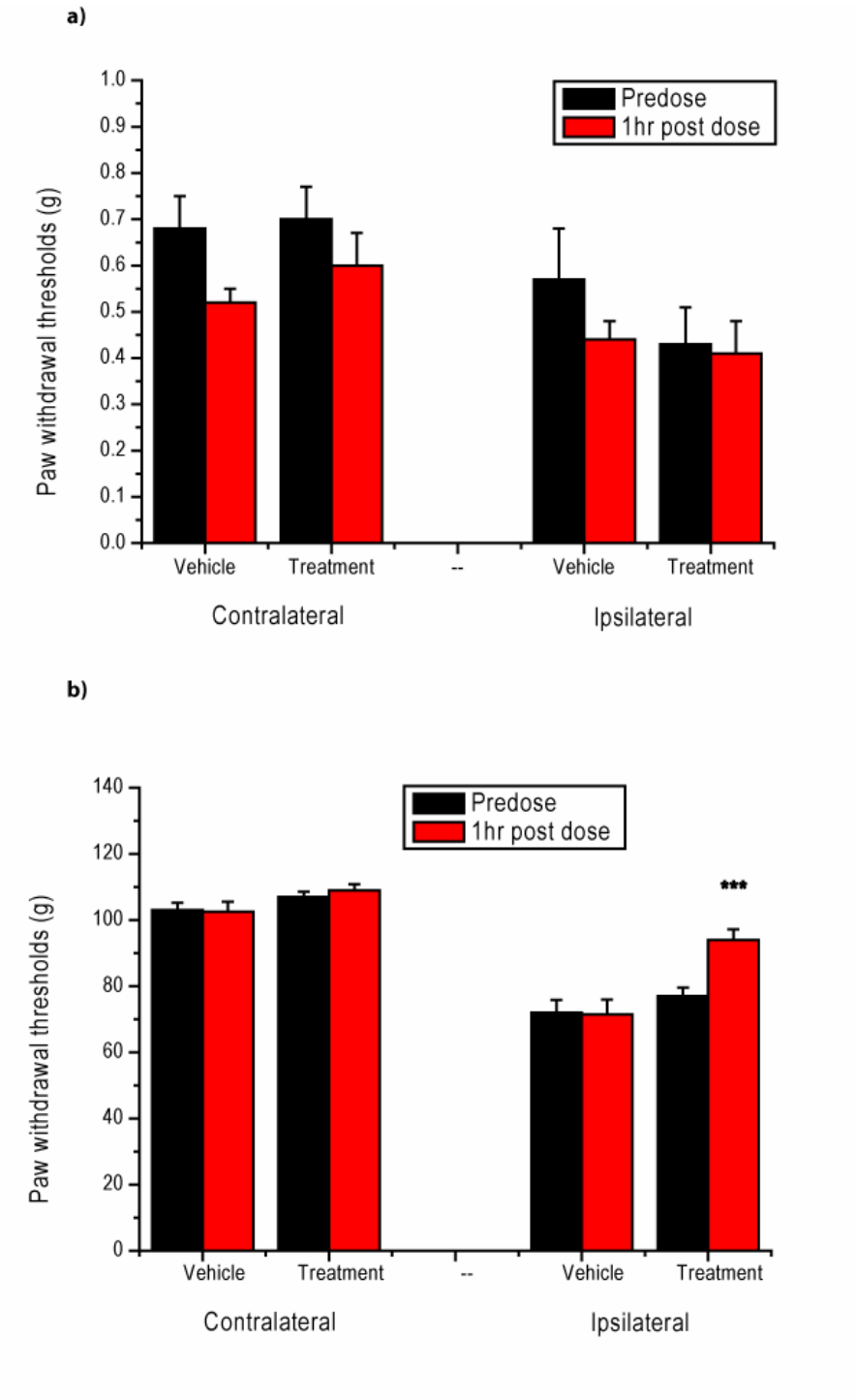


Figure 4-3 The effect of 10mg/kg i.p. diclofenac at day 7 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of diclofenac on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *** P<0.001.

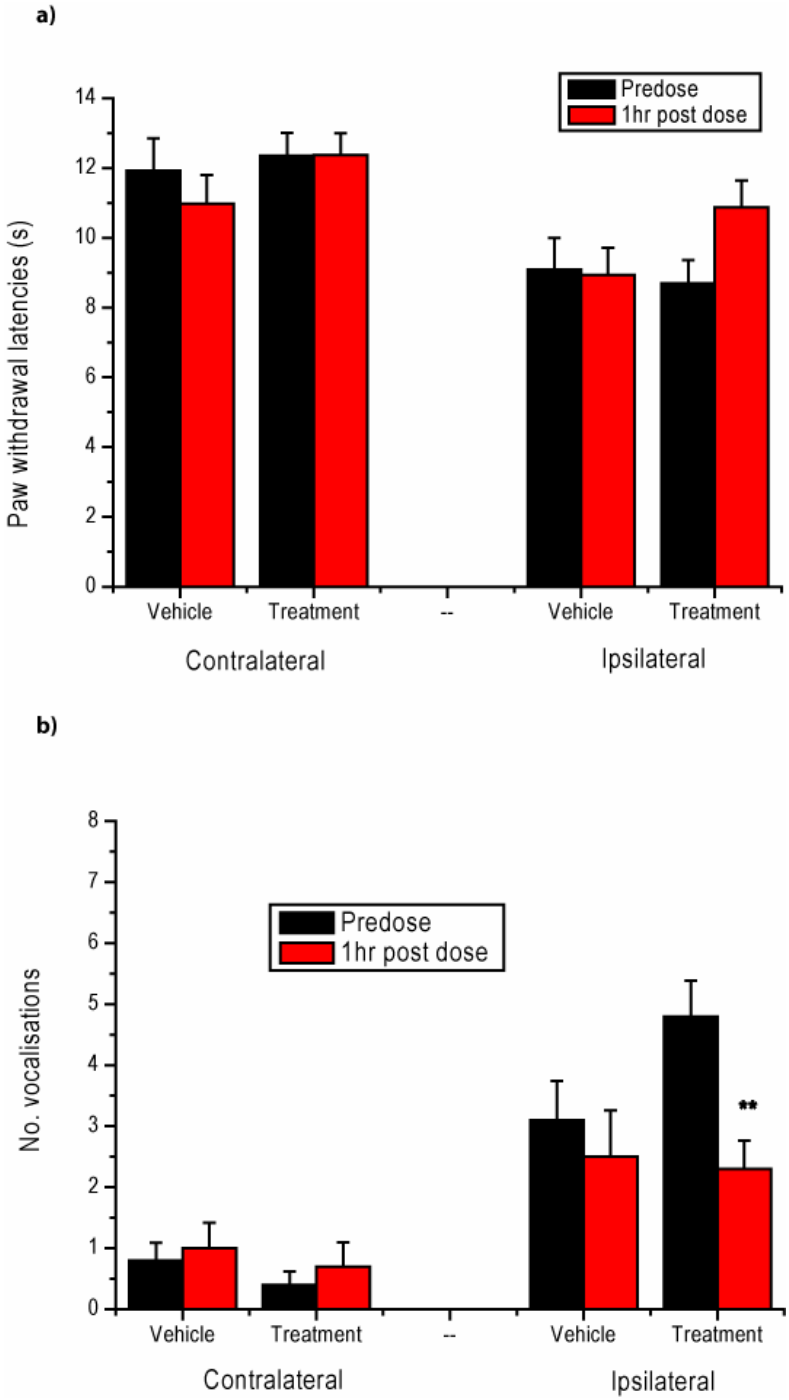


Figure 4-4 The effect of 10mg/kg i.p. diclofenac at day 7 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of diclofenac on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01

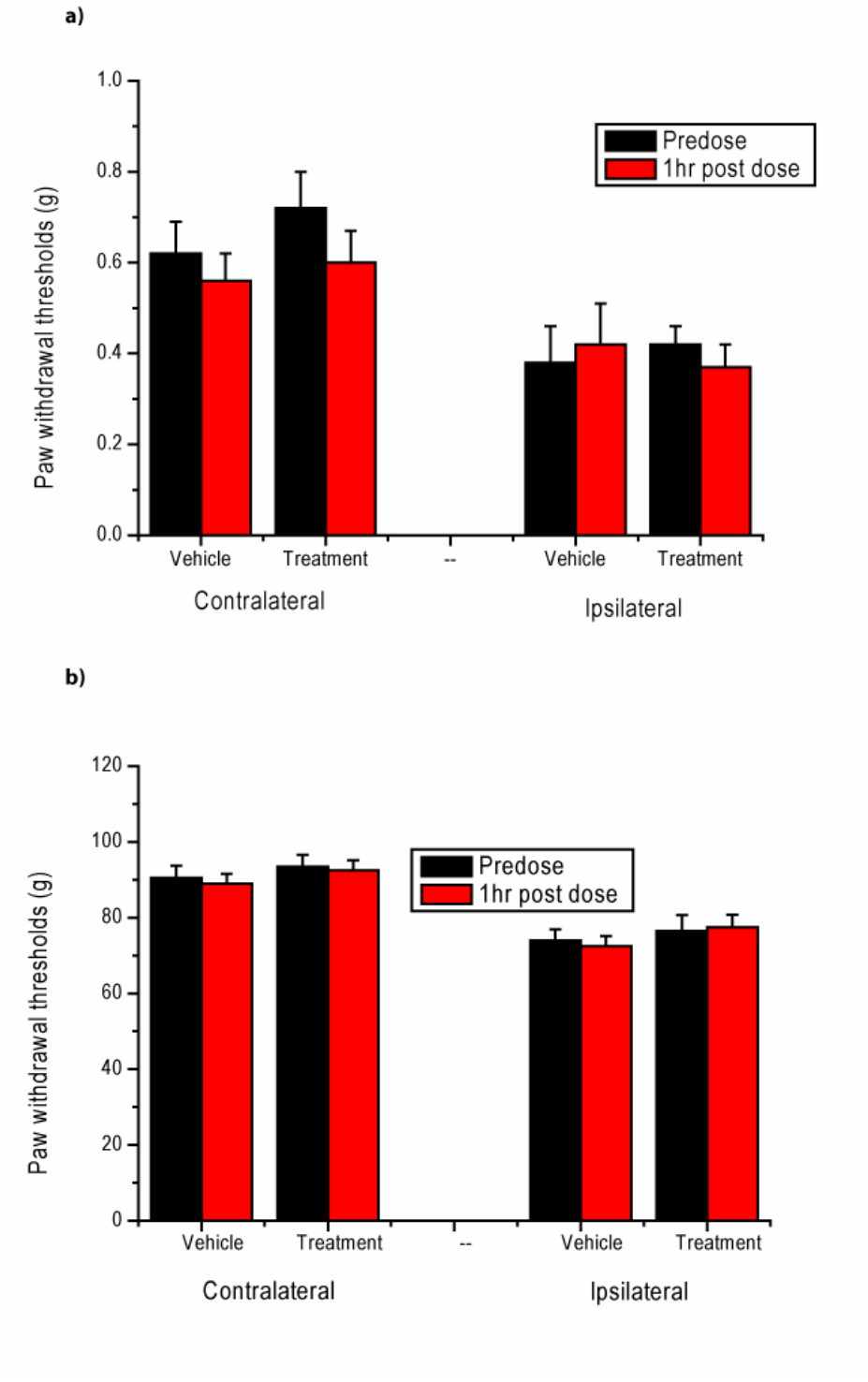


Figure 4-5 The effect of 10mg/kg i.p. diclofenac at day 42 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of diclofenac on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice.

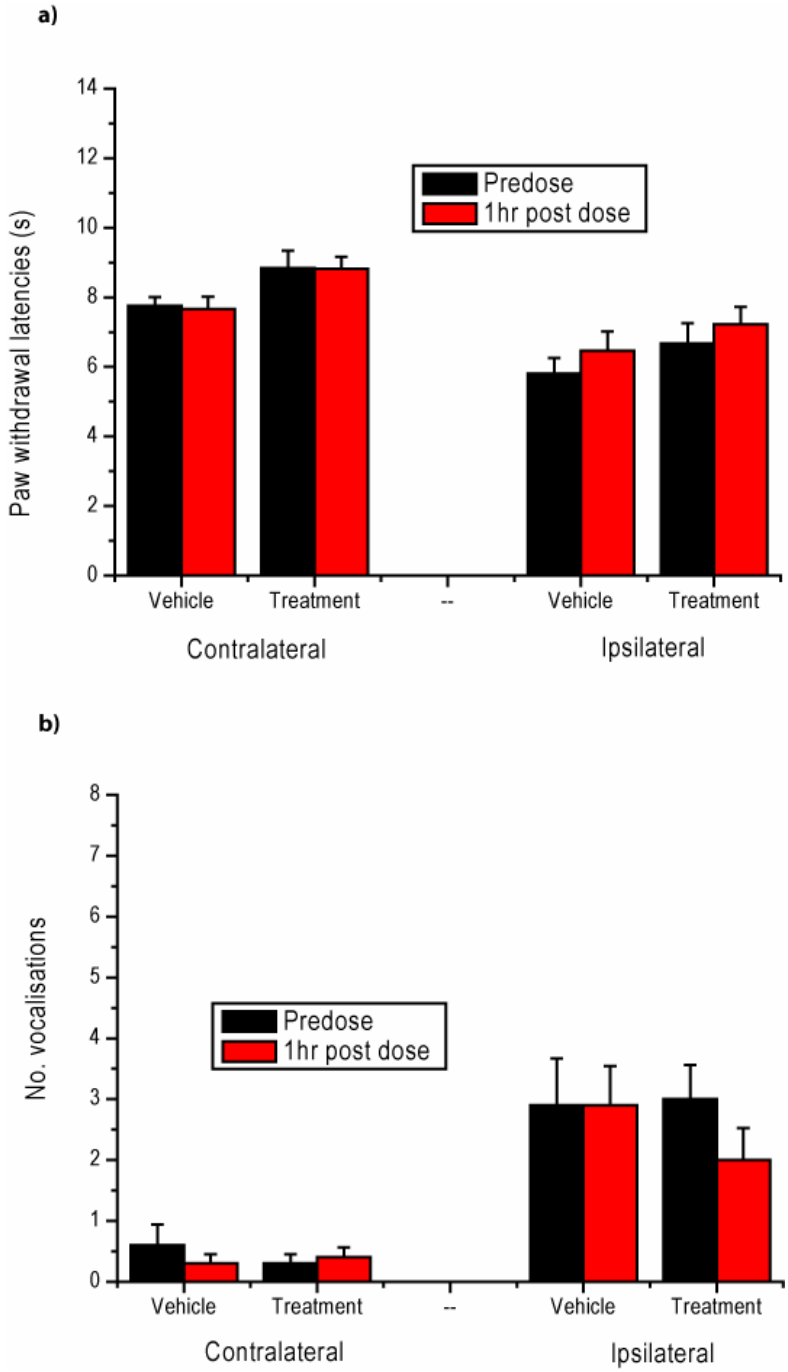


Figure 4-6 The effect of 10mg/kg i.p. diclofenac at day 42 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of diclofenac on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice.

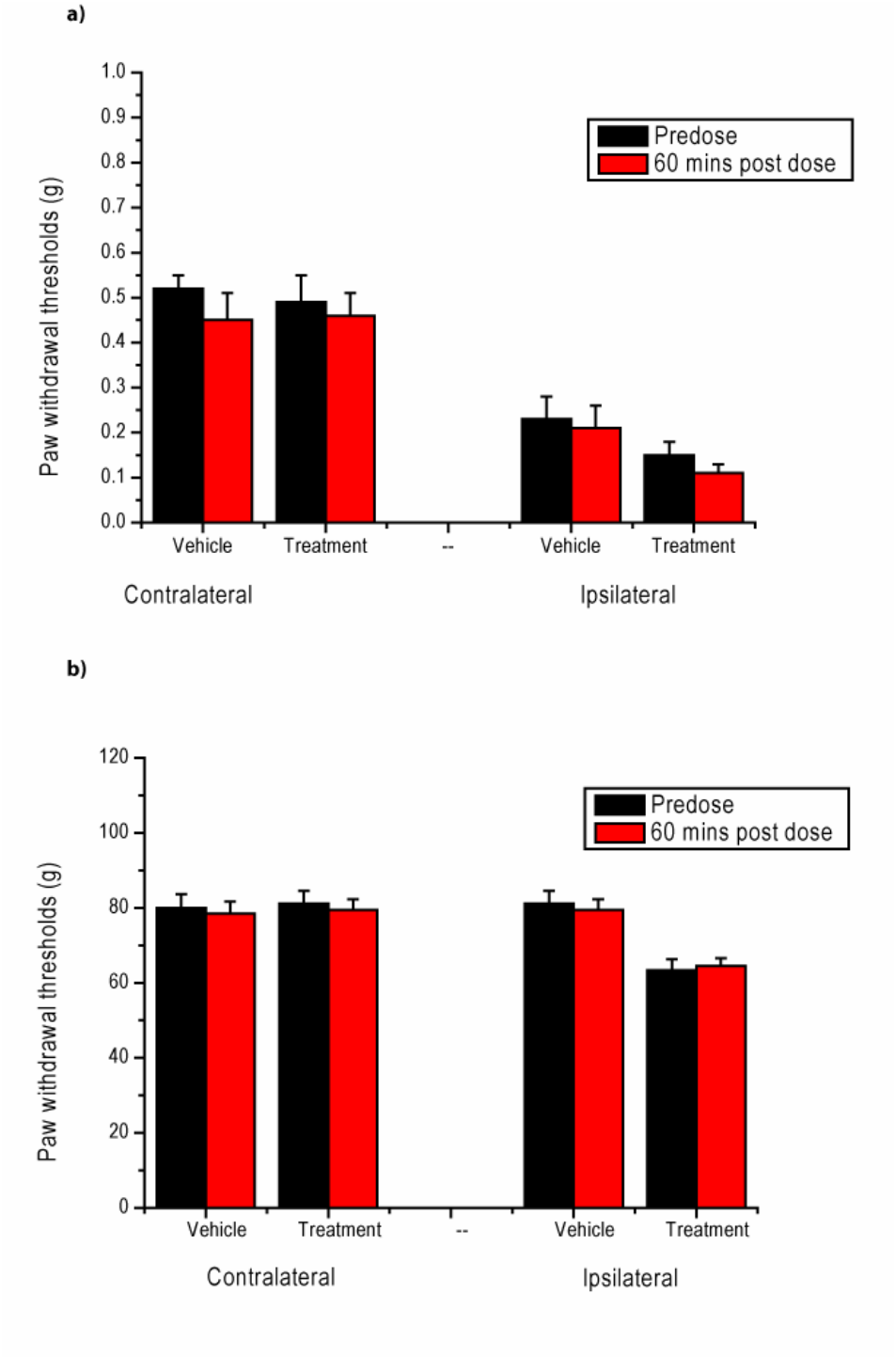


Figure 4-7 The effect of 30mg/kg p.o. celecoxib at day 76 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of celecoxib on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice.

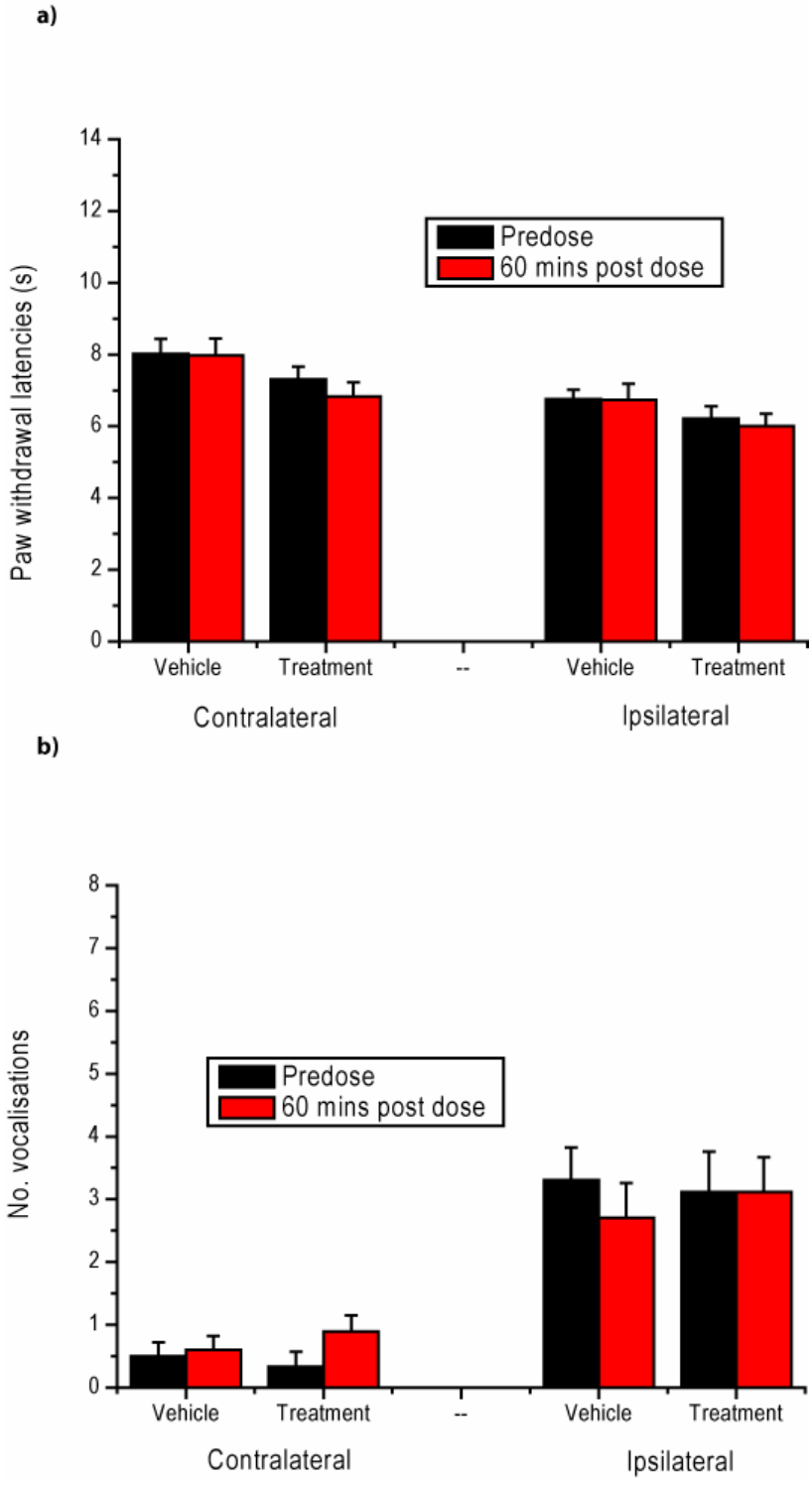


Figure 4-8 The effect of 30mg/kg p.o. celecoxib at day 76 post partial medial meniscectomy of female C57Bl/6 mice.

The effect on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice.

4.3.4 Reversal of pain behaviours by common analgesic drugs

The analgesic drugs chosen for this study (diclofenac, morphine, paracetamol, tramadol, celecoxib) are commonly used for the treatment of chronic pain conditions including OA and have known effects and duration of action in both humans and rodents. A summary table of the results is located in Table 4-1. Mechanical hyperalgesia (paw pressure) was reversed significantly by morphine (4mg/kg s.c., P=0.00018, Figure 4-9b) and paracetamol (300mg/kg s.c., P=0.0083, Figure 4-11b) compared to pre-treatment levels. Cold allodynia was reversed significantly by morphine (P=0.017, Figure 4-10a,) but not by the other drugs. The number of vocalisations in response to knee compressions was reduced significantly by morphine (P=0.0041, Figure 4-10b) and tramadol (50mg/kg s.c., P=0.014, Figure 4-14b). Morphine (P=0.013, Figure 4-9a) and tramadol (P=0.0031, Figure 4-13a) effectively reduced mechanical allodynia.

Meniscectomised mice that received the drug vehicles only, showed no significant alteration in pain behaviours. No sedation, excitation or other noticeable adverse effects were noted following administration of any of the analgesics mentioned above. Administration of morphine (4mg/kg s.c.) did however, produce a significant decrease in mechanical sensitivity in the contralateral limb (P=0.046) indicating a systemic analgesic effect.

Table 4-1 The effect of analgesic drugs on OA pain behaviours.

Early phase represents day 1-14 post surgery. Late phase represents day 42-84 post-surgery. Statistics: Pre-drug versus post drug measurements by unpaired t-test, * P<0.05, ** P<0.01, *** P<0.001

	Mechanical Allodynia PWT (g)		**Mechanical Hyperalgesia PWT (g)**		**Cold Allodynia PWL (s)**		**Knee Compression (no. vocalisations)**	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Early Phase								
Diclofenac 10mg/kg i.p.	0.43 ± 0.08	0.41 ± 0.07	77.00 ± 2.60	**94.00 ± 3.14 *****	8.76 ± 0.66	10.87 ± 0.77	4.80 ± 0.59	**2.30 ± 0.47 ****
Late Phase								
Diclofenac 10mg/kg i.p.	0.42 ± 0.04	0.37 ± 0.05	76.50 ± 4.22	77.50 ± 3.27	6.67 ± 0.59	7.23 ± 0.50	3.00 ± 0.56	2.00 ± 0.52
Morphine 4mg/kg s.c.	0.3 ± 0.05	**0.48 ± 0.04 ****	73.13 ± 2.49	**93.75 ± 3.24 *****	9.33 ± 0.48	**11.5 ± 0.65 ***	1.63 ± 0.42	**0.13 ± 0.13 ****
Paracetamol 300mg/kg s.c.	0.15 ± 0.03	0.19 ± 0.07	70.00 ± 2.79	**81.00 ± 2.45 ****	5.81 ± 0.39	6.33 ± 0.43	2.30 ± 0.37	2.20 ± 0.44
Tramadol 50mg/kg s.c.	0.11 ± 0.01	**0.35 ± 0.07 ****	66.00 ± 3.40	63.5 ± 2.59	5.94 ± 0.28	5.74 ± 0.47	3.10 ± 0.48	**1.30 ± 0.45 ****
Celecoxib 30mg/kg p.o.	0.15 ± 0.03	0.11 ± 0.02	63.33 ± 3.00	64.44 ± 2.12	6.21 ± 0.35	6.00 ± 0.35	3.11 ± 0.65	3.11 ± 0.56

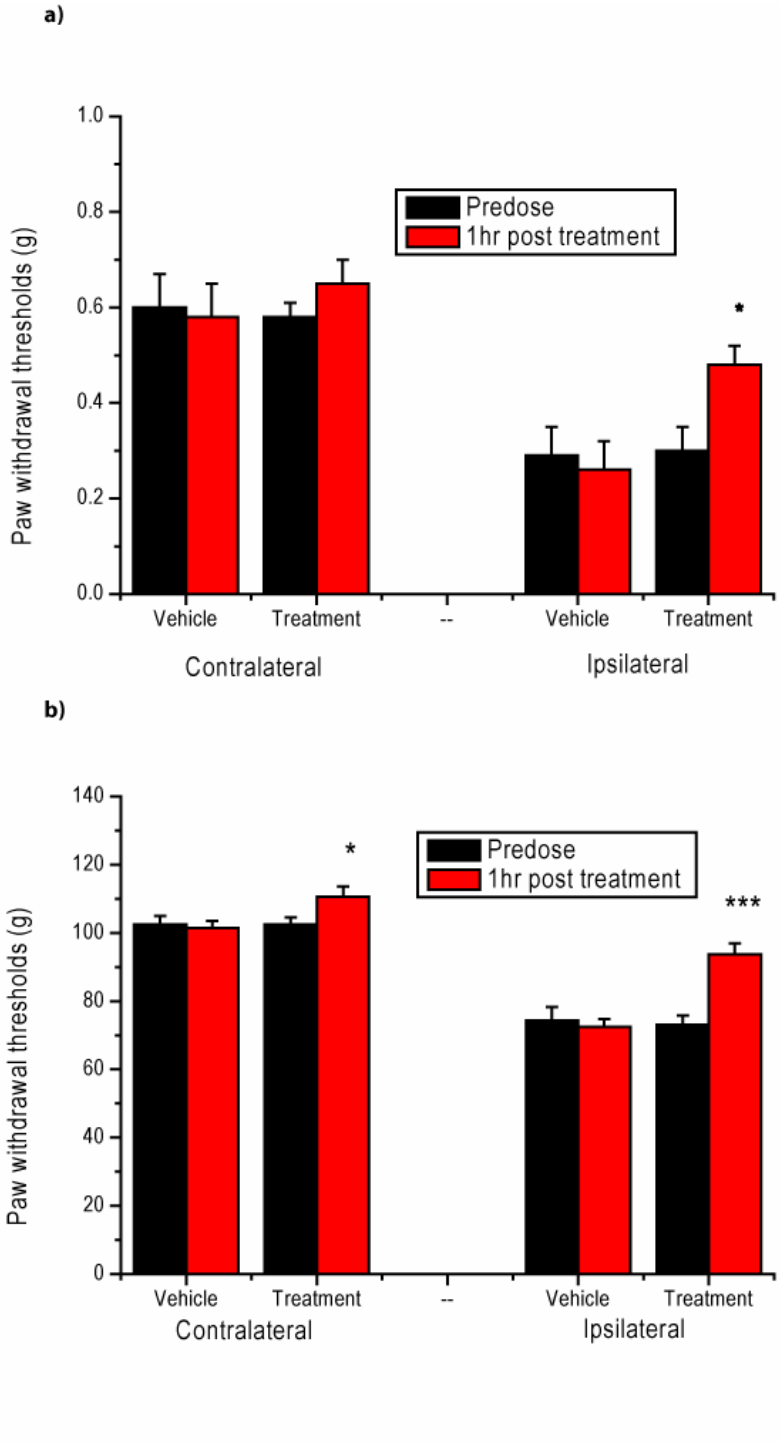


Figure 4-9 The effect of 4mg/kg s.c. morphine at day 42 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of morphine on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, * $P<0.05$, *** $P<0.001$

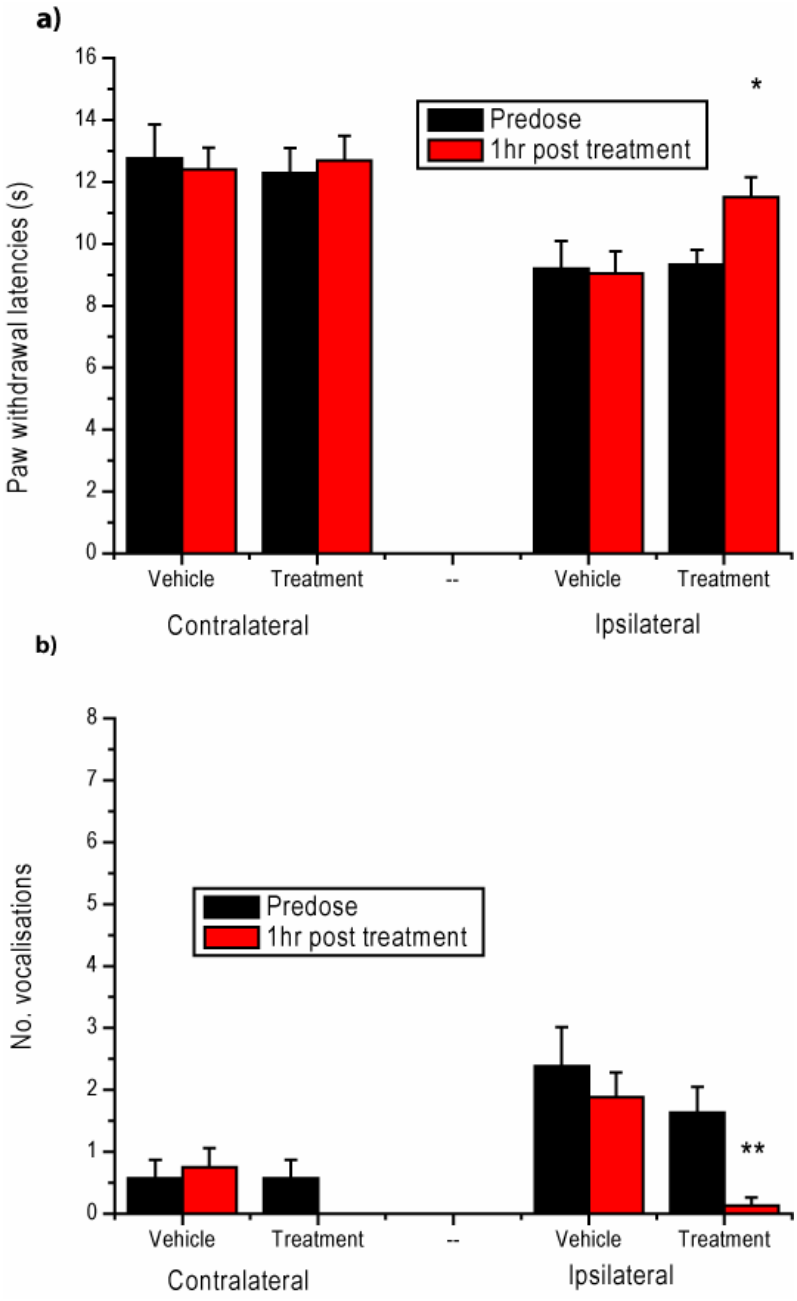


Figure 4-10 The effect of 4mg/kg s.c. morphine at day 42 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of morphine on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, * P<0.05, ** P<0.01.

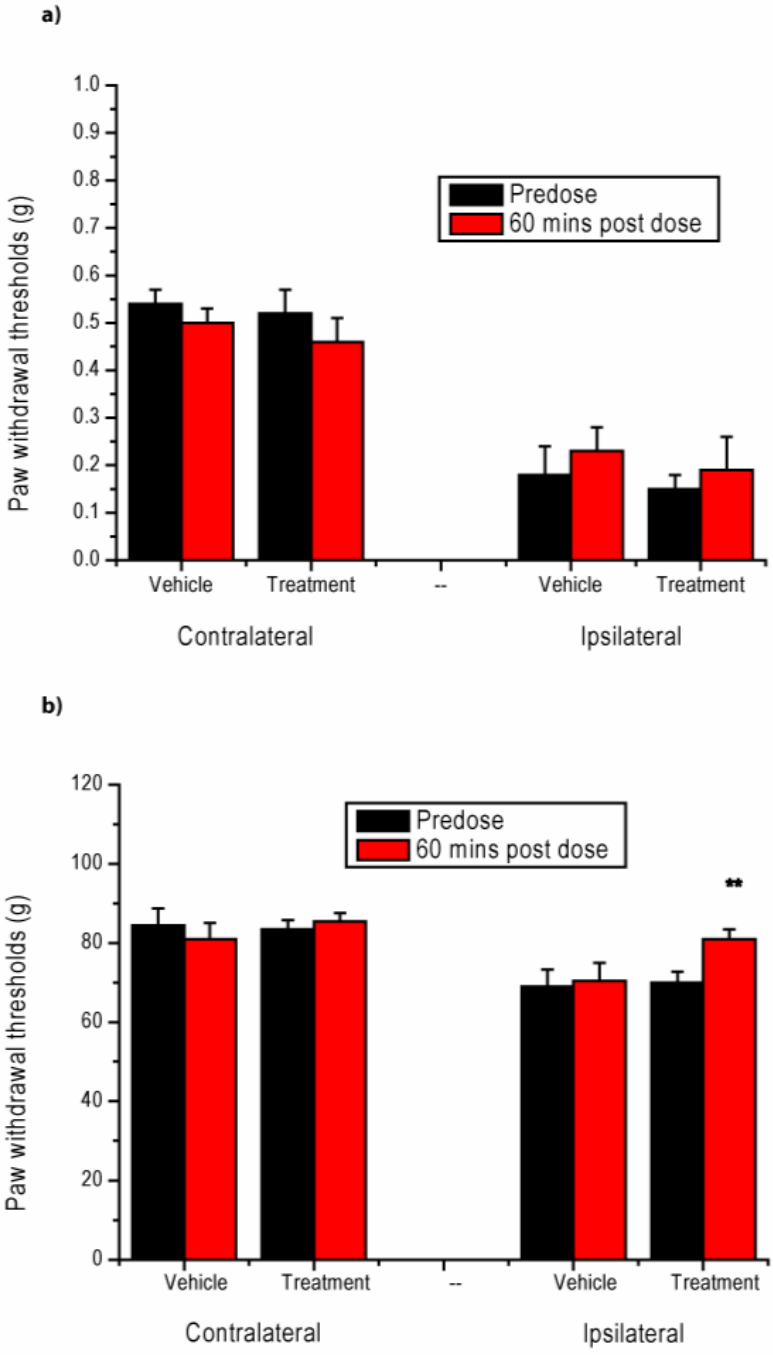


Figure 4-11 The effect of 300mg/kg s.c. paracetamol at day 69 post partial medial meniscectomy of female C57Bl/6 mice.

The effect on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01

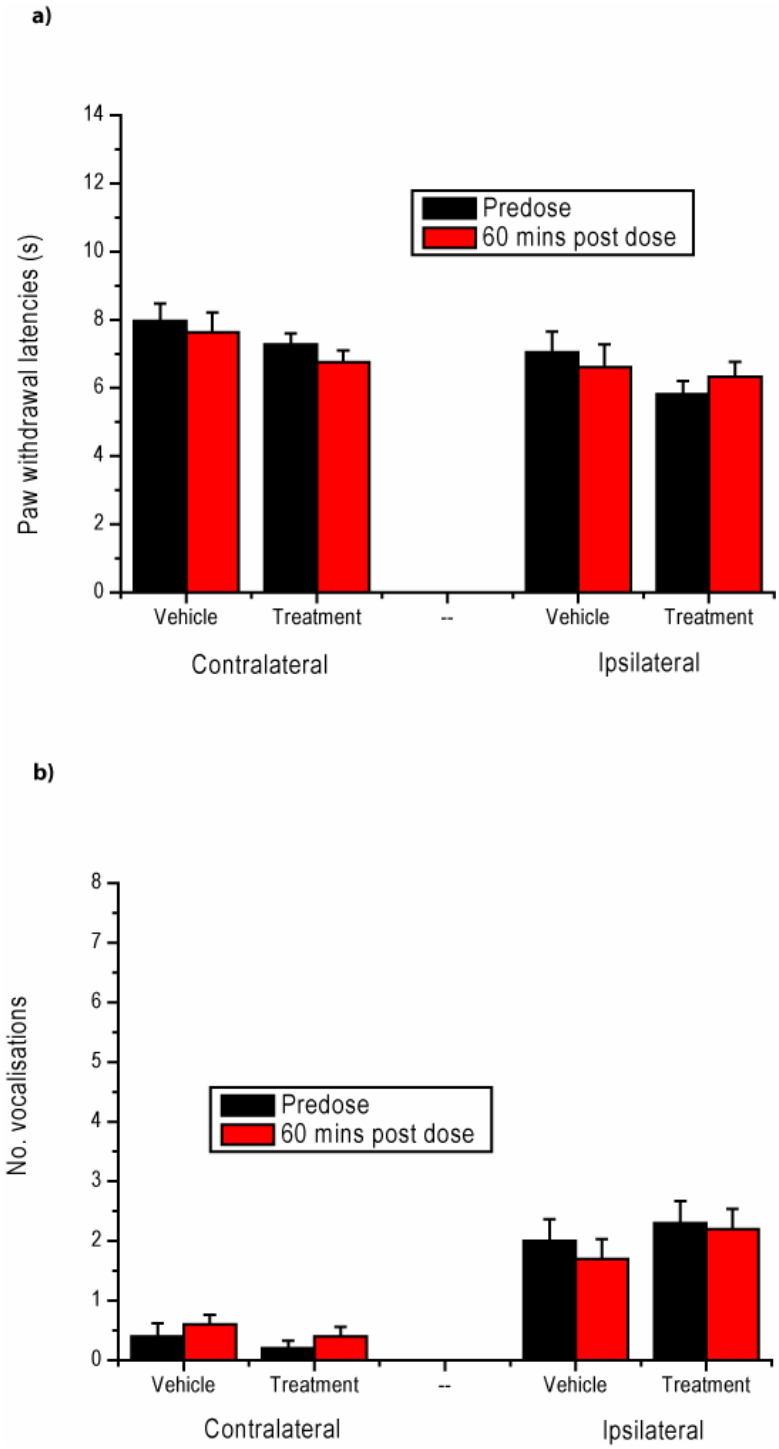


Figure 4-12 The effect of 300mg/kg s.c. paracetamol at day 69 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of paracetamol on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice.

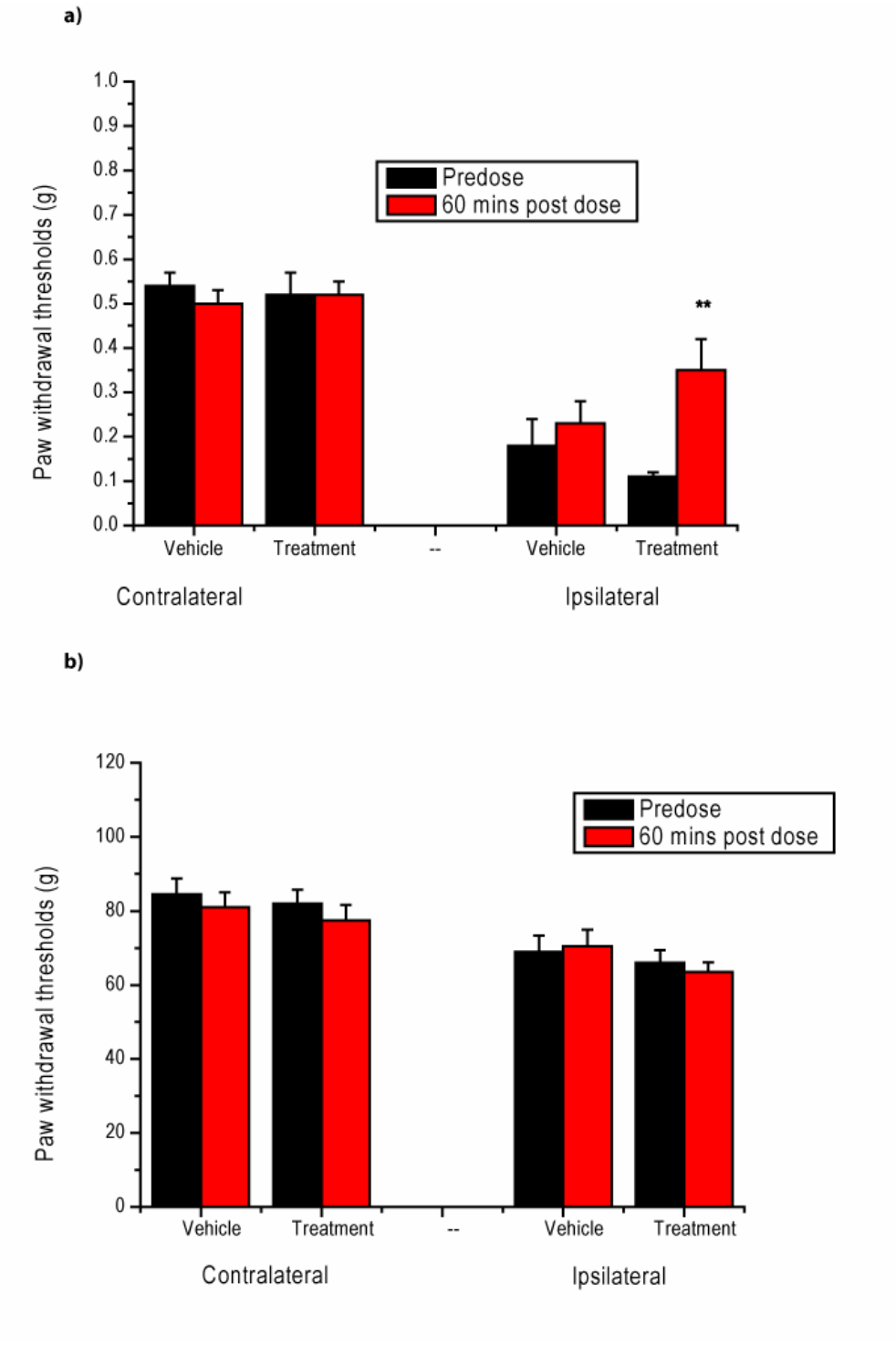


Figure 4-13 The effect of 50mg/kg s.c.. tramadol at day 69 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of tramadol on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01

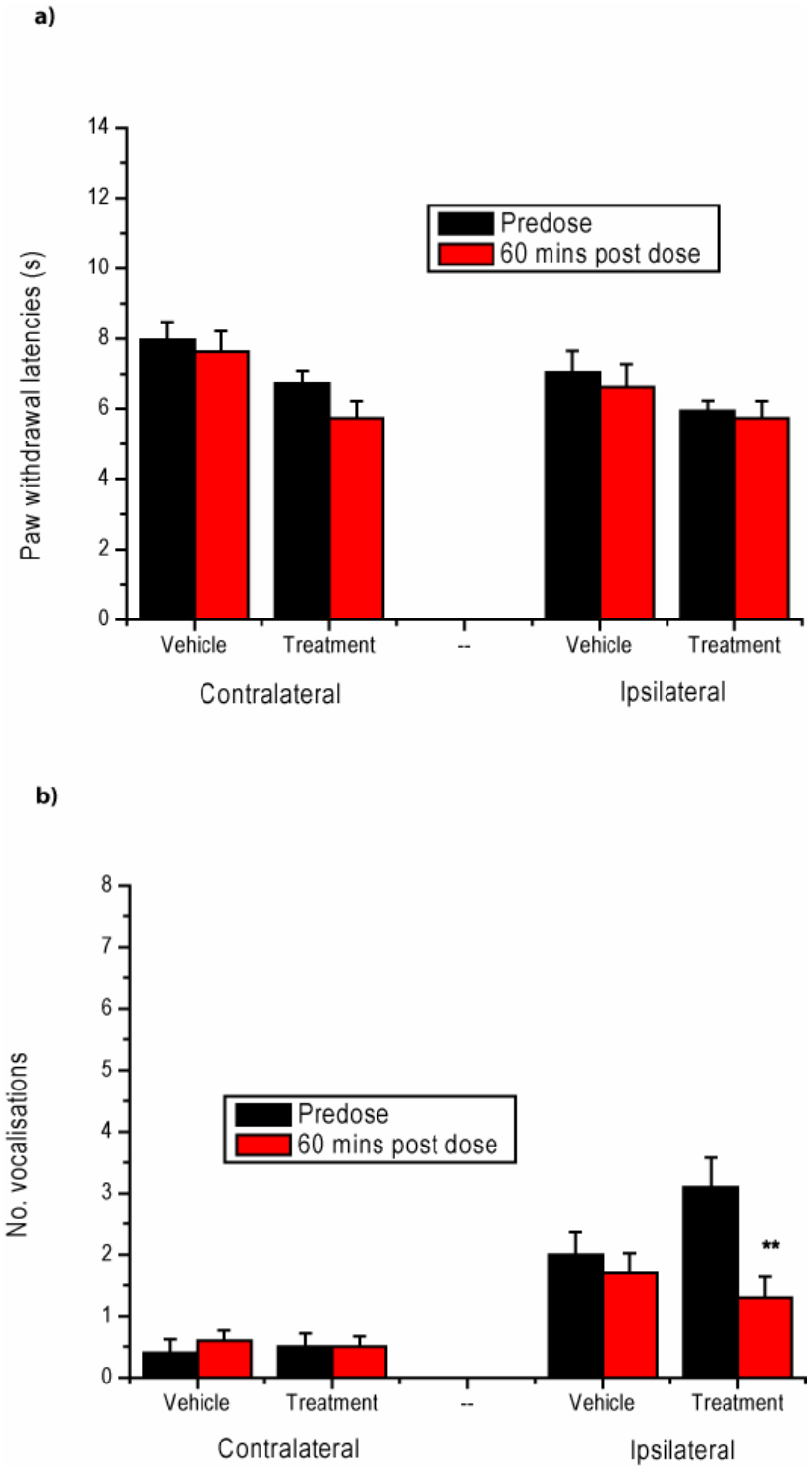


Figure 4-14 The effect of 50mg/kg s.c. tramadol at day 69 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of tramadol on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01

4.3.5 Reversal of pain behaviours by neuropathic pain drugs

Anti-epileptic drugs such as gabapentin and lamotrigine and anti-depressant drugs such as the selective serotonin and norepinephrine reuptake inhibitor duloxetine have been recently investigated for the treatment of neuropathic pain (Segal and Rordorf 1996; Canavero and Bonicalzi 1997; Lunardi, Leandri et al. 1997; Eisenberg, Lurie et al. 2001). A summary table of the effect of these drugs on OA pain behaviours is located in Table 4-2.

Table 4-2 The effect of analgesic drugs on OA pain behaviours.

Early phase represents day 1-14 post surgery. Late phase represents day 42-84 post-surgery. Statistics: Pre-drug measurements compared to post-drug measurements by unpaired t-test, ** P<0.01, *** P<0.001

	Mechanical Allodynia PWT (g)		**Mechanical Hyperalgesia PWT (g)**		**Cold Allodynia PWL (s)**		**Knee Compression (no. vocalisations)**	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Late Phase								
Gabapentin 60mg/kg p.o.	0.18 ± 0.04	**0.56 ± 0.07 *****	73.00 ± 4.84	76.50 ± 5.33	6.94 ± 0.61	7.70 ± 0.50	3.80 ± 0.44	3.20 ± 0.57
Lamotrigine 30mg/kg p.o.	0.34 ± 0.07	0.39 ± 0.05	79.00 ± 6.23	87.00 ± 5.12	6.83 ± 0.69	8.23 ±0.77	2.50 ± 0.48	2.60 ± 0.34
Duloxetine 30mg/kg i.p.	0.21 ± 0.03	**0.50 ± 0.03 *****	80.5 ± 2.73	**54.5 ± 4.5 *****	7.68 ± 0.63	**5.41 ± 0.43 ****	-	-

4.3.5.1 Gabapentin

Single administration of gabapentin (60mg/kg p.o) significantly reversed mechanical allodynia P=0.00014 (Figure 4-15a) of the ipsilateral hind limb when administered at day 49 post-partial medial meniscectomy. It had no significant effect on mechanical hyperalgesia, cold allodynia and vocalisations in response to knee compression (Figure 4-15b, Figure 4-16a and b).

4.3.5.2 Lamotrigine

Lamotrigine, an anti-epileptic drug occasionally prescribed for management of neuropathic pain, was administered to mice at day 49 following partial medial meniscectomy (30mg/kg p.o.). Lamotrigine produced no significant effect on any of the pain behaviours tested (Figure 4-17 and Figure 4-18).

4.3.5.3 Duloxetine

Duloxetine (30mg/kg i.p.) produced a significant reduction in mechanical allodynia ($P<0.00001$) (Figure 4-19a) when administered 42 days post-partial medial meniscectomy. However, once restrained for paw pressure and cold allodynia measurements, the drug treated animals became agitated resulting in unblinding of the groups in the experiment. Results showed a significant increase in mechanical hyperalgesia and cold allodynia (Figure 4-19b, Figure 4-20a), however, lower paw thresholds may be due to intolerance to restraint rather than a specific pain effect. The treated mice could not be restrained sufficiently for the knee compressions to be performed and this measurement was abandoned.

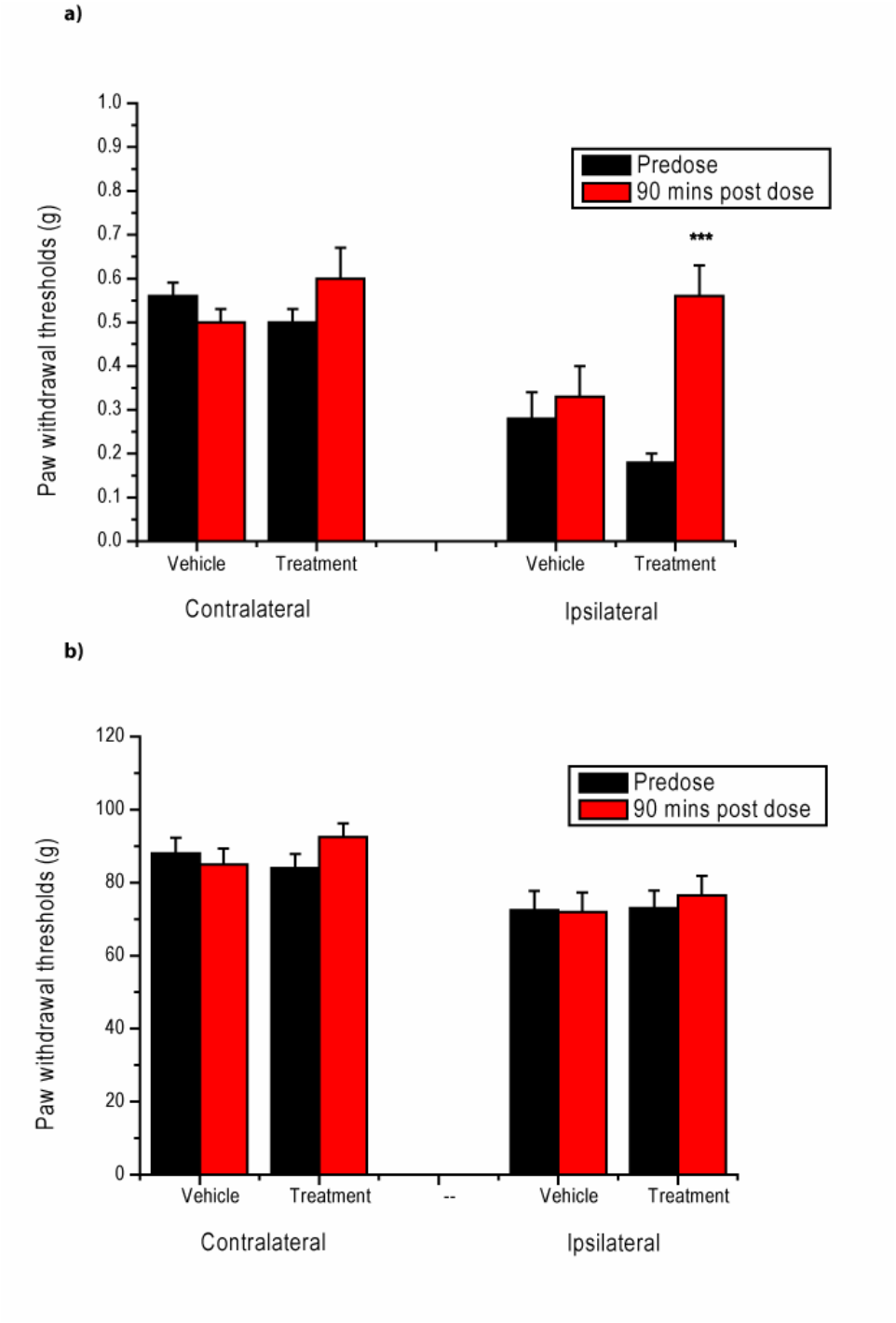


Figure 4-15 The effect of 60mg/kg p.o. gabapentin at day 49 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of gabapentin on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *** P<0.001

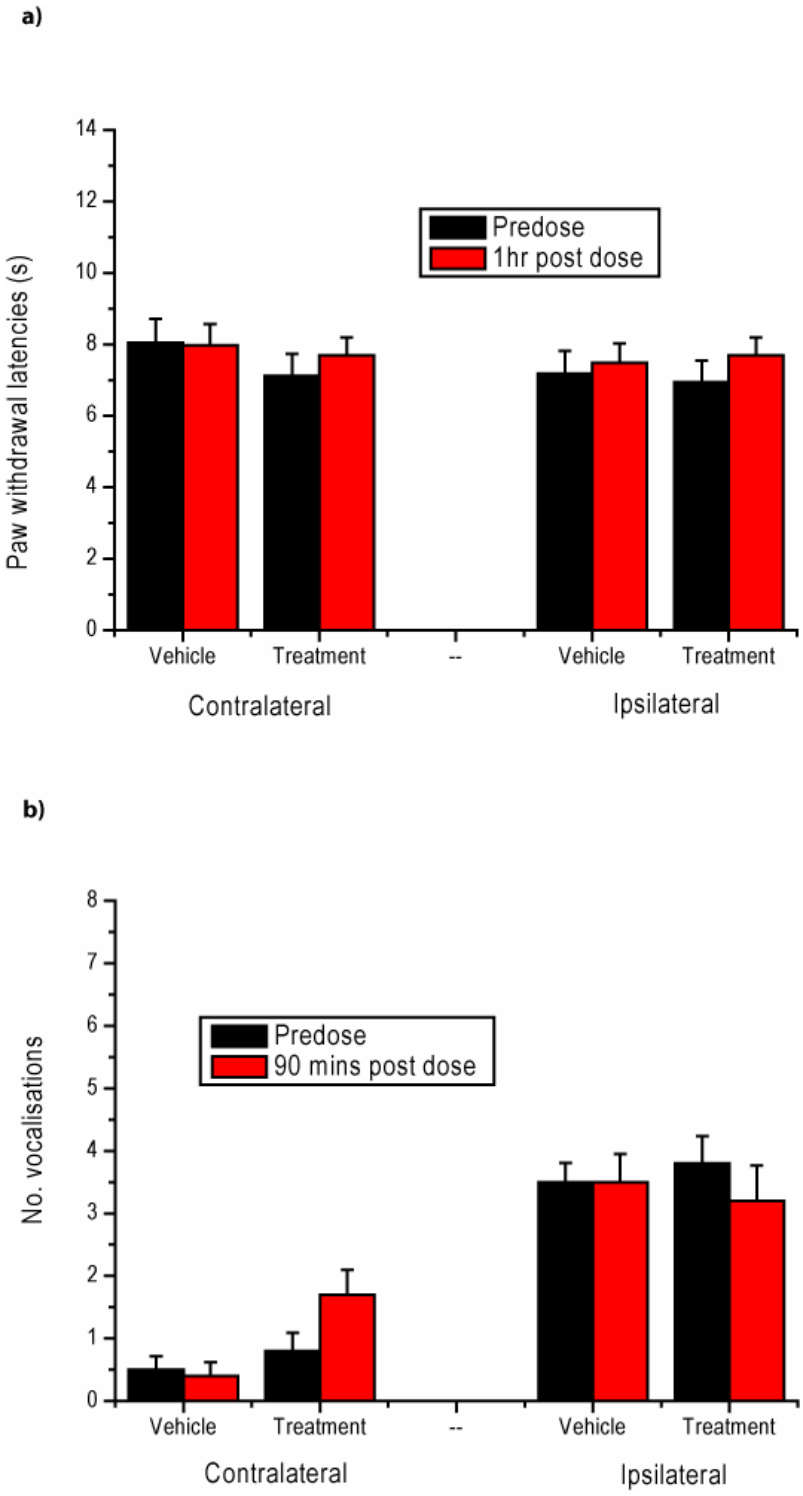


Figure 4-16 The effect of 60mg/kg p.o. gabapentin at day 49 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of gabapentin on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice.

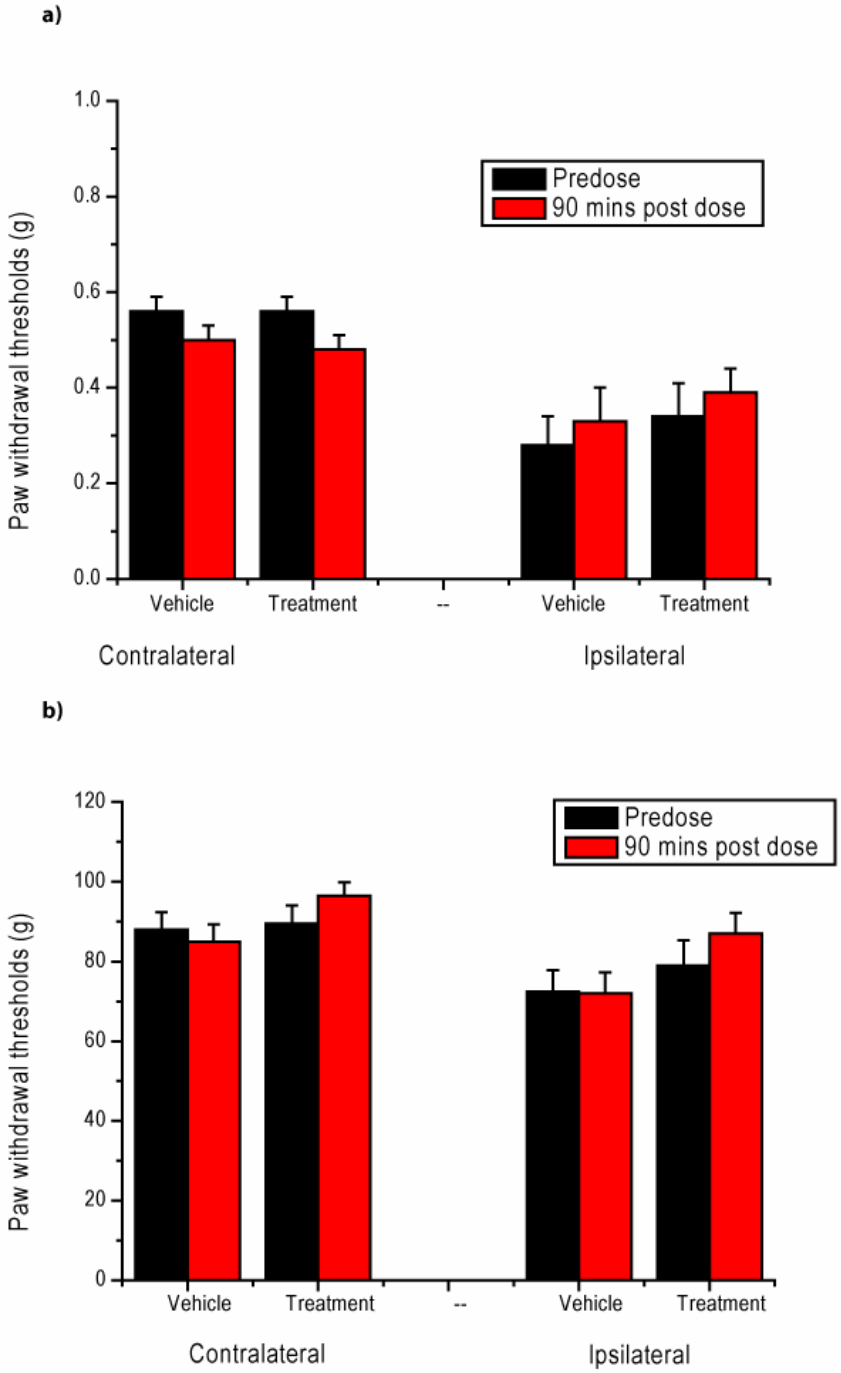


Figure 4-17 The effect of 30mg/kg p.o. lamotrigine at day 49 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of lamotrigine on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice.

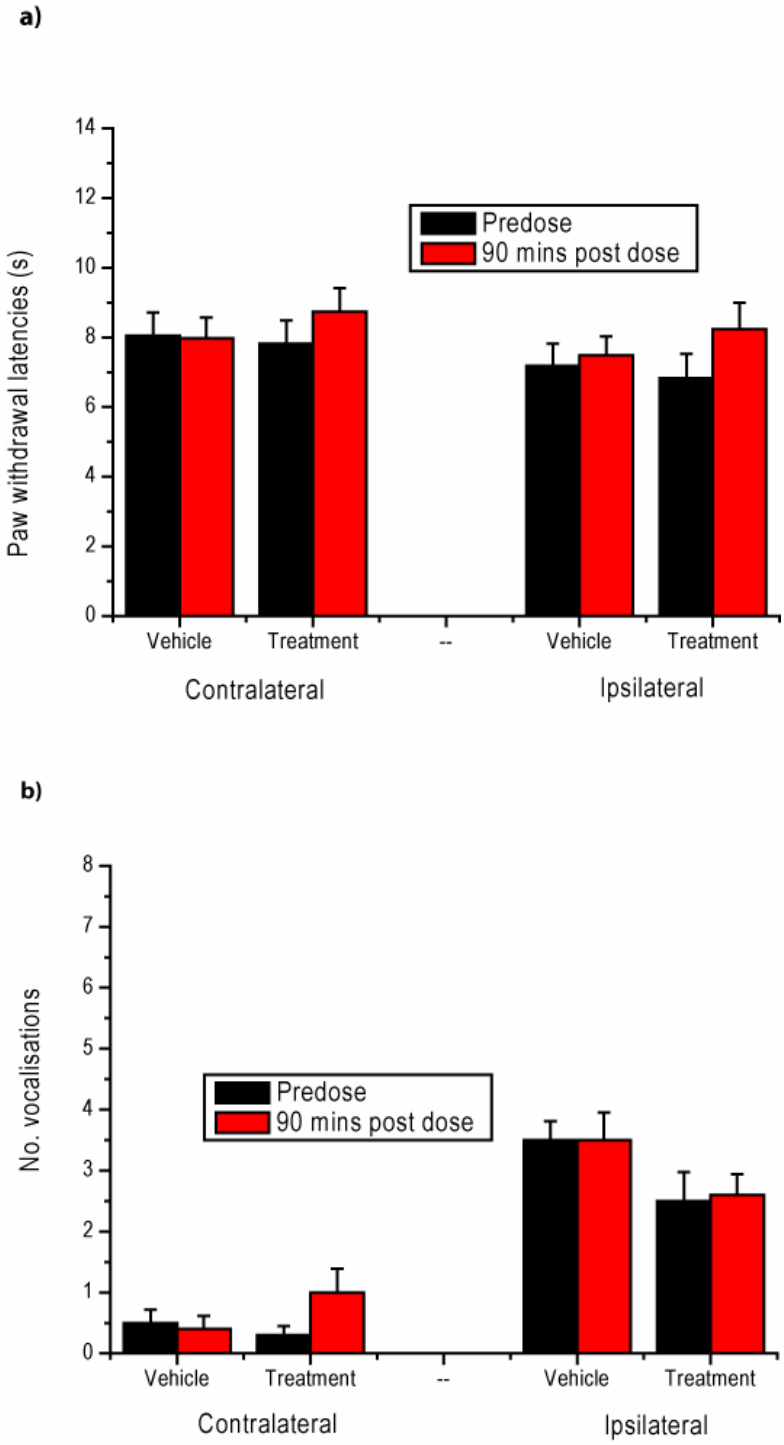


Figure 4-18 The effect of 30mg/kg p.o. lamotrigine at day 49 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of lamotrigine on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice.

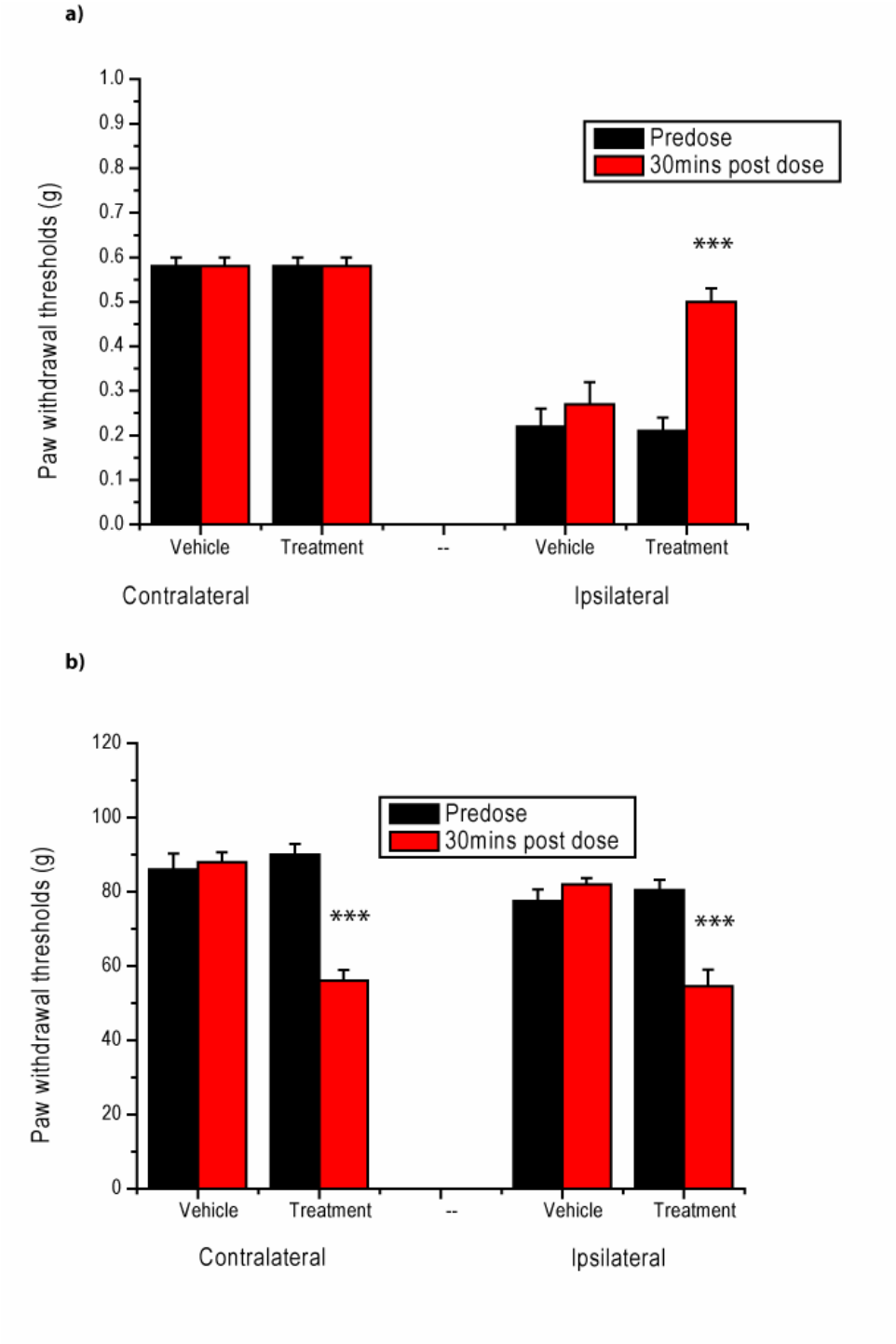


Figure 4-19 The effect of 30mg/kg i.p. duloxetine at day 42 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of duloxetine on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *** P<0.001

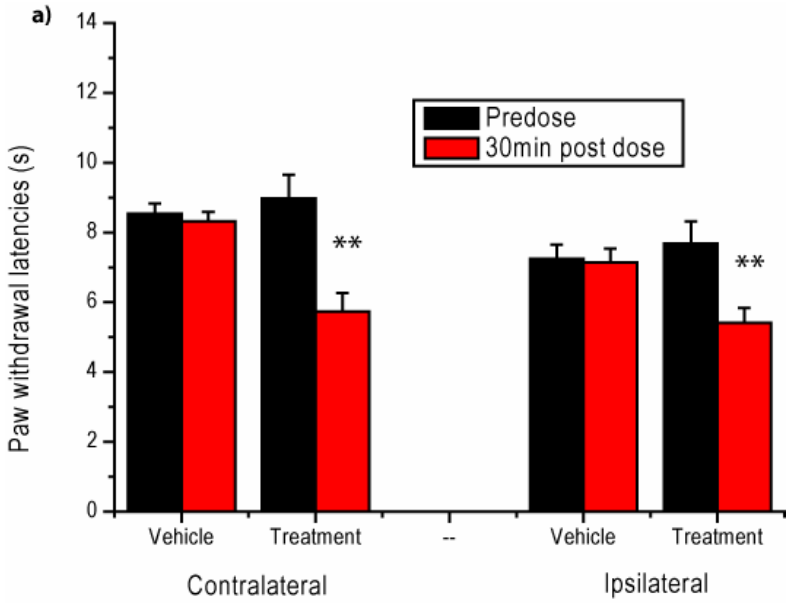


Figure 4-20 The effect of 30mg/kg i.p. duloxetine at day 42 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of duloxetine on cold allodynia a). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01.

4.3.6 Role of the endogenous opioid system in OA

Endogenous opioids have been previously shown to modulate pain behaviours in a murine DMM model of OA pain (Inglis, McNamee et al. 2008). In the partial medial meniscectomy model, 3 of the pain behaviours (mechanical hyperalgesia, cold allodynia and knee compressions) diminished at weeks 3 and 4 following surgery (Figure 3-18, Figure 3-19, Figure 4-1 and Figure 4-2). This rebound effect has also been measured at various time points in the late phase of the model (Figure 4-1 and Figure 4-2). On each occasion, these pain behaviours were greatly reduced (often approaching baseline levels), for 1-2 weeks before returning to previous levels of hypersensitivity. Administration of naloxone at times of reduced sensitivity caused the pain behaviours to return indicating that the reduction in hypersensitivity may be mediated by endogenous opioids (Figure 4-21 and Figure 4-22). Administration of the peripherally restricted opioid antagonist, naloxone methiodide, at day 28 post-surgery produced the same effect as naloxone indicating that the reversal of pain behaviours was due to peripheral rather than centrally acting endogenous opioids (Figure 4-23 and Figure 4-24). Similarly, naloxone methiodide administration at day 84 post-surgery resulted in a significant increase in ipsilateral and contralateral

mechanical hyperalgesia (P=0.00035 and P=0.00019, respectively) and cold allodynia (P=0.014 and P=0.014, respectively) (Figure 4-25). An increased number of vocalisations in response to compression of the ipsilateral knee from 1.6 to 3.5 vocalisations was also noted, however, this was not statistically significant (P=0.052, Figure 4-26). These results suggest that peripherally acting endogenous opioids play a significant role in the temporary reduction of hypersensitivities following partial medial meniscectomy.

In order to characterise the endogenous opioids further, receptor antagonists were used to inhibit the action of the endogenous opioids at mu, kappa and delta receptors. Figure 4-27 and Figure 4-28 show that administration of mu and kappa receptor antagonists (Naloxonazine and GNTI, respectively) produced a significant reduction in ipsilateral PWTs in mechanical hyperalgesia ($P<0.00001$, $P<0.001$) cold allodynia ($P<0.001$, $P<0.05$) and vocalisations in response to knee compressions ($P<0.01$, $P<0.01$). The results indicate that the endogenous opioids involved in modulating the levels of OA pain act predominantly on mu and kappa opioid receptors.

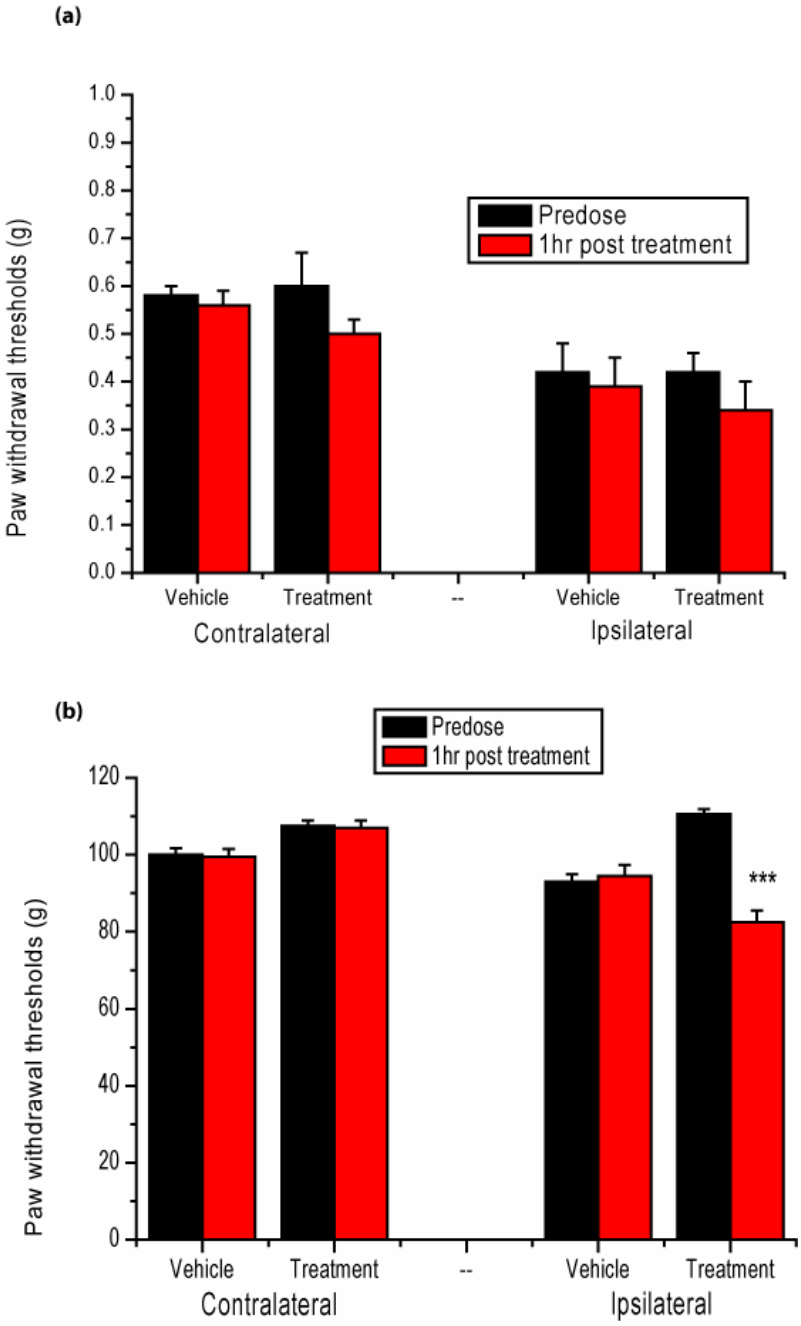


Figure 4-21 The effect of 2.5mg/kg i.p. naloxone at day 21 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of naloxone on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *** P<0.001

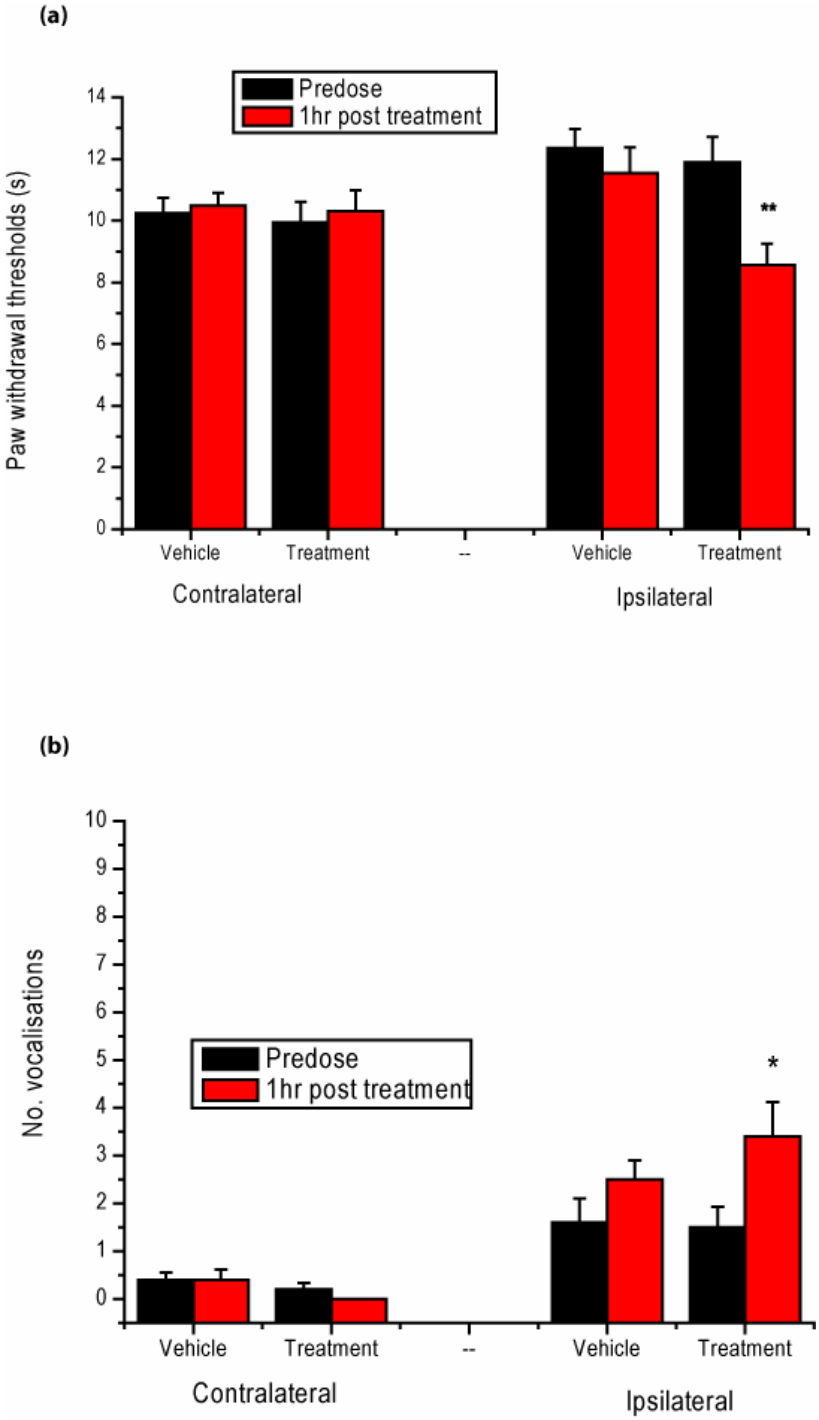


Figure 4-22 The effect of 2.5mg/kg i.p. naloxone at day 21 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of naloxone on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *P<0.05, ** P<0.01.

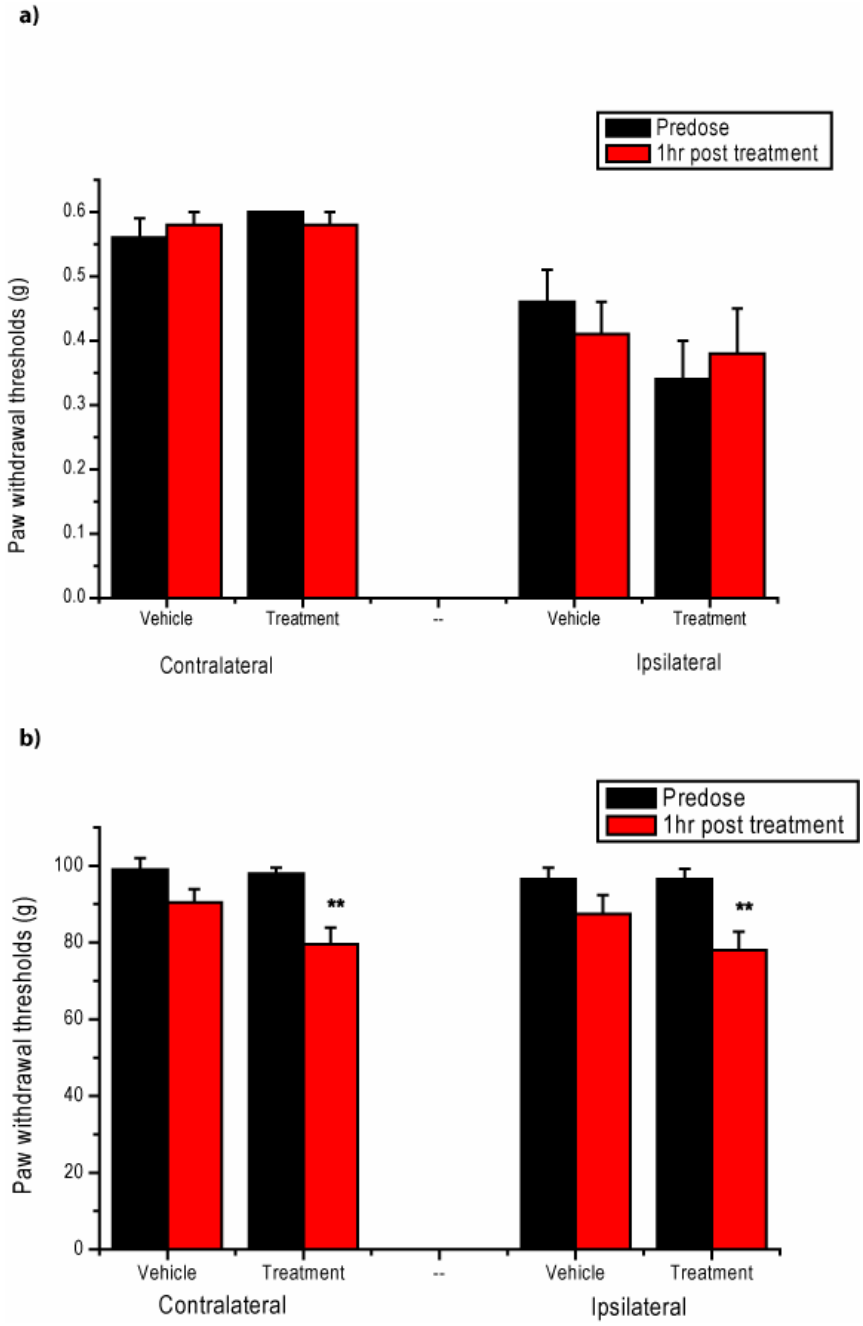


Figure 4-23 The effect of 2.5mg/kg i.p. naloxone methodide at day 28 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of naloxone on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** $P<0.01$.

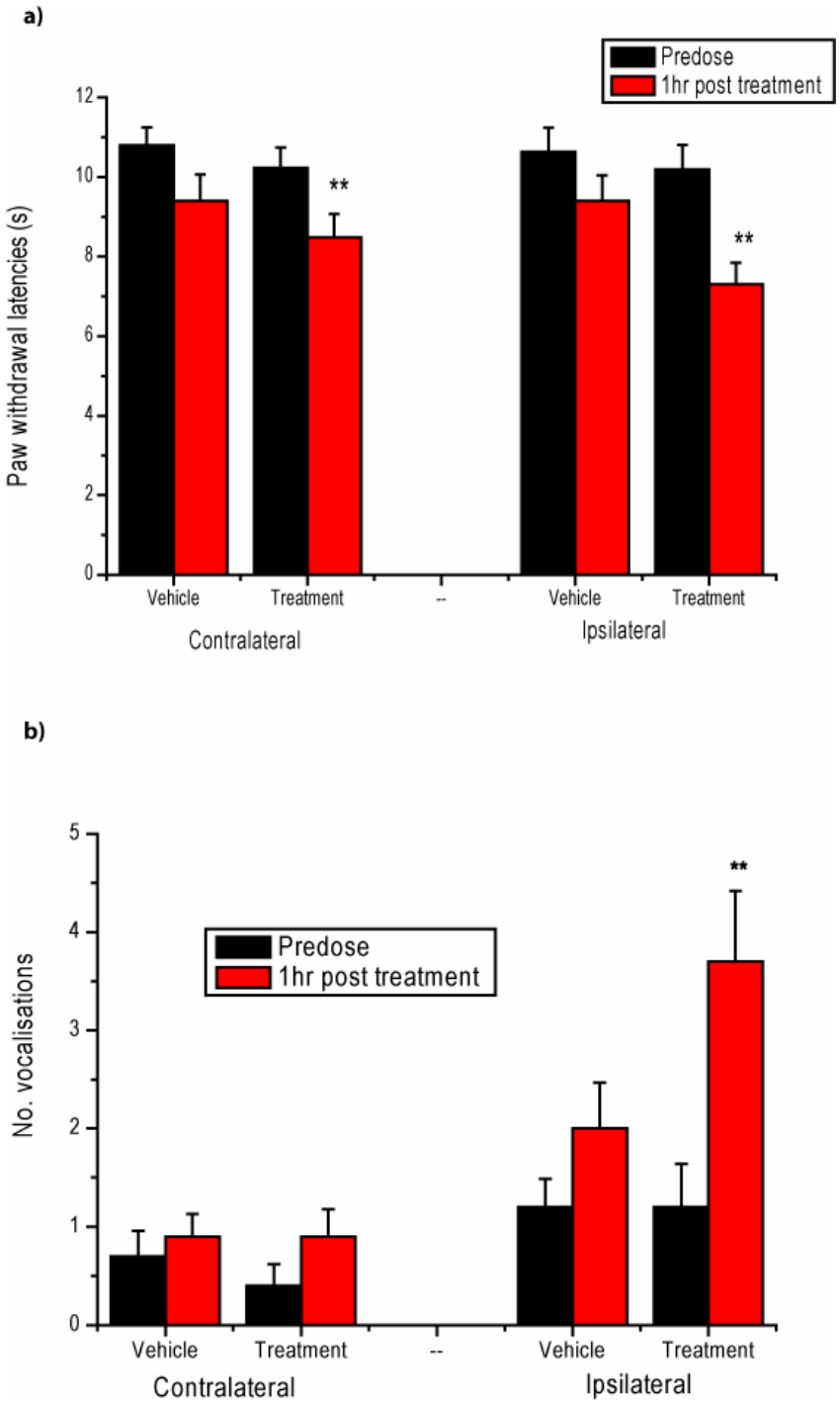


Figure 4-24 The effect of 2.5mg/kg/ i.p. naloxone methodide at day 28 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of naloxone methodide on cold allodynia a) and vocalisations during knee compressions b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, * P<0.05, ** P<0.01

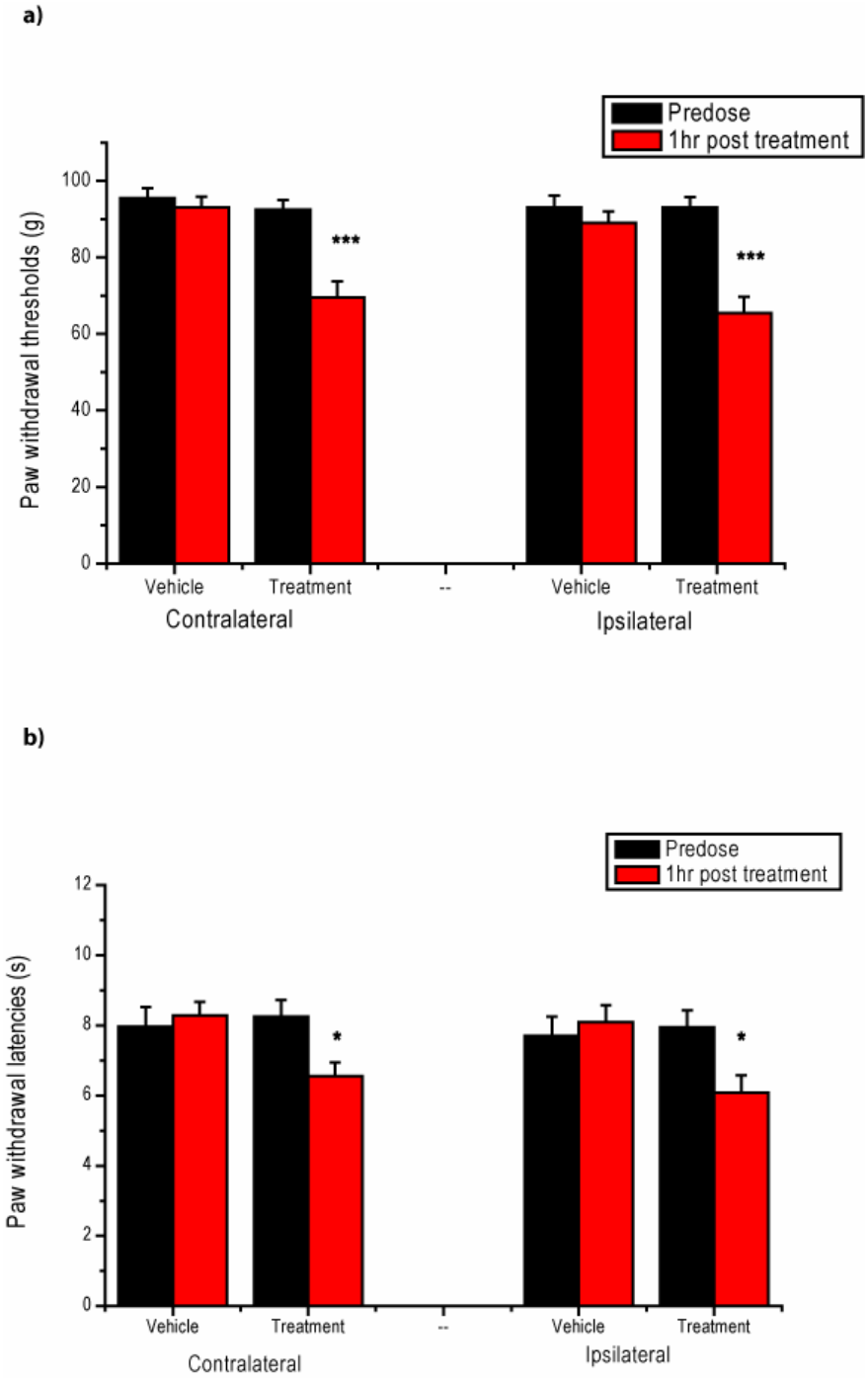


Figure 4-25 The effect of 2.5mg/kg i.p. naloxone methodide at day 84 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of naloxone methodide on mechanical hyperalgesia a) cold allodynia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, * P<0.05, *** P<0.001

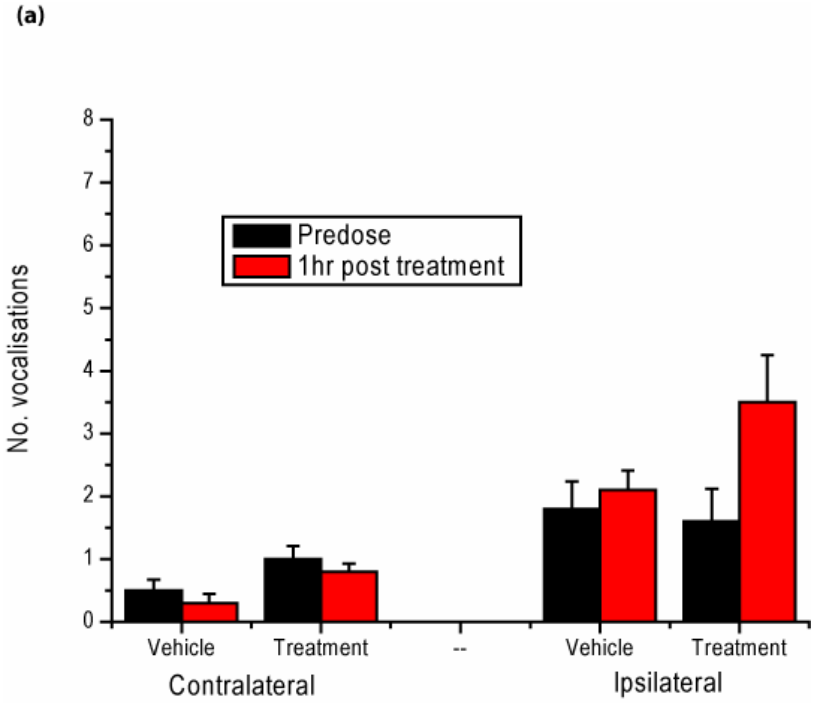


Figure 4-26 The effect of 2.5mg/kg i.p. naloxone methodide at day 84 post partial medial meniscectomy of female C57Bl/6 mice.

The effect of naloxone methodide on vocalisations during ten knee compressions a). Each point represents the mean value ± SEM of ten mice

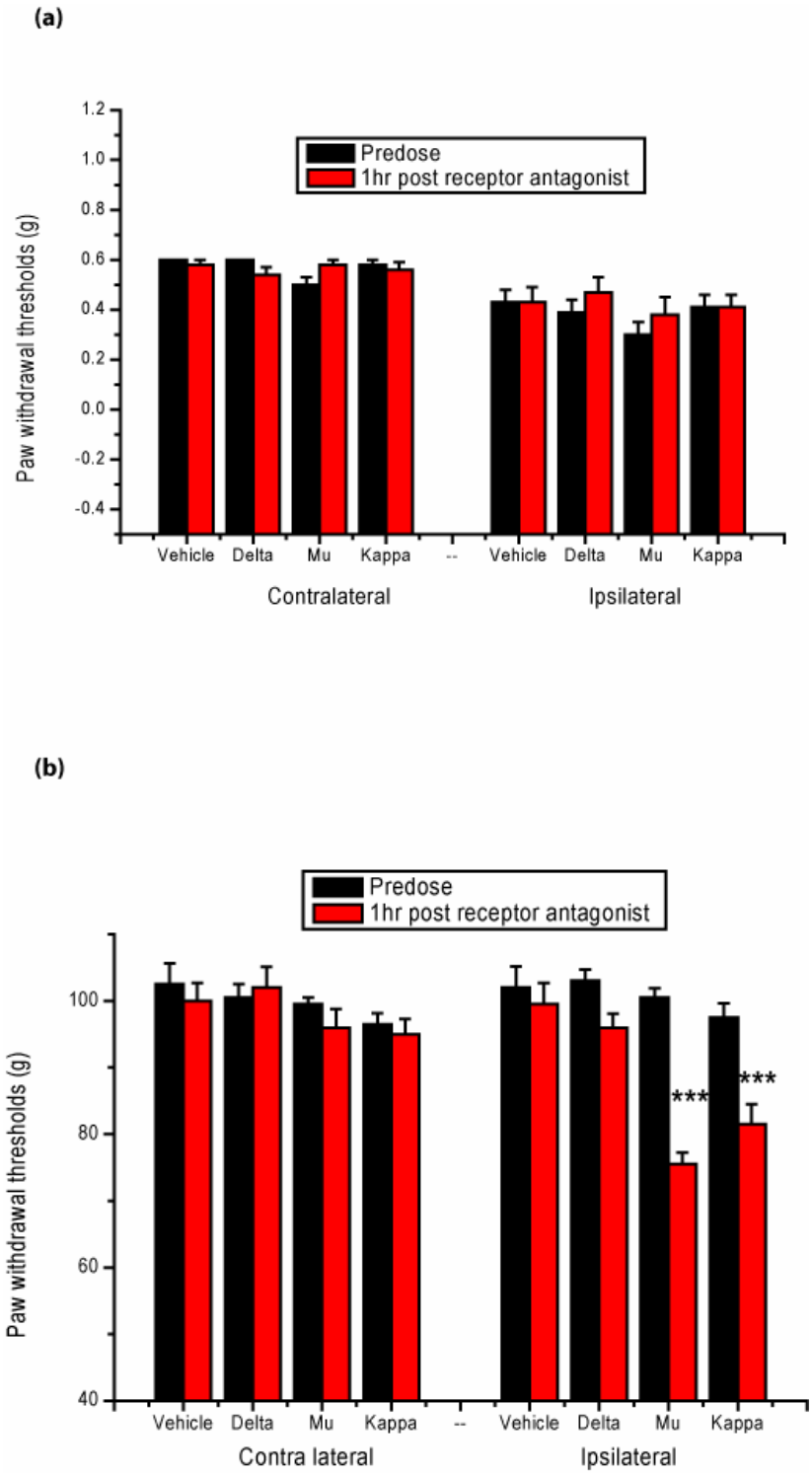


Figure 4-27 The effect of vehicle, δ opioid receptor antagonist naltrindole, μ opioid receptor antagonist naloxonazine, and κ opioid receptor antagonist GNTI on pain behaviours 21 days following partial medial meniscectomy.

The effect on mechanical allodynia (a) and mechanical hyperalgesia (b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, *** P<0.001

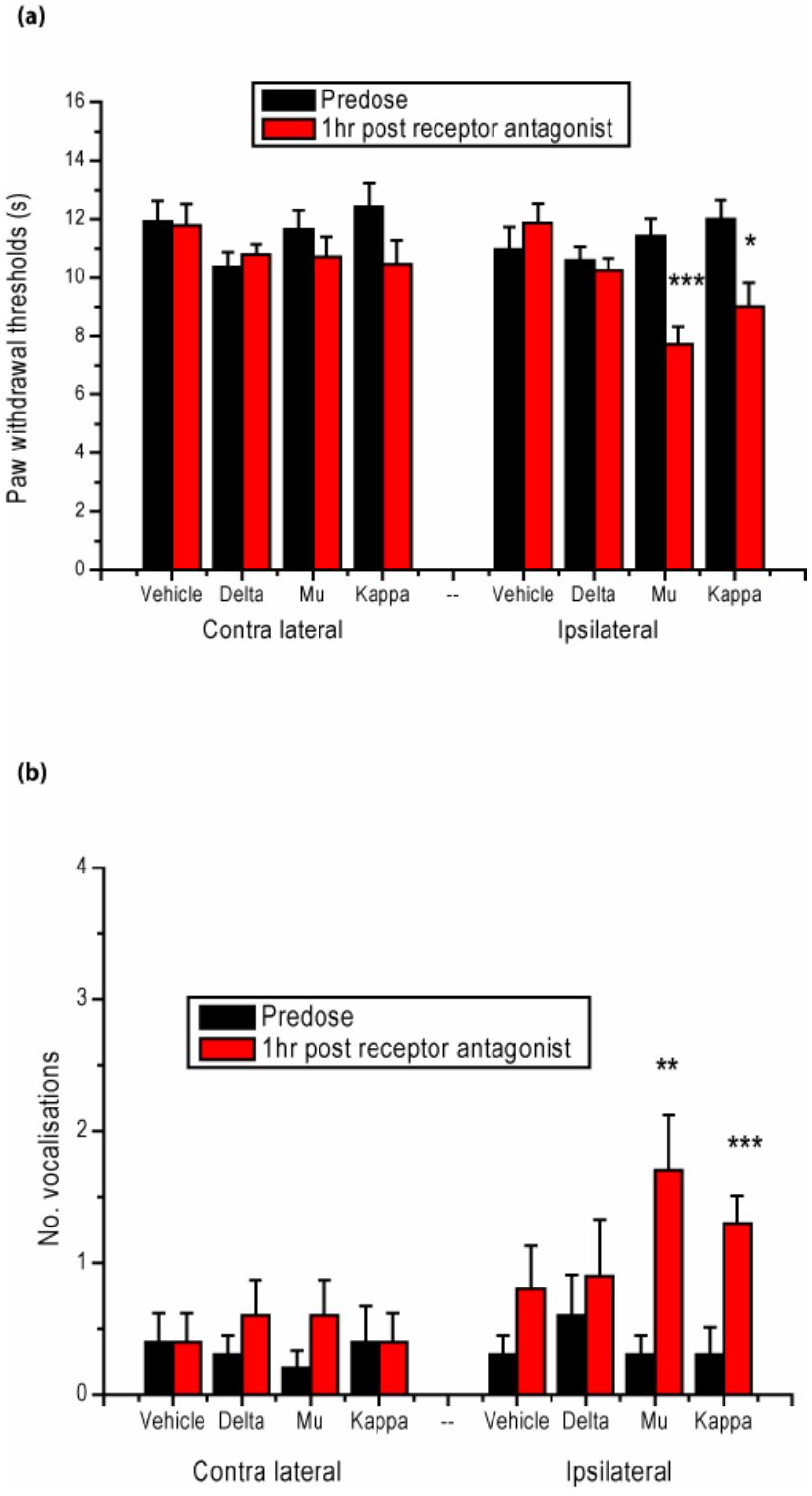


Figure 4-28 The effect of vehicle, δ opioid receptor antagonist naltrindole, μ opioid receptor antagonist naloxonazine, and κ opioid receptor antagonist GNTI on pain behaviours 21 days following partial medial meniscectomy.

The effect on cold allodynia (a) knee compression (b) on the ipsilateral hind limb are shown. Each point represents the mean value ± SEM of ten mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, * P<0.05, ** P<0.01, *** P<0.001

4.3.7 Joint pathology and pain behaviours following partial medial meniscectomy on male, sham ovariectomised female (SOVX) and ovariectomised female (OVX) C57Bl/6 mice

A preliminary experiment was performed to identify whether pain behaviours induced by partial medial meniscectomy would also develop in male C57Bl/6 mice and to study whether the pattern of hypersensitivity would be different in female C57Bl/6 mice that had been ovariectomised at 4 weeks of age (pre-puberty).

4.3.7.1 General

10 male, 4 week old C57Bl/6 mice (12g ± 1g) and 20 female 4 week old C57Bl/6 mice (12g ± 1g) were used for this study. The female mice underwent either bilateral ovariectomy (OVX) or bilateral sham ovariectomy (SOVX) surgery under general anaesthesia at 4 weeks of age. 4 weeks later, behavioural measurements were recorded using von Frey filaments, paw pressure, cold sensitivity and knee compressions prior to partial medial meniscectomy being performed on all thirty mice. Post-surgery behaviour measurements were recorded at weekly intervals. Sham meniscectomised male mice were not used in this study since earlier studies indicated that male mice who underwent sham meniscectomy surgery did not develop pain hypersensitivities in the ipsilateral or contralateral hind limbs (see Figure 5-26 and Figure 5-27).

The male, SOVX and OVX mice maintained good health throughout the study. All groups of mice recovered well from surgery and no complications were noted. Whilst the weights of the 3 groups of mice were comparable at the beginning of the study (12g ± 1g), at age 16 weeks (8 weeks post-partial meniscectomy), male mice (28.9g ± 0.7g) weighed significantly more than SOVX mice (21.9g ± 0.5g, $P<0.001$) and OVX mice (24.9g ± 0.6g, $P<0.001$). Furthermore, OVX mice weighed significantly more than SOVX mice ($P<0.01$).

4.3.7.2 Joint Pathology

At the end of the behavioural study, 5 mice from each group were killed by cervical dislocation and the ipsilateral and contralateral hind limbs removed for histological analysis (Table 4-3 and Table 4-4). A slower method of decalcification of the knee joints was used in these experiments to achieve greater preservation of the subchondral bone and allow for the assessment of osteophytes. At 8 weeks post partial medial meniscectomy, male mice ($P=0.023$), (OVX) mice ($P=0.0015$) and (SOVX) mice ($P=0.0082$) showed significant cartilage pathology in the operated (left) knee compared to the non-operated (right) knee (Figure 4-29). Male mice had the most severe cartilage scores with an average joint score of 8.04 (± 0.5) and

maximal joint score of 11.80 (± 0.4) at 8 weeks post surgery. A representative image is shown in Figure 4-30. SOVX mice had milder cartilage lesions with an average joint score of 4.89 ± 0.06 (Table 4-3) and a maximal joint score of 9.7 ± 0.5 (Table 4-4). OVX mice also had mild-moderate cartilage pathology with an average joint score of 5.39 ± 0.62 and maximal joint score of 9.1 ± 0.1. There was no significant difference between OVX and SOVX mice in this study based on the degrees of cartilage erosion. In all three groups of mice, the most severely affected joint quadrant was the medial tibial plateau (Table 4-4).

In this study, SOVX mice had a lower average joint score of 4.89 ± 0.06 compared to 6.55 ± 1.26 in the previous study of female mice (Table 3-4) and a lower maximal joint score of 9.7 ± 0.5 compared to 11.67 ± 2.45 (Table 3-5). However, the increased number of samples and differences in tissue processing techniques may have had some bearing on joint score making direct comparisons difficult.

The presence or absence of osteophytes in the joint sections was scored according to 2.6.5.1. Osteophytes were observed in the ipsilateral knee of all groups of mice who underwent partial medial meniscectomy and were not identified in the sections of the contralateral knees. OVX mice displayed the greatest degree of osteophytosis with a combined average score of 0.36 ± 0.04 (Table 4-5). Male mice had a combined average score for osteophytes of 0.34 (± 0.02). SOVX mice showed the lowest frequency of osteophytes with a combined average score of 0.22 ± 0.02, although there was no significant difference between the groups.

Table 4-3 Quantitative joint pathology of male, SOVX and OVX C57Bl/6 mice 8 weeks post partial medial meniscectomy.

Average joint scores where L= left knee R= right knee. Values represent the mean value ± SEM of five mice.

	Average LTP (/6)	Average LFC (/6)	Average MTP (/6)	Average MFC (/6)	Combined Average Joint Score (/24)
SOVX Week 8 L	1.10 (± 0.10)	1.05 (± 0.18)	1.42 (± 0.06)	1.32 (± 0.16)	4.89 (± 0.06)
OVX Week 8 L	0.94 (± 0.18)	1.46 (± 0.22)	1.56 (± 0.12)	1.42 (± 0.10)	5.38 (± 0.62)
Male Week 8 L	1.42 (± 0.18)	1.58 (± 0.06)	2.86 (± 0.18)	2.18 (± 0.06)	8.04 (± 0.48)
SOVX Week 8 R	0.46 (± 0.18)	0.25 (± 0.05)	0.18 (± 0.06)	0.24 (± 0.04)	1.13 (± 0.33)
OVX Week 8 R	0.12 (± 0.04)	0.22 (± 0.02)	0.02 (± 0.02)	0.02 (± 0.02)	0.38 (± 0.06)
Male Week 8 R	0.18 (± 0.10)	0.26 (± 0.10)	0.10 (± 0.06)	0.06 (± 0.06)	0.60 (± 0.32)

Table 4-4 Quantitative joint pathology of male, SOVX and OVX C57Bl/6 mice 8 weeks post partial medial meniscectomy.

Maximal joint scores where L= left knee R= right knee. Values represent the mean value ± SEM of five mice.

	Maximal LTP (/6)	**Maximal LFC (/6)**	**Maximal MTP (/6)**	**Maximal MFC (/6)**	**Combined Maximal Joint Score (/24)**
SOVX Week 8 L	2.20 (± 0.00)	2.4 (± 0.04)	2.80 (± 0.20)	2.3 (± 0.10)	9.70 (± 0.50)
OVX Week 8 L	2.3 (± 0.30)	2.1 (± 0.10)	2.30 (± 0.10)	2.4 (± 0.20)	9.1 (± 0.1)
Male Week 8 L	2.40 (± 0.00)	2.40 (± 0.00)	3.90 (± 0.30)	3.10 (± 0.10)	11.80 (± 0.4)
SOVX Week 8 R	1.10 (± 0.10)	1.10 (± 0.10)	0.50 (± 0.10)	0.60 (± 0.20)	3.30 (± 0.30)
OVX Week 8 R	0.40 (± 0.00)	0.50 (± 0.10)	0.00 (± 0.00)	0.20 (± 0.20)	1.10 (± 0.30)
Male Week 8 R	0.80 (± 0.40)	0.9 (± 0.10)	0.60 (± 0.40)	0.40 (± 0.40)	2.70 (± 1.30)

Table 4-5 Quantitative osteophyte score for knee joints of male, SOVX and OVX C57Bl/6 mice 8 weeks post partial medial meniscectomy.

Average joint scores for osteophytosis where L= left knee R= right knee. Values represent the mean value ± SEM of five mice.

	Average LTP (/1)	Average LFC (/1)	Average MTP (/1)	Average MFC (/1)	Combined Average Osteophyte Score (/4)
SOVX Week 8 L	0.00 (± 0.00)	0.12 (± 0.04)	0.00 (± 0.00)	0.10 (± 0.06)	0.22 (± 0.02)
OVX Week 8 L	0.06 (± 0.02)	0.1 (± 0.02)	0.1 (± 0.02)	0.1 (± 0.02)	0.36 (± 0.04)
Male Week 8 L	0.00 (± 0.00)	0.00 (± 0.00)	0.2 (± 0.04)	0.14 (± 0.02)	0.34 (± 0.02)
SOVX Week 8 R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
OVX Week 8 R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
Male Week 8 R	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)

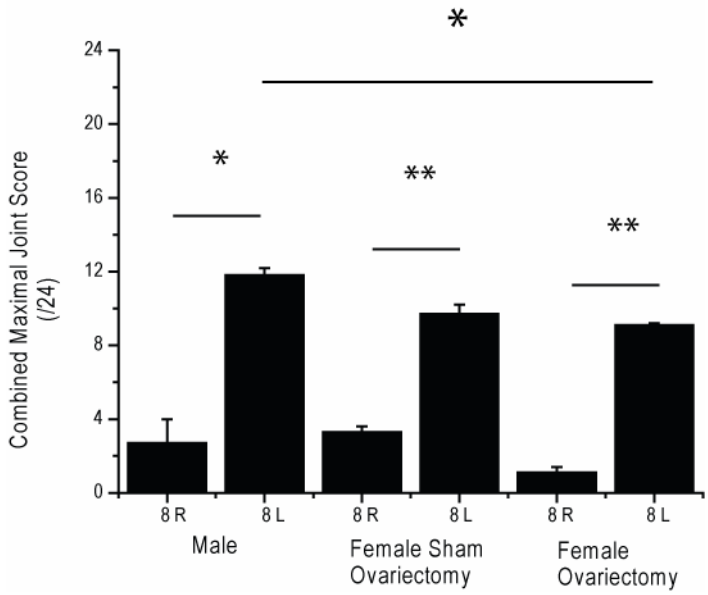


Figure 4-29 Quantitative scoring of osteoarthritic joint pathology 8 weeks post partial medial meniscectomy. Each point represents the mean value ± SEM of five mice where L= operated left leg R= un-operated right leg. Statistics: * P<0.05, ** P<0.01 by unpaired t-test.

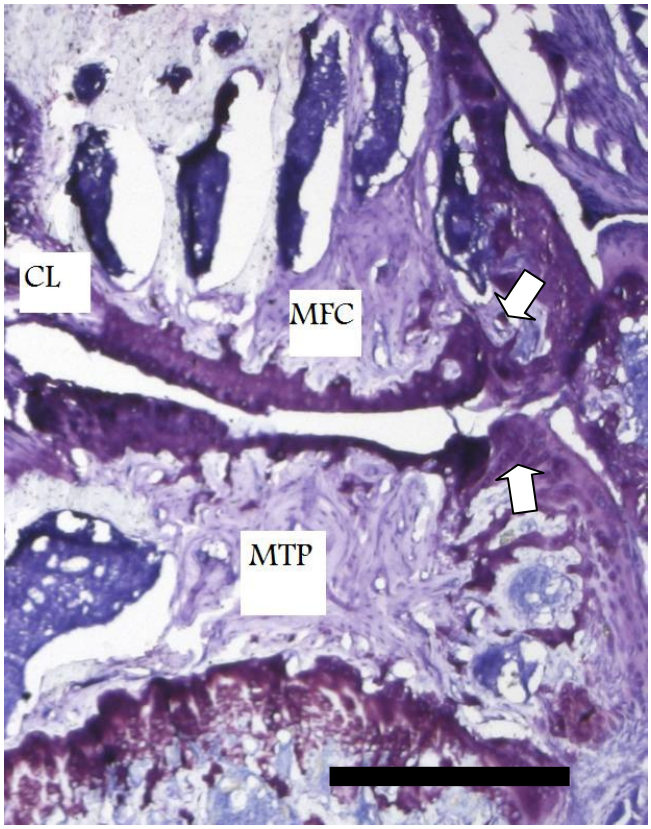


Figure 4-30 Representative 12μm thick section through the medial knee joint of a male C57Bl/6 mouse stained with toluidine blue with cruciate ligament (CL) to the left; medial femoral condyle (MFC) at the top, medial tibial plateau (MTP) at the bottom and osteophytes (⇐) of the ipsilateral knee joint 8 weeks post-meniscectomy. Scale bar: 1 mm.

4.3.7.3 Behaviour

Behavioural tests were performed at weekly intervals following partial medial meniscectomy to determine whether OA pain behaviours were influenced by sex hormones. Results are shown in Figure 4-31 to Figure 4-33.

4.3.7.3.1 Male mice

Male mice developed a persistent mechanical allodynia in the ipsilateral hind limb from day 7 onwards compared to pre-surgery levels ($P<0.001$, Figure 4-31a). The lowest recorded PWT was 0.15g ± 0.03g at day 35 post surgery. Unlike the previous studies with female C57Bl/6 mice, male mice exhibited a significant contralateral allodynia from day 35 ($P<0.001$) onwards with a lowest contralateral PWT of 0.22g ± 0.05g recorded at day 35.

Male mice also developed a waxing and waning mechanical hyperalgesia (Figure 4-31b). However, unlike earlier female studies, no mechanical hyperalgesia was noted at day 7 following surgery. Ipsilateral PWTs decreased significantly at day 14 ($P<0.001$) and 21 ($P<0.001$); these thresholds returned to baseline levels at day 28 and 35. Ipsilateral PWTs were significantly decreased at day 42 ($P<0.001$) but returned to near baseline levels at day 49 and day 56 with a PWT of 94.5g ± 2.93g and 94.5g ± 2.52g, respectively. The lowest ipsilateral PWT was recorded at 61.5g ± 2.69g at day 42 post surgery. No contralateral mechanical hyperalgesia was recorded in this study.

The development of cold allodynia in the ipsilateral hind limb followed a similar pattern to mechanical hyperalgesia with male mice developing a waxing and waning ipsilateral hind paw cold sensitivity (Figure 4-31c). Paw withdrawal latencies were significantly decreased at day 14 ($P<0.001$) and day 21 ($P<0.001$) compared to pre-meniscectomy levels. The lowest recorded ipsilateral PWL was 6.99s ± 0.55s at day 21 post-surgery. Paw withdrawal latencies returned to baseline levels at day 28 and 35. Paw withdrawal latencies decreased to 7.10s ± 0.39s at day 42 ($P<0.001$) and increased at day 49 and 56 although they remained significantly lower than pre-surgery readings at these time points. In contrast to mechanical hyperalgesia readings, a significant contralateral cold allodynia was not recorded at any time point.

Male mice showed significantly increased number of vocalisations in response to knee compression at day 21 ($P<0.001$), day 28 ($P<0.001$) and day 42 ($P<0.01$) post-surgery (Figure 4-31d). The greatest number of vocalisations was 2.5 ± 0.43 at day 28. There was no significant increase in the number of vocalisations from compression of the contralateral knee in male mice.

The potential role of endogenous opioid tone on the absence of pain behaviours at day 56 is described in section 4.3.7.4).

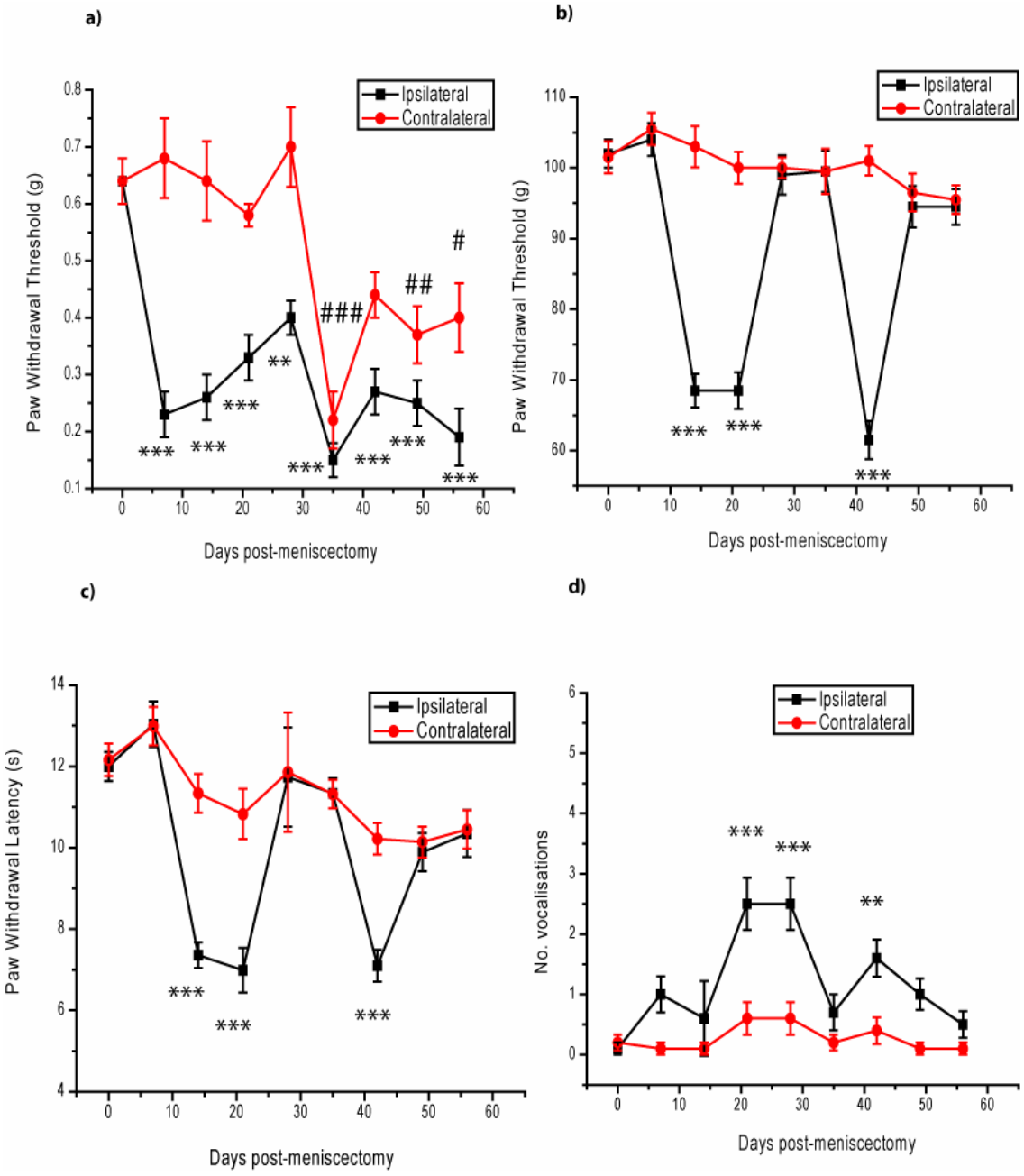


Figure 4-31 The effect of OA induction on behaviour of male C57Bl/6 mice following partial medial meniscectomy.

Test for the development of mechanical allodynia a) mechanical hyperalgesia b) cold allodynia c) and knee vocalisations d). Each point represents the mean value ± SEM of ten mice. Statistics: Ipsilateral reading versus baseline by one way ANOVA followed by Tukey's post hoc Test ** P< 0.01, *** P< 0.001. Contralateral reading versus baseline by one way ANOVA followed by Tukey's post hoc Test, # P<0.05, ## P< 0.01, ### P<0.001.

4.3.7.3.2 Sham ovariectomised mice (SOVX)

The pattern of hypersensitivities in SOVX mice differed dramatically from previous female C57Bl/6 studies with an absence of 2 clear phases of hypersensitivity. No significant pain behaviours were recorded using the paw pressure test, or cold plate test in the period immediately after surgery (day 7 and day 14) and later pain sensitivities did not fluctuate in the same manner.

SOVX mice did develop a persistent mechanical allodynia, however, from day 7 onwards with significantly decreased PWTs following post partial meniscectomy compared to pre-meniscectomy measurements ($P<0.01$, Figure 4-32a). The lowest PWT was 0.15g ± 0.03g recorded at day 56 post surgery. SOVX mice showed a subtle decrease in contralateral PWT in response to von Frey hairs (Figure 4-32a). The lowest contralateral PWT was recorded at 0.48g ± 0.03 at day 35 ($P<0.01$) compared to pre surgery levels which was similar to the lowest contralateral PWT recorded in the initial female partial meniscectomy studies in which the lowest PWT was 0.50g ± 0.06g at day 84 post surgery (Figure 3-17b).

SOVX mice did not develop significant levels of mechanical hyperalgesia in the ipsilateral hind limb until day 21 post surgery (Figure 4-32b). Paw withdrawal thresholds were decreased significantly from day 21 ($P<0.001$) onwards with the lowest PWT of 62.0g (± 2.38g) recorded at day 28. Mechanical hyperalgesia persisted until day 56 where PWTs returned to baseline levels (PWT of 96.0g ± 1.63g). Similarly, SOVX mice did not develop significant levels of cold sensitivity in the ipsilateral hind paw until day 21 post-meniscectomy where PWL was decreased to 7.17s ± 0.52s ($P<0.001$, Figure 4-32c). Significant cold allodynia persisted with lowest PWLs being recorded at day 42 at 6.54s ± 0.36s. At day 56, PWL was increased to 10.07s ± 0.23s and was no longer significantly lower than baseline cold sensitivity readings. During the study, contralateral PWTs decreased slightly compared to baseline levels. The lowest contralateral PWL was 9.30s ± 0.25s recorded at day 28.

SOVX mice exhibited a persistently greater number of vocalisations in response to knee compression of the ipsilateral knee compared to baseline levels from day 7 ($P<0.001$, Figure 4-32d) onwards. At day 49 and day 56 post-surgery, the number of vocalisations reduced to near baseline levels with mice vocalising 0.5 times (± 0.22) by day 56. The greatest number of vocalisations was 3.0 ± 0.3 recorded at day 7 post-meniscectomy. Throughout the study, there was no significant increase in the number of vocalisations from compression of the contralateral knee in SOVX mice compared to pre-surgery readings.

The potential role of endogenous opioid tone on the absence of pain behaviours at day 56 is described in section 4.3.7.4).

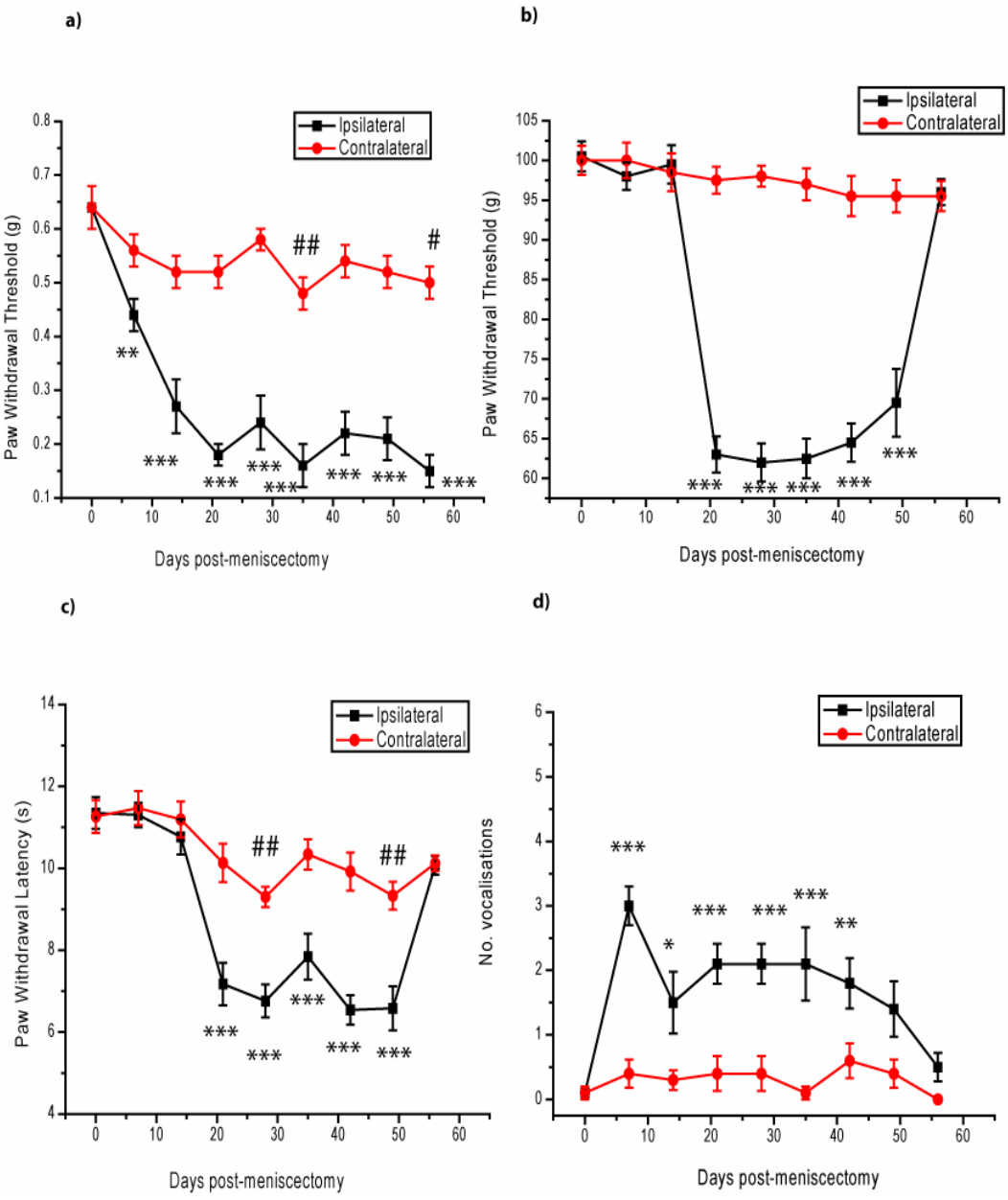


Figure 4-32 The effect of OA induction on behaviour of SOVX female C57Bl/6 mice following partial medial meniscectomy.

Test for the development of mechanical allodynia a) mechanical hyperalgesia b) cold allodynia c) and knee vocalisations d). Each point represents the mean value ± SEM of ten mice. Statistics: Ipsilateral readings versus baseline by one way ANOVA followed by Tukey's post hoc Test, * P<0.05. ** P<0.01, *** P<0.001. Contralateral readings versus baseline by one way ANOVA followed by Tukey's post hoc Test, # P<0.05, ## P< 0.01.

4.3.7.3.3 Ovariectomised mice (OVX)

OVX mice developed a persistent mechanical allodynia from day 7 onwards with significantly decreased PWTs compared to baseline measurements ($P<0.001$, Figure 4-33a). This group of mice showed the greatest level of mechanical allodynia following partial medial meniscectomy with the lowest recorded ipsilateral PWT of 0.09g ± 0.01g at day 56 post meniscectomy surgery (Figure 4-33a). In addition, a persistent contralateral mechanical allodynia was present from day 28 onwards with the lowest contralateral PWT recorded at day 35 of 0.23g ± 0.04g.

OVX mice did not develop a significant persistent mechanical hyperalgesia measured using the paw pressure test in this study, however. Paw withdrawal thresholds were not significantly decreased from baseline levels except at day 21 post-meniscectomy where the PWT was recorded at 92.0g ± 3.89g ($P<0.05$) (Figure 4-33b).

Similarly, OVX mice also did not develop a persistent cold sensitivity in the ipsilateral hind paw. Lowest PWLs were recorded at day 14 of 9.15s ± 0.37s which was the only recording where PWL was significantly decreased from baseline readings ($P<0.05$). Contralateral PWLs did not decrease significantly compared to baseline readings at any time point (Figure 4-33c).

Prior to left partial medial meniscectomy, vocalisations in response to 10 knee compressions was similar in male (0.10 ± 0.10), SOVX (0.10 ± 0.10) and OVX (0.20 ± 0.20) mice (Figure 4-31d, Figure 4-32d, Figure 4-33d). OVX mice vocalised the least frequently during the knee compression test (Figure 4-33d). A significantly increased number of vocalisations were recorded in response to knee compression of the ipsilateral knee at day 7 with 1.9 ± 0.18 vocalisations ($P<0.001$) until day 28 ($P<0.01$) compared to pre-surgery readings of 0.20 ± 0.20. At day 35 onwards, the number of vocalisations was not significantly greater than pre-surgery readings. An increased number of vocalisations from compression of the contralateral knee was recorded at day 21 and day 28 ($P<0.001$) with the greatest number of vocalisations being 1.89 ± 0.35 vocalisations recorded at day 21.

Overall, OVX mice developed the most severe mechanical allodynia, the mildest mechanical hyperalgesia, cold sensitivity and vocalised the least frequently in response to knee compressions following partial medial meniscectomy compared to male mice and SOVX mice tested.

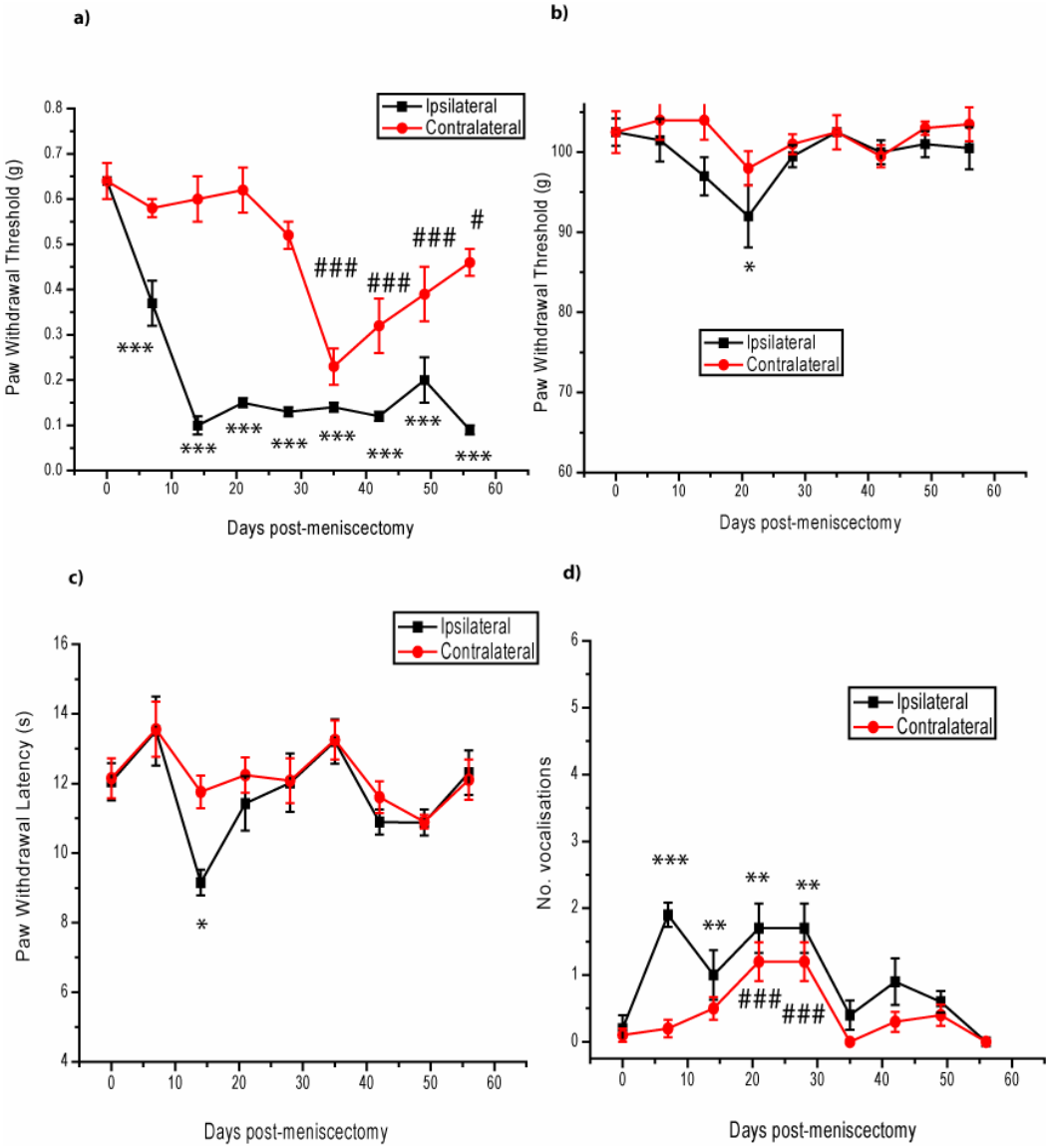


Figure 4-33 The effect of OA induction on behaviour of OVX C57Bl/6 mice following partial medial meniscectomy.

Test for the development of mechanical allodynia a) mechanical hyperalgesia b) cold allodynia c) and knee vocalisations d). Each point represents the mean value ± SEM of ten mice. Statistics: Ipsilateral readings versus baseline by one way ANOVA followed by Tukey's post hoc Test, * P<0.05. ** P<0.01 *** P<0.001. Contralateral readings versus baseline by one way ANOVA followed by Tukey's post hoc Test, # P<0.05, ## P<0.01, ### P<0.001.

4.3.7.4 Role of endogenous opioids in male, OVX and SOVX mice

Mechanical hyperalgesia, cold allodynia and the number of vocalisations in response to knee compressions fluctuated at different time points in the male mice and SOVX mice. Fluctuations were also described in female mice in the previous studies where a reduction of pain sensitivity was related to the influence of endogenous opioids (3.3.2.3.3 and 4.1.2). Therefore, 2.5mg/kg naloxone was administered to male, SOVX and OVX mice at day 56 post partial medial meniscectomy, a time point where all three groups of mice exhibited levels of mechanical and cold sensitivity close to baseline threshold levels. Following the administration of naloxone, male mice showed a significant increase in mechanical hyperalgesia with PWTs decreasing from 94.0g ± 4.85 to 72.0 ± 5.61g ($P=0.018$; Figure 4-34b). Cold sensitivity was also significantly increased in naloxone treated male mice with PWTs decreasing from 10.42s ± 1.02s to 6.48s ± 0.44s ($P=0.0077$; Figure 4-35a). The number of vocalisations increased from 0.40 ± 0.24 to 1.80 ± 0.66 but this was not statistically significant ($P=0.083$; Figure 4-35b).

SOVX mice were similarly affected by the administration of naloxone with significant increases in mechanical hyperalgesia ($P<0.0001$; Figure 4-34b) and cold allodynia ($P=0.00060$; Figure 4-35a) compared to pre-drug levels. The number of vocalisations in response to knee compressions was also significantly increased from 0.60 ± 0.4 to 2.60 ± 0.40 ($P=0.0077$; Figure 4-35b). OVX mice showed no significant change in pain sensitivities in response to the administration of naloxone in any of the behavioural tests (Figure 4-34 and Figure 4-35).

There was no significant change in the pain sensitivities of the contralateral hind limb in naloxone treated mice or in the ipsilateral or contralateral hind limb of vehicle treated mice.

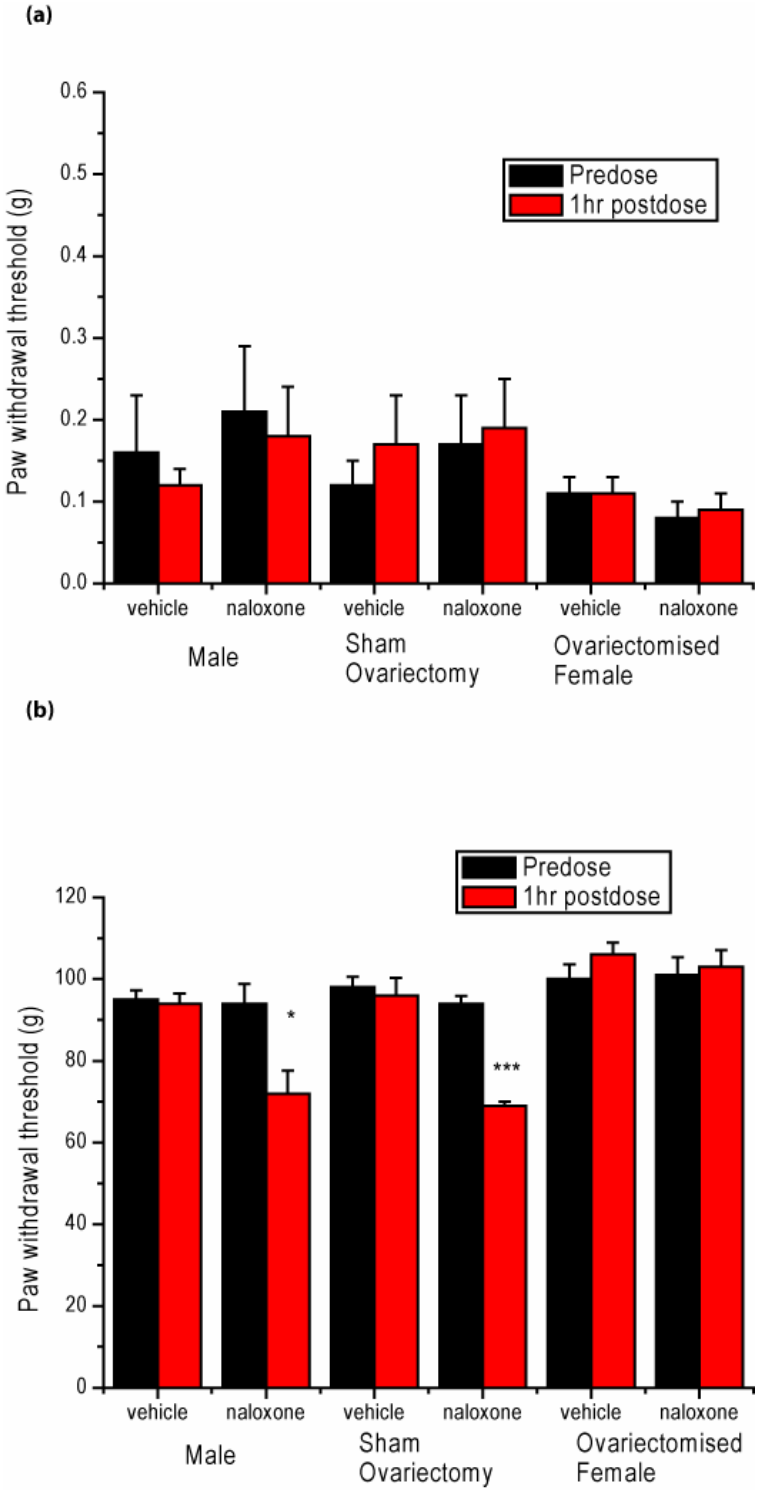


Figure 4-34 The effect of 2.5mg/kg i.p naloxone on C57Bl/6 mice 8 weeks post partial medial meniscectomy. The effect of naloxone on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of five mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, * $P<0.05$. *** $P<0.001$

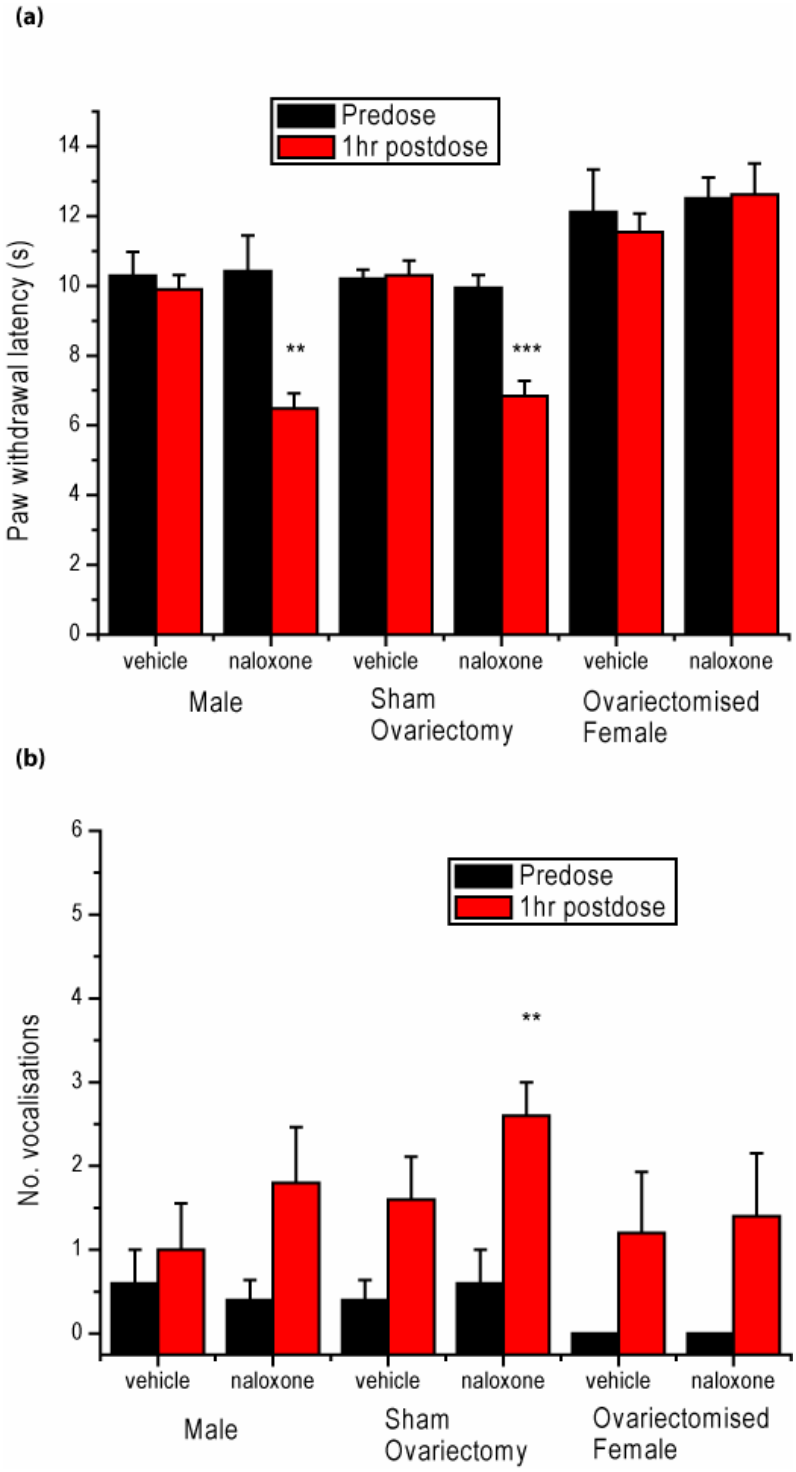


Figure 4-35 The effect of 2.5mg/kg i.p naloxone on C57Bl/6 mice 8 weeks post partial medial meniscectomy.

The effect of naloxone on cold allodynia a) and vocalisations in response to knee compressions b) Each point represents the mean value ± SEM of five mice. Statistics: Pre-drug measurements versus post-drug measurements by unpaired t-test, ** P<0.01. *** P<0.001

4.4 Discussion

4.4.1 Pharmacology

The drugs tested in this study had varying effects with only morphine having a significant effect on all four measures of pain. This correlates well with human OA where single agent drug protocols frequently provide incomplete pain relief (Altman 2004). Similar to this study, other reports have shown morphine to be effective at reversing surgically or chemically induced OA pain behaviours in animal models including weight bearing deficits (Pomonis, Boulet et al. 2005; Inglis, McNamee et al. 2008), mechanical hyperalgesia (Fernihough, Gentry et al. 2004) and mechanical allodynia (Fernihough, Gentry et al. 2004; Pomonis, Boulet et al. 2005). Tramadol, which is used clinically to treat OA pain (Altman 2004), has weak affinity for μ and δ opioid receptors sub-types activity and moderate affinity at κ receptors, and additionally inhibits the uptake of serotonin and noradrenaline (Raffa, Friderichs et al. 1992). Unlike morphine, the effects of tramadol were restricted to reductions in responses to knee compression and mechanical allodynia. The more limited efficacy of tramadol may be related to its weaker opioid receptor activity.

Anti-inflammatory drugs are frequently prescribed for OA pain, however, they are most useful in severe cases of OA or during flare-ups that occur with episodic synovitis, or in inflammatory arthritides such as rheumatoid arthritis (Pincus, Koch et al. 2001). The ability of diclofenac to reverse mechanical hyperalgesia and hypersensitivity to knee compression one week after surgery is consistent with an early inflammatory component. Pain behaviours at later time points were not reversed by single dose treatment with anti-inflammatory drugs diclofenac or celecoxib. NSAIDs have shown greater efficacy in reversing pain behaviours in rodent models of OA when given chronically (Fernihough, Gentry et al. 2004; Pomonis, Boulet et al. 2005; Inglis, McNamee et al. 2008). In models, where single administration of an NSAID has been successful, the analgesia may be attributed to testing within an inflammatory phase of pain behaviours e.g. following intra-articular injection of MIA (Fernihough, Gentry et al. 2004), during ongoing inflammation caused by meniscal tear (Bove, Laemont et al. 2006), or when a destabilized meniscus may be causing a greater level of inflammation within the joint (Inglis, McNamee et al. 2008). The findings in the studies described here suggest that active inflammation does not drive the later stage OA induced pain behaviours produced by partial medial meniscectomy.

Paracetamol is often prescribed to OA patients for long term treatment where the adverse effects of NSAIDs are a concern (Towheed, Maxwell et al. 2006). Paracetamol had a differential effect on the pain behaviours in the current study. Only mechanical hyperalgesia was reversed by paracetamol and other measures were not significantly affected. Paracetamol has been previously shown to reverse mechanical allodynia induced by DMM in CD-1 mice at day 14 post-surgery (Malfait, Ritchie et al. 2010). It is unclear why paracetamol did not have a significant anti-allodynia effect in the studies reported here, however, the difference may be a reflection of the different joint surgeries or mouse strains and subsequent pattern of OA and pain behaviour development.

Anti-epileptics are increasingly utilized in the treatment of neuropathic pain. Lamotrigine and gabapentin are involved in the blockade of voltage-activated ion channels and have been shown to be effective in animal models of neuropathic pain (Blackburn-Munro and Erichsen 2005). Gabapentin was effective at reversing mechanical allodynia but not mechanical hyperalgesia after partial medial meniscectomy, which is consistent with results from studies using other models of OA pain (Fernihough, Gentry et al. 2004; Bove, Laemont et al. 2006). Similar results have been obtained in neuropathic pain models where it has been shown that a single dose of Gabapentin is effective in reversing mechanical allodynia but repeated administration is required to reverse mechanical hyperalgesia (Patel, Naeem et al. 2001; Fox, Gentry et al. 2003). Lamotrigine did not produce a significant reversal of the pain behaviours tested in this study. Chandran et al (2009) also reported poor efficacy of lamotrigine in a rat model of OA where a single dose of lamotrigine had minimal effect on grip force strength in a rat model of OA induced by MIA. It would be useful to perform further studies examining the effect of analgesics (including neuropathic pain medications) on pain behaviours when administered chronically to observe whether there is improved efficacy using chronic dosing regimes.

Duloxetine, a serotonin-norepinephrine reuptake inhibitor, is efficacious in the pre-clinical treatment of acute and chronic inflammatory pain (Jones, Peters et al. 2005) and is used clinically to treat neuropathic pain (Ormseth, Scholz et al. 2011). Duloxetine successfully reversed mechanical allodynia induced by partial medial meniscectomy. However, it also resulted in unacceptable side effects and therefore further investigation is required before conclusions can be drawn on the efficacy in this model of OA pain. Duloxetine has shown moderate analgesic effects in rats with OA induced by MIA which recorded grip force as a measure of pain (Chandran,

Pai et al. 2009) and therefore may have effects on other pain behaviours in the mouse model that could not be evaluated here. In 2009, Sullivan et al. reported some improvement in pain measures in a small clinical trial whereby 17 OA patients received 2 weeks treatment with a placebo followed by 10 weeks treatment with duloxetine (Sullivan, Bentley et al. 2009). More recently, Chappell et al. (2011) reported that duloxetine provided significant pain relief and improved function in patients with knee OA compared to placebo over a 13-week trial period (Chappell, Desaiah et al. 2011) confirming that some OA patients may benefit from treatment with duloxetine either as an adjunct or as an alternative to more traditional therapies such as paracetamol and NSAIDs.

Overall, the poor effect of anti-inflammatory drugs such as diclofenac and celecoxib, and significant effect of morphine (on all pain measures) and gabapentin (on mechanical allodynia) on pain behaviour attributed to degenerative joint disease is in agreement with the concept that chronic OA pain incorporates elements of nociceptive and/or neuropathic pain and lacks a strong inflammatory component (Hochman, French et al. 2010).

The reversal of the pain behaviours induced by partial medial meniscectomy is consistent with a pain condition rather than joint instability. The ability of commonly used analgesics used in the treatment of OA pain to reverse pain behaviours in female C57Bl/6 mice together with the production of progressive degenerative joint disease characterised by cartilage erosion validates this model of OA.

4.4.2 Endogenous opioids

We have confirmed that the absence of pain behaviours in mice following partial medial meniscectomy can be unmasked by the administration of naloxone or naloxone methiodide indicating the role of peripheral endogenous opioids in the modulation of OA pain in this model. Waxing and waning degrees of OA pain behaviours modulated by endogenous opioids were found in both male and female C57Bl/6 mice. The synchronisation of pain behaviours observed in separate groups of male and female mice indicate that the trigger for the production of endogenous opioids is not sex dependent.

The study using specific opioid receptor antagonists, indicated that the endogenous opioids responsible for masking OA pain behaviours act on mu and kappa opioid receptors. It would be useful to perform further studies to examine the role of endogenous opioids in modulating OA pain. These could include measuring specific endogenous opioid levels or by examining opioid receptor expression. In a previous

study of OA pain behaviour induced by destabilization of the medial meniscus (Inglis, McNamee et al. 2008), systemic induction of a μ opioid receptor agonist, endorphin was not observed at any time point assessed post-surgery when measured in sera by enzyme-linked immunosorbent assay (ELISA). Real-time reverse transcription polymerase chain reaction (RT-PCR) of μ opioid receptor did show a 3-fold increase in receptor expression in the ipsilateral DRG at L3/L4 at 8 weeks post-surgery but not at 4 weeks or 16 weeks post-surgery in OA mice compared to sham mice, indicating a transient increase in receptor expression at certain time points following the induction of OA. It would be of value to repeat these studies in the partial medial meniscectomy model including an ELISA for dynorphin, an endogenous opioid receptor agonist acting primarily on kappa opioid receptors. Joint tissue could be used in addition to sera to examine local and systemic levels of endogenous opioids. RT-PCR could also be employed for the study of kappa as well as mu opioid receptors within the DRGs of OA mice compared to sham mice.

Female mice who had received two general anaesthetics (SOVX mice) followed a different time course for the development of OA pain behaviours compared to mice that had received a single surgery (partial medial meniscectomy alone). This may indicate that the anaesthesia or the laparotomy incision itself may have affected the endogenous opioid response so that whilst hypersensitivities developed to the same extent as recorded in previous studies, the waxing and waning effect did not follow the same pattern. Further experiments must be performed to control for the effect of the different conditions to gain an insight into the triggers for the production of endogenous opioids or upregulation of opioid receptors.

4.4.3 Sex differences

Previous studies have described female sex hormones to be cartilage protective in OA models with male mice developing significantly greater cartilage erosion compared to female mice of the same strain (Walton 1977; Mahr, Menard et al. 2003; Ma, Blanchet et al. 2007). Ma et al. (2007) reported OA severity (measured by histopathology) to be markedly greater in males than SOVX 129S6/SvEv mice after DMM surgery and that OVX females developed significantly more severe OA than SOVX mice. In the studies described in this thesis, OVX mice developed the greatest level of osteophytosis, however, there was no significant difference in the cartilage erosion following partial medial meniscectomy between OVX and SOVX mice. Male mice developed the greatest severity of cartilage erosion compared to the groups of female mice, however this was only significant compared to OVX

mice. Differences between these findings and those of Ma et al. may be related to the strain of mice or the age of ovariectomy. Ma et al obtained mice that had been ovariectomised at 6 weeks of age, in contrast to ovariectomizing at 4 weeks of age. The effect of the age of ovariectomy (i.e. pre or post puberty) on the development of OA lesions and pain behaviours using the partial meniscectomy model is yet to be determined and further studies are required to compare the histological and behavioural effects of the induction of OA following ovariectomy in pre-pubertal and mature mice.

In the current studies, baseline measurements, and maximal mechanical and cold sensitivities were very similar for male and SOVX mice indicating that OA pain severity in this model is not sex dependent. OVX mice, however, developed a very different set of pain behaviours compared to the other groups of mice. OVX mice did not appear to develop mechanical hyperalgesia, cold allodynia and vocalised infrequently in response to knee compressions following partial medial meniscectomy. The lack of pain sensitivities did not appear to be due to high endogenous opioid tone or at least was not responsive to the administration of naloxone. These results conflict with population studies of women where the prevalence of OA pain is higher in post menopausal women compared to men (Berkley 1997; Andersen, Crespo et al. 1999; Wijnhoven, de Vet et al. 2006) and women are reported to experience greater OA-related pain and disability (Keefe, Lefebvre et al. 2000; Theis, Helmick et al. 2007; Axford, Heron et al. 2008; Jinks, Jordan et al. 2008; Perrot, Poiraudeau et al. 2009). Furthermore, in laboratory studies, women report higher pain intensity during laboratory studies, especially for mechanical stimuli (Fillingim, King et al. 2009), and demonstrate lower pain thresholds (Otto and Dougher 1985; Brennum, Kjeldsen et al. 1989; Jensen, Rasmussen et al. 1992; Chesterton, Barlas et al. 2003). The mechanisms for the enhanced sensitivity to mechanical stimuli and musculoskeletal pain in women are not fully understood. The influence of gonadal hormones has been substantiated (Aloisi and Bonifazi 2006), however, psychosocial factors are also known to contribute (Otto and Dougher 1985; Levine and De Simone 1991; Sanford, Kersh et al. 2002; Myers, Riley et al. 2003).

Female OVX mice developed a persistent ipsilateral mechanical allodynia from day 7 post partial meniscectomy and a contralateral mechanical allodynia from 4 weeks post partial meniscectomy. Sanoja et al (2005) reported that female C57Bl/6 mice who underwent bilateral ovariectomy developed a significant bilateral mechanical allodynia in the hind limbs at 4 weeks post-ovariectomy surgery when compared to

SOVX mice (Sanoja and Cervero 2005). Therefore the induction of mechanical allodynia in the studies reported here, may not be directly attributable to the OA of the knee joint following partial medial meniscectomy. Further studies whereby mice are ovariectomised but do not undergo partial medial meniscectomy would help determine the role of the ovariectomy surgery for initiating the development of mechanical allodynia compared to knee joint OA.

Preliminary studies on whether pain behaviours are sex dependent in a mouse model of OA have revealed some interesting differences in the pain behaviours exhibited by male, OVX and SOVX. However, further studies are required to enable firm conclusions to be drawn. Informative future studies might include performing a complete study with a full range of control animals including SOVX and OVX mice where partial medial meniscectomy is not performed to assess the influence of sex hormones on baseline pain thresholds. These experiments should include male mice that undergo sham meniscectomy surgery and include male mice who undergo bilateral laparotomy and partial medial meniscectomy to control for the effect of 2 anaesthetics on endogenous opioid tone and therefore pain behaviours. In this way direct comparisons can be made between the groups of mice allowing greater interpretations of the results. It would also be interesting to measure the effect of treatment with oestrogen to mice who had been ovariectomised to see whether pain behaviours become more apparent. Future directions might also include ovariectomizing mice following the development of OA pain behaviours by partial medial meniscectomy in mature female C57Bl/6 mice.

4.5 Conclusion

Partial medial meniscectomy produces a reliable model of OA pain which allows the study of both joint pain and referred pain in the form of mechanical hyperalgesia, cold allodynia and mechanical allodynia.

Chapter 5

Investigation into potential targets for analgesia in OA mice

5 Investigation into potential targets for analgesia in OA mice

5.1 Introduction

Several mediators and ion channels have been proposed to play roles in inflammatory and neuropathic pain and may also influence the development and maintenance of OA pain. These include the ion channels TRPA1 and TRPM8, the inflammatory mediator bradykinin, the cytokine TNFα and the growth factor NGF. Targeting these mediators and ion channels may provide insights into the mechanisms behind the development of chronic pain, allowing rational mechanistic approaches to be taken in the development of new drugs to treat conditions such as OA.

5.1.1 Nerve Growth Factor (NGF)

NGF is a key regulator of sensory neuron excitability and an important mediator of injury-induced nociceptive and neuropathic pain (Sah, Ossipo et al. 2003; Zweifel, Kuruvilla et al. 2005). NGF acts via Tyrosine receptor kinase A (TrkA) and p75 to activate a number of other kinase pathways, leading to altered gene transcription and increased synthesis of sensory neuropeptides (substance P, CGRP), ion channels (TRPV1, NaV1.8, ASIC3), membrane receptors such as bradykinin, and structural molecules (Ro, Chen et al. 1999; Theodosiou, Rush et al. 1999; Hefti, Rosenthal et al. 2006). NGF and TrkA have become attractive targets for attenuating chronic pain. Current strategies for targeting NGF or TrkA include; monoclonal antibodies that sequester NGF (1), monoclonal antibodies that target TrkA and prevent NGF from binding to TrkA (2), small molecule TrkA antagonist therapy (3) and small molecule kinase inhibitors of tyrosine receptor kinases (4). In the studies described in this chapter, TrkA inhibitors were used to gain insight into NGF-TrkA pathways as a mechanism for the development of hypersensitivities associated with OA pain and to further validate the partial medial meniscectomy model.

5.1.2 Tumour Necrosis Factor

Tumour necrosis factor-α (TNFα) is a mediator of inflammatory and neuropathic pain and is one of the major cytokines involved in the pathophysiology of OA. TNFα is produced during the cascade of events initiated by inflammatory stimuli and can activate sensory neurons via TNF receptors TNF1 and TNF2 which initiate inflammatory reactions through the production of interleukins IL1, IL6 and IL 8 (Pollock, McFarlane et al. 2002; Ohtori, Takahashi et al. 2004). Direct

TNFα application in the periphery can induce neuropathic pain behaviour that can be inhibited by ibuprofen and celecoxib (Schafers, Marziniak et al. 2004), and nerve injury can lead to increased TNFα in damaged as well as adjacent undamaged axons (Schafers, Svensson et al. 2003). Anti-TNF has become a common treatment for rheumatoid arthritis (Vinay and Kwon 2011) but has shown poor efficacy in preclinical studies of OA (McNamee, Burleigh et al. 2010) and was only partially effective in a clinical case report of a patient with debilitating knee OA (Grunke and Schulze-Koops 2006). The use of anti-TNF in the partial medial meniscectomy model of OA pain was expected to provide further evidence that TNFα does not play an important role in the maintenance of OA pain behaviours.

5.1.3 Bradykinin

Bradykinin is a peptide that acts on Bradykinin B1 and B2 G protein coupled receptors to exert potent pro-inflammatory effects and is one of the most potent endogenous peripheral mediators of pain (Meini, Cucchi et al. 2011). B1 antagonists have shown anti-hyperalgesic activity in inflammatory and neuropathy models of pain (Gabra and Sirois 2003; Gougat, Ferrai et al. 2004) and were shown to reverse weight bearing deficits in an anterior cruciate ligament transection model of OA (Kaufman, Zaouter et al. 2011). Kaufman et al. also reported disease modifying effects following anterior cruciate ligament transection and treatment with intra-articular B1 antagonist, R-954, with reduced subchondral bone remodelling, greater cartilage thickness and increased levels of cartilage proteoglycans and type II collagen compared to control treated rats. B2 receptor antagonists have also shown anti-hyperalgesic activity in rodent models of inflammatory hyperalgesia, reversing CFA-induced mechanical hyperalgesia in the rat knee joint (Burgess, Perkins et al. 2000) and have been reported to reverse weight bearing deficits in an MIA model of OA in rats (Cialdai, Giuliani et al. 2009; Song, Althoff et al. 2009). B2 antagonist, Icabitant, has also been investigated in a clinical trial using patients with symptomatic knee OA (Song, Althoff et al. 2009). Song et al. reported that treatment by intra-articular injection of Icabitant produced a significant reduction in pain intensity at rest and during activity. Bradykinin receptor antagonists are therefore a useful tool to assess the role of bradykinin in OA pain and may prove to be effective therapeutic drugs.

5.1.4 TRPA1

The non-selective ion channel, TRPA1 (Transient receptor potential ankyrin subfamily, member 1) is highly expressed in sensory neurons of DRGs, nodose ganglia and trigeminal ganglia (Story, Peier et al. 2003; Nagata, Duggan et al. 2005)

and is reported to be co-expressed with TRPV1 (Story, Peier et al. 2003; Bautista, Movahed et al. 2005; Kobayashi, Fukuoka et al. 2005; Nagata, Duggan et al. 2005). TRPA1 is a noxious cold-activated ion channel (≤17ºC), but is also activated by a large number of pungent or irritant compounds, such as cinnamaldehyde, AITC, acrolein, allicin, and formalin, all of which can induce acute pain, hyperalgesia, or neurogenic inflammation in animals and humans (Bandell, Story et al. 2004; Jordt, Bautista et al. 2004; Bautista, Movahed et al. 2005; Macpherson, Geierstanger et al. 2005; McNamara, Mandel-Brehm et al. 2007). TRPA1 ion channels can also be sensitized by inflammatory mediators, including bradykinin, which is known to be significantly elevated in osteoarthritic synovial fluid (Bandell, Story et al. 2004). TRPA1 antagonists have been shown to reduce hypersensitivities induced by injection of AITC or formalin into the paw of rats and in models of chronic inflammatory and neuropathic pain (McNamara, Mandel-Brehm et al. 2007; Eid, Crown et al. 2008; Wei, Hamalainen et al. 2009; Koivisto, Hukkanen et al. 2012). Therefore TRPA1 antagonists may provide insight into the role played by TRPA1 in pain behaviours associated with OA.

5.1.5 TRPM8

Another TRP channel, TRPM8 (Transient Receptor Potential Melastatin 8) is activated by cool temperatures with a threshold for activation in the range 20-30℃. Cold sensitivity (allodynia or hyperalgesia) is prevalent in neuropathic pain conditions such as fibromyalgia (Brederson, Jarvis et al. 2011) and as a manifestation of chronic inflammation e.g. rheumatoid arthritis (Edwards, Wasan et al. 2009; Brederson, Jarvis et al. 2011). The use of *trpm8* $^{-/-}$ mice has indicated that TRPM8 is important for cold sensation and also that TRPM8 mediates acetone cooling sensitivity in a mouse model of neuropathic pain (CCI injury) (Colburn, Lubin et al. 2007; Lashinger, Steiginga et al. 2008). Furthermore, TRPM8 antagonists have been shown to be effective at reducing cold hypersensitivity in inflammatory (CFA) (Knowlton, Daniels et al. 2011) and neuropathic (CCI) pain models in rats and mice (Knowlton, Daniels et al. 2011; Parks, Parsons et al. 2011; Matthews, Qin et al. 2012; Tamayo, Bo et al. 2012). In addition, TRPM8 antagonist, AMTB, was effective in reversing established pain in a model of overactive bladder syndrome (Lashinger, Steiginga et al. 2008). Unpublished studies in the Bevan laboratory using AMTB, and siRNA to reduce TRPM8 expression have indicated that a reduction in TRPM8 activity can lead to lower mechanical as well as cold sensitivity. These pilot results suggest that TRPM8 antagonism could affect joint pain. In the studies described in this thesis, cold sensitivity was observed after the induction of OA in mice following both intra-articular injection of MIA (3.3.2.2.2 and 3.3.2.3.2) and partial medial

meniscectomy (3.3.4.3). Thus, TRPM8 antagonists may be a useful to determine the role of TRPM8 in OA pain.

5.1.6 Genetically modified mice

The in vivo significance of a particular gene can be characterized using genetically modified mice including those with gene deletions or gene over-expression. Studies utilizing available *trpa1*$^{-/-}$ mice were performed to complement pharmacological studies using TRPA1 antagonists since observation of differences between pain behaviours of genetically modified mice and wild type littermates can help determine the role of specific genes.

5.2 Aims

- To identify the importance of selected mediators and ion channels for the transduction/transmission of joint pain *in vivo* using tool compounds and genetically modified mice

5.3 Results

Receptor antagonists are available for a number of targets, including TRPA1, bradykinin B2 and NGF receptors. Where available these were tested for effects on OA pain behaviours. The route of administration depended on the bioavailability of the test compound and previously published data. The dose of administration was based on previous in-house testing, published studies, or pharmacokinetic data supplied by pharmaceutical companies.

In these studies, the tool compounds were tested on female C57Bl/6 mice who underwent left partial medial meniscectomy at 8-10 weeks of age. Pain behaviours were recorded from 6 weeks post-surgery and the compounds tested once the mice had developed pain behaviours that were significant with respect to baseline thresholds and were similar to previous partial medial meniscectomy studies.

5.3.1 TrkA inhibitors

TrkA inhibitors AZ-23 and CE-245677 were obtained from Pfizer Inc, UK. AZ-23 is a small-molecule inhibitor of the Trk tyrosine kinase family. AZ-23 is a potent ATP-competitive inhibitor of the three Trk isoforms (TrkA/B/C). CE-245677 is a prototype inhibitor of the kinase activity of Tyrosine kinase with Immunoglobulin and EGFR-like domains (Tie2) receptor. It has equal potency for TrkA and TrkB. Both compounds were previously pursued for the treatment of cancer but are now being investigated as potential tool compounds for pain.

Dosages for mouse studies were chosen based on pharmacokinetic data provided by Pfizer Inc. These compounds were also tested for any effects on naïve animals. In addition to von Frey, paw pressure, cold plate, knee compression tests, measurements for thermal hyperalgesia were also recorded using a hot plate, since it is known that sensitization of TRPV1 can occur by NGF acting on TrkA (Zhang, Huang et al. 2005).

5.3.1.1 The effect of AZ-23 administration on pain behaviours in naïve female C57Bl/6 mice

Prior to testing AZ-23 on OA pain behaviours, the compound was tested on naïve mice to see whether there was any effect on baseline sensitivities. 30mg/kg AZ-23 w/v in 0.5% methylcellulose/0.1% Tween80 (or the vehicle alone) was administered p.o. to 8 week old female C57Bl/6 mice (n=8 per group). Behavioural measurements were performed prior to treatment and at 2 hours post-treatment. At 2hrs following administration of AZ-23, there was no significant change in von Frey, paw pressure, cold sensitivity or vocalisations in response to knee compressions

compared to pre-drug readings or vehicle treated mice (Figure 5-1 and Figure 5-2). A decrease in PWL was noted with the thermal hyperalgesia test, however (Figure 5-2c). Paw withdrawal latencies decreased in AZ-23 treated mice from 10.89s ± 0.28s to 9.12s ± 0.79s (P=0.055) indicating the unexpected development of a slight sensitivity to noxious thermal stimulus in drug treated mice although this was not statistically significant. Further behavioural testing was performed at 24hrs, 96hrs, 144hrs and 240hrs post-treatment without further drug administration. Surprisingly, from 24hrs to 144hrs following initial drug treatment, a significant mechanical hyperalgesia and cold allodynia was recorded in both hind paws and a significant thermal hyperalgesia was recorded in the left hind paw (the only leg tested using this measurement) in drug treated mice compared to vehicle treated mice (Figure 5-1 and Figure 5-2). At 240hrs post-treatment, pain hypersensitivities were reduced and readings returned to similar levels to vehicle treated mice. Over the course of this study, vehicle treated mice showed some decrease in PWTs for the paw pressure test, cold plate test and hot plate test compared to base line levels which may indicate learned avoidance behaviours since the battery of behavioural tests was performed frequently within the 10 day study period. Paw withdrawal thresholds measured by von Frey filaments and the number of vocalisations from knee compressions remained at near baselines levels for the duration of the study with no significant differences between vehicle and drug treated mice. Due to the unexpected induction of pain hypersensitivities in naïve mice, further experiments using AZ-23 were not performed.

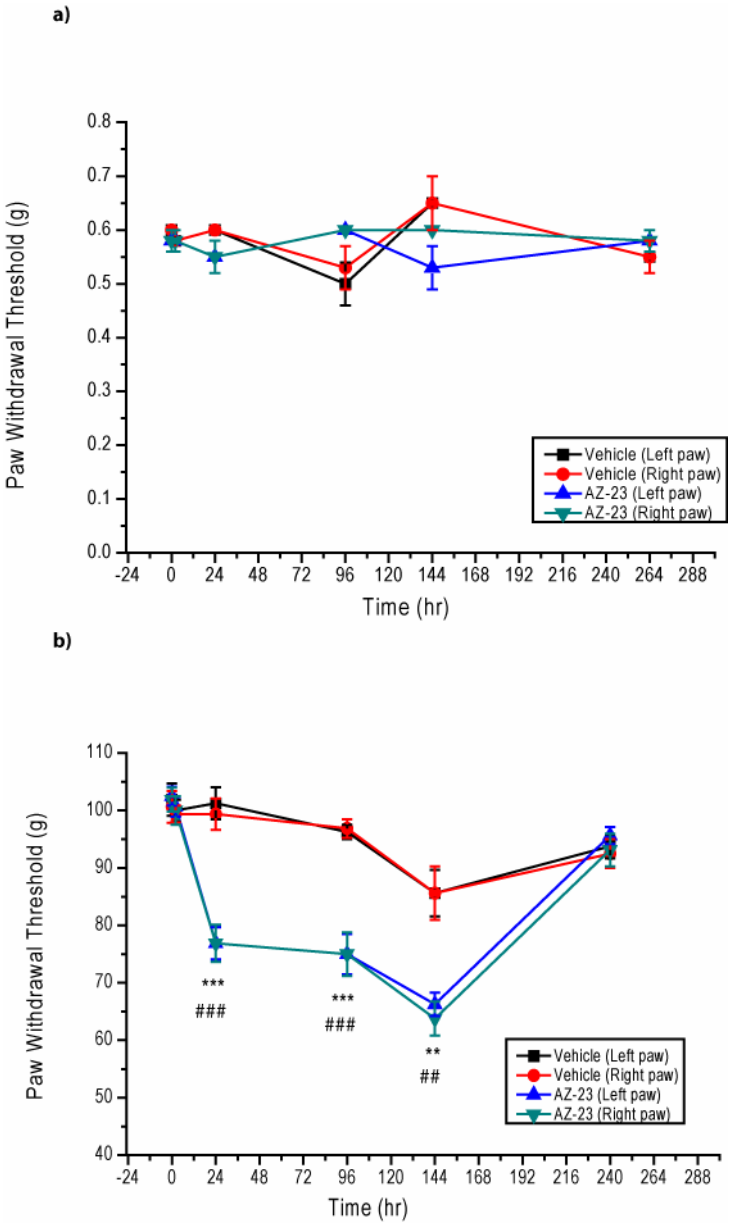


Figure 5-1 The effect of 30mg/kg p.o AZ-23 in 0.5% methyl cellulose/0.1% Tween80 in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.

The effect of AZ-23 on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by Tukey's post hoc test. Vehicle left hind limb versus AZ-23 left hind limb P<0.01**, P<0.001***, Vehicle right hind limb versus AZ-23 right hind limb P<0.01 ##, P<0.001 ###

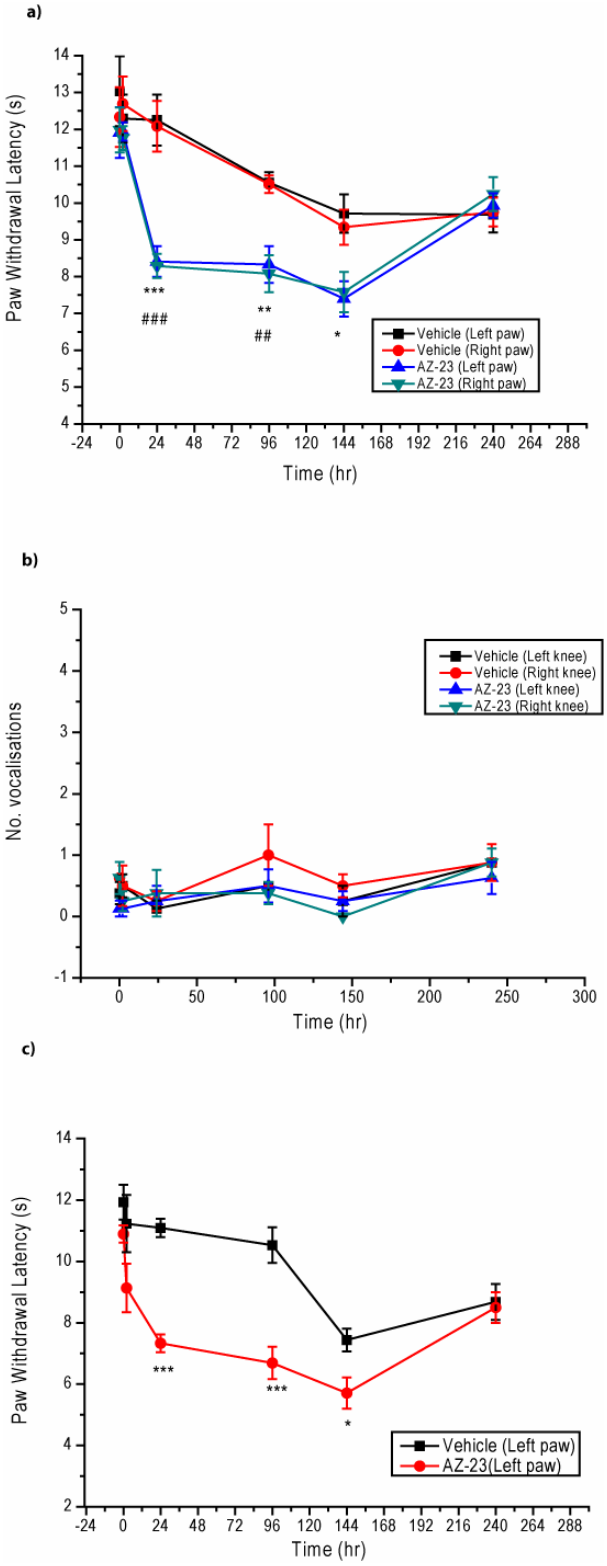


Figure 5-2 The effect of 30mg/kg p.o AZ-23 in 0.5% methyl cellulose/0.1% Tween80 in 8-10 week old naïve female C57Bl/6 mice on pain behaviours. The effect of AZ-23 on cold allodynia a) number of vocalisations b) and thermal hyperalgesia c). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by post hoc Tukey's test. Vehicle left versus AZ-23 left hind limb, P<0.05 *, P<0.01 **, P<0.001 ***. Vehicle right limb versus AZ-23 right hind limb, P<0.001 ###.

5.3.1.2 The effect of CE-245677 administration on naïve female C57Bl/6 mice

Since AZ-23 administration led to the induction of hypersensitivities in the hind limbs of C57Bl/6 mice, CE-245677 was tested on a new group of naïve mice. 30mg/kg CE-245677 w/v in 0.5% methylcellulose was administered p.o. to 8 week old female C57Bl/6 mice (n=8). Behavioural measurements were performed prior to treatment and at 2 hours post-treatment. Results are shown in Figure 5-3, Figure 5-4 and Figure 5-5. Similar to AZ-23 treated naïve mice, there was a significant decrease in PWLs in response to a noxious thermal stimulus at 2hrs post-treatment with CE-245677 from 10.95s ± 0.14s to 7.54s ± 0.45s ($P<0.001$, see Figure 5-5a). From 24hrs to 144hrs following initial drug treatment, a significant mechanical hyperalgesia ($P<0.001$) and cold allodynia ($P<0.001$) was recorded in both hind paws and a significant thermal hyperalgesia ($P<0.001$) was recorded in the left hind paw in drug treated mice compared to vehicle treated mice (Figure 5-3, Figure 5-4 and Figure 5-5). At 264hrs post-treatment pain sensitivities were again similar to vehicle treated mice. Paw withdrawal thresholds measured by von Frey filaments did not show a consistent pattern of sensitivity and the number of vocalisations from knee compressions remained at near baselines levels for the duration of the study.

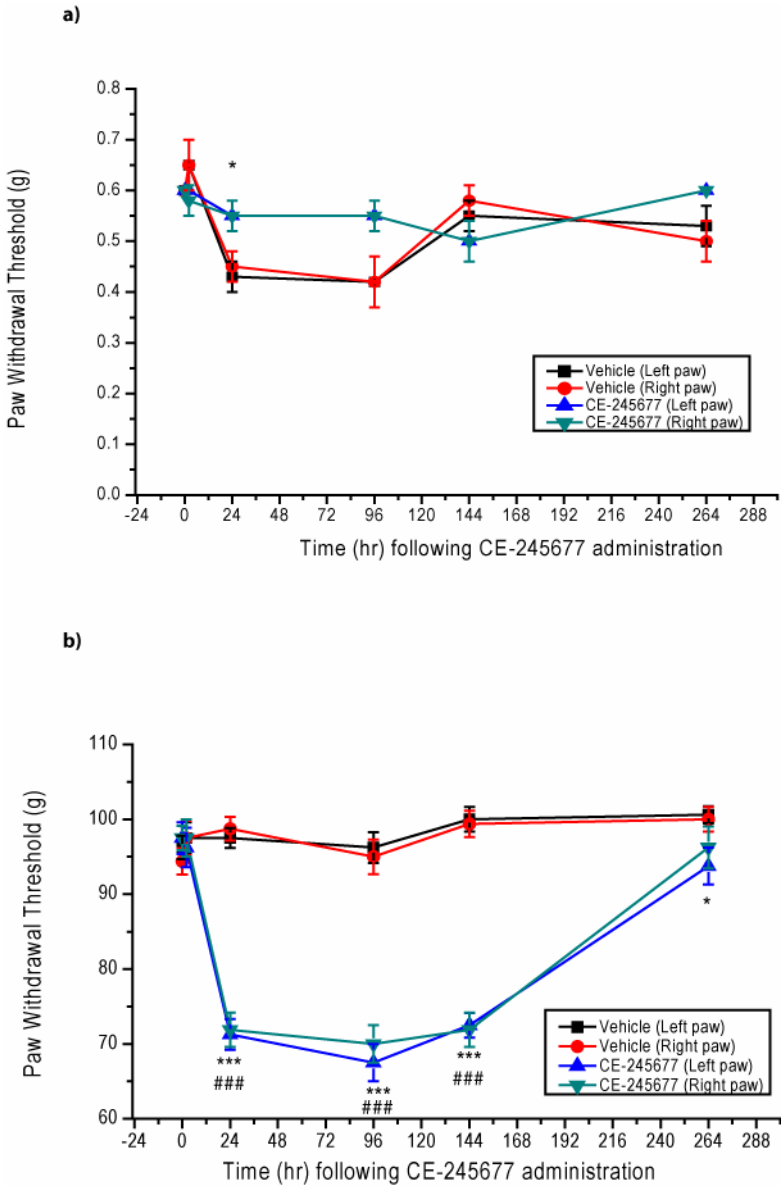


Figure 5-3 The effect of 30mg/kg p.o CE-245677 in 0.5% methyl cellulose in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.

The effect of CE-245677 on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by post hoc Tukey's Test. Vehicle left paw versus CE-245677 left paw P<0.05*, P<0.001 ***. Vehicle right paw versus CE-245677 right paw, P<0.001 ###

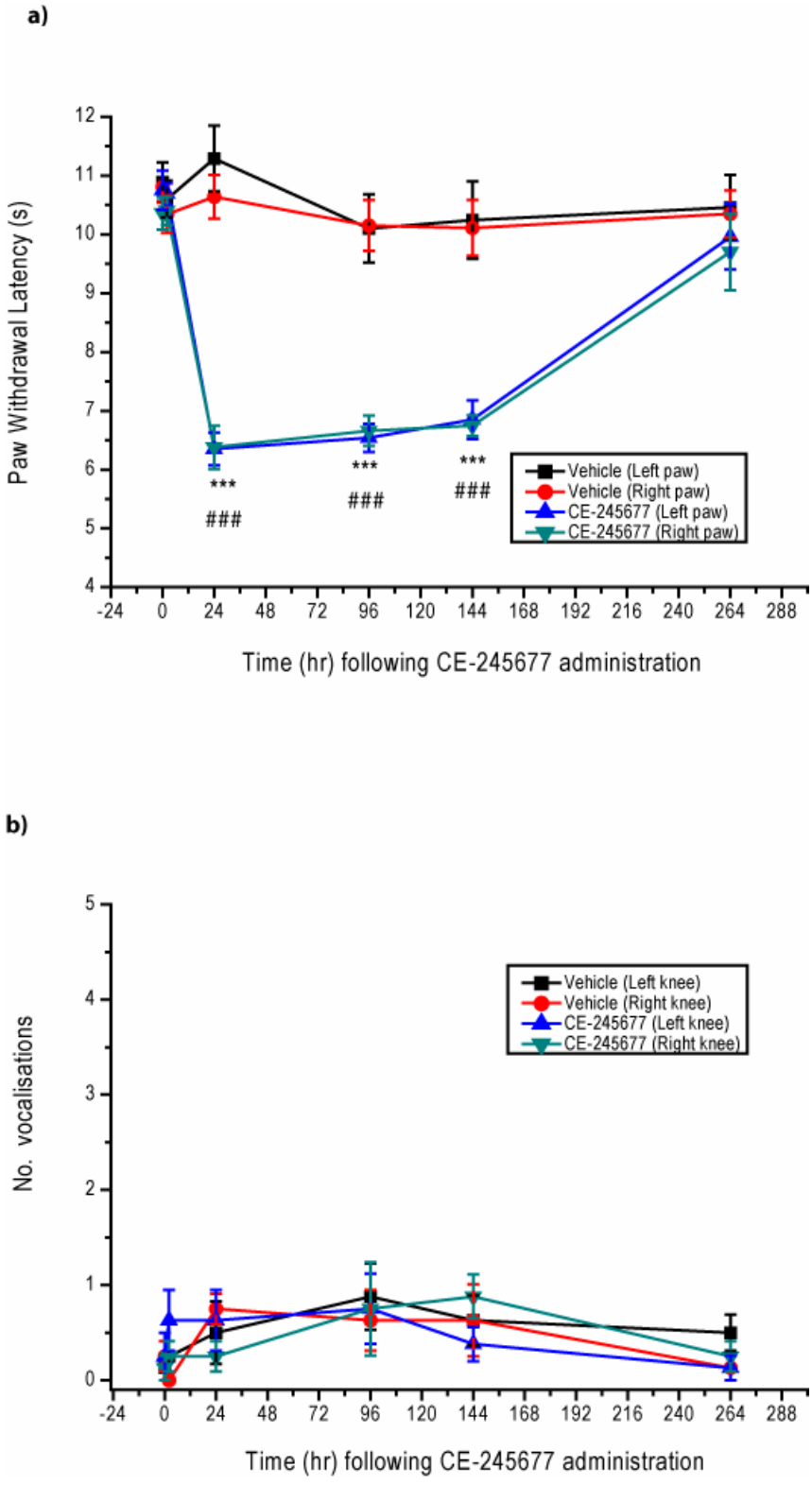


Figure 5-4 The effect of 30mg/kg p.o CE-245677 in 0.5% methyl cellulose in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.

The effect of CE-245677 on cold allodynia a) number of vocalisations in response to knee compression b). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by post hoc Tukey's test. Vehicle left leg versus CE-245677 left leg, P<0.001 ***. Vehicle right leg versus CE-245677 right leg, P<0.001 ###

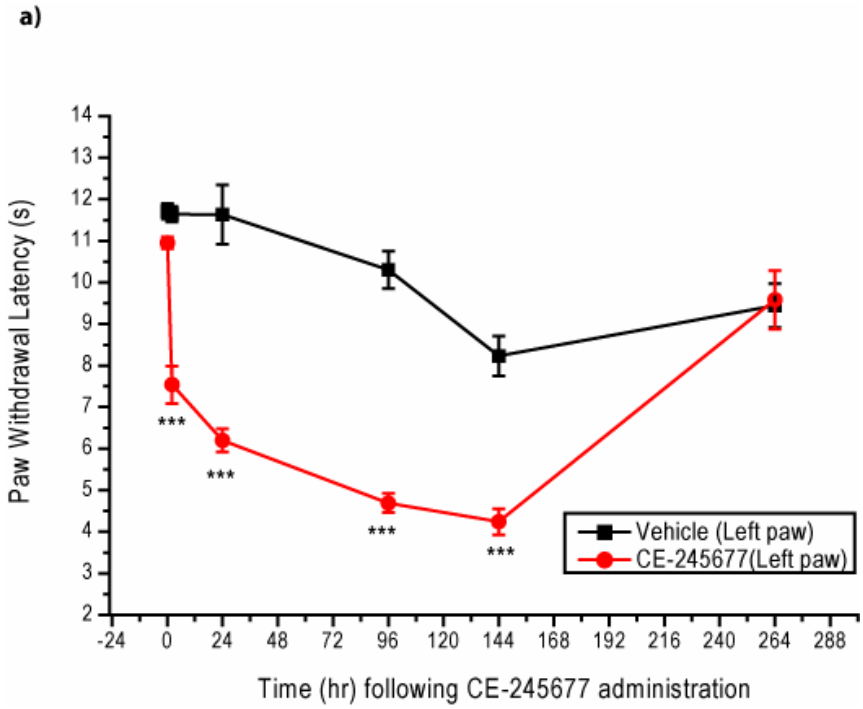


Figure 5-5 The effect of 30mg/kg p.o CE245677 in 0.5% methyl cellulose in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.

The effect of CE-245677 on thermal hyperalgesia a). Each point represents the mean value ± SEM of eight mice. Statistics: Vehicle versus CE-245677 by repeated measures ANOVA followed by Tukey's post hoc test P<0.001 ***.

The development of hypersensitivities following administration of TrkA inhibitor CE-245677 was unexpected and therefore the experiment was performed again to see whether the behavioural responses could be repeated and the results validated. Figure 5-6, Figure 5-7 and Figure 5-8 show the results of the effect of 30mg/kg p.o. of CE-245677 in 0.5% methylcellulose in a new group of naïve C57Bl/6 mice (n=8). The same pattern of hypersensitivity was recorded with significant thermal hyperalgesia (P<0.0001) developing 2hrs post drug administration and significant thermal hyperalgesia (P<0.001), mechanical hyperalgesia (P<0.001) and cold allodynia (P<0.001) present in tested hind paws at 24hrs, 96hrs and 144hrs post initial drug treatment in CE-245677 treated mice compared to vehicle treated mice. As before, there was no significant difference in pain sensitivities for mechanical allodynia and vocalisation in response to knee compressions between the two treatment groups. Pain sensitivities for all measurements were similar in drug treated mice and vehicle treated mice at 264hrs post CE-245677 treatment.

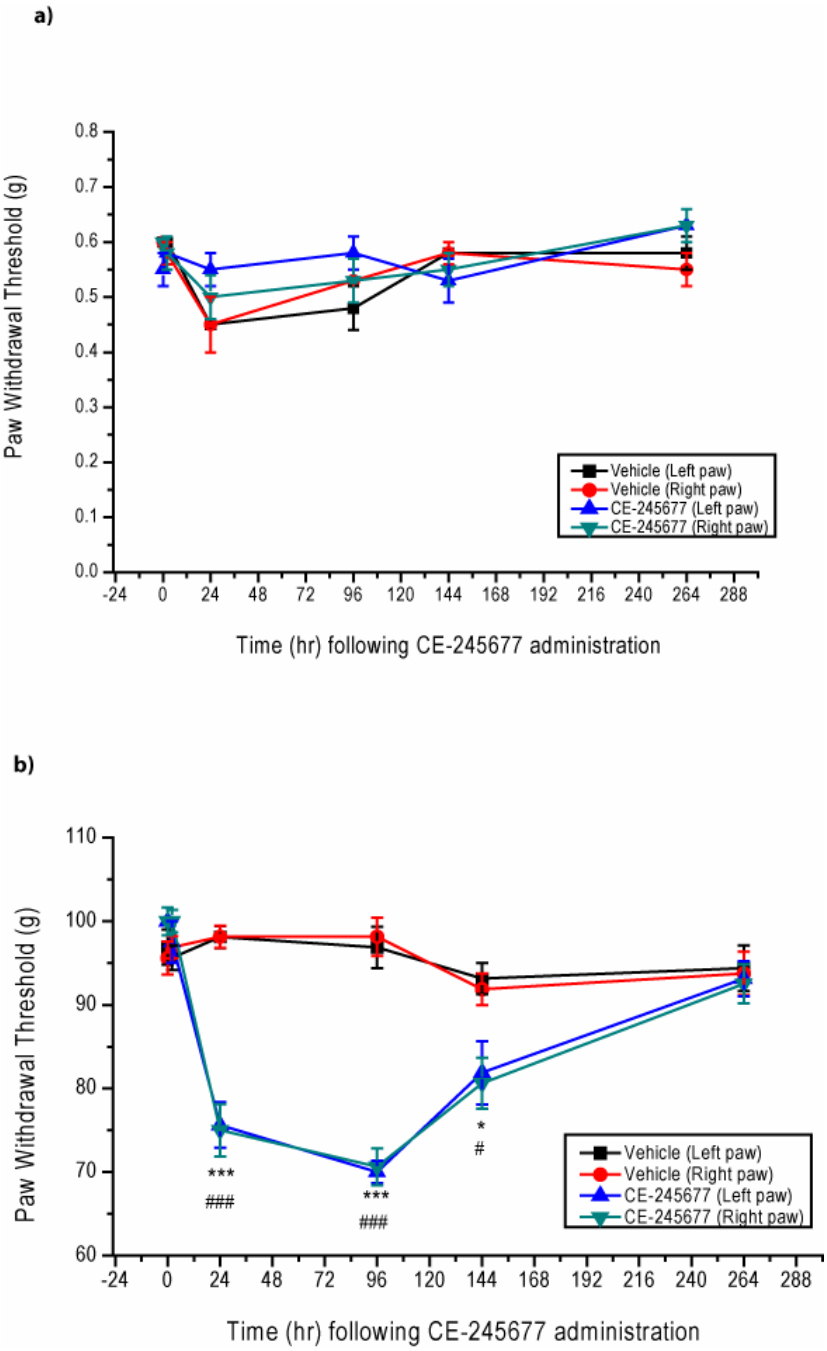


Figure 5-6 Repeat experiment to measure the effect of 30mg/kg p.o CE-245677 in 0.5% methyl cellulose in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.

The effect of CE-245677 on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by Tukey's post hoc test. Vehicle left paw versus CE-245677 left paw $P<0.05$ *, $P<0.001$ ***. Vehicle right paw versus CE-245677 right paw $P<0.05$ #, $P<0.001$ ###

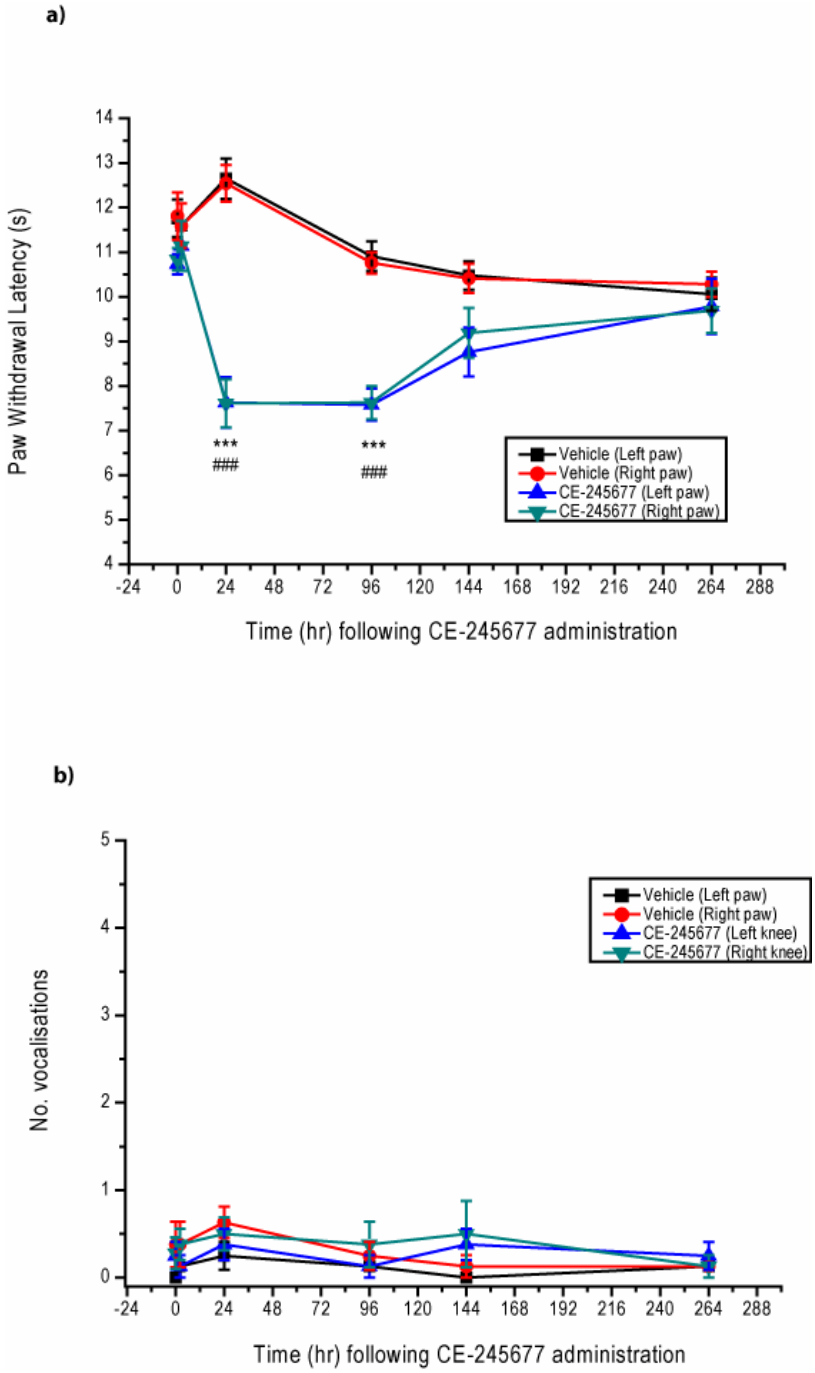


Figure 5-7 Repeat experiment to measure the effect of 30mg/kg p.o CE-245677 in 0.5% methyl cellulose in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.

The effect of CE-245677 on cold allodynia a) number of vocalisations in response to knee compressions b). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by Tukey's post hoc test. Vehicle left leg versus CE-245677 left leg P<0.001 ***. Vehicle right leg versus CE-245677 right leg P<0.001 ###

a)

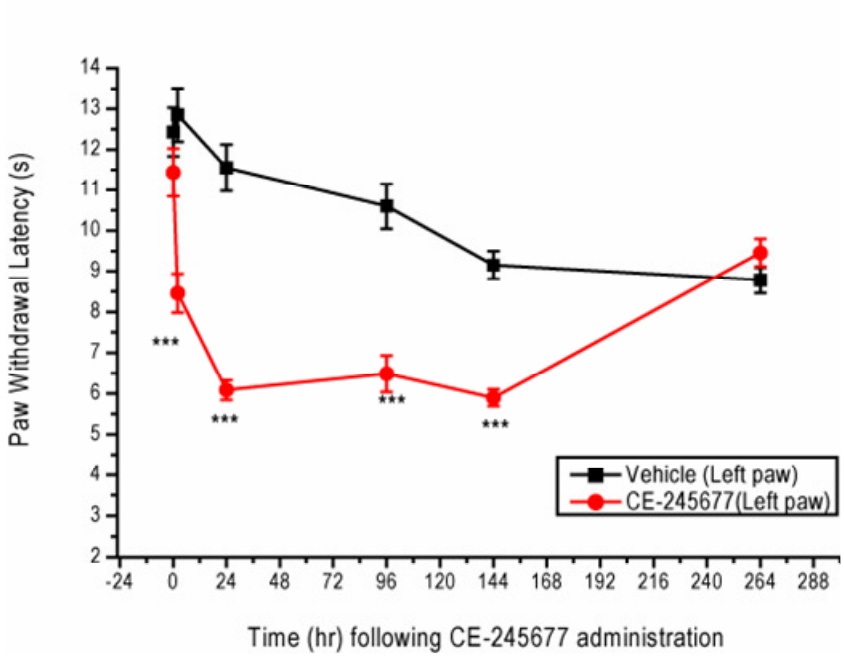


Figure 5-8 Repeat experiment to measure the effect of 30mg/kg p.o CE-245677 in 0.5% methyl cellulose in 8-10 week old naïve female C57Bl/6 mice on pain behaviours.
The effect of CE-245677 on thermal hyperalgesia a). Each point represents the mean value ± SEM of eight mice. Statistics: Vehicle left paw versus CE-245677 left paw by repeated measures ANOVA followed by post hoc Tukey's test P<0.001 ***

5.3.1.3 The effect of CE-245677 administration on CFA treated female C57Bl/6 mice

Administration of trk inhibitor,CE-245677, induced pain hypersensitivities in naïve mice. However, the compound was investigated further in a model of acute inflammation to compare the treatment effect on established pain behaviours in a model of acute inflammation. Baseline behaviour readings were performed in 8-10 week old female C57Bl/6 mice (n=16) by von Frey measurements, paw pressure and thermal hyperalgesia measurements. Cold sensitivity and vocalisations in response to knee compression were not tested since they would not be expected to develop in this model. Acute inflammation was induced in the left hind paw by injection of 1% CFA and 24 hours later, behavioural measurements were repeated. At 24 hours, the mice exhibited significant levels of mechanical allodynia (P<0.0001), mechanical hyperalgesia (P<0.0001) and thermal hyperalgesia

(P<0.0001) in the ipsilateral hind paw compared to baseline readings (Figure 5-9 and Figure 5-10). 30mg/kg CE-245677 p.o. or the drug vehicle was administered to the mice and behaviour readings repeated at 2hrs, 24hrs, 96hrs and 168hrs post-treatment (n=8 mice per group). There was no significant change in PWTs measured by von Frey filaments (mechanical allodynia) at any time point. A very small decrease in mechanical hyperalgesia from 60.0g ± 1.64g to 68.8g ± 2.95g was recorded in the ipsilateral hind limb of drug treated mice at 2hrs following CE-245677 administration compared to pre-treatment levels (P=0.054) and compared to vehicle treated mice (P=0.039). At 24hrs, levels of mechanical hyperalgesia returned with PWTs declining to 63.8g ± 3.5g. Levels of mechanical hyperalgesia persisted at 96hrs (PWT of 67.5g ± 3.4g) and 168hrs (PWT of 65.63g ± 2.40g) post CE-245677 which was not significantly different from vehicle treated mice.

At 24hrs post CE-245677, a contralateral mechanical hyperalgesia was recorded (Figure 5-9b) similar to the studies of CE-245677 in naïve mice (5.3.1.2). Paw withdrawal thresholds decreased from 99.4g ± 1.13g to 69.4g ± 3.05g (P<0.00001). These levels of contralateral mechanical hyperalgesia remained at 96hrs post drug administration but returned to baseline levels at 168hrs.

At 2hrs post CE-245677, thermal hyperalgesia was significantly reversed (P<0.00001, Figure 5-10a) from a PWL of 6.21s ± 0.31s to 10.2s ± 0.41s compared to pre-treatment levels and vehicle treated mice. At 24hrs, levels of thermal hyperalgesia increased with PWLs decreasing to 7.23s ± 0.41s. At 96hrs, and 168hrs PWLs were 9.50s ± 0.42s and 10.0 ± 0.66s which were significantly greater than PWLs for vehicle treated mice (P<0.001, Figure 5-10a).

For vehicle treated mice, there was no significant change in PWTs for mechanical hyperalgesia or PWLs for thermal hyperalgesia for the ipsilateral or contralateral limb at any time point following vehicle administration (Figure 5-9b and Figure 5-10a).

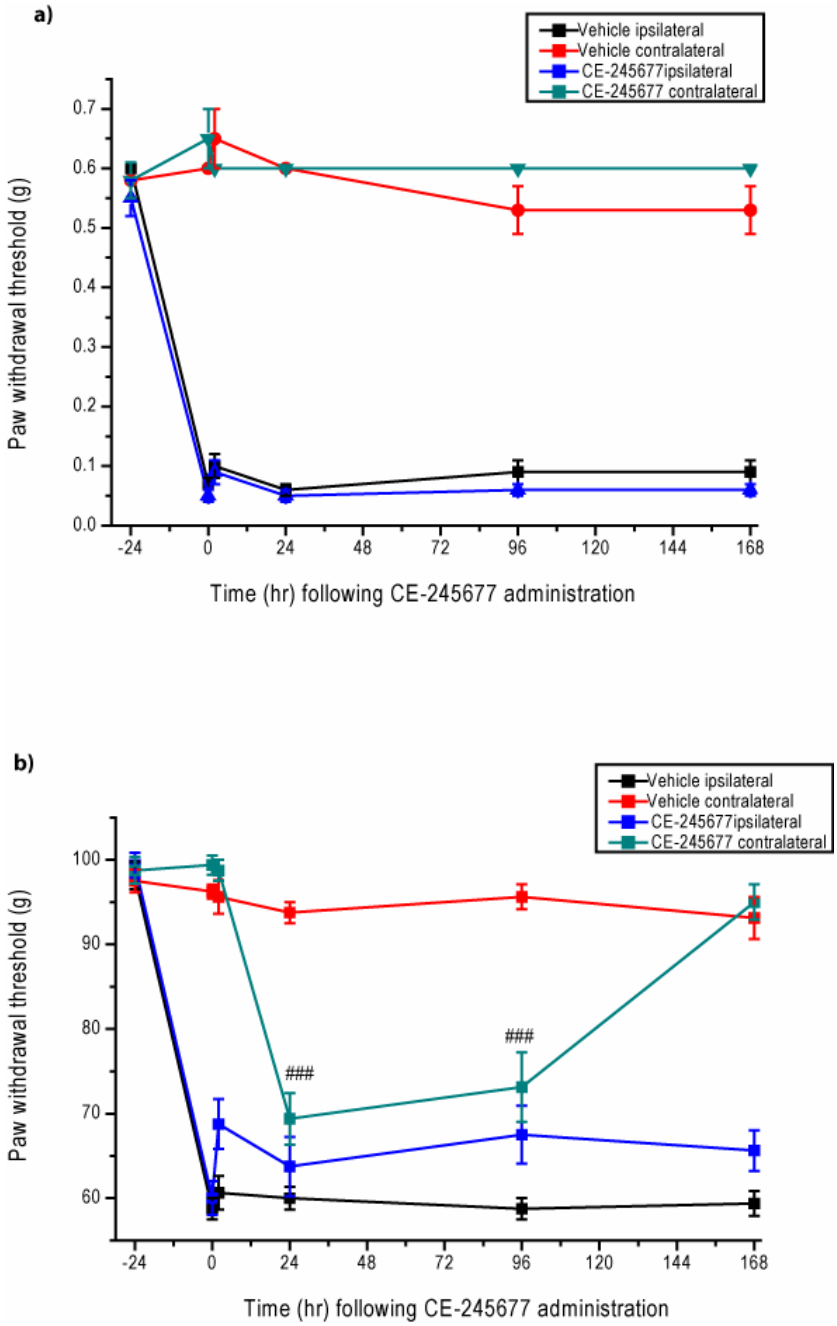


Figure 5-9 The effect of 30mg/kg p.o CE-245677 in CFA treated C57Bl/6 mice on pain behaviours.

The effect on mechanical allodynia (a) mechanical hyperalgesia (b). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by Tukey's post hoc test. Vehicle contralateral paw versus CE-245677 contralateral paw by unpaired t-test $P<0.001$ ###

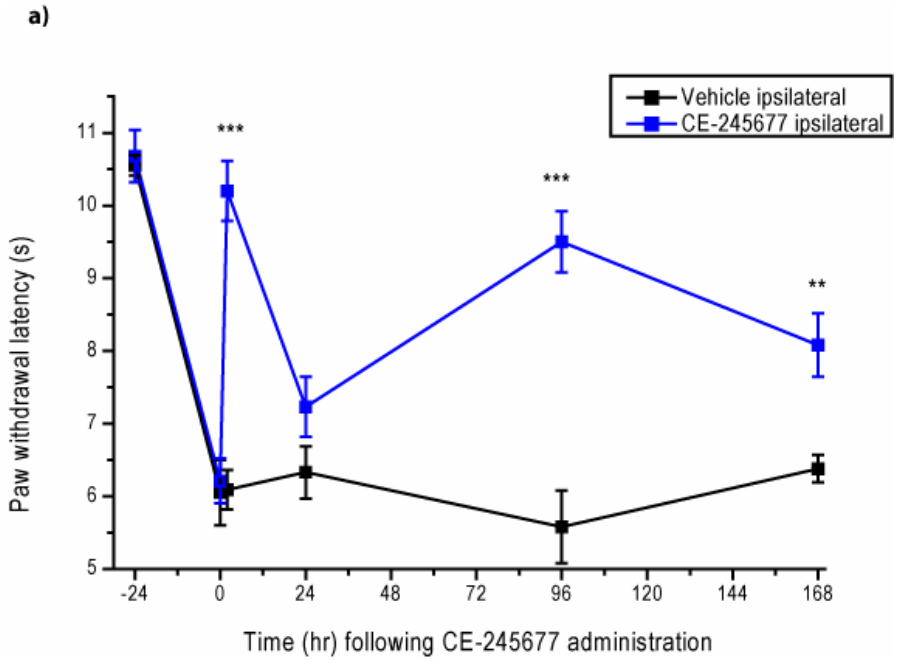


Figure 5-10 The effect of 30mg/kg p.o CE-245677 in CFA treated C57Bl/6 mice on pain behaviours.

The effect on thermal hyperalgesia a). Each point represents the mean value ± SEM of eight mice. Statistics: Repeated measures ANOVA followed by Tukey's post hoc test. Vehicle ipsilateral paw versus CE-245677 ipsilateral paw by unpaired t-test P<0.01**, P<0.001 ***

5.3.1.4 The effect of CE-245677 on OA pain behaviours in female C57Bl/6 mice following partial medial meniscectomy

16 female, 8 week old, C57Bl/6 mice underwent partial medial meniscectomy. Behavioural measurements were performed at regular intervals until pain sensitivities had developed. At week 7 post-surgery, behavioural measurements were performed for von Frey, paw pressure, cold allodynia and vocalisations in response to knee compressions. Thermal hyperalgesia was not tested since we had not previously studied the development of thermal hyperalgesia in OA mice and the repeated measurement of this sensitivity during the development of OA behaviours may have distorted the later post-drug measurements. The mice were treated with 30mg/kg p.o. CE-245677 and behavioural measurements repeated at 2hrs, 24hrs, 96hrs and 144hrs post treatment. At the time of drug administration, OA pain related behaviours were established in the ipsilateral hind limb of the mice with significant levels of mechanical allodynia (P<0.00001), mechanical hyperalgesia (P=0.0001) and cold allodynia (P=0.0001) compared to pre meniscectomy levels (Figure 5-11 and Figure 5-12). The number of vocalisations in

response to knee compressions increased from pre-surgery levels but was not statistically significant (P=0.061, Figure 5-12b).

Following administration of CE-245677, there was no significant change in behavioural readings for the ipsilateral hind limb of either drug treated or vehicle treated mice (Figure 5-11 and Figure 5-12). CE-245677 did not appear to have an overall sensitizing or analgesic effect on the ipsilateral hind limb. Similar to previous studies with CE-245677, contralateral hypersensitivities were recorded at 24hrs post treatment, with significantly decreased PWT to paw pressure measurements with PWT decreasing from 103.8g ± 2.27g (pre drug) to 85.0g ± 3.41g compared to vehicle treated mice (P<0.001, Figure 5-11b). Contralateral PWLs to a cold stimulus also decreased from 13.0s ± 0.63s (pre-drug) to 9.80s ± 0.58s but this was not significant compared to vehicle treated mice (P=0.11, Figure 5-12a). At 144hrs post CE-245677 there was no significant differences between vehicle and drug treated mice in either hind limb.

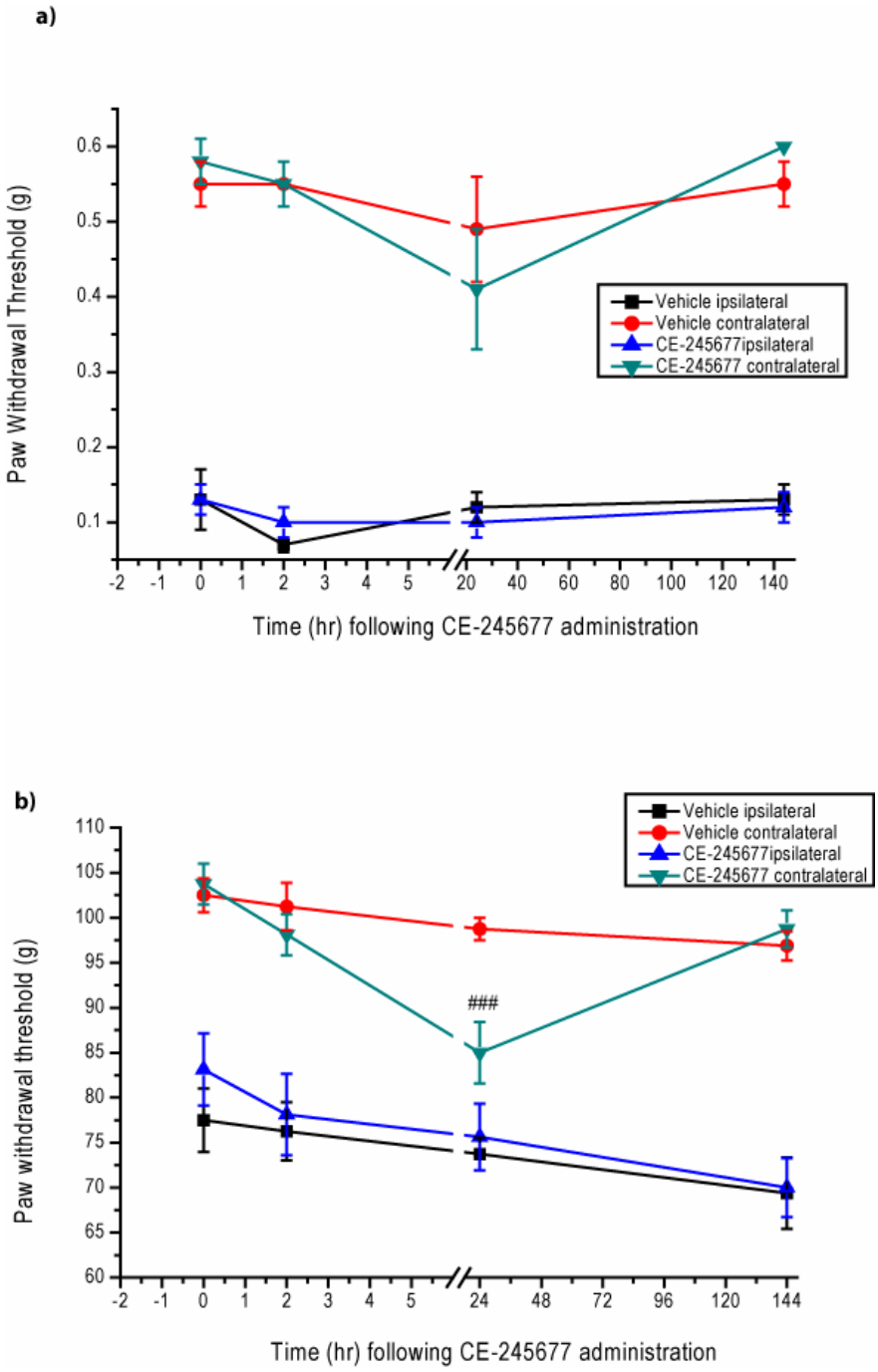


Figure 5-11 The effect of 30mg/kg p.o CE-245677 in OA C57Bl/6 mice on pain behaviours 7 weeks post partial medial meniscectomy.

The effect of CE-245677 on mechanical allodynia (a) mechanical hyperalgesia (b). Each point represents the mean value ± SEM of eight mice. Statistics: Vehicle contralateral paw versus CE-245677 contralateral paw by repeated measures ANOVA followed by tukey's post hoc test $P<0.001$ ###,

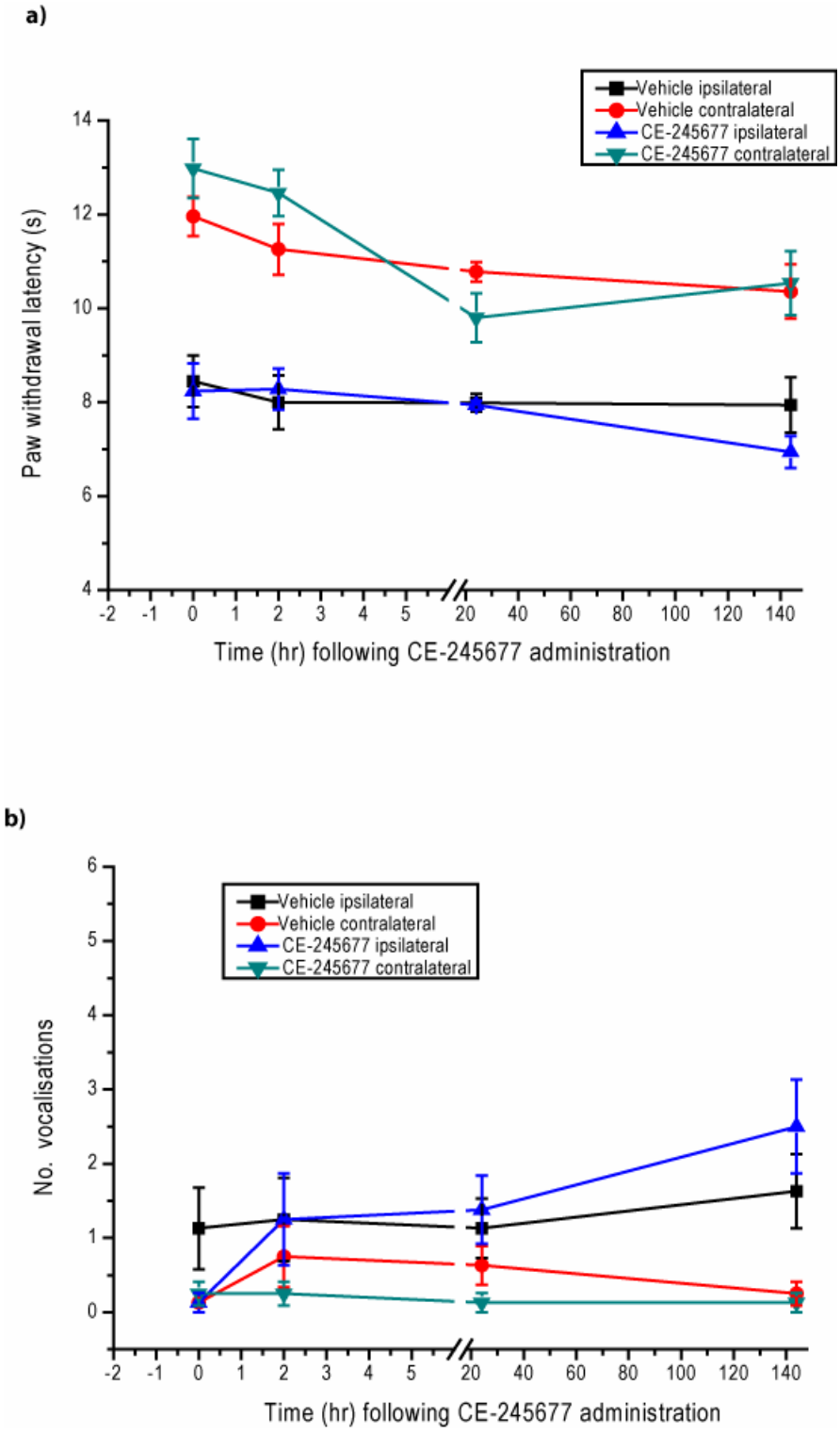


Figure 5-12 The effect of 30mg/kg p.o CE-245677 in OA C57Bl/6 mice on pain behaviours 7 weeks post partial medial meniscectomy.

The effect of CE-245677 on cold allodynia a) vocalisations during knee compressions b). Each point represents the mean value ± SEM of eight mice.

5.3.2 Anti-TNF

5.3.2.1 The effect of administration of anti-TNF on naïve female C57Bl/6 mice

10 week old naïve female C57Bl/6 mice were treated with 200µg per mouse i.p. of murine TNF receptor type II Fc fusion protein (TNFRII.Fc) or a class matched control antibody (anti–Elmeria tenella, muIgG2a; Pfizer Inc., UK), every 48 hours for 4 treatments according to a published treatment schedule (Young, Hegen et al. 2007). Behavioural measurements were performed for von Frey, paw pressure, cold allodynia and knee compressions at day 0 (pre treatment), 24hrs, 72hrs and 168 hours following the initiation of anti-TNF therapy. The results are shown in Figure 5-13 and Figure 5-14. There were no significant alterations in pain behaviour readings following repeated administration of anti-TNF or control antibody during the study period.

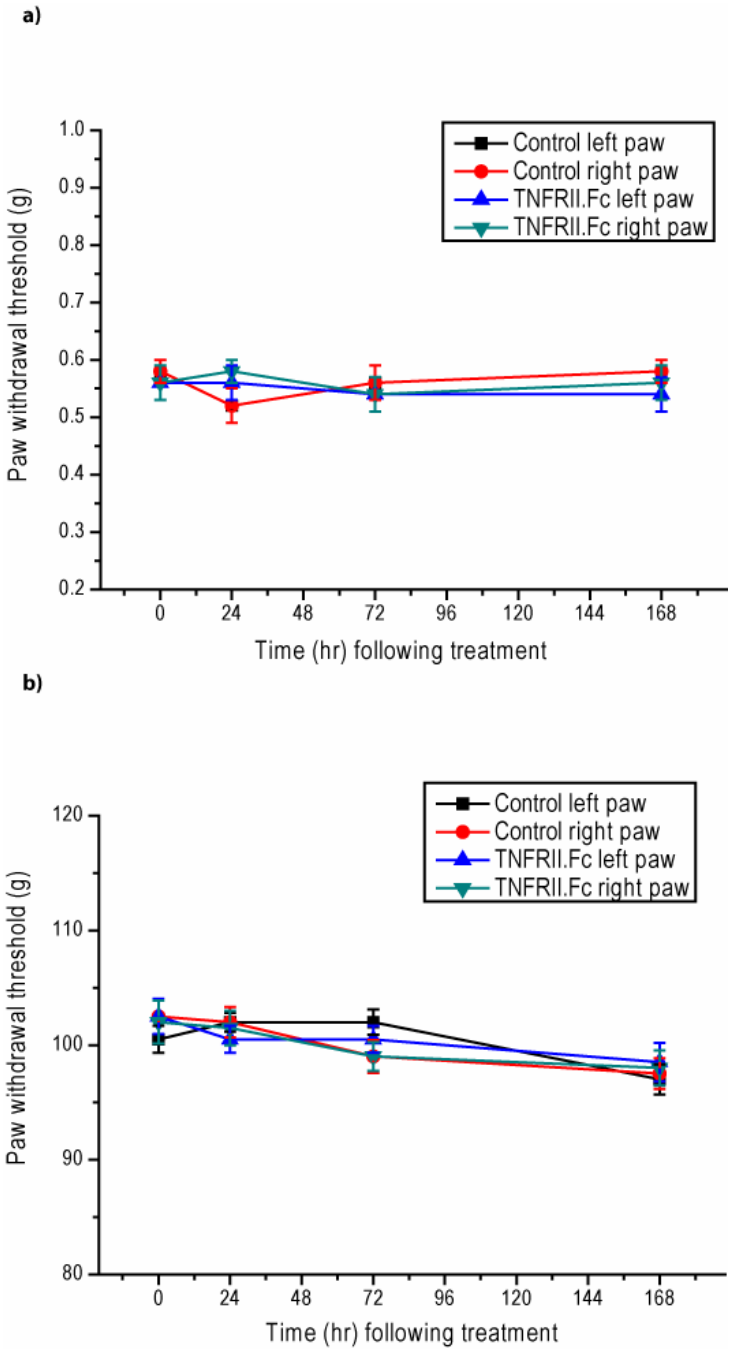


Figure 5-13 The effect of 200µg per mouse i.p. TNFRII.Fc or anti–Elmeria tenella, mulgG2a control antibody every 48hr for 4 treatments in naïve female C57Bl/6 mice on pain behaviours.

The effect on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice.

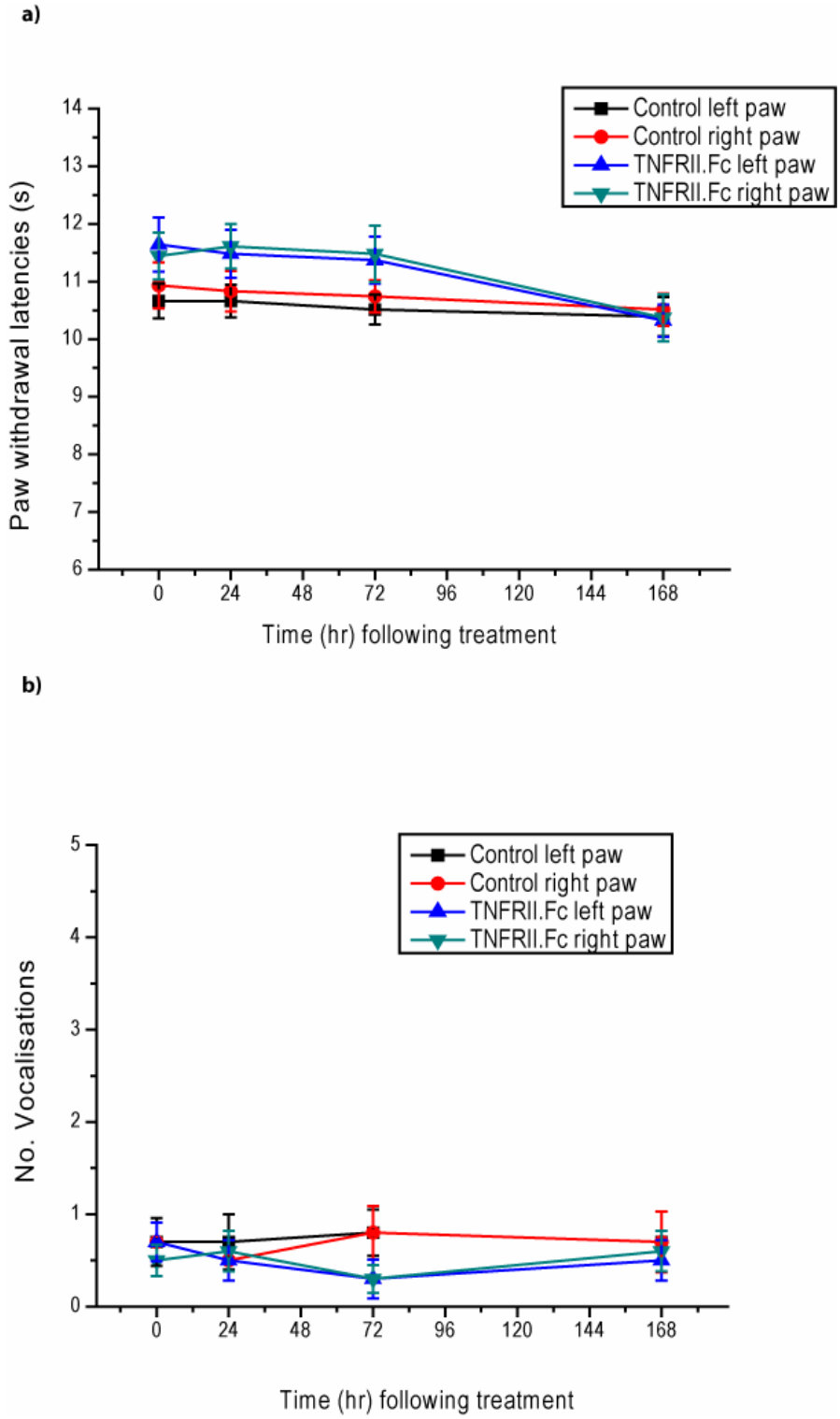


Figure 5-14 The effect of 200µg per mouse i.p. TNFRII.Fc or anti–Elmeria tenella, muIgG2a control antibody every 48hr for 4 treatments in naïve female C57Bl/6 mice on pain behaviours.

The effect on cold allodynia a) knee vocalisations b). Each point represents the mean value ± SEM of ten mice.

5.3.2.2 The effect of administration of anti-TNF on OA pain behaviours in female C57Bl/6 mice

8 weeks post partial medial meniscectomy surgery, significant OA pain behaviours were recorded in the ipsilateral hind limb of female of C57Bl/6 mice. The mice were treated with 200µg per mouse i.p. (0.2ml) of a mouse IgG2a control (anti–Elmeria tenella, muIgG2a; Pfizer Inc., UK), or murine TNF receptor type II Fc fusion protein (TNFRII.Fc) every 48 hours for 4 treatments (n=8 per group). Behavioural measurements were performed for von Frey, paw pressure, cold allodynia and knee compressions at day 0 (pre treatment), 24hrs, 72hrs and 168 hours following the initiation of anti-TNF therapy. The results are shown in Figure 5-15 and Figure 5-16. During the study, there was no significant change in PWTs for von Frey measurements or vocalisations in response to knee compression following repeated administration of anti-TNF or the control antibody. However, changes in mechanical hyperalgesia and cold allodynia were recorded. Prior to drug administration, ipsilateral paw pressure PWTs for the paw pressure test were 64.38g ± 1.75 for the control antibody group and 64.38g ± 2.40g in the anti-TNF group. At 24 hours post-treatment, ipsilateral PWTs unexpectedly rose in both the control antibody (to 108.13g ± 2.30g) and anti-TNF (105.0g ± 2.50g) treated animals. There was no significant difference between the two groups. The presence of mechanical hyperalgesia was diminished at both 24hrs and 72hrs post initial treatment. At 168hours following the initial treatment, mechanical hypersensitivity in the ipsilateral hind limb returned with PWTs for the control antibody group recorded at 61.88g ± 2.82g and 68.33g ± 3.44g for the anti-TNF group. Cold sensitivity followed a similar time course with an initial increase in PWLs (decreased sensitivity) at 24hours post-initial treatment which was recorded in both the control antibody group and anti-TNF treatment group. There was no significant difference between control antibody treated mice and anti-TNF treated mice for any of the pain behaviours during the study period. The phase of diminished pain behaviours may be due to the presence of endogenous opioids masking pain behaviours in both the control treated and anti-TNF treated mice. At 168hrs, the mice had received 4 successive treatments of anti-TNF, yet the pain behaviours were not significantly different from the control antibody treated mice, or from baseline pain behaviours indicating that fluctuations in pain behaviours were not directly related to anti-TNF treatment.

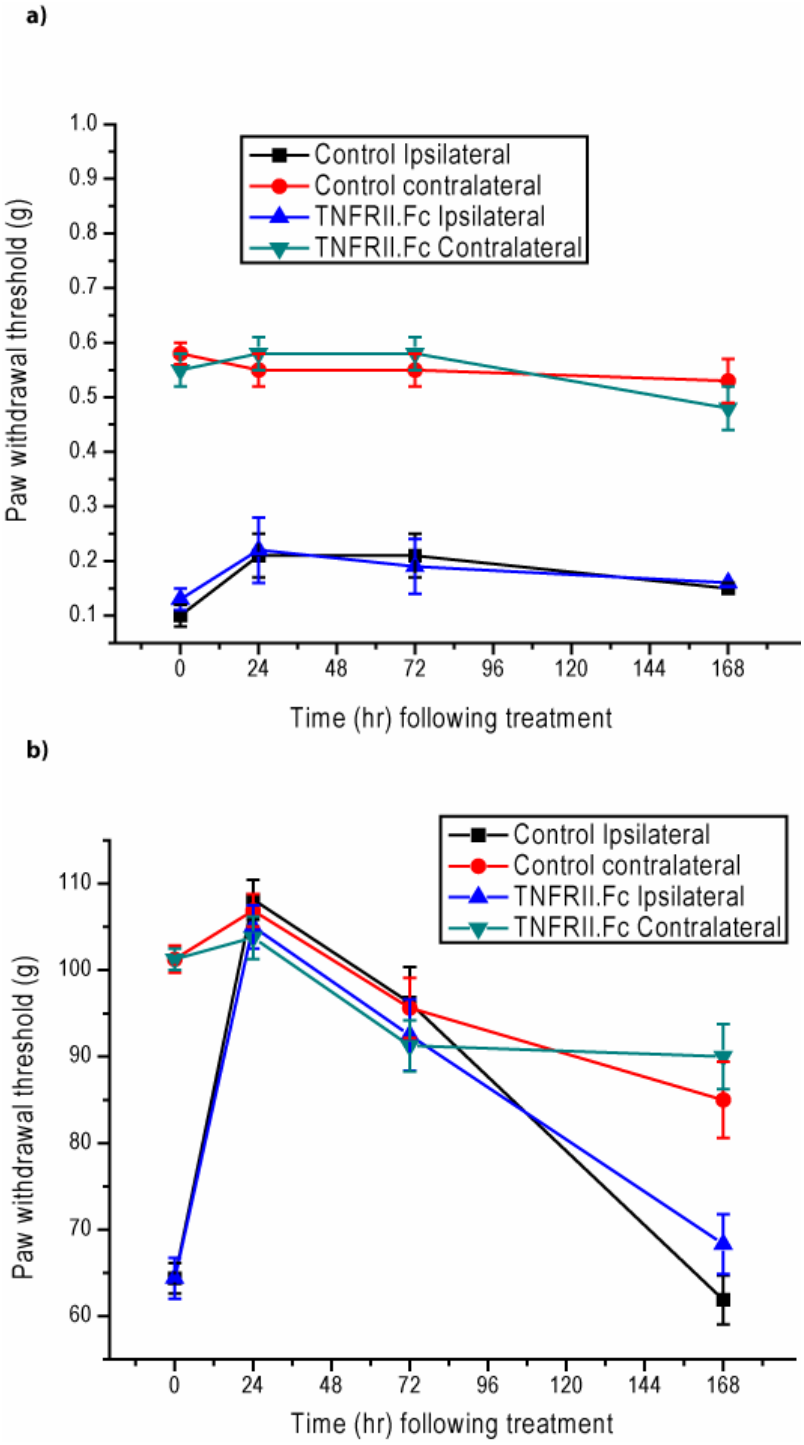


Figure 5-15 The effect of 200µg per mouse i.p. TNFRII.Fc or anti–Elmeria tenella, mulgG2a control antibody in female C57Bl/6 mice every 48hr for 4 treatments on OA induced pain behaviours.

The effect on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of eight mice.

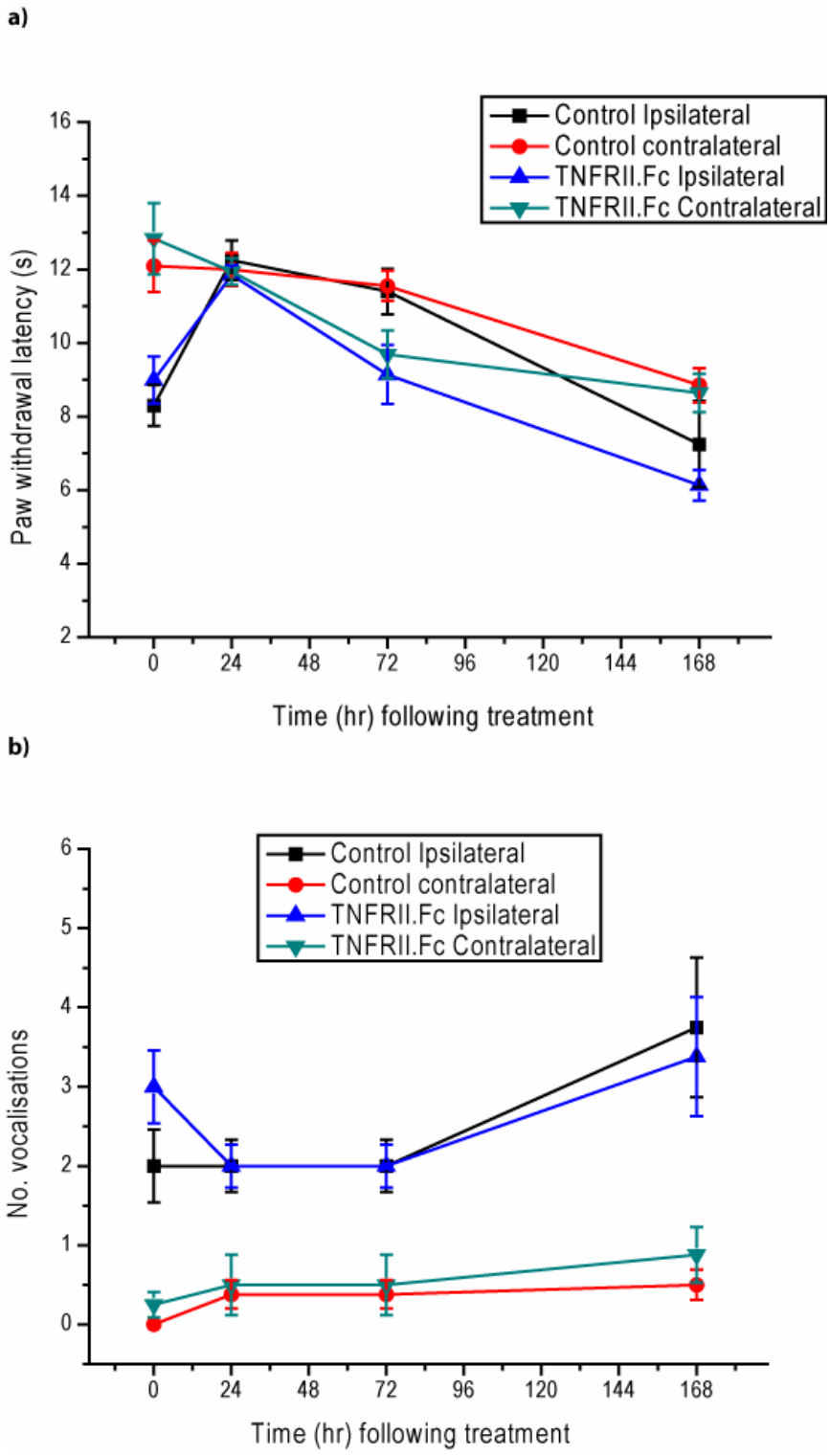


Figure 5-16 The effect of 200µg per mouse i.p. TNFRII.Fc or anti–Elmeria tenella, mulgG2a control antibody every 48hr for 4 treatments in female C57Bl/6 mice on OA induced pain behaviours.

The effect on cold allodynia a) knee vocalisations b). Each point represents the mean value ± SEM of eight mice.

5.3.3 Bradykinin B2 antagonist (Bradyzide)

2mg/kg of bradyzide (a non-peptide B2 receptor antagonist) in 0.5% methyl cellulose or the vehicle alone was administered p.o. to mice with OA pain behaviours and post-treatment behaviour readings recorded after 1 hour (n=10 per group). A significant decrease in ipsilateral mechanical hyperalgesia ($P<0.0001$, see Figure 5-17b), and cold allodynia ($P=0.02$, see Figure 5-18a) was recorded following treatment with bradyzide. No significant difference was recorded in mechanical allodynia measured by von Frey hairs (Figure 5-17a) or the number of vocalisations in response to 10 knee compressions (Figure 5-18b). There was no significant difference recorded in pain behaviours taken from the contralateral limb or in vehicle treated animals.

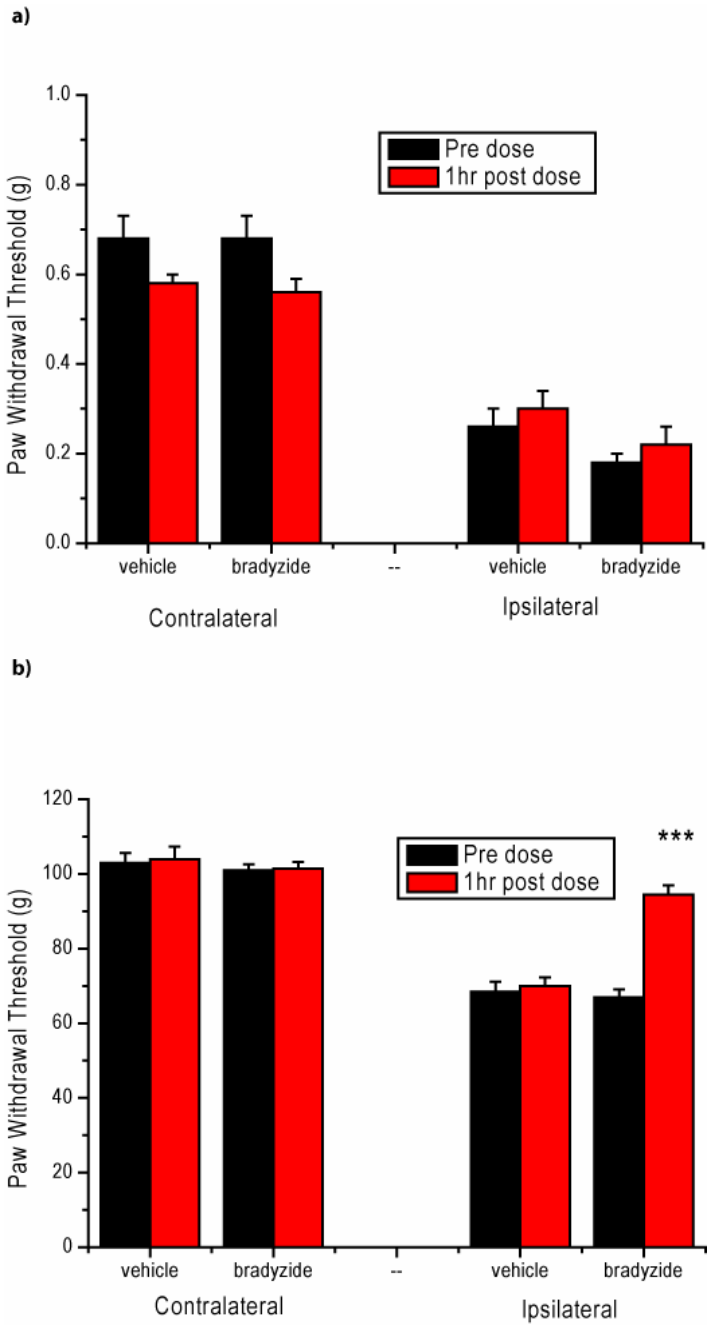


Figure 5-17 The effect of B2 antagonist Bradyzide (2mg/kg p.o.) on OA pain behaviours in female C57Bl/6 mice 8 weeks following partial medial meniscectomy.

The effect of bradyzide on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: pre dose versus post dose by unpaired t-test, *** P<0.001.

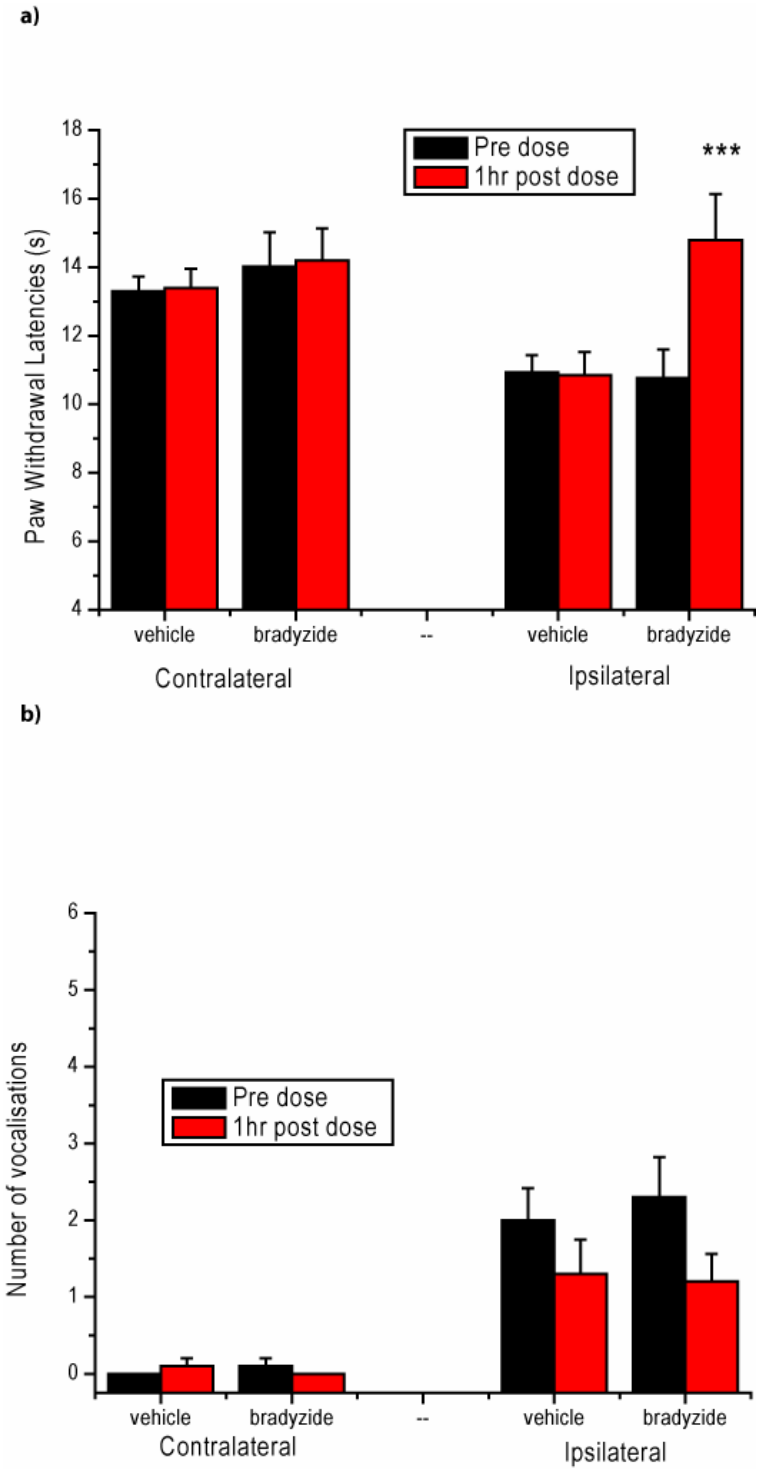


Figure 5-18 The effect of B2 antagonist Bradyzide (2mg/kg p.o.) on OA pain behaviours in female C57Bl/6 mice 8 weeks following partial medial meniscectomy.

The effect of bradyzide on cold allodynia a) knee vocalisations b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre dose versus post dose by unpaired t-test, *** P<0.001.

5.3.4 Transient receptor potential antagonists

5.3.4.1 AMTB – TRPM8 channel blocker

Administration of 10mg/kg AMTB i.p. to mice with OA pain behaviours resulted in a significant decrease in cold allodynia with an increase in PWL from 6.18s ± 0.44s to 7.82s ± 0.5s (P=0.48, see Figure 5-20a) after 1hr. There was no significant effect on mechanical hyperalgesia, mechanical allodynia or vocalisations in response to knee compression (Figure 5-19 and Figure 5-20). There was no significant effect of AMTB on any of the tested modalities on the contralateral limb of AMTB treated mice or the behaviours of vehicle treated mice.

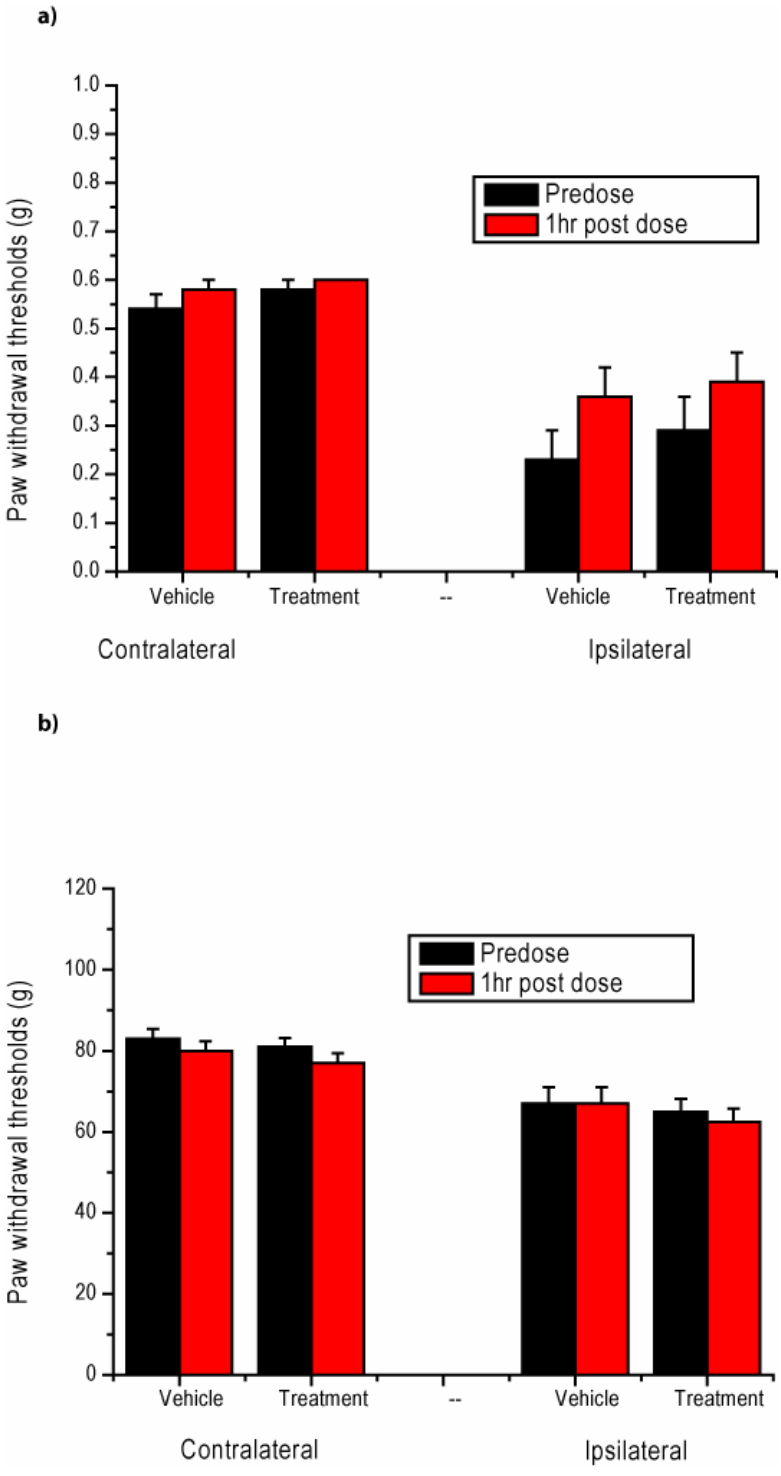


Figure 5-19 The effect of AMTB (10mg/kg i.p.) on pain behaviours at day 56 following partial medial meniscectomy in female C57Bl/6 mice.

The effect of AMTB on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice.

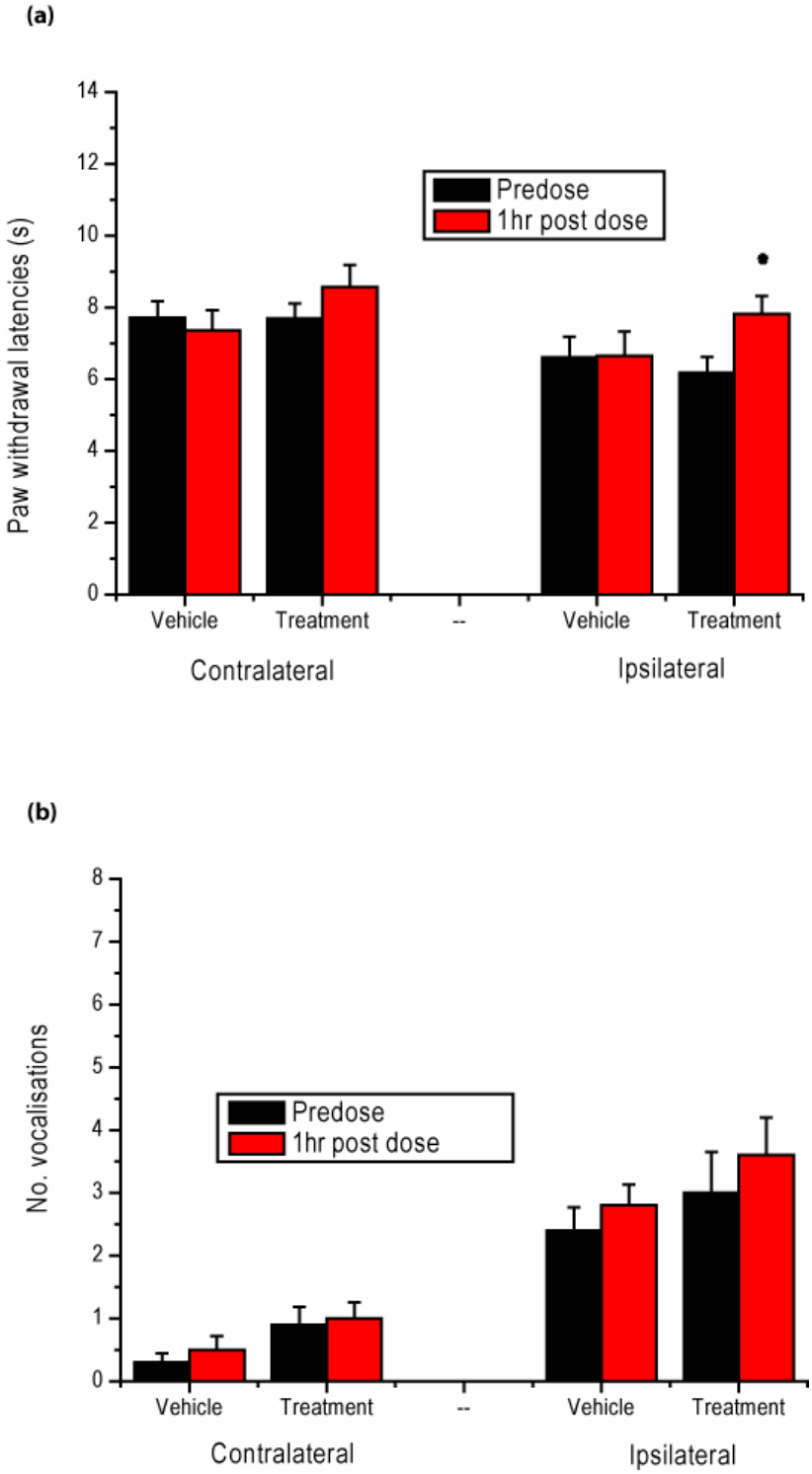


Figure 5-20 The effect of AMTB (10mg/kg i.p.) at day 76 on pain behaviours following partial medial meniscectomy in female C57Bl/6 mice.

The effect of AMTB on cold allodynia a) knee vocalisations b). Each point represents the mean value ± SEM of ten mice. Statistics: pre dose versus post dose by unpaired t-test *P<0.05,

5.3.4.2 HC-030031 – TRPA1 channel blocker

Administration of 300mg/kg HC-030031 p.o. resulted in a significant decrease in mechanical hyperalgesia with an increase in PWT from 62.0g ± 4.2g to 78.5 ± 3.5g (P=0.0071, see Figure 5-21b) after 1hr. A significant decrease in cold allodynia with an increase in PWL from 5.66g ± 0.42g to 7.47g ± 0.18g (P=0.00095, see Figure 5-22a) was also noted. There was no significant effect on mechanical allodynia or vocalisations in response to knee compression (Figure 5-21a and Figure 5-22b). There were also no significant alterations in the measurements recorded from the contralateral hind limb or in the pain behaviours of vehicle treated animals.

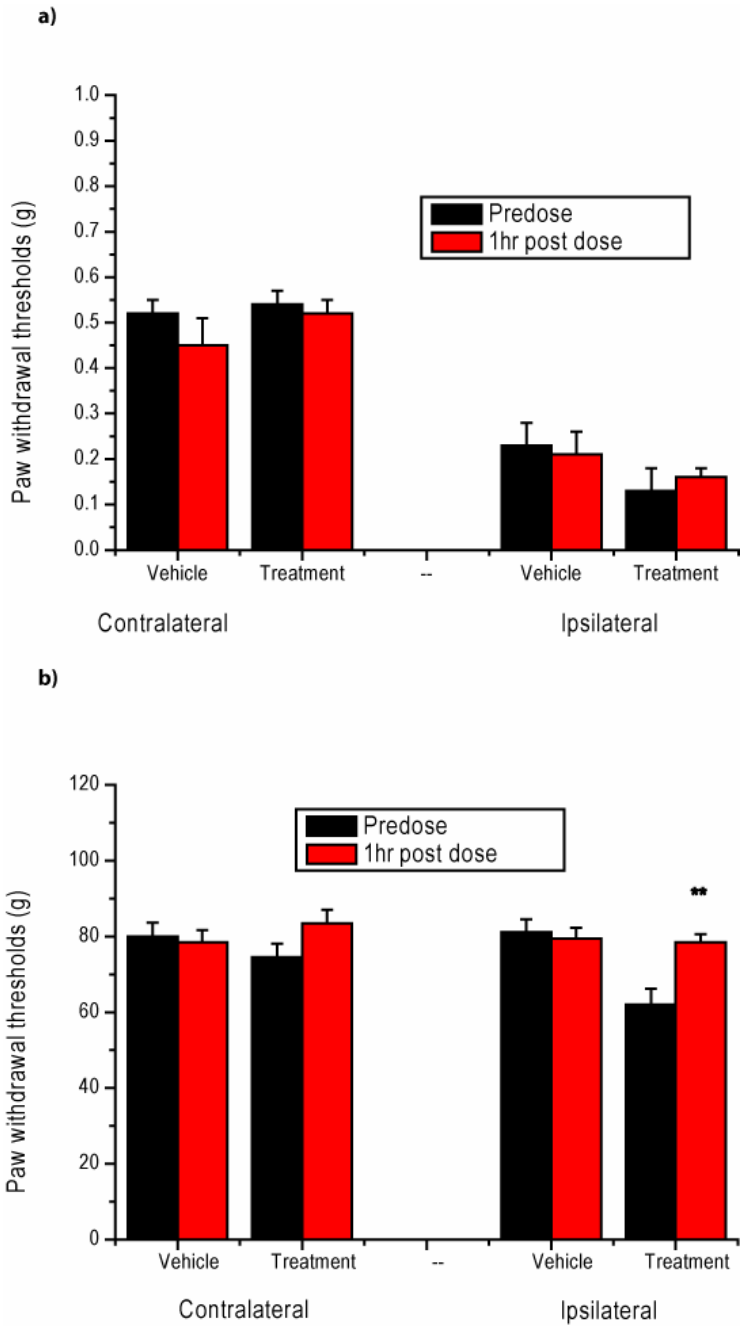


Figure 5-21 The effect of HC-030031 (300mg/kg p.o.) at day 76 following partial medial meniscectomy on pain behaviours in female C57Bl/6 mice.

The effect of HC-030031 on mechanical allodynia a) mechanical hyperalgesia b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre dose versus post dose by unpaired t-test, ** P<0.01.

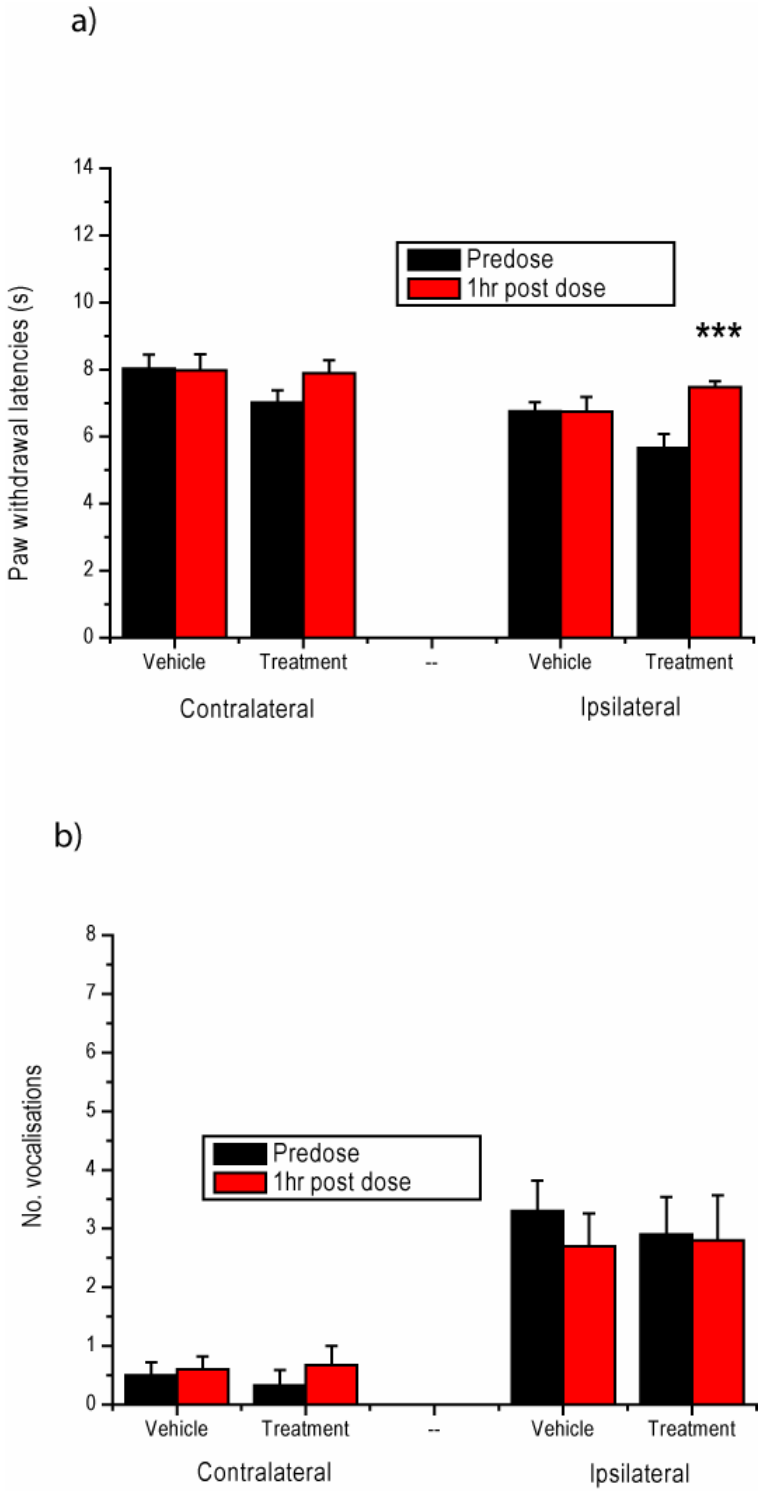


Figure 5-22 The effect of HC-030031 (300 mg/kg p.o.) at day 76 following partial medial meniscectomy on pain behaviours in female C57Bl/6 mice.

The effect of HC-030031 on cold allodynia a) knee vocalisations b). Each point represents the mean value ± SEM of ten mice. Statistics: Pre dose versus post dose by unpaired t-test, *** P<0.001.

5.3.5 *Trpa1* $^{-/-}$ mice

In order to determine the importance of TRPA1 on the development of OA pain behaviours, a group of 8-12 week old *trpa1*$^{-/-}$ mice and *trpa1* $^{+/+}$ mice underwent partial medial meniscectomy and compared to an equal number of t*rpa1*$^{-/-}$ mice and *trpa1* $^{+/+}$ mice who underwent sham surgery. In order to generate sufficient numbers of mice, both male and female mice were used in this study.

5.3.5.1 Pattern of sensitivity in *trpa1*$^{+/+}$ mice following partial medial meniscectomy

Trpa1$^{+/+}$ developed pain behaviours following partial medial meniscectomy similar to those reported in earlier studies of female C57Bl/6 mice except that there was an absence of the first phase of hypersensitivity. Meniscectomised *trpa1*$^{+/+}$ mice developed a significant mechanical allodynia in the ipsilateral hind paw compared to sham *trpa1*$^{+/+}$ mice from day 21 (P=0.0075) onwards with PWTs reaching a lowest value of 0.10g ± 0.02g at day 42 post surgery (Figure 5-23a). *Trpa1*$^{+/+}$ mice developed significant levels of mechanical hyperalgesia (P=0.0014, Figure 5-23b) and cold allodynia (P=0.00096, Figure 5-24a) of the ipsilateral hind paw compared to sham *trpa1*$^{+/+}$ mice from day 28 onwards. The lowest PWT was 64.0g ± 2.21g and lowest PWL was 6.43s ± 0.39s recorded at day 49. The number of vocalisations in response to knee compressions was significantly greater in *trpa1*$^{+/+}$ meniscectomised mice compared to sham mice at day 14 (P=0.048) and day 35 (P=0.00092) during this study (Figure 5-24a).

Compared to initial studies in female C57Bl/6 mice (see 3.3.4.3), there was an absence of the initial phase of hypersensitivity (inflammatory pain phase) in the first two weeks after partial medial meniscectomy. This observation was also recorded in later studies of female C57Bl/6 mice, where mice often displayed no change in pain behaviours at day 7 and day 14 post-surgery (Figure 5-25) and in male C57Bl/6 mice (Figure 4-31) who showed no change in pain behaviours at day 7 post-surgery compared to baseline measurements. The absence of pain behaviours in the immediate post-operative period is likely to be as a result of a more rapid resolution of inflammation and the associated initial pain sensitivities due to greater surgical proficiency.

5.3.5.2 Comparison of pain behaviours between *trpa1*$^{-/-}$ mice and *trpa1*$^{+/+}$ mice following partial medial meniscectomy

Prior to partial medial meniscectomy surgery, *trpa1*$^{+/+}$ mice showed pain thresholds similar to previous studies in C57Bl/6 mice (Figure 5-23 and Figure 5-24). *Trpa1*$^{-/-}$ mice, however had significantly higher baseline thresholds with PWTs measured by

von Frey filaments of 0.72g ± 0.06g compared to *trpa1*$^{+/+}$ mice with PWT of 0.52g ± 0.03g (P=0.030) prior to meniscectomy (Figure 5-23a). *Trpa1*$^{-/-}$ mice who underwent partial medial meniscectomy developed a significant mechanical allodynia in the ipsilateral hind paw compared to sham *trpa1*$^{-/-}$ mice from day 21 (P=0.00024) onwards with PWTs reaching a lowest value of 0.09g ± 0.01g at day 49 post surgery (Figure 5-23a). Following surgery, PWT in *trpa1*$^{-/-}$ and *trpa1*$^{+/+}$ mice were not statistically different from each other except at day 28 when *trpa1*$^{-/-}$ mice had higher PWT (0.33g ± 0.04g) compared to *trpa1*$^{+/+}$ mice (0.13g ± 0.02g, P=0.03, Figure 5-23a). These results suggest that TRPA1 does not play a major role in the development of mechanical allodynia in this model of OA pain.

Baseline PWTs measured by the paw pressure test were significantly greater in *trpa1*$^{-/-}$ mice (140.0g ± 2.24g) compared to *trpa1*$^{+/+}$ mice (98.5g ± 1.07g) (P=0.00016) prior to partial medial meniscectomy (Figure 5-23b). *Trpa1*$^{-/-}$ mice developed significant levels of mechanical hyperalgesia in the ipsilateral hind paw compared to sham *trpa1*$^{-/-}$ mice from day 28 (P=0.00016) onwards (Figure 5-23b). The lowest PWT was 88.0g ± 4.03g at day 28. However, whilst the pattern of hypersensitivity was similar in *trpa1*$^{-/-}$ and *trpa1*$^{+/+}$ mice, *trpa1*$^{-/-}$ had significantly higher PWT for the duration of the 49 day study (P<0.001 at each time point). These results indicate that *trpa1*$^{-/-}$ mice are less sensitive to the application of a noxious stimulus compared to *trpa1*$^{+/+}$ mice, however, TRPA1 is not essential for the development of mechanical hyperalgesia.

Baseline cold sensitivity readings were also higher in *trpa1*$^{-/-}$ mice with PWLs of 26.2s ± 1.1s compared to 10.92s ± 0.38s in *trpa1*$^{+/+}$ mice (P=0.00016) prior to partial medial meniscectomy (Figure 5-24a). *Trpa1*$^{-/-}$ mice developed a significant level of cold allodynia compared to sham mice from day 28 post surgery (P=0.00019) (Figure 5-24a). The lowest PWL was 10.33s ± 0.76s recorded at day 35. Cold allodynia developed a similar pattern of sensitivity in both *trpa1*$^{-/-}$ mice and *trpa1*$^{+/+}$ mice with a decrease in PWL from day 28 and slight increase in PWL at day 42. PWLs remained significantly higher for t*rpa1*$^{-/-}$ mice except at day 35 when PWLs were not statistically different from each other. These results indicate that *trpa1*$^{-/-}$ mice are less sensitive to the application of a cold stimulus compared to *trpa1*$^{+/+}$ mice, however, TRPA1 is not essential for the development of cold sensitivity.

The number of vocalisations in response to knee compressions gradually increased in *trpa1*$^{-/-}$ mice following partial medial meniscectomy over the 49 day study period (**Figure 5-24b**), however, the number of vocalisations produced by *trpa1*$^{-/-}$ mice was not significantly greater than sham *trpa1*$^{-/-}$ mice. There was also no significant

difference in the number of vocalisations between *trpa1*$^{+/+}$ and *trpa1*$^{-/-}$ meniscectomised mice. This study should be repeated before specific conclusions can be drawn on the influence of TRPA1 on the sensitivity of the knee to compressions following partial medial meniscectomy.

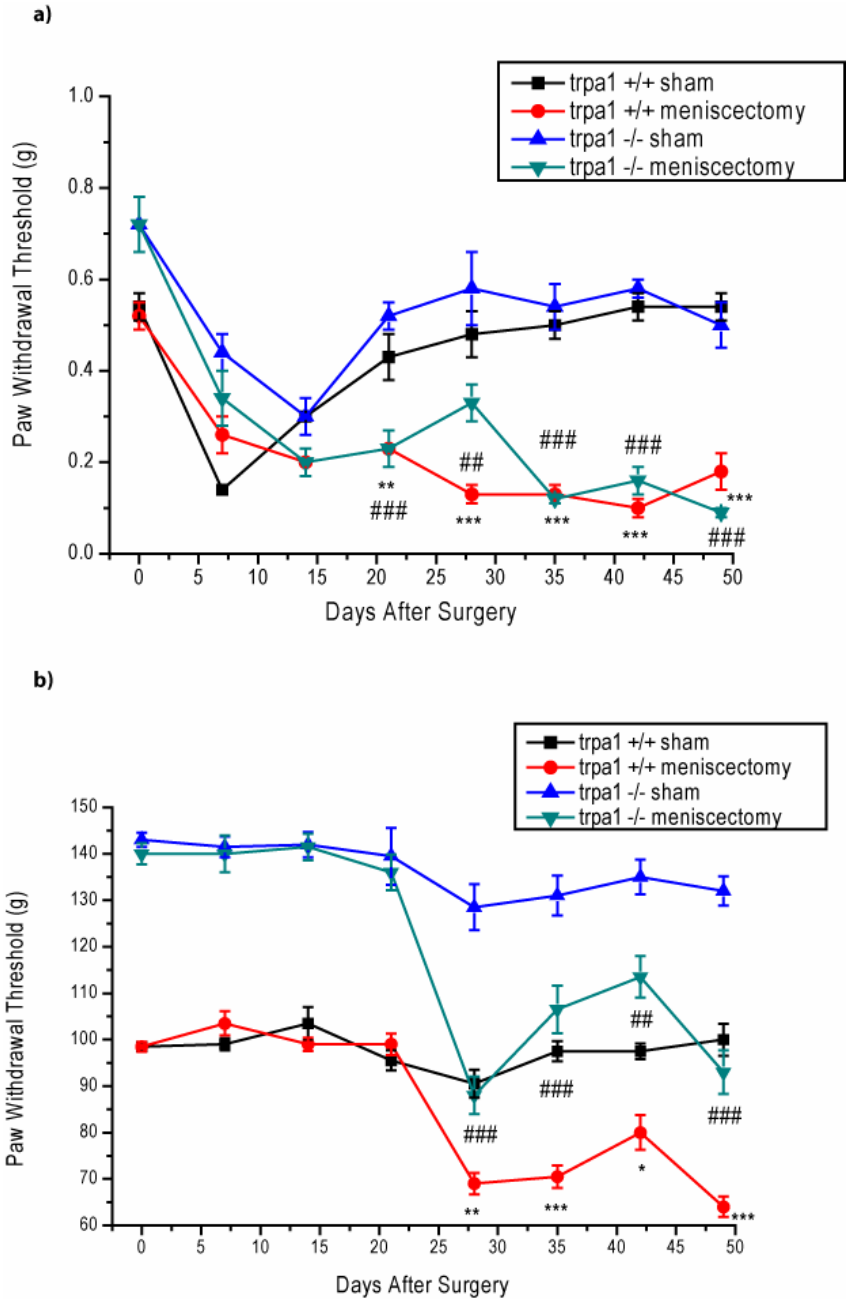


Figure 5-23 The development of pain behaviours following partial medial meniscectomy *trpa1*$^{-/-}$ mice or *trpa1*$^{+/+}$ mice.

The development of mechanical allodynia a) mechanical hyperalgesia b) are shown. Each point represents the mean value ± SEM for the ipsilateral hind limb of six male mice and four female mice. Statistics: *trpa1*$^{+/+}$ sham versus *trpa1*$^{+/+}$ meniscectomy by repeated measures ANOVA followed by Tukey's Test * P<0.05, ** P<0.01, *** P<0.001. *Trpa1*$^{-/-}$ sham versus *trpa1*$^{-/-}$ meniscectomy by repeated measures ANOVA followed by Tukey's Test. ## P< 0.01, ### P<0.001.

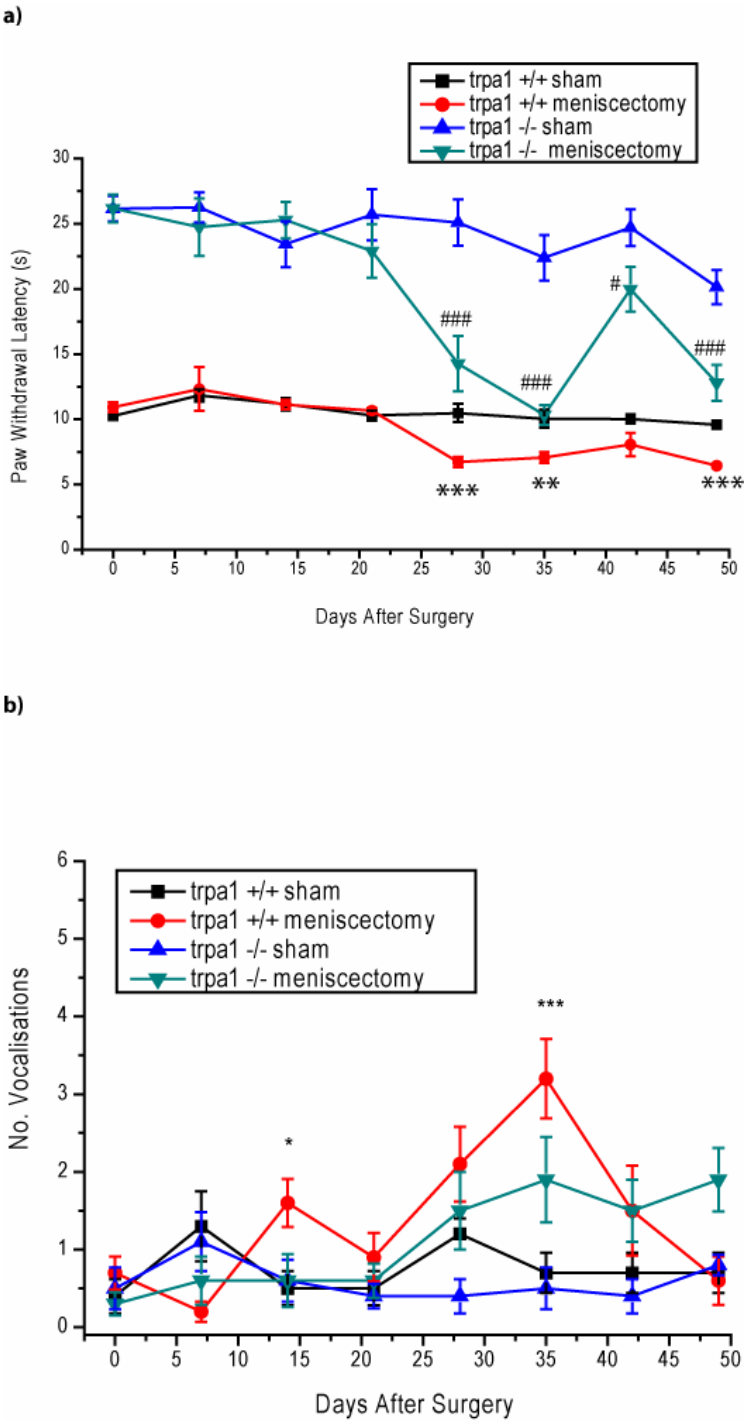


Figure 5-24 The development of pain behaviours following partial medial meniscectomy on *trpa1*$^{-/-}$ mice or *trpa1*$^{+/+}$ mice.

The development of cold allodynia a) knee vocalisations b) are shown. Each point represents the mean value ± SEM for the ipsilateral hind limb of six male mice and four female mice. Statistics: *trpa1*$^{+/+}$ sham versus *trpa1*$^{+/+}$ meniscectomy by repeated measures ANOVA followed by Tukey's Test * P<0.05, ** P<0.01, *** P<0.001. *Trpa1*$^{-/-}$ sham versus *trpa1*$^{-/-}$ meniscectomy by repeated measures ANOVA followed by Tukey's Test. # P< 0.05, ### P<0.001.

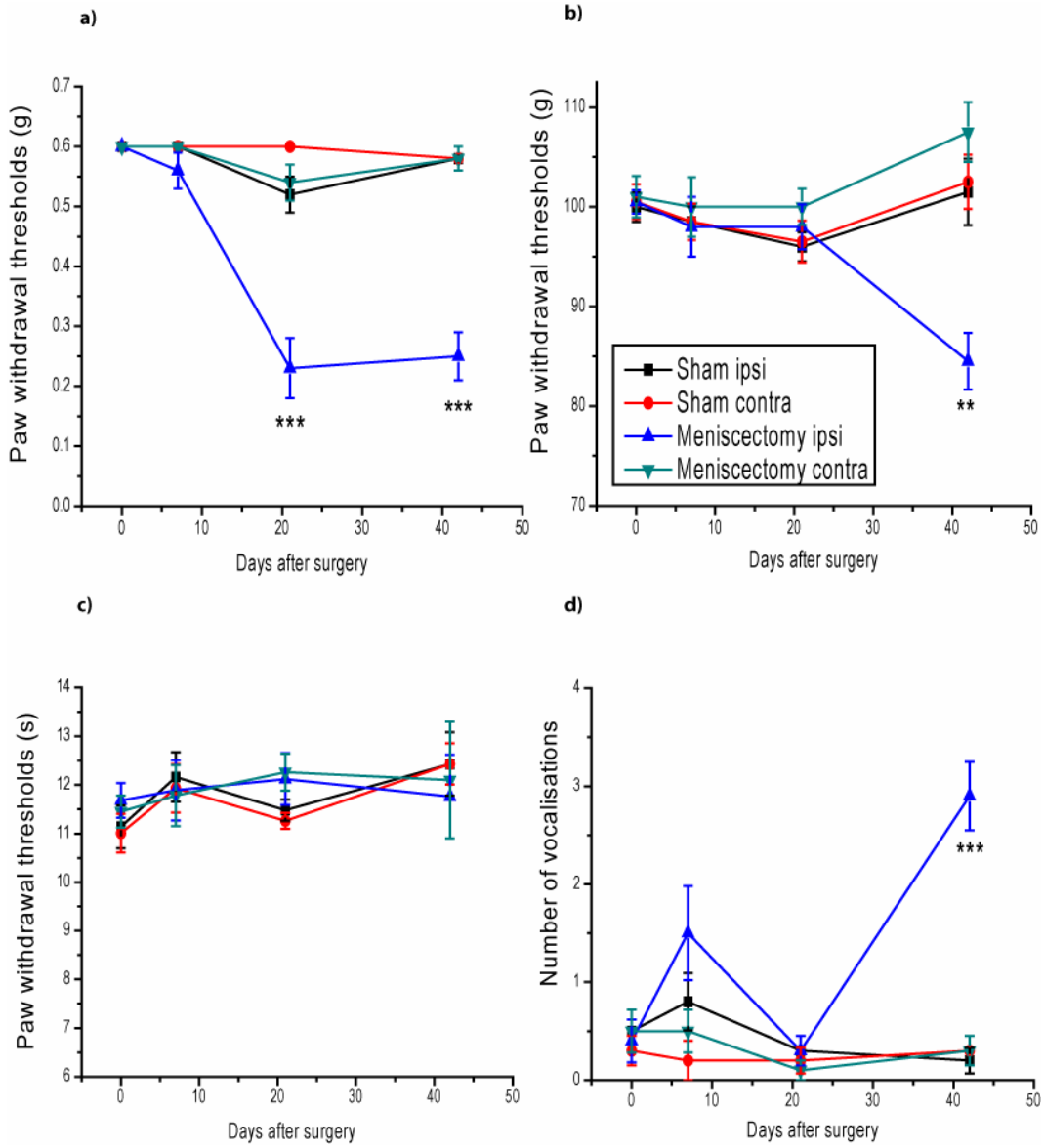


Figure 5-25 The development of pain behaviours following partial medial meniscectomy on female C57Bl/6 mice.

The development of mechanical allodynia a) mechanical hyperalgesia b) cold allodynia c) knee vocalisations d) are shown. Each point represents the mean value ± SEM of ten female mice. Statistics: Ipsilateral sham versus ipsilateral meniscectomy by repeated measures ANOVA followed by Tukey's Test ** P<0.01, *** P<0.001.

5.3.5.3 Sex differences in pain behaviours between male and female *trpa1*$^{+/+}$ and *trpa1*$^{-/-}$ mice.

In order to generate sufficient numbers of mice, both male and female mice were used in this study. Each group contained 6 male mice and 4 female mice. To reveal any marked sex differences in the development of OA pain sensitivities, the pain behaviours for male and female mice were plotted separately (see Figure 5-26, Figure 5-27, Figure 5-28, Figure 5-29). The graphs show that amongst *trpa1*$^{+/+}$ and *trpa1*$^{-/-}$ mice, the pattern of sensitivities that develops following partial medial meniscectomy between male and female mice is very similar for mechanical allodynia, mechanical hyperalgesia and cold allodynia. The main finding between the groups was that male meniscectomised mice vocalize more frequently during knee compressions compared to female mice, however, this was only apparent for 1 time point during the study. At day 35 following partial medial meniscectomy, male *trpa1*$^{+/+}$ mice vocalized an average of 4.33 ± 0.33 times compared to female *trpa1*$^{+/+}$ mice who vocalised 1.50 ± 0.29 times during knee compression (P=0.00045, Figure 5-27b). At day 28 following partial medial meniscectomy, male *trpa1*$^{-/-}$ mice vocalized an average of 2.33 ± 0.61 times compared to female *trpa1*$^{-/-}$ mice who vocalised 0.17 ± 0.17 times during knee compression (P=0.023, Figure 5-29b).

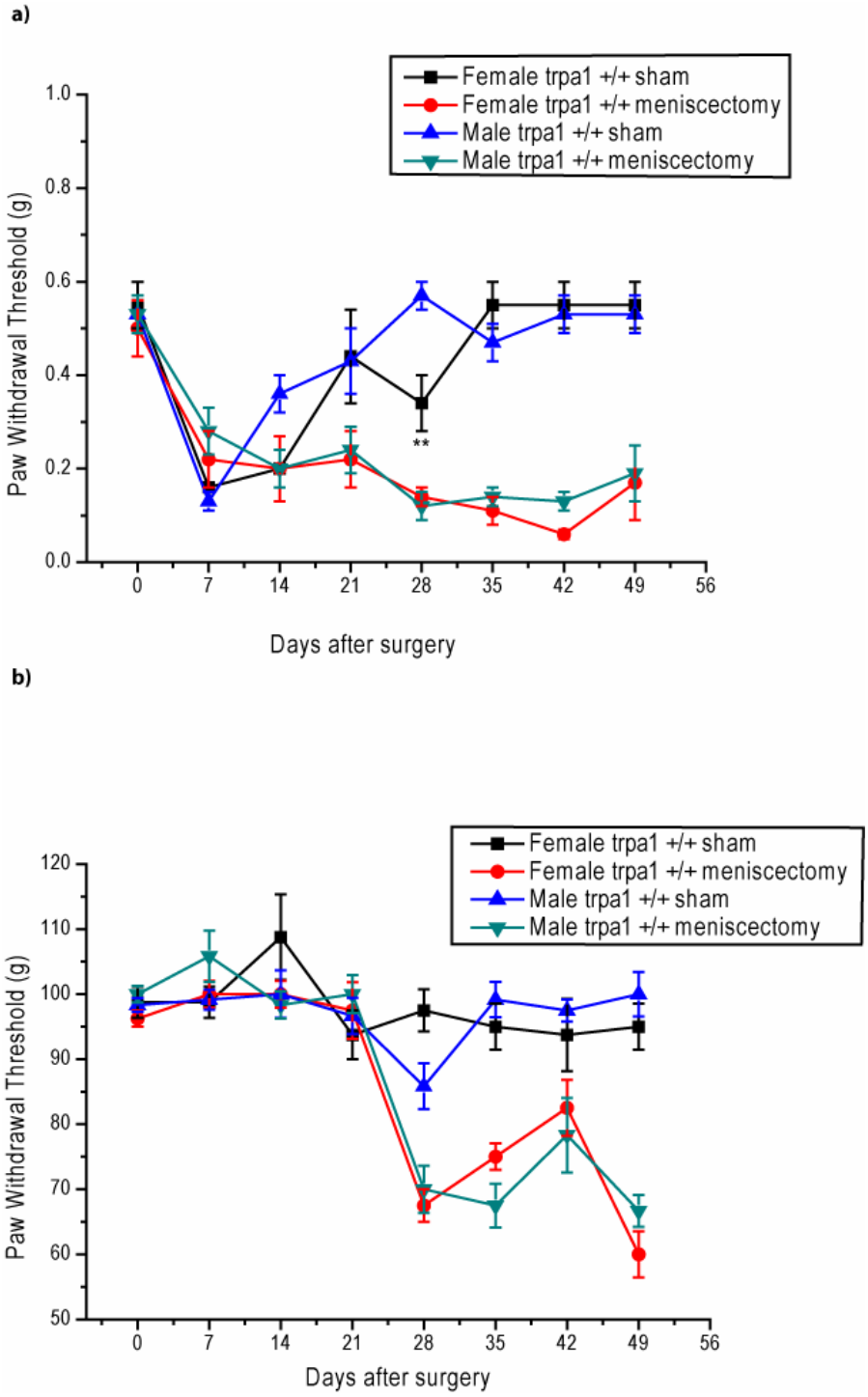


Figure 5-26 The development of pain behaviours following partial medial meniscectomy in male and female *trpa1*$^{+/+}$ mice.

The development of mechanical allodynia a) mechanical hyperalgesia b) are shown. Each point represents the mean value ± SEM for the ipsilateral hind limb of six male mice and four female mice. Statistics: female *trpa1*$^{+/+}$ sham versus male *trpa1*$^{+/+}$ sham by repeated measures ANOVA followed by Tukey's Test ** P<0.01

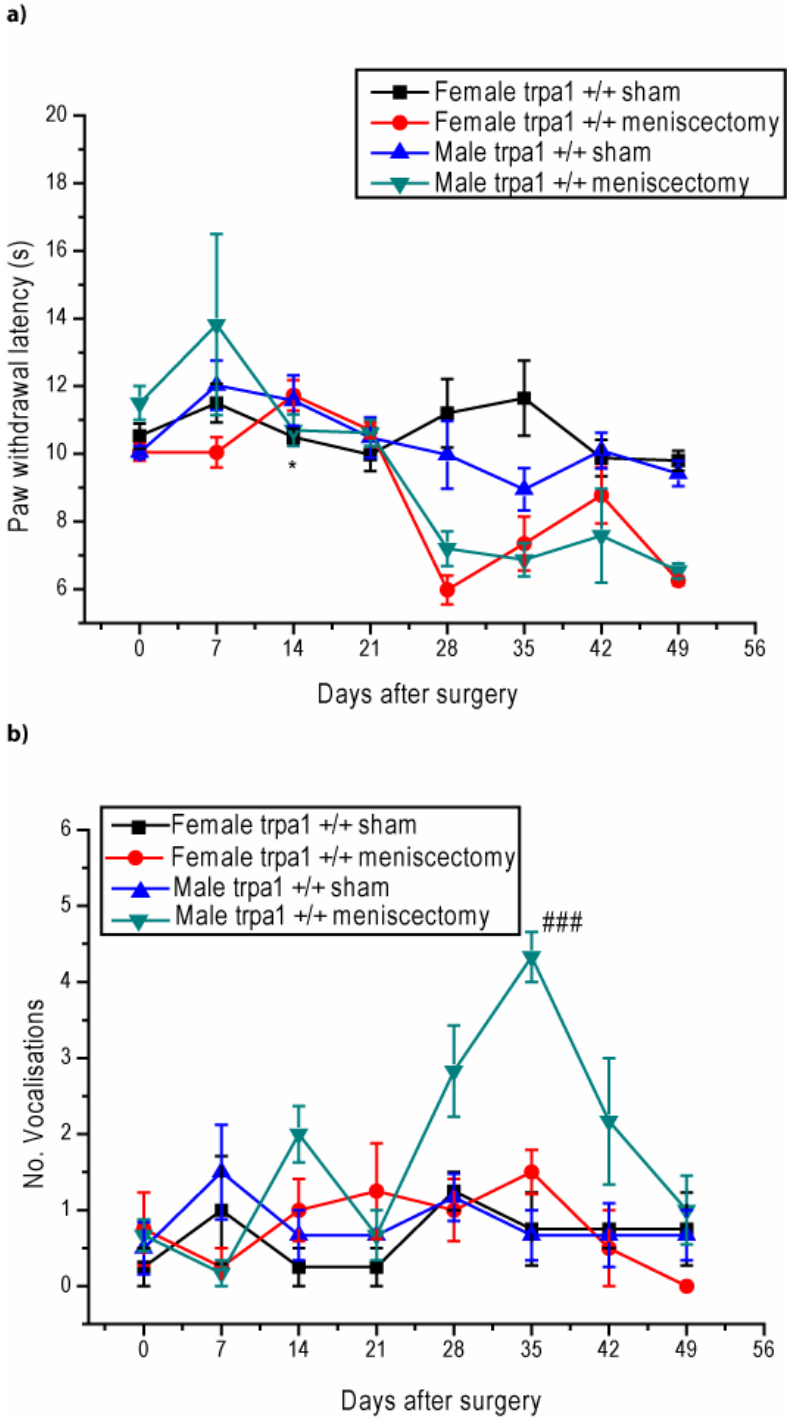


Figure 5-27 The development of pain behaviours following partial medial meniscectomy in male and female *trpa1*$^{+/+}$ mice.

The development of cold allodynia a) knee vocalisations b). Each point represents the mean value ± SEM for the ipsilateral hind limb of six male mice and four female mice. Statistics: Female *trpa1*$^{+/+}$ sham versus male *trpa1*$^{+/+}$ sham by repeated measures ANOVA followed by Tukey's Test, * P<0.05. Female *trpa1*$^{+/+}$ meniscectomy versus Male *trpa1*$^{+/+}$ meniscectomy by repeated measures ANOVA followed by Tukey's Test, ### P<0.001

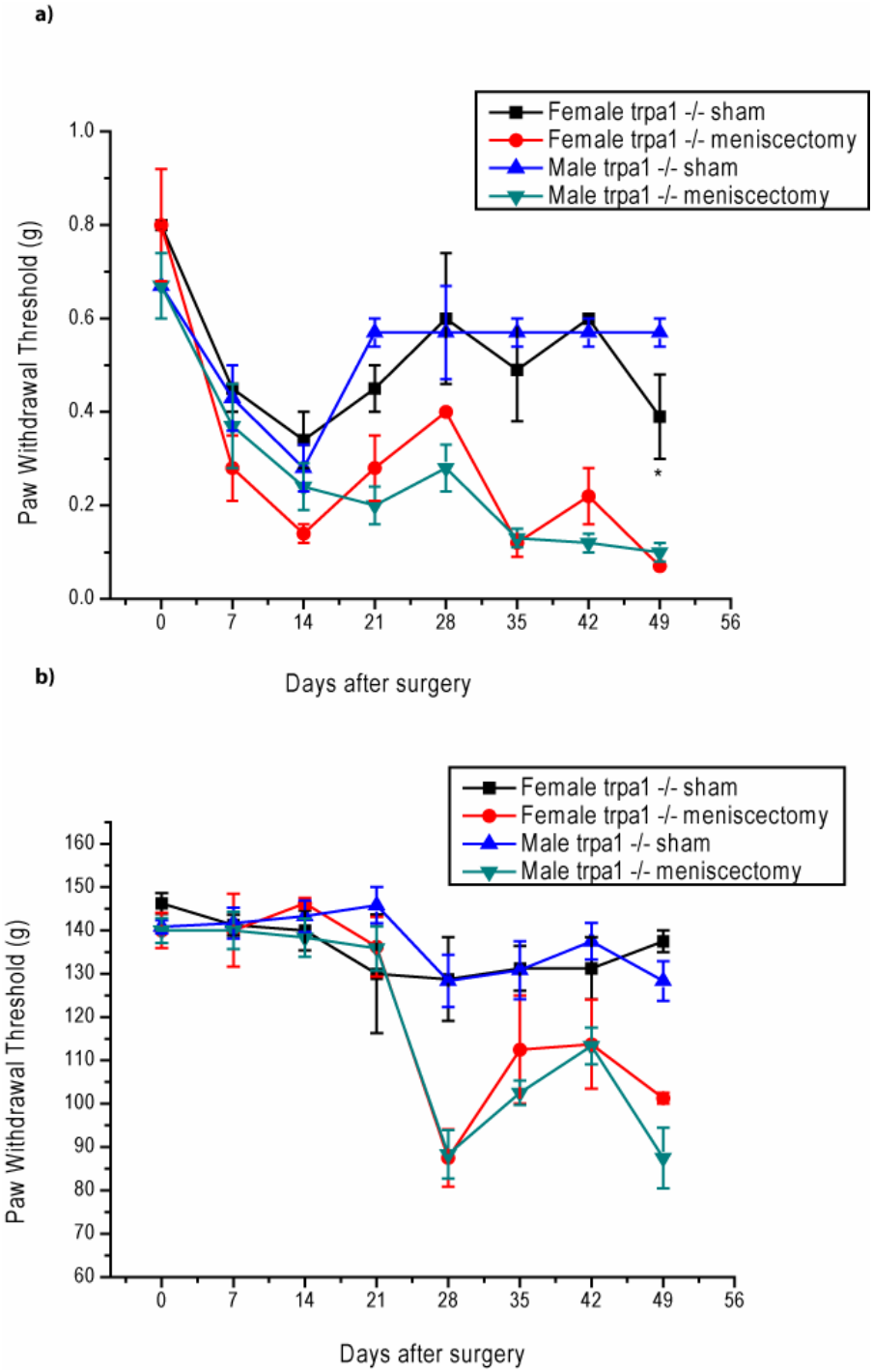


Figure 5-28 The development of pain behaviours following partial medial meniscectomy in male and female *trpa1*$^{-/-}$ mice.

The development of mechanical allodynia a) mechanical hyperalgesia b) are shown. Each point represents the mean value ± SEM for the ipsilateral hind limb of six male mice and four female mice. Statistics: Female trpa1$^{-/-}$ sham versus male trpa1$^{-/-}$ sham by repeated measures ANOVA followed by Tukey's Test, * $P<0.05$.

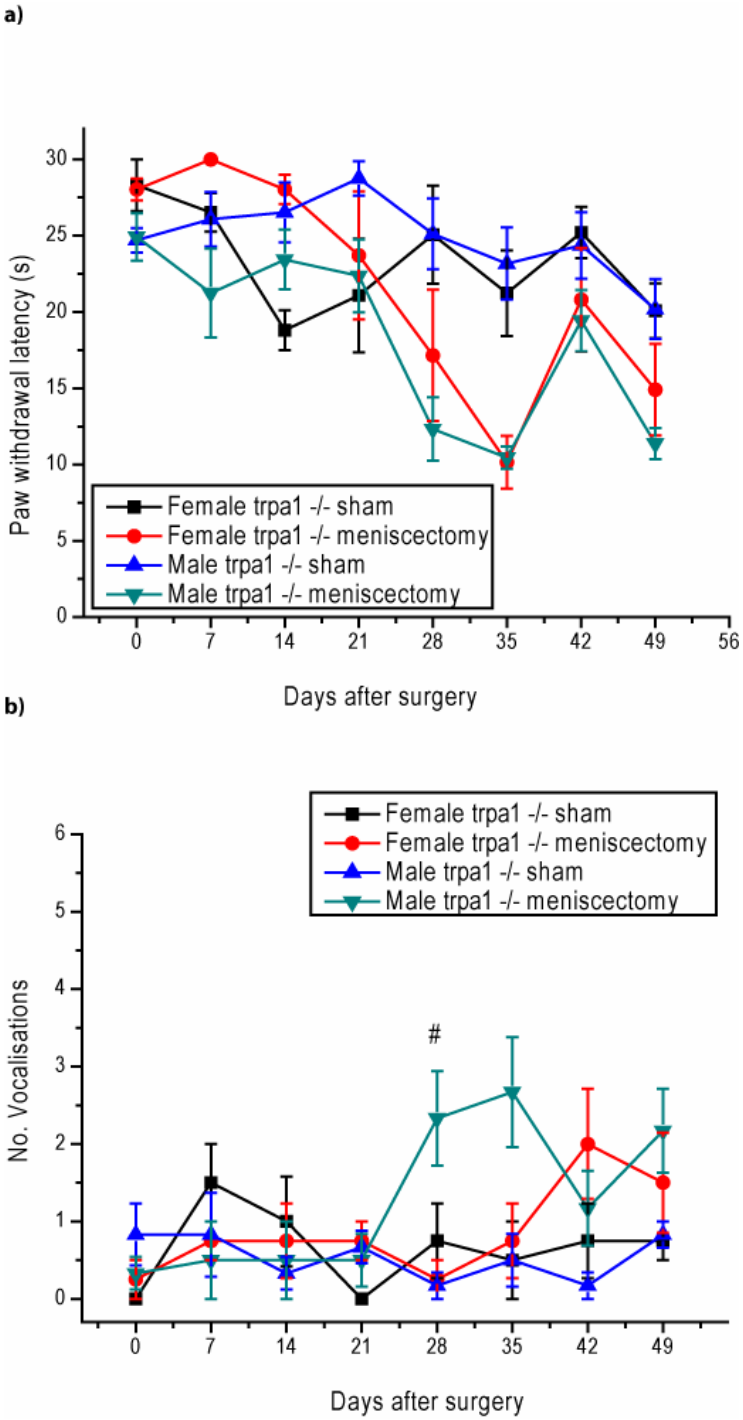


Figure 5-29 The development of pain behaviours following partial medial meniscectomy in male and female trpa1$^{-/-}$ mice.

The development of cold allodynia a) knee vocalisations b) are shown. Each point represents the mean value ± SEM for the ipsilateral hind limb of six male mice and four female mice. Statistics: Female trpa1$^{-/-}$ meniscectomy versus Male trpa1$^{-/-}$ meniscectomy by repeated measures ANOVA followed by Tukey's Test, # P<0.05

5.4 Discussion

5.4.1 TrkA inhibitors AZ-23 and CE-245677

Administration of Trk A inhibitors AZ-23 and CE-245677 in naïve female C57Bl/6 mice led to sensitization with the development of rapid onset thermal hyperalgesia (within 2 hours) in the left hind limb (only hind limb tested) and the development of mechanical hyperalgesia and cold allodynia by 24 hours in both hind limbs which persisted for at least 144 hours. The CE-245677 study was repeated generating highly consistent data. In contrast, CE-245677 administered to mice following the induction of CFA-induced acute paw inflammation caused a significant reduction of established ipsilateral mechanical hyperalgesia and thermal hyperalgesia within 2 hours. These results suggest that in the presence of increased levels of NGF (during acute inflammation), administration of CE-245677 has a short term, local anti-nociceptive effect. From 24 hours onwards, mechanical and thermal hyperalgesia returned in the ipsilateral limb indicating clearance of the drug. The development of a contralateral mechanical and thermal hyperalgesia at 24 hours post-treatment with CE-245677, that persisted for several days (similar to the studies in naïve mice), suggests that CE-245677 activated intracellular signalling cascades resulting in protein expression via translation leading to neuronal sensitization that persisted beyond expected clearance of the drug (<24hrs).

In chronic OA pain following partial medial meniscectomy, CE-245677 did not have a significant effect on established hypersensitivities. However, a contralateral mechanical hyperalgesia was recorded in the contralateral hind limb at 24 hours following CE-245677 which had diminished by 144 hours. To the author's knowledge, pre-clinical studies using compounds that inhibit the action of NGF or Trk A have previously only reported the reversal or prevention of established pain sensitivities in rodent models of acute or chronic pain and there are no reports of hypersensitivities being induced by inhibition of the NGF-TrkA pathway. For example, soluble TrkA receptor, TrkAD5, in mice has been shown to prevent the development of, and reverse established weight bearing deficits following destabilisation of the medial meniscus (McNamee, Burleigh et al. 2010) and pan Trk inhibitor ARRY-470 was shown to significantly reduce spontaneous pain behaviours in a mouse model of bone cancer pain (Ghilardi, Freeman et al. 2010). The development of hypersensitivities following treatment with a Trk inhibitor may be due to the ATP binding site, leading to difficulties obtaining specificity and potentially causing off-target effects on other kinases.

NGF plays a prominent role in acute nociception and in mechanisms behind chronic hypersensitivity providing a clear scientific rationale for targeting NGF-TrkA signalling for pain relief therapeutics. However, more specific tool compounds are needed to explore this pathway for the validation of pain models and for the development of new therapeutic compounds whilst minimizing the potential for adverse effects. Further studies could also include use of other compounds targeting the NGF-TrkA pathway such as the use of anti-NGF antibodies which have proven to be highly effective in inflammatory and neuropathic pain models (Shelton, Zeller et al. 2005; Wild, Bian et al. 2007) and for clinical trials of human OA pain (Lane, Schnitzer et al. 2010).

5.4.2 Anti- TNF

Repeated treatments with anti-TNF or the control antibody had no significant effects on pain behaviours in naïve C57Bl/6 mice. However, following the induction of OA pain by partial medial meniscectomy, pain behaviours did not remain consistent for the duration of the study. Mechanical hyperalgesia and cold allodynia diminished for several days in both the control and anti-TNF treated animals limiting the ability to assess the influence of anti-TNF specifically. The loss of pain hypersensitivities in both groups of mice may be due to fluctuations in endogenous opioid tone which is known to cause waxing and waning of OA pain behaviours following partial medial meniscectomy. At 168hrs into the study, control antibody treated mice and anti-TNF treated mice had regained significant pain hypersensitivities comparable to those immediately prior to the initiation of anti-TNF treatment. At this point, mice had received multiple doses of anti-TNF; however, there was no significant difference between the control and anti-TNF groups indicating that the anti-TNF was not itself effective at reversing OA pain behaviours measured in this study. Anti-TNF is a treatment prescribed for relief of pain in inflammatory conditions such as rheumatoid arthritis (Vinay and Kwon 2011) and has shown poor efficacy in previous reports of OA pain which has a less significant inflammatory component (Magnano, Chakravarty et al. 2007; McNamee, Burleigh et al. 2010). The results described here are consistent with those reported by McNamee et al. (2010) who observed that treating OA mice with a neutralizing soluble receptor to TNF had no effect on weight bearing deficits present at 16 weeks post destabilization of the medial meniscus. Similarly, in an open label pilot study, no significant improvement in pain was found after 12 weeks of anti-TNF treatment (adalimumab) in 12 patients suffering from erosive OA (Magnano, Chakravarty et al. 2007). TNF does not appear to play a significant role in the maintenance of chronic OA pain behaviours

and treatments targeting TNF or its receptors may not prove to be efficacious in the treatment of OA pain.

5.4.3 Bradykinin B2 antagonist

Bradyzide, a selective B2 bradykinin receptor antagonist successfully reversed two of the measured OA pain behaviours (mechanical hyperalgesia and cold allodynia) induced following partial medial meniscectomy. The importance of B2 receptors in OA pain have been previously suggested by reports describing the reversal of weight bearing deficits in rats with OA induced by MIA by B2 receptor antagonists, MEN16132 and Icatibant (Cialdai, Giuliani et al. 2009). Antagonism of the B2 receptor with Icatibant has also been shown to reduce pain intensity in human patients with symptomatic knee OA (Song, Althoff et al. 2009). Song et al. reported a significant reduction in pain intensity at rest and during activity following intra-articular injection of icatibant (Song, Althoff et al. 2009).

The B2 receptor is a valid target that may be exploitable in future drug developments for OA providing an alternative mechanism of action to current treatment with paracetamol or NSAIDs. Further studies aimed at exploring the role of the B2 receptor in OA pain might include testing specific joint tissues or the DRGs for evidence of increased expression of the B2 receptor using western blotting or RT-PCR techniques or the use of mice lacking genes for the B2 receptor.

5.4.4 TRPM8

AMTB is a tool compound available for the evaluation of the role of TRPM8 in animal models. AMTB has been characterized and shown to be selective versus TRPV1 and TRPV4 and effective in reversing established pain in a model of overactive bladder syndrome (Lashinger, Steiginga et al. 2008). In this study, AMTB was used to examine the effect of TRPM8 antagonism on OA related pain behaviours in C57Bl/6 mice. AMTB significantly reversed established cold allodynia induced by partial medial meniscectomy indicating that TRPM8 may be a potential target for the treatment of cold sensitivity in OA pain. Previous reports have also described a role for TRPM8 in cold sensitivities in other pain models. For example, *trpm8*$^{-/-}$ mice failed to develop cold allodynia (measured using the acetone response test) after chronic constriction injury of the sciatic nerve or injection of CFA into the hind paw (Colburn, Lubin et al. 2007; Parks, Parsons et al. 2011) and TRPM8 antagonists, (1-phenylethyl-4-(benzyloxy)-3-methoxybenzyl(2-aminoethyl)carbamate) and 3-[7-Trifluoromethyl-5-(2-trifluoromethyl-phenyl)-1*H*-benzimidazol-2-yl]-1-oxa-2-aza-spiro[4.5]-dec-2-ene Hydrochloride, have been reported to significantly reduce cold allodynia in an inflammatory (CFA) pain model

(Knowlton, Daniels et al. 2011) and chronic constriction injury model of neuropathic pain (Knowlton, Daniels et al. 2011; Parks, Parsons et al. 2011). The current study is the first to describe a role for TRPM8 in cold sensitivities induced by OA. A useful follow up study would be to induce OA by partial medial meniscectomy in *trpm8*$^{-/-}$ mice since cold allodynia would be expected to be less significant compared to *trpm8*$^{+/+}$ mice. This would further validate TRPM8 as a potential target for the relief of cold allodynia in OA.

No effect was identified on any of the other OA hypersensitivities tested, following treatment with AMTB. Previous unpublished studies in the Bevan laboratory have suggested a role of TRPM8 in mechanical sensitivity with siRNA and AMTB treatment leading to a reduction in PWT for paw pressure measurements. However, no significant effect on mechanical sensitivity was recorded in the current studies and therefore, targeting TRPM8 alone would not be expected to provide comprehensive OA pain relief. However, antagonism of TRPM8 may be a useful adjunct in the treatment of chronic pain syndromes such as OA.

Further studies aimed at exploring the role of TRPM8 in OA pain might include testing specific joint tissues or the DRGs for evidence of increased expression using western blotting or RT-PCR techniques. The functional expression of TRPM8 in osteoarthritic mice was studied using calcium imaging techniques and is described in Chapter 6.

5.4.5 TRPA1

The selective TRPA1 receptor antagonist HC-30031 significantly reversed mechanical hyperalgesia and cold allodynia induced by partial medial meniscectomy indicating a role for TRPA1 in cold and mechanical hypersensitivities in OA pain. HC-030031 has been previously shown to decrease similar pain behaviours in other rodent models of chronic pain (McNamara, Mandel-Brehm et al. 2007; Eid, Crown et al. 2008). For example, HC-030031 reduced nocifensive behaviour following AITC or formalin injection into the paw of rats (McNamara, Mandel-Brehm et al. 2007) and decreased mechanical hyperalgesia (measured by the Randall Selitto test) in a model of chronic inflammatory pain (intraplantar injection of CFA), and neuropathic pain (spinal nerve ligation) (McNamara, Mandel-Brehm et al. 2007; Eid, Crown et al. 2008).

In studies reported here, naïve *trpa1*$^{-/-}$ mice displayed deficits in sensing cold stimuli, punctuate cutaneous mechanical stimuli (von Frey) and noxious mechanical (paw pressure) stimuli compared to *trpa1*$^{+/+}$ littermates. Kwan et al. (2006) also

described reduced mechanical sensitivity to von Frey hairs, mechanical pressure applied using Randall Selitto apparatus, reduced sensitivity to intense cold (0ºC cold plate) and cooling via evaporation of acetone applied to the hind paw in naïve *trpa1*$^{-/-}$ mice compared to naïve *trpa1*$^{+/+}$ mice (Kwan, Allchorne et al. 2006). These results suggest that TRPA1 contributes to sensitivity to cold and mechanical stimuli. In contrast, Bautista et al. (2006) reported no significant difference in baseline mechanical (von Frey) or cold (acetone-evoked evaporative cooling) sensitivity between naïve *trpa1*$^{-/-}$ mice and *trpa1*$^{+/+}$ mice (Bautista, Jordt et al. 2006). These results indicate that TRPA1 is not required for normal acute sensitivity to heat or pressure. The reasons for these differences are unclear. The conflicting descriptions of the TRPA1 ion channel as a potential cold and mechanosensor suggest that TRPA1 plays an important role in these functions but other channels also contribute (Brierley, Castro et al. 2011).

Following partial medial meniscectomy, *trpa1*$^{-/-}$ mice and *trpa1*$^{+/+}$ mice showed decreased mechanical thresholds to punctuate cutaneous stimuli with von Frey filaments. This finding indicates that TRPA1 is not involved in the development of mechanical allodynia following the induction of OA which is consistent with previous theories that mechanical hypersensitivity (tactile allodynia) is mediated by low-threshold, large, myelinated, non-peptidergic Aβ fibres that normally sense innocuous stimuli such as touch, hair movement, or gentle pressure (Campbell, Raja et al. 1988; Koltzenburg, Torebjork et al. 1994) and do not express TRPA1 (Kobayashi, Fukuoka et al. 2005). Following partial medial meniscectomy, *trpa1*$^{-/-}$ mice and *trpa1*$^{+/+}$ littermates both developed cold allodynia and mechanical hyperalgesia. However, the PWTs and PWLs remained significantly higher in *trpa1*$^{-/-}$ mice compared to *trpa1*$^{+/+}$ mice for these specific pain behaviours. Similarly, Kwan et al. (2006) reported the development of increased cold sensitivity and mechanical allodynia in *trpa1*$^{-/-}$ mice comparable to that of *trpa1*$^{+/+}$ mice following peripheral nerve injury providing further evidence that TRPA1 is not essential for the development of these pain behaviours.

Antagonism of TRPA1 reversed established mechanical hyperalgesia and cold sensitivity induced by partial medial meniscectomy. *Trpa1*$^{-/-}$ mice showed greater baseline thresholds to cold and mechanical stimulation but still developed hypersensitivities following the induction of OA. Differences in mechanical and cold sensitivity measurements recorded from antagonist treated C57Bl/6 mice and *trpa1*$^{-/-}$ following partial medial meniscectomy may be attributed to genetic compensation of the *trpa1*$^{-/-}$ mice. Further investigations are required to elucidate the mechanisms

by which TRPA1 contributes to cold allodynia and mechanical hyperalgesia. Further studies aimed at exploring the role of the TRPA1 receptor in OA pain might include testing specific joint tissues or the DRGs for evidence of increased expression of the receptor using western blotting or RT-PCR techniques. The functional expression of TRPA1 in osteoarthritic mice was studied using calcium imaging techniques and is described in Chapter 6.

5.4.6 Sex differences in *trpa1*$^{+/+}$ mice

Reports have documented increased sensitivity of female rodents in response to mechanical and thermal stimuli due to the influence of gonadal steroid hormones (Berkley 1997; Craft, Mogil et al. 2004; Cook and Moore 2006; Fillingim, King et al. 2009). There were no significant differences in the development of mechanical allodynia, mechanical hyperalgesia and cold allodynia between male and female C57Bl/6 mice following partial medial meniscectomy, although the lower numbers of mice studied may have masked some mild behavioural differences. Similarly, Malfait et al. (2010) also reported no significant difference in PWTs for mechanical allodynia induced by the DMM model of OA in male and female CD-1 mice.

5.4.6.1 Pattern of sensitivity in *trpa1*$^{+/+}$ mice following partial medial meniscectomy

Unlike our earlier studies of C57Bl/6 mice, *Trpa1*$^{+/+}$ mice did not develop an initial phase of hypersensitivity (inflammatory pain phase) in the first two weeks after partial medial meniscectomy. This observation was also recorded in later studies of female C57Bl/6 and male C57Bl/6 mice. The absence of pain behaviours by day 7 post-surgery is likely to be a result of a more rapid resolution of post-operative inflammation due to decreased soft tissue trauma and enhanced surgical technique. Hence, initial post-surgical inflammation and sensitivity is diminished by day 7 resulting in normal pain behaviours at this time.

5.5 Conclusion

The use of TrkA inhibitors reversed certain pain sensitivities during acute inflammation but did not significantly affect pain sensitivities in the partial medial meniscectomy model of OA. There is a demand for TrkA inhibitors with greater selectivity in order to study the NGF-TrkA pathway and to assist in the validation of pain models.

The administration of anti-TNF provided further evidence that TNFα does not play a significant role in established pain sensitivities in OA. Antagonism of TRPM8, TRPA1 and Bradykinin B2 receptors, however, produced decreases in specific pain behaviours following the induction of OA in female C57Bl/6 mice. The results suggest that these receptors play a role in the transduction of joint pain in OA validating these targets for further investigation and drug development.

Trpa1$^{-/-}$ mice showed decreased sensitivity to cold and mechanical stimulation but developed cold allodynia and mechanical hyperalgesia following the induction of OA. Further investigations are required to elucidate the mechanisms by which TRPA1 contributes to these pain behaviours.

Chapter 6

Use of retrograde neuronal back labelling to explore receptor expression changes in dorsal root ganglia of mice with osteoarthritis

6 Use of retrograde neuronal back labelling to explore receptor expression changes in DRGs of mice with OA

6.1 Introduction

The structural or mechanistic cause of OA pain is unknown. However, there is increasing interest in the role of joint afferents both in the normal functioning of the musculoskeletal system and in the pathophysiology of joint diseases such as OA. So far, the partial medial meniscectomy model of OA in the mouse has been validated with reference to pain behaviour. Here, the model is investigated further using fluorogold and fluoro-emerald back labelling from the articular joint space to identify the cell bodies of primary sensory afferents from the knee joint within lumbar DRGs. Identification of these joint afferents allows calcium imaging techniques to be employed to compare the functional expression of specific ion channel/receptors in normal mice and in mice following the induction of OA by partial medial meniscectomy.

The studies reported so far in this thesis, have suggested a role for TRPM8 and TRPA1 in OA pain and consequently these ion channels were chosen for further research using calcium imaging. In addition, TRPV1, a polymodal ion channel expressed in nociceptive neurons, was also selected. TRPV1 is an important integrator of responses to inflammatory mediators (Marceau, Hess et al. 1998) and the known contribution of TRPV1 to chronic pain has meant that it has become a major target for the development of novel therapeutics (Immke and Gavva 2006). The TRPV1 antagonist, A-889425, has shown efficacy in a rat model of OA pain, alleviating impaired grip force in rats with MIA induced OA (Chu, Chandran et al. 2011). Furthermore, immunohistochemistry studies using back labelled joint sensory neurones have revealed increased expression of TRPV1 in lumbar DRGs of rats with OA compared to control animals (Fernihough, Gentry et al. 2005).

6.2 Aims

- To develop and validate a method for identifying joint afferents within DRGs using retrograde neuronal tracers
- To identify the location of knee joint afferents within lumbar DRGs of mice and determine whether there are any changes between populations of mice with OA
- To use calcium imaging to investigate the functional expression of specific ion channel/receptors (TRPM8, TRPA1, TRPV1) in joint afferents identified using retrograde back labelling techniques and to determine if these are changed in an OA model.

6.3 Results

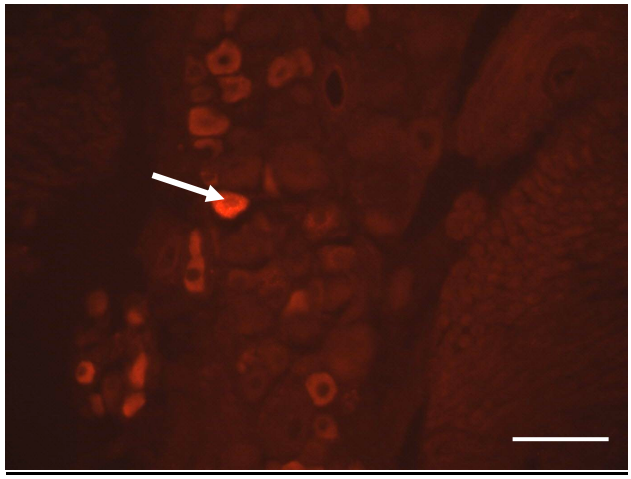
6.3.1 Identification of knee joint afferents using retrograde neuronal back labelling

Intra-articular injection of a neuronal tracer dye into the knee was used to identify the location of cell bodies from joint sensory neurons in the DRGs of rats and mice. Different dyes were explored in order to find an optimal dye that would enable the identification of joint afferents.

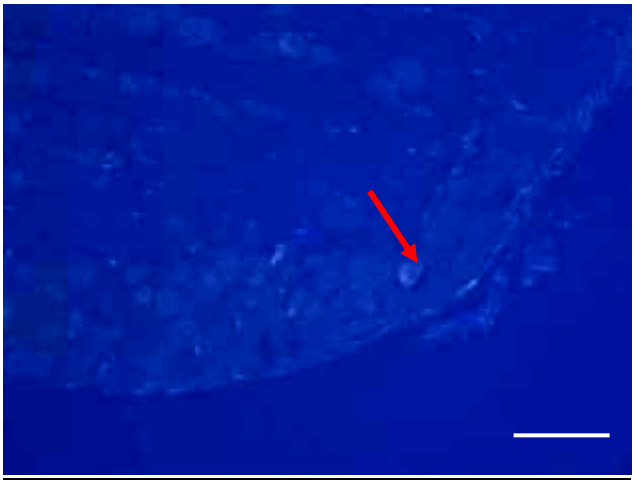
6.3.1.1 Pilot experiment in rats

3 different neuronal tracer dyes were individually injected into the left knee joint of anaesthetized rats to identify the most suitable dye for further studies in mice. Fast Blue, DiI and Fluorogold were detected in the ipsilateral DRGs with UV microscopy 5 days after injection into the knee joint (Figure 6-1). The granular epifluorescence in a number of the somata identify those DRG neurons whose axons projected to the ipsilateral knee joint. Somata stained with Fast Blue and DiI showed weak staining. Fluorogold labelling was of the greatest intensity and was therefore chosen for further experimentation in mice.

A)



B)



C)

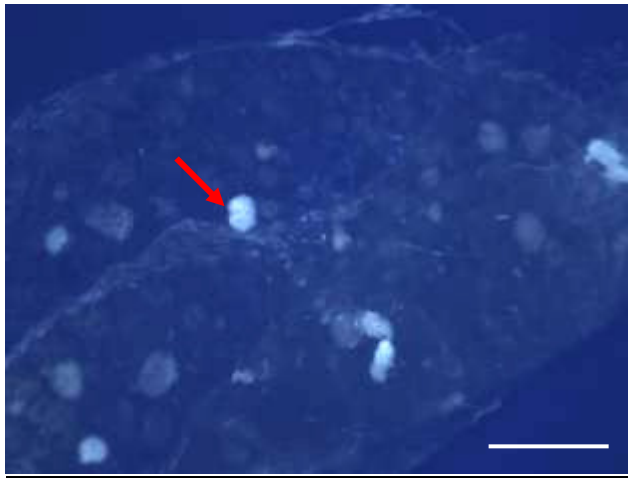
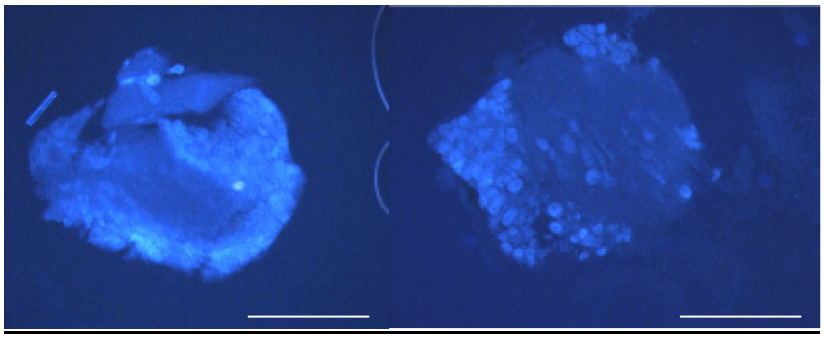


Figure 6-1 Photomicrographs demonstrating the somata of joint afferents of rat DRGs labelled with a fluorescent neuronal marker (↓); A) 2% dil; B) 1% Fast Blue; C) 2% fluorogold. Scale bar: 200µm.

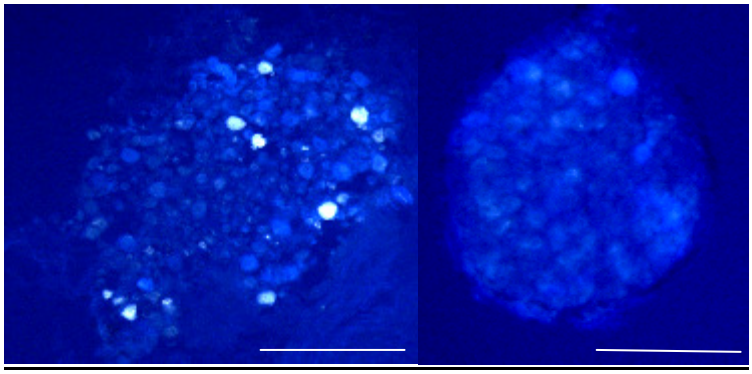
6.3.1.2 Mice

A series of fluorogold concentrations were injected intra-articularly into the knee joints of individual anesthetized mice and the lumbar DRGs collected 3 days later for UV microscopy to determine an optimum dosage for further experiments. All sections of each DRG from L1-L6 were examined and photographed. Representative images are shown in Figure 6-2. At fluorogold concentrations of 0.5%, 1% and 2%, intense fluorogold labelling was apparent under UV microscopy and was visible in DRG somata of all diameters. Little or no fluorogold was evident at a concentration of 0.1%. Fluorogold concentration of 0.5% showed well defined somata with distinct silver blue fluorescence in a number of cell bodies located within the ipsilateral DRG. There was no evidence of fluorogold labelling within the contralateral DRG at this concentration. Examination of sections labelled with fluorogold at 1% and 2% concentrations showed evidence of systemic spread of the dye with high numbers of somata in both the ipsilateral and contralateral DRGs showing characteristic silver blue granular epifluoresence. 0.5% fluorogold was therefore selected for further investigations aimed at determining the location and number of joint afferents within the DRGs of mice.

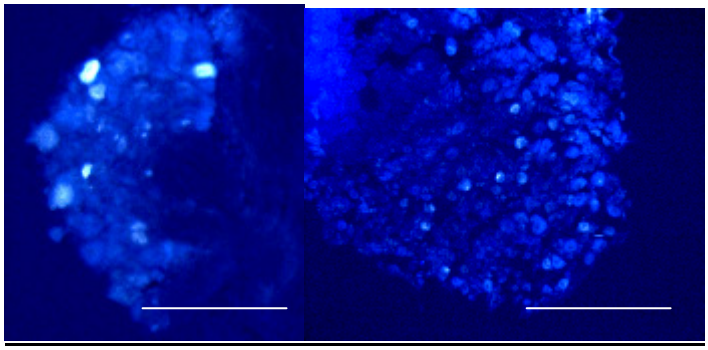
A) 0.1% fluorogold ipsilateral B) 0.1% fluorogold contralateral



C) 0.5% fluorogold ipsilateral D) 0.5% fluorogold contralateral



E) 1% fluorogold ipsilateral F) 1% fluorogold contralateral



G) 2% fluorogold ipsilateral H) 2% fluorogold contralateral

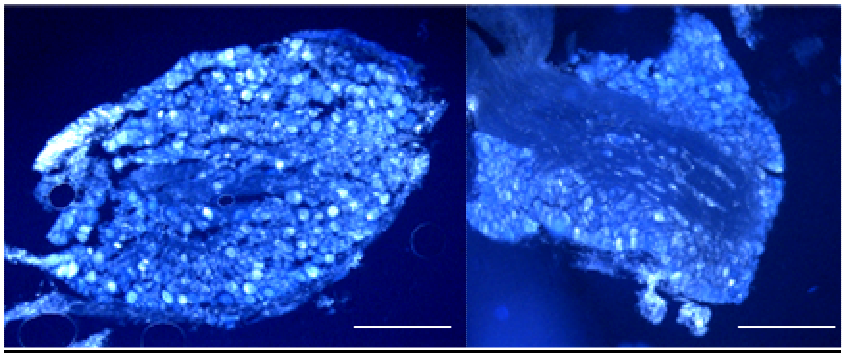


Figure 6-2 Photomicrographs of 10um thick sections of DRGs of mice 3 days after injection of fluorogold into the left knee joint. Scale bar: 500µm

6.3.2 Intra-articular injection validation

Knee joints from several mice were removed at the time of sacrifice and examined under ultraviolet light to determine that the entire joint interior had been exposed to fluorogold and that there was no extra-articular leakage. In all knees examined, Fluorogold staining was seen throughout the synovial lining of the joint and no examples of extra-articular leakage were observed (Figure 6-3).

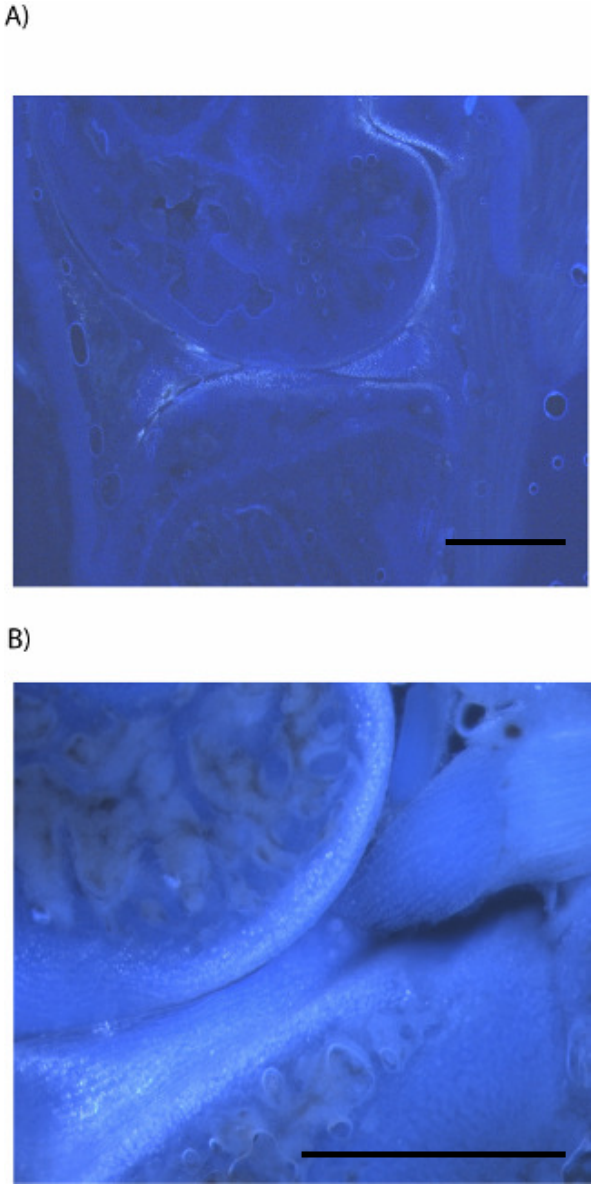


Figure 6-3 Photomicrographs showing the transverse section of the knee joint of a mouse 3 days following intra-articular injection of 0.5% w/v fluorogold in physiological saline. Sections at low power A) and high power B) are shown. White UV fluorescence indicates the presence of fluorogold within the joint cavity. No extra-articular fluorogold is observed. Scale bar: 500µm

6.3.3 Average diameter of a lumbar dorsal root ganglion cell body

A 16 week old female C57Bl/6 mouse was killed by cervical dislocation and the L4 DRG removed by aseptic technique and prepared for histology. The L4 DRG was selected since it is the largest of the lumbar DRG in mice. 10µm sections were stained with toluidine blue stain and photographed. 100 cell bodies were measured with the assistance of a graticule and the mean diameter calculated to be 36.8µm ± 1.08µm. Therefore in further studies examining the number of fluorescently labelled cell bodies within individual DRGs, every 4th 10µm section was counted to provide a representation of labelled somata throughout the DRG.

6.3.4 Location of knee joint afferents within murine DRGs

In order to determine the distribution of knee joint afferents within the lumbar DRGs and whether this was affected by cartilage erosion in a mouse model of OA, an experiment was performed to compare the location of joint afferents in naïve, sham meniscectomised mice and meniscectomised mice 16 weeks post-surgery. 8-10 week old female C57Bl/6 mice underwent either partial medial meniscectomy (n=3), sham surgery (n=3) or no surgery (n=3). At 16 weeks post-surgery, the three groups of mice underwent general anaesthesia and had their left knee joints injected with 10µl of 0.5% fluorogold w/v in physiological saline. 3 of the naïve mice received an intentional extra-articular (instead of an intra-articular) injection of 10µl of 0.5% fluorogold w/v in physiological saline. 3 days later, the mice were killed by cervical dislocation. Ipsilateral and contralateral lumbar DRGs were collected individually and prepared for imaging by fluorescent microscopy. Every 4th section of each DRG was viewed under ultraviolet light using the appropriate filter for fluorogold and photographed. For each group of mice, the cell bodies in each photographed section were counted and the cell bodies containing fluorogold identified and recorded. In order to objectively identify fluorogold containing neurons and non-fluorogold neurons, all of the images were viewed and photographs taken using the same exposure and light intensity. In order to objectively distinguish between a positive and negatively staining cell body, the maximum intensity of auto fluorescence produced by negative control DRG sections from mice that had not been injected with fluorogold was measured from photographs using the same settings. Since the amount of fluorogold fluorescence varied between individual neurons, all neurons fluorescing with intensity greater than this threshold were considered positive for fluorogold. 3 DRGs at each level of the lumbar spinal cord were examined. The results show that only a small proportion of the neurons contained within the lumbar DRGs are knee joint sensory neurons (positive for fluorogold epifluorescence), see Table 6-1. In naïve female C57Bl/6 mice injected

with 0.5% fluorogold, 55.4% of all fluorogold labelled neurons within lumbar DRG L1 to L6 were contained within the DRG at L3 which represented just 8.3% of the neuronal cell bodies identified in this DRG. DRG L4 contained the next greatest proportion of joint sensory neurons with 24.7% of the total fluorogold labelled cell bodies being identified at L4. This represented 4.5% of the total neurons counted in this DRG. Figure 6-4 shows the distribution of fluorogold labelled neuronal cells within the lumbar DRGs of mice injected with intra-articular compared to extra-articular fluorogold. Following extra-articular injection, a much greater proportion of neurons within the DRG showed positive fluorogold staining within each of the lumbar DRGs. For example, following extra-articular injection of fluorogold, 24.9% of L1 neurones were identified as positive for fluorogold compared to 0.3% of neurons within L1 of mice, following intra-articular injection. There was also greater variation in the number of somata that were positive within each DRG for the four mice. This was unsurprising since the peri-articular injection site and subsequent subcutaneous spread of the dye would be expected to have greater variability than intra-articular injection where the dye is confined to the joint space.

A sample of contralateral DRGs taken from experiments using mice injected with intra-articular fluorogold were also viewed under UV microscopy. No evidence of fluorogold staining was visible in these DRGs indicating that systemic spread of the neuronal tracer had not occurred.

There was no significant difference between age matched sham meniscectomised mice, naïve mice or osteoarthritic mice at 16 weeks post-surgery for the number or distribution of fluorogold staining neurons within the DRGs, Table 6-2. This suggests that the presence of cartilage damage due to OA does not significantly affect the uptake of fluorogold by joint sensory neurons. Further studies involving retrograde back labelled DRGs to identify joint sensory neurons focused on L3 and L4 DRGs since these contained the greatest proportion of joint afferents.

Table 6-1 Distribution of articular fluorogold labelled neurons across individual ipsilateral lumbar DRGs (n=3 mice).

Ipsilateral DRGs	L1	L2	L3	L4	L5	L6
Mean no. of cell bodies (± SEM)	630 (191)	202 (33)	958 (116)	787 (65)	745 (171)	396 (144)
% of Fluorogold positive neurons (± SEM)	0.32 (1.0)	3.80 (1.0)	8.28 (1.6)	4.49 (1.0)	2.37 (1.3)	0.34 (0.3)

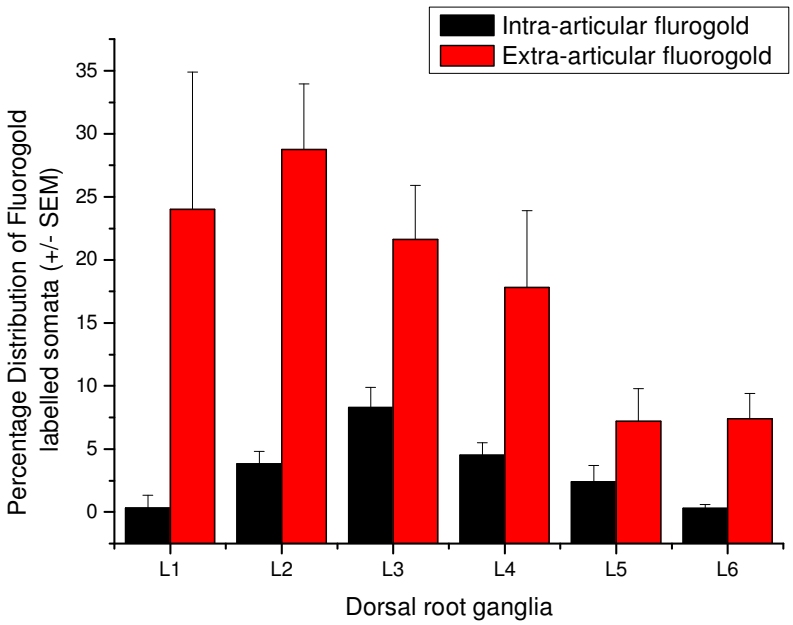


Figure 6-4 Distribution of fluorogold labelled neurons across individual lumbar DRGs identified under ultraviolet microscopy. Each point represents the mean of 3 female C57Bl/6 naïve mice

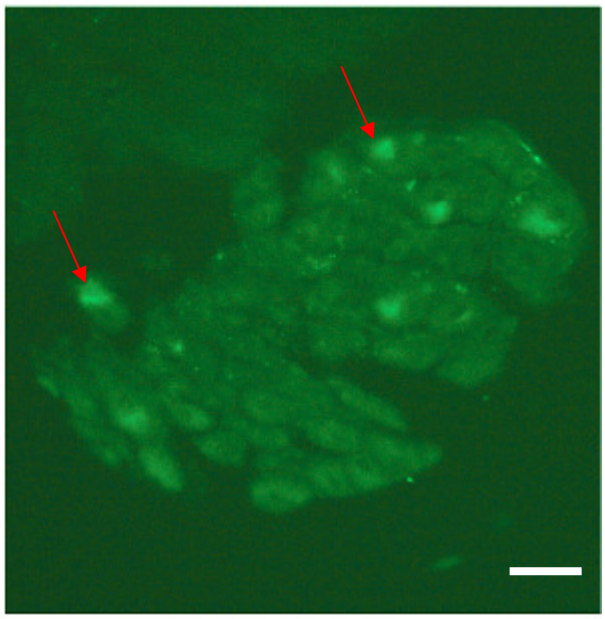
Table 6-2 Distribution of fluorogold labelled neurons across individual ipsilateral lumbar DRGs in female C57Bl/6 mice at 24 weeks of age with naïve mice a) 16 weeks post sham meniscectomy surgery b) and 16 weeks post partial medial meniscectomy surgery c) (n=3 mice per group).

DRGs	L1	L2	L3	L4	L5
a) Naïve					
Mean Number of Flurogold positive cell bodies (± SEM)	2.00 (2.00)	7.67 (0.33)	79.33 (15.3)	35.33 (7.86)	1.33 (1.33)
% of Fluorogold positive neurons at each DRG (± SEM)	0.32 (0.32)	3.8 (0.14)	8.28 (1.7)	4.49 (0.76)	2.37 (1.26)
b) 16 weeks post sham surgery					
Mean Number of Flurogold positive cell bodies (± SEM)	3.33 (0.88)	8.00 (5.13)	60.67 (19.0)	33.67 (18.3)	7.00 (1.86)
% of Fluorogold positive neurons at each DRG (± SEM)	0.53 (0.14)	2.03 (1.30)	5.54 (1.74)	4.93 (2.68)	1.26 (0.33)
c) 16 weeks post partial medial meniscectomy					
Mean Number of Flurogold positive cell bodies (± SEM)	10.00 (2.1)	6.00 (0.58)	81.67 (21.2)	35.00 (14.0)	17.00 (0.50)
% of Fluorogold positive neurons at each DRG (± SEM)	1.84 (0.38)	1.55 (0.14)	7.48 (1.94)	3.72 (1.49)	1.99 (0.06)

6.3.5 Identification of knee joint afferents using fluoro-emerald

Fluoro-emerald is also a dye that can be used to identify neurons using retrograde back labelling. The wavelength at which fluoro-emerald emits light is compatible with fura-2, a commonly used calcium indicator dye. Since there are many advantages to using a ratiometric calcium indicator dye, fluoro-emerald was also validated for the identification of joint afferents for calcium imaging studies. In mice, a series of fluoro-emerald concentrations were used to determine an optimum dosage that would detect joint sensory neurons without systemic spread of the dye. At fluoro-emerald concentrations of 0.5%, 1% and 2%, fluoro-emerald was not visible within the ipsilateral DRG. At 5%, a small amount of fluoro-emerald staining was visible within the somata of the ipsilateral DRG. Rather than increasing the concentration of the dye further, the treatment-dissection interval was increased to 7 days. Using this protocol, UV fluorescence of the joint afferent was visible within the ipsilateral DRG comparable to the percentage of positively staining neurons seen using fluorogold (Figure 6-5). The contralateral DRG showed no evidence of fluoro-emerald staining.

A)



B)

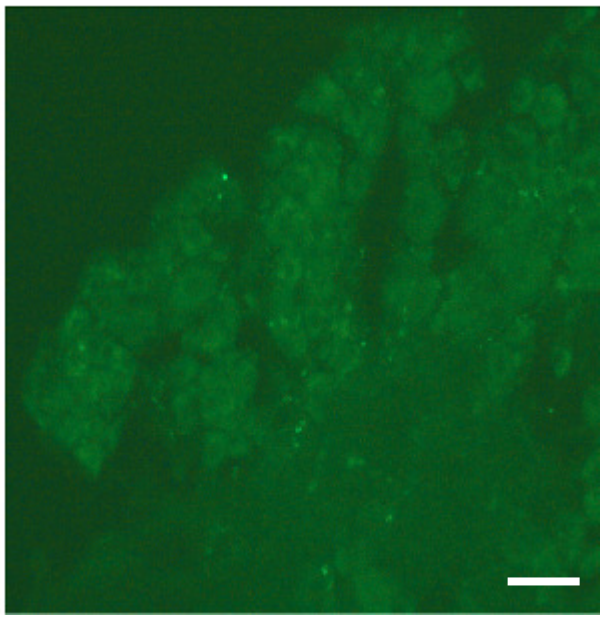


Figure 6-5 Photomicrographs of 10µm thick sections of DRGs of mice 7 days after injection of 5% fluoro-emerald w/v in 0.9% saline into the left knee joint.

The ipsilateral L3 DRG with fluoroemerald stained somata (↑) A) and the contralateral L3 DRG B) are shown. Scale bar: 100µm

6.3.6 Validation of calcium imaging methodology

2 Naïve 8 week old female C57Bl/6 mice were killed and the lumbar DRGs L1-L5 dissected and prepared for calcium imaging. The functional expression of TRPM8, TRPA1 and TRPV1 was explored in these studies by stimulation of the individual receptors using known agonists and the Fura-2 calcium indicator. The concentrations of the agonists applied to the cells were chosen to provide specific stimulation of the receptor of interest. At the end of each experiment DRG neurons were challenged with a high (50mM) concentration of potassium chloride to depolarise the neurons and open voltage gated calcium channels. The consequent robust increase in $[Ca^{2+}]_i$ was used to identify all the viable neurons within the field of study. The transient receptor potential channel agonists were applied sequentially to obtain maximum data from each cover slip. Extracellular physiological solution was applied for a period of 3 minutes between agonists to prevent continued stimulation by the previous agonist exposure and to provide an interval in which the intracellular calcium concentration could return to baseline levels.

To assess the effect of the order of agonist application, the protocol was alternated between each cover slip (see section 2.9.2). The results for protocol A and protocol B were analyzed separately and later pooled. Application of 1μM solution of icilin produced a response in 11.7% and 9.7% of neurons (see Table 6-3) using protocol A and protocol B respectively, consistent with responses due to activation of TRPM8 which is reported to be expressed by 5-10% of DRG neurons (Peier, Moqrich et al. 2002). When AITC (a TRPA1 agonist) was applied prior to capsaicin (a TRPV1 agonist), the percentage of neurons responding to these agonists was 49.0% and 50.0%, respectively (Figure 6-6). However, when capsaicin was applied first, 64.9% of neurons showed an increase in intracellular calcium in response to this agonist and only 18.6% of neurons produced recordable responses to a subsequent challenge with AITC (Figure 6-7). The calcium concentration remained high following exposure to capsaicin and did not return to baseline, occluding responses of the neurons to AITC. Performing separate experiments with individual agonists would have greatly increased the number of mice required for generating calcium imaging data. Therefore, it was decided to maximise the data generated by using both protocol A and protocol B for further studies, alternating the protocols between cover slips.

Table 6-3 Number of neurons responding with an increased [Ca^{2+}] following application of icilin, AITC, capsaicin or potassium chloride in neurons from the lumbar DRGs of 2 naïve 8 week old female C57Bl/6 mice. Number of viable neurons identified by responses to potassium chloride.

Protocol A	**Total number of responses to each agonist**	**%**
Icilin	**101**	11.7
AITC	**424**	49.0
Capsaicin	**432**	50.0
Neurons	**866**	
Protocol B	**Total number of responses to each agonist**	**%**
Icilin	**81**	9.7
Capsaicin	**541**	64.9
AITC	**155**	18.6
Neurons	**833**	

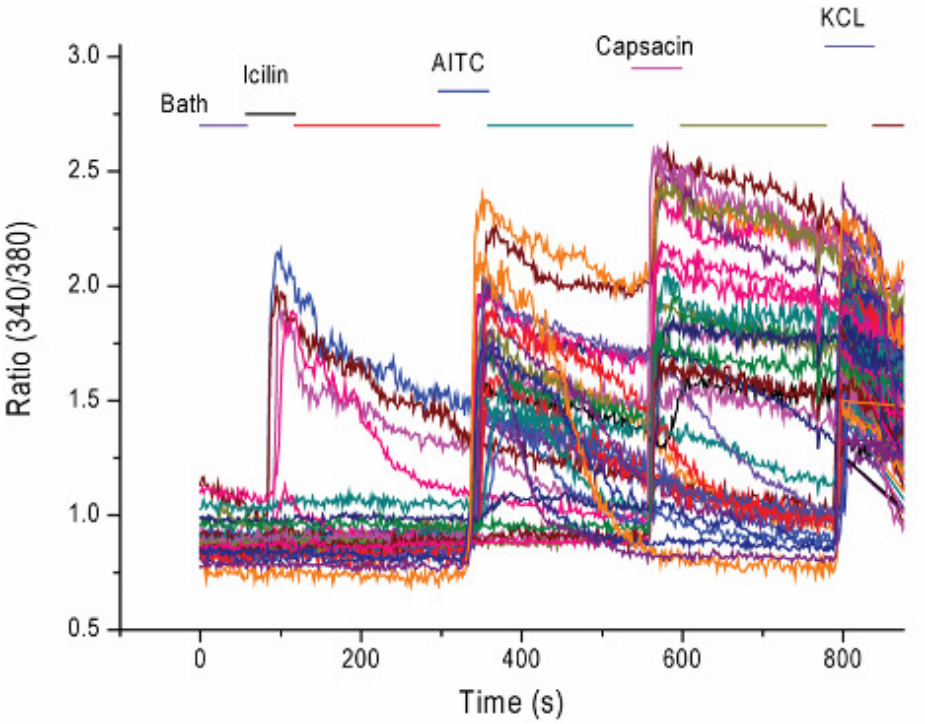


Figure 6-6 Representative time course showing changes in [Ca^{2+}] indicated by a change in the ratio of fluorescence intensity at 340nm and 380nm of Fura-2AM following the application of four agonists applied sequentially to cultured lumbar DRG neurons taken from 8 week old female C57Bl/6 mice.

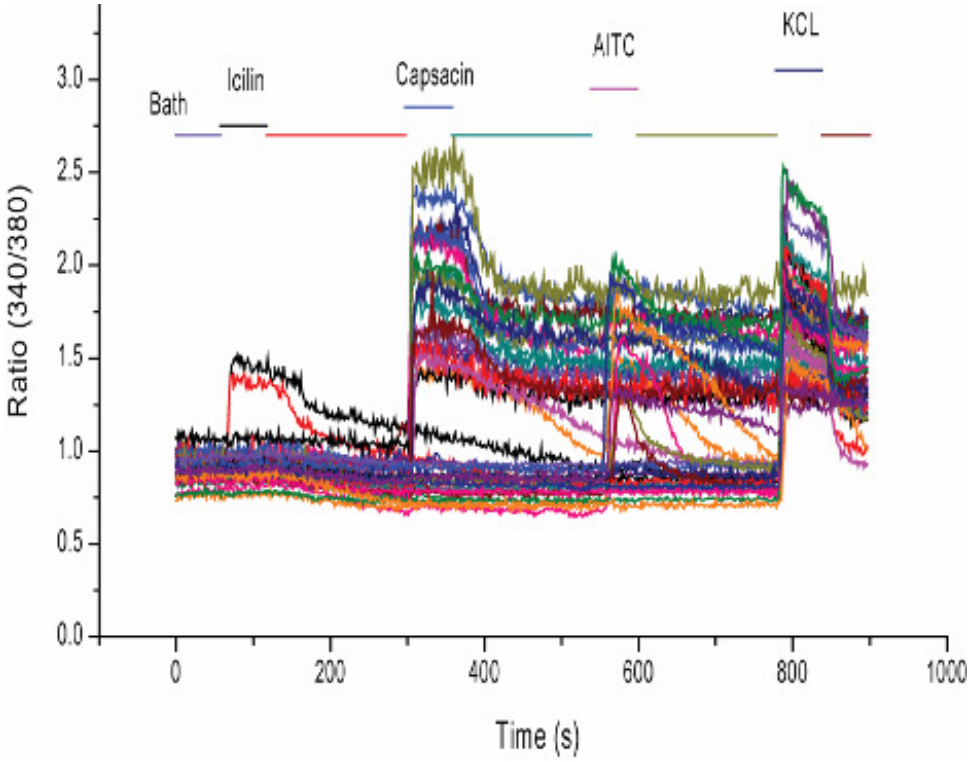


Figure 6-7 Representative time course showing changes in [Ca^{2+}] indicated by a change in the ratio of fluorescence intensity at 340nm and 380nm of Fura-2AM following the application of four agonists applied sequentially to cultured lumbar DRG neurons taken from 8 week old female C57Bl/6 mice.

Table 6-4 shows the pooled data for the two protocols. Since application of icilin did not influence the neurons' reponses to further agonists, the results for icilin using protocol A and B were combined. The results for protocol A were used to record AITC responses, and the results for protocol B were used to record capsaicin responses. 10.7% of murine lumbar DRG neurons responded to icilin. 49.0% of neurons responded to AITC and 64.9% of neurons responded to capsaicin. Previous reports indicate a wide range for the expression of TRP channels in neurons of rodent DRGs depending on the species and specific location of the DRGs being examined. Generally the studied TRP channels are found to be expressed in approximately 5-10% for TRPM8, 30-50% for TRPA1 and 40-60% for TRPV1 which is consistent with these findings (McKemy, Neuhausser et al. 2002; Hwang, Oh et al. 2005; Nagata, Duggan et al. 2005; Peier, Moqrich et al. 2002; Mishra, Tisel et al. 2011; Kobayashi, Fukuoka et al. 2005).

Table 6-4 Number of neurons responding with an increased [Ca^{2+}] following application of icilin, AITC, capsaicin in neurons from the lumbar DRGs of naïve 8 week old female C57Bl/6 mice. Number of viable neurons identified by responses to potassium chloride.

	Number of responses	Number of Neurons	%
Icilin	182	1699	10.7
AITC	424	866	49.0
Capsaicin	541	833	64.9

6.3.7 Identification of TRPA1 expressing neurons that do not express TRPV1

TRPA1 is currently thought to be co-expressed with TRPV1 in rodent DRGs (Story, Peier et al. 2003; Bautista, Movahed et al. 2005; Kobayashi, Fukuoka et al. 2005; Nagata, Duggan et al. 2005), however, application of the agonist capsaicin prior to AITC, revealed a population of neurons that were not responsive to capsaicin but were responsive to AITC (Figure 6-7). Analysis of the calcium imaging data showed that 541 neurons out of 833 responded to capsaicin when this agonist was applied first. Of the 292 neurons that did not respond to capsaicin, 129 responded to AITC. These results suggest that approximately 15.5% of neurons express TRPA1 but not TRPV1.

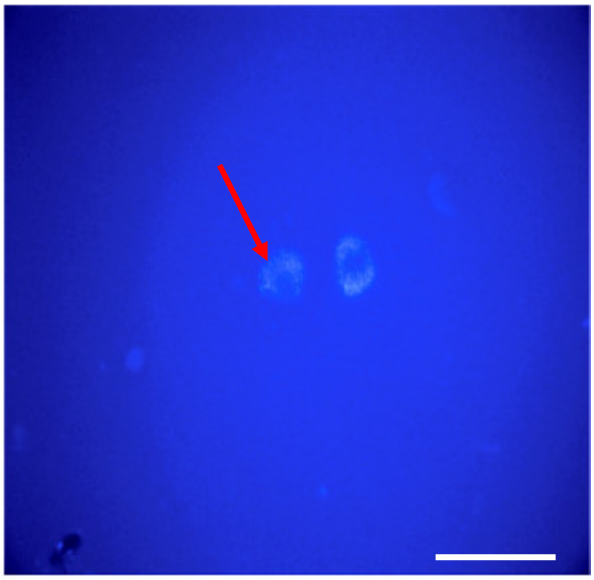
Similar findings were observed in later studies with 18.4%, 17.8%, and 13.4% of neurons (from naïve mice, OA mice and sham mice respectively) failing to respond to capsaicin but subsequently responding to challenge with AITC. These results provide further evidence to suggest that there is a population of neurons where TRPA1 is not always co-expressed with TRPV1.

6.3.8 Identification of joint afferents for calcium imaging studies

It was necessary to ensure that the fluorogold and fluoro-emerald back labelling dyes remained within neuronal cells following dissociation and culture of DRG neuronal cells prior to calcium imaging studies. Mice that had received an intra-articular injection of fluorogold 3 days previously or fluoro-emerald 7 days previously were killed by cervical dislocation and the lumbar DRG dissected and prepared for

culture. Figure 6-8 shows the fluorogold or fluoro-emerald staining within the neurons in the culture. The dye was clearly visible when viewed using the calcium imaging microscope using appropriate UV filters. An excitation scan was performed to identify the excitation wavelength that produced the maximum fluorescence intensity for fluorogold and fluoro-emerald since variations are known to occur with different conditions such as pH. The peak intensity for fluorogold was found to be 370nm and for fluoro-emerald was 490nm.

A)



B)

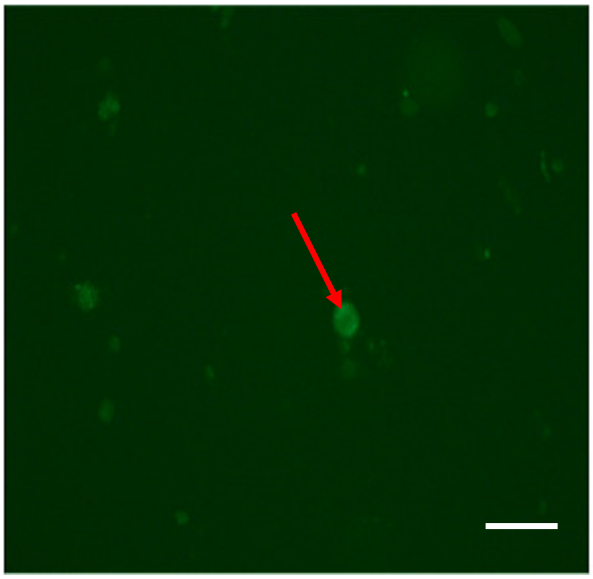


Figure 6-8 Photomicrographs using appropriate UV filters showing DRG neuronal cells containing UV fluorescent retrograde neuronal tracer dye staining following intra-articular injection of the knee joint.

Somata within cultured ipsilateral lumbar DRG neurons containing Fluorogold A) or Fluoro-emerald B) are shown. White UV fluorescence of fluorogold and green UV fluorescence indicates the presence of the neuronal tracer within the cells. Scale bar: 100µm

6.3.9 Validation of exposure times for identification of fluorescent indicator dyes in calcium imaging studies

Insufficient exposure times will lead to decreased sensitivity in the identification of neuronal cells that contain the fluorescent neuronal tracer dyes. High exposure times can reveal auto fluorescence of neuronal cells and decreased specificity. Therefore an experiment was performed to measure the maximum exposure times of neuronal cells at the wavelengths required for the identification of fluorogold and fluoro-emerald before auto fluorescence could be detected. Wells containing plated DRG neuronal cells with no fluorescent neuronal tracer were imaged using a range of exposure times at 370nm and 490nm. Auto fluorescence of the cells was visible at 370nm at exposure times greater than 400ms. Auto fluorescence of the cells was visible at 490nm at exposure times greater than 800ms. Therefore, these were the maximum exposure times used for the identification of joint sensory neurons stained with retrograde neuronal tracers within dissociated DRG neurons in calcium imaging studies.

6.3.10 Validation of Fluorogold/fluo-4 in calcium imaging and Fluoro-emerald/fura-2 in calcium imaging

2 naïve 8 week old female C57Bl/6 mice underwent general anaesthesia and received an intra-articular injection of either fluorogold or fluoro-emerald (n=3 mice per group). After the appropriate interval, the ipsilateral lumbar DRGs (L2-L4) were prepared for calcium imaging.

Visualization of the cells from the fluorogold injected mice at 370nm required an exposure time of 300ms. The cells were loaded with Fluo-4 and could be visualised within the neuronal cells at 480nm and exposure time of 300ms. Visualization of the cells from the fluoro-emerald injected mice at 490nm required an exposure time of 600ms. The cells were loaded with Fura-2 and this could be visualised within the neuronal cells at 340/380nm at 200ms. On average 2-3 fluorogold or fluoro-emerald stained neuronal cells were visible per field with approximately 100 non-fluorogold stained cells visible. The greater exposure time required by fluoro-emerald produced an image with a poor resolution compared to that obtained with fluorogold. Despite the advantages of being able to use a ratiometric calcium indicator dye with fluoro-emerald, flurogold was used for further studies since the identification of joint afferents was easier with this dye. This was essential as joint afferents comprised a very small number of the neurons within the lumbar DRG.

6.3.11 Functional expression of TRPM8, TRPA1 and TRPV1 in fluorogold labelled neurons within lumbar DRGs following intra-articular injection of fluorogold in 20 week old naïve female C57Bl/6 mice.

12 twenty week old naïve female C57Bl/6 mice received an intra-articular injection of fluorogold and 3 days later were killed by cervical dislocation and the lumbar DRGs (L2-L4) dissected and prepared for calcium imaging studies. Table 6-5 shows the number of fluorogold neurons responding with an increased [Ca^{2+}] following application of each agonist; these comprised 1.8% of the total neurons studied. 10.7% of the fluorogold labelled neurons responded to icilin compared to 10.2% of non-fluorogold labelled neurons (P=0.92). 46.2% of fluorogold labelled neurons responded to AITC compared to 47.0% of non-fluorogold labelled neurons (P=0.95). 73.3% of fluorogold labelled neurons responded to capsaicin compared to 61.7% of non-fluorogold labelled neurons (P=0.52). The response of the fluorogold and non-fluorogold labelled neurons to icilin and AITC were consistent with previous reports for the expression of TRPM8 and TRPA1 in rodent DRGs (McKemy, Neuhausser et al. 2002; Hwang, Oh et al. 2005; Nagata, Duggan et al. 2005; Peier, Moqrich et al. 2002; Mishra, Tisel et al. 2011; Kobayashi, Fukuoka et al. 2005).

Table 6-5 Number of neurons responding with an increased [Ca^{2+}] following application of icilin, AITC, or capsaicin in neurons from the lumbar DRGs of naïve 20 week old female C57Bl/6 mice (n=12). Viable neurons identified by responses to potassium chloride. Data represents the total of 3 identical experiments performed on separate days (4 mice per experiment). Total number of neurons studied = 1545.

	Flurogold labelled neurons			Non-flurogold labelled neurons		
Agonist	**Number of responses**	**Number of neurons**	**%**	**Number of responses**	**Number of neurons**	**%**
Icilin	3	28	10.7	154	1517	10.2
AITC	6	13	46.2	398	846	47.0
Capsaicin	11	15	73.3	414	671	61.7

6.3.11.1 Functional expression of TRPM8, TRPA1, and TRPV1 in joint sensory neurons did not differ significantly between OA, sham and naïve female C57Bl/6 mice

8-10 week old female C57Bl/6 mice underwent partial medial meniscectomy or sham surgery under general anaesthesia. After 12 weeks, significant OA related pain behaviours were recorded in the ipsilateral knee joint. The mice received an intra-articular injection of fluorogold and 3 days later, the lumbar DRGs were prepared for calcium imaging studies. Table 6-6 shows the responses of fluorogold labelled neurons in mice 12 weeks following sham surgery or the induction of OA by partial medial meniscectomy. Fluorogold labelled neurons comprised 3.9% of the cultured lumbar DRG neurons studied in sham mice and 3.3% of the studied neurons in OA mice. 11.9% of fluorogold labelled neurons from sham mice responded to icilin consistent with the previously described expression of TRPM8, compared to 13.1% of fluorogold labelled neurons from OA mice (P=0.84). 51.2% of fluorogold labelled neurons responded to AITC from sham mice compared to 36.7% of fluorogold labelled neurons from OA mice (P=0.32). 70.8% of fluorogold labelled neurons from sham mice responded to capsaicin compared to 64.5% of fluorogold labelled neurons from OA mice (P=0.83). There were no significant differences between responses of the fluorogold labelled neurons in OA mice compared to sham mice. The proportion of neurons responding to capsaicin was greater in fluorogold labelled neurons than non-fluorogold neurons in all of the groups studied (naïve, sham and OA mice) although this was not statistically significant. The large proportion of TRPV1 expressing neurons back labelled from the knee joint suggests that the knee joint is innervated by a large proportion of nociceptive neurons compared to other tissues with cell bodies contained within the L3 and L4 DRG.

Table 6-6 Number of neurons responding with an increased [Ca^{2+}] following application of icilin, AITC, or capsaicin in neurons from the lumbar DRGs of 20 week old female C57Bl/6 mice 12 weeks post sham surgery a) or partial medial meniscectomy b) (n=20 mice per group). The number of viable neurons were identified by responses to potassium chloride. Data represents the total of 4 identical experiments performed on separate days for each group (5 mice per experiment).

a) Sham (Total number of neurons studied =1727)						
	Flurogold labelled neurons			**Non-flurogold labelled neurons**		
Agonist	**Number of responses**	**Total number of neurons**	**%**	**Number of responses**	**Total number of neurons**	**%**
Icilin	8	67	11.9	242	1640	14.8
AITC	22	43	51.2	604	1032	58.5
Capsaicin	17	24	70.8	387	608	63.7
b) Partial medial meniscectomy (Total number of neurons studied =1837)						
Agonist	**Number of responses**	**Total number of neurons**	**%**	**Number of responses**	**Total number of neurons**	**%**
Icilin	8	61	13.1	228	1776	12.8
AITC	11	30	36.7	429	769	55.8
Capsaicin	20	31	64.5	656	1007	57.5

6.4 Discussion

Fluorogold and fluoro-emerald back labelling was used successfully to identify neurons innervating the joint following intra-articular injection of the tracer dye. The fluorescent tracer dyes could be used to identify joint sensory neurons within DRG cell cultures and were compatible with the calcium indicator dyes Fluo-4 or Fura-2 for calcium imaging studies. Fluorogold was superior for the identification of labelled cells due to the greater fluorescence intensity which enabled easier identification of joint afferents from within dorsal root ganglion cells. Calcium imaging studies could thus be used to examine differences in TRP channel distribution in OA and naïve mice.

The reliability of the data in this study was dependent on the specific staining of joint afferents following intra-articular injection of a fluorescent retrograde neuronal tracer. In these studies joint afferents formed a very small population of neurons contained within lumbar DRGs with extra-articular injection of fluorogold producing a much larger population of fluorescent cell bodies within the DRG. UV microscopy of knee joints following intra-articular injection also did not show signs of leakage of the tracer dye to the surrounding tissues suggesting that the injection of fluorogold into the knee joint was accurate and consistent.

The finding that L3 lumbar DRG contained the greatest proportion of back labelled neurons following intra-articular injection of fluorogold is consistent with previous work (Salo and Tatton 1993) and is in contrast to the rat whereby the greatest proportion of joint sensory neurons is contained in the L4 DRG (Fernihough, Gentry et al. 2005; Lim, Chan et al. 2011). In these studies, we made no attempt to accurately quantify the number of neurons within each dorsal root ganglia, however, the numbers of fluorogold stained DRG neurons were counted across each DRG (every 4^{th} section) to provide a reflection of the distribution of fluorogold labelled neurons in the lumbar DRGs. 1.8%-3.9% of cultured DRG neurons were identified as containing fluorogold during calcium imaging studies. This meant that only a small number of fluorogold staining neurons could be studied for their responses to known agonists of the TRP channels TRPM8, TRPA1 and TRPV1. The current tudies did not show significant differences between the neuronal responses of naïve, sham and partially meniscectomised mice. However, an immunohistochemistry study by Fernihough et al. (2005), reported increased expression of TRPV1 in DRGs back labelled by intra-articular injection of Fast Blue from rats with OA (induced by MIA) compared to control animals. Small differences may have been hidden by the low power of the study with low numbers of cells identified as joint

afferents within the lumbar DRG. To obtain a greater number of cells for study, a greater number of mice would be required. Alternatively, techniques such as laser scanning cytometry or laser capture technology could be employed to identify the fluorogold containing DRG neurons so that a greater percentage of the total population of joint sensory neurons could be studied.

This is the first report of calcium imaging studies used for the study of the functional expression of identified joint sensory neurons. The back labelling and calcium imaging techniques used here, however, can be adopted to study other populations of sensory neurons innervating other structures in the body, particularly where the population of neurons comprise a larger percentage of the total neurons within a specific DRG. Recently, Christianson et al. (2010) reported the use of retrograde back labelling of colonic visceral afferents to the lumbar sacral DRG and performed calcium imaging studies to measure TRPV1 and TRPA1 responses to application of capsaicin and AITC, respectively. Retrograde back labelling and calcium imaging studies represent useful tools for the study of the functional expression of specific receptors/ion channels in populations of sensory neurons innervating target tissues.

Whilst calcium imaging studies did not find significant differences in the responsiveness to TRPM8, TRPA1 and TRPV1 agonists between the groups of mice, some interesting findings were recorded. Calcium imaging studies revealed that 15.5% of the neurons within the DRG of naïve mice did not respond to capsaicin but were responsive to stimulation with AITC indicating that TRPA1 is not always co-expressed with TRPV1. This population of neurons was identified in all of the calcium imaging studies with a mean of 16.3% (± 1.14%) of neurons responding to AITC despite not responding to capsaicin. This population of neurons has not previously been documented. Also, the number of responses to capsaicin (a TRPV1 agonist) was greater in the fluorogold labelled neurons compared to the non-fluorogold labelled neurons suggesting that the neurons innervating the joint have a higher proportion of nociceptive neurons compared to the remaining neurons within the L2-L4 DRG, although this finding was not statistically significant.

The functional expression of TRPM8, TRPA1 and TRPV1 were assessed using retrograde neuronal back labelling and calcium imaging techniques. Retrograde neuronal back labelling in conjunction with immunohistochemistry could also be used to compare the expression of these ion channels in joint or DRG tissues in OA and control mice, such as the study reported by Fernihough et al (2005). However, this technique relies on the sensitivity and specificity of primary and secondary antibodies and the limited availability of specific antibodies for TRP channels

suitable for use in mice restricted the use of immunohistochemistry in the studies reported in this thesis.

6.5 Conclusion

Retrograde back labelling of sensory neurons is a useful tool to study the functional expression of specific receptors. TRPM8, TRPA1 and TRPV1 have been shown to contribute to the development of pain behaviours associated with OA in addition to other chronic pain conditions. However, no significant differences were found in the functional expression of key TRP channels in joint sensory neurons between naïve, sham and partial medial meniscectomised mice.

Chapter 7

General Discussion

7 General Discussion

OA is the most common cause of persistent musculoskeletal pain and can be a serious disabling disease. Current therapies provide incomplete relief and side effects associated with currently available analgesic drugs limit treatment strategies. The addition of new drugs has the potential to provide additional relief for many OA patients. In recent years, the development of animal models has provided a valuable contribution to our expanding knowledge on the mechanisms of chronic pain such as neuropathic pain disorders and OA. However, there has been a lack of clinically relevant mouse models of OA pain. In this thesis, the development of degenerative joint disease lesions and a variety of accompanying pain behaviours following spontaneous, chemically induced and surgically induced OA in mice have been studied. Partial medial meniscectomy and intra-articular injection of MIA into the knee joint both produced progressive cartilage erosion and accompanying pain behaviours in C57Bl/6 mice. STR/Ort mice also developed spontaneous cartilage erosion, although significant levels of pain were not apparent which made this particular model unsuitable for further studies investigating the effects of different analgesic drugs and tool compounds on OA pain behaviours. It is extremely important to match an animal model as closely as possible to the human disorder so that it may be able to predict the outcome of a novel therapeutic intervention. The intra-articular injection of the metabolic toxin, MIA, may lead to non-specific effects on tissues within the knee joint and thus was considered to be a less clinically relevant method for OA induction compared to the other models studied. The partial medial meniscectomy model was selected for further study since it mirrors a clinical scenario in humans whereby the early onset of OA occurs secondary to meniscal tear and treatment by partial meniscectomy. The production of progressive degenerative joint disease characterised by cartilage erosion and the presence of osteophytes mimics many structural changes seen in human OA. On this basis, the model is likely to show neuronal and other changes that can occur in humans with similar structural damage. Reversal of pain behaviours induced by partial medial meniscectomy is consistent with a pain condition rather than joint instability and the similar response of the pain behaviours to clinically used analgesics used in human OA patients validates the partial medial meniscectomy model of OA pain. We will not know whether this new model of joint pain is predictive of clinical outcomes, however, until novel therapies developed on the basis of efficacy in this model are tested in humans.

An effective animal model benefits from being robust, readily reproducible and easy to use so that it can enable a high throughput of studies. Partial medial meniscectomy is a quick and relatively simple surgery to perform although the development of persistent pain behaviour may take up to 6 weeks to develop. Variation in the pain behaviours between cohorts of mice and fluctuation in pain behaviours due to the influence of endogenous opioids limits the application of the model to longer term studies whereby trials can be scheduled according to the individual time course of pain behaviours for a particular cohort of mice. Nevertheless, the model offers great potential for further studies targeted at elucidating the pathophysiology behind OA pain and the development of new therapeutics.

Initial studies were completed examining the response of OA pain behaviours to novel analgesics targeting specific ion channels and mediators. Antagonists of TRPM8, TRPA1 and Bradykinin B2 receptors produced decreases in specific pain behaviours following the induction of OA in female C57Bl/6 mice indicating that these ion channels/mediators play a mechanistic role in the transduction of joint pain in OA. The induction of hypersensitivities following the administration of TrkA receptor inhibitors highlights the importance of obtaining selective compounds to avoid off target effects. Further studies using specific pain receptor agonists/antagonists will help to further our understanding of the pathophysiology of OA pain. We have examined the development of OA pain in *trpa1*$^{-/-}$ mice, however, many other colonies of genetically modified mice are available for study which will also help to further our understanding of specific pain receptors and aid the identification of future therapeutic targets.

Fluorescent retrograde back labelling was used to identify joint sensory neurons innervating the knee joint within the dorsal root ganglia for calcium imaging. This is the first time calcium imaging has been reported for the study of joint sensory neurons using fluorescent retrograde back labelling. Whilst no significant differences were found in the functional study of TRPV1, TRPA1 and TRPM8 expressing neuronal cells in mice with OA compared to control mice, the techniques employed, may be extremely useful for the functional studies of other populations of neurons particularly where neurons innervating a particular structure form a larger proportion of cell bodies within individual ganglia.

In summary, the results in this thesis provide histological and pharmacological characterisation of a new model for the induction of OA pain in mice which will facilitate the development of new therapeutics and a greater understanding of the pathophysiology behind OA pain.

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